2020

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Phytotaxa 433 (2): 081–093
https://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.433.2.1

Nostoc oromo sp. nov. (Nostocales, Cyanophyceae) from Ethiopia: a new species based on morphological and molecular evidence

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Abstract

In the course of study of the cyanobacterial flora of Ethiopian soil crusts, we isolated a distinctive strain most closely resembling *Chroococcidiopsis kashayi,* and at first considered it to be a new *Chroococcidiopsis* species. However, when placed in nitrogen-free medium for an extended time period this strain developed heterocytes, and consequently was placed in the genus *Nostoc*. It is morphologically distinct from all other *Nostoc* species due to its consistent formation of microscopic few-celled colonies lacking clear filamentous organization, which release smaller colonies from the mother colony, leaving behind a persistent thin firm sheath that resembles cell wall material in the light microscope. Analysis of 16S rRNA and 16S-23S ITS sequence data confirmed its uniqueness among numerous strains of soil *Nostoc*. We are describing it as *Nostoc oromo*, named for the nationality of the people from the region of its origin.

Introduction

The genus *Nostoc* is one of the oldest cyanobacterial genera to be recognized taxonomically (Geoffroy ex Linnaeus pro synon. 1753: 1157) due to the macroscopic nature of many of its species, including the long recognized type species *N. commune* Vaucher ex Bornet et Flahault (1888: 203). It is distinguished from other heterocytous cyanobacteria by having trichomes with heterocytes and akinetes in a colonial common mucilage. It is defined as the heterocytous taxon with almost no morphological synapomorphies, and was long thought to be a basal lineage in the Nostocales (the order containing all heterocytous taxa). Recent phylogenetic analyses have demonstrated that it occupies a more derived position in the order and is polyphyletic (Řeháková *et al.* 2007, Bohunická *et al.* 2015, Hauer *et al.* 2014, Johansen *et al.* 2014, Komárek *et al.* 2014). Some close genera have been separated from *Nostoc* based upon molecular evidence in an attempt to make the genus less polyphyletic, including *Mojavia* Řeháková et Johansen (Řeháková *et al.* 2007: 490), *Desmonostoc* Hrouzek et Ventura (Hrouzek *et al.* 2013: 211), *Trichormus* (Ralfs ex Bornet et Flahualt 1888: 226) Komárek et Anagnostidis (1989: 304), *Halotia* Genuario *et al.* (2015: 667), *Aliinostoc* Bagchi, Dubey et Singh (2017: 3307), *Compactonostoc* F. Cai et R. Li in Cai *et al.* (2019: 202), and *Desikacharya* Saraf, Dawda et Singh (2019: 107). However, even with these taxa removed, existing phylogenetic analyses show strains attributed to *Nostoc* outside of the well-supported clade containing the generitype (Bohunická *et al.* 2015, Johansen *et al.* 2014, Miscoe *et al.* 2016). *Nostoc sensu stricto* has been frequently sequenced, and consists mostly of soil taxa (microscopic and macroscopic) and lichen phycobionts (Řeháková *et al.* 2007).

Biological soil crusts have been extensively studied in recent years due to the significant ecological roles that they play in the soils of semi-arid and arid lands. One of the key ecologically significant taxa in these microbial associations is *Nostoc*, which is an important contributor to ecosystem function of arid lands through nitrogen fixation. Both free-living and lichenized *Nostoc* are widespread and abundant (Evans & Johansen 1999). *Nostoc* lives on the surface of the soil, and typically has a heavily pigmented sheath that protects the cells from photo-damage, particularly from UV radiation. Due to high similarity in cell sizes, the *Nostoc* in soils were long considered to be a single species (*N. commune*) or a few species (*N. commune, N. punctiforme* Hariot 1891: 31*, N. paludosum* Kützing ex Bornet et Flahault 1886: 191*, N. muscorum* C. Agardh ex Bornet et Flahault 1886: 200 - see Johansen 1993, Flechtner *et al.*

Accepted by Jeffery Hughey: 11 Jan. 2020; published: 17 Feb. 2020

1998, 2008). With the advent of molecular methods, researchers have realized the genus has more diversity, and new species have been described (Řeháková *et al.* 2007, Suradkar *et al.* 2017). Certainly more species remain to be isolated, characterized and described.

The study of soil cyanobacteria in Africa is limited. Study has been conducted on biological soil crusts in South and Southwestern Africa, where crusts are common and well developed (Büdel *et al.* 2009, Dojani *et al.* 2011, 2013, Weber *et al.* 2012, 2015, 2018, Dumack *et al.* 2016, Maier *et al.* 2018, Tamm *et al.* 2018). However, these studies are primarily ecological in nature, are confined to the southern part of the African continent, and do not describe new species. Work in the northern part of Africa is much less common (Guidez *et al.* 2015), and no work on soil cyanobacteria has been done in Ethiopia in northeastern Africa.

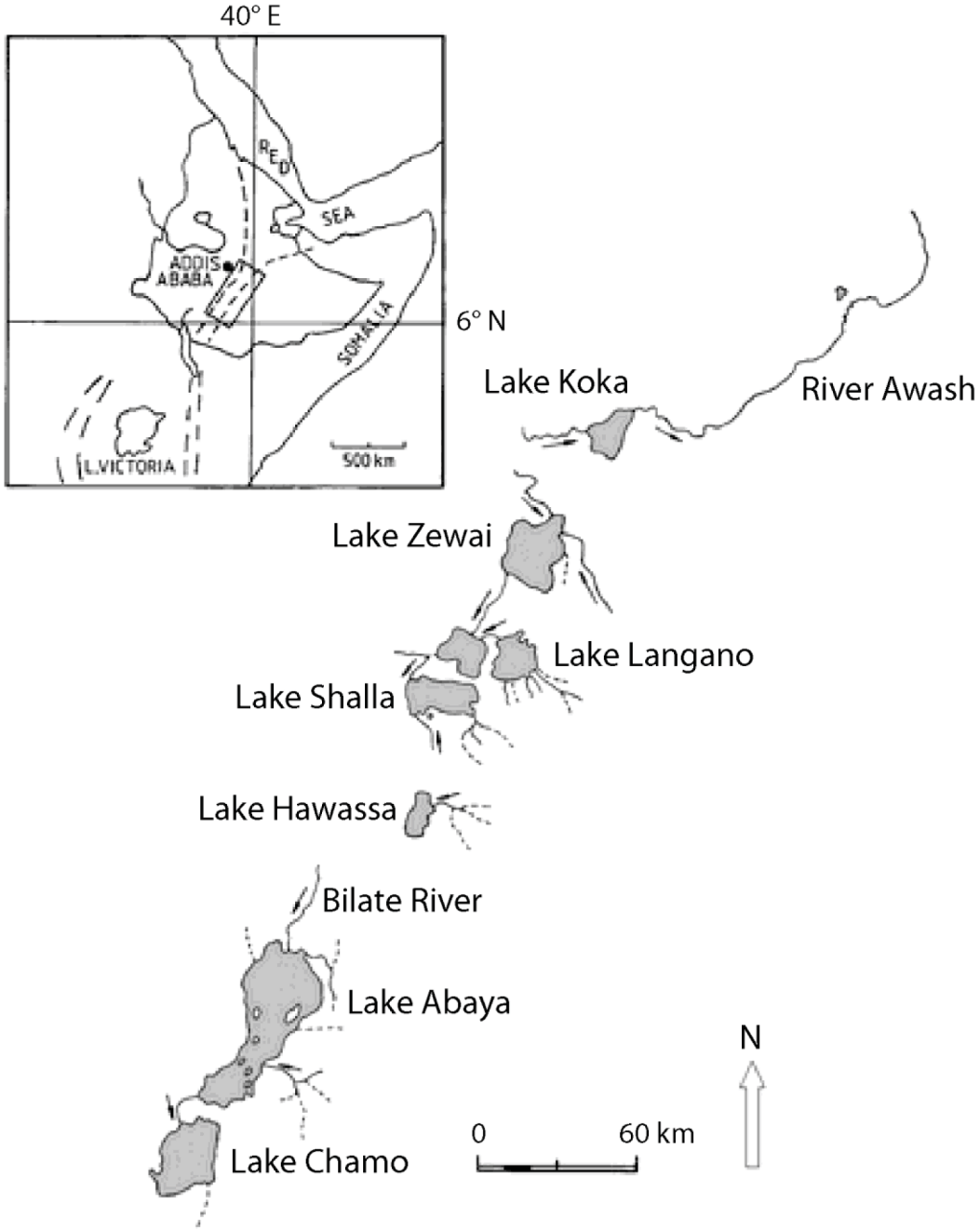

FIGURE 1. Map of region of collection, showing Ethiopian Rift Valley Lake System, including Hawassa and Zewai areas of collection. The type locality for *Nostoc oromo* was slightly northwest of the town Zewai. Inset: Lower magnificaion map of Rift Valley system in context of Addis Ababa, Somalia, and the Red Sea; box in valley represents area of Ethiopian Rift Valley Lake System in main map (adapted from Kebede *et al.* 1994)

As part of an examination of biological soil crusts in the Hawassa and Zewai regions of Ethiopia (Fig. 1) we isolated a unique cyanobacterium which we initially identified as *Chroococidiopsis* Geitler (1933: 625). It had tightly

compact cells in a firm investment that we interpreted to be a mother cell. No heterocytes were observed over a period of well over a year. However, upon transferring the strain to nitrogen-free medium, the strain produced heterocytes (although infrequently and then after several months on nitrogen-free medium). We concluded it was *Nostoc*, and molecular analysis confirmed its position in *Nostoc sensu stricto*. In this paper we describe this new species based on morphology, phylogeny, and characterization of the 16S-23S ITS region of the ribosomal operon. This species will be referred to as *Nostoc oromo* throughout the remainder of this paper.

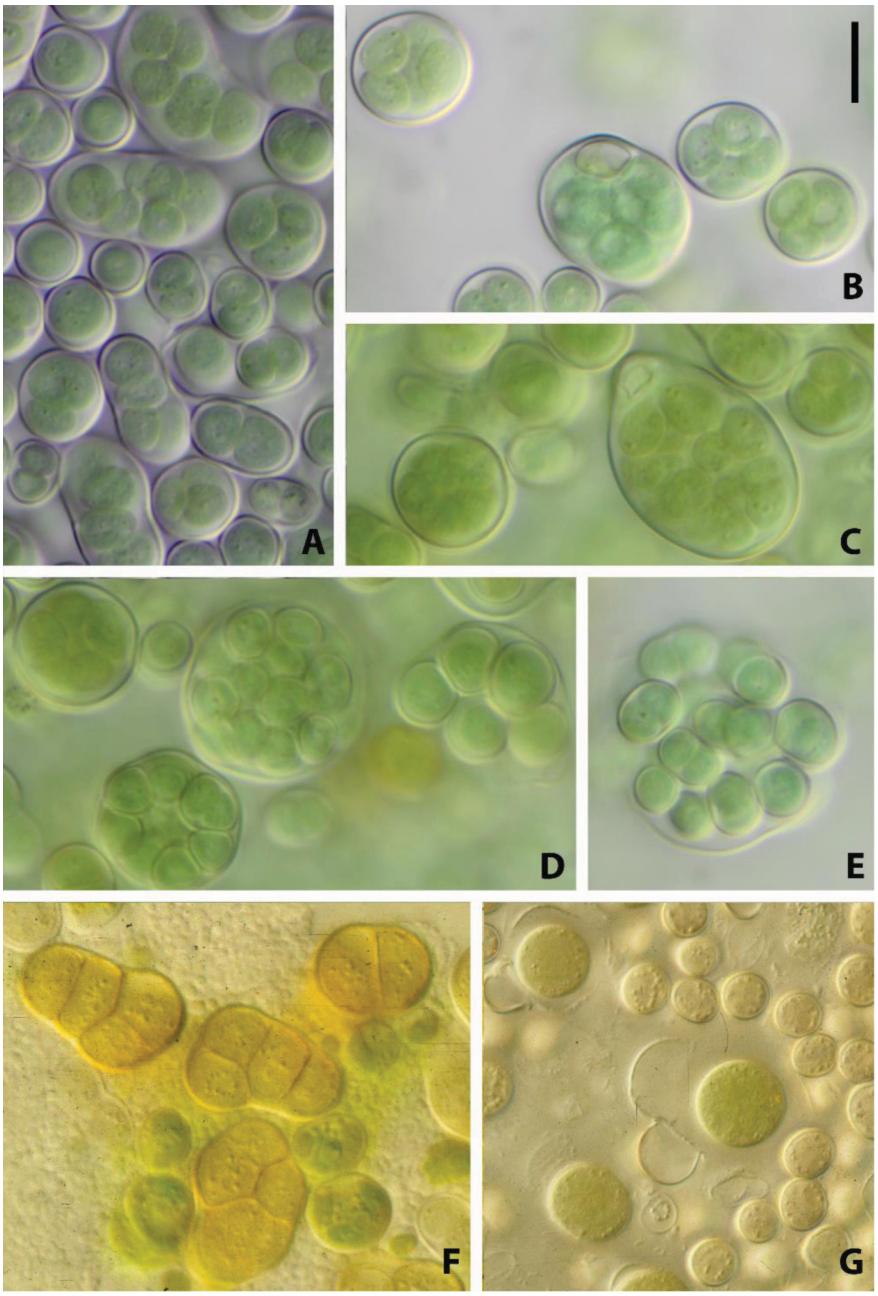

FIGURE 2. *Nostoc oromo* at various life-cycle stages. A. Phase of very small colonies devoid of heterocytes. B, C. Small colonies similar to *Chroococcidiopsis*, but with heterocytes visible in central colonies (nitrogen-free medium). D, E. Release of 1–2 celled hormocytes. F. Few-celled colonies in scenescent culture, with tight, rigid, yellow sheaths. G. Release of single cells from thickened walls of akinetes. Scale = 10 μm for all panels.

Methods

Study sites and sampling protocol:—The study areas are located in the southern part of the Ethiopian Rift Valley System in the vicinity of the towns of Hawassa (Daro Road, 7.115973°N, 38.554396°E) and Zewai (Koshe Road,

7.946518°N, 38.639054°E), respectively (Fig. 1). Both sites are in semi-arid regions and have annual temperature and annual precipitation ranging from 13–27 °C and 760–1200 mm, respectively (FAO 1984, Belete 2013). They are characterized as a mosaic of grassland and acacia woodlands characteristic of savanna. The dominant vegetation in the region includes *Faidherbia albida*, *Olea europaea*, *Acacia tortilis*, *Acacia seyal, Balanites aegyptiaca*, *Euphorbia candelabrum, Croton dychogamus*, and *Solanum schimperianum* (Zerihun & Mesfin 1990). This part of the Rift System has climate and physical and chemical properties of soil capable of supporting microbiotic crusts when protected from mechanical disturbances (trampling by livestock and humans). We sampled relatively undisturbed crusts from communal lands of both regions, and they were the source material for *Nostoc oromo*. A total of 10 samples of microbiotic crust were collected from both locations. *N. oromo* was found in all 10 samples, but was sequenced only from Zewai site 4, and this is the source of the reference strain and holotype. A paratype collected from Zewai site 2 was also designated.

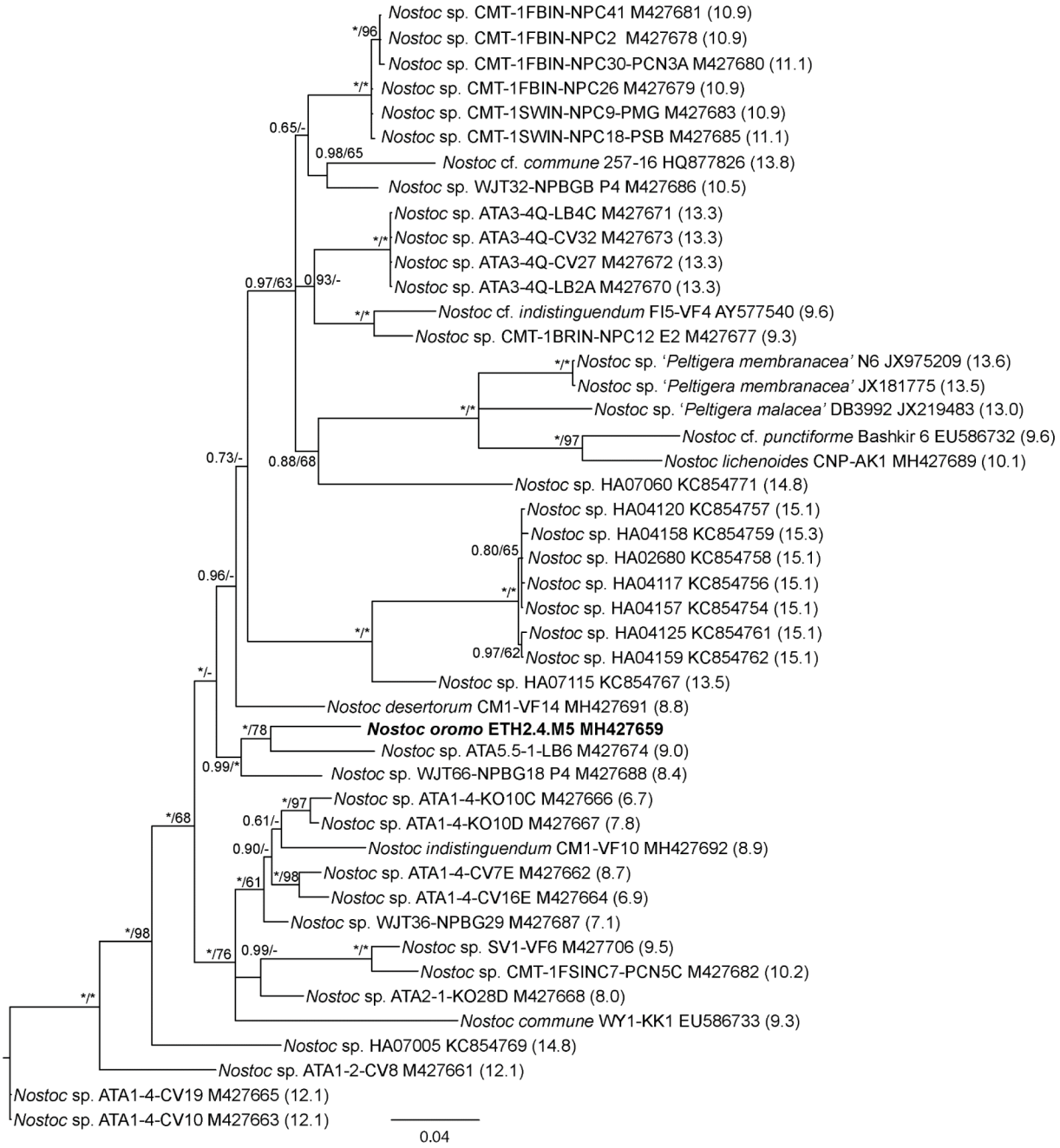

FIGURE 3. Bayesian Inference phylogeny based on 16S rRNA and 16S-23S ITS sequence data, with node support representing BI posterior probabilities/maximum parsimony (MP) bootstrap values. Numbers in parentheses after the accession numbers are percent dissimilarity values between the taxon and *Nostoc oromo*.

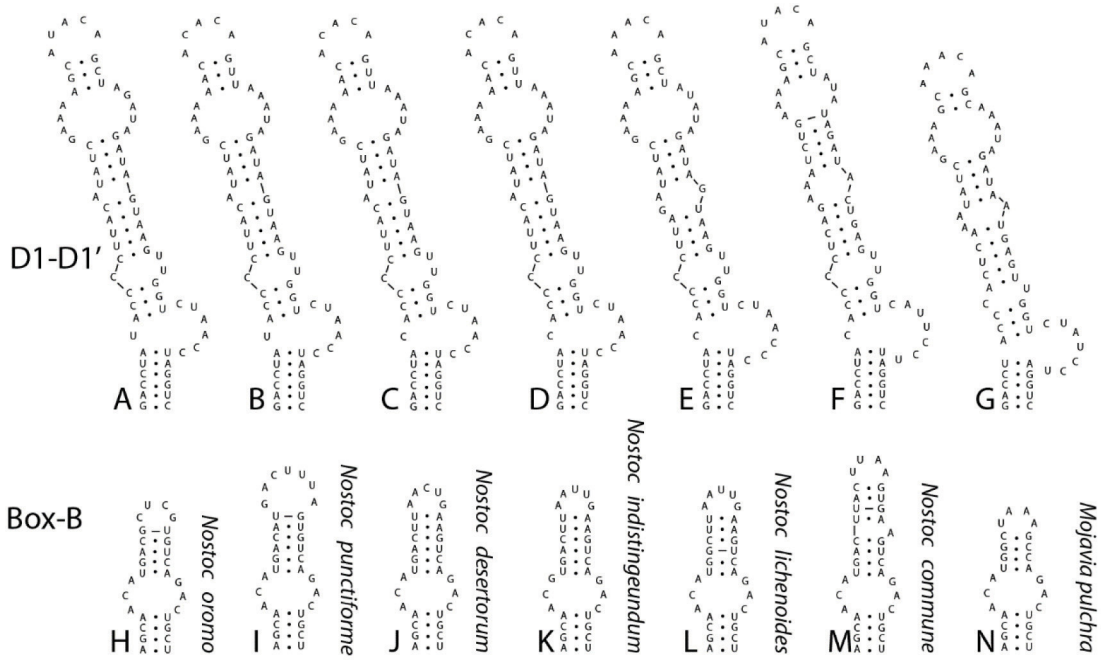

FIGURE 4. Secondary structure of conserved domains of the 16S-23S ITS region. A–G. D1-D1' helix. H–N. Box-B helix. Species labels apply to both structures in a column. Strain designations and accession numbers for the species are as follows: *Nostoc oromo* ETH. 2.4. M.5 MH427659, *Nostoc punctiforme* Bashkir 6A EU586732, *Nostoc desertorum* CM1-VF14 MH427691, *Nostoc indistinguendum* CM1-VF10 MH427692, *Nostoc lichenoides* ANP-AK1 MH427689, *Nostoc commune* WY1-KK1 EU586733, and *Mojavia pulchra* JT2-VF2 AY579903.

Isolation and characterization of strains:—Enrichment plates on agar-solidified Z-8 medium (Carmichael 1986) were prepared in triplicate at 10^{-3} and 10^{-4} dilutions, and then incubated at 23°C in 16:8 hr light dark cycle at 200 µmol m^{-2} s^{-1}. The cyanobacterial isolates made from the plates were studied and identified using a Ziess Axioskop Photomicroscope with Nomarski DIC optics at 1000X magnification. Filament and cell dimensions were taken as well as images throughout the life cycle. The *Nostoc oromo* culture was tested on nitrogen-free Z-8 medium to stimulate heterocyte formation. *Nostoc oromo* was checked against all described *Nostoc* species described from all geographic locations and biotopes in the world using Komárek (2013), a recent compendium of nomenclaturally valid heterocytous taxa and found to not fit any existing taxa.

The holotype was prepared by filtering young, healthy cultures of *N. oromo* onto glass-fiber filters. The filter was allowed to air dry, attached to lichen herbarium cardstock, and placed in herbarium envelopes. Two isotypes were additionally preserved in 2% glutaraldehyde and stored in glass vials. All materials were deposited in the Herbarium of Nonvascular Cryptogams, Monte L. Bean Museum, Provo, Utah (USA). The reference culture is available on request from the John Carroll Algal Culture Collection.

Molecular characterization:—DNA was extracted from unialgal culture using the Mo-Bio Microbial Isolation Kit. Cells were mechanically broken using a cell homogenizer (Minibeadbeater, Biospec.). The DNA extractions were stored at -20 °C. Polymerase Chain Reaction (PCR) was used to amplify a partial 16S rRNA region (bp 327 to end, ~1160 nucleotides) and the associated 16S-23S ITS region following the methods of Johansen *et al.* (2014). Briefly, we used primers 1 and 2 (Boyer *et al.* 2001) adapted from Wilmotte (1993), and Nübel *et al.* (1997): Primer 1: 5'-CTCTGTGTGCCTAGGTATCC-3' and Primer 2: 5'-GGGGAATTT TCGCAATGGG-3'. The internal primers for sequencing were: Primer 5: 5'-TGTACACACCGGCCCGTC-3'; Primer 7: 5'-AATGGGATTAGATACCCCAGTAGTC-3'; and Primer 8: 5'-AAGGAGGTGATCCAGCCACA-3'

Each reaction contained 10 µl 10x buffer (Promega), 0.5 µl of each dNTP (G, A, T, C) at 10 mM, 0.5 µl primer 1 and 0.5 µl primer 2, 0.5 µl Taq polymerase (Promega), and 1µl of genomic DNA, and sufficient sterile water to bring reaction to 100 µl. The most commonly used profile for the initial long PCR reaction using primers 1 and 2 was 94 °C for 1 minute, 57 °C for 1 minute, 72 °C for 4 minutes (35 cycles), followed by a 10 minute extension at 72 °C and then 4 °C dwell. Reactions were carried out using Thermolyne's amplitron thermal cycler (Barnstead International, Dubuque, IA, USA). The presence of PCR products was detected by standard agarose gel electrophoreses with ethidium bromide staining.

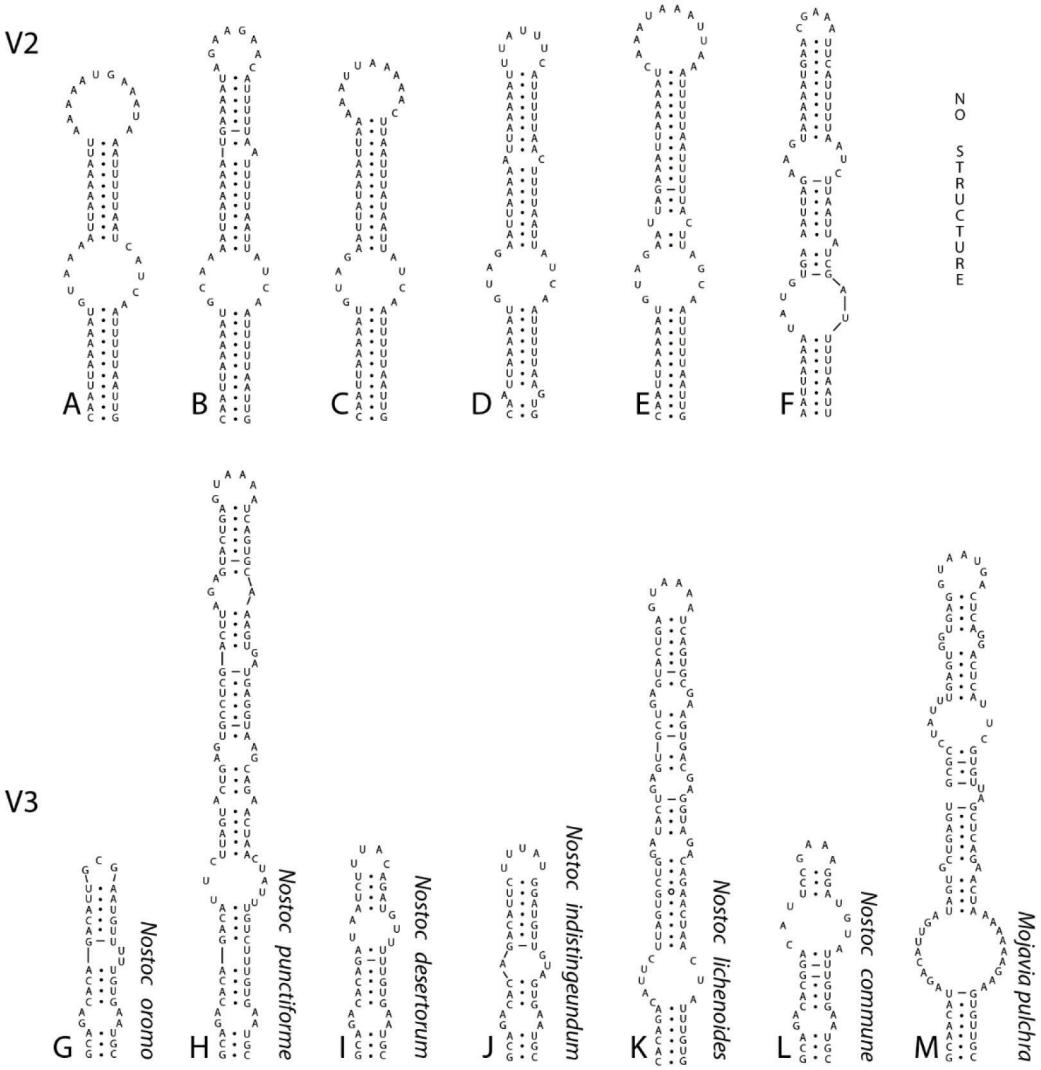

FIGURE 5. Secondary structure of conserved domains of the 16S-23S ITS region. A–F. V2 helix. G–M. V3 helix. Species labels apply to both structures in a column. Strain designations and accession numbers for the species are as follows: *Nostoc oromo* ETH. 2.4. M.5 MH427659, *Nostoc punctiforme* Bashkir 6A EU586732, *Nostoc desertorum* CM1-VF14 MH427691, *Nostoc indistinguendum* CM1-VF10 MH427692, *Nostoc lichenoides* ANP-AK1 MH427689, *Nostoc commune* WY1-KK1 EU586733, and *Mojavia pulchra* JT2-VF2 AY579903.

The PCR product was cloned into plasmids containing the sites for universal primers M13 forward and M13 reverse on either side of the insert site using Invitrogen's TOPO TA Cloning Kit for Sequencing, Version A (Invitrogen Corp. Carlsbad, CA, USA). Plasmid DNA was purified and obtained from the resultant clones using Qiagen's QiaPrep Spin Kit. In the case of clones containing PCR products generated with primers 1 and 2; nine minipreps were digested with EcoRI enzyme and run on a long gel to visualize the size of the inserts. Three clones from the nine minipreps were chosen for sequencing in an attempt to obtain multiple operons, and used to produce a single consensus sequence. Automatic sequencing with the universal primers M13 forward and M13 reverse and internal primers VF5, VF7, and VF8 was performed by Cleveland Genomics.

Subsequently, the beginning portion of the 16S rRNA gene was amplified using the 16S promoter forward primer as first reported in Lukešová *et al.* (2009) (5'-GGATATATTGGATAAGTGCC-3'), and the reverse primer to primer 2 above. PCR conditions were 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min; a 5 min extension at 72°Cand 4°C hold followed. Final concentrations of reagents in the reactions were 13 Taq polymerase buffer (USB, Cleveland, Ohio), 1.5 mM MgCl2, 2.5 pmol ml21 of each primer, 1 ml of template DNA (100–200 ng total), 0.2 mM dNTPs (USB), and 1.25 units Taq polymerase (USB). Amplification of the 23S-5S ITS was performed by using the 23S end (5'-GCTGAAAGCATCTAAGTGGG-3') and 5S reverse (5'-CCTGGCRTCGAGCTATTT-3') primers. Reaction conditions were 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s followed by a 72°C extension for

5 min and a 4°C incubation. Reagent concentrations were the same as indicated above. GenBank accession numbers for the consensus 16S rRNA+16S-23S ITS and the 23S-5S ITS are MH427659 and MH427660, respectively.

Sequences were aligned using ClustalW, and position of indels was manually corrected based upon secondary structure (Řeháková *et al.* 2014). Bayesian Inference (BI) analysis was implemented using MrBayes 3.2.6. (Ronquist *et al.* 2012). A preliminary BI analysis of 79 OTUs of *Nostoc* and Nostocales was used to identify a list of 46 sequences of *Nostoc sensu stricto* in a highly supported clade (data not shown). These *Nostoc* sequences all had 16S-23S ITS sequence data available in addition to the 16S rRNA gene. Furthermore, all of the 16S-23S ITS sequences possessed both the $tRNA^{Ile}$ gene and $tRNA^{Ala}$ gene, ensuring that operons used in the subsequent analyses were orthologous. BI analysis was performed on a concatenated alignment of both the 16S rRNA gene (bp 343–1537) and associated 16S-23S ITS region (728 characters). Indels (253 characters) in the ITS region were coded 0 (absent) and 1 (present). The evolutionary model was chosen using J ModelTest2 on XSEDE and the BI analysis was subsequently conducted using the GTR+Γ+I evolutionary model, with both analyses being run in the CIPRES Science Gateway supercomputing facility (Miller *et al.* 2015). The analysis was run for 50 million generations discarding the first 25% of samples as burn in with NST=6 and Rates=Gamma. The final average standard deviation of split frequencies was 0.009 and the average potential scale reduction factor (PSRF) for all parameter values was 1.000.

A second analysis utilizing parsimony was run in PAUP Version 4b10 (Swofford 1998) on the same alignment, but with indel coding excluded. Indels were treated as a fifth base in this analysis. Bootstrap values were determined with 1000 replicates. The tree reported from this round of analyses is the BI analysis, with bootstrap values from the parsimony analysis mapped on to the Bayesian tree.

Secondary structures of the conserved domains of the 16S-23S ITS region (D1-D1', Box B, V2, and V3 helices) were determined using Mfold (Zuker 2003). These structures were then redrawn for clarity in Adobe Illustrator 15.0.0 in CS5. Percent dissimilarity based on both 16S rRNA and 16S-23S ITS was calculated with the SHOWDIST command in PAUP v. 4b10, and was calculated based on p-distance.

Results

***Nostoc oromo* Mesfin et Johansen *sp. nov.* (Fig. 2):—**Colony bright blue green to olive, spherical to oblong or irregular, up to 50 μm in diameter, fragmenting to release one-, two- or four-celled colonies, filamentous nature never apparent in colonies, which are always densely packed with cells. After transfer, some colonies will fragment to release few-celled, immotile trichomes. Sheath firm, colorless, in some old colonies becoming compartmentalized and yellowish. Trichomes curved or bent, never straight, never motile, few-celled (up to 16 cells long at most), 3–4 μm wide. Vegetative cells often with a single large central granule, when enclosed in firm yellow sheath having the appearance of division in two planes, 4–6 μm long. Akinetes thin walled, minutely granular, distinguished primarily by their larger size, oval to spherical, 4–7 μm wide, 6–7.5 μm long. Heterocytes very rare, colorless to yellowish, compressed hemispherical, with a single polar nodule, 4–6 μm wide, 3–4 μm long.

Diagnostic features:—This strain resembles *Chroococcidiopsis kashayi* Friedmann (1961). Heterocytes are very rare, and were only seen in 6 month-old cultures on nitrogen-free medium. The filamentous nature is likewise consistently absent and only seen in very young cultures. The short trichomes released were not motile, and did not resemble typical *Nostoc* hormogonia. The release of many few-celled, nonfilamentous propagules from aging colonies was very distinctive (Fig. 2 E), and we have not seen similar release in other *Nostoc* we have kept in culture.

Type locality: Microbiotic crust on sandy loam of the Ethiopian Rift Valley System, Zewai, Ethiopia. Koshe Road, 7.946518°N, 38.639054°E.

Holotype here designated: BRY C37795, Dried material prepared from reference strain ETH. 2.4. M.5, Monte L. Bean Museum, Provo, Utah, USA.

Isotypes here designated: BRY C37796, BRYC37797, Preserved samples in 2% glutaraldehyde, prepared from reference strain ETH. 2.4. M.5, Monte L. Bean Museum, Provo, Utah, USA.

Paratypes here designated: BRY C37798, BRYC37799, BRYC37800, Preserved samples in 2% glutaraldehyde, prepared from reference strain ETH. 2.4. M.5, Monte L. Bean Museum, Provo, Utah, USA.

Reference strain: ETH. 2.4. M.5, John Carroll University Algal Culture Collection, University Heights, Ohio, USA.

Reference DNA sequence: GenBank accession number for holotype 16S rRNA+16S-23S ITS and the 23S-5S ITS is MH427659.

Etymology: Named after the nationality of people living in the region, the Oromo.

Phylogenetic Analyses:—Genetic similarity based on 16S rRNA gene sequence data was very high among the *Nostoc* strains examined in this study, with almost all isolates being >98.0% similar, and many being >99.0% similar. The BI and MP analyses had highly similar topologies. All of the *Nostoc* taxa in our analysis were isolated from soil or soil lichens. *N. oromo* was in a highly supported clade with a strain isolated from the Atacama Desert in Chile (ATA5.5-1-LB6) and a strain from the Mojave Desert, CA in USA (WJT66-NPBG18) (Fig. 3).

16S-23S ITS Region:—The percent dissimilarity among aligned ITS sequences between *Nostoc oromo* and the 45 other *Nostoc* species for which ITS sequence with both tRNA genes was available averaged 12.75%, with a range of 6.72–18.51%. Values for the nine taxa most similar to *N. oromo* ranged 6.72–8.95%, with the phylogenetically closest strain, ATA5.5-LB6, actually having the highest dissimilarity in this group at 8.95% (Table 1). This alone provides strong evidence for *N. oromo* being in a species separate from all other *Nostoc* in our analyses, as identical species typically have ITS p-distances <3.0%, with the mean intraspecies percent dissimilarity usually being <1.0% (Erwin and Thacker 2008, Osorio-Santos *et al.* 2014, Pietrasiak *et al.* 2014).

TABLE 1. Percent dissimilarity based on 16S-23S ITS sequence for operons containing both tRNA genes among *Nostoc oromo* and closest sister taxa (OTUs with <9.0% dissimilarity to *N. oromo*).

		1	2	3	4	5	6	7	8	9
1	*Nostoc oromo* ETH2.4.M5									
2	*Nostoc* sp. ATA1-4-KO10C	6.7								
3	*Nostoc* sp. ATA1-4-CV16E	6.9	5.0							
4	*Nostoc* sp. WJT36-NPBG29	7.1	5.3	3.7						
5	*Nostoc* sp. ATA2-1-KO28D	8.0	6.2	5.5	3.8					
6	*Nostoc* sp. WJT66-NPBG18	8.4	10.0	9.7	9.5	7.7				
7	*Nostoc* sp. ATA1-4-CV7E	8.7	4.4	2.8	3.2	4.3	9.9			
8	*Nostoc desertorum* CM1-VF14	8.8	8.9	9.0	8.8	7.3	5.9	10.0		
9	*Nostoc indistinguendum* CM1-VF10	8.9	5.6	6.4	6.2	7.8	10.1	6.8	9.3	
10	*Nostoc* sp. ATA5.5-1-LB6	8.9	10.7	11.5	9.7	10.0	11.8	10.3	12.5	12.4

The domain lengths of the conserved regions of the ITS were variable among most strains (Table 2). While some domains are invariant in length (leader, both tRNA genes), none of the strains in Table 2 have exactly matching lengths in all domains. The phylogenetically closest taxon based on ITS sequence data, ATA5.5-LB6, was different in length of five of the ten variable-length domains. The taxon with the most similar domain length was FI5-VF4, but this strain had very divergent 16S rRNA sequence. *N. oromo* had the shortest V3 helix of any of the taxa examined (Table 2).

The secondary structures of conserved ITS domains in *Nostoc* are highly similar (Figs 4, 5), particularly in the D1-D1' helices which were structurally identical in *N. oromo, N. punctiforme, N. desertorum*, and *N. indistinguendum* (Fig. 4 A–D). *N. oromo* had 4–5 different nucleotides in the D1-D1' helix in comparison with the structurally most similar strains (Fig. 4 A–D), and up to 13 different nucleotides in comparison with *N. commune* (Fig. 4 F). The Box-B helices for *Nostoc* were all very similar in the basal part of the helix, but differed in sequence and structure in the terminus (Fig. 4 H–N). The secondary structure of the Box-B helix for *N. oromo* differed from all ITS sequences in our analyses.

The V2 helices also shared common basal sequence and structure, but were all different in their terminal regions (Fig. 5 A–F). The V3 helices were similar only in the basal clamp, and varied considerably in both structure and length (Fig. 5 G–M).

TABLE 2. Lengths of conserved domains of the 16S-23S ITS. ITS domains with same length as the *N. oromo* ITS domains are shaded gray for ease of comparison.

Strain	Leader	D1-D1' Helix	Spacer + D2	D3 + Spacer	tRNA–Ile	V2 Helix Region	tRNA–Ala	Spacer	Box-B Helix	Spacer + Box–A	D4 + Spacer	V3 helix	D5 region
Nostoc oromo ETH2.4_M5	9	67	45	10	74	76	73	39	28	28	26	35	28
Nostoc CMT-1FBINC26	9	67	45	11	74	80	73	39	29	28	26	36	28
Nostoc HA04159.0001	9	67	47	11	74	81	73	42	28	28	26	39	26
Nostoc 07115.0001	9	68	47	11	74	84	73	43	29	28	26	39	28
Nostoc ATA1-2CV8	9	67	45	10	74	85	73	42	29	28	26	40	25
Nostoc HA07060.0001	9	67	47	11	74	66	73	46	38	28	26	40	29
Nostoc commune 257.16	9	68	45	10	74	80	73	38	28	28	26	39	23
Nostoc CMT-1FSINC7	9	67	46	10	74	80	73	37	28	28	26	39	29
Nostoc WJT32-NPBGB	9	67	46	10	74	80	73	39	31	28	26	39	28
Nostoc CMT-1SWINC18	9	67	47	11	74	80	73	39	29	28	26	36	28
Nostoc JX975209	9	67	50	11	74	80	73	40	28	28	26	93	41
Nostoc JX219483	9	67	47	10	74	80	73	41	35	28	26	107	23
Nostoc lichenoides CNP-AK1	9	68	47	11	74	88	73	40	31	28	26	99	29
Nostoc punctiforme Bashkir6A	9	67	39	10	74	88	73	40	31	28	26	99	28
Nostoc ATA3-4Q-CV27	9	65	47	10	74	80	73	39	31	27	26	39	28
Nostoc FI5-VF4	9	67	45	10	74	76	73	39	28	28	26	39	29
Nostoc CMT-1BRINC12	9	65	47	10	74	76	73	39	28	28	26	39	28
Nostoc ATA1-4CV19	9	65	47	10	74	81	73	42	29	28	26	39	28
Nostoc HA07005.0001	9	67	47	10	74	81	73	42	31	27	26	36	28
Nostoc SV1-VF6	9	67	45	11	74	94	73	46	28	28	26	39	29
Nostoc CMT-1FBINC41	9	68	46	10	74	94	73	39	29	28	26	36	28
Nostoc WJT66-NPBG18	9	67	45	10	74	88	73	38	28	28	26	39	28
Nostoc desertorum CM1-VF14	9	67	45	10	74	79	73	39	31	28	26	39	27
Nostoc ATA2-1KO28	9	69	47	10	74	83	73	41	31	28	26	40	29
Nostoc commune WY1-KK1	9	67	46	10	74	89	73	50	35	28	26	39	28
Nostoc ATA1-4KO10	9	67	47	10	74	83	73	43	31	27	26	39	28
Nostoc indistinguendum CM1-VF10	9	67	45	10	74	83	73	43	31	28	26	39	28
Nostoc ATA5.5-1-LB6	9	67	45	10	74	86	73	39	27	27	25	39	28
Most common lengths	*9*	*67*	*47*	*10*	*74*	*80*	*73*	*39*	*31*	*28*	*26*	*39*	*28*

Discussion

Nostoc oromo is morphologically distinct from all other species of *Nostoc*. During most of the life cycle, it lacks filamentous stages, having mostly single, separated cells or cell pairs in minute colonies. Without molecular sequence data we would have concluded it was a *Chroococcidiopsis* species, and did not see heterocytes until it was placed on nitrogen-free medium, which were still very rare even after an extended period on agar lacking a nitrogen source. The

release of small, one-two celled propagules from minute colonies was highly unusual, especially in the instance when single cells were released from akinetes, which then increased in size before dividing. The apparent cell division in two planes was also a unique character. Consequently, unlike many *Nostoc* species, *N. oromo* is morphologically easily distinguishable from all other species in the genus.

The length of 16S-23S ITS domains also set this species apart from all other sequenced strains. While the D1-D1' helix was highly similar to other strains and taxa, the Box-B was shorter than most other Box-B helices. *N. oromo* had the shortest V3 helix in our comparator group of over 50 strains.

The morphologically closest *Nostoc* species to *N. oromo* in the literature is *N. imperfectum* Schwabe et El Ayouty (1966: 532–533, figs. 15–22), described from soils in India. *N. imperfectum* shares the characteristic of rare hormogonium production, tightly packed small colonies, tight sheaths, and obscure trichome formation in colonies. *N. oromo* differs in that the small colonies are much smaller, mostly spherical to broadly oval (Fig. 2 B–D), and even less likely to form visible trichomes (Fig. 2 A). Instead of the release of hormogonia, small spherical colonies of one to two cells are typically released (Fig. 2 E). Akinete structure and germination also differ (Fig. 2 G). The apparent cell division in more than one plane was not observed in *N. imperfectum* (Schwabe et El Ayouty 1966). *N. imperfectum* also forms large, elongated colonies in culture, a feature never seen in *N. oromo*. Cell sizes of both vegetative cells and heterocytes in *N. oromo* had larger maximum dimensions than cells in *N. imperfectum*. Ecologically, there are some similarities between the two species. Both are in semi-arid climates that never experience freezing temperatures and have peak precipitation in June-August. The Rift Valley in the Zewai region has more constant and stable temperatures (mean = 19.2°C) than the area of India from which *N. imperfectum* was isolated (Kanpur, Uttar Pradesh, India; mean = 25.6°C)(Cheung *et al.* 2008).

N. oromo is phylogenetically in a clade of mostly microscopic *Nostoc* isolated from desert soils in North and South America (Figs. 3). The locations from which these *Nostoc* were isolated (CMT, CM1, FI, SV, WJT strains from Mojave Desert and ATA strains from Atacama Desert) differ climatically in that they do experience freezing temperatures every year, and are much more arid. The yellowish compartmentalized colonies seen rarely in *N. oromo* (Fig. 2 F) are similar to the compartmentalized colonies seen in *N. desertorum* Řeháková et Johansen in Řeháková *et al.* (2007, fig. 28), although this latter taxon never appeared to have cell division in two planes.

In summary, *N. oromo* is distinct morphologically, ecologically, geographically, and genetically from all other described and/or sequenced *Nostoc* species, and we feel that it will stand as a distinct species even as more morphologically described species within the genus are sequenced in the future. It is apparent from this study that desert *Nostoc* taxa are likely diverse, with a number of cryptic species separable by phylogeny and molecular distance in the 16S-23S ITS regions. This interesting group requires more intensive study.

Acknowledgements

We thank Tesfaye Chaka, who collected the soil samples in Ethiopia for us. Support for molecular sequencing of desert *Nostoc*, including *N. oromo*, was provided by the National Science Foundation grant number DEB-9870201. Isolates from the Atacama Desert used in this study were collected with support from NSF grant number DEB-0842702. Any opinions, findings, conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. Recent research support to Johansen enabling completion of the project was provided by grant No. 15-11912S from the Czech Science Foundation.

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