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Fissurina inabensis (Graphidaceae, Ascomycota), a new record to Russia from Shikotan Island

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Keywords: distribution, lichen, phylogeny, Russian Far East, Sakhalin Region, stictic acid.

Summary. During the study of the lichen biota of Shikotan Island, *Fissurina inabensis* (Vain.) M. Nakan. et Kashiw. was identified on the basis of morphological and anatomical data. It is the first record to Russia both at a species and at genus level. The phylogenetic analysis based on the ITS sequences of the studied specimens supported the close relationship with *F. insidiosa* C. Knight et Mitt. belonging to the subfamily *Fissurinoideae*. A detailed description of the morphology, anatomy and secondary metabolites of the studied specimens is given. This species was previously known from Japan, Taiwan, and Thailand, and the area is extended to Shikotan Island where are the northernmost localities of the species. Detection of stictic acid might be variable depending on researchers because of the small amount of the substance. It was well detected using HPLC in this study. Differences in the number and size of ascospores in specimens from Shikotan Island and those from Japan described in the literature are discussed.

Fissurina inabensis (Graphidaceae, Ascomycota) – новый вид для России с о. Шикотан

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Ключевые слова: лишайник, распространение, Российский Дальний Восток, Сахалинская область, стиктовая кислота, филогения.

Аннотация. В ходе изучения лихенобиоты о. Шикотан на основании морфологии и анатомии был выявлен новый для России вид – *Fissurina inabensis* (Vain.) M. Nakan. et Kashiw. Филогенетический анализ, основанный на последовательности ITS изученного образца, подтвердил его близкое родство с *F. insidiosa* C. Knight et Mitt. и принадлежность к подсемейству *Fissurinoideae*. Приведено подробное описание морфоло-

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гии, анатомии и вторичных метаболитов изученных образцов. Ранее *Fissurina inabensis* был известен из Японии Тайваня и Тайланда, в настоящее время его ареал простирается до о. Шикотан, где находятся самые северные места обитания. Выявление стиктовой кислоты может быть затруднено из-за небольшого ее количества. В этом исследовании стиктовая кислота была обнаружена с помощью ВЭЖХ. Обсуждаются различия в числе и размерах спор у образцов с о. Шикотан и из Японии, описанные в литературе.

Introduction

According to the most recent classification of lichenized fungi (Lücking et al., 2017), Graphidaceae Dumort. is the largest crustose lichen family, with more than 2160 species and the dominant element in tropical crustose lichen communities (Rivas Plata et al., 2008). Despite the dominance of Graphidaceae in the tropical zone, some representatives are able to grow in the temperate zone due to high humidity in some regions. For example, ten genera (*Diploschistes* Norman, *Echinoplaca* Fée, *Graphis* Adans., *Gyalectidium* Müll. Arg., *Gyalidea* Lettau ex Vězda, *Gyalideopsis* Vězda, *Leptotrema* Mont. et Bosch, *Phaeographis* Müll. Arg., *Phyllogyalidea* Lücking et Aptroot, and *Thelotrema* Ach.) from the family Graphidaceae are currently known on the territory of Russia (Urbanavichus, 2014). Most representatives of these genera are mainly distributed in the Caucasus, Southern Siberia, and southern part of the Russian Far East (Urbanavichus, 2010). Only a few species, for example *Diploschistes muscorum* (Scop.) R. Sant., *D. scruposus* (Schreb.) Norman, *Graphis scripta* (L.) Ach., *Gyalideopsis piceicola* (Nyl.) Vězda et Poelt, were noted in more northern regions (Zhurbenko, 2000; Poryadina, 2005; Hermansson et al., 2006; Urbanavichus et al., 2008).

During the study of the biodiversity of lichens on Shikotan Island, we discovered *Fissurina inabensis* (Vain.) M. Nakan. et Kashiw. which is reported here for the first time for Russia.

The genus *Fissurina* Fée was established by Fée (1824) for the species with fissurine, lirelline ascomata and ovoid ascospores with a halo. For many years it was subsumed within *Graphis* Adans. and *Graphina* Müll. Arg., chiefly on the basis of its hyaline, transversely septate or muriform ascospores (e. g. Zahlbruckner, 1923; Redinger, 1935; Eriksson, Hawksworth, 1998). Thanks to B. Staiger, a more natural classification of the family Graphidaceae was defined and generally accepted, and the genus *Fissurina* reinstated (Staiger, Kalb, 1999; Staiger, 2002). In phylogenetic studies (Staiger et al., 2006; Mangold et al., 2008) it stands distinct amongst the known genera within Graphidaceae. The diagnostic characters of the genus include a pale to yellow-brown to olive green (rarely whitish), mostly smooth and glossy thallus, fissurine, simple to branched, immersed to prominent lirellae, uncarbonized or rarely carbonized proper exciple, clear hyaline non-amyloid hymenium, 1–8-spored asci, and hyaline, oval or narrowly to broadly ellipsoid, trans-septate to muriform, amyloid to non-amyloid, thick-walled, mostly halonate ascospores (Archer, 2009; Sharma et al., 2012). *Fissurina* occurs mainly in the tropics and temperate regions (e. g. Nakanishi, 1966; Nakanishi et al., 2001, 2003; Staiger, Kalb, 2004; Makhija, Adawadkar, 2007; Kantvilas, 2010; Sharma et al., 2012; Joshi et al., 2013, 2015; Aptroot, 2014; Komposch, 2016; Singh, Singh, 2017) and presently comprises more than 100 species worldwide, many of which were added as a result of the molecular and phylogenetic revision of the Graphidaceae (Rivas Plata et al., 2012, 2013; Sharma et al., 2012).

Materials and Methods

Field and herbarium study. – Specimens were collected by Liudmila Konoreva on the Shikotan Island in the Sakhalin Region of Russia in 2017. The specimens were deposited in the lichen herbaria of the Komarov Botanical Institute of the Russian Academy of Sciences (LE) and the Altai State University (ALTB). The material was examined by the authors in the Laboratory of Lichenology and Bryology of Komarov Botanical Institute, using standard microscopic techniques (Smith et al., 2009; Stepanchikova, Gagarina, 2014). Photographs of the species were taken with a stereoscopic microscope Motic SMZ-171-LED with an attached MotiCam S6 camera and Axio Scope.A1 with Axiocam 506 color camera. Geographical coordinates are given in spatial reference system WGS 1984.

Chemical analyses. – The presence or absence of lichen substances was checked by spot tests with 10 % of KOH (K), $Ca(ClO)_2$ (C) and $C_6H_4(NH_2)_2$ (P) (Smith et al., 2009; Stepanchikova, Gagarina, 2014) and high performance liquid chromatography (HPLC) on an Agilent 1200 instrument. For separation, a ZORBAX SB-C18 (80 Å, 100 × 3 mm, 1.8 μm) column was used. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B)

in a ratio of 20:80. Elution carried out in the isocratic mode. The analysis was conducted for 50 min at a flow rate of 100 μl/min and a column temperature of 25 °C. The injection volume was 1 μl. The detection wavelength was 254 nm. Quantification of the lichen substances was carried out using authentic reference standards of lichen substances from Komarov Botanical Institute collection.

Molecular data generation and analyses. – DNA extraction was performed with the PhytoSorb kit (Syntol, Russia). The ITS region was amplified with the primer pair ITS1f (Gardes, Bruns, 1993) and ITS4 (White et al., 1990). PCR was run according to program, starting with 2 minutes at 95 °C, followed by a 35 cycles schedule using a denaturation temperature of 95 °C for 1 min, an annealing temperature of 55 °C for 1 min, and an extension temperature of 72 °C for 1 min. Chromatograms were edited and aligned using the ClustalW algorithm in the Unipro UGENE (Okonechnikov et al., 2012). The new sequence was uploaded to NCBI (GenBank). Our resulting ITS sequences were aligned along with sequences members of the family Graphidaceae available from GenBank (Table). Species of the genus *Coenogonium* Ehrenb. and *Gyalecta* Ach. were chosen as an outgroup. The DNA sequence evolution model for the ITS locus was selected using the ModelTest-NG v 0.1.6 program (Darriba et al., 2020) using the Akaike Information Criterion (AIC). The alignment was subjected to RAxML-HPC BlackBox on XSEDE (CIPRES gateway) for phylogenetic tree construction using "-m GTRCAT". Branches were statistically supported by bootstrap analysis of 1000 pseudo-replicas. The whole original alignment (including ambiguously aligned regions) was used in the analysis; gaps were treated as missing data.

Table

GenBank accession numbers and additional information for the specimens used in the phylogenetic analysis in Fig. 1. Newly generated sequence is in bold

Taxon name	Location	Source	Collector and Herbarium	ITS
Coenogonium isidiatum	Russia, Iturup	Konoreva et al. (2018)	Konoreva LE-L-14437	MH179136
Coenogonium luteum		Schmull et al. (2011)	Aftol 352	HQ650710
Coenogonium pineti	Canada, Ontario	Telfer et al. (2015)		KT695346
Cruentotrema thailandicum	Myanmar	Ohmura et al. (2020)	Ohmura 12327	LC573996
Fissurina insidiosa	USA, Alaska	Resl et al. (2015)	Spribille 39035, GZU	KR017123
Fissurina inabensis	**Russia, Shikotan**	**this paper**	**Konoreva LE L-18645**	**OP901516**
Glyphis cicatricosa 1	Thailand	Ekanayaka et al. (2019)	Ekanayaka, HD070, MFLU 18-0686	MK499350
Glyphis cicatricosa 2	Jamaica	unpublished	Dal Forno 3198, US	MT553331
Graphis betulina	Poland	Singh et al. (2019)	Kukwa et Lubek, s.n.	MN387113
Graphis caribica	China	unpublished	Jia AH17163, LCU	MF588529
Graphis scripta 1	USA, North Carolina	McDonald et al. (2013)	McDonald 1518	KC592274
Graphis scripta 2	United Kingdom, Wales	unpublished	Cannon s.n., K(M):202343	MZ159599
Gyalecta hypoleuca		Schmull et al. (2011)	Aftol 380	HQ650711
Gyalecta jenensis		Schmull et al. (2011)	Aftol 361	HQ650712
Gyalecta ulmi		Schmull et al. (2011)	Aftol 362	HQ650713
Phaeographis cf. *brasiliensis*	Jamaica	unpublished	Dal Forno 3188a, US	MT553329
Phaeographis dendritica	United Kingdom: England	unpublished	Cannon s.n., K(M):253800	MZ159751
Phaeographis elliptica	USA, Florida	McDonald et al. (2013)	Gaya 16.03.08-8 EGB12, DUKE	KC592276

Results and Discussion

Although the phylogenetic analyses of the Graphidaceae were usually based on three loci (mtSSU, nuLSU, RPB2) (e. g., Rivas Plata et al., 2013; Lumbsch et al., 2014), ITS sequence was uncommon and small numbers of them were registered in GenBank. We generated one new ITS sequence from a sample identified as *Fissurina inabensis* collected on the Shikotan Island. The sequence was formed in a strongly supported clade (ML BP: 99) within subfamily *Fissurinoideae* where is grouped together with the sequence of *F. insidiosa* C. Knight et Mitt. (Fig. 1). Thus, phylogeny based on ITS sequences was sufficient to support our assumption, based on morphology and anatomy, that the specimens belong to the *Fissurina*. Unfortunately, there are no sequences of *F. inabensis* in the GenBank, so here molecular data cannot be used to confirm the identification of our samples that was based on morphological data. Below we provide a taxonomic treatment of *F. inabensis*, including a description of the material from the Russian Far East.

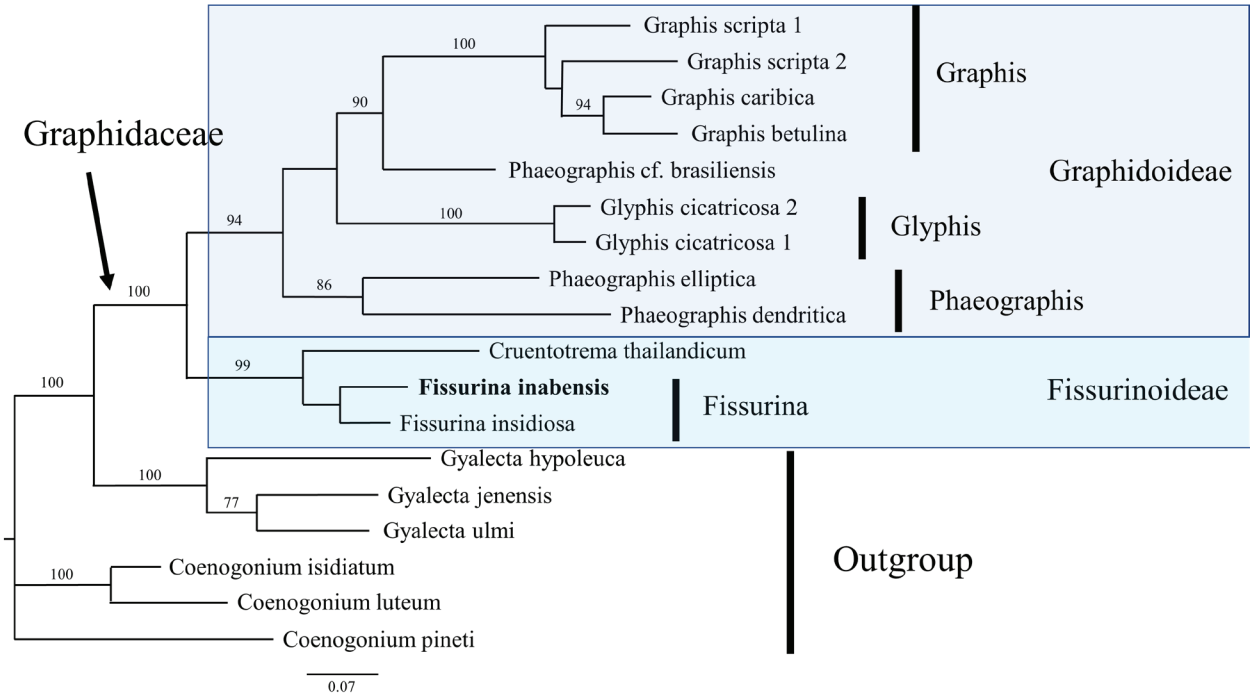

Fig. 1. A phylogenetic tree for *Fissurina inabensis* and the related taxa in Graphidaceae inferred from ITS sequences. Numbers on branches represent maximum likelihood bootstrap values (≥ 70 %). Newly sequenced samples are indicated in bold.

Fissurina inabensis (Vain.) M. Nakan. et Kashiw., 2003, in Nakanishi, Kashiwadani et Moon, Bull. Natl. Sci. Mus., Tokyo, Ser. B 29(2): 86. ≡ *Graphis inabensis* Vain., 1918, Bot. Mag., Tokyo 32: 161. ≡ *Graphina inabensis* (Vain.) Zahlbr., 1923, Cat. Lich. Univers. 2: 409.

Type: "Japan, Honshu, Prov. Inaba: lwai. Ad corticem arborum. 4 August 1914. Y. lkoma" (herb. A. Yasuda 47-isotype in TNS, not seen) (Fig. 2).

Description of studied specimens. – Thallus corticolous, epiphloeodal, corticate, to ca. 100 µm thick, glossy, verrucose to smooth, green, olive green to pale green; medulla with calcium oxalate crystals small to moderately large; prothallus not seen. Vegetative propagules not seen. Ascocarps numerous, scattered, lirelliform, conspicuous, immersed to sessile, slightly open, raised and paler than the thallus, straight, curved or sinuous, branched, 1–3 mm long, disc slit-like, narrow, scarcely open in mature apothecia, flesh colored. Thalline margin concolorous with thallus, thick. Proper exciple poorly to well developed in mature fruits, hyaline to pale or straw-colored. Epihymenium hyaline, indistinct. Hymenium hyaline, clear, to 100–120 µm high, non-amyloid (I–). Paraphyses straight to bent, ±interwoven, unbranched, with slightly thickened tips, moderately to distinctly conglutinated, 1–2 µm thick. Subhymenium indistinct, hyaline, 20–30 µm high. Asci clavate,

mainly 8-spored (Fig. 2C), less often 2–4-spored (Fig. 2B). Ascospores hyaline, submuriform with 4–6 transverse and 3–5 longitudinal septs, oblong to ellipsoidal, with ±rounded ends, non-amyloid, gelatinous sheath present, (20–)23–32(–45) × (8–)10–16 µm (Fig. 2D and E).

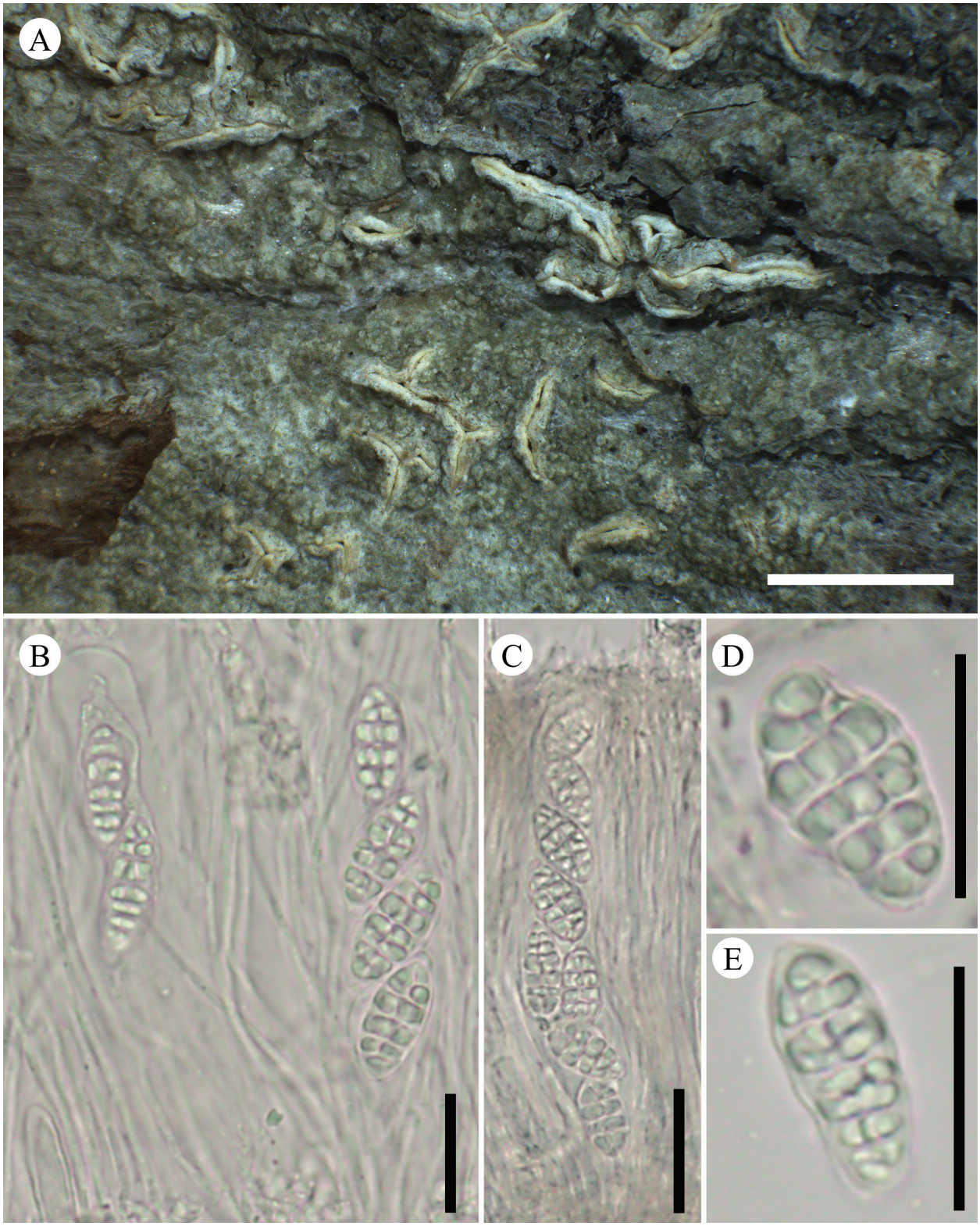

Fig. 2. *Fissurina inabensis* (LE L-18645): A – lirelliform apothecia and thallus; B – 2 and 4-spored asci on the same section; C – 8-spored asci; D, E – submuriform ascospores. Scale bar: A = 3 mm, B–E = 25 µm.

Chemistry. One specimen (LE L-18690) contained trace amounts of stictic acid, which could only be found using HPLC; in the other (LE L-18645), no lichen substances were found. Spot tests with K, C, and P are negative for all specimens.

Distribution and ecology. The species was first described from Japan (Wainio, 1918) where it is one of the most common *Fissurina* species (Nakanishi, 1966). Later it has been reported for Taiwan and Thailand (Nakanishi et al., 2001). It was collected from bark tree in mountain forests. On the Shikotan Island, the species was found in the forests at the foot of the Shikotan and Notoro mountains on the trunks of coniferous trees.

Specimens examined: "Sakhalin Region, Yuzhno-Kurilsky District, Shikotan Island, neighborhood of Malokurilsk village, 43°52'16.1"N, 146°50'36.9"E, 96 m a. s. l., fir-yew-birch forest with maple, on bark of *Taxus cuspidata*. 11 VI 2017. Leg. L. A. Konoreva 293" (ALTB, LE L-18645), not found lichen substances detected by HPLC; "*ibid.*, neighborhood of Notoro Mountain, 43°46'50.4"N, 146°42'16.9"E, 72 m a. s. l., birch forest with maple undergrowth, on bark of *Abies sachalinensis*. 17 VI 2017. Leg. L. A. Konoreva 437" (LE L-18690), stictic acid in trace amounts.

Notes. According to the protologue of *Graphis inabensis* (Wainio, 1918), this species is characterized by prominent fissurine ascocarps, well-developed straw-colored exciples covered with thalloid margins, periphysoids, 8-spored asci, submuriform ascospores with 4–6 transverse and 3–5 longitudinal septa. Wainio (1918) reported a "fuscescens" reaction with KOH that indicating stictic acid content. However, Nakanishi (1966) reported about negative reactions (K–, P–) for the species. Later, when creating a new combination for *Graphina inabensis* (Vain.) Zahlbr., Nakanishi et al. (2003) indicated the presence of stictic acid as a characteristic feature. Apparently, the presence or absence of stictic acid is related to the lighting. Thus, our specimen with stictic acid grew in sunlit birch forest, while the specimen without stictic acid was found in a mixed coniferous forest under shaded conditions. Also, Wainio (1918) and Nakanishi (1966) treated 8-spored asci as an important feature. In our material, and sometimes on the same section, it was possible to observe both 2–4-spored and 8-spored asci. At the same time, ascospore sizes also differed. So ascospore sizes in 8-spore asci are more suitable for Wainio's description: our material – 20–25 × 8–10 μm, according to Wainio (1918) – 21–24 × 8–10 μm. Ascospores in 2–4-spored asci larger and more suitable for Nakanishi's description: our material – 23–32(–45) × 12–16 μm, according to Nakanishi (1966) – 21–30 × 10–14 μm.

The closest species to *Fissurina inabensis* is *F. undulata* (Müll. Arg.) M. Nakan. et Kashiw. *Fissurina undulata* also appears to produce stictic acid but differs in slightly larger sizes of ascospores (30–38 × 15–18 μm according to Nakanishi (1966)) and their morphology (ascospores muriform and consist of a large number of septa and locules, see Nakanishi (1966)).

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