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Genotypic and Phenotypic Diversity of Cyanobacteria in Biological Soil Crusts of the Succulent Karoo and Nama Karoo of Southern Africa

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Abstract Biological soil crusts (BSCs) are communities of cryptogamic organisms, occurring in arid and semiarid regions all over the world. Based on both morphological identification and genetic analyses, we established a first cyanobacterial inventory using the biphasic approach for BSCs within two major biomes of southern Africa. The samples were collected at two different sites in the Succulent Karoo and one in the Nama Karoo. After cultivation and morphological identification, the 16S rRNA gene was sequenced from the cyanobacterial cultures. From the soil samples, the DNA was extracted, and the 16S rRNA gene sequenced. All the sequences of the clone libraries from soil and cultures were compared with those of the public databases. Forty-five different species were morphologically identified in the samples of the Succulent Karoo (observatories of Soebatsfontein and Goedehoop). Based on the genetic analyses, 60 operational taxonomic units (OTUs) were identified for the Succulent Karoo and 43 for the Nama Karoo (based on 95 % sequence similarity). The cloned sequences corresponded well with the morphologically described taxa in cultures and sequences in the public databases. Besides known species of typical crust-forming cyanobacterial genera (*Microcoleus*, *Phormidium*, *Tolypothrix* and *Scytonema*), we found sequences of so far undescribed species of the genera *Leptolyngbya*, *Pseudanabaena*, *Phormidium*, *Oscillatoria*, *Schizothrix* and *Microcoleus*. Most OTUs were restricted to distinct sites. Grazed soils showed lower taxa numbers than undisturbed soils, implying the presence of early successional crust types and reduced soil surface protection. Our combined approach of morphological identification and genetic analyses allowed both a taxa inventory and the analysis of species occurring under specific habitat conditions.

Introduction

Biological soil crusts (BSCs) consist of cryptogamic organisms, i.e. cyanobacteria, green algae, lichens, bryophytes, fungi and other bacteria in varying proportions [5]. Living in the upper few millimetres of the soil, they are found in arid and semiarid environments around the world [5]. BSCs contribute considerably to the biomass of low-productivity regions [12]. In arid and semiarid regions, they are mostly initiated and very often also dominated by cyanobacteria [5]. Cyanobacteria in BSCs enhance soil stability and water capacity, and protect soils against erosion [3, 10, 32]. Additionally, the nitrogen pool of soils is enriched by the activity of nitrogen-fixing cyanobacteria [4, 19, 22, 23, 38]. Because disturbances, such as grazing and trampling, regularly cause alteration in diversity and distribution of BSCs, species inventories of crust-forming cyanobacteria allow us to draw conclusions with respect to ecosystem stability and recovery [66, 67].

Determination of cyanobacterial diversity can be accomplished by two general methods: (1) phenotype diversity using cultivation and morphological identification, and (2) genotype diversity with PCR amplification, cloning and sequencing of DNA. Cultivation allows for recording of multiple morphological and ecological parameters. However, with culture-based methods, fast-growing strains ('weeds') can suppress

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the growth of other cyanobacteria, and cyanobacteria that do not grow under the applied conditions are easily missed. Genotypic information gained without prior culturing lacks data on the growth preferences and ecology of the cultured organism, and is prone to bias from DNA extraction [28, 64]. In order to minimise these effects, we decided to implement a polyphasic approach by combining both the phenotypic and the genotypic approaches as its usefulness has already been described in a number of studies, e.g. [15, 28, 48].

Our study investigated two major biomes of southern African drylands: the Succulent Karoo biome, renowned as one of the world centres of endemism and biodiversity with mild climatic conditions within the winter rainfall region, and the Nama Karoo biome, belonging to the summer rainfall area and characterised by sparse vegetation of shrubs and grasses with taller trees, which are more or less restricted to river beds. Out of these biomes, the three sites Soebatsfontein, Goedehoop and Duruchaus were chosen, which are characterised by different land use intensities and climatic conditions. At Soebatsfontein and Goedehoop, we assessed the morphological diversity by microscopic analyses of free living and cultured material and molecular diversity by sequencing the 16S rRNA genes of the characterised strains and soil. At Soebatsfontein, Goedehoop and Duruchaus, the 16S rRNA genes of soil DNA were amplified, cloned and sequenced. Together with putatively related sequences from public databases, all the sequences were analysed by creating operational taxonomic units (OTUs) with 95 or 97 % similarity, respectively, and by using neighbour joining.

This combined approach is firstly applied here to determine the cyanobacterial diversity in BSCs, leading to a greater knowledge gain by the combined detection of morphological species and genotypic OTUs. The investigation of site-specific species inventories influenced by abiotic habitat parameters and disturbance effects allows the inclusion of ecological aspects and the detection of so far unknown species. Additionally, the results provide important information on the efficacy of detection methods for different genera and strains.

Methods

Sampling Sites

Within the framework of the long-term project 'BIOTA' (www.biota-africa.org), 33 observatories covering an area of 1×1 km each were established in southern Africa [35]. In our study, we assessed the cyanobacterial diversity near the observatories Soebatsfontein (no. 22) and Goedehoop (no. 26), both located in the Succulent Karoo biome of South Africa, and Duruchaus (no. 40), located in the Nama Karoo biome of Namibia. The soils of the Succulent Karoo biome are characterised by a high diversity, driven by abiotic (geology) and biotic (bioturbation) factors. The soils of the Nama Karoo are shallow, weakly structured and skeletal. At all sites, the soils are typically nitrogen-depleted, and the occurrence of intermediate and late successional crusts has already been reported [13, 14]. Crust samples were taken between vascular plant vegetation, with the distance to the next plant varying between a few centimetres and about 1 m.

The Soebatsfontein site (So) is characterised by broad valleys and undulating hills, with some granite inselbergs and local quartz outcrops [31]. The predominant soil reference groups are Durisols and Cambisols with a pH of about 3.8 to 10.4 and a texture ranging from sandy to silty loam [73]. Vegetation is dominated by leaf succulent dwarf shrubs. The average annual rainfall is around 130 mm with the main amount precipitating between March and August [31]. The observatory is utilised by small stock (sheep and goat) farming of moderate intensity. In 2003, an ungrazed exclosure, which was moderately grazed before, was established next to the observatory.

The Goedehoop observatory (Gh) is located in a gently undulating plain [31], being characterised by extensive areas covered with translucent quartz pebbles. The dominant soil groups are Cambisol, Solonchak and Leptosol, and the pH values range between 4.0 and 9.0. The occurring textures are silt, silty loam and sandy silt [73]. The quartz fields accommodate a unique vegetation, dominated by compact leaf-succulent dwarf shrubs of extraordinary biodiversity and a high rate of endemism. The average annual rainfall is around 120 mm, with regularly occurring fog and dewfall events [31]. The observatory is moderately grazed by sheep and goats.

The area of the Duruchaus observatory is situated in the Khomas region and is nearly flat. Calcisol, as the dominant soil group, shows pH values between 7.4 and 8.7. The texture is sandy loam dominated by fine sand and coarse silt [73]. It is characterised by an average annual precipitation of around 290 mm, falling from December to April. After the site was extensively grazed in the preceding years, in 2004, two exclosures were established. One of them is ungrazed (DuUn), while the other one is still grazed by livestock during the rainy summer and the dry winter season (DuGra).

Sampling

Soebatsfontein

Based on the standardised observatory sampling technique [70], the observatory was divided into 100 hectare plots. A stratified sampling design was applied to rank the hectare plots, thus defining the sequence of hectare plots being analysed [35]. In 2002, we took one sample each out of three hectare plots for the phenotypic approach. At the fourth hectare plot, we took four samples in order to test for species saturation within one plot. For the genotypic approach, the soil

samples were taken within an exclosure next to the BIOTA observatory. The sampling sites, measuring 0.36 m^2 each, were randomly established during a study on the succession of BSC after disturbance [18] in March 2006. One sample each was taken from the centre of five randomly chosen undisturbed plots. For each sample, the lower lid of a petri dish (5.3-cm diameter) was lined with cellulose paper and pressed into the upper 2 cm of the soil. Then, a trowel was pushed below the lid, and the sample was lifted and turned around carefully. Finally, the petri dish was closed and sealed with taping band [14].

Goedehoop

Based on the standardised observatory sampling technique, three hectare plots were sampled once in March 2002. All the samples were used for morphological investigation of cyanobacterial diversity. For genotype analyses, subsamples of the soil sample of 1-ha plot were used to extract DNA.

Duruchaus

In March 2006, three randomly chosen plots were sampled once in both the grazed and ungrazed area of the site for the genotypic analyses.

Morphological Approach

Of each BSC, sample axenic cultures were established. Eight subsamples covering an area of 1 cm^2 each were randomly taken from each petri dish using forceps. Each subsample was grown under different culture conditions, i.e. different media and growth conditions. As the media for cyanobacterial growth, BG 11 and Z-454 were used both as liquid medium and solidified with 1 % agar (after 7, 55, 69] and 75), resulting in the four different types of media. The organisms were grown both at 20 and 17 °C at a light intensity of approximately 100 μmol PAR m^{-2} s^{-1} at a 12:12 h light/dark regime. After the cyanobacteria initially started to grow, they were transferred to 17 °C in order to reduce the growth of contaminating fungi and bacteria. The raw cultures were examined microscopically, and small amounts of cells of each observed cyanobacterial taxon were transferred into fresh medium. This was conducted several times until pure strains were obtained. After purification, the taxa were identified by light microscopy. The identification was based on Geitler [29], Komárek [39] and Komárek and Anagnostidis [41, 42]. The isolated cyanobacteria were compared to the strains of the culture collection of the University of Kaiserslautern (Department of Plant Ecology and Systematics) and the 'Sammlung für Algenkulturen' (SAG) of the University of Göttingen.

Molecular Approach

Cyanobacterial Cultures

The DNA was extracted from 62 pure cultures using the Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany). The amplification of the 16S rRNA gene, including the adjacent ITS region and a part of the 23 S rRNA gene, was done using primers PCR 1 (5′-GAG TTT GAT CCT GGC TCA G-3′) and PCR 18 (5′-TCT GTG TGC CTA GGT ATC C-3′) *Escherichia coli* position 204, both from Eurofins MWG Operon (Ebersberg, Germany) and modified after Wilmotte et al. [72]. As shown in the results of the BLAST search, the used primers were cyanobacteria specific. PCR was conducted in a Mastercycler (Eppendorf AG, Hamburg, Germany) using HotStarTaq-DNApolymerase (Quiagen, Hilden, Germany) under the following conditions: initial denaturation, 15 min at 95 °C; 35 cycles of denaturing, 95 °C for 45 s; annealing, 60 °C for 45 s; and extension, 72 °C for 90 s. Incubation for 8 min at 72 °C finalised the PCR. To minimise PCR bias, three 50 μl reactions of each sample were carried out.

Fragment length and DNA yield were measured before and after purification using a 1 % agarose gel. The purification was made using the MinElute PCR purification Kit (Quiagen). The 16S rRNA genes were sequenced with primers PCR 1, 6, 7, 8, 10, 11, 12, 13, 14, 16, 17 and 18 as described by Wilmotte et al. [72] at the Labor für Immunbiologie und Genetik, Kaiserslautern, Germany. Contiguous sequences were compiled using the software Sequencer (Gene Codes Corporation, Ann Arbor, MI, USA). Accession numbers (KC463174-KC463235), sequence lengths and abbreviations for own cultures are given in the Online Resource 1. To check the chimeric sequences, the Bellerophon chimera checking tool available at the greengenes website was used (http://greengenes.lbl.gov) [34].

Clone Libraries from Soil Samples

The soil samples were ground with liquid nitrogen in 2-ml reaction tubes, and the DNA extraction and amplification were carried out as described above. Prior to amplification, the extracts from each site were merged and purified with the DNA Wizard Kit (Promega Corporation, Madison, USA). The resulting DNA fragments were cloned with the Topo TA cloning kit (Invitrogen, Life Technologies GmbH, Darmstadt, Germany). The DNA was extracted from *E. coli* colonies carrying the correct insert with the extraction kit of PerkinElmer chemagen Technology GmbH (Baesweiler, Germany) and sequenced with primer PCR 1 and further primers as described by Wilmotte et al. [72] at the Labor für Immunbiologie und Genetik, Kaiserslautern, Germany. Compilation of contiguous sequences and the check for chimera were done as described above. The alignment of the 16S

rRNA gene sequences was performed using the clustal algorithm by Seaview [30]. The resulting sequences were submitted to BLAST (http://blast.ncbi.nlm.nih.gov) [1] to detect similar sequences. The sequences differ in length between 535 and 1,499 bp with two sequences being shorter than 300 bp. The sequences can be found in GenBank under the accession numbers KC463059-KC463173 and KC463236-KC463696.

Genotypic Reference Strains

In addition to the most similar sequences, representative GenBank sequences of different taxa expected to be found were added, resulting in 140 reference sequences from GenBank. We also used pure cultures gained from the observatories Wlotzkasbaken (Namib Desert biome); Niko North, Karios (both Nama Karoo biome); Soebatsfontein, Goedehoop, Remhogte, Paulshoek, Numees, Ratelgat, Knersvlakte communal (Succulent Karoo biome) and Sonop (Woodland Savannah biome); and Otjiamongombe, Alpha (both Thornbush Savanna biome). These reference strains are listed also in Table 1, but did not occur at the four study sites. For a description of the sites, see Büdel et al. [14] and Haarmeyer et al. [31]. The accession numbers and the abbreviations for the GenBank sequences are given in the Online Resource 2.

OTUs and Neighbour-Joining Tree

To investigate the species inventory, OTUs were built, and a bootstrapped neighbour-joining tree was calculated. Distance matrices were calculated using PHYLIP (evolution.genetics.washington.edu/phylip/getme.html) [25, 26]. The OTUs were determined with mothur (www.mothur.org) [56]. The OTUs were calculated for all thresholds, and the attribution of sequences was examined. For an interpretation at the genus level, thresholds for similarity of 95 % were chosen as proposed by Ludwig et al. [44] and Komarek [39]. The similarity level related to species was set to 97 %. Stackebrandt and Goebel [58] showed that species with more than 70 % DNA similarity, measured as DNA–DNA reassociation of the 16S rRNA gene, have more than 97 % sequence identity.

The neighbour-joining tree was generated with PAUP 4.0b10 [63] using a HKY85 likelihood model [37]. One thousand bootstrap replicates were used to estimate the statistical support for internal branches [24]. Two sequences of *Gloeobacter violaceus* were chosen as out-group [45].

The OTU diversity was calculated for the various sites using the Simpson index. The Chao 1 coefficient was calculated using the software mothur to assess the species richness. As the sequences resulting from the pure cultures were not derived by cloning of the DNA extracts, we excluded them from the calculation of diversity coefficients.

Results

Most genera were identified by both the phenotypic and the genotypic approaches. The presence of the genera *Stigonema* and *Hormoscilla* was exclusively detected by the molecular approach, whereas some species of the genera *Leptolyngbya*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Scytonema*, *Tolypothrix*, *Trichocoleus* and *Pseudanabaena*, as well as the genera *Calothrix* and *Lyngbya* were identified only by the morphological approach (Table 1). The species, which are listed but did not occur at the four study sites, were identified at the other observatories along the transect of the BIOTA project and were used as reference strains.

Phenotypic Species and OTUs Based on Cultures

Based on the morphological data, presented in Table 1, we detected 33 species from 12 genera at Soebatsfontein, while 24 species belonging to 13 genera were identified at Goedehoop. The following information is based on the 95 % similarity level. Some sequences of the cultured strains revealed OTUs that showed only low similarity with known species. We assume them to be new species of the genera *Leptolyngbya* (OTUs 2, 5, 6 and 81), *Trichocoleus*/*Pseudanabaena* (OTU 4), *Phormidium* (OTU 70, 81), *Oscillatoria* (OTU 7), *Schizothrix* (OTU 9) and *Microcoleus* (OTUs 10 and 32).

Genotypic Diversity Based on Clone Libraries

In total, 578 clones were analysed, resulting in 309 sequences for Soebatsfontein, 28 partial sequences for Goedehoop, 126 for Duruchaus ungrazed, and 115 for Duruchaus grazed. Eighty-one OTUs (95 % similarity) and 125 OTUs (97 % similarity) included, respectively, sequences from clones and cultures. Thirty-two OTUs (97 % similarity) contained clones which could not be related to any reference taxon.

The number of the sampled clones did not reach the estimated amount of OTUs given as Chao 1 values presented in Table 2 (Soebatsfontein 84 %, Goedehoop 64 %, Duruchaus ungrazed 80 % and Duruchaus grazed 86 %), but the number of OTUs on 95 % level exceeded the number of morphologically described species for Soebatsfontein (49 OTUs). For Goedehoop, the species numbers lie within the same range as OTU numbers with a low coverage of the clone library. In general, the cloned sequences corresponded well with many of the morphologically described taxa in the cultures and with the sequences in public databases, e.g. representatives of the genera *Scytonema*, *Tolypothrix*, *Microcoleus*, *Leptolyngbya*, *Phormidium* and *Chroococcidiopsis*.

Table 1 Comparison of taxa found by morphologic determination and/or molecular investigation of the 16S rRNA gene sequence from own cultured strains or cloned sequences originating from DNA extracts of soil samples. Genotypic determination on 95 % similarity level

	Morphologic determination		Genotypic determination by 16S rRNA gene sequences				Sequences confirmed by
	So	Gh	So	Gh	DuUn	DuGra	
Taxon							
Calothrix sp.	+	−	−	−	−	−	−
Chroococcidiopsis	−	+	+	+	−	−	a, b, c
Hormoscilla pringsheimii	−	−	+	−	+	−	b, c
Leptolyngbya Albertano–Kovacik green/(*Phormidium priestleyi*)	+	−	+	−	−	−	a, b[a], c
Leptolyngbya Albertano–Kovacik green/*Pseudophormidium*	−	−	+	−	−	−	a, b[a], c
Leptolyngbya cf. *compacta* (2 OTUs)	+	+	+	+	−	−	a
Leptolyngbya compacta/*subtilissima*	−	−	−	−	−	−	a[b]
Leptolyngbya crispata/*Plectolyngbya hodgsonii*	−	−	−	−	+	−	b, c
Leptolyngbya schmidlei/Albertano–Kovacik green	+	−	+	−	+	−	a, b[a], c
Leptolyngbya scottii/Albertano–Kovacik green/*Leptolyngbya foveolarum*	+	+	+	+	+	−	a, b[a], c
Leptolyngbya sp./*Pseudanabaena* sp. (OTU 1)	+	+	+	+	+	+	a, b, c
Leptolyngbya sp./*Pseudanabaena* sp. (OTU 2)	+	+	+	+	+	+	a, b
Leptolyngbya subtilissima/(*Pseudanabaena*)	+	+	+	+	−	−	a, b
Lyngbya cf. *semiplena*	−	+	−	−	−	−	−
Microcoleus chthonoplastes	−	−	+	−	−	−	a, c
Microcoleus chthonoplastes/*Trichocoleus* cf. *delicatulus* (Gb)	−	−	−	−	−	−	a, b[b]
Microcoleus paludosus (OTU 1)	+	−	+	−	−	−	a, c
Microcoleus paludosus (OTU 2)	−	−	+	+	+	+	b, c
Microcoleus sp.	−	+	+	+	+	−	b, c
Microcoleus steenstrupii/sp.	−	−	+	+	+	−	a, b, c
Microcoleus vaginatus	+	+	+	+	+	+	a, b, c
Nostoc cf. *calcicola*	+	−	−	−	−	−	−
Nostoc cf. *punctiforme*	+	−	−	−	−	−	−
Nostoc sp.	+	+	+	+	+	−	b, c
Oscillatoria limosa	+	−	−	−	−	−	−
Oscillatoria subbrevis	+	−	−	−	−	−	−
Oscillatoria tenuis	+	+	+	+	−	−	a
Phormidiaceae	−	−	+	+	+	+	b, c
Phormidium cf. *aerugineo-caeruleum*	+	+	+	+	+	+	a, c
Phormidium cf. *caerulescens*	+	−	−	−	−	−	−
Phormidium cf. *nigrum*	+	−	−	−	−	−	−
Phormidium chlorinum/*Leptolyngbya* sp.	−	−	+	+	+	+	a, c
Phormidium murrayii	−	−	+	−	−	+	b, c
Phormidium sp.	+	+	−	−	−	−	−
Phormidium vulgare	−	+	−	−	−	−	−
Pseudanabaena cf. *frigida*	−	+	−	−	−	−	−
Pseudanabaena cf. *starmachii*	+	−	−	−	−	−	−
Pseudanabaena cf. *tenuis*	−	+	−	−	−	−	−
Pseudanabaena minima	+	+	−	−	−	−	−
Pseudanabaena sp.	+	+	−	−	−	−	−
Pseudophormidium hollerbachianum	+	−	+	−	−	−	a
Schizothrix cf. *arenaria*	+	−	+	−	−	−	a

Table 1 (continued)

	Morphologic determination		Genotypic determination by 16S rRNA gene sequences				Sequences confirmed by
	So	Gh	So	Gh	DuUn	DuGra	
Schizothrix lardacea	+	+	−	−	−	−	−
Schizothrix cf. sp./*Pseudophormidium* cf. sp./*Leptolyngbya* cf. sp.	+	+	+	+	−	−	a, c
Scytonema cf. *millei*	+	−	−	−	−	−	−
Scytonema hofmanni	+	−	−	−	−	−	−
Scytonema ocellatum	−	+	−	−	−	−	−
Scytonema sp.	+	−	+	+	+	+	b, c
Scytonema sp./*hyalinum*/*ocellatum*	−	+	+	+	+	+	a, b, c
Stigonema ocellatum	−	−	−	+	−	−	b, c
Symplocastrum cf. *friesii*	−	+	−	−	−	−	−
Tolypothrix bouteillei	+	−	−	−	−	−	−
Tolypothrix distorta	−	+	+	+	+	+	a, b, c
Tolypothrix sp.	+	−	−	−	−	−	−
Trichocoleus cf. *cavanillesii*	+	−	−	−	−	−	−
Trichocoleus cf. *delicatulus*	−	+	−	−	−	−	−
Trichocoleus sociatus	+	+	−	−	−	−	−
Trichocoleus sp./*Pseudanabaena* sp.	+	−	+	−	−	−	a
Sum phenotype genera/species[c]	12/33	13/24					
Sum OTUs in site (95/97 % similarity)			56/91	21/40	37/44	28/38	
OTUs exclusively found at site (95 % similarity)			27	3	6	2	
Sum OTUs in biome (95 % similarity)			60		43		
OTUs exclusively found in biome (95 % similarity)			33		16		

So Soebatsfontein, *Gh* Goedehoop, *DuUn* Duruchaus ungrazed site, *DuGra* Duruchaus grazed site, *a* assigned sequences confirmed by extract from culture, *b* assigned sequences confirmed by GenBank, *c* assigned sequences confirmed by clones

[a] Only the genus is confirmed by sequences from GenBank or morphologic determination

[b] Reference sequence based on samples originating from other BIOTA observatories

[c] The sum of phenotypic taxa is altered because of the presence of more than one OTU for some morphospecies

Table 2 Number of OTUs observed per habitat based on clonal analyses (isolates from cultured strains were ignored) and diversity coefficients

Site	Similarity per OTU (%)	Observed OTUs	Chao 1			Coverage[a] (%)	Simpson index		
			Estimated OTUs	lCI	hCI		Coefficient	lCI	hCI
So	97	71	109	87	163	65	0.056	0.042	0.07
	95	49	59	52	81	84	0.067	0.053	0.08
Gh	97	17	35	22	92	48	0.045	0.016	0.074
	95	16	25	18	56	64	0.048	0.02	0.076
DuUn	97	44	87	59	163	51	0.066	0.041	0.092
	95	37	46	38	75	80	0.07	0.045	0.095
DuGra	97	38	61	45	112	62	0.055	0.039	0.071
	95	28	33	29	51	86	0.06	0.044	0.075

lCI lower bound of confidence interval (95 %), *hCI* upper bound of confidence interval (95 %), *So* Soebatsfontein, *Gh* Goedehoop, *DuUn* Duruchaus ungrazed site, *DuGra* Duruchaus grazed site

[a] Coverage was calculated as percentage of observed OTUs related to the expected amount of OTUs

Site-Specific Taxon Inventory

OTU inventories differed between the sites and biomes: based on 95 % sequence similarity values, 60 OTUs occurred in the Succulent Karoo, and 43 in the Nama Karoo, with 33 and 16 OTUs, respectively, being unique within each biome (Table 1). In Soebatsfontein, the highest number of site-specific OTUs occurred (Table 1). The highest diversity given as Simpson index was calculated for the ungrazed site of Duruchaus, followed by Soebatsfontein, the grazed site of Duruchaus and Goedehoop (Table 2).

Some OTUs (95 %) comprised sequences from different habitats. But for several OTUs at 97 % level, the sequences were divided into groups representing the different habitats, e.g. OTU 45, comprising sequences from Soebatsfontein and Duruchaus grazed (Fig. 1). Only in the Succulent Karoo we found sequences of the genera *Trichocoleus* (OTUs 1 and 4), *Stigonema* (OTU 11) and *Chroococcidiopsis* (OTU 36), as well as representatives of the cosmopolitan genus *Oscillatoria* (OTUs 7 and 63), known also from benthic habitats (Fig. 1, Table 1).

The genera *Leptolyngbya*, *Microcoleus*, *Phormidium* and *Scytonema* showed a high species diversity at all sites (Fig. 2, Table 3). The representatives of the genus *Pseudanabaena* were missing in the samples of both sites in Duruchaus, and the diversity within the genus was higher at Goedehoop as compared to that at Soebatsfontein. In contrast, the diversity within the genus *Scytonema* was higher at Soebatsfontein as compared to that at Goedehoop. The grazed site of Duruchaus showed a higher species diversity within the genera *Phormidium* and *Scytonema* compared to the ungrazed site, whereas for the genera *Leptolyngbya a*nd *Microcoleus*, the species diversity was higher at the ungrazed site than in the grazed site of Duruchaus (Fig. 2, Table 3).

Comparison of OTUs Composition and Neighbour-Joining Tree

In most cases, the OTU composition corresponded well with the results of the neighbour-joining tree, shown as a collapsed tree in Fig. 1 and in the Online Resources 3 and 4. Clades 1, 5, 7 (related to *Leptolyngbya*) and 2 (related to *Microcoleus*) are shown in Fig. 3. Clades 8 (related to *Scytonema*), 9 (related to *Tolypothrix*) and 10 (related to *Nostoc*) are shown in Fig. 4. For some groups, the results of the OTU analysis and the neighbour-joining tree were quite different, as e.g. OTUs 50 and 65 hosted sequences that were spread over the tree.

The full tree, the list showing all OTUs, a detailed description of the results and a comparison of both the neighbour-joining tree and the OTUs are given in the Online Resources 3, 4 and 5. The following genera were identified by OTUs at the 95 % similarity level and placed in the neighbour-joining tree:

1. Genus: *Microcoleus*

   The sequences assigned to *Microcoleus* were found in multiple clades of the tree. The following species could be identified and confirmed by OTUs and the neighbour-joining tree:

   (a) *Microcoleus vaginatus*: clades 2, 3 and 4, OTU 40 and 63; the characteristic 17 bp consensus insert [28] was found in all sequences.
   (b) *Microcoleus paludosus*: OTU 62, clades 12, 13 and 14, and *Microcoleus* cf. *paludosus*: OTU 32, clade 31.
   (c) *Microcoleus* cf. *steenstrupii*: OTU 80, clades 15, 16 and 17. OTU 75, clades 18, 19 and 20.
   (d) *Microcoleus* cf. *chthonoplastes*: OTU 10, clade 32.
2. Genus: *Scytonema*

   Scytonema hyalinum: OTU 73, clade 8. The second copy of the 16S rRNA gene [27, 74] was found in OTU 65. In the tree, the sequences of OTU 65 were spread over several clades.
3. Genus: *Tolypothrix*

   (a) *Tolypothrix distorta*: OTU 49, clade 9. The reference sequences originated from *Spirirestis rafaelensis* and *T. distorta*. A differentiation between both taxa based on their 16S rRNA genes was impossible due to their sequence similarity of about 99 % [27].
4. Genus: *Nostoc*

   (a) *Nostoc* sp.: OTU 55, clade 10.
5. Genus: *Leptolyngbya*

   The sequences correlating to known *Leptolyngbya* sequences were found at several positions in the tree and in several different OTUs, i.e. OTU 81, clade 1, OTU 53, clade 5, OTU 19, clade 7 (Fig. 1).
6. Genus: *Chroococcidiopsis*

   (a) *Chroococcidiopsis* sp.: OTU 36, clade 28.
7. Genus: *Hormoscilla*

   (a) *Hormoscilla pringsheimii*: OTU 69, clade 29
8. Genus: *Phormidium*

   We recognised a separation of the different OTUs containing sequences from morphologically described *Phormidium* strains into different clades in the tree (Fig. 1).

   (a) *Phormidium aerugineo-caeruleum*: OTU 70, clades 21, 22, 23 and 24
   (b) *Phormidium murrayii*: OTU 48, clades 26 and 27. Interestingly, the corresponding reference strain was collected in Antarctica [65].
9. Genus: *Oscillatoria*

   (a) *Oscillatoria tenuis*: OTU 7, clade 37

Discussion

Diversity of Cyanobacteria in BSCs

Most of the identified genera are known to colonise BSCs in arid and semiarid ecosystems, and are part of the orders Oscillatoriales, Pseudanabaenales and Nostocales without the dominance of a single group. The genus *Microcoleus*, found in all sites, is dominant in most desert BSCs [28, 71] and represents a key species in early successional state crusts [33]. The three genera *Schizothrix*, *Trichocoleus* and *Microcoleus* are known to form multifilamentous ropes with thick sheaths stabilising the soil and protecting it against erosion by wind and water [6]. Particularly, species of the genus *Microcoleus* are considered being ecosystem engineers, gluing together soil particles and thus initiating BSCs (Fig. 2, Table 3) [32]. The crust-forming genus *Phormidium* and the nitrogen-fixing genera *Tolypothrix* and *Scytonema* were found in all sites. Nitrogen-fixing cyanobacteria grow preferably in habitats with low nitrogen soil content, and their presence is essential to the ecosystem function with respect to nitrogen allocation into the adjacent vegetation [4, 19, 22, 23, 61]. The presence of genera like *Nostoc* and *Scytonema* is not surprising as they are highly adapted to life in arid environments and able to sustain high light intensities and UV radiation. The synthesis of scytonemin, mycosporines and other photoprotective pigments enables them to shade not only themselves but also the surrounding microbial community [17]. The presence of *Lyngbya* and *Symplocastrum* is not typical for BSC-forming cyanobacteria of arid soils, as *Lyngbya* is frequently found on wet walls, whereas *Symplocastrum* is known from forest soils or old wooden substrates.

Comparison of the Sites

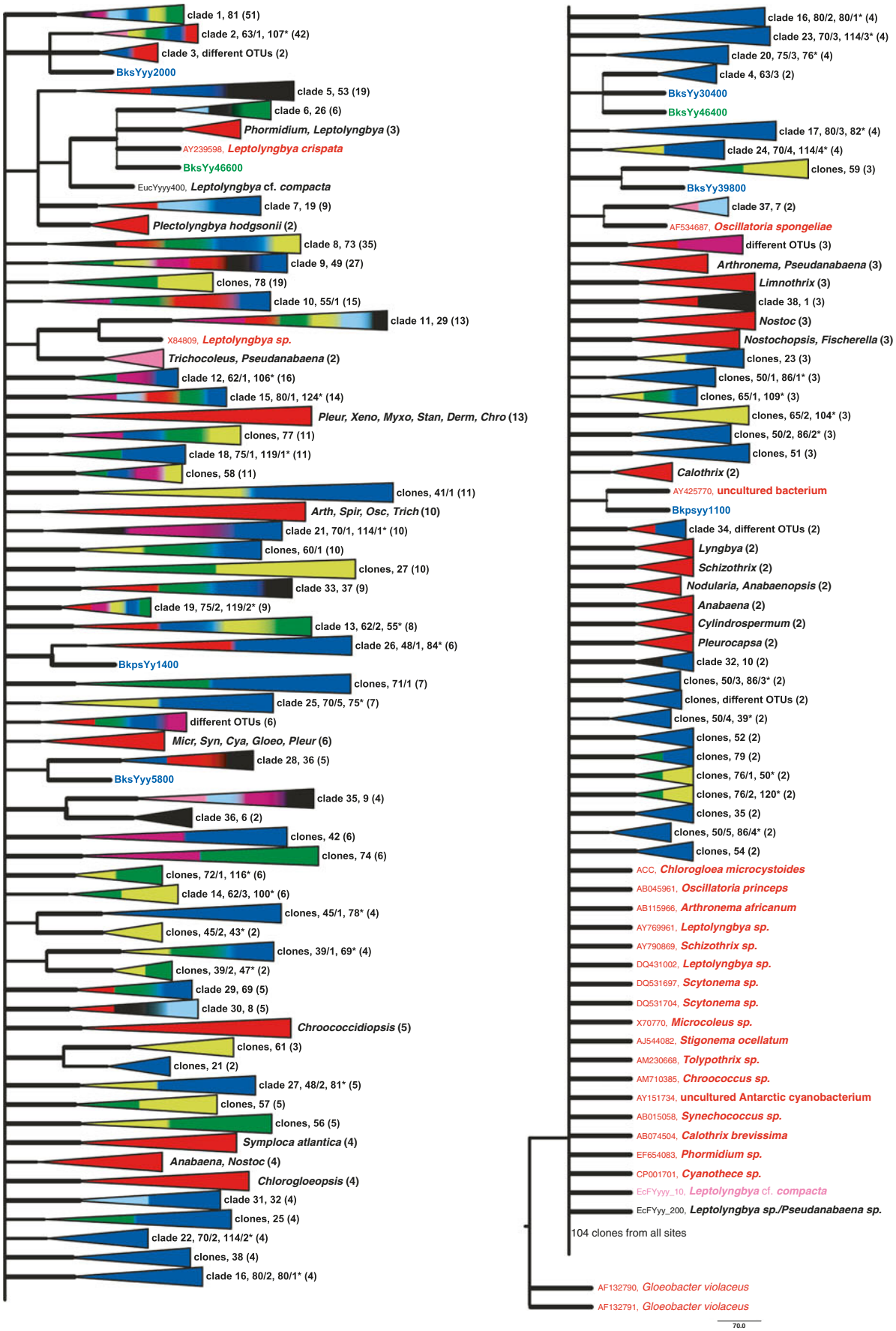
Morphological similarities of different cyanobacteria led to the assumption that microorganisms like cyanobacteria are cosmopolitan and spread over different habitats. For some species, this may be true, as clones corresponding to sequences originating from benthic habitats or Antarctic strains were found (*Oscillatoria tenuis* [42] and *Phormidium murrayi* [65]), thus reflecting the presently insufficient taxonomical status of cyanobacteria. Contrastingly, our data show that 10 OTUs occur in all habitats, but 38 OTUs (both at 95 % similarity) and e.g. the taxa *Calothrix*, *Trichocoleus*, *Chroococcidiopsis*, *Oscillatoria*, *Pseudanabaena* and *Schizothrix* were only found in specific sites or biomes (Table 1). However, the sequences of the genus *Schizothrix*, a typical taxon colonising solid substrates with low sedimentation rates and calcium carbonate precipitation [60], which are characteristics for Duruchaus soils [60, 73], are absent in the Nama Karoo samples. Consequently, a growth limitation caused by geographic boundaries is visible, and we expect evolutionary differentiation processes of cyanobacteria depending on habitat conditions. According to the Chao 1 index, the highest richness of OTUs was expected for Soebatsfontein and Duruchaus ungrazed, followed by Duruchaus grazed and Goedehoop (Table 2).

Fig. 1 Neighbour-joining tree calculated using PAUP (Swafford, 1998) with a HKY85-likelihood model [37] with clades collapsed. One thousand bootstrap replicates were used [24]. Bootstrap values exceeding 70 % are indicated by *bold lines*. First, the clade number is given, followed by the number of the operational taxonomic unit (OTU) (95 % similarity level). The number of the OTU with 97 % similarity is marked by an *asterisk*. In *brackets*, the amount of sequences participating in the clade is given. Clades and their respective taxa are coded by site and origin of the sequences as follows: Soebatsfontein: clones: *dark blue*, pure cultures: *light blue*; Goedehoop: clones: *violet*, pure cultures: *pink*; Duruchaus: ungrazed: *dark green*, grazed: *yellow-green*; pure cultures originating from different sites: *black*; reference sequences from GenBank: *red*. The sequences can be found in GenBank under the accession numbers KC463059 to KC463696

Cyanobacterial diversity of the two Succulent Karoo sites is difficult to compare. Aside from the different coverage of the clone libraries, variable sequence lengths and different soil substrates [46], the sampling sites were grazed differently, and the samples were taken in different years (2002 and 2006, respectively). Goedehoop and Soebatsfontein show quite different ecological niches, and aside from that, the soil at Soebatsfontein is strongly influenced by fossil termite mounds, causing a high variability of soil parameters [43]. Nevertheless, two thirds of the OTUs occurring in the Succulent Karoo were found both at Soebatsfontein and Goedehoop, and the set of the phenotypic genera was nearly identical, with the nitrogen-fixing genus *Calothrix* being the only one occurring exclusively on Soebatsfontein soil. The higher taxonomic diversity of some genera (e.g. *Leptolyngbya*, *Nostoc* and *Trichocoleus*) at Soebatsfontein may be caused by the presence of a late successional crust [33]. At Goedehoop, a high coverage of quartz pebbles promotes hypolithic growth of low light tolerant taxa like *Chroococcidiopsis* [11, 67, 68]. Besides that, the presence of *Lyngbya* and *Symplocastrum* occurring exclusively in Goedehoop underlines the differences between the two sites of the Succulent Karoo.

Effects of Land Use

Apart from the climatic and habitat parameters, land use is known to destroy BSCs and reduce the diversity of cyanobacteria, e.g. [3, 66, 67]. This is well in accordance with our results for the Duruchaus site, where we identified 37 OTUs on the ungrazed plot, whereas only 28 OTUs (both 95 % similarity) were determined on the grazed plot. The decreased species richness within the genus *Leptolyngbya*, the lack of *Nostoc*, which seems to be sensitive to trampling, and the presence of the large motile cyanobacterium


clade 1, 81 (51)
clade 2, 63/1, 107* (42)
clade 3, different OTUs (2)
BksYyy2000
clade 5, 53 (19)
clade 6, 26 (6)
Phormidium, Leptolyngbya (3)
AY239598, Leptolyngbya crispata
BksYy46600
EucYyyy400, Leptolyngbya cf. compacta
clade 7, 19 (9)
Plectolyngbya hodgsonii (2)
clade 8, 73 (35)
clade 9, 49 (27)
clones, 78 (19)
clade 10, 55/1 (15)
clade 11, 29 (13)
X84809, Leptolyngbya sp.
Trichocoleus, Pseudanabaena (2)
clade 12, 62/1, 106* (16)
clade 15, 80/1, 124* (14)
Pleur, Xeno, Myxo, Stan, Derm, Chro (13)
clones, 77 (11)
clade 18, 75/1, 119/1* (11)
clones, 58 (11)
clones, 41/1 (11)
Arth, Spir, Osc, Trich (10)
clade 21, 70/1, 114/1* (10)
clones, 60/1 (10)
clones, 27 (10)
clade 33, 37 (9)
clade 19, 75/2, 119/2* (9)
clade 13, 62/2, 55* (8)
clade 26, 48/1, 84* (6)
BkpsYy1400
clones, 71/1 (7)
clade 25, 70/5, 75* (7)
different OTUs (6)
Micr, Syn, Cya, Gloeo, Pleur (6)
clade 28, 36 (5)
BksYyy5800
clade 35, 9 (4)
clade 36, 6 (2)
clones, 42 (6)
clones, 74 (6)
clones, 72/1, 116* (6)
clade 14, 62/3, 100* (6)
clones, 45/1, 78* (4)
clones, 45/2, 43* (2)
clones, 39/1, 69* (4)
clones, 39/2, 47* (2)
clade 29, 69 (5)
clade 30, 8 (5)
Chroococcidiopsis (5)
clones, 61 (3)
clones, 21 (2)
clade 27, 48/2, 81* (5)
clones, 57 (5)
clones, 56 (5)
Symploca atlantica (4)
Anabaena, Nostoc (4)
Chlorogloeopsis (4)
clade 31, 32 (4)
clones, 25 (4)
clade 22, 70/2, 114/2* (4)
clones, 38 (4)
clade 16, 80/2, 80/1* (4)
clade 16, 80/2, 80/1* (4)
clade 23, 70/3, 114/3* (4)
clade 20, 75/3, 76* (4)
clade 4, 63/3 (2)
BksYy30400
BksYy46400
clade 17, 80/3, 82* (4)
clade 24, 70/4, 114/4* (4)
clones, 59 (3)
BksYy39800
clade 37, 7 (2)
AF534687, Oscillatoria spongeliae
different OTUs (3)
Arthronema, Pseudanabaena (3)
Limnothrix (3)
clade 38, 1 (3)
Nostoc (3)
Nostochopsis, Fischerella (3)
clones, 23 (3)
clones, 50/1, 86/1* (3)
clones, 65/1, 109* (3)
clones, 65/2, 104* (3)
clones, 50/2, 86/2* (3)
clones, 51 (3)
Calothrix (2)
AY425770, uncultured bacterium
Bkpsyy1100
clade 34, different OTUs (2)
Lyngbya (2)
Schizothrix (2)
Nodularia, Anabaenopsis (2)
Anabaena (2)
Cylindrospermum (2)
Pleurocapsa (2)
clade 32, 10 (2)
clones, 50/3, 86/3* (2)
clones, different OTUs (2)
clones, 50/4, 39* (2)
clones, 52 (2)
clones, 79 (2)
clones, 76/1, 50* (2)
clones, 76/2, 120* (2)
clones, 35 (2)
clones, 50/5, 86/4* (2)
clones, 54 (2)
ACC, Chlorogloea microcystoides
AB045961, Oscillatoria princeps
AB115966, Arthronema africanum
AY769961, Leptolyngbya sp.
AY790869, Schizothrix sp.
DQ431002, Leptolyngbya sp.
DQ531697, Scytonema sp.
DQ531704, Scytonema sp.
X70770, Microcoleus sp.
AJ544082, Stigonema ocellatum
AM230668, Tolypothrix sp.
AM710385, Chroococcus sp.
AY151734, uncultured Antarctic cyanobacterium
AB015058, Synechococcus sp.
AB074504, Calothrix brevissima
EF654083, Phormidium sp.
CP001701, Cyanothece sp.
EcFYyyy_10, Leptolyngbya cf. compacta
EcFYyy_200, Leptolyngbya sp./Pseudanabaena sp.
104 clones from all sites
AF132790, Gloeobacter violaceus
AF132791, Gloeobacter violaceus
70.0

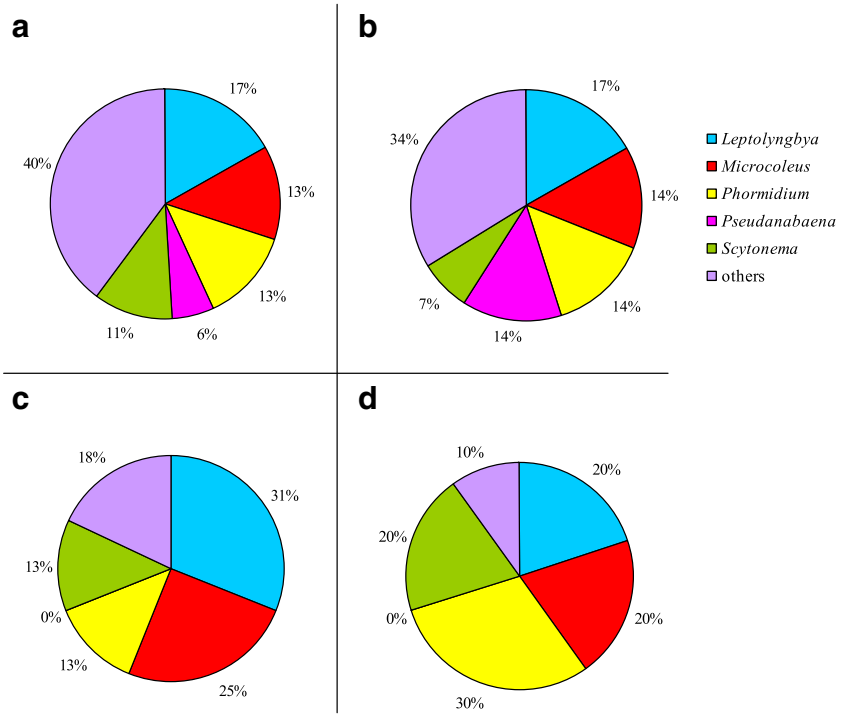


Fig. 2 Percentage of species from the most species-rich genera related to the overall amount of species. Included in the figure are morphologically determined species and OTUs that can be clearly related to a species. **a** Soebatsfontein, **b** Goedehoop, **c** Duruchaus ungrazed site, **d** Duruchaus grazed site

Microcoleus in the more heavily grazed site of Duruchaus lead to the conclusion that an early successional crust type exists here [2, 14]. As the complete Duruchaus site faced heavy land use before 2004, it is reasonable that the observed species richness is lower than that in Soebatsfontein.

The Polyphasic Approach

The polyphasic approach proved to be successful as our study extends the knowledge about cyanobacterial diversity in BSCs of southern Africa and helps identify species and genera that would not have been detected by the individual methods alone. The advantage of a polyphasic approach is the discovery of organisms that either do not grow under the applied culture conditions or are untraceable via molecular approaches. For example, in our study, the presence of the genera *Stigonema* and *Hormoscilla* was exclusively proven by the molecular approach. As we know from earlier studies, representatives of the genus *Stigonema* grow on nitrogen-depleted media like the one we used for this study. We consider that the amount of cells was not high enough for a growth in culture or the genus appears only occasionally in the samples. The morphological approach enabled us to detect representatives of the genera *Pseudanabaena*, *Leptolyngbya*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Scytonema*, *Tolypothrix*, *Trichocoleus*, *Lyngbya* and *Symplocastrum*, which were not identified by molecular methods (Table 4). Since no references exist for many of the identified OTUs, they cannot be assigned to any known taxon. Thus, it is of interest to complement the morphological and genetic descriptions of further taxa in order to assign a taxon name to these non-defined OTUs. Some clades of our study (e. g. clade 30) contained only sequences from cultured cyanobacteria, which is in accordance with Garcia-Pichel et al. [28] who showed that a DNA analysis of field samples failed to detect the presence of cyanobacteria which were identified in cultures. We can exclude the effects of the media or chemicals due to the positive results obtained from the control samples. Various artefacts occurring at every single step of sampling procedure (like DNA extraction, purification, PCR, cloning and sequencing) reduce the chance to detect all species colonising the soil. Especially, the efficiency of DNA extraction is known to depend on species inventory and the applied extraction method, e.g. [47, 57].

Table 3 Amount of assigned species per genus for the most species-rich genera. Data based on morphological identification and OTUs that can be assigned to a specific taxon

	So	Gh	DuUn	DuGra
Leptolyngbya	8	5	5	2
Microcoleus	6	4	4	2
Phormidium	6	4	2	3
Pseudanabaena	3	4	0	0
Scytonema	5	2	2	2
Amount of assigned species	47	29	16	10

So Soebatsfontein, *Gh* Goedehoop, *DuUn* Duruchaus ungrazed, *DuGra* Duruchaus grazed

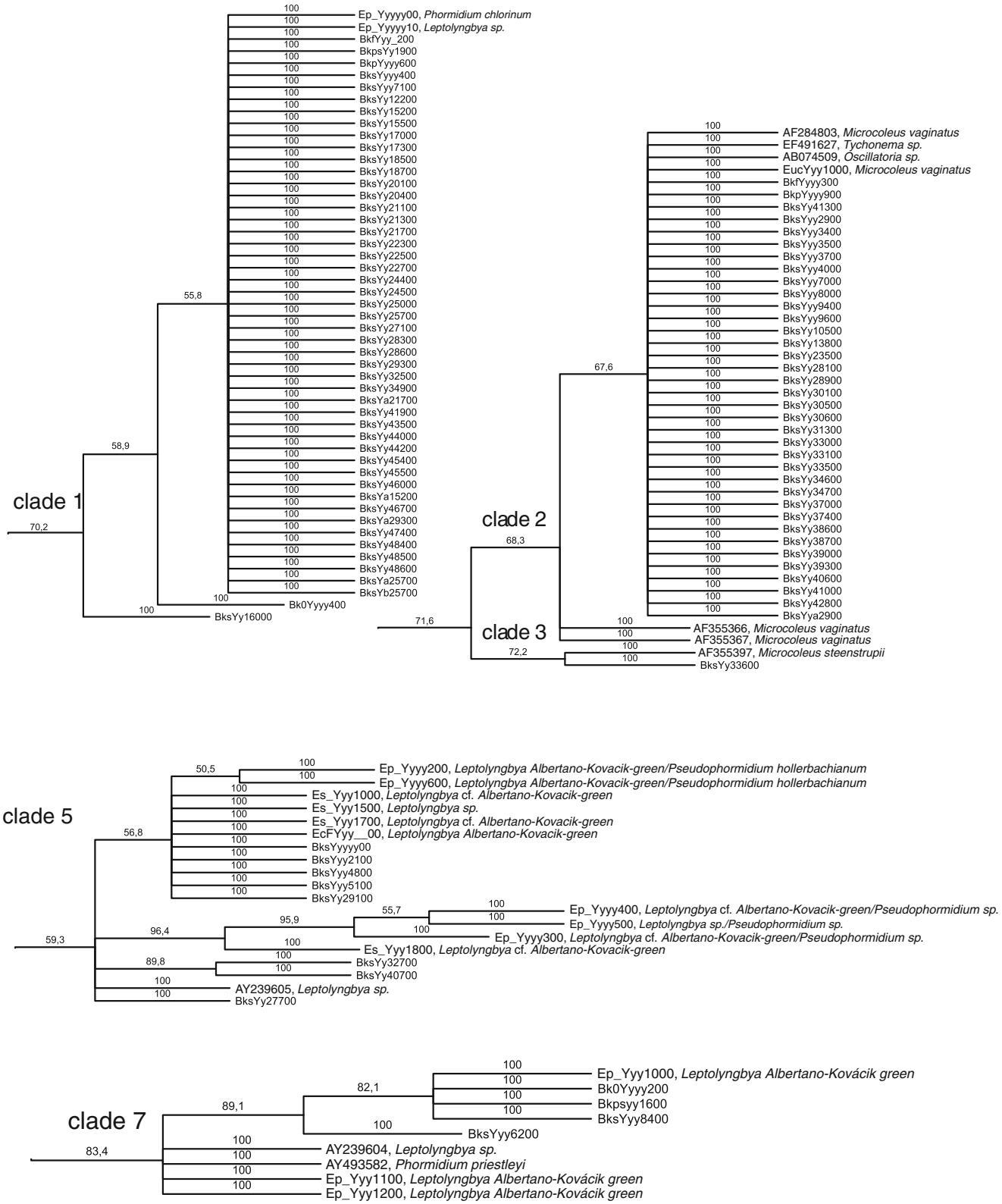


Fig. 3 Clades 1, 5, 7 (*Leptolyngbya*), 2 and 3 (*Microcoleus*) from the neighbour-joining tree; boostrap values are shown above the *branches*. The sequences can be found in GenBank under the accession numbers KC463059 to KC463696

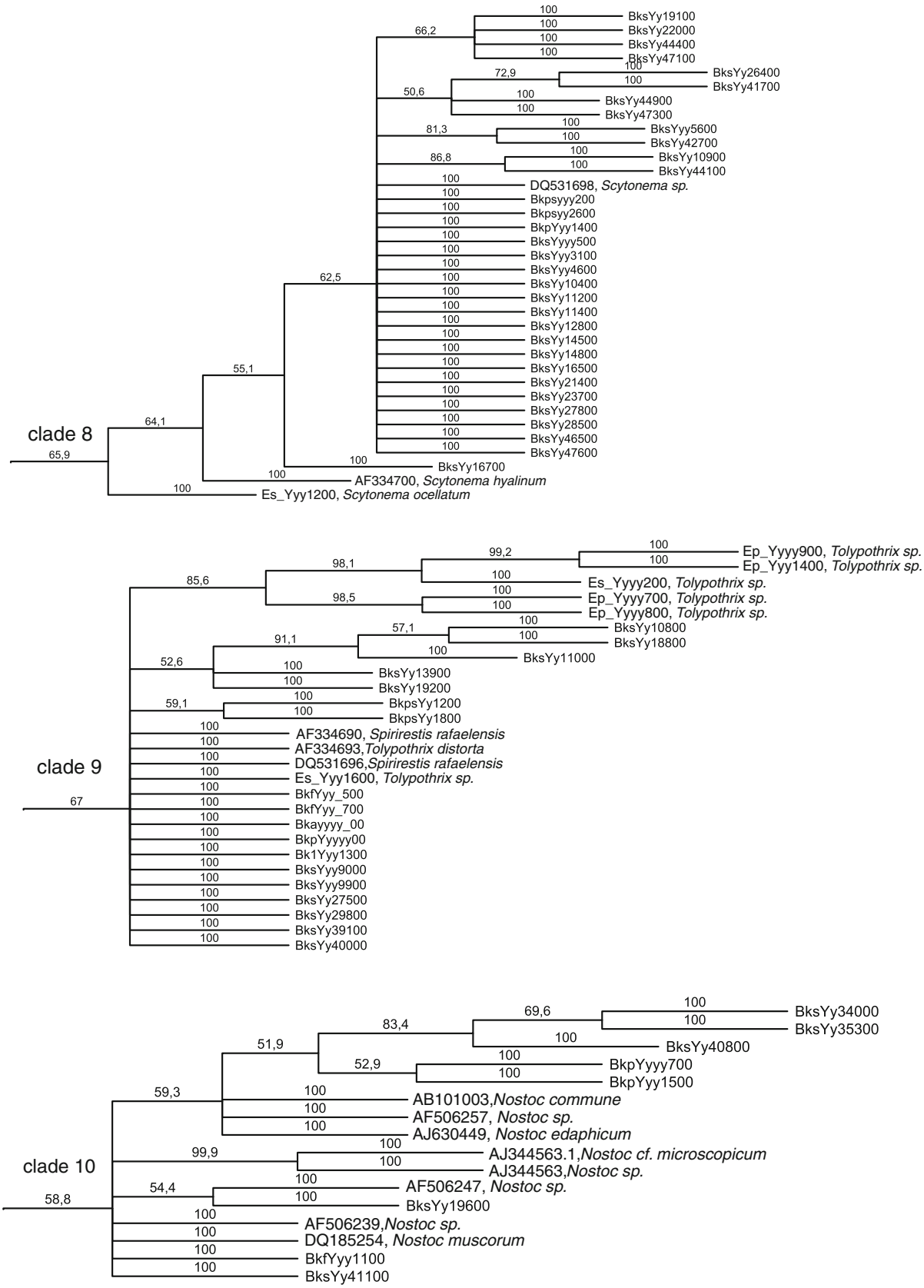


Fig. 4 Clades 8 (*Scytonema*), 9 (*Tolypothrix*) and 10 (*Nostoc*) from the neighbour-joining tree; boostrap values are shown above the branches. The sequences can be found in GenBank under the accession numbers KC463059 to KC463696

The correct identification of cyanobacterial taxa is essential in order to correlate the species inventory with ecosystem function. The morphological determination of species is often difficult due to the lack of observable characteristics, fluid transitions from one shape to another as well as its dependence on environmental factors and culture conditions [16, 20, 21, 50]. The percentage of misidentifications of strains in culture collections is estimated to be higher than 50 % [40]. This affects not only the phenotypic approach itself but also the assignment of species to genotypes as provided by public databases. Consequently, the misidentification of reference strains alters the results of the molecular approach as well, which emphasises the need for a profound expertise of the individual classifier and a sound and coherent taxon concept.

The survey of sequence data from cultured strains and their relation to GenBank data enabled us to compare sequence similarity data with morphological data and data resulting from the neighbour-joining approach. The sequence similarity of 95 % is often accepted as corresponding to the genus level [44]. Several of our examples showed that correspondence of sequence similarities of 16S rRNA genes with taxonomic units like genera or species varied within the groups of the investigated cyanobacteria. For example, our data support the results of Rikkinen et al. [53], Svenning et al. [62] and Rehakova et al. [52] that *Nostoc commune*, *Nostoc punctiforme* and symbiotic *Nostoc* strains are monophyletic and originate from a common ancestor. All of the collected sequences were part of OTU 55 with 95 % similarity, even if the reliability of phylogenetic analyses can be limited by short sequences, resulting in low bootstrap values [15]. Two reference sequences of the genus *Pleurocapsa* were part of OTU 55 (95 % similarity); with higher similarity values, they separate into a new OTU (OTU 58, 97 % similarity). Because of the differences between the similarity based grouping of the sequences (OTUs) and the neighbour-joining algorithm, the results can be different. As seen in the neighbour-joining tree (Online Resource 3), representatives of the genus *Pleurocapsa* build a different clade than the sequences of the genus *Nostoc*. For a more detailed comparison of the results from the phylogenetic tree and the OTU calculation, see Online Resources 3, 4 and 5.

The sequences of the genus *Microcoleus* were found in different clades of the phylogenetic tree and in different OTUs (95 % sequence similarity). The division of clades into several OTUs and the occurrence of *M. steenstrupii*-related sequences at different places within the tree and in different OTUs lead to the conclusion that subspecies of *M. steenstrupii* exist, supporting the proposal of Boyer et al. [9]. Our data also show that the genus *Leptolyngbya* is polyphyletic [49, 51]. Furthermore, taxon assignment based on sequence similarities is challenged by the presence of two different copies of the 16S rRNA gene occurring, e. g. in *S. hyalinum* [27, 74]. In the phylogenetic tree, the sequences of copy 1 are monophyletic and part of OTU 73, whereas the sequences of the second copy are part of OTU 65 (both 95 % similarity) and widespread over the tree (Fig. 1, Online Resource 3). From the comparison of the amount of sequences found at each site, we cannot exclude that more taxa contain multiple different copies of 16S rRNA genes. In 2006, Stackebrandt and Evers [59] proposed a 16S rRNA similarity range above 98.7 % to ensure 'the genetic uniqueness of a novel isolate'. Since we do not describe new taxa but identify species and genera, the application of a threshold value is necessary to classify the environmental sequences into a taxonomic concept. As there is no linear correlation between DNA similarity, measured as DNA–DNA reassociation, and 16S rRNA homology and we expect the thresholds for different taxa to be different, it seemed reasonable to focus on the 97 % threshold.

For flagellates, ciliates and chlorophyta, it has been shown that morphologically almost identical organisms can differ with respect to phylogeny and ecological aspects [8, 36, 54]. Our study supports that morphologically identical cyanobacteria, e.g. of the genus *Leptolyngbya*, are found in different clades within the phylogenetic tree and in different OTUs. On the other hand, the cyanobacterium *S. rafaelensis* is morphologically clearly different from *T. distorta*, but cannot be distinguished based on 16S rRNA gene analysis [27]. This apparent conflict might be caused by the fact that evolutionary pressure on the 16S rRNA gene sequence is different from the pressure affecting the morphological

Table 4 Summary of the main genera detected by morphology and/or 16S rRNA gene sequences

Genus	Species detected by morphology and the 16S rRNA gene sequence	Species detected by morphology only	OTUs detected by the 16S rRNA gene sequence only
Calothrix		1	
Chroococcidiopsis	1		
Hormoscilla			1
Leptolyngbya	7		3
Lyngbya		1	
Microcoleus	3		3
Nostoc	1	2	
Oscillatoria	1	2	
Phormidium	1	4	1
Pseudanabaena		5	
Pseudophormidium	1		
Schizothrix	2	1	
Scytonema	2	3	
Stigonema			1
Tolypothrix	1	2	
Trichocoleus	1	3	

characters. As genetic diversity is thus not necessarily reflected by detectable morphological characters and the amount of such 'cryptic' species is still unknown, the advantage of a polyphasic approach is obvious.

Conclusion

In African BSCs, we found typical representatives for BSCs of arid soils such as *Microcoleus*, *Phormidium*, *Tolypothrix* and *Scytonema*. Grazing had an effect as cyanobacterial diversity was reduced at the grazed site of Duruchaus. Especially, the differences in the diversity of *Leptolyngbya* species suggested reduced development of late successional crusts in the grazed area. The presence of multiple *Microcoleus* species in the formerly and presently grazed Duruchaus sites confirms the pioneer character of this genus, while the absence of *Nostoc* spp. at the grazed site may be due to their high sensitivity to trampling effects. As 38 OTUs (Table 1) contained sequences from particular sites, we postulate that the differentiation processes correlated with the habitat conditions. The polyphasic approach revealed that the correlation of DNA similarity values with morphologic units varies within the different groups of cyanobacteria. A distinct identification of, e. g. *Pseudanabaena* was only possible using the morphological approach, whose diversity results were exceeded by the diversity of OTUs, underlining the importance of both approaches. The need for a tool allowing the proper placement of cyanobacteria into a taxonomic construct combining morphology and sequence information is evident especially with respect to the aspects of ecosystem function, as the value of sequence data lacking morphological and ecological background is strongly limited.

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