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Standard Paper

A new species and a new record of the genus *Squamulea* (*Teloschistaceae*, lichenized *Ascomycota*) from Pakistan

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Abstract

A novel species in the genus *Squamulea*, *S. chikarensis,* is described from Himalayan moist temperate forest in Pakistan. The morphology, chemistry and ITS sequences support its distinction from other species of this genus. The taxon is characterized by a pale green to yellow thallus, large apothecia up to 0.8–1.8 mm wide, pale yellow to yellow-orange apothecial discs, a hymenium 70–110 µm high, large ascospores (12–20 × 5–11 µm) and a narrow ascospore septum (1.5–3 µm). In addition, *S. flakusii* is reported as new to Pakistan and Eurasia.

Key words: *Caloplacoideae*, Himalayas, *Huriella*, lichen diversity, *Xanthorioideae*

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Introduction

In addition to the *Teloschistes*-type asci, most species of the lichen family *Teloschistaceae* are characterized by the presence of anthraquinones, which impart a yellowish orange colour to the thallus and/or apothecia. The widespread and well-delimited family currently comprises *c.* 110 genera and is estimated to contain over 1500 species (Arup *et al.* 2013; Kondratyuk *et al.* 2013, 2014, 2015*a*, *b*, *c*, *d*, 2016, 2017*a*; Søchting *et al.* 2014*a*, *b*; Wilk *et al.* 2021). The family is divided into three subfamilies, *Caloplacoideae*, *Teloschistoideae* and *Xanthorioideae* (Gaya *et al.* 2012; Arup *et al.* 2013) or four subfamilies (Kondratyuk *et al.* 2015*d*). The genus *Squamulea* is most similar to *Huriella* and is characterized by small, squamulose or areolate thalli containing anthraquinones, a saxicolous habit, and by producing apothecia with a paraplectenchymatous proper margin and hypothecium (Wilk 2020). From Pakistan, only one species of the genus *Squamulea* (namely *Squamulea subsoluta* (Nyl.) Arup *et al.* as *Caloplaca irrubescens* (Nyl. ex Arnold) Zahlbr. in Aptroot & Iqbal (2012)) has been previously identified.

During our exploration of the lichen diversity of Pakistan, collections of the genus *Squamulea* were made from Khyber Pakhtunkhwa Province and various sites in Azad Jammu and Kashmir, Pakistan. Using molecular analyses, as well as morphological and chemical characters, we were able to confirm the presence of one new species and one new record of the genus *Squamulea* in Pakistan, which are presented here.

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Materials and Methods

Morphological and chemical studies

Collections were made during a lichen survey of Azad Jammu and Kashmir, Kaghan Valley and Khanspur in 2021. The specimens were examined macro and micromorphologically using a stereomicroscope (EMZ-5TR, Meiji Techno, Japan) and compound microscope (SWIFT M4000-D) with a 9MP camera system, respectively. For anatomical investigation, sections of apothecia were made by hand and mounted in water and 10% KOH (K). A minimum of 20 measurements in water were made for each diagnostic feature from five specimens. The collected specimens were deposited in the herbarium of the Institute of Botany, University of the Punjab, Lahore (LAH). Secondary chemistry was analyzed using spot tests which were performed using 10% KOH and sodium hypochlorite solution (C). Thin-layer chromatography was carried out using solvent system C, following standard methods (Orange *et al.* 2010).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted directly from a portion of thallus with apothecia from each specimen using a modified 2% CTAB method (Gardes & Bruns 1993). The primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) was used to amplify the internal transcribed spacer (ITS) regions, following the amplification protocol of Khan *et al.* (2018). PCR products were sent to BGI (Beijing Genomics Institute), China, where both strands were sequenced.

Sequences were assembled using BioEdit (Hall 1999). BLAST analyses (https://blast.ncbi.nlm.nih.gov/Blast.cg) were used for initial verification of their identities and to retrieve highly similar ITS sequences. The newly generated sequences and additional sequences retrieved from GenBank were used in an initial

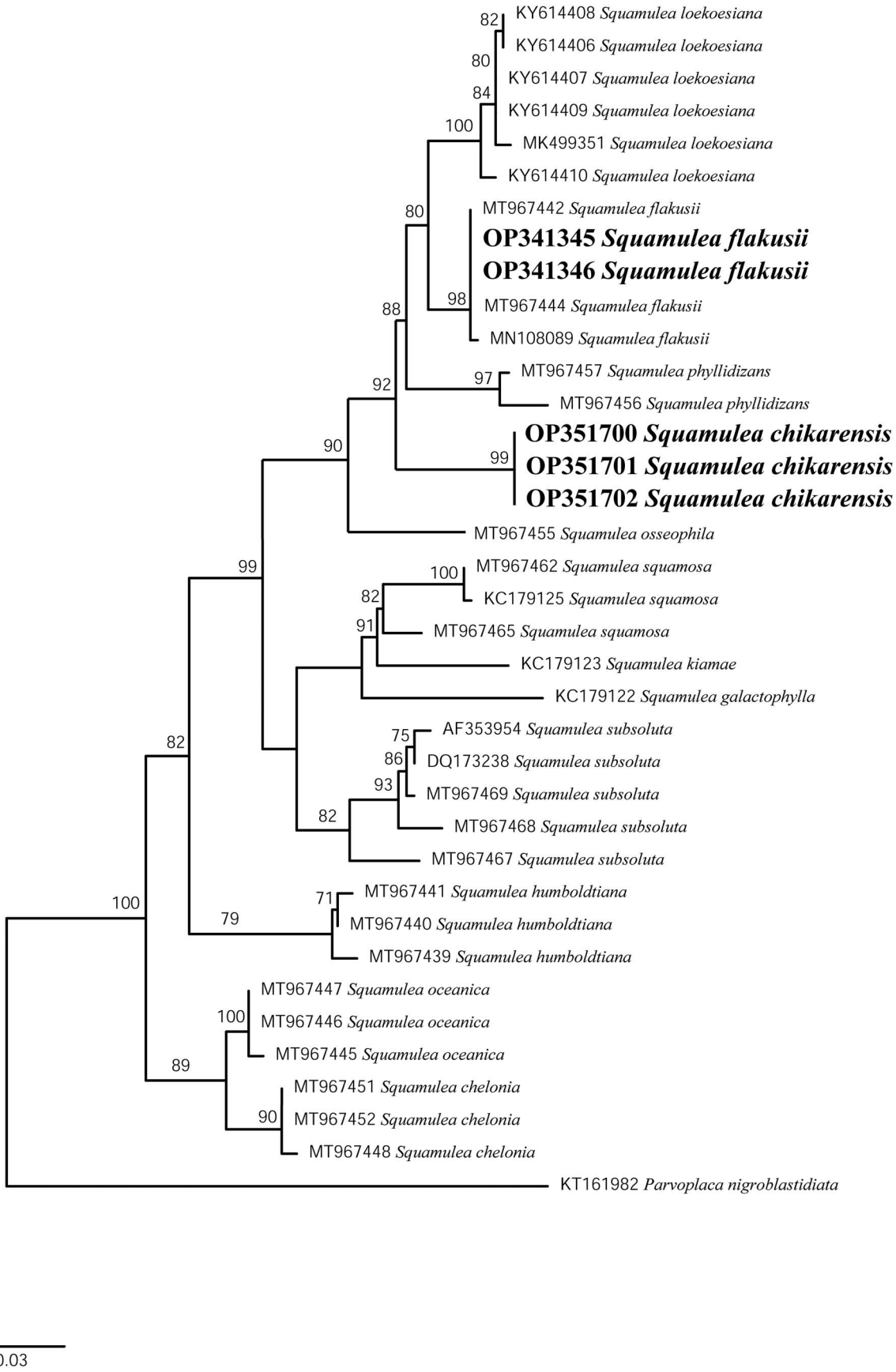

Fig. 1. Phylogenetic relationships within *Squamulea* based on a maximum likelihood (ML) analysis of the ITS region. The ML bootstrap values ≥70 obtained from a RaxML analysis are shown above internal branches. New sequences generated in this study are in bold. *Parvoplaca nigroblastidiata* was used as outgroup.

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Table 1. Sequences used in the ITS phylogenetic analysis of *Squamulea* species, with GenBank Accession numbers and voucher information. New sequences generated in this study are in bold.

Name of species	Country/Origin	Voucher specimen	GenBank Accession no.
Parvoplaca nigroblastidiata	USA	42983 (BG)	KT161982
Squamulea chelonia	Ecuador	*Bungartz* 6146 (CDS 34358)	MT967452
S. chelonia	Ecuador	*Bungartz* 9251 (CDS 46069)	MT967451
S. chelonia	Ecuador	*Bungartz* 4521 (CDS 28607)	MT967448
***S. chikarensis* sp. nov.**	**Pakistan**	***Afshan* et al. s. n. (LAH37546, holotype)**	**OP351700**
***S. chikarensis* sp. nov.**	**Pakistan**	***Afshan* et al. s. n (LAH37547)**	**OP351701**
***S. chikarensis* sp. nov.**	**Pakistan**	***Afshan* & *Iftikhar* s. n. (LAH37548)**	**OP351702**
S. flakusii	Ecuador	*Bungartz* 4131 (CDS 28162)	MT967442
S. flakusii	Ecuador	*Bungartz* 65261 (CDS 31847)	MT967444
S. flakusii	Peru	*Flakus* 9263 & *Cykowska* (KRAM-L-70242)	MN108089
S. flakusii	**Pakistan**	***Afshan* & *Iftikhar* s. n. (LAH37549)**	**OP341345**
S. flakusii	**Pakistan**	***Afshan* et al. s. n. (LAH37550)**	**OP341346**
S. galactophylla	USA	*Morse* 10997 (LD)	KC179122
S. humboldtiana	Saint Kitts and Nevis	*Buck* 29560 (MIN)	MT967439
S. humboldtiana	Ecuador	*Bungartz* 9985 (CDS 47354)	MT967441
S. humboldtiana	Ecuador	*Bungartz* 4711 B (CDS 56235)	MT967440
S. kiamae	Australia	*Kondratyuk* 20480 (LD, isotype)	KC179123
S. loekoesiana	South Korea	KoLRI 40238	KY614410
S. loekoesiana	South Korea	KoLRI 40141	KY614408
S. loekoesiana	South Korea	KoLRI 15423	KY614406
S. loekoesiana	-	HKAS 102112	MK499351
S. loekoesiana	South Korea	KoLRI 40236	KY614409
S. loekoesiana	South Korea	KoLRI 19017	KY614407
S. oceanica	Ecuador	*Bungartz* 9857 (CDS 47195)	MT967447
S. oceanica	Ecuador	*Bungartz* 10152 (CDS 47571)	MT967446
S. oceanica	Ecuador	*Bungartz* 2023 (CDS 48373)	MT967445
S. osseophila	Ecuador	*Bungartz* 65489 (CDS 32078)	MT967455
S. phyllidizans	Ecuador	*Bungartz* 4710 (CDS 28808)	MT967457
S. phyllidizans	Ecuador	*Bungartz* 65468 (CDS 32057)	MT967456
S. squamosa	Ecuador	*Bungartz* 7428 (CDS 37915)	MT967465
S. squamosa	Mexico	*Moberg* 8782 (UPS)	MT967462
S. squamosa	USA	*Kärnefelt* AM960105 (LD)	KC179125
S. subsoluta	Austria	*Arup* 97072	AF353954
S. subsoluta	Italy	*Arup* L97829	DQ173238
S. subsoluta	Canada	L89634 (LD)	MT967469
S. subsoluta	Canada	L89634 (LD)	MT967468
S. subsoluta	Ecuador	11884 (CDS 37243)	MT967467

alignment, which was then trimmed and realigned using webPRANK with default settings (Löytynoja & Goldman 2010). On the CIPRES Web Portal (Miller *et al.* 2010), the HYK + G + I model was selected using jModelTest (Posada 2008). A maximum likelihood analysis (ML) was also implemented using RAxML-HPC2 v. 8.1.11 (Stamatakis 2014) on CIPRES, with 1000 bootstraps for rapid bootstrapping. *Parvoplaca nigroblastidiata* Arup *et al.* (KT161982) was used as outgroup. A tree displaying the phylogeny reconstruction from the ML analysis was generated in FigTree v. 1.4.3 (Rambaut *et al.* 2014).

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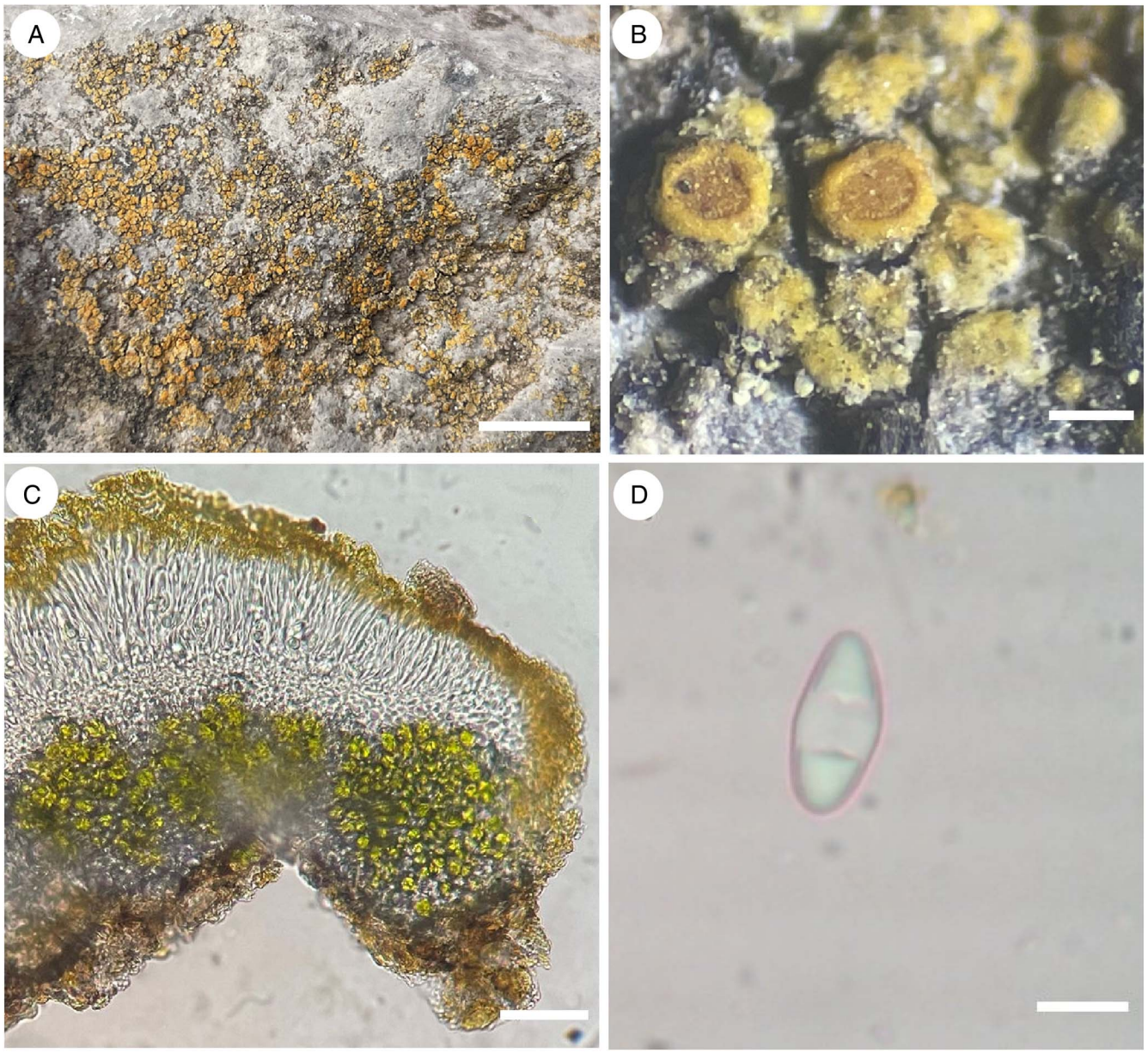

Fig. 2. *Squamulea chikarensis* (holotype-LAH37546). A, squamulose thallus in natural habitat. B, apothecia. C, transverse section of apothecium showing paraplectenchymatous proper margin and hypothecium. D, ascospore. Scales: A = 1 cm; B = 1.5 mm; C = 100 µm; D = 10 µm. In colour online.

Results

Phylogenetic analyses

The final ITS dataset consisted of 35 sequences, representing 12 species of *Squamulea* and *Parvoplaca nigroblastidiata* (see Table 1 for voucher details). The aligned ITS1-5.8S-ITS2 region comprised 565 sites, of which 282 were conserved and 155 variable; 128 sites were parsimony-informative. Our phylogeny recovers *S. chikarensis* in a clade together with *S. loekoesiana* (S.Y. Kondr. & Upreti) Arup *et al.*, (KY614406, KY614407, KY614408, KY614409, KY614410, MK499351), *S. flakusii* (Wilk) Arup *et al.*, (MT967442, MT967444, MN108089, OP341345, OP341346), *S. phyllidizans* (Wetmore) Søchting & Bungartz (MT967456, MT967457) and in a basal position in the clade, *S. osseophila* Søchting & Bungartz (MT967455). This clade contains species from a wide geographical range and diverse substrata, including species with blastidia, microsquamules and no asexual propagules. Molecular methods are often required in *Squamulea* to distinguish species reliably. The ITS sequence of the holotype of *S. chikarensis* is identical to the sequences of additionally studied specimens (i.e. OP351701, OP351702). Two further newly generated sequences nested within the clade of *S. flakusii* previously known from Ecuador (Galapagos Islands) and Peru. We therefore report *S. flakusii* for the first time from Pakistan and Eurasia.

Taxonomy

Squamulea chikarensis Afshan, Fayyaz, Iftikhar & Khalid sp. nov.

MycoBank No.: MB 845620

The taxon is characterized by a pale green to yellow thallus, large apothecia up to 0.8–1.8 mm wide, pale yellow to yellow-orange

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apothecial discs, hymenium 70–110 µm high, large ascospores (12–20 × 5–11 µm) and a narrow ascospore septum (1.5–3 µm).

Type: Pakistan, Azad Jammu and Kashmir, Chikar, moist temperate forest, 36°23′N, 75°47′E, 3850 m alt., on rock, 2 October 2020, *N. S. Afshan, I. Fayyaz & F. Iftikhar* (MK-11) (holotype—LAH37546).

(Fig. 2)

Thallus areolate to subsquamulose, areoles/squamules are scattered to crowded, flat to concave, pale green to yellow, slightly pruinose thallus irregular in outline, up to 2–4 cm diam., not delimited by a prothallus. *Cortex* paraplectenchymatous, even, 14–22 µm thick. *Algal layer* continuous, 53–65 µm thick. *Photobiont cells* more or less globose, trebouxioid, 9–15 µm. *Medulla* white, 60–75 µm thick.

Apothecia mostly sparse, erumpent to sessile, circular, (0.8–)1.0–1.4–1.6(–1.8) mm diam., zeorine. *Disc* concave to flat, pale yellow to yellow-orange, slightly contrasting against thallus, pruinose. *Margin* paler than disc, slightly prominent in young apothecia, then level with disc. *Proper exciple* thin, paraplectenchymatous. *Thalline exciple* thick, slightly/partly reduced, algae abundant, in a continuous layer. *Epihymenium* brownish orange, granular inspersed, K+ purple, 13–18 µm thick. *Hymenium* hyaline, 70–110 µm high. *Paraphyses* simple, septate, 2–3 µm wide, tips up to 5 µm. *Hypothecium* hyaline, not inspersed, paraplectenchymatous. *Asci* 8-spored, clavate, *Teloschistes*-type, 58–65 × 13–17 µm. *Ascospores* polarilocular, oblong to broadly ellipsoid, (12.0–)13.5–15.7–18.3(–20.0) × (5.0–)6.2–7.3–9.6(–11.0) µm, septa (1.5–)1.9–2.2–2.7(–3.0) µm wide.

Pycnidia not seen.

Spot test. Thallus and apothecia: K+ reddish brown, C–, KC+ dark red.

Chemistry. Thallus and apothecia with a large proportion of parietin and smaller proportions of teloschistin, fallacinal, parietinic acid and emodin (chemosyndrome A *sensu* Søchting (1997)).

Etymology. The specific epithet '*chikarensis*' (Latin) refers to the type locality Chikar, where the specimen was collected.

Habitat and distribution. The new species was collected in the Himalayas of Pakistan, on siliceous rocks in moist temperate forests, dominated mainly by *Pinus roxburghii*, *Pyrus pashia*, *Quercus oblongata* and *Q. glauca*. The maximum daily temperature of the region varies from 20–30 °C during the summer and averages 4 °C during the winter, with moderate rainfall.

Additional specimens examined. **Pakistan:** *Khyber Pakhtunkhwa*: Kaghan Valley, moist temperate forest, 34°30′N, 73°18′E, 2500–3000 m alt., on rock, 2021, *N. S. Afshan & F. Iftikhar* (SG-20) (LAH37548). *Azad Jammu and Kashmir*: Chikar, moist temperate forest, 36°23′N, 75°47′E, 3850 m alt., on rock, 2021, *N. S. Afshan, I. Fayyaz & F. Iftikhar* (CKT-39) (LAH37547).

Squamulea flakusii (Wilk) Arup, Søchting & Bungartz

In Bungartz, Søchting & Arup, *Plant and Fungal Systematics* **65**, 564 (2020).

(Fig. 3)

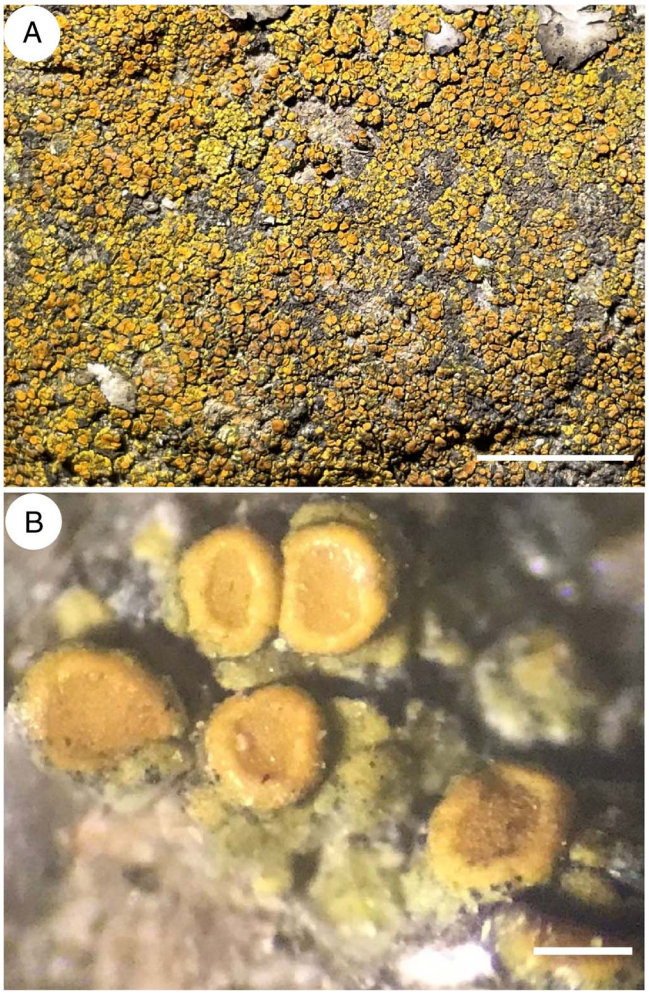

Fig. 3. *Squamulea flakusii* (LAH37549). A, squamulose thallus in natural habitat. B, apothecia. Scales: A = 1 cm; B = 1 mm. In colour online.

Thallus squamulose or strongly reduced and almost invisible, 2–3 cm diam., orbicular to irregular in outline, pale yellow to dark yellow, pruinose, squamules flat to convex. *Prothallus* absent. *Cortex* thin to thick (in well-developed thallus), 27–50 µm thick, paraplectenchymatous. *Algal layer* continuous. *Photobiont cells* trebouxioid, more or less globose, 9–14 µm diam.

Apothecia abundant, somewhat crowded and aggregated, rounded, angular, old apothecia distinctly undulate, adnate, zeorine, (0.5–)0.8–1.0–1.2(–1.4) mm wide. *Disc* plane to strongly convex, yellow-orange to pale orange, pruinose, often cracked (especially in old apothecia). *Proper margin* thin, slightly prominent in young apothecia, then level with disc, slightly paler than disc. *Thalline margin* 65–90 µm thick, conspicuous, much reduced, algal cells abundant, forming a continuous layer. *Epihymenium* light brown, 25–38 um thick. *Hymenium* 60–78 µm thick. *Paraphyses* simple to slightly branched, 2–3 µm broad at base, with upper cells slightly thickened, up to 4 µm. *Hypothecium* 80–140 µm thick, paraplectenchymatous, hyaline. *Asci* 8-spored, clavate, 50–63 × 12–22 µm. *Ascospores* polarilocular, usually broadly ellipsoid, 12–18 × 7–11 µm, septum (1.0–)1.3–2.0–2.6(–3.0) µm wide.

Pycnidia not seen.

Spot test. Thallus and apothecia: K+ purple, C–, KC+ purple.

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Table 2. A comparison of morphological and anatomical features of *Squamulea chikarensis* sp. nov. with selected *Squamulea* species.

Characters	*S. chikarensis*	*S. flakusii* (Pakistan)	*S. flakusii* (Peru)	*S. flakusii* (Galapagos)	*S. kiamae*	*S. loekoesiana*	*S. osseophila*	*S. parviloba*	*S. phyllidizans*	*S. squamosa*	*S. subsoluta*
Thallus morphology	areolate to subsquamulose	squamulose or strongly reduced	squamulose	more conspicuously squamulose	rosettes of radiating areoles	areolate	barely subsquamulose	areolate to subsquamulose; areoles with small lobules	subsquamulose to minutely squamulose	squamulose	areolate to subsquamulose
Thallus colour	pale green to yellow	pale yellow to dark yellow	orange	deep orange	brownish orange	dull yellow-brownish, yellow-greenish	deep yellow	yellow-orange to orange	yellowish orange	yellow-orange to orange or reddish orange	yellow-orange to orange or reddish orange
Apothecium type	zeorine	zeorine	zeorine	-	lecanorine/ zeorine	zeorine	lecanorine	zeorine	lecanorine	zeorine	usually biatorine/ zeorine
Apothecium diam. (mm)	0.8–1.8	0.5–1.4	0.2–1.0	-	0.3–0.7	0.2–0.5	-	0.2–0.5	0.8	0.5–2.0	0.1–1.1
Apothecial disc	pale yellow to yellow-orange	yellow-orange to pale orange	reddish	-	reddish to reddish brown	dull yellow to bright yellow	deep orange	darker orange than thallus	deep orange	darker orange than thallus	darker orange than thallus
Hymenium (µm)	70–110	60–78	70–85	-	75–80	50–60	-	40–70	-	65–80	60–80
Ascospore size (µm)	12–20 × 5–11	12–18 × 7–11	10–15 × 5.0–9.5	-	10–16 × 6–8	8.5–12 × 4.5–6	7.6–17.3 × 4.4–7.4	10–14 × 5.5–7.0	8.3–12.8 × 3.5–8.2	9.5–14.0 × 5.5–7.0	9.5–14.0 × 5.5–7.0
Ascospore septum (µm)	1.5–3	1–3	2–4	-	1–4	4–7	1.7–4.1	3–4	1.7–5.9	3–4	3–4
Distribution	South Asia (Pakistan)	South Asia (Pakistan)	South America (Peru)	South America (Galapagos)	Australia	Eastern Asia (South Korea)	South America (Galapagos)	south-western North America	south-western North America and South America	south-western North America	North America and Europe
References	This paper	This paper	Wilk 2020	Bungartz *et al.* 2020	Kondratyuk *et al.* 2007	Kondratyuk *et al.* 2017*b*	Bungartz *et al.* 2020	Wetmore 2003	Bungartz *et al.* 2020	Wetmore 2003	Wetmore 2003

Specimens examined. **Pakistan:** *Khyber Pakhtunkhwa*: Kaghan Valley, moist temperate forest, 34°30′N, 73°18′E, 2500–3000 m alt., on rock, 2021, *N. S. Afshan* & *F. Iftikhar* (SG-45) (LAH37549). *Azad Jammu and Kashmir*: Garhi Dupatta, moist temperate forest, 34°36′N, 73°35′E, 817 m alt., on rock, 2021, *N. S. Afshan, I. Fayyaz* & *F. Iftikhar* (CKR-07) (LAH37550).

Discussion

The species *Squamulea chikarensis* is characterized by minute squamules, usually scattered, large apothecia and large ascospores with rather thin septa. The comparison of the new species with its close relatives is presented in Table 2. Morphologically the new species resembles *S. subsoluta*, a widespread species also occurring in Pakistan (under the name *Caloplaca irrubescens*; Aptroot & Iqbal 2012). However, *S. subsoluta* differs from the new species in having a yellow-orange to orange or reddish orange thallus, a blackish prothallus, a darker orange apothecial disc and smaller ascospores, 9.5–14.0 × 5.5–7.0 µm (Wetmore 2003). *Squamulea flakusii*, newly recognized in the lichen biota of Pakistan, differs from *S. chikarensis* by having a pale yellow to dark yellow thallus, apothecia that are 0.5–1.4 mm wide, a hymenium 60–78 µm high and smaller ascospores, 12–18 × 7–11 µm.

In the present study, *Squamulea flakusii* is morpho-anatomically and molecularly characterized, and confirmed as a new record for Pakistan. The morphological features of the Pakistani collections agree with the published description of *S. flakusii* (from South America, Peru) (Wilk 2020), with the exception of the following (features in brackets refer to the Peru collections): less reduced thallus (vs more reduced thallus), pale yellow to dark yellow thallus (vs orange thallus), less crowded and aggregated apothecia (vs more crowded and aggregated apothecia), larger apothecia up to 0.5–1.4 mm wide (vs 0.2–1.0 mm), yellow-orange to pale orange apothecial discs (vs reddish apothecial discs), larger ascospores, 12–18 × 7–11 µm (vs 10–15 × 5.0–9.5 µm) and a narrower ascospore septum, 1–3 µm (vs 2–4 µm). The morphology of Pakistani specimens of *S. flakusii* is, however, quite different to specimens from the Galapagos. Pakistani specimens have a pale yellow to dark yellow thallus (vs deep orange thallus) and are collected at an elevation of 817–3000 m alt. (vs above 1000 m alt. for Galapagos specimens). For a comparison of *S. flakusii* with *S. chikarensis* and other taxa, see Table 2 and the discussion under *S. chikarensis*.

Although *Squamulea flakusii* has been described from South America (Peru) and was then recorded only from the Galapagos Islands (Bungartz *et al.* 2020; Wilk 2020), its distribution in the Northern Hemisphere is possible. This could indicate that the species may be a widespread taxon, as in the case of *S. subsoluta* known also from Austria. To date, due to the uncertainty regarding phenotypic characters, phylogenetic analysis of DNA sequences is almost the only reliable way to distinguish species and to study the geographical distribution of *Squamulea* species (Bungartz *et al.* 2020). Therefore, our knowledge of those species should increase in the future with sampling and analysis of further *Squamulea* species. In Pakistan, *S. flakusii* was collected in the high mountain area, at 817–3000 m alt., in humid forest habitat, on siliceous rock, which more or less corresponds with the previous reports from South America. One Pakistani specimen sequenced (OP341346), however, was collected at *c.* 817 m alt., in moist temperate forest. The genus is probably more abundantly represented in Pakistan's lichen biota than is currently known.

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