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Willdenowia
Annals of the Botanic Garden and Botanical Museum Berlin-Dahlem

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# A new corticolous *Megaspora* (*Megasporaceae*) species from Armenia

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**Abstract:** The corticolous species *Megaspora cretacea* is described as new for science. The species is characterized by a thick, cretaceous thallus and a pale bluish, rather coarse soredia covering most of the thallus. It grows on *Juniperus* bark in open arid woodlands in Armenia. A key to the three species included in the genus *Megaspora* is presented. Phylogenetic analysis based on nrITS sequences revealed that *M. cretacea* clustered within the *Megaspora* clade as sister species to *M. rimisorediata* with high support.

**Key words:** lichens, *Megasporaceae*, *Megaspora*, taxonomy, new species, sorediate, *Juniperus*, South Caucasus, Armenia, Khosrov Forest State Reserve, ITS

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## Introduction

According to recent phylogenetic studies, *Megasporaceae* Lumbsch is monophyletic (Nordin & al. 2010). They are mostly saxicolous crustose lichens (Valadbeigi & al. 2011). In Armenia, they are among the more common lichen families, in species diversity but especially in abundance, covering large parts of most siliceous rock faces and also present on limestone. The genus *Megaspora* (Clauzade & Cl. Roux) Hafellner & V. Wirth is closely related to the genus *Circinaria* Link (Nordin & al. 2010). It is an exception within the family, in that it is predominantly corticolous, with two species on trees, one of which is also occasionally terricolous. Both currently accepted species, *M. rimisorediata* Valadbeigi & A. Nordin and *M. verrucosa* (Ach.) Hafellner & V. Wirth (Valadbeigi & al. 2011), occur in Armenia (Gasparyan & Sipman 2013; Harutyunyan & al. 2011).

During a lichenological excursion to Armenia, organized by the second author, we collected a sorediate crustose lichen at the bases of trees of *Juniperus polycarpos* K. Koch in the Khosrov Forest State Reserve. The territory of the Reserve was already considered as a protected area in the fourth century C.E. by the Armenian king Khosrov Kotak (Khanjyan 2004). In 1958, the Khosrov Forest was officially declared as a state reserve (Anonymous 2008). The natural landscapes of phryganoid vegetation, open arid forests and montane steppes have high biological diversity and are recognized as a priority area for conservation. So far, 1849 species of vascular plants (including 24 endemic species) and 176 lichenized and lichenicolous fungi have been registered in the reserve (Anonymous 2008; Gasparyan & al. 2015).

While in the field it was not possible to recognize the collected specimens as representatives of *Megasporaceae*; rather they gave the impression of a species of the

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*Caloplaca albolutescens* (Nyl.) H. Olivier / *C. teicholyta* (Ach.) J. Steiner group or, less likely, a species of *Lepraria* Ach., but subsequent examination of the material revealed a few black apothecia immersed in the thallus, with large, thin-walled ascospores and a greenish epihymenium, suggesting *Megasporaceae*.

In the framework of a phylogenetic study of Asian *Megasporaceae*, the first author sequenced the material and found that it clusters inside *Megaspora* as a sister species to *M. rimisorediata.* Therefore, we describe it as a new species in this genus.

*Megaspora rimisorediata* has a restricted distribution. It was described from Iran (Valadbeigi & al. 2011) and later found also in S Armenia (Gasparyan & Sipman 2013; Gasparyan & al. 2015). *Megaspora verrucosa* has been reported from Europe, Africa, Asia, North and South America, New Zealand and Antarctica (Smith & al. 2009).

Currently, Armenia is the centre of diversity of the genus, with all three currently known species present. The new species has been reported from two localities. Further comprehensive studies are required to explore distributional and ecological patterns of the new species.

## Material and methods

Identification and descriptive work was carried out in Soest and BGBM using an Olympus SZX7 stereomicroscope and an Olympus BX50 compound microscope with interference contrast, connected to a Nikon Coolpix digital camera. Sections were mounted in tap water, in which also all measurements were taken. The specimens from this study are preserved in ABL and B (herbarium codes after Thiers 2016+). The chemistry of the type specimen was investigated by thin-layer chromatography (TLC) using solvent A (Orange & al. 2001).

*DNA extraction* — We used nuclear ITS1-5.8S-ITS2 rDNA sequences of specimens in the molecular study because it has been shown that among the regions of the ribosomal cistron, the internal transcribed spacer (ITS) region has the highest probability of successful identification for a range of fungi (Schoch & al. 2012; Divakar & al. 2015). Total DNA was extracted from freshly collected material according to Park & al. (2014). We followed the instructions given in that paper except for the following steps: we used a 1 × 1 $mm^2$ piece of medulla and mixed it with beadbeader without liquid nitrogen; instead of chloroform we used Roti®-C/I (chloroform/isoamyl alcohol at a ratio of 24:1); and at the end we used only 30 µL TE buffer instead of 100 µL because of the low quantity of DNA.

*PCR amplifications and sequencing* — The primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White & al. 1990) was used for the PCR amplifications. PCR amplifications were performed in a 12.5 µL volume containing 2 µL undiluted DNA, 0.5 µL of each primer (10 mM), 6.4 µL of sterile water, 1 µL dNTP (2 mM), 1 µL s-buffer, 1 µL MgCl2, 0.1 µL Taq-polymerase. Thermal cycling parameters were initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 sec at 95 °C, 30 sec at 54 °C, and 1 min at 72 °C; following the last cycle a final extensions for 3 min at 72 °C was included. Amplification product was viewed by electrophoresis on 1% agarose gels and stained with ethidiumboromide and was purified by adding 2 µL ExoSAP-IT™ (Exonuclease 1-shrimp alkaline phosphatase) to 5 µL of the PCR products, followed by a heat treatment of 15 min at 37 °C and 15 min at 80 °C. The PCR product was sequenced in both directions by Bik-F Laboratory in Frankfurt am Main. For the reconstruction of a phylogenetic tree, all ITS sequences of *Megasporaceae* from Valadbeigi & al. (2011) were used as well as seven accessible sequences of *Megaspora* from NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Two sequences were obtained from the new species and submitted to the NCBI GenBank (Table 1). The sequences were aligned through the Muscle V4 program web server (Edgar 2004) with the default settings. The aligned sequences were adjusted manually in PhyDE software (Müller & al. 2010). Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) was used to eliminate ambiguously aligned positions, applying settings allowing for smaller final blocks, gap position within the final blocks and less strict flanking position (Castresana 2000).

*Phylogenetic analyses* — MrModeltest (Nylander 2004) was used to determine the most appropriate model using AIC, with GTR + I + G found to be the best-fitting model of nucleotide evolution. Bayesian inference of phylogeny with Markov chain Monte Carlo sampling was performed on the Bayesian inference of phylogeny with Markov chain Monte Carlo sampling was performed on the 477 unambiguously aligned nucleotide positions. Bayesian analyses were conducted with MrBayes v. 3.2.2 (Ronquist & Huelsenbeck 2003) using the GTR model of nucleotide substitution including a proportion of invariable sites and a discrete gamma distribution with six rate categories. Two independent runs, each with four Metropolis-Coupled Markov Chain Monte Carlo chains and a temperature of 0.2 were initiated and run for 1 000 000 generations, with tree and parameter sampling every 100 generations. Burn-in was set to discard 25 % of samples. Maximum parsimonious trees (MPs) were reconstructed in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2003) using the heuristic search option with 100 random sequence additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Alignment gaps were treated as missing and all characters were unordered and of equal weight. The robustness of the trees obtained was evaluated by 1000 bootstrap replications with ten random sequence additions. Molecular Evolutionary Genetics Analysis software (MEGA ver-

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Table 1. Voucher specimens and NCBI GenBank accession numbers of the ITS sequences used in the phylogenetic analyses.

| Taxon | Locality, voucher | GenBank acc. no. |
|---|---|---|
| *Aspicilia cinerea* | Sweden, Dalarna, *Hermansson 13275* (UPS) | EU057899 |
| *Aspicilia indissimilis* | Sweden, *Nordin 5943* (UPS) | EU057909 |
| *Aspicilia laevata* | Sweden, *Tibell 23659* (UPS) | EU057910 |
| *Circinaria calcarea* | Sweden, *Nordin 5888* (UPS) | EU057898 |
| *Circinaria contorta* | Sweden, *Nordin 5895* (UPS) | EU057900 |
| *Circinaria leprosescens* | Sweden, *Nordin 5906* (UPS) | EU057911 |
| *Lobothallia melanaspis* | Norway, *Own-Larsson 8943a* (UPS) | JF825524 |
| *Lobothallia radiosa* | Sweden, *Nordin 5889* (UPS) | JF703124 |
| *Megaspora cretacea* | Armenia, *Aptroot 73835* (B) | KX253974 |
| *Megaspora cretacea* | Armenia, *Gasparyan 600199170* (B) | KX253975 |
| *Megaspora rimisorediata* | Iran, *Valadbeigi 2250* (TARI) | JF825525 |
| *Megaspora rimisorediata* | China, Xinjiang, *XJU 20116002* | KT443790 |
| *Megaspora rimisorediata* | China, Xinjiang, *XJU 20136001* | KT443789 |
| *Megaspora rimisorediata* | China, Xinjiang, *XJU 20111617* | KT443788 |
| *Megaspora rimisorediata* | China, Xinjiang, *XJU 91815043* | KT443787 |
| *Megaspora verrucosa* | Austria, *Trinkaus* (GZU) | AF332121 |
| *Megaspora verrucosa* | Austria, *Hafellner 48544 & Ivanova* (GZU) | AF332122 |
| *Megaspora verrucosa* | China, Xinjiang, *XJU 200753* | KT443786 |
| *Megaspora verrucosa* | China, Xinjiang, *XJU 20000724* | KT443785 |
| *Megaspora verrucosa* | U.S.A., Colorado, *St. Clair C54042* (BRY) | KC667053 |
| *Sagedia mastrucata* | Norway, Troms, *Nordin 5708* (UPS) | EU057913 |
| *Sagedia simoensis* | Sweden, *Own-Larsson 9000* (UPS) | EU057926 |
| *Sagedia zonata* | Sweden, *Nordin 5932* (UPS) | EU057949 |
| *Ochrolechia parella* | France, Brittany, *Feige* (ESS-20864) | AF329174 |

sion 7.0) was used to reconstruct the Maximum Likelihood phylogenetic tree based on the GTR + I + G model (Nei & Kumar 2000; Kumar & al. 2016). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A GTR model of nucleotide substitution including a proportion of invariable sites and a discrete gamma distribution with five rate categories (GTR + I + G) were used in Maximum Likelihood approach.

## Results

### *Phylogeny*

The maximum parsimony analysis resulted 12 most parsimonious trees with 513 steps, consistency index (CI) = 0.591, retention index (RI) = 0.690, rescaled consistency index (RC) = 0.407 and homoplasy index (HI) = 0.409. The Maximum Likelihood analysis resulted a tree with the highest log likelihood (-2014.3274). Majority rule consensus tree for maximum parsimony analysis was congruent with the tree obtained by Bayesian and maximum likelihood phylogenetic inference. The majority rule consensus tree of Bayesian analysis is shown here (Fig. 1) with posterior probabilities

Table 2. The main distinguishing characteristics of *Megaspora cretacea*, *M. rimisorediata* and *M. verrucosa*.

| | ***Megaspora cretacea*** | ***Megaspora rimisorediata*** | ***Megaspora verrucosa*** |
|---|---|---|---|
| Thallus | whitish grey, irregularly delimited to almost lobate | ochraceous to bluish grey, dense net of elongate cracks over thallus | white to grey-white, continuous to areolate to verrucose |
| Soredia | pale bluish grey, covering most of thallus, c. 100 µm in diam. | dark bluish green, produced along sides of elongate cracks, 50–70 µm in diam. | absent |
| Hymenium | not inspersed, c. 150 µm high | not inspersed, to 150 µm high | inspersed at times, 200–250 µm high |
| Paraphyses | unbranched | branched and anastomosing | branched but not anastomosing |
| Asci | 125–140 × 25–31 µm | c. 145 × 46 µm | 200–230 × 45–50 µm |
| Ascospores per ascus | 4 | 4–8 | 8 |
| Ascospores | 27–31 × 18–21 µm | 35–42 × 23–27 µm | 30–60 × 21–42 µm |
| Substrate | bark of *Juniperus* sp. | bark of *Juniperus* sp., *Quercus* sp. | soil, mosses, plant remains on calcareous rocks, bark |

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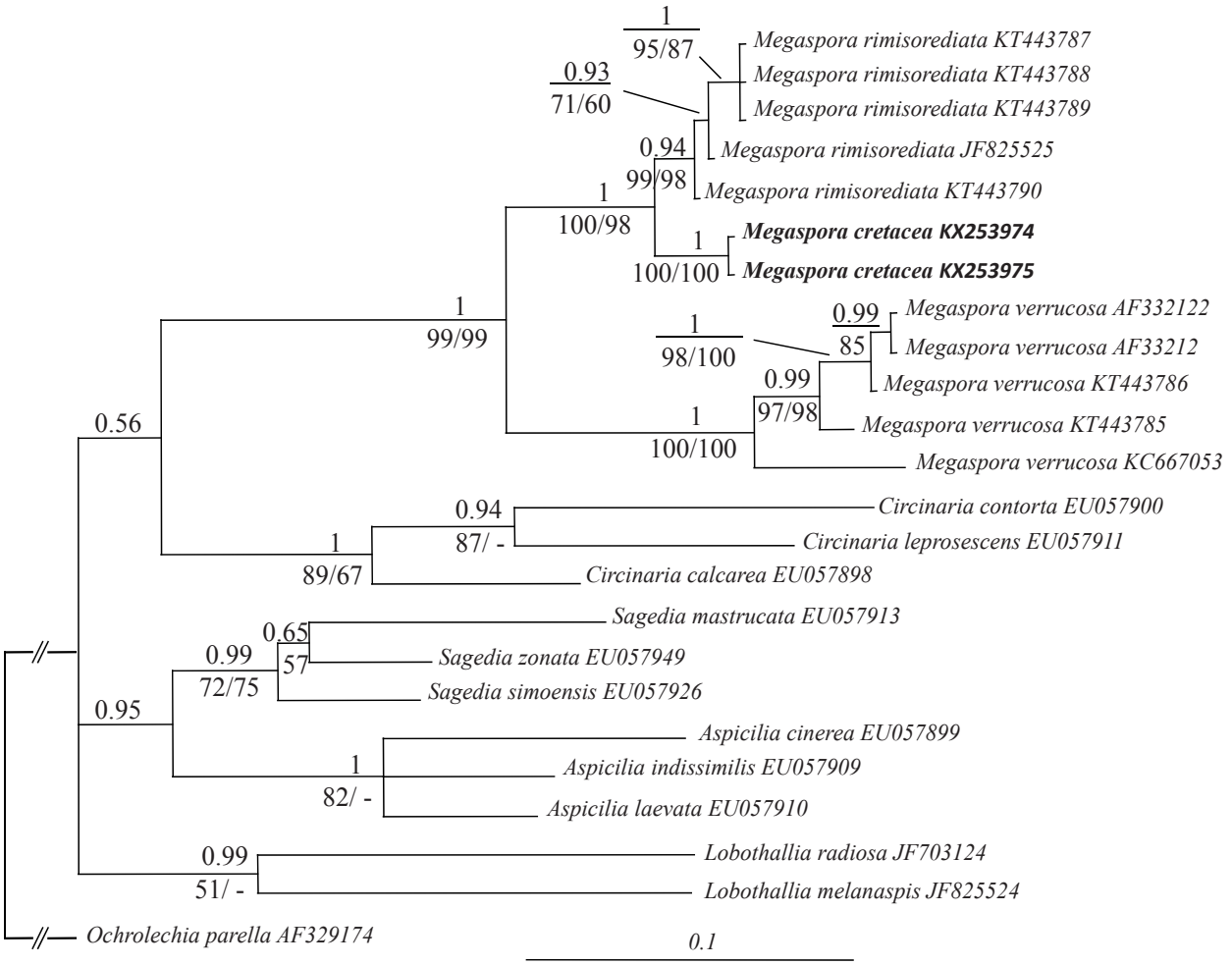



Fig. 1. Phylogenetic relationships of family *Megasporaceae* showing the consensus tree of the Bayesian analysis of the ITS dataset. Bayesian posterior probabilities are shown above the branches and MP/ML bootstrap values ≥ 50 are shown below the lines adjacent to the branches. Distance of outgroup and ingroup root is shortened three times.

of Bayesian analysis and bootstrap numbers of Maximum Parsimony and Maximum Likelihood analysis.

The molecular phylogenetic results confirmed affiliation of the new species to the genus *Megaspora*. It is clusters in a phylogenetic tree in *Megaspora*, as sister to *M. rimisorediata* (PP = 1; MP/ML BS= 100/100). The phylogenetic trees resulting from the three different analyses also confirmed *Megaspora* clade as a monophyletic group even after adding the new species samples (*M. cretacea*) with a high posterior probability and bootstrapping values (PP = 1; MP/ML BS = 99/99). Monophyly of species *M. verrucosa* and *M. rimisorediata* were confirmed with a high supporting values (PP = 1; MP/ML BS = 100/100 for *M. verrucosa* and PP = 0.94; MP/ML BS = 99/98 for *M. rimisorediata*).

### *Taxonomy*

***Megaspora cretacea*** Gasparayan, Zakeri & Aptroot, **sp. nov.** – MycoBank #817072 – Fig. 2A–C.

Holotype: Armenia, Ararat, Vedi, Urtsadzor, Khosrov Forest State Reserve, 40°00'42"N, 44°54'04"E, 1600 m, on *Juniperus polycarpos* bark, 17 Jun 2015, *A. Aptroot 73835* (B 600200932; isotypes: ABL, GLM).

*Diagnosis* — *Megaspora* with thallus whitish grey, cretaceous, fully sorediate with soredia c. 0.1 mm in diam.; apothecia sparse, immersed; ascospores 4 per ascus, broadly ellipsoid, 27–31 × 18–21 µm, hyaline, thin-walled.

*Description* — *Thallus* whitish grey, crustose, ecorticate, to 0.2 mm thick, irregularly delimited to almost lobate, occupying areas up to 5 cm in diam. *Medulla* white, cretaceous. *Soralia* covering most of thallus surface, pale bluish grey; soredia c. 100 µm in diam. *Photobiont* chlorococcoid. *Apothecia* sparse, dispersed, immersed in thallus, round, 0.3–0.5 mm in diam.; disc black, concave; margin black, raised above disc, incurved, c. 0.1 mm wide, with some crenations. *Hymenium* IKI+ blue, c. 150 µm high, not inspersed with oil droplets. *Subhymenium* hyaline. *Epihymenium* greenish, colour unchanged in KOH. *Hypothecium* hyaline. *Paraphyses* 2–2.5 µm thick, not branched. *Asci* clavate, 125–140 × 25–31 µm. *Ascospores* 4 per ascus, broadly ellipsoid, 27–31 × 18–21 µm, hya-

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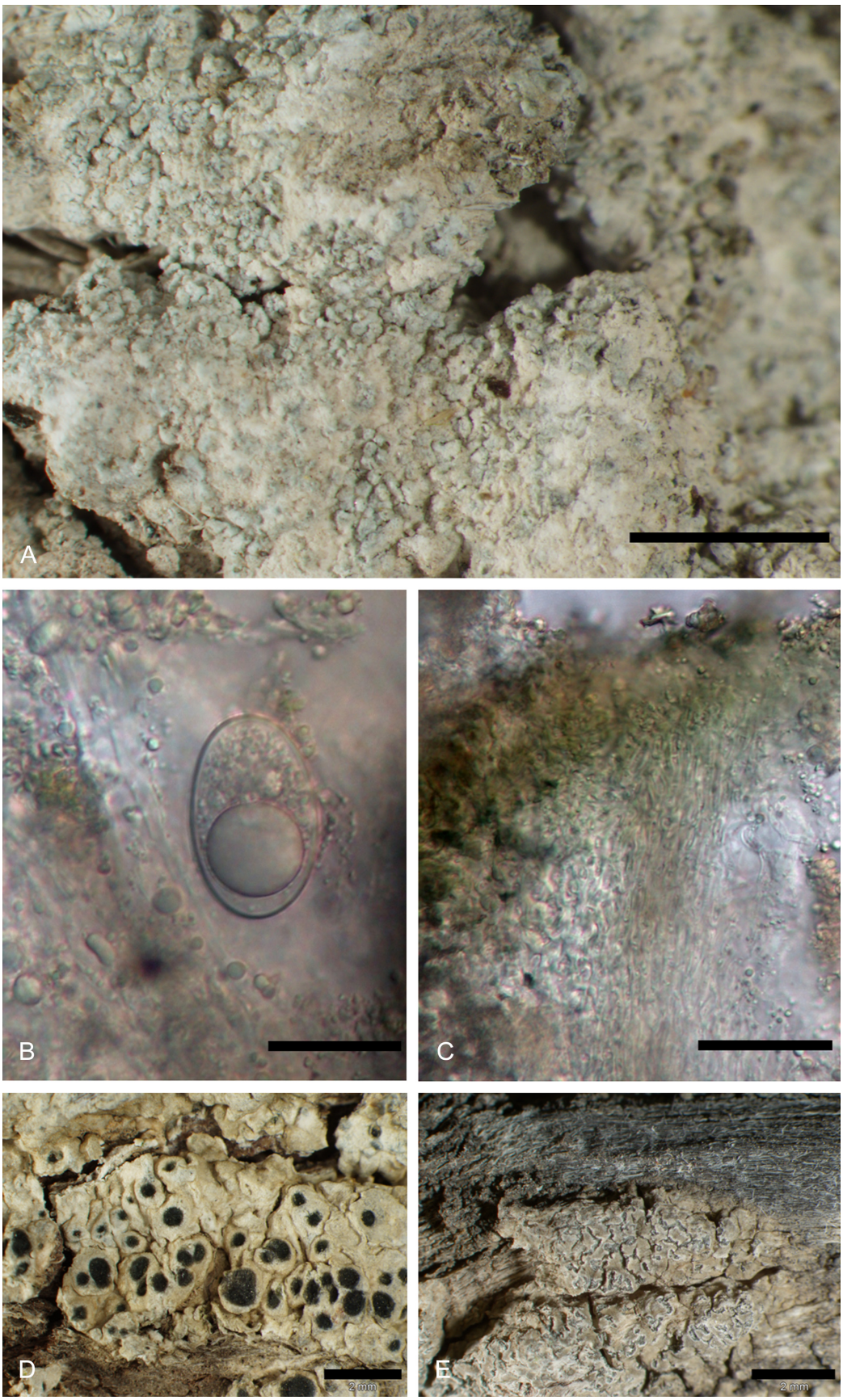



Fig. 2. A–C: *Megaspora cretacea*, holotype; A: thallus with soredia and apothecia; B: ascospore; C: hymenium (with excipulum at left). – D: *M. verrucosa* thallus with apothecia. – E: *M. rimisorediata* thallus with net of cracks and soredia. – Scale bars: A, D, E = 2 mm; B, C = 20 µm.

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line, thin-walled (less than 1 µm). *Pycnidia* not observed. *Conidia* not observed.

*Chemistry* — Thallus KOH-, C-, Pd-, UV-. TLC: No lichen substances detected.

*Distribution and ecology* — The species is known from two separate localities within the Khosrov Forest State Reserve, Armenia. It occurs on bases of trees of *Juniperus polycarpos* K. Koch in arid, open, montane forests. The forest ecosystems in the Khosrov Forest State Reserve, at 1400–2300 m, are generally dominated by oak trees (*Quercus macranthera* Fisch. & C. A. Mey. ex Hohen.) and sparse juniper (*J. polycarpos*) formations, accompanied by *Fraxinus excelsior* L., *Sorbus aucuparia* L., and species of *Acer* L. and *Pyrus* L. (Khanjyan 2004).

*Etymology* — The epithet is derived from word *cretaceus* (resembling chalk) in reference to the colour and texture of the thallus.

*Additional specimen examined* — Armenia: Ararat, Vedi, Urtsadzor, Khosrov Forest State Reserve, 39°59'07"N, 44°53'51"E, 1390 m, on *Juniperus polycarpos* bark, 17 Jun 2015, *A. Gasparyan* (B 600199170).

## Discussion

*Megaspora cretacea* is a morphologically distinctive species, from which the two other species of the genus, *M. verrucosa* (Fig. 2D) and *M. rimisorediata* (Fig. 2E), can be separated as follows (Table 2): *M. verrucosa* has no soredia, whereas *M. cretacea* and *M. rimisorediata* are both sorediate; the closely related *M. rimisorediata* differs from *M. cretacea* by the presence of a dense net of elongate cracks over the thallus, dark bluish green soredia, branched paraphyses and larger ascospores.

**Key to the species of *Megaspora***

1. Soredia absent . . . . . ***M. verrucosa***
– Soredia present . . . . . **2**
2. Thallus ochraceous to bluish grey with a dense net of elongate cracks; soredia produced along sides of elongate cracks, dark bluish green . . . . . ***M. rimisorediata***
– Thallus whitish grey, irregularly delimited to almost lobate; soredia covering most of thallus, pale bluish grey . . . . . ***M. cretacea***

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