



# First insights into genetic diversity and relationships of European taxa of *Solenopsora* (Catillariaceae, Ascomycota) with implications for their delimitation

ANNA GUTTOVÁ<sup>1\*</sup>, JUDITA ZOZOMOVÁ-LIHOVÁ<sup>1</sup>, EINAR TIMDAL<sup>2</sup>, JAROMÍR KUČERA<sup>1</sup>, MAREK SLOVÁK<sup>1</sup>, KATARÍNA PIKNOVÁ<sup>1</sup> and LUCA PAOLI<sup>3</sup>

<sup>1</sup>*Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 9, SK-845 23 Bratislava, Slovakia*

<sup>2</sup>*Botanical Museum, University of Oslo, Sars' gate 1, N-1162 Oslo, Norway*

<sup>3</sup>*Department of Life Science, University of Siena, via Mattioli 4, IT-53100 Siena, Italy*

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The lichen genus *Solenopsora* occurs predominantly in temperate and subtropical regions of the world, and the centre of diversity and distribution is in the Mediterranean, Macaronesian, and Madrean floristic regions. Taxonomic treatment of several taxa has varied over time and the concepts lack clarity. Focusing on multilocus sequence data, morphology, anatomy, chemistry, and ecological preferences, the present study investigates European *Solenopsora* taxa to obtain the first insights into their genetic variation and relationships. Our results show discrepancy between the number of currently recognized taxa in Europe and the number of genetic entities identified. We recognize eight species in the genus in Europe: *Solenopsora candicans*, *Solenopsora cesatii* (including *Solenopsora carpatica*), *Solenopsora grisea*, *Solenopsora holophaea*, *Solenopsora liparina*, *Solenopsora marina*, *Solenopsora olivacea*, and *Solenopsora vulturiensis*. We gathered evidence to recognize *S. liparina*, an edaphic vicariant of *S. candicans* confined to ultramafic rocks, as a separate species. We disclosed a previously unknown sister relationship between *S. grisea* and *S. vulturiensis*. Taxonomic synopses, geographical distribution, and an identification key for the treated taxa are provided. Phylogenetic analyses revealed two major lineages among the European *Solenopsora* spp., differentiated by thallus organization, the presence/absence of rhizines on lower side of the thallus, secondary chemistry, and anatomy of upper cortex. The generic circumscription and phylogenetic position, however, appear problematic and additional studies with increased sampling including related genera are needed. © 2014 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2014, 176, 203–223.

**ADDITIONAL KEYWORDS:** fungal nonreducing PKS – lichens – nrDNA ITS region – replication licencing factor *Mcm7* gene – species tree.

## INTRODUCTION

The circumscription of species in lichen-forming fungi has largely been based on morphological and chemical characters. Lichens display few taxonomically useful characters and many of these are widely variable, and the homology of character states within and between systematic groups is difficult to assess (Printzen, 2010). Nevertheless, accurate species delimitation is crucial in biodiversity assessments and conservation biology. Molecular markers provide an invaluable tool for robust species delimitation (Lumbsch & Leavitt, 2011) but, despite advanced methods and employment

of genetic data in lichen taxonomy, many lichen-forming families and genera still receive little attention, resulting in conflicting taxonomies or uncertain species circumscriptions.

One of the poorly known lichen genera with conflicting taxonomy is *Solenopsora* A.Massal. It comprises approximately 20 species (Gilbert, Purvis & James, 2009) and predominantly occurs in temperate and subtropical regions of the world, with the exception of South America and polar regions (Ryan & Timdal, 2002). The centre of diversity and distribution is in the Mediterranean, Madrean, and South-West Australian floristic regions (Takhtajan, 1986). Despite having a relatively small genus, its thallus morphology, anatomy, and chemistry are complex.

\*Corresponding author. E-mail: anna.guttova@savba.sk

Thalli can be placodioid, squamulose, effigurate or crustose. The upper cortex consists of an epinecral layer of different thickness and various types of pseudocortex or eucortex. A lower cortex may be present or absent. Lecanorine apothecia with persistent thalline margins or immarginate (pseudobiatorine) when adult are, depending on the taxon, regularly or rarely present. Taxa with clavate asci lacking an ocular chamber (*Catillaria*-type), one-septate, hyaline ascospores, and bacilliform, pleurogenous conidia are included in the genus (Ryan & Timdal, 2002). Further characters traditionally used for distinguishing the species are the colour of the thallus (white, pale grey, olive–grey, grey, brown, red–brown), pruina on upper surface (thick, thin, marginal, laminal), and lobe shape (flattened and/or convex). The species reproduce sexually and through vegetative propagules (blastidia, soredia). Secondary metabolites in *Solenopsora* are produced through the polyketide pathway ( $\beta$ -orcinol *para*-depsides atranorin and briarimontin,  $\beta$ -orcinol depsidones pannarin, and lobaric acid) and mevalonic acid pathways (terpenoid zeorin and several unknown terpenoids) (Ryan & Timdal, 2002). Members of the genus inhabit rock and soil, and rarely colonize the bark of trees and shrubs. They grow on calcareous (limestone, dolomite), siliceous (Palaeozoic metamorphic rocks, sandstone-quartzite breccia with low carbonate cement), volcanic (basalt), and serpentine substrates of natural and man-made origin (e.g. rock fences, walls, gravestones) (Van den Boom & Ryan, 2004). *Solenopsora* is placed in Catillariaceae, a poorly resolved lichen-forming lineage (Hafellner, 1984; Printzen, 2010; Schmutz *et al.*, 2011; Miadlikowska *et al.*, 2014), which traditionally includes six genera (Kotlov, 2004).

Biological complexity of the genus is reflected in the different taxonomic concepts that have been applied (see Supporting information, Table S1). The description of the genus was based on *Solenopsora holophaea* (Mont.) Samp. Now *Solenopsora* also includes species historically classified in different genera; for example, *Biatora* Fr. (Fries, 1825), *Candelariella* Müll. Arg. (Zahlbruckner, 1928), *Catillaria* A.Massal. (Bouly de Lesdain, 1914), *Diphrotora* Trevis. ex Jatta (Bouly de Lesdain, 1924), *Haematomma* A.Massal. (Dodge in Fineran, 1969), *Lecania* A.Massal. (Steiner, 1896; Szatala, 1941), *Lecanora* Ach. in Luyken (Nylander, 1876), *Lichen* P.Micheli (Dickson, 1793), *Parmelia* Ach. (Montagne in Berthelot & Webb, 1839–1841), *Placolecania* (J.Steiner) Zahlbr. (Zahlbruckner, 1907, 1932; Herre, 1910), and *Ricasolia* A.Massal. (Massalongo, 1853; Baglietto, 1862). On the other hand, some species treated under *Solenopsora* in the past were recently excluded from this genus; for example, *Lecania hassei* (Zahlbr.) W.J.Noble, *Gyalidea lecanorina* (C.Knight) P.James or *Coscinocladium gadita-*

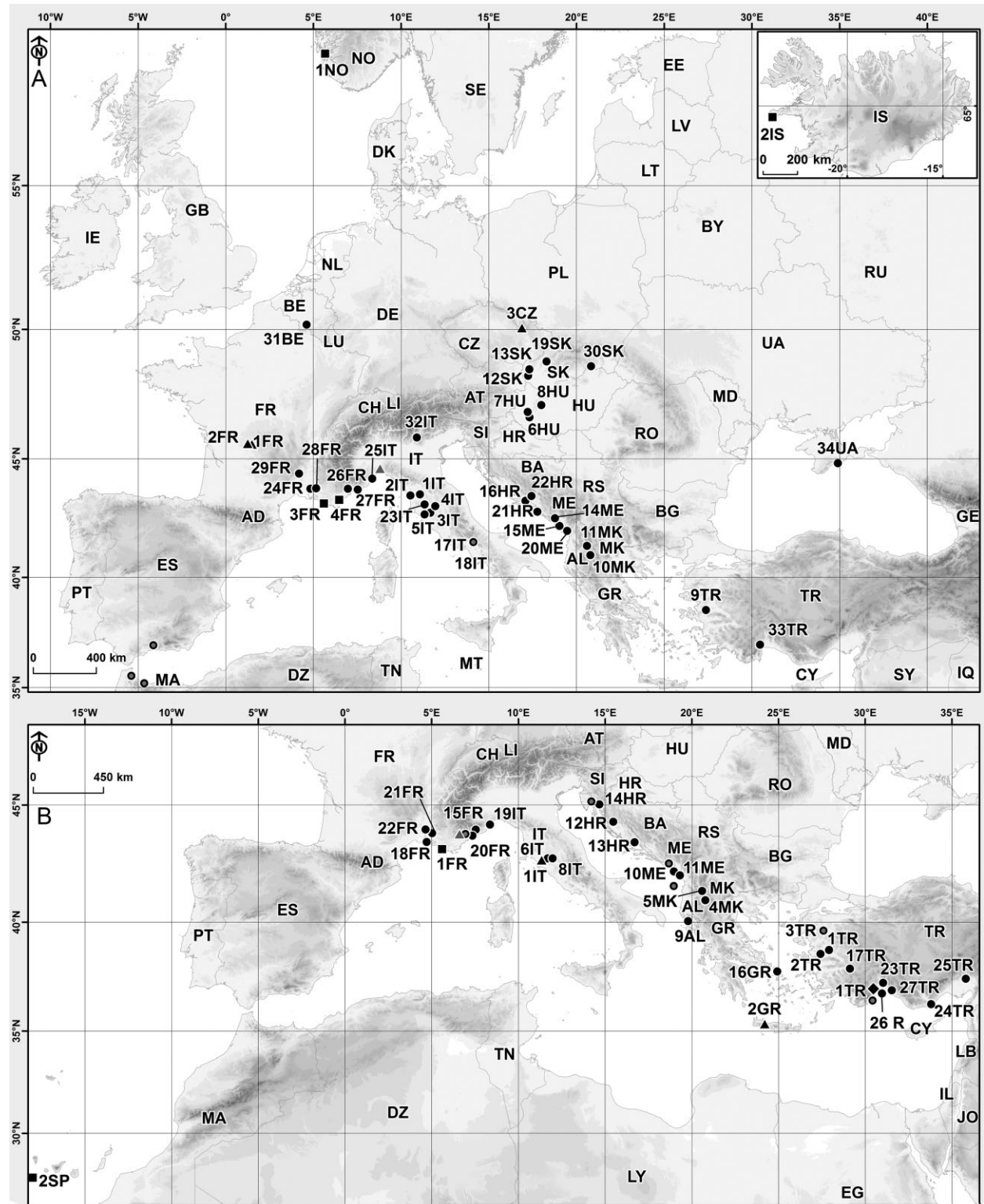
*num* (Clemente) A.Crespo, Llimona & D.Hawksw. No overall revision of the genus, especially using modern taxonomic tools, has been made to date. Some taxa are known only from type material [e.g. *Solenopsora asperatula* (J.Steiner) Zahlbr., *Solenopsora fumosula* (Zahlbr.) Zahlbr., *Solenopsora tasmanica* Kantvilas] or original descriptions with type material not having been recovered (e.g. *Solenopsora caliacrae* Cretz., *Solenopsora paskovskiana* Cretz.). The classification of several taxa, especially described from Europe, has varied over time and the delimitation and/or systematic position of six of them, *Solenopsora carpatica* Pišút & Vězda, *Solenopsora cesatii* (A.Massal.) Zahlbr., *Solenopsora grisea* (Bagl.) Kotlov, *Solenopsora liparina* (Nyl.) Zahlbr., *Solenopsora olivacea* (Dufour ex Fr.) Kiliás, and *Solenopsora olivacea* (Dufour ex Fr.) Kiliás subsp. *olbiensis* (Nyl.) Clauzade & Cl.Roux, remains ambiguous. The present study addresses the taxa occurring in Europe, where *Solenopsora* species diversity and abundance are particularly important in the Mediterranean and Atlantic regions.

Focusing on multilocus DNA sequence data, morphology, anatomy, chemistry, and ecological preferences, we aim to revise taxonomic positions and relationships between the *Solenopsora* taxa described from Europe and the Canary Islands. We pay special attention to the taxonomically most problematic entities: *S. carpatica*, *S. cesatii*, *S. grisea*, *S. liparina*, and *S. olivacea* s.l. (see Supporting information, Table S1). In this regard, we address four specific questions: (1) is there any support for the recognition of *S. carpatica* as a separate species and do the collections of this taxon, known from the Western Carpathians, the Sudety Mts and the Alpi Bergamasche mountains, represent the same taxon; (2) is *S. cesatii* a complex of several infraspecific taxa confined to the Mediterranean; (3) is there any justification for classification of *S. liparina* at species level; and (4) do fertile (subsp. *olivacea*) and sterile (subsp. *olbiensis*) variants in *S. olivacea* represent a species pair?

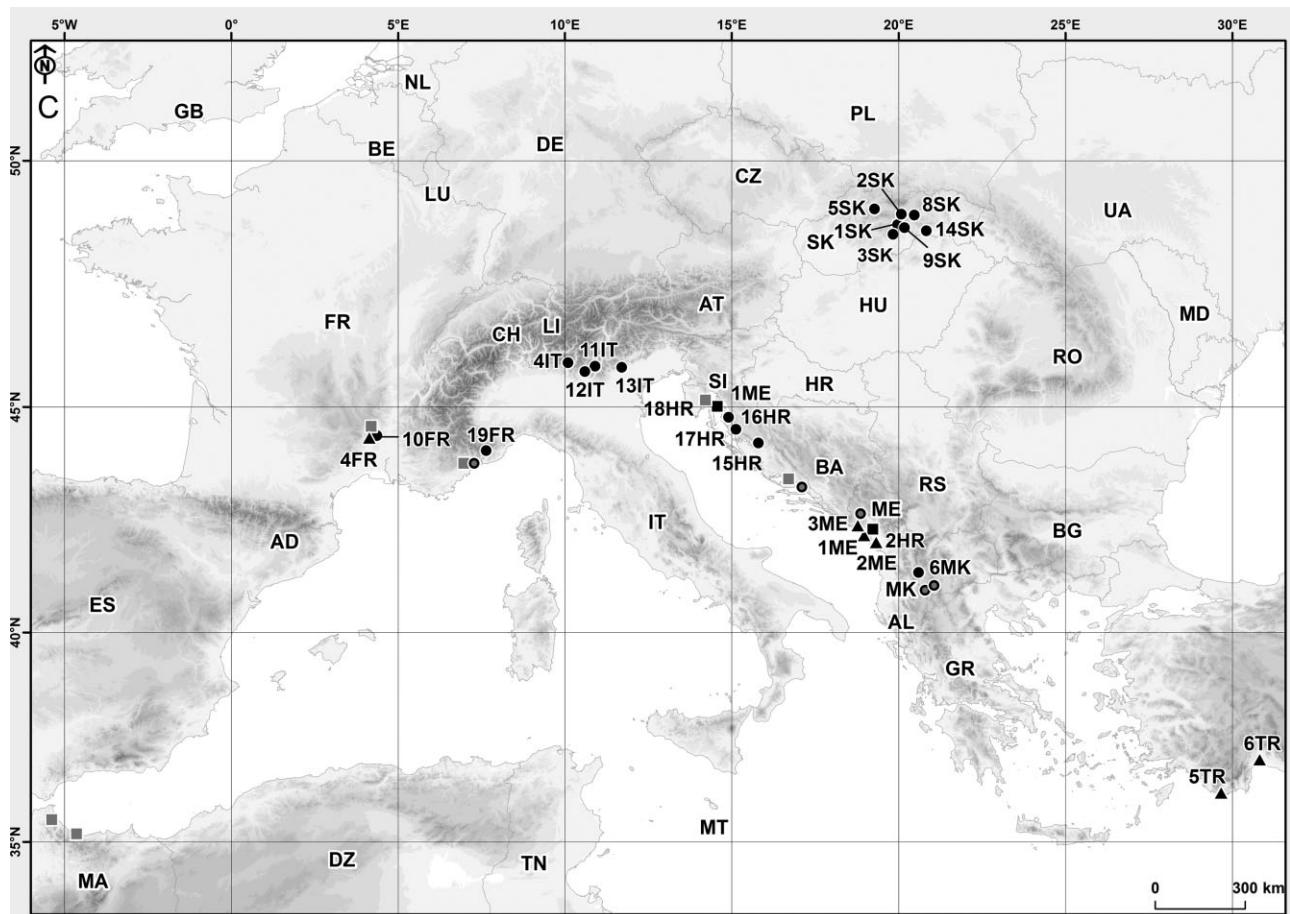
## MATERIAL AND METHODS

### TAXON SAMPLING

The distribution ranges of the *Solenopsora* taxa described from Europe and Canary Islands were assessed from herbarium specimens (BG, BP, BR, BRA, CBFS, GZU, H-NYL, O, PRA, PRC, PRM, SAV, TO, VER, W) and relevant literature sources (see Supporting information, Doc. S1). Based on these data, during 2010–2013, we sampled material representing all but three taxa (see below) reported from Europe. Each taxon was sampled from multiple sites, including type localities when possible (see Fig. 1; see also Supporting information, Doc. S2).



**Figure 1.** A, B, C, geographical distribution of *Solenopsora* specimens sampled for molecular analyses (black symbols). Grey symbols indicate the specimens used only for morphological and chemical studies. A, dot, *Solenopsora candicans*; triangle, *Solenopsora liparina*; square, *Solenopsora vulturienensis*. B, dot, *Solenopsora grisea*; triangle, *Solenopsora olivacea* subsp. *olbiensis*; square, *Solenopsora holophaea*; diamond, *Solenopsora* sp. 1. C, dot, *Solenopsora cesatii*; square, *Solenopsora olivacea* subsp. *olivacea*; triangle, *Solenopsora marina*. For locality details, see Supporting information (Doc. S1).



**Figure 1.** *Continued*

Additional material from the USA was obtained and used for molecular analyses (herbarium MSC). Voucher specimens are deposited in SAV and O. The list of additional specimens examined only for morphology, anatomy, chemistry, and ecological requirements is indicated in the Supporting information (Doc. S3). We did not include *S. caliacrae*, described from Bulgaria, and *S. paskovskiana*, described from Ukraine, because they are known only from protologues (Cretzoiu, 1940) and the type material has not been found in relevant collections in Romania (BUC, BUCM, CL). We also did not find *S. caliacrae* in its type locality during our field research in 2013. We do not treat *S. fumosula*, described from Istria, because the species is known so far only from the type locality and we did not find it during field work. We only have the historical type material at our disposal (W). Based on the available information on the studied group, a member of Catillariaceae (Printzen, 2010), *Catillaria lenticularis* (Ach.) Th. Fr. (see Supporting information, Doc. S2), was chosen as the outgroup.

#### SELECTED LOCI, POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION AND SEQUENCING

Total genomic DNA was extracted from peripheral parts of thalline lobes (*Solenopsis* sp. div., up to 2 mm from lobe tips), and apothecia (*Catillaria lenticularis*), using a DNeasy® Plant Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The quality of the extracted DNA was checked on 1.5% TAE-agarose gels. The quantity and purity of the isolated genomic DNA was also determined spectrophotometrically (NanoDrop 2000; Thermo Scientific). An initial screening of multiple primer combinations amplifying diverse nuclear target regions was performed using three different species. As a result, three regions of different molecular nature were selected for the subsequent analyses: the commonly applied multicopy internal transcribed spacer (ITS) region of nuclear ribosomal DNA (ITS1-5.8S-ITS2), the ketosynthase (KS) domain of the polyketide synthase gene (PKS, nonreducing fungal type I; Schmitt *et al.*, 2005), and a single-copy protein-coding nuclear gene *Mcm7*

**Table 1.** Markers and primers used in the present study

Locus	Primer		Reference
	Name	Sequence (5' to 3')	
Internal transcribed spacer region of nuclear ribosomal DNA (ITS1-5.8S-ITS2)	ITS5	GGA AGT AAA AGT CGT AAC AAG G	White <i>et al.</i> (1990)
	ITS4	TCC TCC GCT TAT TGA TAT GC	White <i>et al.</i> (1990)
DNA replication licencing factor ( <i>Mcm7</i> )	MCM7-709for	ACI MGI GTI TCV GAY GTH AAR CC	Schmitt <i>et al.</i> (2009)
	MCM7-1348rev	GAY TTD GCI ACI CCI GGR TCW CCC AT	Schmitt <i>et al.</i> (2009)
	MCM7-solF	CYG ARA TCT TCC AGY CCG TCA	Present study
	MCM7-solR	CCA TRA GGC ARA YGT TGA TG	Present study
Polyketide synthase type I (PKS) – ketosynthase (KS) domain	LC1-Im	GAC CCG MGG TTY TTY AAY ATG	Schmitt <i>et al.</i> (2005)
	LC2c-Im	GTG CCG GTG CCR TGC ATY TC	Schmitt <i>et al.</i> (2005)

(replication licencing factor) recently shown to be phylogenetically informative (Schmitt *et al.*, 2009; Raja *et al.*, 2011; Truong *et al.*, 2013). The primers used are listed in Table 1. The published *Mcm7* primers (Schmitt *et al.*, 2009) performed poorly in some samples and *Solenopsora*-specific primers were therefore developed to increase PCR specificity and efficiency (Table 1). Standard PCR was performed in 25- $\mu$ L reaction volumes (Mastercycler® ep gradient S; Eppendorf). For amplification of the ITS and KS regions, the PCR mix contained 0.625 U of DreamTaq polymerase (5 U  $\mu$ L<sup>-1</sup>; Thermo Scientific, Fermentas), 2.5  $\mu$ L of buffer (including MgCl<sub>2</sub> at 2 mM in the final volume), 0.2 mM each dNTPs, 0.2  $\mu$ M each primer, and 1  $\mu$ L template in a final reaction volume of 25  $\mu$ L. The PCR cycle profile for KS amplification followed that described by Winka, Ahlberg & Eriksson, (1998) with modifications: 94 °C for 4 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, elongation at 72 °C for 5 min, and cooling to 4 °C. For ITS amplification, a touchdown cycle (Reese Næsberg, Ekman & Tibell, 2007) was used: 94 °C for 4 min, six cycles of 94 °C for 1 min, 62 °C (decreasing 1 °C per cycle) for 1 min, 72 °C for 1 min 45 s, 34 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 1 min 45 s (increasing by 3 s per cycle), final elongation at 72 °C for 10 min, and cooling to 4 °C. For amplification of *Mcm7*, the PCR mix contained 0.625 U of either AmpliTaq Gold (5 U  $\mu$ L<sup>-1</sup>; Life Technologies) or HOT FIREPol (5 U  $\mu$ L<sup>-1</sup>; Solis BioDyne) polymerase with supplied buffers, 2 mM MgCl<sub>2</sub>, 0.2 mM each dNTP, 0.2  $\mu$ M each primer, and 1  $\mu$ L of template in a final reaction volume of 25  $\mu$ L. The *Mcm7* PCR cycling conditions followed those described by Schmitt *et al.* (2009) with modifications: 95 °C for 15 min, 35 cycles of 95 °C for 45 s, 56 °C for 50 s, 72 °C for 1 min, final elongation at 72 °C for 5 min, and cooling to 4 °C. In cases where these PCR conditions failed to amplify the targeted loci efficiently,

we used Qiagen Multiplex PCR kit (Qiagen). The PCR cycling parameters were the same as those described above; in KS and ITS cycle profiles, just an initial 15-min step at 94 °C was added to activate the polymerase. PCR products were purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) in accordance with the manufacturer's instructions. Cycle-sequencing reactions were carried out using the original PCR primers at the BITCET Consortium at the Department of Molecular Biology, Comenius University, Bratislava. Products were electrophoresed on an ABI 3130xl Genetic Analyzer.

#### SEQUENCE ALIGNMENT

Sequence identity was confirmed using the BLAST search tool in GenBank (Wheeler *et al.*, 2006). Electropherograms were carefully inspected in CHROMAS LITE, version 2.01 (Technelysium Pty Ltd) for double peaks indicating heterozygous positions (*Mcm7*) or sequence variation among the multiple copies of the KS and ITS regions. Intra-individual polymorphic sites were coded using International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes. Sequences were aligned manually in BIOEDIT, version 7.1.3.0 (Hall, 1999). Manual alignments of the KS and ITS regions were improved in GENEIOUS, version 5.6.4 (Biomatters Ltd) using MAFFT, version 6.814b (Katoh *et al.*, 2002) plugin. A highly ambiguous indel region observed at the 5' end of ITS1 was excluded. All sequences generated were deposited in GenBank (for accession numbers, see Supporting information, Doc. S1).

#### PHYLOGENETIC ANALYSIS

Phylogenetic trees were inferred using Bayesian and maximum likelihood (ML) analyses. Best-fit models of nucleotide substitutions were selected in JMODEL-

**Table 2.** Summary of the nucleotide alignments used in the present study

Locus	Number of sequences/unique sequences	Alignment length (bp)	Number/percentage of variable sites	Evolutionary model	Character partitions
ITS of nuclear ribosomal DNA*	101/40	539	195/36%	TIM3 + I + G	Two partitions (ITS1 + ITS2, 5.8S gene)
KS of PKS	102/31	681	222/33%	TIM2 + I + G	Four partitions (one intron, three codon partitions of two exons)
<i>Mcm7</i>	100/34	509	158/31%	TIM2 + I + G	Three codon partitions
Concatenation	94/67	1,729	559/32%	–	Three locus partitions

Number of sequences/unique sequences (both excluding outgroups), alignment length, number/percentage of variable positions, nucleotide substitution models selected based on Akaike information criterion in JMODELTEST, and partitions used in MrBayes analyses. ITS, internal transcribed spacer; KS, ketosynthase; PKS, polyketide synthase gene.

\*Excluding the ambiguous indel region at 5' end of ITS1.

TEST, version 0.1.1 (Guindon & Gascuel, 2003) using Akaike information criterion (Akaike, 1974) and implemented in the Bayesian and ML computations. For the KS domain that included a short intron region, intron and exon sequences were defined as different partitions, specifying also the first, second, and third codon positions of the exons and unlinking model parameters across the defined partitions of the data. For the *Mcm7* data that encompassed a single exon region, we also defined and unlinked three character partitions based on the first, second, and third codon positions. In the ITS dataset, two partitions corresponding to the 5.8S gene and ITS1 + ITS2 were defined (Table 2). Bayesian analyses based on the Markov chain Monte Carlo algorithm (MCMC; Huelsenbeck & Ronquist, 2001) were run in MrBayes, version 3.2.1 at the Biportal at the University of Oslo (<http://www.biportal.uio.no>, Kumar *et al.*, 2009). Two parallel runs with four chains of MCMC were conducted for five million generations, with a sampling frequency of every 100 generations. The settings corresponding to the best-fit evolutionary models were specified; otherwise, default priors were used. Convergence of all parameters was verified by inspecting the graphical output from the Biportal website. The average standard deviation of split frequencies between the two simultaneous runs was checked to fall below 0.01, and the potential scale reduction factor to be close to 1.0 for each parameter. For each run, the first 5000 trees (500 000 generations) were discarded as the burn-in period, and the consensus tree was generated from the remaining trees from both runs, computing also the Bayesian posterior probabilities (BPP) for each node. ML analyses were computed in GARLI, version 2.0 (Zwickl, 2006) setting five million generations, and with multiple independent replicate runs. Searches were per-

formed with random starting trees and by setting the program to stop after 20 000 generations if no improvement of the log-likelihood was detected ( $\leq 0.01$ ), with a maximum of 50 000 generations. A check was made that the topologies of the resulting trees obtained from the replicate runs were the same. Branch support was assessed by 500 bootstrap replicates under the same settings as described above.

Before combining the three datasets, topological congruence between the gene trees was examined using 500 replicates of ML bootstrapping under the same models described above, on each locus separately (Mason-Gamer & Kellogg, 1996). Because there was no conflict detected using a 70% reciprocal threshold (with only a slightly higher value, 74%, for a single clade in the KS gene tree), the alignments were concatenated. We conducted a Bayesian analysis (MrBayes, version 3.2.1) of a concatenated dataset of all three markers. In this case, each locus was defined as a separate partition with the rest of settings as specified above. In addition to the concatenation approach that, in some cases, may lead to poor estimation of the species tree (Kubatko & Degnan, 2007), we also employed a Bayesian coalescent-based approach to estimate a species tree using \*BEAST implemented in BEAST, version 1.7.4 (Heled & Drummond, 2010). \*BEAST infers the species tree directly from the sequence data and assumes that the incomplete lineage sorting/deep coalescence is the main source of inconsistency between gene trees and species trees. BEAUti, version 1.7.4, was used to generate the input file for BEAST, setting three data partitions (corresponding to the three loci), the best-fit evolutionary model for each dataset (determined in JMODELTEST, see above), assuming uncorrelated lognormal clock, a birth–death model for the species tree prior, and the remaining parameters set to

default. Four independent MCMC analyses were run each for 10 million generations, sampling every 1000th generation. Convergence of all parameters to the stationary distribution in each run was assessed using TRACER, version 1.5 (Rambaut & Drummond, 2007). TREEANNOTATOR, version 1.7.4, (<http://beast.bio.ed.ac.uk/TreeAnnotator>) was used to set the burn-in (discarding the first 2000 trees) and to calculate the maximum clade credibility tree.

In the concatenated and coalescent-based approaches, *S. holophaea* was not included (*Mcm7* sequences could not be retrieved here) and *S. olivacea* subsp. *olbiensis* was represented by a single sample only (ITS sequence could not be retrieved from the sample *olbiensis* 2 GR). All other taxa were represented by multiple accessions (see Supporting information, Doc. S1).

#### MORPHOLOGICAL, ANATOMICAL, CHEMICAL, AND ECOLOGICAL ANALYSES

For all studied taxa, we scored selected morphological, chemical and ecological traits (not only in the specimens listed in the Supporting information, Doc. S1, but also for those marked in Doc. S2). We focused on thallus morphology, mode of reproduction, ascospore size, hymenium and hypothecium heights, and the presence of secondary metabolites. For ecological preferences, we studied obligate and facultative substrate and microhabitat preferences related to light and humidity. The examined features and preferences are summarized in Figure 3 and in Table 3. Microscopical examinations (light microscopy) were performed on hand-cut sections mounted in water and 10% KOH. The measurements of thallus and apothecia given in the description of the species are based on the selected examined material. Cryotome sections were prepared to study the anatomy of the cortex in detail. Chemistry was studied by thin-layer chromatography (TLC), in solvent systems A, B' and C (Culberson & Kristinsson, 1970; Culberson, 1972; Culberson & Johnson, 1982; Orange, James & White, 2001). Microextracts of herbarium specimens with TLC determined chemistry (pannarin, zeorin, atranorin) were used as standards. Terminology regarding morphology, and anatomy of the lichen thallus follows that of Ryan, Bungartz & Nash (2002) and Ryan *et al.* (2012).

## RESULTS

#### SEQUENCE DATA CHARACTERISTICS

Details of nucleotide alignments are summarized in Table 2, including the best-fit models of evolution selected with JMODELTEST and notes on the data partitions used. In the ITS dataset, variation in the 5.8S gene was negligible, whereas ITS1 and ITS2 were

highly variable, requiring the introduction of numerous (mainly 1- or 2-bp, or longer but overlapping) indels. Separate indel scoring, however, appeared ambiguous. In the KS alignment, one short intron region with four 1–4-bp indels was identified. Two indels supported two of the main clades of the resulting gene tree (clades B and IV of A; see below) and two were singletons, each present in a single sample. The scarcity of indels contrasts here with a high number of SNPs in the alignment and implies that their phylogenetic information is negligible in this case. Thus, in both datasets, the indel characters were treated as missing data. The *Mcm7* alignment included only the exon sequences with no indel variation.

#### GENE TREES VERSUS CONCATENATED PHYLOGENY AND COALESCENT-BASED SPECIES TREES

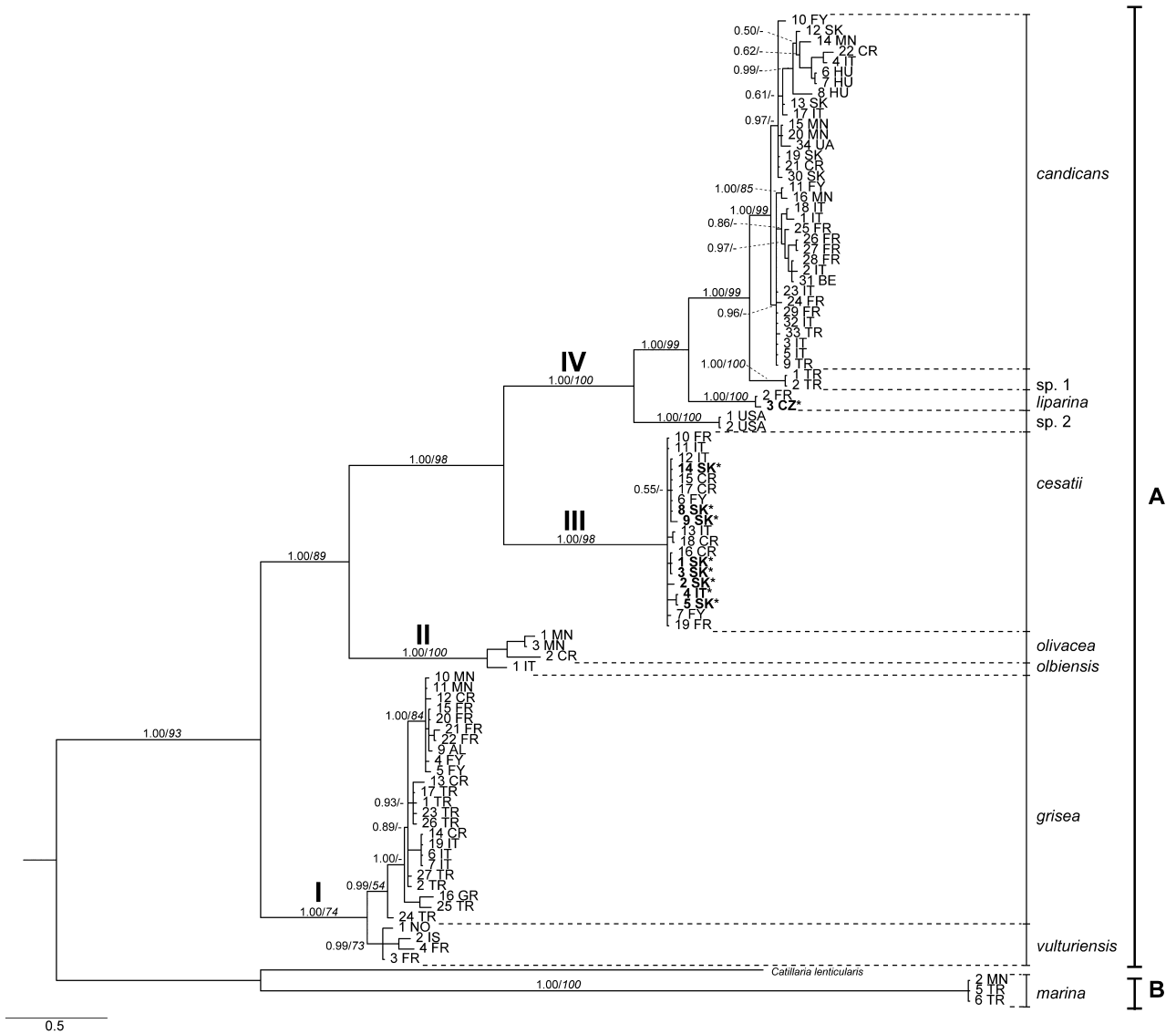
Bayesian phylogenetic trees based on KS, *Mcm7* and ITS (see Supporting information, Figs S1, S2, S3) yielded similar topologies, showing that two species, *Solenopsora holophaea* (present in KS and ITS trees) and *S. marina* (denoted as clade B), are divergent from the rest of the species (clade A) that form four major clades (I–IV): clade I, *Solenopsora grisea* and *S. vulturienensis* (BPP<sub>KS</sub> = 0.96, BPP<sub>*Mcm7*</sub> = 0.99, BPP<sub>ITS</sub> = 1.00); clade II, *S. olivacea*, including subsp. *olivacea* and subsp. *olbiensis* (BPP = 1); clade III, *S. cesatii* (BPP = 1); and clade IV, *S. candicans*, *S. liparina*, and two potential new species that need further study, tentatively labelled here as *Solenopsora* sp. 1 and *Solenopsora* sp. 2 (BPP = 1). Accessions of *S. carpatica* (indicated in bold and with an asterisk in Fig. 2; see also Supporting information, Figs S1, S2, S3) appear in the clades of *S. cesatii* (samples from the Western Carpathians and the Alpi Bergamasche Mts) and of *S. liparina* (a sample from the Sudety Mts) in all three gene trees. Resolution of the gene trees, however, was insufficient in some parts. They did not resolve the relationship between *S. grisea* and *S. vulturienensis* (clade I). The ITS tree (see Supporting information, Fig. S3) suggested an alternative branching pattern for clade II (here sister to clade I), albeit with a weak support (BPP = 0.97, bootstrap support = 52%). Similarly, the sister relationship between *S. olivacea* subsp. *olivacea* and subsp. *olbiensis* was supported only in the KS tree (BP = 1; see Supporting information, Fig. S1), whereas it remained unresolved in the *Mcm7* and ITS trees (see Supporting information, Figs S1, S3). ML trees (not shown) displayed topologies similar to the Bayesian ones. Bootstrap support obtained from the ML analyses is depicted on the respective Bayesian trees (see Supporting information, Figs S1, S2, S3). The same two main clades (A and B) and four clades (I–IV) in clade A and relationships among them were observed for each gene tree, with topological

**Table 3.** Summary of morphological and chemical traits observed in the treated European *Solenopsis* taxa

Taxon	Thallus	Reproduction	Ascospores (µm)	Hymenium (µm)	Hypotecium (µm)	Secondary chemistry
<i>candicans</i>	Placodioid, white, pruinose, of single rosettes (diameter up to 4–5 cm), lobes flat, branched	Sexual: apothecia frequent, thalline margin visible, ± persisting, disc brown, black-brown, diameter up to 1.5 mm, faintly pruinose or not	(10-)12–16 × 2.5–5	50–70	110–140	Pannarin, zeorin (both major)
<i>cesatii</i>	Placodioid, blue-grey, greyish, smooth, whitely pruinose, of single rosettes (diameter up to 2 cm) or when centres die away of concentric radiating circles or arcs (diameter up to 15 cm), lobes undulate, folded, crisped, with round, frequently entire margins of branches	Sexual: apothecia frequent, thalline margin visible, ± persisting, disc dark brown, blackish, diameter up to 1.2 mm, faintly pruinose or not	(7-)8–11 × 2.5–4	40–60	100–110	Pannarin, zeorin (both major), minor unidentified substances
<i>grisea</i>	Placodioid, of continuous, irregular patches (diameter up to 8–10 cm), marginal lobes to 1 mm wide; central parts green/grey-green, glaucous, margins of lobes whitely pruinose; central lobes raised when producing blastidia	Sexual: apothecia rare, diameter up to 1.5 mm, juvenile with crenulate thalline margin and flat disc, at maturity becoming convex, faintly pruinose or not, thalline margin retreating; vegetative: blastidia, soralia like structures	(10-)14–18 × 2.5–4	60–70	80–110	Complex: terpenoids, unidentified substances, atranorin (occasionally)
<i>vulturienis</i>	Squamulose-granular, central parts green/grey-green, glaucous, lobe margins whitely pruinose; central lobes raised when producing blastidia	Sexual: apothecia rare, diameter up to 0.6 mm, juvenile with crenulate thalline margin and flat disc, faintly pruinose or not; vegetative: blastidia, soralia like structures	9–11 × 4–5	50–60	80–110	Complex: terpenoids, unidentified substances, atranorin (occasionally)
<i>holophaea</i>	Squamulose, epruinose, of shiny, red-brown, greenish-brown squamules (to 2.5 mm wide) with rounded, entire margin; central parts attached to the substrate, outer lobes loose, flexuose, recurved	Sexual: apothecia frequent, diameter up to 1.5 mm, sessile, often shortly stipitate, disc red-brown/blackish	12–18 × 4–5	50–60	130–140	Complex: terpenoids, unidentified substances
<i>liparina</i>	Placodioid, grey-green, mostly of single rosettes (diameter up to 2.5 cm), or forming clusters or arcs, lobes loosely attached to substrate, lobe ends round, whitish pruinose	Sexual: apothecia frequently present, diameter up to 1 mm, thalline margin visible, ± persisting, disc brown, bluish-whitish pruinose	13–16 × 3–4	60–70	80–90	Pannarin, zeorin (both major)
<i>marina</i>	Squamulose, ± distinctly rosetted, rosettes or irregular patches of up to 5–6 cm in diameter, squamules pale greenish to green, at margins lobate, white pruinose, central parts attached to substrate, outer lobes loose, flexuose, ± folded	Sexual: apothecia frequently present, light to medium brown, sessile, at maturity frequently globose, diameter up to 1 mm	9–12–16 × 3–3.5	60–70	90–100	Complex: terpenoids, unidentified substances
<i>olivacea</i> subsp. <i>olivacea</i>	Crustose, forming continuous, irregular patches (diameter up to 10 cm), green/brown-green, marginal squamules effigurate	Sexual: apothecia frequent, diameter up to 0.5 mm, juvenile with hardly visible thalline margin and flat disc, at maturity becoming convex, light to dark brown; vegetative: pycnidia immersed in thallus, conidia colourless, simple, bacilliform, 3.5–4.5 × 0.5–0.9 mm	(10-)12–16 × 2.5–4	35–40	50–90	Pannarin, zeorin (both major)
<i>olivacea</i> subsp. <i>olbiensis</i>	Crustose, of continuous, irregular patches (diameter up to 10 cm), green/brown-green, marginal squamules effigurate	Vegetative: soralia light-green, excavate, rounded, or contiguous	NA	NA	NA	Pannarin, zeorin (both major)

NA, not available.





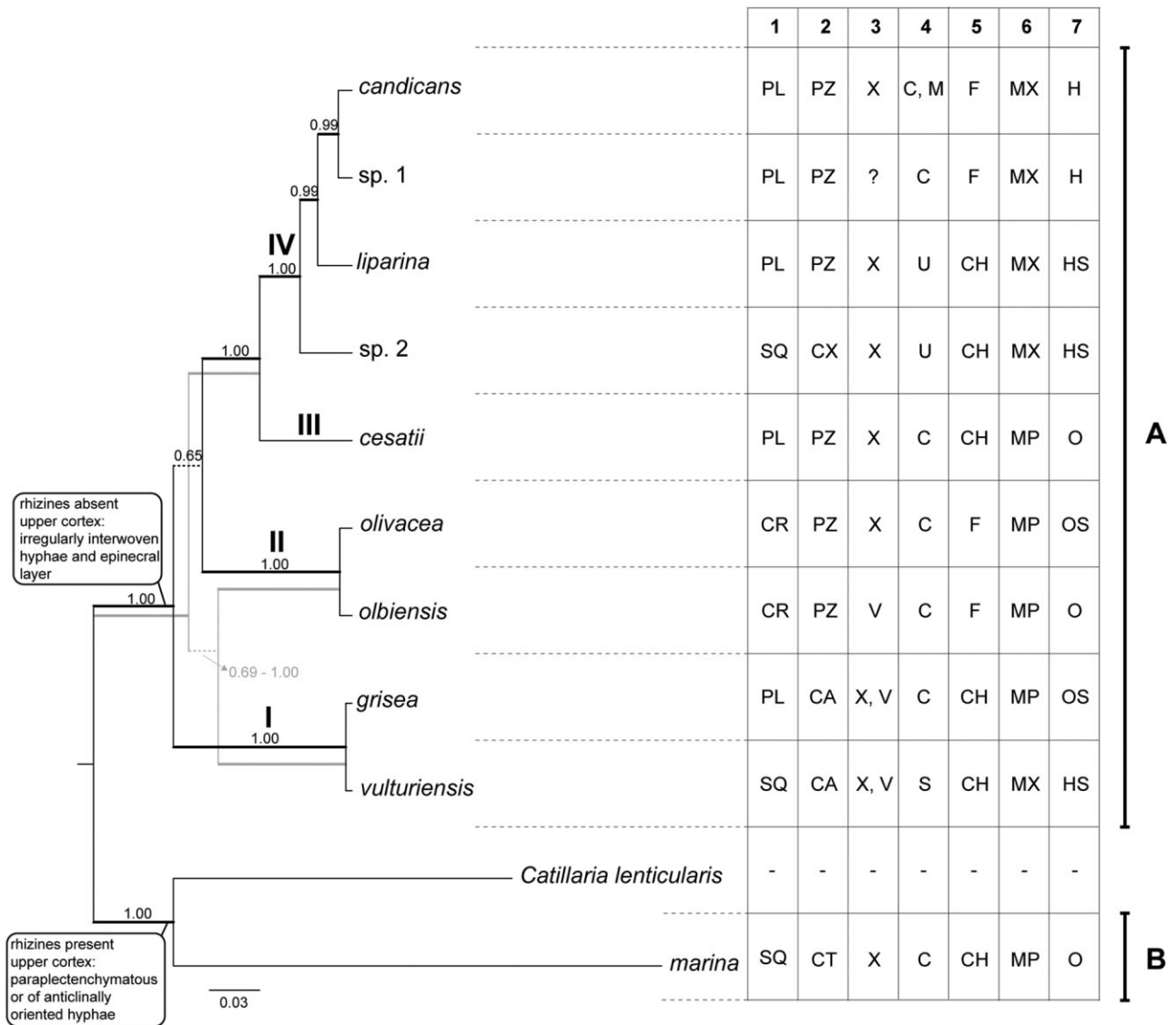
**Figure 2.** Bayesian majority-rule consensus tree with mean branch lengths based on the concatenated sequences from all three loci (ITS, KS, *mcm7*). The posterior probability values are indicated above the branches (before slash); italicized values (after slash) show bootstrap support > 50% assessed by maximum likelihood analyses. A and B denote two main lineages, and I-IV indicate the clades referred to in the text. The codes of accessions follow Doc. S1. The codes marked in bold and accompanied by asterisk indicate the accessions classified as *Solenopsora carpatica*.

differences regarding more terminal subclades with lower BPP support.

A Bayesian phylogenetic tree inferred from the concatenated alignment showed a topology congruent with the PKS and *Mcm7* gene trees in respect of the position of clade II (Fig. 2). In the coalescent-based species tree, the same four clades (all BPP = 1) and topology were obtained, except for the position of clade II that remained uncertain (Fig. 3). In the replicate runs, it was either resolved in the same position as in the concatenated tree but with low node support (BPP = 0.65) or as sister to the clade I as also seen in

the ITS tree (with BPP = 0.69–1, depending on the run; Fig. 3).

Sister relationships of closely related taxon pairs *S. grisea* and *S. vulturienis* (clade I) and *S. olivacea* subsp. *olivacea* and *S. olivacea* subsp. *olbiensis* (clade II) are confirmed in both the concatenated and species trees. The topology of the clade IV consisting of four species (*S. candicans*, *S. liparina*, *Solenopsora* sp. 1 and sp. 2) was congruent in the concatenated gene tree and the species tree, being identical to that in the ITS tree, and resolved the uncertainties in the KS and *Mcm7* trees (see Supporting information, Figs S1, S2).



**Figure 3.** The maximum clade credibility species tree obtained from Bayesian inference in \*BEAST based on the three studied DNA loci (ITS, KS, *Mcm7*). The posterior probability values are indicated above the branches; thick branches indicate high support ( $\geq 0.99$ ). The dashed branch indicates low support [Bayesian posterior probabilities (BPP) = 0.65] and incongruence between four independent Markov chain Monte Carlo algorithm runs: the alternative topology is indicated by grey branches with the BPP range of replicate runs being 0.69–1.00. A and B denote two main lineages, and I–IV indicate the clades referred to in the text. The species characteristics depicted on the right-hand side of the tree include: 1 thallus form: SQ – squamulose, PL – placodioid, CR – crustose; 2 secondary chemistry: PZ – pannarin and zeorin major, CA – complex of terpenoids with occasional presence of atranorin, CT – complex of terpenoids, CX – complex chemistry; 3 reproduction: X – sexual, V – vegetative; 4 habitat: C – calcareous, S – siliceous, U – ultramaphic, M – muscicolous; 5 microhabitat: F – plane rock face, CH – chasmophyte; 6 humidity: MX – mesoxerophyte, MP – mesophyte; and 7 light: H – heliophyte to helioscophyte, O – ombrophyte, OS ombro-scyophyte, HS – helioscophyte.

*Catillaria lenticularis*, used as the outgroup, was resolved in the species tree in the sister position to *S. marina* with a high support (BPP = 1), although this relationship had no support in the concatenated gene tree (Figs 2, 3).

#### MORPHOLOGY, CHEMISTRY, AND ECOLOGICAL PREFERENCES

The studied taxa share similar dorsiventral body plans with three different thallus growth forms. In

clade B, we observe foliose-squamulose thalli with sparse, pale rhizines on the lower side, whereas, in the core clade A, placodioid or crustose thalli without rhizines prevail (two accessions of foliose/squamulose thallus occurs in clade IV – *S. vulturien-sis* and *S. sp. 2*).

Pruina is present in the taxa of both main clades. It might cover the entire thalline surface (e.g. *S. candidans*, *S. cesatii*, *S. sp. 1*) or it can be limited to the thalline margins (e.g. *S. marina*, *S. vulturien-sis*, *S. liparina*, *S. sp. 2*). We noted differences in pruina cover in *S. grisea*. Thalli on rocks more exposed to light were whitish on their entire surfaces, whereas, on shaded rocks (in woodlands), the pruina was limited to lobe ends. This feature might have been a source of misidentifications of *S. grisea* in the past, especially when a small, marginal piece was available for closer examination.

The taxa in the B clade are characterized by a well developed upper cortex, either paraplectenchymatous or composed of anticlinally oriented, gelatinized hyphae, and sexual reproduction. Clade A comprises taxa with upper cortex of irregularly interwoven hyphae with a continuous epinecral layer, and both sexual and vegetative modes of reproduction (blastidia, soralia). Both reproduction modes occur in *S. grisea*, *S. vulturien-sis*, and *S. olivacea* subsp. *olbiensis* (the type species forming apothecia and soralia; Nylander, 1876; *H-NYL29314*).

Clade B includes samples containing unknown terpenoids (products of the mevalonic acid pathway). In clade A, we found a combination of polyketide pathway products (pannarin, atranorin) and mevalonic acid pathway products (zeorin, unknown terpenoids).

The characters described above are summarized in Table 3 and depicted on the species tree (Fig. 3). It is apparent that characters such as squamulose thallus organization, pruina on the thalline surface, ascospore size, hymenium and hypothecium height, and complex secondary chemistry are homoplasious.

Each *Solenopsora* taxa has peculiar, unique microhabitat preferences (e.g. light, humidity), thus occupying specific microniches in the same outcrop. They are mostly saxicolous, growing on calcareous sedimentary rocks, such as limestone, dolomite, and conglomerate, or on siliceous igneous rocks, such as basalt or ultramafic rocks. With respect to ecological requirements and distribution, we can divide them into two groups. The first group includes the species with narrow ecological amplitudes confined to the Mediterranean climate: *S. grisea*, *S. marina*, *S. olivacea* subsp. *olivacea*, and *S. olivacea* subsp. *olbiensis*. The second group comprises the taxa with wider ecological amplitudes and distribution ranges: *S. candidans*, *S. cesatii*, *S. vulturien-sis*, and *S. holophaea*.

Two taxa (*S. liparina* and *S. sp. 2*) feature a particular position because they are confined to ultramafic rocks and appear as edaphic vicariants of *S. candidans* that typically grows on calcareous rocks. These three taxa are part of clade IV (Fig. 3).

## DISCUSSION

### MULTILOCUS APPROACH

Fungi in general, unlike other pluricellular organisms, have a relatively limited set of taxonomically useful morphological traits, and the specific genetic control underlying most diagnostic characters is currently unknown (Leavitt *et al.*, 2011). Furthermore, as a result of the small size of the fungal chromosomes, there are no karyological data that are significant in the taxonomy of vascular plants (Crespo & Pérez-Ortega, 2009).

Molecular data provide an excellent basis for the critical evaluation of phenotypic characters, and help to trace previously overlooked differences (Lumbsch & Huhndorf, 2007). They are also extremely useful for assessing whether the investigated traits are reliable or show homoplasy (Fontaine, Ahti & Piercey-Normore, 2010; Muggia *et al.*, 2011) and revealing cases of cryptic speciation (Schmitt *et al.*, 2005; Vondrák *et al.*, 2009). The present study is largely based on inferences from DNA sequences of three tested nuclear regions. Morphological and chemical data were also gathered and interpreted in the light of the presented genetic data. Both the gene tree and coalescent-based species tree approaches were employed to infer a robust phylogenetic inference of the studied taxa. The ITS region is the most popular genetic marker for mycological systematics at low taxonomic levels and has been used for barcoding (Nilsson *et al.*, 2012). This is mainly because of straightforward amplification, even with partly or severely degraded DNA, and the availability of universal primers. Phylogenetic inference based on ITS can, however, be biased and misled by the orthology/paralogy conflation (e.g. ITS heterogeneity reported from *Physcia aipolia* (Ehrh. ex Humb.) Fürnr. and *Physcia stellaris* (L.) Nyl.; Simon *et al.*, 2005), intra- and interlocus sequence homogenization, a higher level of homoplasy, and alignment uncertainty mostly as a result of indel accumulation (Álvarez & Wendel, 2003). Alignments with low confidence have posed problems especially among representatives of higher taxonomic categories (Peršoh, Beck & Rambold, 2004), which we also encountered during the present study, when the highly ambiguous 5' end of the ITS1 had to be excluded. The exclusive use of ITS has therefore been abandoned, and it has been suggested that more robust insights can be achieved by a

multilocus approach including also single- or low-copy nuclear genes (Álvarez & Wendel, 2003, Hofstetter *et al.*, 2007; Truong *et al.*, 2013). Multicopy nuclear PKS (KS) genes have been shown to be reliable markers (Schmitt *et al.*, 2005). The KS region, although less variable than ITS, has proved useful in some phylogenetic studies (e.g. on species of *Lecanora*: Grube & Blaha, 2003; and the *Cladonia gracilis* group: Fontaine *et al.*, 2010). *Mcm7* is a single-copy, protein-coding nuclear gene, recently recognized as a useful marker to infer higher and lower level taxonomic relationships (Raja *et al.*, 2011; Truong *et al.*, 2013). Although it may be more difficult to amplify by PCR, it can provide a better phylogenetic resolution than the other markers (e.g. ITS; Truong *et al.*, 2013). Our results show that KS and *Mcm7*-based trees provided largely congruent branching patterns with minor discrepancies in the topology and resolution in more terminal clades only, whereas the ITS-based tree suggested a certain discordance in a different placement of the clade II. Importantly, both the concatenated gene tree and the species tree provided more resolution, and also resolved the sister positions of two pairs of closely-related taxa (*S. olivacea* subsp. *olivacea* and subsp. *olbiensis*, clade II; *S. grisea* and *S. vulturiensis*, clade I), which were inconclusive in the individual gene trees. However, the position of clade II (*S. olivacea*) remained uncertain, and the source of this conflict needs to be finally resolved by additional sampling or markers (other nuclear low copy or mitochondrial regions).

#### RELATIONSHIPS AMONG THE TREATED TAXA AND THEIR TAXONOMIC DELIMITATIONS

Phylogenetic analyses have recently confirmed either monophyly (e.g. *Diploschistes* Norman: Fernández-Brime *et al.*, 2013) or polyphyly of the studied lichen genera (e.g. *Lecania*: Reese Næsberg *et al.*, 2007; *Hypocenomyce* M.Choisy: Bendiksby & Timdal, 2013). In the present study, the inferred phylogenetic trees reveal two divergent lineages (clades A and B) among European representatives of *Solenopsora*. The two lineages exhibited highly divergent sequences for all three markers and appeared as two well-supported clades. This division is also supported by several diagnostic features, such as thallus organization, rhizines on lower side of the thallus, secondary chemistry, and anatomy of upper cortex (Fig. 3). At this point, we consider that it would be premature to outline firm phylogenetic or taxonomic conclusions regarding the genus circumscription because our focus was on the European taxa only. Related genera and *Solenopsora* taxa from the other parts of the world need to be included to revise the generic concept of the studied group; for example, *Solenop-*

*sora chihuahuana* B.D.Ryan & Timdal, *Solenopsora cladonioides* B.D.Ryan & Timdal, *Solenopsora crenata* (Herre) Zahlbr., and *Solenopsora cyathiformis* (Szatala) Van den Boom from North America (Ryan & Timdal 2002, 2011) and *Solenopsora elixiana* Verdon & Rambold, *Solenopsora sordida* (C.W.Dodge) D.J.Galloway, and *S. tasmanica* Kantvilas from the Southern Hemisphere (Galloway, 2004; Kantvilas, 2004; Van Den Boom *et al.*, 2011).

In the lineage A, clade I is resolved as the first-branching; it includes *S. grisea* and *S. vulturiensis* (Fig. 3). Although the individual gene trees did not resolve their relationships, the concatenated and species trees confirm here for the first time the sister position of these two taxa. Among the synapomorphies that these species share the presence of both sexual and vegetative (blastidia) reproduction modes and complex chemistry. The species differ in thallus morphology and size (see key to the treated *Solenopsora* taxa), substrate preferences (calcareous versus siliceous rocks), and distributional range [strictly Mediterranean climate (*S. grisea*) versus wider occurrence to temperate to boreal zones of the Northern and Southern Hemispheres (*S. vulturiensis*; see Supporting information, Doc. S3)].

Our results suggest that the crustose species *S. olivacea* (clade II) includes two infraspecific lineages, which we consider subspecies rather than varieties (McNeill *et al.*, 2012: chapter 1, art. 4). They differ in reproduction mode, with the nominate subspecies *olivacea* reproducing sexually and subspecies *olbiensis* obligately reproducing vegetatively via soredia (apothecia are exceptionally recorded, cf. Nylander, 1876). The two subspecies also differ in ecological preferences: *S. olivacea* subsp. *olivacea* grows on rock faces in shaded and open habitats, whereas *S. olivacea* subsp. *olbiensis* is an obligatory chasmophyte; if it covers perpendicular rock faces, then these are situated in humid and shaded forests.

The *S. cesatii* clade (clade III) is well supported in all the analyses, although it also includes the samples so far assigned to the taxon *S. carpatica* from the Western Carpathians (Slovakia) and the Alpi Bergamasche (Italy) (see Supporting information, Figs S1, S2, S3). These results confirm Kotlov's concept (Kotlov, 2004), that *S. carpatica* is a synonym of *S. cesatii* (see Supporting information, Table S1). The revealed variation patterns do not favour recognizing infraspecific taxa within *S. cesatii*.

Clade IV includes two well-delimited species: *S. candicans* and *S. liparina* (Figs 2, 3). *Solenopsora liparina* also includes a sample until now identified as *S. carpatica* from the Sudety Mts (Czech Republic). Our data do not support the systematic position of *S. liparina* as a variety of *S. cesatii* [*S. cesatii* var. *olivacea* (Bagl.) Kotlov; Kotlov, 2004] (see Supporting

information, Table S1). The phylogenetic analyses disclosed also two separate, so far unrecognized/unidentified entities. *Solenopsora* sp. 1 was collected from perpendicular calcareous rock faces in Turkey. *Solenopsora* sp. 2 grows on silica-carbonate rocks in California, USA (Rajakaruna *et al.*, 2012). We consider it premature at this stage to describe these two new species formally because more material would be desirable to sample and analyze, considering also the other, non-European taxa of the genus.

#### MORPHOLOGICAL, REPRODUCTIVE, AND CHEMICAL TRAITS

Lichen genera are often delimited on the basis of thallus growth form, which is a highly variable character (Printzen, 2010). Several characters, thalline form including, that are homoplasious at a certain level (e.g. family) can be differential or synapomorphic at lower levels such as genera or species (Crespo & Pérez-Ortega, 2009). The point is that these forms, having particular anatomical structure and function (Grube & Hawksworth, 2007), are optimal to the successful colonization of particular ecological niches. Foliose-squamulose thallus is present in lineage B, with two occurrences recorded also in lineage A (*Solenopsora* sp. 2 and *S. vulturiensis*). The squamules/folioles of the taxa in lineage B are partly detached from the substrate, corticate on the upper surface (cortex well developed, either paraplectenchymatous or of anticlinally oriented, gelatinized hyphae) and with rhizines on lower surface. Variation in the morphology and anatomy of the lichen thallus has been shown to control water storage capacity and evaporative resistance (Pintado, Valladares & Sancho, 1997). Well developed upper cortex promotes thallus hydrated for longer period (Fos *et al.*, 1999) and limits CO<sub>2</sub> exchange necessary for photosynthesis and respiration (Green, Snelgar & Brown, 1981). Development of rhizines was shown to be a mechanism for increasing water storage capacity in *Parmelina pastillifera* (Harm.) Hale (Tretiach & Brown, 1995). Grube & Hawksworth (2007) raised the hypothesis that the cortex imposes a physical necessity to let the thallus grow as a detached form. These traits, in case of *S. holophaea* and *S. sp. 2* (cluster IV), may help to keep the thallus hydrated longer and decrease too high irradiation because these species grow in open habitats. *Solenopsora marina*, however, is confined to shaded and humid sites. Non-rhizinate placodioid thallus of brittle areoles and lobes attached to the substrate dominate in lineage A clades I, III, and IV. Clade II includes taxa with crustose thallus (*s.s.*, not forming lobes). The studied entities demonstrate that various types of thallus categories may occur in closely related taxa, as is the case of *S. grisea* (placodioid

thallus) and *S. vulturiensis* (crustose-squamulose thallus).

Pruina is a concept widely used in lichenology. It includes many types of surface coverings (e.g. calcium oxalate, anthraquinones, dead mycobiont tissues) and its occurrence has been applied as an important species character in some genera (Timdal, 1984; Heiðmarsson, 1996; Wei & Wei, 2012). Different hypotheses about the ecological significance have been summarized by Wadsten & Moberg (1985) and Heiðmarsson (1996). Giordani, Modenesi & Tretiach (2003) provided experimental evidence that formation of calcium oxalate minerals in lichens is at least partially biologically controlled. Metabolic capability of forming calcium oxalate may be lost during thallus development (pruina limited to the younger parts of thalli), or retained (pruina covering entire thalline surface). Disappearance/dissolution of calcium oxalate as a result of its metastability in humid and warm habitats, creating different light conditions for the bionts, may allow the formation of soredia in mature thalline parts (Modenesi *et al.*, 2001). *Solenopsora* taxa exhibit characteristic traits regarding surface coverings. The presence of pruina appears homoplastic in the studied group and suggests that we may be dealing with an ecologically plastic character, which should be interpreted with caution.

Both reproduction modes (sexual and vegetative) occur in the studied group, with two apparent complementary functions: long-distance dispersal of potentially novel genotypes via sexually derived ascospores and rapid spread of locally-adapted genotypes via asexual propagules (Seymour, Crittenden & Dyer, 2005). The taxa in lineage B are characterized by sexual reproduction. Lineage A comprises taxa with both sexual and vegetative modes of reproduction. We observe a primarily vegetative reproduction mode in *S. grisea* and *S. vulturiensis* (blastidia or soralia like structures) and in *S. olivacea* subsp. *olbiensis* (soralia), with occasionally occurring apothecia. Ascospore morphology is frequently a key character to distinguish genera in lichens. Molecular evidence indicates that most types have evolved several times independently in Lecanoromycetes and constitute a highly homoplasious character (Printzen, 2010). Members of *Solenopsora* have one-septate, ellipsoid ascospores, with subtle variation in the ascospore apex shape: acute-acuminate, obtuse, pointed (Verdon & Rambold, 1998; Kantvilas, 2004). Spore-size and shape did not segregate the studied taxa into certain groups (Table 3). Moreover, similar to the observations of Kantvilas (2004), the studied fertile specimens yielded few, mature, well-developed ascospores, therefore making a detailed assessment of spore shape difficult (Kantvilas, 2004). Further study is also needed to explore this element in

*Solenopsora* and to clarify whether spores have reproductive function.

The widespread occurrence of most secondary metabolites across the lichenized ascomycetes indicates that they convey little information on phylogenetic relationships, although some have proved useful when distinguishing species or even at higher levels (Printzen, 2010) or at least suggest general trends (Leavitt, Johnson & St. Clair, 2011). Secondary chemistry is one of the characters distinguishing the two main lineages identified in the present study. Lineage B features complex secondary chemistry of accessory type compounds (terpenoids, unknown substances), whereas the taxa in lineage A produce either chemosyndromes with pannarin and zeorin as major substances, or accessory type compounds (terpenoids, unknown substances) with occasional records of atranorin, as in *S. grisea* (Table 3). Moreover, other *Solenopsora* spp. not included in the present study produce peculiar major compounds [lobaric acid in *S. elixiana* (Verdon & Rambold, 1998; Van Den Boom *et al.*, 2011); brialmontin in *S. tasmanica* (Kantvilas, 2004); and gangaleoidin in *S. cyathiformis* (Van den Boom & Ryan, 2004)]. Additionally, *S. chihuahuana*, *S. cladonioides*, and *S. crenata* produce pannarin and zeorin as major substances (Ryan & Timdal, 2002, 2011). The phylogenetic position of these taxa and the evolutionary significance of their secondary compounds need further exploration.

#### BIOGEOGRAPHICAL AND ECOLOGICAL CONSIDERATIONS

Lichens are broad-ranging organisms and are sensitive to air humidity. They can contribute to outline the bioclimatic features of an area (Nimis & Losi, 1984). They often have large geographical ranges, with many species showing high potential for long-distance dispersal, also across strong physical barriers (mountains, ocean, sea). One of the most striking distribution models found in lichens is the biogeographical Mediterranean pattern, in which several species are distributed across different regions with a Mediterranean climate on different continents (Crespo & Pérez-Ortega, 2009). Although the total distribution range of all *Solenopsora* spp. is still not sufficiently explored, and for some species only scarce records exist (e.g. *S. chihuahuana*, *S. crenata*, *S. cladonioides*), the present study indicates that the treated taxa are a good example of this kind of distribution. The taxa studied here are successful and widespread in the circum-Mediterranean and Atlantic Europe, whereas their occurrence is limited in continental parts of Europe. According to the data available until now, we can characterize four taxa as strictly Mediterranean, reaching the Canary Islands: *S. grisea*, *S. marina*,

*S. olivacea* subsp. *olivacea*, and *S. olivacea* subsp. *olbiensis*. Siliceous *S. vulturiensis* grows along the Atlantic coast of Portugal, Great Britain and reaches south-west Norway (Jørgensen & Nordin, 2009). We include here a recent outlying finding in western Iceland. *Solenopsora candicans* and *S. cesatii* were able to disperse to more continental habitats in Europe with oro-Mediterranean conditions. However, additional collecting is necessary in the Carpathians and Alps (where they frequently grow on places that are difficult to access) to complement the picture of the distribution of these species in Europe.

*Solenopsora* taxa are mostly saxicolous, growing on calcareous sedimentary rocks or siliceous igneous rocks. Several lichens collected from ultramafic (serpentinite) substrates in Europe have been described as being new to science, even though it is unclear whether these are truly ultramafic endemics or species that are rare and were collected only from those substrates (Favero-Longo, Isocrono & Piervittori, 2004). Our results show that *S. liparina* and *S. sp. 2* growing on serpentinites form two genetically distinct and well-supported entities. This supports observations that the stressful edaphic conditions of ultramafic substrates can promote speciation and the evolution of ultramafic endemism (Rajakaruna *et al.*, 2012).

#### CONCLUSIONS

In the present study, multilocus DNA sequence data revealed two major lineages that are differentiated by thallus organization, rhizines on lower side of the thallus, secondary chemistry, and anatomy of the upper cortex. Our results clarified the relationships between the taxa treated under *Solenopsora* occurring in Europe. We recognize eight species in Europe: *S. candicans*, *S. cesatii*, *S. grisea*, *S. holophaea*, *S. liparina*, *S. marina*, *S. olivacea*, and *S. vulturiensis*. We showed that there is no support for recognition of *S. carpatica*; the collections from the Western Carpathians, including the type, and from the Alpi Bergamasche represent the species *S. cesatii*. The name *S. carpatica* thus falls into synonymy of *S. cesatii*. Our results confirm the concept of Kotlov (2004) proposing that *S. carpatica* is a synonym of *S. cesatii* and, provide supporting molecular, morphological, chemical, and ecological arguments, which were missing so far. *Solenopsora cesatii* is not a complex of infraspecific taxa, as it was treated in the past (see Supporting information, Table S1). It represents a well-delimited, genetically homogeneous entity. The collection from the Sudety Mts was grouped with the specimens representing *S. liparina*. We gathered molecular, morphological, chemical, and ecological evidence to recognize *S. liparina* as a sepa-

KEY TO THE TREATED *SOLENOPSORA* TAXA

- 1a. Thallus formed of ± distinctly rosetted squamules or thallus foliose, not placodioid; upper cortex well developed with patchy or significant epinecral layer; apothecia regularly present; vegetative propagules absent; rhizines present; secondary chemistry complex – terpenoids, unknown substance ..... 2
- 1b. Thallus placodioid or crustose forming irregular patches; upper cortex thinner, of irregularly interwoven hyphae with continuous epinecral layer; apothecia or/and vegetative propagules present; rhizines absent ..... 3
- 2a. Squamules pale greenish to green, at margins lobate, white pruinose, central parts attached to substrate, outer lobes loose, flexuose, ± folded, upper cortex paraplectenchymatous; frequently fertile, apothecia light/medium brown, sessile, at maturity frequently globose; on calcareous substrates (rocks, fissures), in humid, shaded situations; resembling *Squamarina lentigera* ..... *S. marina*
- 2b. Foliose thallus of epruinose, shiny, red–brown, greenish–brown squamules with rounded, entire margin; central parts attached to the substrate, outer lobes loose, flexuose, recurved, upper cortex of anticlinally oriented, gelatinized hyphae; frequently fertile, apothecia dark red–brown/blackish, sessile, often shortly stipitate, disc flat; on basic siliceous soil and rock fissures (siliceous breccia, basalt), in open or slightly sheltered situations; resembling *Psora globifera* or *Romjularia lurida* (growing on calcareous substrates) ..... *S. holophaea*
- 3a. Thallus placodioid, forming continuous, irregular patches or single rosettes, or concentric radiating circles and arcs; apothecia or/and vegetative propagules present; on calcareous or ultrabasic rocks ..... 4
- 3b. Thallus crustose or diminutive squamulose–granular–crustose, apothecia or/and vegetative propagules present; on calcareous or siliceous (siliceous breccia, basalt) rocks ..... 9
- 4a. On calcareous rocks ..... 5
- 4b. On ultrabasic rocks (serpentines) ..... 8
- 5a. Secondary chemistry complex with occasional records of atranorin ..... 6
- 5b. Secondary chemistry – major substances pannarin and zeorin ..... 7
6. Thallus forming continuous, irregular patches (diameter up to 8–10 cm), central parts green/grey–green, glaucous, margins of lobes whitely pruinose; central lobes raised when producing blastidia or breaking into soralia like structures; apothecia not frequent, juvenile with crenulate thalline margin and flat disc, at maturity becoming convex, thalline margin retreating; in open or slightly sheltered situations ..... *S. grisea*
- 7a. Thallus of either single rosettes (diameter up to 2 cm) or when centres die away forming concentric radiating circles or arcs (diameter up to 15 cm); blue–grey, greyish, smooth, whitely pruinose, lobes undulate, folded, crisped, with round, margins; in fissures, in humid and sheltered situations also on perpendicular faces ..... *S. cesatii*
- 7b. Thallus of rosettes (diameter up to 5 cm) white, pruinose; lobes flat, adpressed to substrate, isitomically branched; apothecial disc light brown to brown/dark-brown, frequently epruinose; on calcareous rocks, on vertical and horizontal faces, in open as well as sheltered situations ..... *S. candicans*
8. Thallus of rosettes (diameter up to 2 cm), grey–green, lobe ends whitish pruinose; apothecial disc dark, bluish–whitish pruinose; often in fissures, in shaded situations also on perpendicular faces; resembling *Lecanora muralis* ..... *S. liparina*
- 9a. On calcareous rocks ..... 10
- 9b. On siliceous rocks ..... 11
- 10a. Thallus green/brown–green, forming continuous, irregular patches (diameter up to 10 cm), marginal squamules effigurate; apothecia present (diameter up to 0.5 mm), juvenile with hardly visible thalline margin and flat disc, at maturity becoming convex, light to dark brown; on rock faces in sheltered and open situations ..... *S. olivacea* subsp. *olivacea*
- 10b. Thallus green/brown–green, forming continuous, irregular patches; sorediate, soralia round, light green (turning yellowish in herbarium specimens); apothecia rarely found; in fissures and rock faces in humid, shade habitats (e.g. forests) ..... *S. olivacea* subsp. *olbiensis*
11. Thallus diminutive, composed of single, crowded or scattered squamules, forming irregular patches; central lobes raised when producing blastidia or breaking into soralia like structures; apothecia rare, juvenile with crenulate thalline margin and flat disc, thalline margin retreating; marginal lobes to 0.5 mm wide, whitely pruinose; on rock, in fissures of rocks, in open or slightly sheltered situations; resembling *Lepraria* sp. .... *S. vulturiensis*

rate species, being an edaphic vicariant of *S. candicans*, confined to ultramafic rocks. We disclosed so far unknown sister relationship between *S. grisea* and *S. vulturiensis*. Moreover, we disclosed two new entities, *Solenopsora* sp. 1 collected from perpendicular

calcareous rock faces in Turkey as a sister taxon to *S. candicans*, and *Solenopsora* sp. 2 collected on silica-carbonate rocks in California (Rajakaruna *et al.*, 2012), being a closest relative to *S. liparina*. However, we consider it premature at this stage to

describe these new species, before more material is made available for analysis.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Bayesian majority-rule consensus tree with mean branch lengths based on the ketosynthase (KS) domain of PKS genes. The posterior probability values are indicated above the branches (before slash); thick branches indicate high support (> 0.90). Italicized values (after slash) show bootstrap support > 50% assessed by maximum likelihood analyses. I–IV denote the main clades referred to in the text. The codes of accessions follow Doc. S1. The codes marked in bold and accompanied by asterisk indicate the accessions classified as *Solenopsora carpatica*.

**Figure S2.** Bayesian majority-rule consensus tree with mean branch lengths based on the *Mcm7* gene. The posterior probability values are indicated above the branches (before slash); thick branches indicate high support (> 0.90). Italicized values (after slash) show bootstrap support > 50% assessed by maximum likelihood analyses. I–IV denote the main clades referred to in the text. The codes of accessions follow Doc. S1. The codes marked in bold and accompanied by asterisk indicate the accessions classified as *Solenopsora carpatica*.

**Figure S3.** Bayesian majority-rule consensus tree with mean branch lengths based on the ITS (ITS1-5.8S-ITS2) region of nuclear ribosomal DNA. The posterior probability values are indicated above the branches (before slash); thick branches indicate high support (> 0.90). Italicized values (after slash) show bootstrap support > 0.50 assessed by maximum likelihood analyses. I–IV denote the main clades referred to in the text. The codes of accessions follow Doc. S1. The codes marked in bold and accompanied by asterisk indicate the accessions classified as *Solenopsora carpatica*.

**Table S1.** Overview of different taxonomic concepts applied in genus *Solenopsora* over time.

**Document S1.** List of specimens sequenced in the present study. Species name, voucher information [geographical location, collector(s), collection number, herbarium acronym] and GenBank accession numbers for ITS, KS, and *Mcm7* regions are indicated, respectively. A dash (–) denotes missing information.

**Document S2.** List of additional specimens examined for morphology, anatomy, chemistry (\*), and ecological requirements.

**Document S3.** Synopsis of the European *Solenopsora* taxa.