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# New records of the genus *Ramalina* (Lecanorales, Ascomycota) in Dagestan with a key to species

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***Keywords*:** biodiversity, broad-leaved forest, Caucasus, DNA barcoding, HPTLC, lichenized fungi, molecular phylogeny, protected area.

***Summary*.** Based on morphological, chemical and molecular evidences, *Ramalina europaea*, *R. lacera*, and *R. obtusata* are reported for the first time for Dagestan. *Ramalina lacera* is the first confirmed record for the Russian part of the Caucasus and the second one for Russia. ITS of nuclear ribosomal DNA barcodes for Caucasian specimens of *R. lacera* and *R. obtusata* are generated for the first time. Specimen descriptions for firstly revealed species are presented. A key for identification of the 13 known taxa of the genus *Ramalina* in Dagestan is provided.

# Новые находки в роде *Ramalina* (Lecanorales, Ascomycota) в Дагестане с ключом к известным видам

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***Ключевые слова*:** биоразнообразие, ДНК-штрихкодирование, Кавказ, лихенизированные грибы, молекулярная филогения, охраняемая территория, широколиственный лес, HPTLC.

***Аннотация*.** Основываясь на морфологических, химических и молекулярных данных, виды *Ramalina europaea*, *R. lacera* и *R. obtusata* впервые приводятся для Дагестана. Находка *R. lacera* – первое подтвержденное указание вида для российского Кавказа и второе для России. Для кавказских образцов *R. lacera* и *R. obtusata* впервые получены последовательности ярДНК. Приводится описание образцов впервые выявленных видов. Составлен ключ к 13 известным в Дагестане таксонам из рода *Ramalina*.

## Introduction

The genus *Ramalina* Ach. is a globally distributed genus with large ecological amplitude (found also in Antarctica) and currently combined up to 230 taxa. It is included in 50 largest genera of lichenized fungi being the 18th most diverse (Lücking et al., 2016). In general, the genus is characterized by fruticose

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thallus with flat to cylindrical, solid to hollow branches, often with pseudocyphellae, hyaline 1-septate ascospores and producing usnic acid (Smith et al., 2009).

Species of *Ramalina* occur in a large variety of habitats. Most of them are predominantly corticolous but frequently saxicolous in oceanic climates. Savannas, tropical-montane, Mediterranean, temperate and boreal forests are the richest habitats for the genus. Significant number of *Ramalina* species has a geographically restricted distribution, such as endemics of oceanic islands (Krog, Østhagen, 1980; Krog, 1990; Blanchon et al., 1996; Aptroot, Bungartz, 2007; Pérez-Vargas, Pérez-Ortega, 2014; Sparrius et al., 2017).

In the work "A checklist of the lichen flora of Russia" (Urbanavichus, 2010) this genus is represented by 39 species, of them 18 species are recorded for the Russian Caucasus. Among them, *R. elegans* (Bagl. et Carestia) Jatta, *R. montana* Barkh., and *R. panizzei* De Not. are known within Russia only from the Caucasus. Later, in virgin mixed coniferous-broad-leaved forest in the Western Caucasus *R. subgeniculata* Nyl. was found for the first time for Russia (Urbanavichus, Urbanavichene, 2014). Further studies on lichens in different Russian regions and taxonomic studies have expanded the list of *Ramalina* to 44 species (Kataeva, 2014; Gasparyan et al., 2017; Davydov, Yakovchenko, 2023).

In Dagestan the genus *Ramalina* was presented by 10 taxa which grows as epiphytes in various forest communities (*R. asahinana* Zahlbr., *R. calicaris* (L.) Fr., *R. farinacea* (L.) Ach., *R. fastigiata* (Pers.) Ach., *R. fraxinea* (L.) Ach., *R. pollinaria* (Westr.) Ach., *R. sinensis* Jatta) and on siliceous rocks in open habitats (*R. capitata* var. *capitata* (Ach.) Nyl., *R. capitata* var. *digitellata* (Nyl.) Nimis, *R. polymorpha* (Lilj.) Ach.) (Ismailov, Urbanavichus, 2013; Urbanavichus, Ismailov, 2013, 2016; Ismailov, 2018).

During the fieldwork as continuing studies of lichens in broad-leaved forests (Ismailov et al., 2017; Ismailov, 2020) at lowland and in the foothills of Dagestan we found several corticolous specimens of the genus *Ramalina*. Determination of taxonomic status of specimens revealed new species for Daghestan. In this paper, we provide detailed information on new records with a key for all *Ramalina* taxa occurring in Dagestan.

## Materials and Methods

Light microscopy (LOMO MSP-2 microscope), chemical spot tests and UV light were used for specimen investigations. The secondary metabolites of species were identified using high performance thin layer chromatography (HPTLC) in solvent system A according to the methods summarized by Arup et al. (1993). Camag glass plates with F254 layer were used.

Genomic DNA was amplified directly from thalli pieces of dried *Ramalina* specimens with the Phire Plant Direct PCR Master Mix Kit (Thermo Fisher Scientific, Lithuania) without prior DNA purification according to the manufacturer's instructions. The internal transcribed spacer regions of the nuclear ribosomal DNA (ITS) were amplified with the primers pair ITS1F/ITS4 (White et al., 1990; Gardes, Bruns, 1993). Products of amplification were visualized by 1 % agarose gel electrophoresis, and then purified using the CleanMag DNA (Evrogen, Russia) purification kit. Sequencing was performed on an ABI model 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). Raw data were edited and assembled in MEGA X (Kumar et al., 2018). The newly obtained sequences were deposited in GenBank.

The sequences were aligned with ten additional sequences retrieved from GenBank database (Table), using a MAFFT version 7 web tool (Katoh et al., 2019) with the E–INS–I option. The maximum-likelihood phylogenetic analysis using the IQ-TREE web server (Trifinopoulos et al., 2016) with 1000 ultra-fast bootstrap repeats was performed to identify the phylogenetic position of newly sequenced specimens.

## Results and discussion

Based on morphological, anatomical and chemical identification of specimens supported by molecular analysis results, three *Ramalina* species new to Dagestan – *R. europaea* Gasparyan, Sipman et Lücking, *R. lacera* (With.) J. R. Laundon, and *R. obtusata* (Arnold) Bitter – have been revealed. For *R. obtusata*, we notice a minor concentration of obtusatic acid as a result of not favourable habitat conditions.

Molecular study on *Ramalina* species has been carried out using data on ITS nrDNA sequences. In this research, two complete ITS sequences for *Ramalina lacera* and *R. obtusata* were generated based on collections made in the Republic of Dagestan. Based on a megablast search of NCBIs GenBank nucleotide database, our *R. lacera* sequence showed 100 % identity (97 % query coverage) with all of 18 ITS sequences of *R. lacera* originated from Spain,

Table

Voucher information and GenBank accession numbers for ITS sequences used in this study

| Species | Specimen | Origin (country, region) | GenBank accession No. | References |
|---|---|---|---|---|
| *Ramalina canariensis* J. Steiner | HBG 17040 | Spain | FJ871072 | Unpublished |
| *Ramalina canariensis* J. Steiner | Sipman, Raus 63658 (B 60 0203171) | Greece | MN989290 | Unpublished |
| *Ramalina lacera* (With.) J. R. Laundon | Nimis, Tretiach 26272 (TSB) | Italy | MG926016 | Kistenich et al. (2018) |
| *Ramalina lacera* (With.) J. R. Laundon | G. Salvà (Salines_1) | Spain, Ibiza | MH252130 | Garrido-Benavent et al. (2022) |
| ***Ramalina lacera* (With.) J. R. Laundon** | **A. Ismailov DAG 1470** | **Russia, Republic of Dagestan** | **OR289700** | **Present study** |
| *Ramalina mollis* Krog et Østh. | HBG 17074 | Spain | FJ871092 | Unpublished |
| *Ramalina obtusata* (Arnold) Bitter | PRA-JV24089 | Czech Republic | OK333014 | Vondrák et al. (2022) |
| *Ramalina obtusata* (Arnold) Bitter | PRA-Vondrak24397 | Czech Republic | OQ718046 | Unpublished |
| ***Ramalina obtusata* (Arnold) Bitter** | **A. Ismailov DAG 1469** | **Russia, Republic of Dagestan** | **OR289699** | **Present study** |
| *Ramalina pollinaria* (Westr.) Ach. | A. Gasparyan B 60 0201017 (epitype) | Sweden | KY362419 | Gasparyan et al. (2017) |
| *Ramalina pollinaria* (Westr.) Ach. | O-L-200850 | Sweden | MK812114 | Marthinsen et al. (2019) |
| *Niebla robusta* (R. Howe) Rundel et Bowler | D. Ertz 12446 (BR) R605 | USA, California | GU827336 | Sérusiaux et al. (2010) |

Note. The sequences obtained in this study are in bold face.

and our *R. obtusata* sequence had 100 % identity (98 % query coverage) with OK333014 (voucher PRA-JV24089) from Czech Republic.

For phylogenetic analysis, a total of nine nrITS reference sequences of *Ramalina* species presented in recent molecular studies (Gasparyan et al., 2017; Kistenich et al., 2018; Marthinsen et al., 2019; Garrido-Benavent et al., 2022; Vondrák et al., 2022) and the sequence of *Niebla robusta* (R. Howe) Rundel et Bowler as an outgroup (GU827336) were chosen. The final dataset contained 12 nrITS sequences and consisted of 852 characters, including gaps. Tree topology obtained using Maximum Likelihood method for phylogenetic analysis is presented in Fig.1. Our nrITS phylogeny supports separate clades corresponded to different *Ramalina* morphological species. Among them, there are two well-supported lineages *Ramalina lacera* and *R. obtusata* that include sequences originated from the Russian part of the Caucasus and generated for the first time from *Ramalina* specimens collected in riparian forests of the Republic of Dagestan on the territory of Samurskiy National Park. These molecular data are congruent to morphological and ecological features of species revealed.

Detailed morphological descriptions of specimens collected complied with the information on their substrates, distribution and biochemical features are presented below.

***Ramalina europaea*** Gasparyan, Sipman et Lücking, 2017, Lichenologist 49(4): 306 (Fig. 2).

**Description:** thallus corticolous, fruticose, yellowish-green to green (greyish green in herbarium), erect to subupendulous, up to 3 cm long and 4 cm wide. Lobes flattened, up to 3 mm wide and 1–2 cm long, solid, developing numerous tiny, irregular, sometimes spine-like lateral lobules, on top of which developing soralia as punctiform, granular structures. Soralia lateral and terminal, never

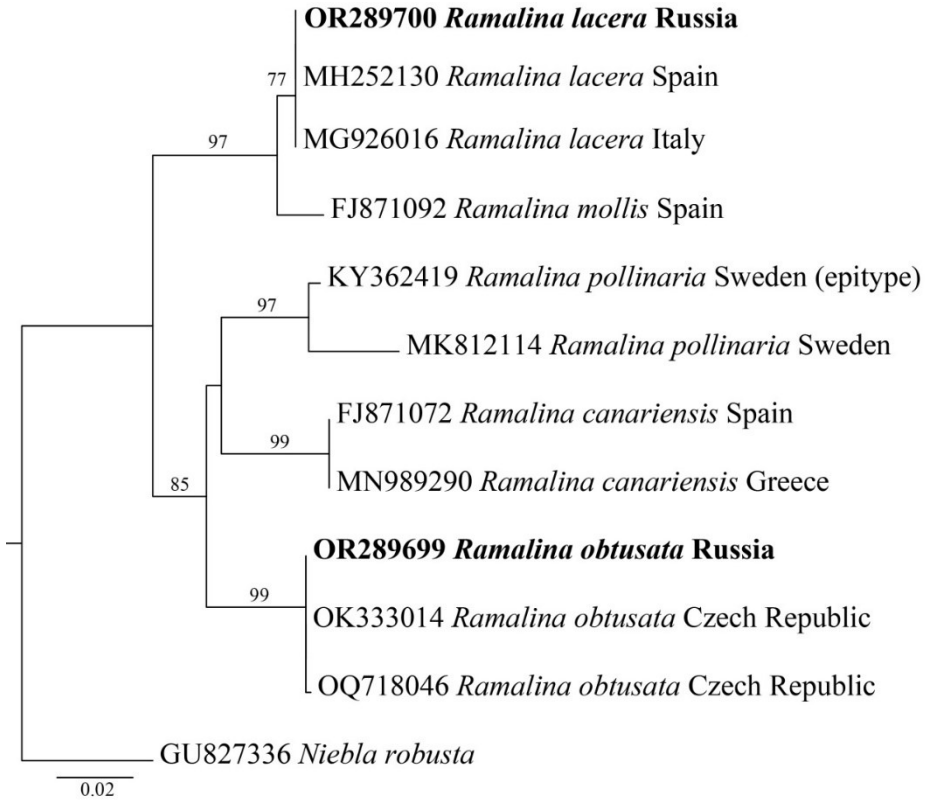


Fig. 1. Phylogenetic position of ITS of nrDNA *Ramalina* sequences newly generated in this study (in bold face) based on Maximum Likelihood method. Bootstrap support values are shown above branches (BS ≥ 75 %). The scale bar represents the expected number of nucleotide changes per site.

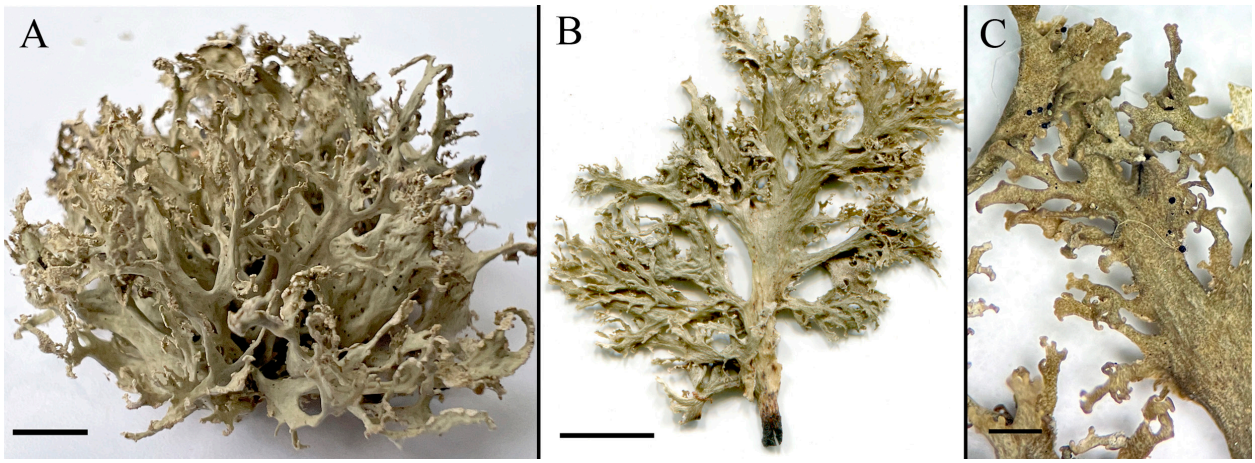


Fig. 2. Habitus (A), single lobes (B) and lateral lobules (C) of *Ramalina europaea*. Scale: A, B – 0.5 cm; C – 1 mm.

becoming large and labriform. Soredia granular. Medulla white, compact. Apothecia not observed in Dagestan material. Photobiont chlorococcoid. Spot tests: all negative.

**Substrate and ecology:** on trunk of *Fagus orientalis* Lipsky in broad-leaved beech-hornbeam forest in the gorge of the Akhtichay River.

**Secondary metabolites:** usnic and evernic acid.

**Specimen examined:** "Russia, Republic of Dagestan, Kazbekovskiy district, vicinity of Almak village, beech forest in gorge of the Akhtichay River, on *Fagus orientalis*, 950 m above the sea level. 42°57′51″N, 46°34′53″E. 08 V 2018. A. B. Ismailov" (DAG 1435).

**Distribution:** species with Eurasian distribution. Widespread in Europe. Easternmost locality is known from the south of the Ural Mountains (Gasparyan et al., 2017). Also common in broad-leaved forests of the Caucasus.

**Notes:** From close *Ramalina pollinaria* it differs by the punctiform soralia borne terminally on tiny

spine-like, lateral branches, and by the granular soredia. Our specimens are close in morphology to other Caucasian and Russian specimens summarized in study describing this species (Gasparyan et al., 2017).

***Ramalina lacera*** (With.) J. R. Laundon, 1984, Lichenologist 16(3): 221 (Fig. 3).

≡ *Lichen lacerus* With., 1776, Bot. arr. veg. Gr. Brit. 2: 716.

**Description:** Thallus fruticose, shrubby, subpendent, yellowish green, dull, up to 4 cm long and 5 cm wide, palmately or irregularly branched, weakly to clearly scrobiculate (especially on the lower surface), flattened laciniae growing from a common holdfast; pseudocyphellae scarce. Main laciniae with numerous, small, marginal secondary branches 1–2 mm wide; soralia on lower parts of lacinia, at first marginal then becoming laminal, mainly in upper parts of thallus. Laminal soralia growing mainly on top of the wrinkle. Cortex very thin, of a single prosoplectenchymatous layer, chondroid tissue absent, medulla white, dense. Apothecia and pycnidia not observed in Dagestan material. Photobiont chlorococcoid. Spot tests: cortex K–, C–, KC+ yellow, P–, UV–; medulla all spot test negative.

**Substrate and ecology:** on twigs of *Pinus eldarica* Medw. within manmade pine plantings and *Crataegus pentagyna* Walds. et Kit. in open areas of natural broad-leaved liana forest on the Caspian Sea coast in the delta of the Samur River.

**Secondary metabolites:** usnic and bourgeanic acid.

**Specimen examined:** "Russia, Republic of Dagestan, Magaramkentskiy district, Samurskiy National Park, on twigs of *Crataegus pentagyna* among sparse vegetation along forest road, 23 m b. s. l. 41°52′52″N, 48°31′08″E. 07 V 2023. A. B. Ismailov" (DAG 1470); ibid., "manmade forest plantations of *Pinus eldarica*, on twigs, 36 m b. s. l. 41°53′43″N, 48°29′56″E. 19 IX 2022. A. B. Ismailov" (DAG 1471).

**Distribution:** mainly Mediterranean-Atlantic species with broad but somewhat spotted distribution. Known from Europe, west of Asia, Africa, southwest of North America, South America (Gumboski et al., 2014). For Russia and the Caucasus, *R. lacera* is known from several references. Within Russia previously reported only from Crimea (Krasnaya kniga, 2015). Doubtful record of this species from Western Caucasus (Krivorotov, 1997), because the report was not confirmed by the specimen. For the East Caucasus *R. lacera* is known only by our record.

**Notes:** *R. lacera* is polymorphic species with a wide ecological amplitude. It is growing on trees and rocks in areas with a humid-warm climate, in rather dry, well-lit sites of manmade habitats, with strong coastal affinities (Gumboski et al., 2014; Nimis, 2016). Habitus variates from deep lacerate to more or less foliose-fruticose forms. Our specimens with a deep lacerate thallus grew as epiphyte in habitats with sparse vegetation along the forest roads and in manmade pine plantings on the Caspian Sea coast.

***Ramalina obtusata*** (Arnold) Bitter, 1901, Jb. wiss. Bot. 36: 435 (Fig. 4).

≡ *Ramalina minuscula* var. *obtusata* Arnold, 1875, Verh. Kaiserl.-Königl. zool.-bot. Ges. Wien 25: 472.

**Description:** Thallus fruticose, greenish grey, shrubby, 1 cm high and 2 cm wide, growing from a narrow holdfast. Branches several arising from the base. Laciniae fistulose, inflated, ridged, foveolate, with some side branches. Medulla hollow. Soralia common, labriform to helmet-shaped, developed within terminal or subterminal vesicles. Soredia farinose. Apothecia not observed in Dagestan material. Photobiont chlorococcoid. Spot tests: all negative.

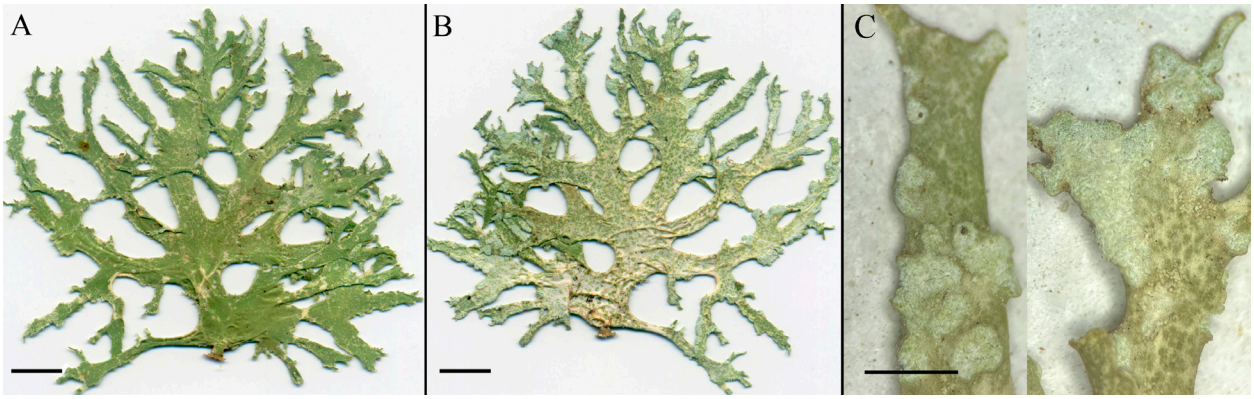


Fig. 3. Upper (A), lower (B) surface of thallus and soralia (C) of *Ramalina lacera*. Scale: A, B – 0.5 cm; C – 1 mm.

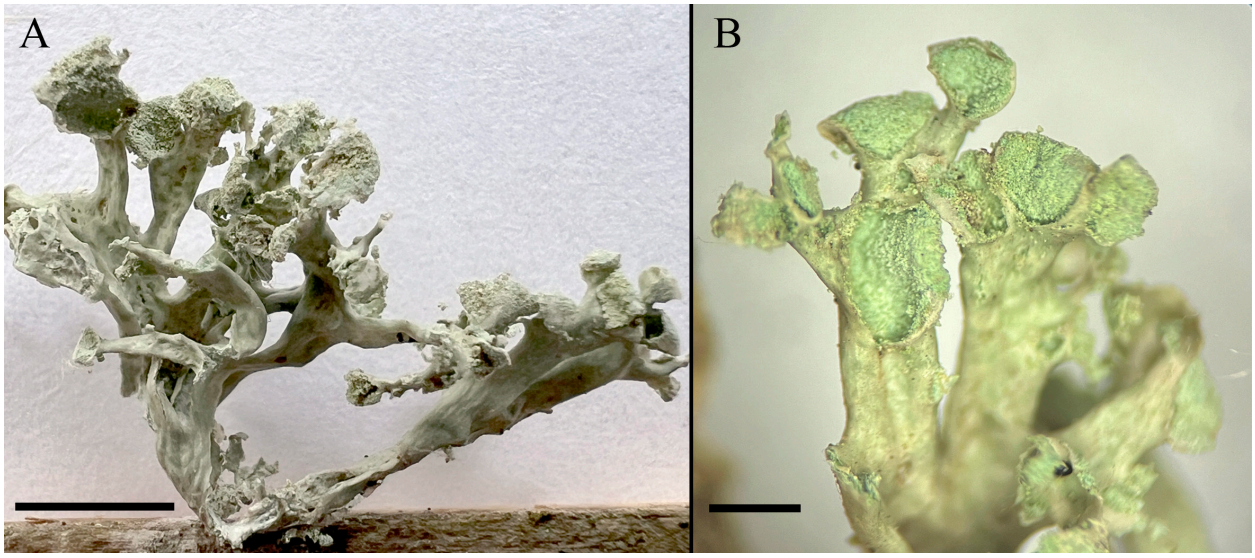


Fig. 4. Habitus (A) and soralia (B) of *Ramalina obtusata*. Scale: A – 0.5 cm, B – 1 mm.

**Substrate and ecology:** on fallen decorticated trunk of *Pinus eldarica* in dry conditions of manmade pine plantings on the Caspian Sea coast.

**Secondary metabolites:** evernic, obtusatic (minor) and usnic acid.

**Specimen examined:** "Russia, Republic of Dagestan, Magaramkentskiy district, Samurskiy National Park, manmade forest plantations of *Pinus eldarica*, on fallen trunk, 36 m b. s. l. 41°53′43.4″N, 48°29′56.5″E. 19 IX 2022. A. B. Ismailov" (DAG 1469).

**Distribution:** a cool-temperate to southern boreal species widespread in Europe and North America (Walker, Pystina, 2005) and quite common in Russia (Urbanavichus, 2010). Location in Dagestan is the southernmost in the European part of Russia.

**Notes:** *Ramalina obtusata* mainly grows in well-preserved humid boreal forests, more rarely in cold-moist, open montane forests. Probably, our record from dry, lowland conditions is extreme habitat for the species. This is also indicated by the small size of the thallus of our specimen and minor concentration of obtusatic acid. *R. obtusata* is morphologically and chemically similar to close *R. baltica*, but the latter one doesn't occur within the Caucasus.

Including new records presented in this paper, the genus *Ramalina* in Dagestan comprises 13 taxa. The key for all taxa known in Dagestan is given below.

**An identification key to *Ramalina* taxa registered in Dagestan**

1. Thallus with soredia and sometimes with isidia- like granules. Apothecia often absent ..... 2

+ Thallus without vegetative diaspores. Apothecia often frequent ..... 9

2. On rock ..... 3

+ On bark ..... 5

3. Thallus with isidia-like granules originating inside numerous, elongated, laminal pseudocyphellae ..... *R. polymorpha*

+ Thallus sorediate, isidia-like granules absent .. 4

4. Soralia mostly capitate (some labriform soralia may be present), terminal ..... *R. capitata* var. *capitata*

+ Soralia located on the lower side of thin, short, finger-like apical branches of lobes ..... ..... *R. capitata* var. *digitellata*

5. Thallus up to 15 cm long, pendent, richly dichotomous branched. Lobes narrowed, (0.5)1–2(3) mm wide, tapering towards the tips, flattened or slightly canaliculated. Soralia numerous, round to elongate, lateral, sometimes terminal .... *R. farinacea*

+ Thallus 1–3(5) cm long, erect to pendent, soralia with different structure ..... 6

6. Thallus, pendent, yellowish green, palmately branched, lacerate, reticulately ridged especially on the lower surface. Soralia diffuse, laminal or marginal, on lower side of lacinia. Coastal forestry and sparse vegetation habitats ..... *R. lacera*

+ Thallus erect to subpendent, greenish to greyish, weakly or richly branched. Soralia laminal, marginal or terminal ..... 7

7. Thallus 1–2 cm high, consists of 1–2 laciniae, lobes hollow. Soralia labriform to helmet-shaped, terminal ..... *R. obtusata*

+ Thallus shrubby, richly branched from a narrow or expanded holdfast, lobes solid. Soralia laminal, marginal or terminal ..... 8

8. Soralia well-delimited, round, laminal, lateral or terminal. Branch tips predominantly obtuse, bearing irregular labriform soralia on the lower side. On bark, rarely on stone, soil and bryophytes ..... ..... *R. pollinaria*

+ Soralia punctiform, often developing on numerous tiny, irregular, somehow spine-like lateral or terminal branchlets. On bark ..... *R. europaea*

9. Thallus monophylous, pendent, weakly or strongly dissected into several wide and obtuse lobes, tips with short branchlets. Apothecia apical and marginal. Spores broad ellipsoid, (12.5)13–14(15) × 6–7.5 µm ..... *R. asahinana*

+ Thallus richly branched ..... 10

10. Apothecia terminal ..... *R. fastigiata*

+ Apothecia laminal or subterminal ..... 11

11. Lobes definitely canaliculate rather narrow 1–2(3) mm wide, dichotomous branched, branch tips attenuate, apothecia subterminal. On bark in humid broadleaved forests of high-mountainous Dagestan .. ..... *R. calicaris*

+ Lobes flattened, not canaliculated. Distribution more wider ..... 12

12. Thallus 2–20 cm long. Lobes flattened, unbranched, with a reticulate-folded surface; apothecia numerous, pedunculated, located on the upper surface of the lobes. Pseudocyphellae pale, round or oval ..... *R. fraxinea*

+ Thallus 2–7 cm long, erect to pendent. Lobes often strongly or slightly branching, flat, rather thin, with outgrowths along the edges, lower surface with protruding ribs and slit-like white pseudocyphellae between them. Apothecia terminal or subterminal. Spores broad ellipsoid 12–14 × 4–6 µm ... *R. sinensis*

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