Turczaninowia 26, 1: 136–144 (2023)
DOI: 10.14258/turczaninowia.26.1.14
http://turczaninowia.asu.ru

ISSN 1560–7259 (print edition)
TURCZANINOWIA
ISSN 1560–7267 (online edition)

УДК 582.293.22:581.95(4/5+470)

The poorly known in Russia sporodochial lichen genus *Sporodophoron* (Arthoniaceae) with one species new to the country and continental Eurasia

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Keywords: conidia, HPLC, hyphomycetes, mrSSU, new records, North East Asia, Primorye, Russian Far East, sporodochia.

Summary. The genus *Sporodophoron* (Arthoniaceae, lichenized Ascomycota) is characterized by whitish, convex, and usually discrete or rarely becoming confluent sporodochia and conidia formed in zigzag-shaped and occasionally branched chains. It consists of four species, and only *S. primorskiense* was known in Russia, in a single locality in the Primorye Territory. Here we report the second locality of *S. primorskiense* and two localities of new to Russia *S. gossypinum*, all from the deciduous and mixed forests of the very south of the Primorye Territory. Morphology and chemistry of our specimens are briefly discussed in comparison with the literature data. Using HPLC-UV-MS, in *S. gossypinum* we detected several minor and trace compounds previously unknown to the species, namely confluentic acid, 4-*O*-methylolivetolcarboxylic acid, hyperlatolic acid, and perlatolic acid. Using mass-spectrometry, we found that "lepraric high unknown" substance has the same molecular formula and fragmentation pattern as lepraric acid. The find of *S. gossypinum* is also confirmed by the mrSSU sequence.

Малоизвестный в России род спородохиальных лишайников *Sporodophoron* (Arthoniaceae) с одним видом, новым для России и континентальной Евразии

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Ключевые слова: ВЭЖХ, гифомицеты, конидии, новые находки, Приморский край, российский Дальний Восток, Северо-Восточная Азия, спородохии, mrSSU.

Поступило в редакцию 31.10.2022
Принято к публикации 14.04.2023

Submitted 31.10.2022
Accepted 14.04.2023

Аннотация. *Sporodophoron* – небольшой род лишайников (Arthoniaceae, лихенизированные Ascomycota), характеризующийся беловатыми выпуклыми и обычно раздельными, реже сливающимися спородохиями и конидиями в форме зигзагообразных, иногда ветвящихся, цепочек. Род состоит их четырёх видов, но только *S. primorskiense* был известен в России, в одном локалитете на юге Приморского края. В настоящей работе мы приводим данные о втором местонахождении *S. primorskiense* и двух местонахождениях нового для России *S. gossypinum*. Все три новые находки сделаны в широколиственных и смешанных лесах на самом юге Приморского края. Кратко обсуждаются и сравниваются с литературными данными морфология и биохимия собранных нами образцов. С помощью метода ВЭЖХ-УФ-МС у *S. gossypinum* были обнаружены несколько минорных и следовых вторичных метаболитов, ранее неизвестных для этого вида: конфлуэнтовая, 4-*O*-метилоливетолкарбоксиловая, гиперлатоловая и перлатоловая кислоты. С помощью масс-спектрометрии было обнаружено, что вещество, известное под названием «lepraric high unknown», имеет ту же молекулярную формулу и спектр характеристических фрагментов, что и лепраровая кислота. Определение *S. gossypinum* подтверждено с использованием молекулярного маркера – последовательности митохондриальной рибосомальной ДНК mrSSU.

Introduction

Lichens characterized by presence of sporodochia (open conidiomata in the form of discrete tufts of conidiophores on the thallus) are not numerous and quite rare in Russia. These are three species of the genus *Cheiromycina* B. Sutton (e. g., Printzen, 2007) and *Reichlingia leopoldii* Diederich et Scheid (Urbanavichus et al., 2020), for which no well-developed sexual stages have been reported so far, and *Micarea adnata* Coppins (e. g., Konoreva et al., 2019) with both apothecia and sporodochia. The species of *Cheiromycina* are easily recognizable due to peculiar, palmately branched, multiseptate conidia and globose or ellipsoid conidiogenous cells (Printzen, 2007; Muggia et al., 2017). *Reichlingia leopoldii* is characterized by the leprose thallus bearing irregular or almost confluent brown sporodochia with irregularly branched, multiseptate dark brown conidia (Diederich, Scheidegger, 1996), whereas *M. adnata* has simple, cylindrical to ellipsoid conidia and cylindrical conidiogenous cells (Coppins, 1983).

Sporodophoron Frisch et al. is another genus of crustose sporodochial lichens recently proposed by Frisch et al. (2015), and one species, *S. primorskiense* Frisch et Y. Ohmura, was reported in Russia. It is morphologically and chemically similar to *Inoderma* (Ach.) Gray and forms the sister clade to that genus on the phylogeny of Arthoniaceae (Frisch et al., 2015). The main difference between these genera is the presence of sporodochia in *Sporodophoron* instead of pycnidia in *Inoderma*. Sporodochia of *Sporodophoron* are whitish, convex, and usually discrete or becoming confluent in the thallus centre; conidia formed in zigzag-shaped and occasionally branched chains. Apothecia are known from the single species (*S. gossypinum* Frisch et al.). *Sporodophoron* has distinctive thallus chemistry including 2'-*O*-methylperlatolic acid, the lepraric high unknown and/or supposedly related trace compounds (Frisch et al., 2015).

The genus *Sporodophoron* consists of four species distributed in the temperate Northern Hemisphere. *Sporodophoron americanum* (Lendemer et al.) Ertz et Frisch is known in the eastern North America (Frisch et al., 2015; Gockman et al., 2020), *S. cretaceum* (Hue) Ertz et Frisch – in the western continental Europe, Great Britain and Ireland (Frisch et al., 2015), *S. gossypinum* Frisch et al. – in a few localities in Japan (Frisch et al., 2015), and *S. primorskiense* Frisch et Y. Ohmura – in two localities, in the Primorye Territory of Russia and in Japan (Frisch et al., 2015; Ohmura, Frisch, 2016).

Here we present the records of the second locality in Russia and the third locality in the world of *Sporodophoron primorskiense*, and the first two localities of *S. gossypinum* found outside Japan, in the Russian Far East.

Material and methods

The specimens were collected by the first author in the Primorye Territory of Russia in 2020 and deposited in LE and the personal herbarium of I. Frolov.

Phenotype evaluation

All microscopic anatomical observations are based on hand-cut sections mounted in water, without chemical treatments. Results for measurements, which were repeated at least 10 times, are given as (min.)–mean–(max.) [n], where min. / max. are extremes, mean is an arithmetic mean of all measurements, and n is the number of measurements.

DNA extraction, amplification and sequencing

DNA was extracted with a CTAB-based protocol (Aras, Cansaran, 2006). Amplification was made of the small subunit of the mitochondrial ribosomal RNA gene (mrSSU). Primers for PCR amplification were mrSSU1 (Zoller et al., 1999) and MSU7 (Zhou, Stanosz, 2001). The PCR settings followed Ekman (2001). The sequence obtained was uploaded to the NCBI database (GenBank).

Identification of secondary metabolites (TLC and HPLC-UV-MS analyses)

For thin-layer chromatography (TLC) and high performance liquid chromatography with UV detection coupled with mass spectrometry (HPLC-UV-MS), two air-dried samples of *S. gossypinum* and one of *S. primorskiense* were ground. The secondary substances from each sample were extracted with 0.3 ml of acetone. Extraction was carried out with constant stirring for 12 h at 20–25 °C. The obtained extracts were centrifuged 10 min at 6000 g and kept at 4 °C until analysis.

TLC was performed on plates TLC Silica gel 60 Merck (Germany) using solvent system C (toluene : acetic acid = 170 : 30 v/v) according to Huneck, Yoshimura (1996). A control, containing authentic atranorin, norstictic acid, lepraric acid and stictic acid, was used. After chromatographic development, the plates were examined under UV light (254 and 366 nm), then sprayed with a 10 % sulphuric acid solution and heated at 100 °C for 15 min. Finally, the plates were cooled to room temperature and studied in daylight.

HPLC-UV-MS analyses were performed with a 1290 Series Agilent chromatograph with UV detection. For chromatographic separation, a Thermo Hypersil-Keystone C18, column (150 × 2.1 mm × 5 μm) was used. The mobile phase consisted of (A) water : acetonitrile : formic acid (95 : 5 : 0.1 v/v), and (B) acetonitrile : water : formic acid (90 : 10 : 0.1 v/v). Analyses were performed at 30 °C and a flow rate of 0.3 ml min^{-1} in the gradient elution mode, the percentage of B was programmed as follows: 5 % (2 min) – 50 % (5 min) – 70 % (15 min) – 100 % (25 min) – 100 % (35 min). The volume of the injected sample was 5 μL. Spectra of eluting substances were recorded in UV at 250 nm. After separation, the samples were also analyzed with a quadrupole time-of-flight mass spectrometer (6538 Series, Agilent, USA). Ionization was achieved by electrospray in the negative mode. Voltage on the capillary was 2.5 kV, capillary temperature 350 °C, atomizing gas pressure 45 psi, desiccant gas (nitrogen) temperature 225 °C, drying gas flow rate 5 L/min. Mass spectra were recorded in the range 100–1000 m/z. The resulting chromatograms were processed with the MassHunter WorkStation v. B.07.00 software package (Agilent, USA). To identify lichen substances, we compared their polarity related to their retention time (Rt), molecular formula from accurate molecular weight measurement, along with pseudo-molecular and fragment ions with the authentic standards from the V. L. Komarov Botanical Institute collection.

Results

Sporodophoron gossypinum Frisch, Y. Ohmura et G. Thor, 2015, Lichenologist 47(4): 250.

Holotype: "Japan, Hokkaido, Ikutahara-Kiyosato, Engaru-cho, Monbetsu-gun, on shady rock wall in deciduous forest, 290 m a. s. l., 43°51'13"N, 143°29'11"E. 29 V 2012. A. Frisch, Y. Ohmura 12/Jp186" (TNS, not seen) (Fig. 1A–C).

Morphology. The detailed description was given by Frisch et al. (2015). Phenotypic characters of our material mainly agree with the description. The specimens have whitish to olivaceous grey fissured-areolate epilithic ca. 100–225 μm thick thallus with a felty surface and trentepohlioid photobiont, white to creamy white sporodochia (0.25)–0.53–(0.90) mm diam. [20] and 0.23–0.35 mm tall with conidia in zigzag-shaped and occasionally branched chains constricted at the septa, that disintegrate into fragments of irregular shape 8.0–12.2–18.0 × 4.0–4.9–7.0 μm [21]; conidia densely covered with granules ca. 1 μm diam.

Unlike the Japanese material, both of our specimens are sterile and have slightly darker and thicker (up to 225 μm vs. up to 120 μm) thallus, sporodochia with larger diameter (0.25–0.90 mm vs. 0.5–0.8 mm) and slightly longer fragments of conidia (8.0–18.0 μm vs. 6.0–14.0 μm).

Chemistry. Six secondary metabolites were identified based on the TLC and HPLC-UV-MS data (Figs. 2, 3A). Their detailed MS data are shown in Table. 2'-*O*-methylperlatolic acid (detected by both TLC and HPLC, major), lepraric high unknown (TLC, HPLC, major), confluentic acid (TLC, HPLC, minor), unknown fatty acid (TLC), 4-*O*-methylolivetolcarboxylic acid (HPLC, trace), hyperlatolic acid (HPLC, trace), perlatolic acid (HPLC, trace). Sporodochia K+ lemon yellow and thallus greenish yellow.

Molecular data. The mrSSU sequence was obtained from the specimen Frolov 3705 (GB accession number OQ756128). It is identical to other

sequences of *S. gossypinum* available in GenBank, apart from several indels missing in these sequences and occasionally occurring in some sequences of *S. cretaceum* and *S. primorskiense*, and, for example, in *Inoderma byssaceum*. These indels are very similar between sequences regardless of the species, to which the sequence belongs, and possibly represent ancestral polymorphism.

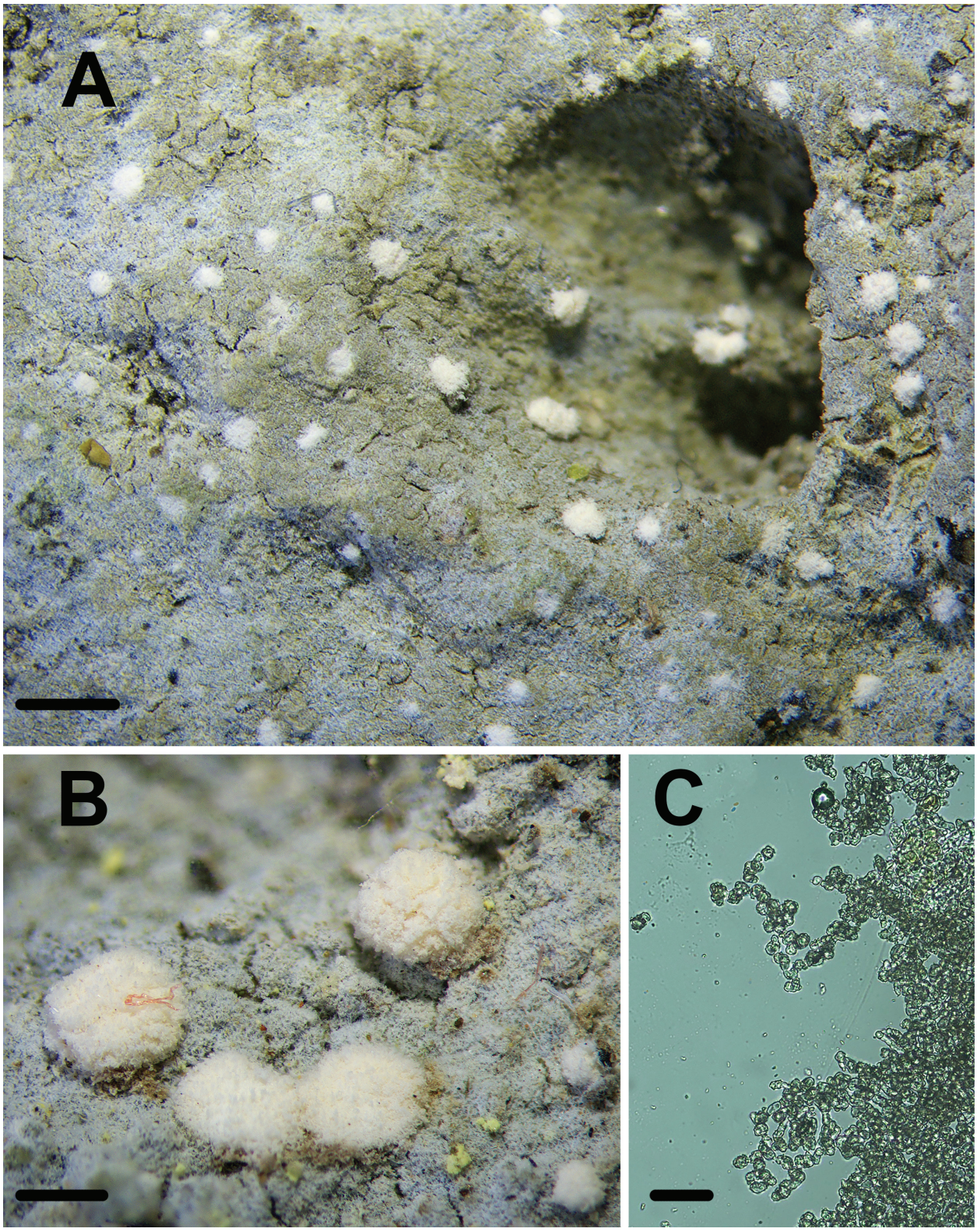

Fig. 1. *Sporodophoron gossypinum*: A – Crustose thallus with sporodochia on tuff (Frolov 3704); B – Sporodochia (Frolov 3705); C – Sporodochial conidia (Frolov 3704). Scales – 1 mm (A), 0.5 mm (B) and 25 μm (C).

Table

Identification of lichen substances in *Sporodophoron gossypinum* by HPLC-UV-MS

Peak no.	t_R (min)	Molecular formula	$[M-H]^-$, *m/z*	MS fragments, *m/z*	Identification
1	11.0	$C_{18}H_{18}O_8$	723.1911*	143	Lepraric high unknown
2	17.0	$C_{13}H_{18}O_4$	237.1134	193	4-*O*-Methylolivetolcarboxylic acid
3	20.5	$C_{28}H_{36}O_8$	499.2347	237, 193	Confluentic acid
4	24.3	$C_{26}H_{34}O_7$	457.2247	237, 193	2'-*O*-methylperlatolic acid
5	26.4	$C_{25}H_{32}O_7$	443.2075	223, 205	Perlatolic acid
6	28.7	$C_{27}H_{36}O_7$	471.2324	251, 207	Hyperlatolic acid

*– a deprotonated dimer ion $[2M-H]^-$ was observed as major detectable mass peak.

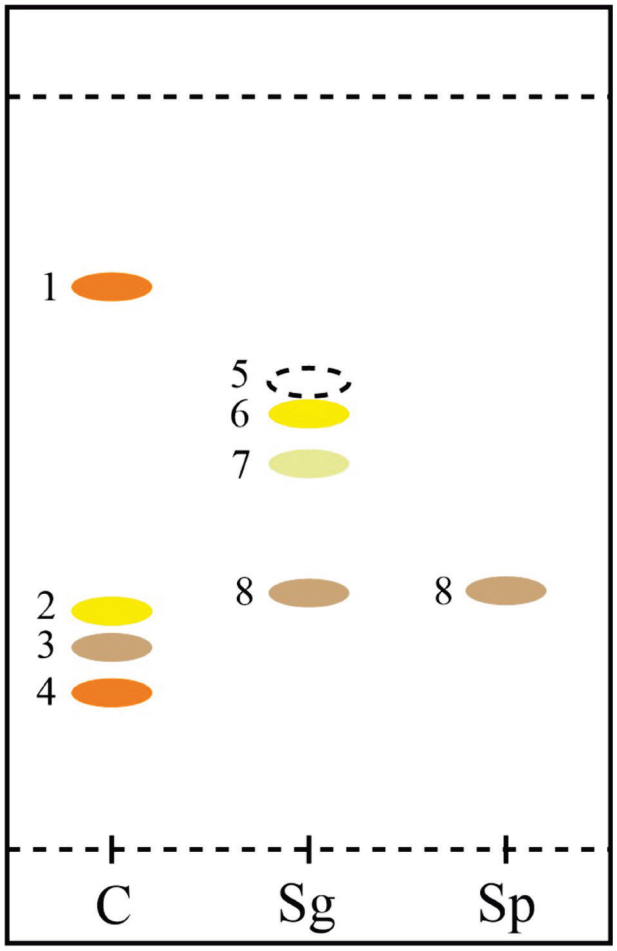

Fig. 2. TLC analysis in solvent C (toluene : acetic acid = 170 : 30 v/v): C – Controls; Sg – *Sporodophoron gossypinum*; Sp – *S. primorskiense*. 1 – atranorin; 2 – norstictic acid; 3 – lepraric acid; 4 – stictic acid; 5 – unidentified fatty acid; 6 – 2'-*O*-methylperlatolic acid; 7 – confluentic acid; 8 – lepraric high unknown. In daylight after spraying with 10 % sulphuric acid and drying at 100 °C for 10 min.

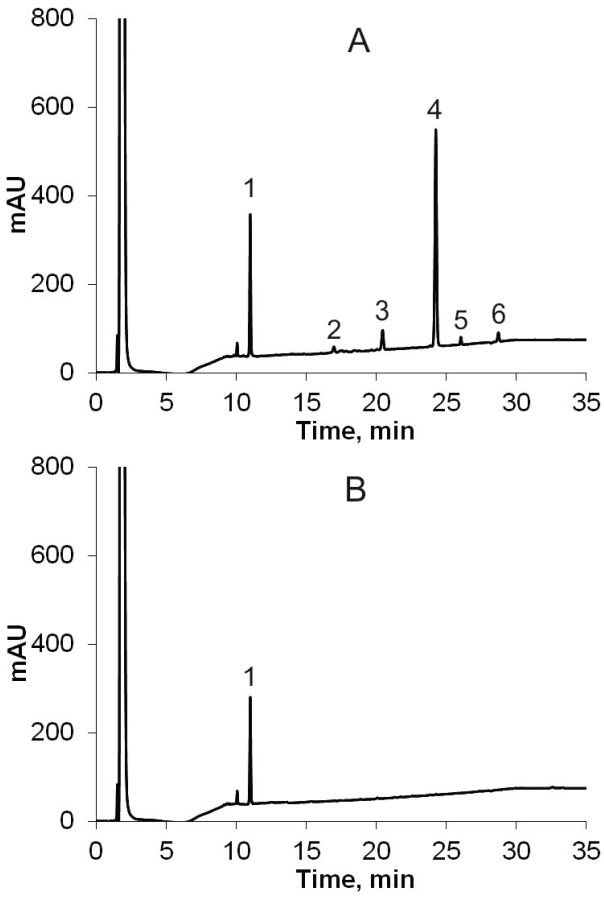

Fig 3. HPLC-UV chromatograms (250 nm) of acetone extracts of *Sporodophoron gossypinum* (A) and *S. primorskiense* (B): 1 – lepraric high unknown; 2 – 4-*O*-methylolivetolcarboxylic acid; 3 – confluentic acid; 4 – 2'-*O*-methylperlatolic acid; 5 – perlatolic acid; 6 – hyperlatolic acid.

Distribution and ecology. The species was known from five localities on Hokkaido and Honshu, Japan (Frisch et al., 2015; Fig. 4). We found two additional localities in the very south of the Primorye Territory of Russia (Fig. 4). These are the first records of the species in the country and in continental Eurasia as well. In Japan, *S. gossypinum* grows on steep, more or less rain-shaded siliceous rocks in deciduous to mixed forests at up to 1020 m elevation (Frisch et al., 2015). Specimens in Russia were collected in similar

conditions on the vertical rain-shaded surfaces of siliceous outcrops in deciduous forests at 50 m and 100 m a. s. l.

Specimens examined: "Russia, Primorye Territory, Khasan District, Kedrovaya Pad' Nature Reserve, on siliceous outcrops in forest with *Quercus mongolica*, *Acer pseudosieboldianum*, *Tilia* sp. on S slope to Kedrovaya River, 100 m a. s. l., 43°6'37.670"N, 131°31'57.630"E. 19 X 2020. Frolov 3705" (LE [L-18456]; hb. Frolov); "Nadezhdinsky District, 1 km N of Terekhovka, old volcano Baranovsky, on tuff outcrops in *Quercus mongolica* forest on E slope to Razdol'naya River, 50 m a. s. l., 43°39'6.466"N, 131°55'6.388"E. 25 X 2020. Frolov 3704" (hb. Frolov).

Fig. 4. Geographical distribution of *Sporodophoron gossypinum* (squares) and *S. primorskiense* (triangles). Black icons – our data; white icons – literature data. Arrows point at the type localities of the species. The white square on Hokkaido combines two localities of *S. gossypinum*.

Sporodophoron primorskiense Frisch et Y. Ohmura, 2015, Lichenologist 47(4): 251.

Holotype: "Russia, Primorye Territory, Chandolaz, ca. 13 km W of Novitskoye, on bark of broadleaf deciduous tree, 220 m a. s. l., 43°03'02"N, 133°01'04"E. 20 IX 2013. Y. Ohmura 10509" (TNS, not seen) (Fig. 5A–C).

Morphology. The detailed description was given by Frisch et al. (2015), with additional information by Ohmura, Frisch (2016). Phenotypic characters of our material mainly agree with these descriptions. The specimen has olivaceous grey endophloedal ca. 85 µm thick thallus with trentepohlioid photobiont, white sporodochia (0.23)–0.27–(0.30) mm diam.

[10] and 0.15–0.25 mm tall with conidia in zigzag-shaped and occasionally branched chains constricted at the septa, that disintegrate into fragments of irregular shape 6.0–9.9–14.0 × 4.0–4.9–6.0 µm [12]. Apothecia are absent.

Our specimen slightly differs from those described by Frisch et al. (2015) and Ohmura, Frisch (2016) by thinner thallus (85 µm vs. up to 170 µm), smaller sporodochia (0.23–0.30 mm diam. vs. 0.25–0.50 mm), and longer conidia fragments (up to 14.0 µm vs. up to 11.0 µm).

Chemistry. See Figs. 2 and 3B. Lepraric high unknown (TLC, HPLC). Sporodochia K+ lemon yellow and thallus greenish yellow.

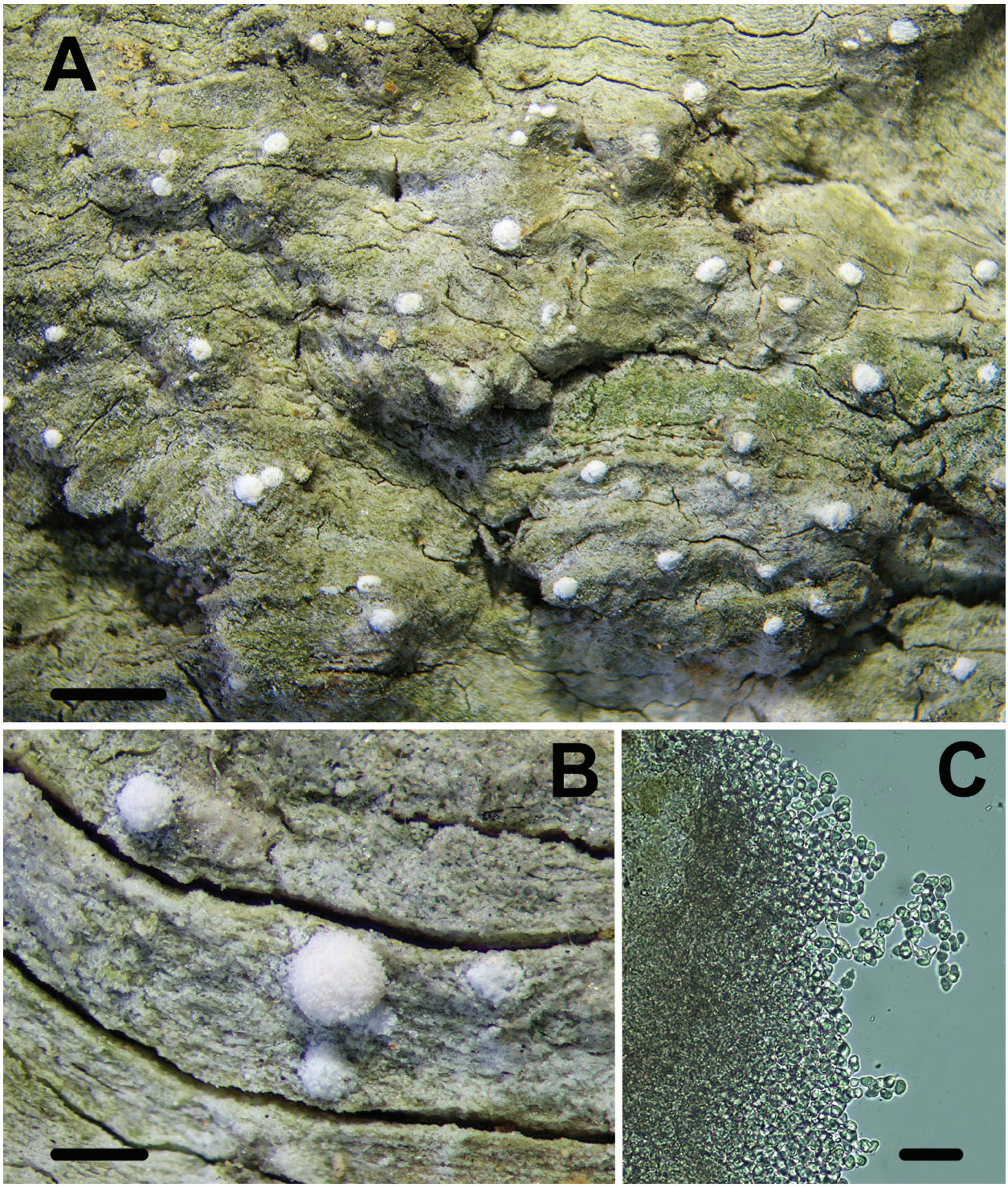

Fig. 5. *Sporodophoron primorskiense* (Frolov 2825): A – Crustose thallus with sporodochia on bark of *Carpinus cordata*; B – Sporodochia; C – Sporodochial conidia. Scales – 1 mm (A), 0.3 mm (B) and 25 µm (C).

Molecular data. Unfortunately, the mrSSU sequence we obtained was of poor quality. However, an online NCBI BLAST search using the sequence demonstrated that it was more closely related to *Sporodophoron* than to other genera.

Distribution and ecology. The species was described from a single locality in the Primorye Territory of Russia in a mixed forest dominated by broad-leaved deciduous trees of *Betula* sp. and *Quercus* sp. at 220 m elevation (Frisch et al., 2015; Fig. 4). Shortly after it was found in Japan (Honshu) in a mixed old-growth forest equally dominated by broad-leaved deciduous and coniferous trees at ca. 1000 m elevation (Ohmura, Frisch, 2016; Fig. 4). Our new find is located in the Primorye Territory of Russia ca. 70 km northwest of the type locality (Fig. 4) in a mixed forest dominated by coniferous trees of *Abies holophylla* and *Pinus koraiensis* at 120 m elevation. In all these localities *Sporodophoron primorskiense* was collected on bark of broad-leaved deciduous trees – on *Carpinus cordata* in our new locality, on *Fagus crenata* in Japan, and on unidentified broad-leaved deciduous tree in the type locality.

Specimen examined: "Russia, Primorye Territory, ca. 30 km NE Vladivostok, S outskirts of Artyom, mixed forest dominated by *Abies holophylla* and *Pinus koraiensis* on slope, on bark of *Carpinus cordata*, 120 m a. s. l., 43°18'52.528"N, 132°12'46.145"E. 24 VI 2020. Frolov 2825" (LE [L-18457]; hb. Frolov).

Discussion

Sporodophoron gossypinum and *S. primorskiense*, two sympatric sporodochial Arthoniaceae species occurring in deciduous and mixed forests of north-eastern Asia, are morphologically similar and form sister lineages on the phylogeny (Frisch et al., 2015). Nevertheless, they can be reliably separated by the saxicolous vs. corticolous habit, the size of sporodochia (always less than 0.50 mm diam. in *S. primorskiense* and up to 0.90 mm diam. in *S. gossypinum*) and absence of 2'-*O*-methylperlatolic acid and some other compounds in *S. primorskiense*. Like *S. primorskiense*, the North American *S. americanum* growing on both bark and rocks has small sporodochia 0.2–0.5 mm diam. and lacks 2'-*O*-methylperlatolic acid. The latter species differs from *S. primorskiense* by its pattern of unknown trace compounds below the lepraric high unknown on the TLC plates (Frisch et al., 2015; Ohmura, Frisch, 2016). Western European *S. cretaceum* differs from the other *Sporodophoron* species by the confluent sporodochia covering most of the thallus surface (Frisch et al., 2015). Also, Frisch et al. (2015) noted that the species of *Sporodophoron* can be confused with *Tylophoron hibernicum* forming convex to subglobose very pale ochraceous sporodochia 0.3–0.6(–1.0) mm diam. (Hawksworth et al., 1979). This species, however, differs by the 0–1-septate conidia not being formed in long zigzag-shaped and occasionally branched chains, and by the presence of lecanoric acid (C+ weakly red reaction; Ertz et al., 2011).

During our chemical investigation of *Sporodophoron gossypinum* using HPLC-UV-MS, we detected several minor and trace compounds previously unknown to the species, namely confluentic acid, 4-*O*-methylolivetolcarboxylic acid, hyperlatolic acid, and perlatolic acid. All these substances are biosynthetically related with 2'-*O*-methylperlatolic acid. Confluentic acid is also known from the genus *Inoderma* [*I. subabietinum* (Coppins et P. James) Ertz et Frisch], which is closely related to *Sporodophoron* (Frisch et al., 2015). Unlike the results of Frisch et al. (2015) and Ohmura, Frisch (2016), confluentic acid was also visible on our TLC plate (Fig. 2). We assume that this is due to the larger amount of the material we used for the analysis and, as a result, the higher concentration of the compound on the TLC plate. Similar to Frisch et al. (2015), we did not observe the unknown trace compounds below the lepraric high unknown obtained on the TLC plates for *S. gossypinum* by Ohmura, Frisch (2016). The additional minor substances newly detected by HPLC (4-*O*-methylolivetolcarboxylic acid etc.) do not correspond to these trace compounds, since their spots on the TLC plate should be located between confluentic acid and lepraric high unknown.

Additionally, using mass-spectrometry we found that lepraric high unknown has the same molecular formula ($C_{18}H_{18}O_8$) and fragmentation pattern as lepraric acid. Moreover, as it was shown by Frisch et al. (2015) and confirmed by us, both substances give the same colour spot reactions. The above suggests that lepraric high unknown is probably a structural isomer of lepraric acid. However, we were unable to determine the structural formula of the compound, because of the scarcity of the available material.

Acknowledgements

The first author would like to thank Kirill Korznikov (Vladivostok) for his hospitality, Yuriy Mamontov (Moscow) and particularly Liudmila Konoreva (Saint Petersburg) for their help during the field trips, and the employees of the Kedrovaya Pad' Nature Reserve for the opportunity to work on the territory of the reserve. Vladimir Kukarskikh

(Yekaterinburg) kindly provided his photographic equipment. The second author thanks Alexey Shavarda (Saint Petersburg) for his help with HPLC-MS. Ivan Frolov worked within the framework of the national project of the Institute Botanic Garden (Russian Academy of Sciences, Ural Branch) and the project 122040800089-2 of the Botanical Garden-Institute FEB RAS. Ilya Pokopiev worked in the framework of the institutional research projects (no. AAAA-A18-118032390136-5) of the Komarov Botanical Institute of the Russian Academy of Sciences and Institute for Biological Problems of the Cryolithozone of Siberian Branch of Russian Academy of Sciences (no. AAAA-A21-121012190035-9).

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