

cryptogamie

Mycologie

2021 • 42 • 1

Analysis of lichen secondary chemistry doubled
the number of *Cetrelia* W.L. Culb. & C.F. Culb.
species (Parmeliaceae, lichenised
Ascomycota) in Hungary

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Cryptogamie, Mycologie est indexé dans / *Cryptogamie, Mycologie is indexed in:*

- Biological Abstracts
- Current Contents
- Science Citation Index
- Publications bibliographiques du CNRS (Pascal).

Cryptogamie, Mycologie est distribué en version électronique par / *Cryptogamie, Mycologie is distributed electronically by:*

- BioOne® (<http://www.bioone.org/loi/crym>)

Cryptogamie, Mycologie est une revue en flux continu publiée par les Publications scientifiques du Muséum, Paris
Cryptogamie, Mycologie is a fast track journal published by the Museum Science Press, Paris

Les Publications scientifiques du Muséum publient aussi / *The Museum Science Press also publishes: Adansonia, Geodiversitas, Zoosystema, Anthropozoologica, European Journal of Taxonomy, Naturae, Cryptogamie sous-sections Algologie, Bryologie, Comptes Rendus Palevol.*

Diffusion - Publications scientifiques Muséum national d'Histoire naturelle

CP 41 - 57 rue Cuvier F-75231 Paris cedex 05 (France)

Tél.: 33 (0)1 40 79 48 05 / Fax: 33 (0)1 40 79 38 40

diff.pub@mnhn.fr / <http://sciencepress.mnhn.fr>

© Publications scientifiques du Muséum national d'Histoire naturelle, Paris, 2021

ISSN (imprimé / print): 0181-1584/ ISSN (électronique / electronic): 1776-100

Analysis of lichen secondary chemistry doubled the number of *Cetrelia* W.L. Culb. & C.F. Culb. species (Parmeliaceae, lichenised Ascomycota) in Hungary

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Submitted on 3 April 2019 | Accepted on 6 January 2021 | Published on 2 February 2021

Farkas E., Biró B., Varga N., Sinigla M. & Lőkös L. 2021. — Analysis of lichen secondary chemistry doubled the number of *Cetrelia* W.L. Culb. & C.F. Culb. species (Parmeliaceae, lichenised Ascomycota) in Hungary. *Cryptogamie, Mycologie* 42 (1): 1-16. <https://doi.org/10.5252/cryptogamie-mycologie2021v42a1>. <http://cryptogamie.com/mycologie/42/1>

ABSTRACT

The distribution patterns of lichen secondary metabolites are often taxon specific. They represent cryptic chemical diversity additional to morphological-anatomical biodiversity. *Cetrelia* W.L. Culb. & C.F. Culb. species (*c.* 200 specimens) were checked and revised by thin-layer chromatography. Soredia, pseudocyphellae, rhizines, features of lower surface are the main morphological characters analysed against the presence of cortical pigment, atranorin and medullary α -alectoronic acid, anziaic acid, α -collatolic acid, β -alectoronic acid, β -collatolic acid, imbricatic acid, 4-O-demethylimbricatic acid, olivetoric acid, perlatolic acid, physodic acid and 4-O-methylphysodic acid. The European occurrence of *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. – described from America – is confirmed on the basis of identical secondary metabolite composition by seven lichen substances. Four species were revealed and mapped in Hungary. *Cetrelia chicitae* and *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb. are new for the Hungarian lichen flora. From the originally known two taxa *C. cetrarioides* (Delise) W.L. Culb. & C.F. Culb. proved to be rare, *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. is less frequent than regarded earlier. Currently *C. monachorum* is the most frequent *Cetrelia* species in Hungary. *Cetrelia chicitae* and *C. cetrarioides* may need protection measures. *Parmelia cetrarioides* f. *pseudofallax* (Gyeln.) Gyeln. is lectotypified here.

KEY WORDS

Chemotaxonomy,
geographical distribution,
high performance thin-layer
chromatography (HPTLC),
lichen-forming fungus,
lichen secondary
metabolites (LSMs),
lectotypification.

RÉSUMÉ

L'analyse de la chimie secondaire du lichen double le nombre d'espèces de Cetrelia W.L. Culb. & C.F. Culb. (Parmeliaceae, Ascomycota lichénisées) en Hongrie.

Les modèles de distribution des métabolites secondaires des lichens sont souvent des taxons spécifiques. Cela représente une diversité chimique qui s'ajoute à la biodiversité morphologique et anatomique. Les espèces de *Cetrelia* W.L. Culb. & C.F. Culb. (environ 200) furent contrôlées et révisées avec une chromatographie sur couche mince. Soredia, pseudocyphellae, rhizinae et les caractéristiques de la surface inférieure sont les principaux caractères morphologiques analysés par rapport à la présence de pigment cortical, d'atranorine et d'acide médullaire (acide α -alectoronique, acide anziaque, acide α -collatolique, acide β -alectoronique, acide β -collatolique, acide imbricarique, acide 4-O-déméthylimbricarique, acide olivetorique, acide perlatorique, acide physodique, acide 4-O-méthylphysodique) qui peuvent être trouvés dans les médullas. La présence de l'espèce *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. (décrite en Amérique) en Europe est prouvée sur la base de l'apparition de sept composants de lichens différents. Nous avons démontré et cartographié la présence de quatre espèces en Hongrie. Les espèces *C. chicitae* et *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb. sont nouvelles pour la flore lichenique Hongroise. L'espèce connue auparavant *C. cetrarioides* (Duby) W.L. Culb. & C.F. Culb. devient rare, et l'espèce *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. est moins fréquente qu'elle ne l'était. De nos jours *C. monachorum* est l'espèce la plus fréquente de ce genre en Hongrie. Les espèces *C. chicitae* et *C. cetrarioides* doivent être protégées. *Parmelia cetrarioides* f. *pseudofallax* (Gyeln.) Gyeln. est lectotypifié ici.

MOTS CLÉS

Chimiotaxonomie, distribution géographique, chromatographie en couche mince haute performance (CCMHP), lichen formant des fungus, lichen secondaire métabolite (LSM), lectotypification.

INTRODUCTION

Lichens produce approximately 1050 secondary metabolites, most of which are bioactive and unique to lichen-forming fungi (Stocker-Wörgötter 2008, 2015; Molnár & Farkas 2010). These chemically diverse (aliphatic and aromatic) lichen substances are produced by the mycobiont (Elix 1996; Huneck 1999). They are accumulated in the cortical (e.g. atranorin) and medullary layers (e.g. imbricariac, perlatic or olivetoric acid and others) as extracellular crystals on the outer surfaces of the hyphae. Lichen secondary chemistry analyses (e.g. Bjerke *et al.* 2005; Wang *et al.* 2009; Duong *et al.* 2017; Brakni *et al.* 2018) – and study of their relation to environmental conditions (cf. Armaleo *et al.* 2008; Hauck *et al.* 2009; Hauck 2011) – clarify the background of the chemical diversity of the studied taxa. The production of lichen secondary metabolites (LSMs) is genetically controlled (Culberson & Culberson 2001), and often correlated with morphology and geography at the species and genus levels at different scales (Egan 1986; Zhou *et al.* 2006; Matteucci *et al.* 2017). The distribution patterns of taxa characterised by LSMs have been widely used in lichen taxonomy and systematics since they represent cryptic chemical diversity additional to morphological-anatomical biodiversity (Crespo & Lumbsch 2010). Thanks to standardised chromatographic studies (TLC – Culberson & Kristinsson 1970; Culberson 1972, 1974; HPTLC – Arup *et al.* 1993) the knowledge of LSMs has developed worldwide largely since the 1970s. Application of HPTLC was introduced for analysing samples of Hungarian herbaria later in 1998 (Farkas *et al.* 1998) contributing to morphological investigations during identifications and revisions (Biró *et al.* 2015; Farkas & Biró 2015; Farkas *et al.* 2016).

The role of LSMs in taxonomic identification of lichens has already been studied by scientists of the 19th century (cf.

Nylander 1866a, b; Hawksworth 1976; Carlin 1987). Since the occurrence and composition of secondary metabolites are often taxon specific, they have been widely used in lichen taxonomy and systematics in several groups of lichens (e.g. Culberson 1969, 1970, 1986a, b; Schmitt & Lumbsch 2004; Nelsen & Gargas 2008; Leavitt *et al.* 2011). Though the number of LSMs produced by lichens varies from 1–3 to 12, not the number, but the composition of LSMs characterises them better (Elix 1996). The presence/absence and composition of LSMs were considered at various levels of taxa from chemosyndromes, chemical races – via species – to families (Hawksworth 1976; Nourish & Oliver 1976; Randlane *et al.* 2009; Osyczka & Skubala 2011; Lendemmer 2012).

Differences in secondary chemistry within the genus *Cetrelia* W.L. Culb. & C.F. Culb. (Parmeliaceae, lichenised Ascomycota) were also considered at various taxonomic levels of taxa from chemical varieties to species level. Though their treatment as species is becoming widely accepted by now, there are other opinions in publications until quite recent years. Thell & Kärnefelt (2011) treated all three Nordic *Cetrelia* species under one name, *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb., thus the chemically different species *C. cetrarioides* (Duby) W.L. Culb. & C.F. Culb. and *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb. were listed as synonyms. Olivetoric acid (“*C. olivetorum* s. str.”), perlatic acid (“*C. cetrarioides*”) and imbricariac acid (“*C. monachorum*”) containing chemotype specimens are mentioned in the paragraph “Chemistry”. Similar taxonomic status and nomenclature are followed by Roux *et al.* (2017) for chemotypes, as *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. chémo. *olivetorum*, *C. olivetorum* chémo. *cetrarioides*, *C. olivetorum* chémo. *monachorum*, and *C. olivetorum* chémo. *chicitae*.

In Britain (Smith *et al.* 2009) similarly only *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. *s.l.* is mentioned with four

chemotypes characterised by spot reactions. However, Wirth *et al.* (2013) characterise four species mentioning them together as *C. olivetorum* s.l. from Germany. Stenroos *et al.* (2011, 2016) differentiated two species, *C. cetrarioides* and *C. olivetorum* on morphological (pseudocyphellae, soredia) and chemical basis (spot reactions, LSMs) in Finland. Degtjarenko *et al.* (2018) reported – with illustrations of TLC plates – that three species (*C. cetrarioides*, *C. monachorum*, *C. olivetorum*) are known from Estonia after revising specimens kept under different names.

According to the latest knowledge, the genus *Cetrelia* includes 18 species worldwide (Randlane *et al.* 2013; Mishra & Upreti 2015). Some of them are morphologically close to *Parmotrema*, *Platismatia* and *Punctelia* species. *Cetrelia* species are characterised by foliose thallus with laminal pseudocyphellae on the grey upper cortex, and on the mostly black and brown lower cortex with few rhizines. Apothecia are often lacking, therefore vegetative propagules (soredia, isidia), pseudocyphellae, rhizines, features of lower surface are the main morphological characters available for differentiating taxa (Fig. 1A–C). However, the genus is chemically diverse containing cortical pigment, atranorin and medullary substances: α -alectoronic acid, anziaic acid, α -collatolic acid, β -alectoronic acid, β -collatolic acid, imbricatic acid, 4-O-demethylimbricatic acid, microphyllinic acid, olivetoric acid, 4-O-methylolivetoric acid, perlatolic acid, physodic acid and 4-O-methylphysodic acid. Following the combined chemical and morphological species concept of Culberson & Culberson (1968) several revisions have been published from various parts of Europe (Randlane & Saag 1991; Obermayer & Mayrhofer 2007; Kukwa & Motiejūnaitė 2012; Kukwa *et al.* 2012; Wirth *et al.* 2013; Bely *et al.* 2014). Molecular genetic studies treating cetrarioid taxa confirmed, to some extent, chemical species of *Cetrelia* (Thell *et al.* 2002; Nelsen *et al.* 2011). Randlane *et al.* (2013) compiled a list of cetrarioid lichens, including all chemical species of *Cetrelia*, with updated phylogenetic information, Mishra & Upreti (2015), treating *Cetrelia* from India, prepared a worldwide key for the 18 *Cetrelia* species.

Recently Mark *et al.* (2018) analysed a dataset of four nuclear markers (ITS, IGS, Mcm7, RPB1) from 62 specimens within Bayesian and maximum likelihood frameworks and justified that chemotypes, distinguished according to the major medullary substance, clearly correlated with clades recovered within *Cetrelia*. The traditional species were generally monophyletic, with the exception of *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. and *C. pseudolivetorum* (Asahina) W.L. Culb. & C.F. Culb. However, delimiting *Cetrelia* species based only on reproductive morphology was not supported phylogenetically.

Since *C. chicitae* was described from the United States (West Virginia, Pocahontas Co., Gaudiner Knob., Hale, Lich. Amer. Exs. 56 [holotype-DUKE]) and no American specimens were studied by Mark *et al.* (2018) American and European herbarium specimens available in BP and VBI were included together in the present study.

It seems that *Cetrelia cetrarioides*, *C. chicitae*, *C. monachorum* and *C. olivetorum* occur in a considerable part of Europe, at least in the so far studied geographical regions in various

distance and direction from Hungary. The above-mentioned European studies contain very few or no data from Hungary. Therefore, our main aim was to improve the knowledge on the European distribution of *Cetrelia* species with Hungarian records via taxonomic revision of the c. 150 available Hungarian specimens. Up to now a former taxonomic concept (Wirth 1980) was followed in Hungary with two taxa treated at the level of variety of *C. olivetorum* (Verseghy 1965, 1973, 1988, 1994; Kiszelyné-Vámosi 1983; Seaward *et al.* 1985; Farkas 1990; Lőkös & Farkas 1997; Lőkös *et al.* 1997; Lőkös 2009, 2010; Lőkös & Balogh 2016). We were also interested if all the four species occur also in Hungary or the surrounding high mountains (Alps and Carpathians) make a kind of barrier for the distribution of some of these species. Since only spot reactions (hypochlorite probe: C+/-) were applied during identification process earlier, which allowed only an uncertain identification of olivetoric acid, present in *C. olivetorum* var. *olivetorum* and lacking in *C. olivetorum* var. *cetrarioides* (Fig. 1D), HPTLC method was essential for the chemical revision in this study.

The commonest substrate of these species is bark, both of deciduous and coniferous trees, but they seldom occur also on various, mossy rocks. Our further aim was to establish if there is any difference in habitat or substrate preferences of the species in Hungary.

Obermayer & Mayrhofer (2007) gave a detailed description of morphological characters of the species based on Central European, mostly Austrian specimens. Concerning to morphological characters we were mostly focused on the range of soredium size, since, according to Obermayer & Mayrhofer (2007), soredia differ between species, i.e., *C. cetrarioides* has fine soredia, *C. chicitae* and *C. monachorum* have coarse soredia, whereas both types are reported for *C. olivetorum*.

Before the revision, *Cetrelia olivetorum* (including both of these varieties at that time) was regarded a vulnerable species (Lőkös & Tóth 1997), thus we also planned to make an improved evaluation of *Cetrelia* species in red list categories or to make suggestions for legal protection in Hungary.

MATERIAL AND METHODS

THE RESEARCH OBJECT

The genus *Cetrelia* is characterised in detail in, for example, Smith *et al.* (2009), Wirth *et al.* (2013), Kukwa *et al.* (2012) and Obermayer & Mayrhofer (2007). The Hungarian *Cetrelia* species were originally identified under various names: e.g. *Parmelia cetrarioides* Delise, *Parmelia cetrarioides* var. *typica* Du Rietz, *Parmelia cetrarioides* f. *pseudofallax* (Gyeln.) Gyeln., *Parmelia cetrarioides* f. *sorediosa* (DC.) Fr., *Parmelia olivaria* Th. Fr., *Parmelia perlata* (Huds.) Ach. or *Parmelia trichotera* Hue (cf. Hazslinszky 1868, 1884; Borbás 1879; Boros 1925, 1929, 1931; Szatala 1925, 1926; Timkó 1925; Gyelnik 1926, 1928, 1931, 1933, 1934, 1937; Fóris 1938, 1940; Kiszely 1968; Gallé 1975). From the 1980s they were kept in herbaria based on nomenclature applied in Verseghy (1994) under “*C. cetrarioides*” (as *C. olivetorum* var. *cetrari-*

TABLE 1. — Rf values of lichen secondary metabolites (LSMs) related to norstictic acid (N) and atranorin (A) in solvent systems A, B and C (cf. Arup *et al.* 1993).

LSM	Rf _{LSM} /Rf _N , Rf _A	Rf _{LSM} /Rf _N , Rf _A	Rf _{LSM} /Rf _N , Rf _A
	in solvent system A	in solvent system B	in solvent system C
–			
atranorin	36	30	28
perlatolic acid	30	32	24
imbricatic acid	30	32	22
4-O-methylphysodic acid	–	19	21
α -collatolic acid	24	21	18
anziaic acid	–	–	16
4-O-demethylimbricatic acid	–	21	15
β -collatolic acid	–	–	13
olivetic acid	27	24	11
physodic acid	18	18	10
α -alectoronic acid	21	16	7
β -alectoronic acid	–	–	3

oides) and “*C. olivetorum*” (as *C. olivetorum* var. *olivetorum*). Specimens of bcMRDS, BP, BRA, BTM, CL, DE, EGR, JPU, SAMU, SZE, and VBI, herbaria [abbreviations mainly according to Thiers (continuously updated)] were revised by HPTLC and microcrystal tests. Altogether 166 specimens were studied from Hungary, 98 specimens were kept in the lichen collection BP (Budapest), furthermore 68 specimens were investigated from the other herbaria. One specimen of GZU mentioned from Hungary in literature (Obermayer & Mayrhofer 2007) was also considered. A further nine specimens of *C. chicitae* (8 BP, 1 VBI) from United States and Europe were investigated.

MORPHOLOGICAL INVESTIGATIONS

Soredia, pseudocyphellae, rhizines, as well as features of lower surface were investigated as the main morphological characters by stereo microscope following Obermayer & Mayrhofer (2007).

The morphology and anatomy were studied by using a NIKON Eclipse/NiU (DIC, epifluorescence) compound microscope, Nikon SMZ18 stereo microscope as well as Olympus SZX9 and Olympus BX50 (DIC) microscopes. Micrographs were prepared by Olympus E450 camera (with Quick Photo Camera 2.3 software) and Nikon DS-Fi1c and Fi3 camera (with NIS-Elements BR ML software) with the above-mentioned microscopes. If it was possible, 5–10 specimens by species were checked for measurements of soredia. Usually 15 micrographs of their sorediate lobe margins were taken. Diameters of soredia ($n = 20$) were measured on each views. Mean and standard deviation values were also established.

PRESENCE OF LICHEN SECONDARY METABOLITES

The presence of cortical pigment, atranorin and medullary LSMs: α -alectoronic acid, anziaic acid, α -collatolic acid, β -alectoronic acid, β -collatolic acid, imbricatic acid, 4-O-demethylimbricatic acid, olivetic acid, perlatolic acid, physodic

acid and 4-O-methylphysodic acid were analysed by HPTLC (Fig. 2) and TLC (Fig. 3).

The cortical pigment atranorin is usually present in all species, but in some specimens this substance is present in trace amount (e.g. *C. cetrarioides*, coll. G. Matus, 2013, DE; *C. monachorum*, coll. M. Sinigla, 2015, BTM; coll. L. Lőkös, 2015, BP; coll. G. Matus, 2016) or lacking (e.g. *C. cetrarioides*, coll. E. Farkas and L. Lőkös, 1983, VBI, *C. chicitae*, coll. A. Feichtinger, 1875, BP; coll. A. Vězda, 1990, VBI).

CHEMICALS

All chemicals were of analytical or higher grade. HPLC acetone (VWR) was applied to extract LSMs from intact lichen samples for chromatographic analysis. Glycerine (ACIDUM-2), toluene (CARLO ERBA), acetic acid (LACH-NER), dioxene (REANAL, Sigma Aldrich), cyclo-hexane (LACH-NER), methyl-tert-butyl ether (Fisher Scientific UK), formic acid (LACH-NER), anisaldehyde (Sigma Aldrich), methanol (REANAL) and sulphuric acid (CARLO ERBA) were obtained from Reanal for TLC and HPTLC investigations and microcrystal tests.

MICROCRYSTAL TESTS

Microcrystal tests were prepared in GE solvent (glycerine – acetic acid, 3:1 v/v) following the methods described in Orange *et al.* (2010: 41–44). Microscopical views were compared to those published by Obermayer & Mayrhofer (2007) and Huneck & Yoshimura (1996).

Application of microcrystal test was especially useful for confirmation of the presence of imbricatic acid (Fig. 4A) and perlatolic acid (Fig. 4B). We could identify also olivetic acid (Fig. 4C) and atranorin (Fig. 4D) by this method.

HPTLC METHOD

HPTLC analysis was carried out according to standard methods for analysing lichen samples described by Arup *et al.* (1993) and Molnár & Farkas (2011). CAMAG horizontal chamber of 10 × 10 cm, CAMAG TLC Plate Heater III, 10 × 10 cm HPTLC plates (Merck, Kieselgel 60 F254) were used. The usual solvent systems (A: toluene – dioxene – acetic acid, 45:15:2 v/v/v; B: cyclo-hexane – methyl-tert-butyl ether – formic acid, 6.5:5:1 v/v/v) and most often solvent system C (toluene – acetic acid, 20:3 v/v) were applied. Plates were investigated under UV 254 nm and UV 366 nm after development, then fatty acids and water-repellent substances were studied, while sprayed with water, finally it was followed by spraying with 10% sulphuric acid and spots were observed at daylight.

The presence of α -alectoronic acid, anziaic acid, α -collatolic acid, atranorin, β -alectoronic acid, β -collatolic acid, imbricatic acid, 4-O-demethylimbricatic acid, olivetic acid, perlatolic acid, physodic acid and 4-O-methylphysodic acid was investigated. Rf values of some of these LSMs are not given or slightly differing in Table 1 of Arup *et al.* (1993), therefore our data are summarised in Table 1 below. The presence of above LSMs in *Cetrelia* species is summarised in Table 2.

TABLE 2. — Presence of LSMs in *Cetrelia* W.L. Culb. & C.F. Culb. species (cf. Obermayer & Mayrhofer 2007). + = present; (+) = present in minor amount.

LSM/Species	<i>C. cetrarioides</i> (Delise)	<i>C. chicitae</i> (W.L. Culb.)	<i>C. monachorum</i> (Zahlbr.)	<i>C. olivetorum</i> (Nyl.)
	W.L. Culb. & C.F. Culb.	W.L. Culb. & C.F. Culb.	W.L. Culb. & C.F. Culb.	W.L. Culb. & C.F. Culb.
atranorin (atr)	+	+	+	+
perlatolic acid (perl)	+	–	(+)	–
imbricatic acid (imbr)	(+)	–	+	–
4-O-methylphysodic acid (4mp)	–	+	–	–
α -collatolic acid (α -col)	–	+	–	–
anziaic acid (anz)	+	–	+	–
4-O-demethylimbricatic acid (4dmi)	–	–	+	–
β -collatolic acid (β -col)	–	+	–	–
olivetoric acid (ol)	–	–	–	+
physodic acid (phys)	–	+	–	–
α -alecoronic acid (α -alec)	–	+	–	–
β -alecoronic acid (β -alec)	–	(+)	–	–

TLC METHOD

TLC analysis was carried out according to standard methods for analysing lichen samples described by Orange *et al.* (2010). 20 × 20 cm thin-layer chromatographic plates (Merck, Kieselgel 60 F254) were used. Solvent system C (toluene – acetic acid, 170:30 v/v) was applied according to Mietzsch *et al.* (1994) and Elix (2014). Plates were investigated under UV 254 nm and UV 366 nm after development, it was followed by spraying with 10% anisaldehyde/sulphuric acid and spots were observed at daylight. The presence of α -alecoronic acid, anziaic acid, α -collatolic acid, atranorin, β -alecoronic acid, β -collatolic acid, imbricatic acid, 4-O-demethylimbricatic acid, olivetoric acid, perlatolic acid, physodic acid and 4-O-methylphysodic acid was investigated. Rf values were compared to published data (Orange *et al.* 2010 and Elix 2014). The presence of above LSMs in *Cetrelia* species is summarised in Table 2. Special attention was given to the *C. chicitae* samples.

DISTRIBUTION MAPS

Distribution maps were constructed by the computer program for geographical information system, QGIS 3.8 ‘Zanzibar’, released in 2019, applying an adaptation of the Central European grid system (Niklfeld 1971; Borhidi 1984). The symbols (dots, circles) illustrated represent units of c. 5 × 6 km areas. Maps presenting distribution before and after revision were compared.

ABBREVIATIONS

LSM lichen secondary metabolite;
Rf retardation factor – here applied according to Arup *et al.* (1993): travelling distance expressed in mm for the studied LSM (Rf_{LSM}) compared to controls norstictic acid (Rf_N) and atranorin (Rf_A).

RESULTS AND DISCUSSION

Our results, based on morphological characters and LSMs, show that four species occur in Hungary as in several other European countries: *Cetrelia cetrarioides* (Fig. 5A, 16 specimens),

C. chicitae (Fig. 5B, two specimens), *C. monachorum* (Fig. 5C, 100 specimens) and *C. olivetorum* (Fig. 5D, 29 specimens). *Cetrelia chicitae* and *C. monachorum* were discovered as new species for the Hungarian lichen flora.

Cetrelia cetrarioides (Delise) W.L. Culb. & C.F. Culb.
(Figs 1C; 2; 5A; 6; 7; 12)

It is characterised by the presence of atranorin, perlatolic acid (major), \pm imbricatic acid (minor) and anziaic acid. The lack of 4-O-demethylimbricatic acid is obvious and well observable already under UV 254 nm after developing the HPTLC plate (Fig. 2). Its soredia are fine [25–35(–40) μ m in diam. – Obermayer & Mayrhofer (2007)], $32.3 \pm 3.4 \mu$ m in Hungarian samples. Not or slightly raised pseudocyphellae of various size (c. 30–300 μ m) occur on upper cortex, smaller ones on lower cortex. Its specimens were found most frequently on oak (*Quercus* sp. – 38%), beech (*Fagus sylvatica* L. – 15%) and other tree species (46%) at lower elevations than in Austria (Obermayer & Mayrhofer 2007), mostly at 200–600 m a.s.l., though in Belarus it occurs at even lower elevation (Bely *et al.* 2014). This species proved to be less frequent than expected (Figs 6; 7), and was previously confused with *C. monachorum*. *Cetrelia cetrarioides* is considered here as a critically endangered (CR) species in Hungary.

C. chicitae (W.L. Culb.) W.L. Culb. & C.F. Culb.
(Figs 1A; 2; 3; 5B; 8; 12)

It is easy to recognise by a here revised characteristic composition of LSMs detected by HPTLC and TLC: atranorin, 4-O-methylphysodic acid, α -collatolic acid, β -collatolic acid, physodic acid, α -alecoronic acid, β -alecoronic acid. Since this species was described from the United States (West Virginia, Pocahontas Co., Gaudiner Knob, Hale, Lich. Amer. Exs. 56 – holotype-DUKE) and has not been confirmed from Europe by Mark *et al.* (2018), special attention was paid to its identification. All specimens available in BP and VBI were studied by TLC and HPTLC (Fig. 2). In addition to the

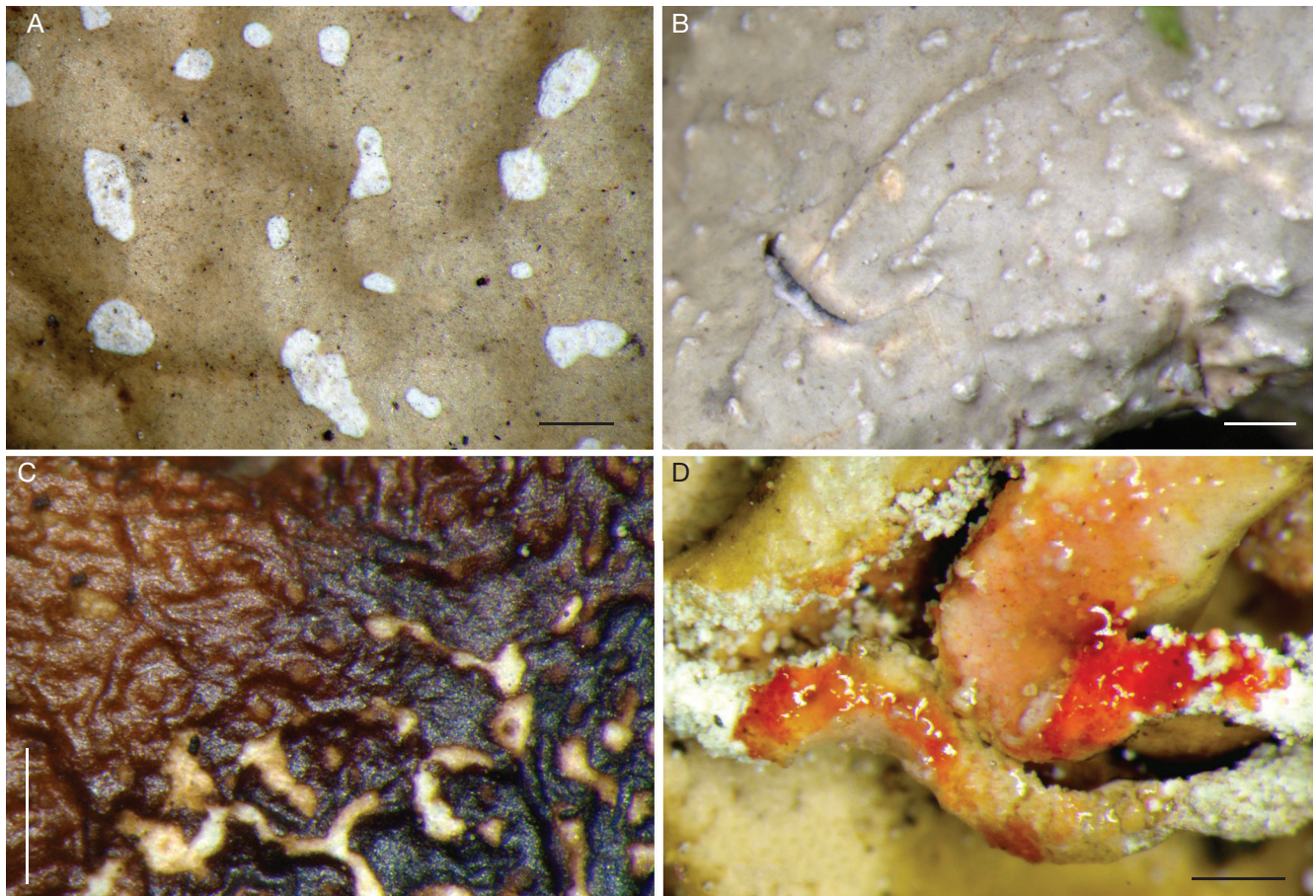


Fig. 1. — Differential morphological and chemical features in *Cetrelia* W.L. Culb. & C.F. Culb. species: **A**, large, not raised pseudocyphellae on upper cortex of *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb.; **B**, small, raised pseudocyphellae on upper cortex of *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb.; **C**, brown lower cortex of *C. cetrarioides* (Delise) W.L. Culb. & C.F. Culb. without rhizines; **D**, C+ reaction by NaOCl on marginal soralia of *Cetrelia olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. Scale bars: 500 µm.

previously studied compounds (cf. Culberson & Culberson 1968; Obermayer & Mayrhofer 2007), the identification of α -collatolic acid, β -collatolic acid, α -alectoronic acid and β -alectoronic acid (i.e., β -collatolic acid and β -alectoronic acid as newly identified substances of the above list) was possible by using an anisaldehyde/sulphuric acid spray on regular TLC plates (Fig. 3). Based on the seven substances above it was found that specimens from Hungary, Poland, Ukraine, Romania, Italy had the same pattern of substances as the three specimens from the United States, containing one from Pocahontas County (BP 91365), collected very near the type locality. Most of these specimens contained one or two additional unidentified minor substances, observable only under UV 366 prior to sulphuric acid treatment in position below atranorin and above 4-O-methylphysodic acid. The specimen collected near the type locality (BP 91365) contained both of these substances and a third one. This third unidentified component had the same travelling distance on the plate as that of 4-O-methylphysodic acid. One of the two Hungarian specimens (BP 49905) contained the upper unknown, the other (BP 71276) the lower one. The Polish sample (BP 21508) and one of the Ukrainian samples (BP

21504) contained both of these unknowns. Furthermore, the Italian (VBI 1742) and a part of a Ukrainian specimen (BP 22781) was lacking atranorin. The occurrence of above-mentioned unknown substances within specimens might be explained by the possibly non-monophyletic origin of *C. chicitae* suggested by Mark *et al.* (2018). It needs further studies, since the specimens from Virginia (United States) (BP 91365, BP 93416 and BP 75822) are not homogeneous either in the presence of these unknown substances.

Apart from the chemical composition, the somewhat twisted, wavy soralia are also characteristic features. Its soredia are coarse, 46.1 ± 7.3 µm in diam. (cf. (35-)40-55 µm in Obermayer & Mayrhofer 2007). Pseudocyphellae are large (150-500 µm), not raised on upper cortex, lacking from lower cortex.

Both Hungarian specimens of *C. chicitae* were found on beech (*Fagus sylvatica*) at 450-600 m a.s.l., one from the Bükk [BP 71276 sub *Parmelia cetrarioides* var. *typica*] and the other in the Zemplén Mts [BP 49905 sub *Parmelia cetrarioides*] (as *P. cetrarioides*) (Fig. 8). This is the rarest *Cetrelia* species in Hungary. As it has not been collected since 1961, *Cetrelia chicitae* is here proposed as critically endangered (CR(PE)) lichen species in Hungary.

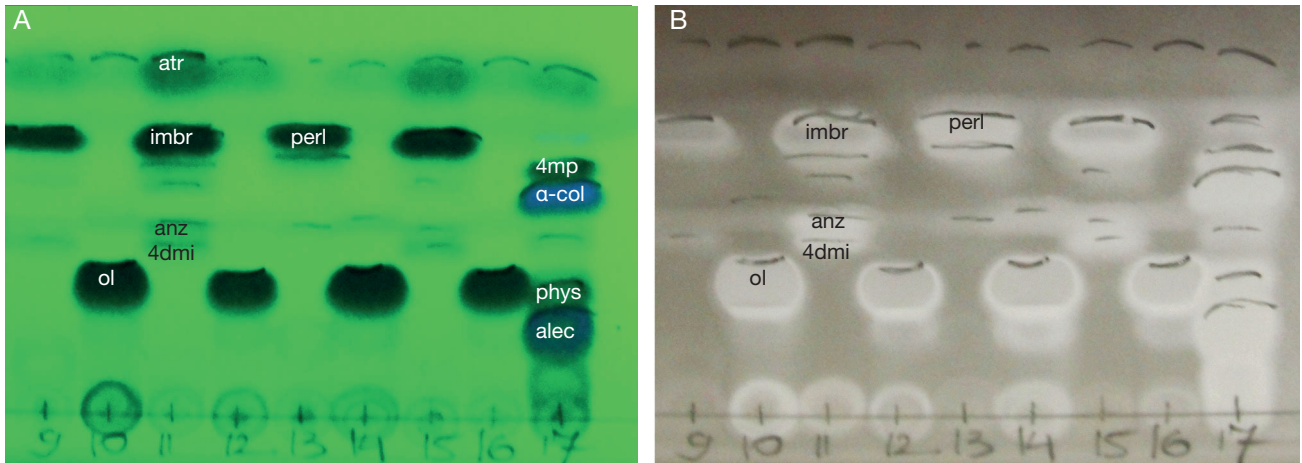


FIG. 2. — A detail of the chromatographic plate HPTLC nr 74/2014 developed in solvent system C presenting all species under UV 254 nm (A), and sprayed with water (B). Specimens in positions A10-A17 are A10: *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. (BP 21529), A11: *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb. (BP 85318), A12: *C. olivetorum* (BP 84893), A13: *C. cetrarioides* (Delise) W.L. Culb. & C.F. Culb. (BP 21538), A14: *C. olivetorum* (BP 22787), A15: *C. monachorum* (BP 45013), A16: *C. olivetorum* (BP 21508), A17: *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. (BP 93416). Abbreviations of LSMs are according to Table 2.

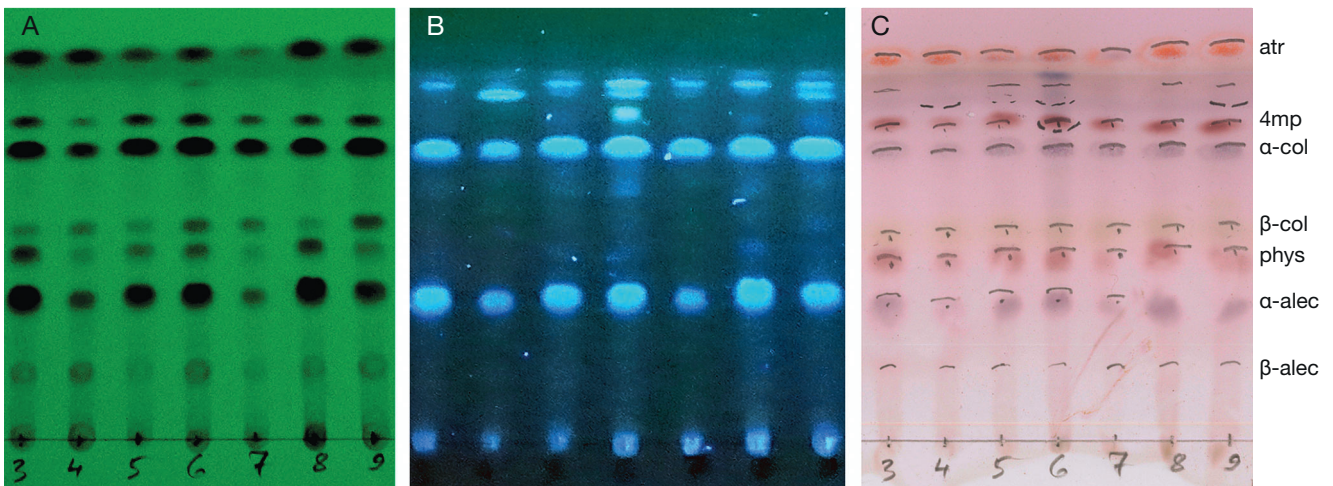


FIG. 3. — A detail of the chromatographic plate TLC nr 1903 developed in solvent system C presenting *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. specimens under UV 254 nm (A), UV 366 nm (B) and sprayed with anisaldehyde/sulphuric acid (C). Specimens in positions 3-9 are: 3, from Page County, Virginia, United States (BP 75822); 4, from the Bükk Mts, Hungary (BP 71276); 5, from the Zemplén Mts, Hungary (BP 49905); 6, from Pocahontas County, West Virginia, United States (BP 91365); 7, from Ukraine (BP 44999); 8, from Romania (BP 85320); 9, from Poland (BP 21508). Abbreviations of LSMs are according to Table 2.

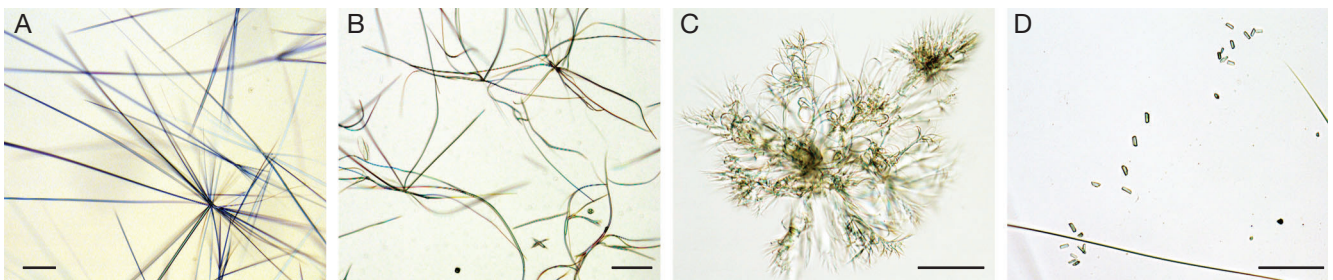


FIG. 4. — Microcrystal tests of A, imbricarin; B, perlatolic; C, olivetoric acids and D, atranorin in GE solvent (glycerine – acetic acid, 3:1 v/v). Scale bars: 50 μm.

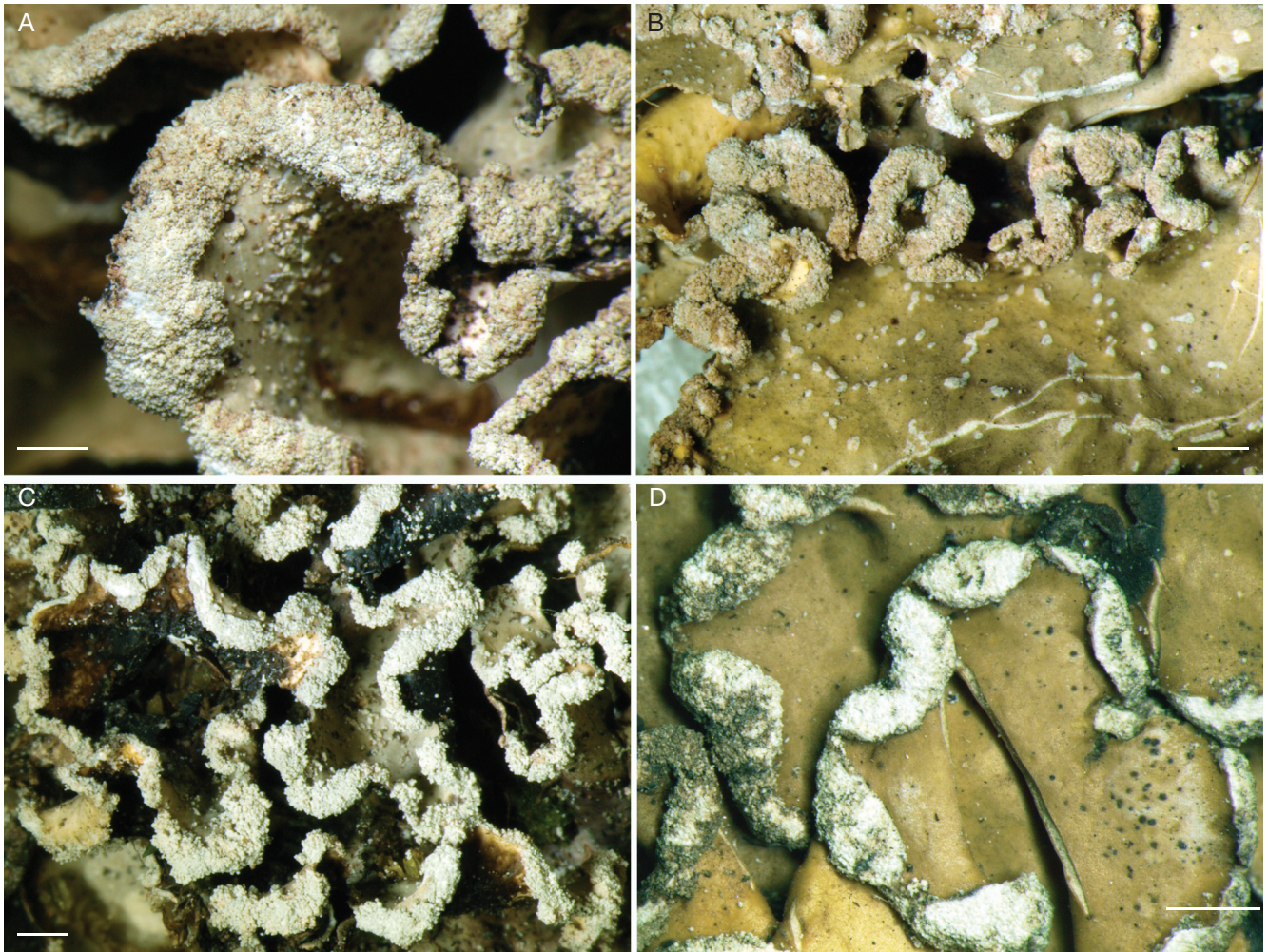


Fig. 5. — Thallose lobes with marginal soralia of **A**, *Cetrelia cetrarioides* (Delise) W.L. Culb. & C.F. Culb.; **B**, *C. chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb.; **C**, *C. monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb.; and **D**, *C. olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. Scale bars: 1 mm.

Cetrelia monachorum (Zahlbr.) W.L. Culb. & C.F. Culb.
(Figs 2; 5C; 9; 12)

It was not known before this revision as it was repeatedly misidentified before as either *C. cetrarioides* or *C. olivetorum*. It now turns out to be the most frequent *Cetrelia* species in Hungary (Fig. 9).

The presence of atranorin, imbricatic acid (major) ± perlatolic acid (minor), anziaic acid and 4-O-demethylimbricatic acid is characteristic. *Cetrelia sayanensis* Otnyukova, Stepanov & Elix (Otnyukova *et al.* 2009), a closely related species, has a slightly different chemical composition containing atranorin (minor), imbricatic acid (major), perlatolic acid (minor), divaricatic acid (minor), anziaic acid (minor), 4-O-demethylimbricatic acid (minor), glomelliferic acid (trace) and loxodellic acid (trace). However, it has pustulate-capitate soralia with farinose soredia, while *C. monachorum* has only seldom laminal, capitate soralia and is further characterised by coarse soredia, $52.7 \pm 5.6 \mu\text{m}$ in Hungarian samples [vs (35)40-55 μm diam. in Obermayer & Mayrhofer (2007)],

small (50-150 μm), raised pseudocyphellae on upper cortex, but very rare or lacking on lower cortex.

Cetrelia monachorum is most frequently collected from rocks (70%), but also grows on *Quercus* (14%), *Fagus* (6%), *Carpinus* (2%), *Acer pseudoplatanus* L. (1%) and on unidentified bark (7%) between 100-1000 m a.s.l. reaching the highest possible elevation in Hungary (Mátra Mts). The species is proposed for the category near threatened (NT) in Hungary.

Cetrelia olivetorum (Nyl.) W.L. Culb. & C.F. Culb.
(Figs 2; 5D; 10-12)

It is the easiest of the four species to identify because olivetoric acid produces a characteristic, water repellent spot on the chromatographic plate, although a wider species concept previously (Wirth 1980; Versegly 1988, 1994, Smith *et al.* 2009) also was applied for specimens not containing olivetoric acid (here revised mostly as *C. monachorum* by HPTLC). Cortical atranorin is also present. This species forms soredia varying

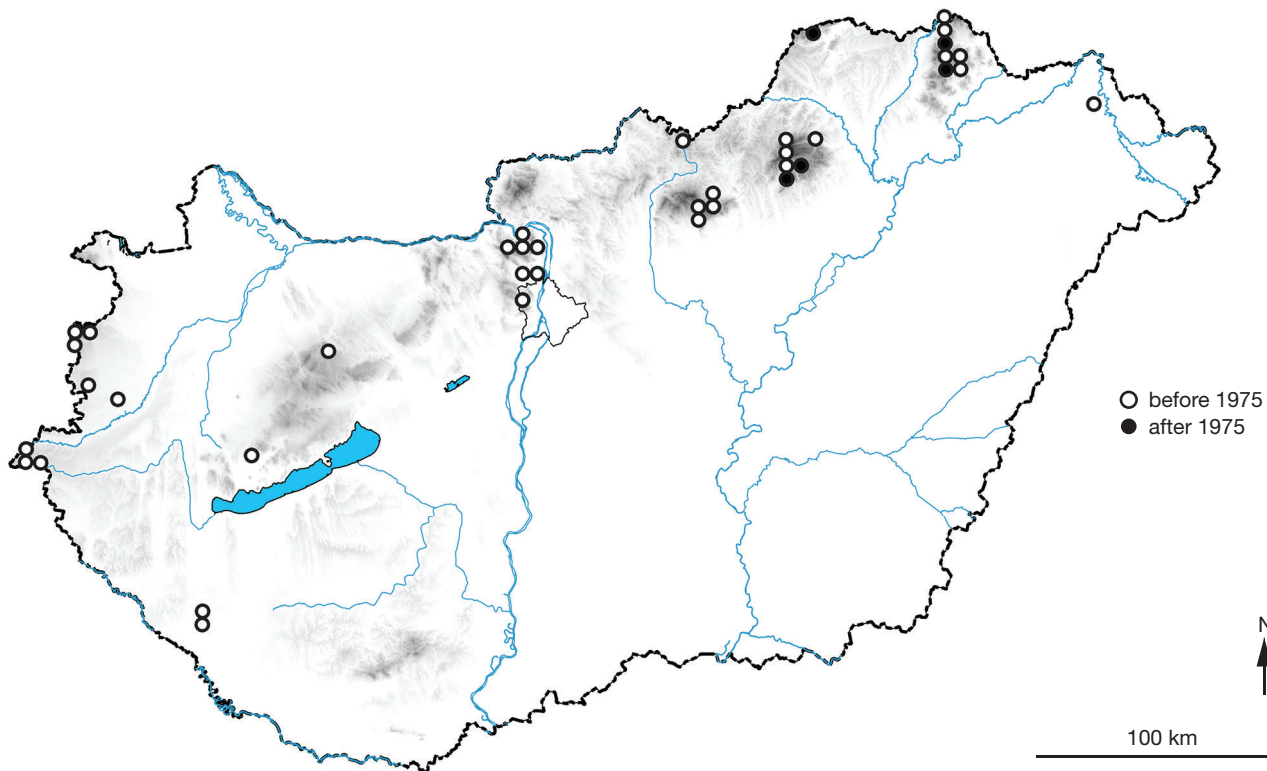


FIG. 6. — Distribution of *Cetrelia cetrarioides* (Delise) W.L. Culb. & C.F. Culb. in Hungary before revision.

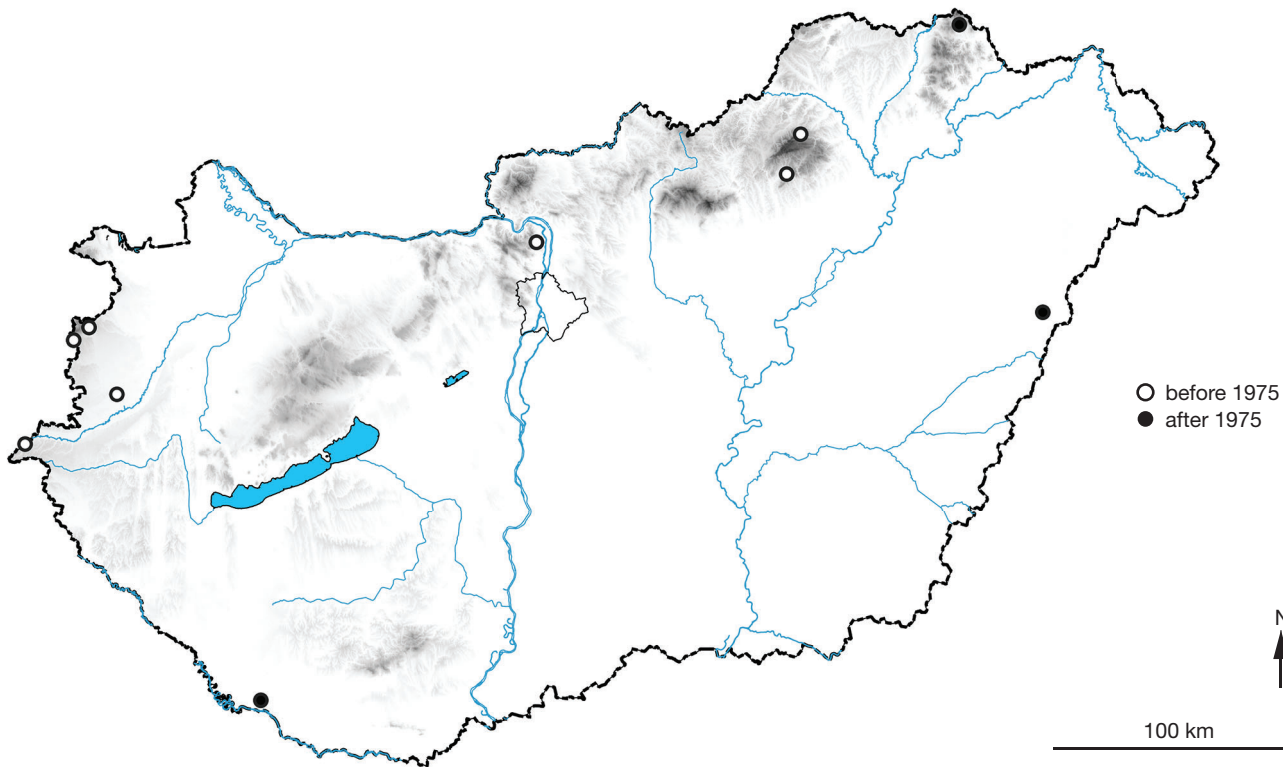


FIG. 7. — Distribution of *Cetrelia cetrarioides* (Delise) W.L. Culb. & C.F. Culb. in Hungary after revision.

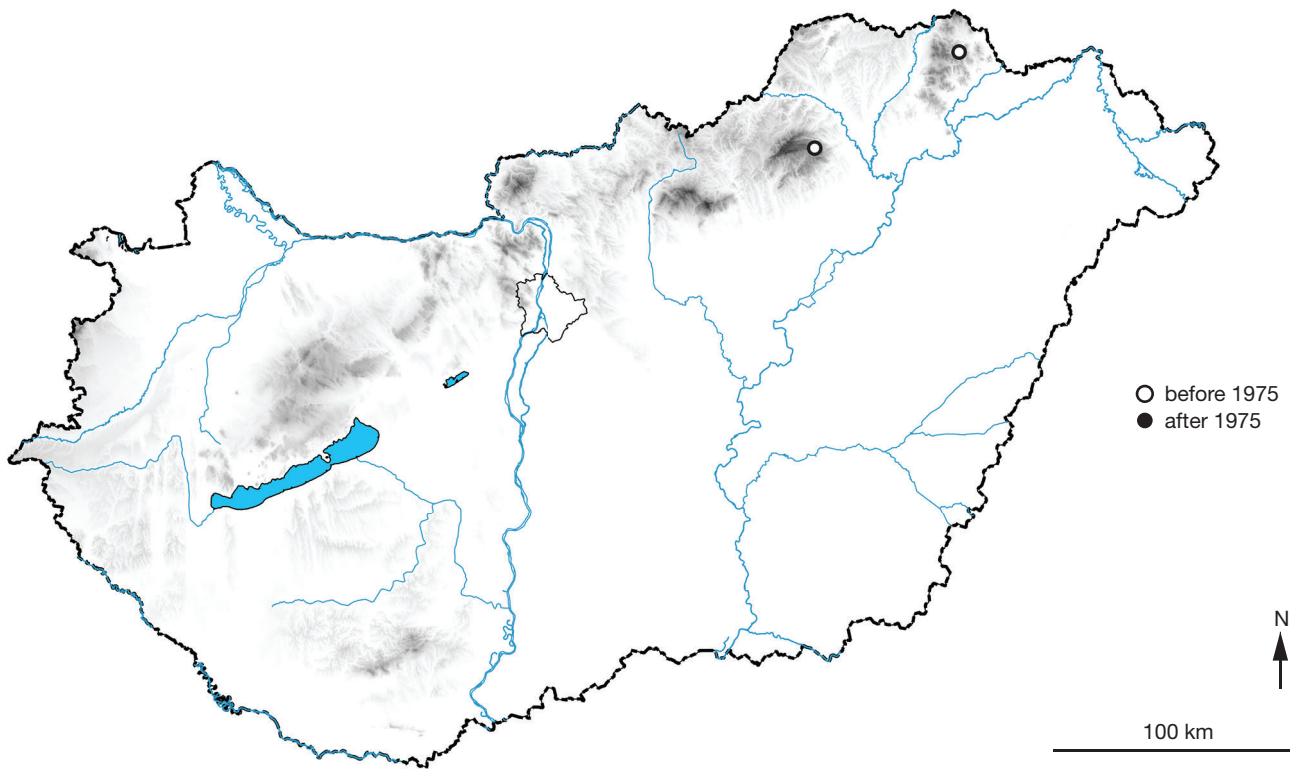


FIG. 8. — Distribution of *Cetrelia chicitae* (W.L. Culb.) W.L. Culb. & C.F. Culb. in Hungary.

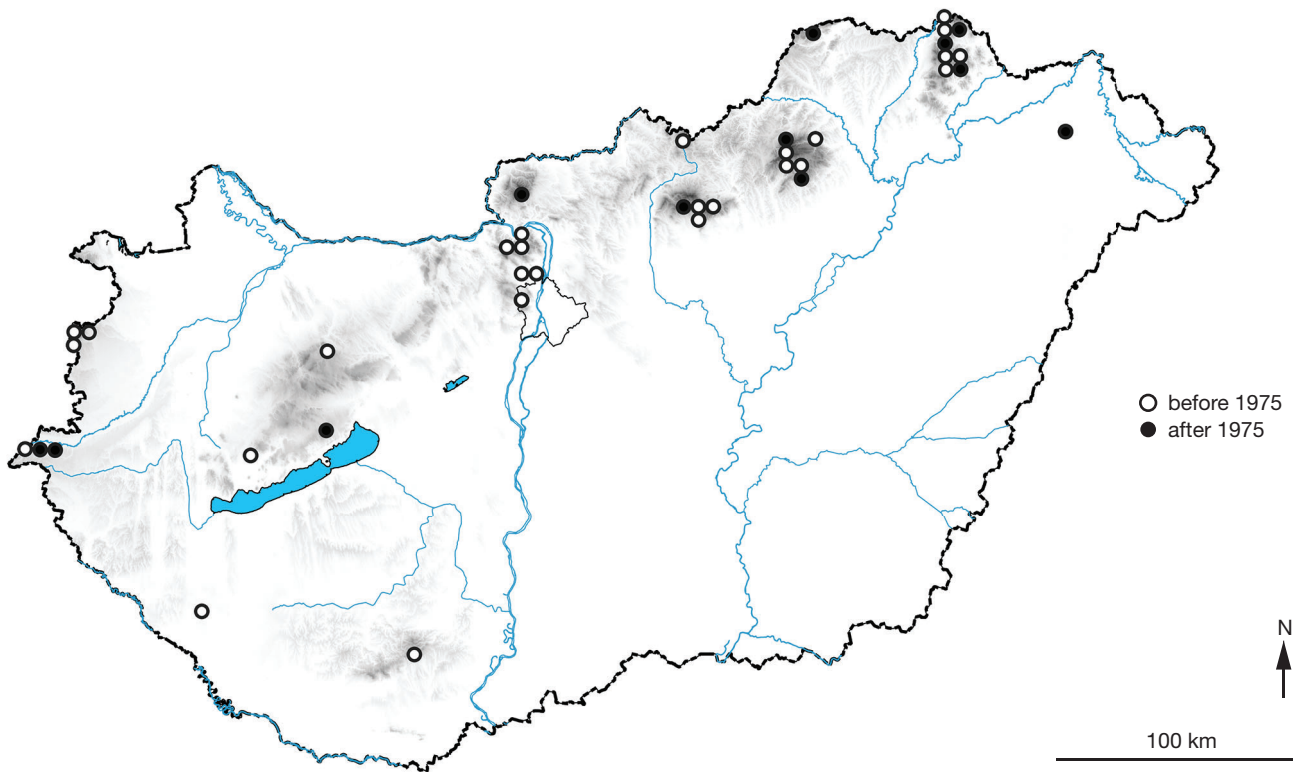


FIG. 9. — Distribution of *Cetrelia monachorum* (Zahlbr.) W.L. Culb. & C.F. Culb. in Hungary.

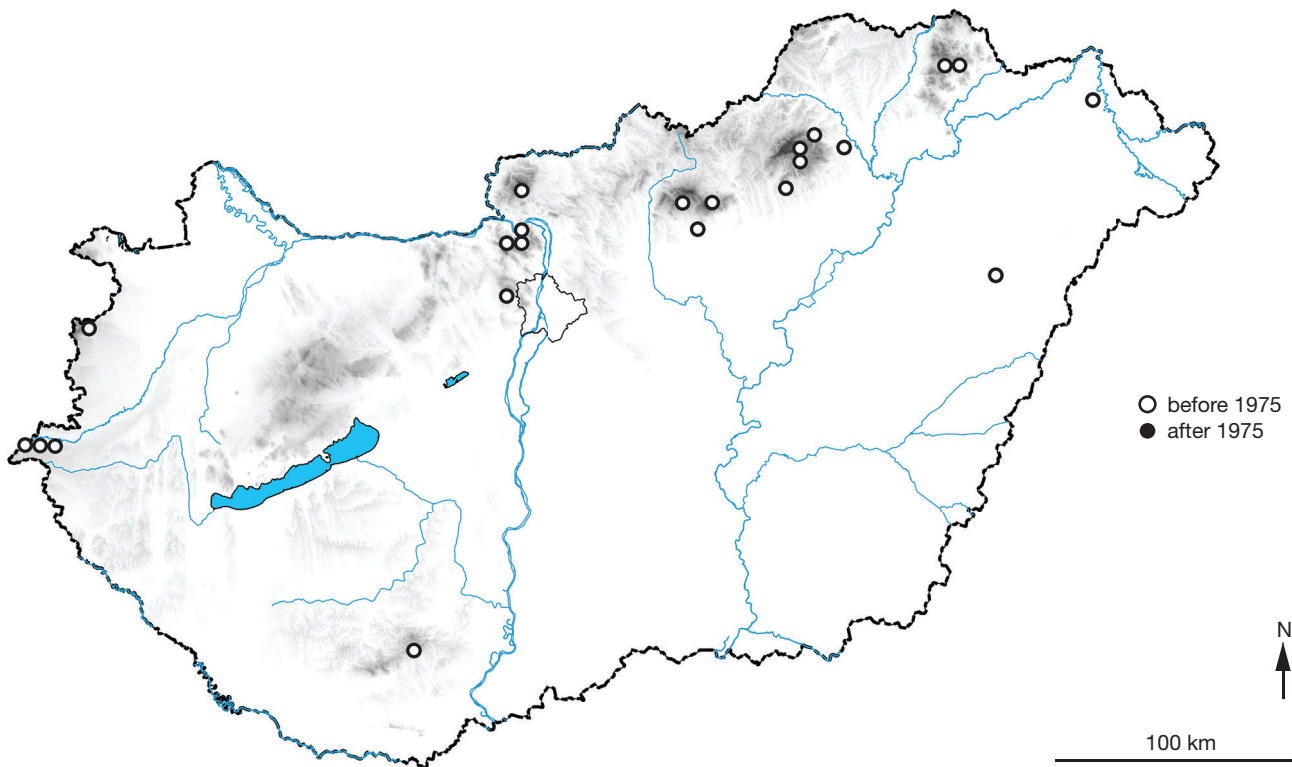


FIG. 10. — Distribution *Cetrelia olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. in Hungary before revision.

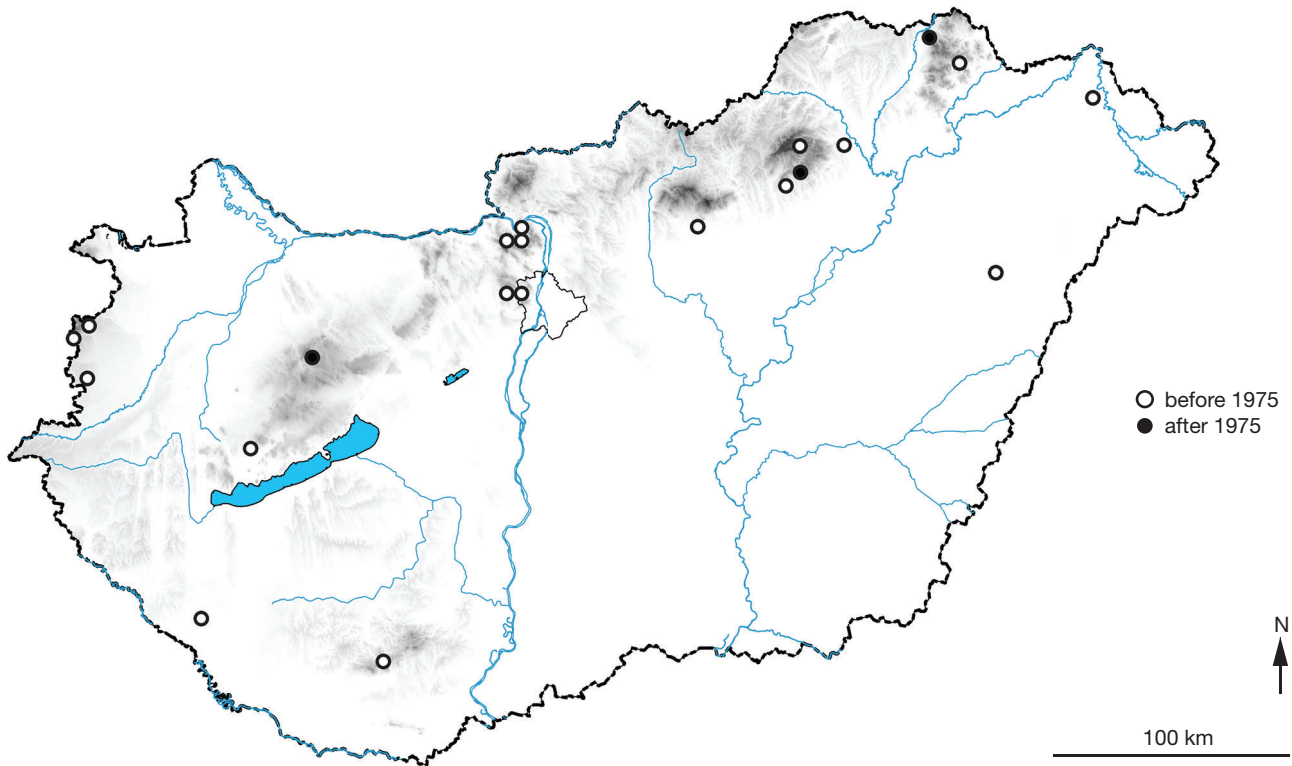


FIG. 11. — Distribution of *Cetrelia olivetorum* (Nyl.) W.L. Culb. & C.F. Culb. in Hungary after revision.

from fine to coarse, $36.4 \pm 9.5 \mu\text{m}$ in Hungarian samples [versus 25–55 μm diam. in Obermayer & Mayrhofer (2007)], while pseudocyphellae – if any – are rare on both upper and lower cortex and small (20–50 μm).

This species was collected from rocks (39%), but was mostly found on bark of *Quercus* (13%), *Fagus* (9%) and various unidentified trees (39%) at 100–800 m a.s.l. It is the second most frequent species of *Cetrelia*, known from a moderate 26 collections (Figs 10; 11). *Cetrelia olivetorum* is proposed as vulnerable (VU) in the Hungarian red-list.

TAXONOMIC REMARKS

Lectotypification of Parmelia cetrarioides Delise f. pseudofallax
All three specimens (BP 21541 from the Caucasus, BP 21492 from Hungary, BP 21540 from Croatia) served as type material of *Pseudoparmelia pseudofallax* cited in the protologue (Gyelnik 1933) were localised and revised. After our revision one of them proved to be *Cetrelia cetrarioides* (BP 21541), and the other two were determined as *C. monachorum* (BP 21492, BP 21540). Unfortunately, the species *Pseudoparmelia pseudofallax* was never validly described. Instead, Gyelnik (1935) published a later description of the taxon where he treated it as a form of *Parmelia cetrarioides*, referring to the former publication with the three specimens. In 1937 he issued an exsiccatum of the same taxon (as *Parmelia cetrarioides f. pseudofallax*) from another Hungarian locality (Haláp), which was not cited in the protologue (Gyelnik 1937). One specimen of this set was selected as syntype and revised as *Parmelia cetrarioides* by Ödön Szatala in 1956, later confirmed by Mason E. Hale in 1961. This syntype specimen was included in the type catalogue of Verseghy (1964) as syntype of *Pseudoparmelia pseudofallax* and it was later revised and listed as *Cetrelia olivetorum* var. *cetrarioides* by Verseghy (1988) as *Parmelia pseudofallax*. According to our revision the syntype specimen and 12 available duplicates also proved to be *C. monachorum*, except for one *C. olivetorum*. *Parmelia cetrarioides f. pseudofallax* is lectotypified here based on the most typical Hungarian specimen corresponding well with Gyelnik's concept:

Parmelia cetrarioides Delise f. pseudofallax Gyelnik

Revue bryologique et lichénologique 7: 220 (1935).

MYCOBANK NO. — MB435214.

Cetrelia monachorum (Zahlbr.) W.L. Culb. & C.F. Culb, *Systematic Botany* 1 (4): 326 (1977).

LECTOTYPE. — (designated here under no. MBT392402): Hungary. Mohás trachyttufa sziklán. “Keserús-hegy” c. 500 m. Dömös mel., Esztergom vm.; leg.: Timkó Gy., 12.X.1913. (BP 21492 sub *Parmelia cetrarioides*).

IDENTITY OF THE TYPE MATERIALS FOR *PARMELIA OLIVARIA*
VAR. *SUBVENOSA* GYELN.

Type specimens (holotype, isotype and one additional authentic specimen) of *Parmelia olivaria* var. *subvenosa* Gyeln. were also checked and evaluated. The holotype (BP 22803, T 636/a) specimen was confirmed as *Cetrelia olivetorum* (Culberson &

Culberson 1968), however the isotype (BP 22802, T 636/b) and an additional specimen from the same place and date (BP 21493) proved to be *C. monachorum* after the revision.

CONCLUSIONS

The characteristic composition of Lichen Secondary Metabolites (LSMs) described in literature was supported by HPTLC and TLC analysis of 166 specimens of *Cetrelia* from Hungary; an additional nine specimens of *C. chicitae* were investigated from United States (3) and Europe (6). The specimens showed homogeneous secondary chemistry on the basis of 7 LSMs (including two substances as newly identified from this species). Thus the presence of *C. chicitae* (described from America) in Europe was confirmed, based on chemistry. Although in *C. monachorum* slightly larger soredia were measured in Hungarian specimens than given by Obermayer & Mayrhofer (2007), the subtle differences in soredium sizes of the four species was generally confirmed by our measurements. With the relatively wide range in diameter of the soredia, the large standard deviation in *C. olivetorum* seems to be an important feature of the species.

The current knowledge on the frequency of species considerably changed after the chemotaxonomic revision. *Cetrelia* species are predominantly distributed in the mountainous woodland areas of Hungary, especially the Inner Western Carpathians (Zemplén Mts, Bükk Mts, Mátra Mts, Börzsöny Mts, etc.), the Transdanubian Mountains and the eastern foothills of the Alps. *Cetrelia chicitae* was found only in the NE part of Hungary, however only slight differences or tendency in distribution, ecology or habitat preference were recognised in Hungary between the four species. While in Central Europe frequency maxima of *Cetrelia* species are around 600 and 1000 m a.s.l., it is at 300 and 500 m in case of the same species in Hungary (Fig. 12; cf. Obermayer & Mayrhofer 2007). These lichens most probably find their adequate habitat conditions also at lower elevations due to various microclimatic effects. Consequently, the analysis of Hungarian specimens largely contributed to the knowledge on ecological requirements of these species.

The increased number of species in different parts of Hungary after the revision is shown in a map (Fig. 13). Before the revision, the genus *Cetrelia* was thought to be represented exclusively by varieties of *C. olivetorum* in Hungary, the analysis of LSMs revealed the chemical diversity of this taxonomic group and the hidden chemical diversity, together with morphological characters, confirmed presence of four different species. However, the current status of *C. chicitae* in Hungary is uncertain, since it was collected only twice, in 1933 and 1961, and has not been observed since then. Global climatic change is a major risk for species extinction also in Hungary (cf. Thomas *et al.* 2004; Thuiller *et al.* 2005; UNFCCC 2013; Woodruff 2001). All existing *Cetrelia* species are rare in Hungary. There is a limited number of recent collections. Since there is only a low number of recently collected

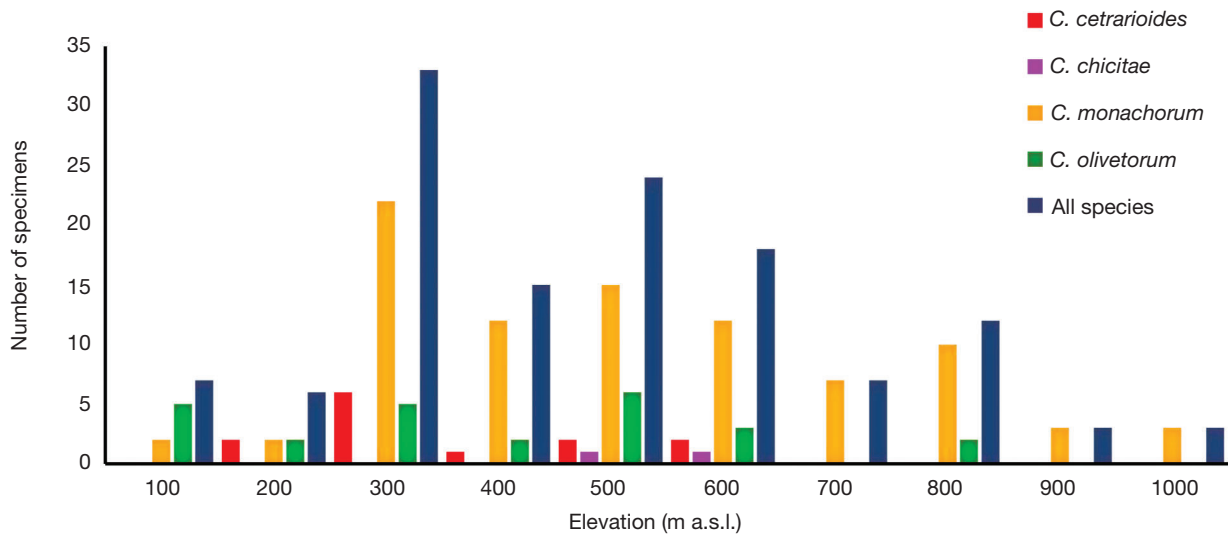


Fig. 12. — Altitudinal distribution of *Cetrelia* W.L. Culb. & C.F. Culb. species in Hungary.

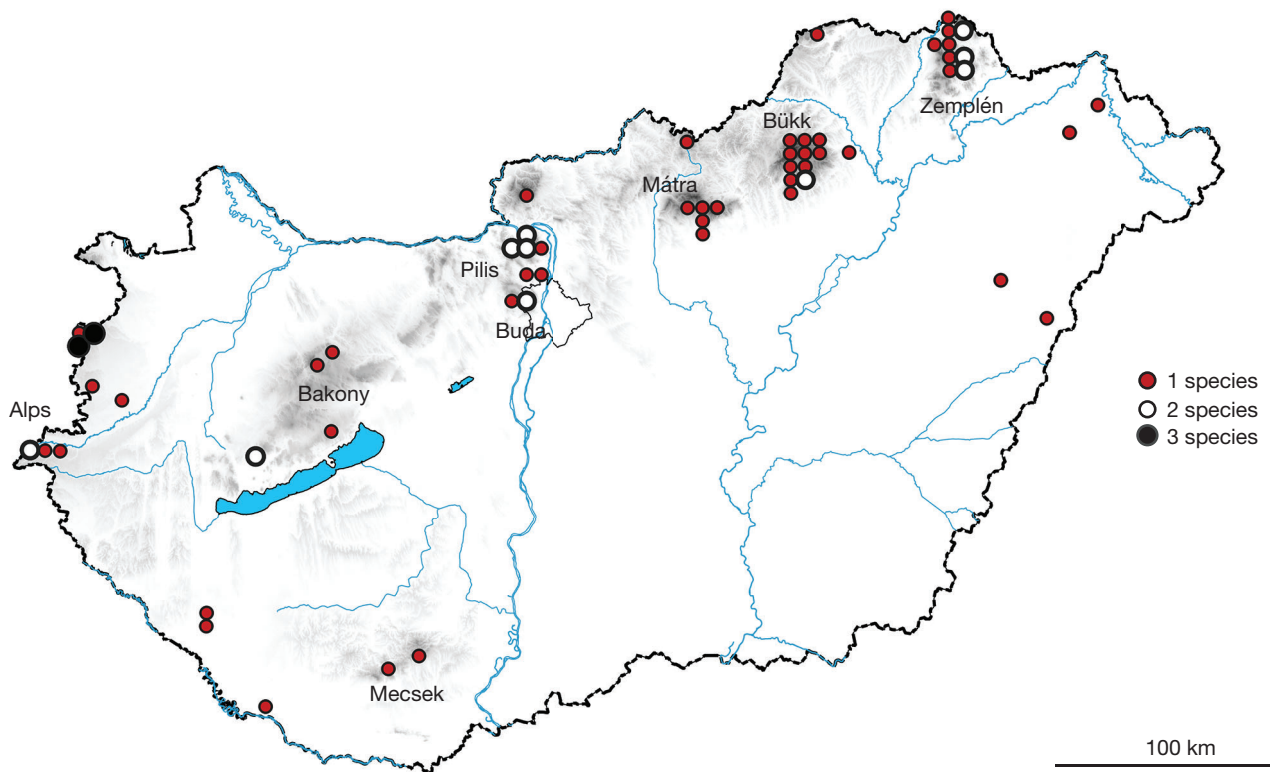


Fig. 13. — The number of *Cetrelia* W.L. Culb. & C.F. Culb. species after revision in various parts of Hungary.

specimens for *C. cetrarioides*, it is worthy of legal protection, together with *C. chicitae*. Current populations of all species of the genus should be further studied to be able to estimate their size and establish their IUCN Red List categories more precisely (IUCN 2018).

Acknowledgements

Authors are grateful to Dr Katalin Molnár (Komárom) and Katalin Veres (Vácrátót) for reading the manuscript. We highly appreciate comments of two anonymous reviewers on a former version of the manuscript and the current thorough

reviews by Dr Walter Obermayer (Graz) and Dr Arne Thell (Lund). Prof Éva Szőke and László Kátai are thanked for useful advise concerning to the application of reagent anisaldehyde/sulphuric acid. The curators of various herbaria (Ivona Kautmanová, Bratislava; Dr Mihai Puscas, Cluj; Dr Gábor Matus, Debrecen; Dr Andrea Sass-Gyarmati, Eger; Dr János Csiky, Pécs; András Varga, Szeged; Dr Lajos Balogh, Szombathely) are thanked for being helpful in searching lichen specimens. French abstract was translated by Ludmilla Dési and Dr Katalin Török. Technical help is thanked for Adrienn Szabó (Szeged) and Krisztina Szabó (Vácrátót). The work was supported by the Hungarian Scientific Research Fund OTKA 81232 and the project NKFI K 124341 financed by the National Research Development and Innovation Fund.

The travel grant of the British Lichen Society made a contribution towards a poster presentation on this research during IAL8 2016.

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Submitted on 3 April 2019;
accepted on 6 January 2021;
published on 2 February 2021.