Standard Paper

Squamarina subcetrarioides comb. & stat. nov. (Stereocaulaceae), a separate species from the type species of Squamarina

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Abstract

The type species of *Squamarina* has two varieties, *S. gypsacea* var. *gypsacea* and *S. gypsacea* var. *subcetrarioides*. In this study, a phylogenetic and taxonomic analysis of these two varieties shows that *S. var. subcetrarioides* merits treatment as a species separate from *S. gypsacea*. Therefore, we raise this variety to species level as *S. subcetrarioides* (Zahlbr.) Y. Y. Zhang. *Squamarina subcetrarioides* is phylogenetically not closely related to *S. gypsacea* and differs from that species in the thallus forming rosettes when young, later becoming cracked and irregular in outline, and consisting of numerous small squamules.

Keywords: alpine lichen; Mediterranean; Squamarinoideae; taxonomy

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Introduction

Squamarina Poelt is a lichen genus established by Poelt (1958) with *S. gypsacea* (Smith) Poelt as the type species. The species has two varieties, *S. gypsacea* var. *gypsacea* and *S. gypsacea* var. *subcetrarioides* (Zahlbr.) J. Nowak & Tobol. (Nowak & Tobolewski 1975). The latter was originally described as *Lecanora fragilis* Zahlbr. var. *subcetrarioides* Zahlbr. from Slovakia (Keissler 1925), and later invalidly (Art. 41.5) combined into *Squamarina* by Pišút (1971).

Squamarina gypsacea var. gypsacea and S. gypsacea var. subcetrarioides have the same secondary metabolites but are very different in thallus morphology, and this difference was attributed to their occurrence in different habitats (Poelt & Krüger 1970). Squamarina gypsacea var. gypsacea is mainly distributed in the Mediterranean zone and characterized by a squamulose thallus. The squamules are roundish, originally scattered and later contiguous (Poelt 1958; Timdal 1983).

Squamarina gypsacea var. subcetrarioides, on the other hand, is restricted to alpine regions and is characterized by a lobed to squamulose thallus that deeply and frequently divides into small squamules (Poelt 1958; Poelt & Krüger 1970). Although *S. gypsacea* is the type species of its genus, molecular phylogenetic studies on this species are still very limited (Zhang *et al.* 2020). In this study, we investigated material of the two varieties of *S. gypsacea* from Austria, Greece, Norway, Spain and Switzerland, and generated DNA sequence data of *S. gypsacea* var. subcetrarioides for the

first time. Our morphological and phylogenetic results show that the two varieties should be treated as two different species.

Materials and Methods

Materials and morphological observation

Fifteen specimens of the two varieties of *Squamarina gypsacea* were studied here. Specimens were deposited in the following herbaria: Senckenberg Forschungsinstitut und Naturmuseum (FR), Kunming Institute of Botany, Chinese Academy of Sciences (KUN), Botanische Staatssammlung München (M) and the University of Oslo (O). Morphological features were studied using a Nikon SMZ745T dissecting microscope. Apothecia and thalli were sectioned by hand with a razor blade and microscopic traits were observed and measured using a Nikon Eclipse Ci-S microscope. Lugol's iodine (I) was used to examine ascus apical structures and 10% KOH (K) to test whether the granules dissolved in the apothecia and thalli. Secondary metabolites were detected by 1,4-Phenylenediamine (P) spot reactions and thin-layer chromatography (TLC) in solvent system C (Orange *et al.* 2001).

DNA isolation, PCR and phylogenetic analysis

Genomic DNA was extracted from thallus fragments of dry specimens using the AxyPrep Multisource Genomic DNA Miniprep Kit 50-prep (Qiagen). Polymerase chain reactions (PCR) were performed in an automatic thermocycler (C 1000TM). The PCR settings and the primers for ITS, nrLSU, *RPB1*, *RPB2* and mtSSU follow Zhao *et al.* (2015). PCR products were sequenced by TsingKe Biological Technology (Kunming, China). To examine the phylogenetic relationships of the two varieties *S. gypsacea* var. *gypsacea* and *S. gypsacea* var. *subcetrarioides*,

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				Accession number				
Species	Locality	Voucher specimens	ITS	nuLSU	RPB1	RPB2	mtSSU	
Herteliana schuyleriana*	USA	Lendemer 37806 (NY)	KT823750	NA	NA	NA	KT823746	
H. gagei*	Ireland	Hertel 39599 (UPS)	NA	AY756351	AY756385	NA	AY756369	
Squamarina cartilaginea	Norway	L-194212 (O)	ON062954	ON074705	ON166931	ON166935	ON074744	
S. cartilaginea	Norway	L-194557 (O)	ON062953	ON074704	ON166930	ON166934	ON074743	
S. gypsacea	Greece	L-59266 (O)	NA	MK778019	NA	NA	NA	
S. gypsacea	Greece	L-196255 (O)	NA	MK778020	NA	NA	NA	
S. gypsacea	Greece	L-196249 (O)	NA	MK778021	NA	NA	NA	
S. gypsacea	Spain	L-16444 (O)	NA	MK778022	NA	NA	NA	
S. kansuensis	China	18-59260 (KUN)	MK778062	MK778026	MK766428	MK766449	ON074746	
S. kansuensis	China	18-59306 (KUN)	ON062957	ON074708	ON166932	ON166936	ON074745	
S. lentigera	Sweden	F268003 (S)	ON062955	ON074706	ON166933	NA	NA	
S. lentigera	Spain	L-16441 (O)	ON062956	ON074707	NA	NA	NA	
S. lentigera	Norway	Haugan & Timdal 4801 (O)	NA	AY756363	AY756425	NA	AY756377	
S. oleosa	China	19-66399 (KUN)	MN904893	MN904897	MN923189	MN923192	MN911318	
S. oleosa	China	19-66401 (KUN)	MN904894	MN904898	MN923190	MN923193	MN915136	
S. subcetrarioides	Switzerland	<i>Scheidegger</i> 10149 (hb. C. Scheidegger)	NA	ON074712	ON166940	ON166941	NA	
S. subcetrarioides	Austria	ZYY48 (KUN)	NA	ON074709	ON166937	NA	ON074747	
S. subcetrarioides	Austria	ZYY49 (KUN)	NA	ON074710	ON166938	NA	NA	
S. subcetrarioides	Austria	ZYY50 (KUN)	NA	ON074711	ON166939	ON166942	ON074748	
S. subcetrarioides	Slovakia	L-204635 (O)	NA	ON505831	NA	ON553177	NA	

Table 1. Voucher information and GenBank Accession numbers for sequences of *Squamarina* used in the phylogenetic analyses in study. Newly obtained sequences are in bold. NA = not available; * = outgroup.

topologies based on the 5-locus matrix of *Squamarina*, including all molecular sequences of the available species and our newly generated sequences, were established. According to previous studies, we selected two species of *Squamarinoideae*, *Herteliana schuyleriana* Lendemer and *H. gagei* (Sm) J. R. Laundon, as outgroup (Lendemer 2016; Lumbsch & Leavitt 2019; Zhang *et al.* 2020).

Geneious R8 was used to assemble the raw sequences and generate a single matrix of the five markers. The matrices for each marker were individually aligned with MAFFT using the web service (http://mafft.cbrc.jp/alignment/server/index.html). Single-gene trees were reconstructed using IQ-TREE (http://iqtree.cibiv.univie. ac.at/) to assess the conflict amongst individual genes and no significant incongruence was detected. We concatenated the matrices of each marker using SequenceMatrix v. 1.7.8 and PartitionFinder 2 (Lanfear et al. 2017) was used to estimate the best-fitting substitution model for each dataset for maximum likelihood (ML) and Bayesian inference (BI) analyses. The models selected were: TIM + G for ITS1 (pos. 1-221) and ITS2 (pos. 381-586); TrN + I + G for 5.8S (pos. 222-380), nrLSU (pos. 587-1297), RPB1-B codon 1 (pos. 1348-1419\3), RPB1-B codon 3 (pos. 1350-1419\3), intron 2 (pos. 1420–1484); K80 + G for intron 1 (pos. 1298–1347), intron 3 (pos. 2691-2730), RPB1-C codon 3 (pos. 1487-1994\3) and RPB2-7 codon 3 (pos. 1997-2690\3); F81+I for mtSSU (pos.

2731-3477), RPB1-B codon 2 (pos. 1349-1419\3), RPB2-7 codon 2 (pos. 1996-2690\3) and RPB1-C codon 2 (pos. 1486-1994\3); F81 for RPB1-C codon 1 (pos. 1485-1994\3) and RPB2-7 codon 1 (pos. 1995-2690\3). Bayesian reconstructions of phylogenies were performed with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001), using four Markov chains running for two million generations, and trees were sampled every 100 generations. The first 25% of runs was discarded as burn-in. For model parameters allowed to vary, we used flat Dirichlet priors on substitution rates and nucleotide frequencies, a flat beta on transition-transversion rates, a gamma Dirichlet with mean of 10 on tree and branch lengths, a uniform (0, 200) on the gamma distributed rate heterogeneity across sites, a uniform (0, 1) on the proportion of invariant sites, and a uniform distribution on tree topologies. Subset rates were modelled as fixed and equal. We considered the sampling of the posterior distribution to be adequate when the average standard deviation of split frequencies was < 0.01. Tracer v. 1.6 (Rambaut & Drummond 2003) was used to assess chain convergence by checking the effective sampling size (ESS > 200). ML analyses were performed with RaxmlHPC, using the General Time Reversible model of nucleotide substitution (GTR). Support values were inferred from the 70% majority-rule tree of all saved trees obtained from 1000 non-parametric bootstrap replicates. Trees were visualized in FigTree v. 1.4.0 (Rambaut 2012).



Figure 1. Phylogenetic tree of Squamarina generated from maximum likelihood (ML) analysis based on the concatenated matrix of ITS, nrLSU, RPB1, RPB2 and mtSSU sequence data. ML bootstrap values and posterior probabilities (PP) from the Bayesian analysis are given adjacent to nodes (ML/PP). The two previous varieties of the type species of Squamarina are highlighted with different coloured shapes. In colour online.

Results and Discussion

Thirty-five sequences of ITS, nrLSU, RPB1, RPB2 and mtSSU were newly generated in this study for the taxa Squamarina cartilaginea (With.) P. James, S. kansuensis (H. Magn.) Poelt, S. lentigera (Weber) Poelt and S. gypsacea var. subcetrarioides (Table 1). Our topologies recovered samples of S. gypsacea var. subcetrarioides in a highly supported clade (ML = 99, BI = 1.00) that was phylogenetically distant from S. gypsacea var. gypsacea (Fig. 1). The former was recovered as sister to S. oleosa (Zahlbr.) Poelt, whereas S. gypsacea var. gypsacea clustered with S. lentigera and S. kansuensis. The two varieties also differ in morphology and geographical distribution. Squamarina gypsacea var. gypsacea is mainly distributed in the Mediterranean region, and is characterized by a squamulose thallus that is scattered when young and contiguous with age (Timdal 1983). Whereas S. gypsacea var. subcetrarioides is mainly distributed in the alpine zone (Poelt 1958; Poelt & Krüger 1970), and is characterized by the lobed thallus that is contiguous when young then frequently cracked with numerous small squamules growing from the margin of cracks. Therefore, we propose to raise S. gypsacea var. subcetrarioides to the species level as S. subcetrarioides (Zahlbr.) Y. Y. Zhang. The sister species S. oleosa differs from S. subcetrarioides in the light yellowish apothecial disc with distinct concolorous pruina, the absence of isousnic acid, and in the restricted distribution in Yunnan Province, China.

Taxonomy

Squamarina subcetrarioides (Zahlbr.) Y. Y. Zhang comb. & stat. nov.

MycoBank No.: MB 843548

Lecanora fragilis Zahlbr. var. subcetrarioides Zahlbr. Annalen des Naturhistorischen Museums in Wien **38**, 143 (1925).—Squamarina gypsacea (Sm.) Poelt var. subcetrarioides (Zahlbr.) J. Nowak & Tobol., Porosty Polskie, 1119 (1975).

Type: Slovakia, ad rupes calcareas conglomeratas prope vicum Sulov, elev. 350–400 m, *H. Suza* s. n., distributed as *Krypt. Exs. Vindob.* No. 2856 (W-0207523—holotype; M-0163618, GZU-000294606, GZU-000294607, O-L-211871—isotypes!).

(Fig. 2A–D)

Thallus lobate to squamulose, relatively loosely attached to the substratum, 2-7 cm wide. The growth form of the thallus differs between immature and mature stages. Immature thallus rosettes centrally continuous, green, epruinose; marginally lobed, lobes deeply and frequently divided, paler than the centre, whitish pruinose, margins having a white rim. Apothecia usually not present at this stage. Mature thalli irregular in outline, becoming transversely cracked to appear squamulose, numerous small, white-rimmed and dissected squamules growing from the mature thallus. Apothecia commonly present at this stage. Lower surface white to pale brown, with scattered rhizinose strands. Rhizinose strands dark brown, non-branched, thick, carbonized and fragile. Upper cortex 45-55 µm thick, filled with pale brown granules (dissolving in 10 % KOH (K)); epinecral layer gelatinized, continuous, filled with pale brown granules (dissolving in K); medulla white, with numerous calcium oxalate crystals; lower cortex absent.

Apothecia common, scattered, rounded, 2–6 mm diam.; *disc* pale ochraceous with indistinct white pruina, slightly concave; thalline margin entire, partly pruinose, persistent. *Hymenium* 75–80 μ m, inspersed with pale brown granules (dissolving in K); *epihymenium* 10–17 μ m, with pale brown granules (dissolving in K) and calcium oxalate crystals, subhymenium 20–27 μ m, containing calcium oxalate crystals in groups; *hypothecium* 150–200 μ m, with pale brown granules (dissolving in K); cortex of thalline margin identical with upper cortex of thallus, algal layer

Figure 2. A–D, *Squamarina subcetrarioides* (Zahlbr.) Y. Y. Zhang comb. & stat. nov. A, immature thallus (KUN66844). B, mature thallus and apothecia (KUN66843). C & D, ascospores and apex structure of ascus in Lugol's solution (KUN66843). E & F, *Squamarina gypsacea*. E, immature thallus and apothecia (O-059266). F, mature thallus and apothecia (O-059266). F, mature thallus and apothecia (O-016444). Scales: A, B, E & F = 5 mm; C & D = 5 μm. In colour online.

not extended above hypothecium. Asci subcylindrical, $57-75 \times 10-15 \mu$ m, apex Porpidia-type; paraphyses simple, c. 2 μ m thick; ascospores subfusiform to ellipsoid, $11-17 \times 5-7 \mu$ m.

Chemistry. Medulla P+ yellow; containing isousnic, usnic, psoromic and 2'-O-demethylpsoromic acids (detected by TLC).

Ecology and distribution. Growing on calcareous soil in the alpine zone. World distribution: European Alps and Carpathians.

Notes. Squamarina subcetrarioides is characterized by the rosetteforming thallus when immature, becoming fragmented and irregular in outline with age, the presence of numerous small squamules, the pale ochraceous disc with indistinctive white pruina, and the white to pale brown lower surface with scattered rhizinose strands. This species was treated as a variety of the type species, S. gypsacea, but the latter differs in the squamulose thallus and the roundish squamules that are slightly or not divided, scattered when young, then continuous to irregularly overlapping (Poelt 1958; Timdal 1983) (Fig. 2E & F). Apothecia can be present in the very early stages, while they are usually missing in immature thalli of S. subcetrarioides. Squamarina concrescens (Müll. Arg.) Poelt is also similar to S. subcetrarioides in the thallus morphology but differs in its lobes or squamules having an upturned margin with exposed white medullary hyphae, and in its restricted distribution in the Mediterranean zone (Poelt 1958; Poelt & Krüger 1970).

Additional specimens examined. Squamarina subcetrarioides. Austria: Salzburg: Lungau, Tamsweg, Grossek-Speiereck, 47° 7'54"N, 13°38'17"E, 2162 m, on soil, 2019, Yanyun Zhang ZYY48 (KUN 66843), ZYY49 (KUN 66844), ZYY50 (KUN 66845). Upper Austria: Gamskarkogel, Höllengebirge, O. Ö., Kalkalpen, 1700 m, an Karbonatgestein, 1967, F. Grims 8382 (FR-0058553), 8383 (FR-0058554).—Slovakia: Zilina, Súl'ovské skaly, 1.5 km NW of Súl'ov-Hradná Village, 49°17'8"N, 18° 58'59"E, 580 m, on steep rock wall, calcareous rock, 23 v 2016, S. Rui & E. Timdal (O-L-204635).—Switzerland: Graubünden: Scuol, Vald' Assa, Truoi Nov, 2040 m, on Trockene Kalkfelsflur, 2017, C. Scheidegger 10149 (hb. C. Scheidegger).

Squamarina gypsacea. **Greece**: Kavála, Thassos, along dirt road from Maries to Theologos, near Vatos, 40°70′16″N, 24°66′16″E, 590 m, on E-facing limestone wall in/above steep pine forest, 31 v 2000, *S. Rui & E. Timdal* (O-59266).—**Spain:** *Alicante*: between Callosa d'en Sarrià and Confrides, 38°68′33″N, -0°22′66″E, 260 m, 6 x 1985, *E. Timdal* (O-16444).

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