

## First record of earth tongue *Hemileucoglossum pusillum* (*Geoglossales*) in the Czech Republic

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In 2015, during a visit to Řeka Nature Reserve, an interesting earth tongue fungus was found in a fen meadow. It was later identified as *Hemileucoglossum pusillum*, a recently described species. Its occurrence on the site was also confirmed in 2021, when abundant material was collected. In the Czech Republic, it is the only known locality of this rare species, previously reported from Slovakia, Norway and Spain only. Its identity was verified both morphologically and molecularly by sequencing the ITS and LSU regions of nuclear ribosomal DNA. Its macro- and microscopic characters are presented and the variability, distribution, ecology and phenology of the species are discussed. A Czech name is proposed for the species.

**Key words:** *Ascomycota*, *Geoglossaceae*, Řeka Nature Reserve, fen meadows, distribution.

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V roce 2015 byl během terénní exkurze v přírodní rezervaci Řeka na slatinné louce nalezen zajímavý jazourek, později ztotožněný s nedávno popsáním druhem *Hemileucoglossum pusillum*. Jeho výskyt na lokalitě se podařilo potvrdit také v roce 2021, kdy byl sebrán bohatý materiál. Je to dosud jediná lokalita tohoto vzácného druhu v České republice, jinak je známý pouze ze Slovenska, Norska a Španělska. Identita druhu byla ověřena jak morfologicky, tak molekulárně pomocí sekvenace ITS a LSU regionů jaderné ribosomální DNA. Je uveden popis makroskopických a mikroskopických znaků a diskutována variabilita, rozšíření, ekologie a fenologie druhu. Je navrženo české jméno jazourek zakrslý, odpovídající latinskému epitetu (*pusillus* = zakrslý, nízký, maličký, drobný).

## INTRODUCTION

*Hemileucoglossum pusillum* V. Kučera, Fedosova et Arauzo was described recently from Slovakia (Crous et al. 2017). It belongs to the genus *Hemileucoglossum* Arauzo, which is characterised by (1) black sporophores with setose hairs (up to 160 µm long) localised only on the stipe (mainly in the upper part) which are visible with the naked eye or a lens in fresh conditions, (2) relatively long (> 50 µm in most of the species), septate ascospores, remaining hyaline for a long time and becoming tan to brownish with age, and (3) paraphyses agglutinated by a brown amorphous matrix which precipitates in 5% KOH and forms dark spots on the paraphyses. Six species are currently assigned to this genus, three of which occur in Europe [*H. littorale* (Rostr.) Arauzo, *H. elongatum* (Starbäck ex Nannf.) Arauzo, and *H. pusillum*], two in North America [*H. alveolatum* (E.J. Durand ex Rehm) Arauzo and *H. intermedium* (E.J. Durand) Arauzo], and two in Asia (again *H. alveolatum* and also *H. kelabitense* V. Kučera, Fedosova et Sochorová) (Crous et al. 2020). Morphologically *H. pusillum* is most similar to *H. littorale*, which differs mainly by the presence of long, dark brown, branched hyphae on the stipe, creating a network-like structure (Fadnes et al. 2021). Furthermore, *H. littorale* prefers sandy shores of oligotrophic lakes with *Littorella uniflora*, and sometimes grows in fen meadows, while *H. pusillum* prefers to grow near mineral springs of fens or in peat-bogs, but can be found on sandy river banks and in grasslands as well (Crous et al. 2017, Kučera et Fedosova 2017, Fadnes et al. 2021).

The distribution of *H. pusillum* is insufficiently explored. In Slovakia, it grows besides the type locality [Močiar Nature Reserve (further: NR)] at one adjacent site (Rojkovské rašelinisko NR) only. Since its description in 2017, the species was refound only in 2020 at the first of the listed localities (leg. V. Šišková). Apart from Central Europe, the fungus is known from three other sites in Spain (Arauzo et Iglesias 2014, reported as *H. littorale*) and a single locality in Norway (Fadnes et al. 2021). Both in Spain and Norway, the species was recorded in the same place for several years.

As part of the work on a bachelor's thesis, Kamila Čížková visited Řeka NR (Fig. 1) to make an inventory of the funga in 2015, when an unusual earth tongue fungus appeared in the collected material, which was identified as *H. littorale*. However, after repeated collections at the same site in 2021, the identification was corrected to *H. pusillum*. Řeka NR represents the first known locality of the species in the Czech Republic and the third within Central Europe. The aim of our study is to present the new locality of this unusual fungus and its natural environment in the Czech Republic.



**Fig. 1.** Collection site of *Hemileucoglossum pusillum* (BRNU 680005, BRNU 680006, and SAV F-11789) in Řeka Nature Reserve, 27 August 2022. Photo D. Dvořák.

## MATERIAL AND METHODS

Macromorphological characters were studied on fresh material. Micromorphological features were studied both on fresh and dry specimens using an Axio Scope A1 light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Distilled water, 5% KOH, and Melzer's reagent were used as observation media. Dimensions of microstructures and Q value (length to width ratio) are presented as the mean  $\pm$  SD of 30 measurements with the minimal and maximal values in parentheses. The identification was based on Crous et al. (2017). The exact location of the sporophores found is georeferenced by coordinates in the WGS 84 system. The description is made on the basis of material collected in 2021. Dried specimens are stored in the herbaria of Masaryk University in Brno (BRNU) and the Botanical Institute of SAV in Bratislava (SAV). Parts of the specimens were sent to the commercial laboratory ALVALAB in Spain, which provided extraction, amplification, and sequencing of the ITS and LSU nrDNA regions. The obtained sequences were deposited in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>).

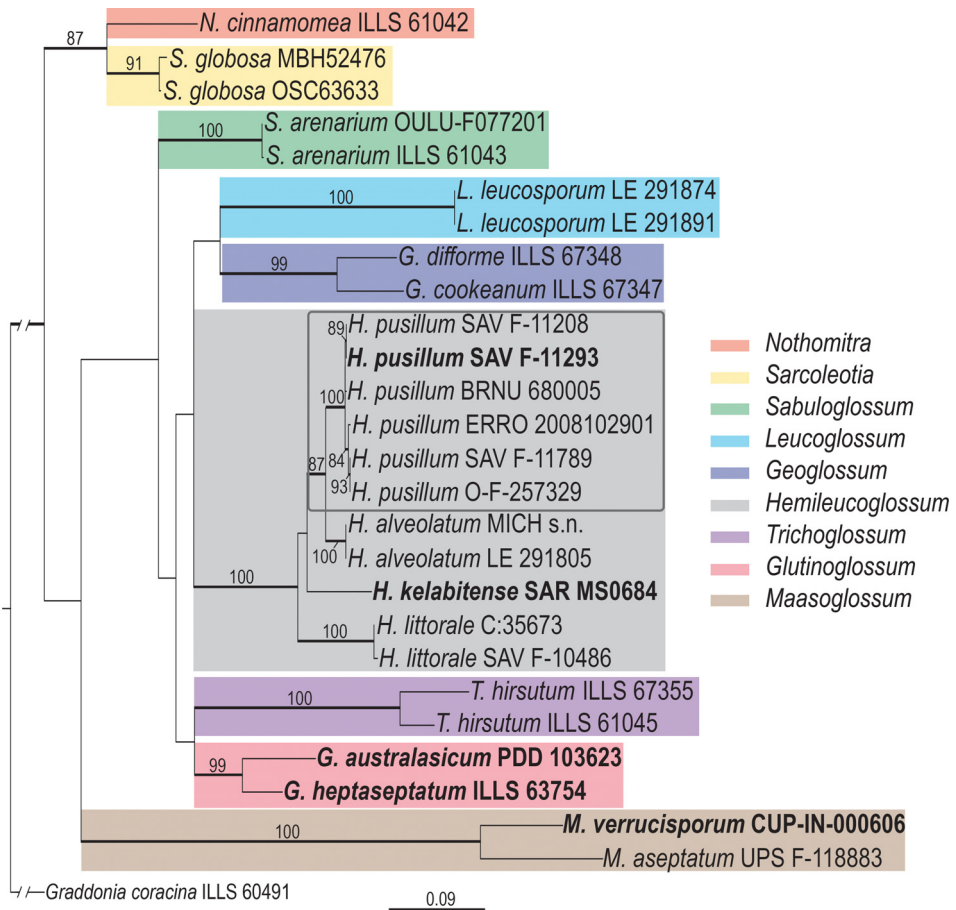
In total, 27 ITS and 25 LSU sequences were selected for phylogenetic analyses, including sequences from holotype of *H. pusillum* and four other type specimens as well as four newly generated sequences (two ITS and two LSU, Tab. 1). *Graddonina coracina* (Bres.) Dennis was taken as an outgroup. Individual ITS and LSU datasets were created in MEGA v.7 (Kumar et al. 2016) and then were aligned in MAFFT v.7 Web tool (Katoh et al. 2019) with G-INS-1 strategy and default settings for other options. The final alignment of ITS region was corrected by eye in MEGA v.7. Ambiguous regions were eliminated from individual alignments using TrimAl v.1.2b (Capella-Gutiérrez et al. 2009) with default settings. The best-fit AICc-selected model of evolution (SYM+I+G for ITS and GTR+I+G

**Tab. 1.** Specimens and NCBI GenBank accession numbers of DNA sequences used in phylogenetic analysis. Newly generated sequences are in bold.

Species	Country	Voucher no.	GenBank accession no.		References
			ITS	LSU	
<i>Geoglossum cookeanum</i>	Czech Republic	ILLS 67347	KC222122	KC222135	Hustad et al. 2013
<i>Geoglossum difforme</i>	USA	ILLS 67348	KC222123	KC222136	Hustad et al. 2013
<i>Glutinoglossum australasicum</i> , holotype	New Zealand	PDD 103623	KP690088	KP690100	Hustad et Miller 2015a
<i>Glutinoglossum heptaseptatum</i> , holotype	Czech Republic	ILLS 63754	KC222130	KC222143	Hustad et al. 2013
<i>Graddonia coracina</i> , outgroup	USA	ILLS 60491	JQ256423	JN012009	Hustad et al. 2011, Hustad et Miller 2011
<i>Hemileucoglossum alveolatum</i>	USA	MICH s.n.	KP657560	KP657565	Hustad et Miller 2015b
<i>Hemileucoglossum alveolatum</i>	Russia	LE 291805	MF353087	-	Crous et al. 2017
<i>Hemileucoglossum kelabitense</i> , holotype	Borneo	SAR MS0684	MT021979	MT021912	Crous et al. 2020
<i>Hemileucoglossum littorale</i>	Denmark	C:35673	KP657561	KP657566	Hustad et Miller 2015b
<i>Hemileucoglossum littorale</i>	Slovakia	SAV F-10486	MF353089	MF353092	Crous et al. 2017
<b><i>Hemileucoglossum pusillum</i></b>	<b>Czech Republic</b>	<b>BRNU 680005</b>	<b>OM272955</b>	<b>OM272958</b>	<b>This study</b>
<b><i>Hemileucoglossum pusillum</i></b>	<b>Czech Republic</b>	<b>SAV F-11789</b>	<b>OM272956</b>	<b>OM272959</b>	<b>This study</b>
<i>Hemileucoglossum pusillum</i>	Norway	O-F-257329	MW295710	MW295713	Fadnes et al. 2021
<i>Hemileucoglossum pusillum</i> , holotype	Slovakia	SAV F-11293	MF353090	MF353093	Crous et al. 2017
<i>Hemileucoglossum pusillum</i>	Slovakia	SAV F-11208	MF353088	MF353091	Crous et al. 2017
<i>Hemileucoglossum pusillum</i> (as <i>H. littorale</i> )	Spain	ERRO 2008102901	KP144108	-	Arauzo et Iglesias 2014
<i>Leucoglossum leucosporum</i>	Russia	LE 291891	KP272112	KP272113	Fedosova et Kovalenko 2015
<i>Leucoglossum leucosporum</i>	Russia	LE 291874	KP272114	KP272115	Fedosova et Kovalenko 2015
<i>Maasoglossum aseptatum</i>	Sweden	UPS F-118883	KP657562	KP657567	Hustad et Miller 2015b
<i>Maasoglossum verrucisporum</i> , isotype	Bhutan	CUP-IN-000606	KP657563	KP657568	Hustad et Miller 2015b
<i>Nothomitra cinnamomea</i>	France	ILLS 61042	JQ256424	JQ256439	Hustad et al. 2011
<i>Sabuloglossum arenarium</i> (as <i>Thuemenidium arenarium</i> )	Netherlands	ILLS 61043	JQ256426	JQ256440	Hustad et al. 2011
<i>Sabuloglossum arenarium</i> (as <i>Thuemenidium arenarium</i> )	Finland	OULU-F077201	GU324765	GU324764	Ohenoja et al. 2010
<i>Sarcoleotia globosa</i>		OSC63633	AY789410	AY789409	Wang et al. 2005
<i>Sarcoleotia globosa</i>		MBH52476	AY789429	AY789428	Wang et al. 2005
<i>Trichoglossum hirsutum</i>	Czech Republic	ILLS 61045	JQ256428	JQ256442	Hustad et al. 2011
<i>Trichoglossum hirsutum</i>	USA	ILLS 67355	KC222132	KC222145	Hustad et al. 2013

for LSU) was calculated by PartitionFinder 2 (Lanfear et al. 2017) which includes PhyML program (Guindon et al. 2010), and using greedy algorithm described in Lanfear et al. (2012).

A maximum likelihood (ML) phylogenetic analysis was run in RAxML v. 7.2.6 (Stamatakis 2006) implementing the search protocol for the best-fit model of evolution. Clades with a bootstrap support (BS) value  $\geq 80\%$  were considered significant. The Bayesian analyses (BA) employing a metropolis coupled Markov Chain Monte Carlo (MCMCMC) algorithm was performed using MrBayes v. 3.2.7 (Ronquist et al. 2012). Four independent chains were run one million generations under the appropriate model parameters with trees sampled every 100 generations. To evaluate the quality of a sample



**Fig. 2.** Phylogenetic tree generated by means of maximum likelihood analysis based on ITS-LSU sequence data. The numbers below the branches indicate BS values  $\geq 80\%$ , branches in bold indicate PP value  $\geq 0.95$ . Type collections are marked in bold. Affiliations to genera of the family *Geoglossaceae* are highlighted. The clade *H. pusillum* is indicated in a box. The scale bar represents the number of nucleotide changes per site.

from the posterior and the continuous parameters, effective sample size (ESS) based on the standard deviation of split frequencies produced by BA was estimated in Tracer v. 1.7.1 (Rambaut et al. 2018). The clades with posterior probability (PP) value  $\geq 0.95$  were considered to be significantly supported.

Individual alignments of ITS and LSU were checked for potential conflict before concatenation into a single dataset. Incongruence of individual gene phylogenies was assumed to be significant if clades with BS  $\geq 80\%$  in ML or PP  $\geq 0.95$  in BA were conflicting in the individual tree topologies (Lutzoni et al. 2004). Conflicting clades among the individual gene topologies were not found; therefore, ITS and LSU regions were combined. The concatenated ITS-LSU data matrix had an aligned length of 1354 bp, which was reduced to 1216 bp after elimination of 138 bp by TrimAl. Further ML and BA phylogenetic analyses were performed on the concatenated dataset as described above.

The MCMCMC algorithm in BA was run with four chains for 10 million generations with tree sampling every 100 generations. To remove the pre-stationary posterior probability distribution, a burn-in of 21% (ESS = 1134.5) was estimated with Tracer to be sufficient. Visualisation and editing of the trees were performed in FigTree v. 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>). Alignments with obtained phylogenies were deposited in TreeBASE (<http://treebase.org>) under submission ID 29182.

## RESULTS

### MOLECULAR STUDY

The most likely tree topology produced by the ML analysis of the combined ITS–LSU dataset is illustrated (Fig. 2). The phylogenetic analyses revealed that *H. pusillum* forms a strongly supported clade (BS = 100%, PP = 1) which includes specimens from the Czech Republic, Norway, Slovakia, and Spain. The closest sister clade to *H. pusillum* is *H. alveolatum*.

### MORPHOLOGICAL STUDY

***Hemileucoglossum pusillum*** V. Kučera, Fedosova et Arauzo, in Crous et al., *Persoonia* 39: 305, 2017 Figs 3–5

Sporophores represented by apothecial ascomata scattered or clustered in groups of 3–4, showing a distinct sterile stipe and a fertile part, 10–60 mm high, dry, black. Fertile part lanceolate, flattened, clavate, smooth or vertically wrinkled, usually clearly delimited from the stipe, 5–15 mm long (up to 1/4 of the sporophore length). Stipe cylindrical, slender to robust, distinctly hairy, appearing scaly when dry; setose hairs mostly present in upper part of the stipe, clustered, more visible on fresh material.

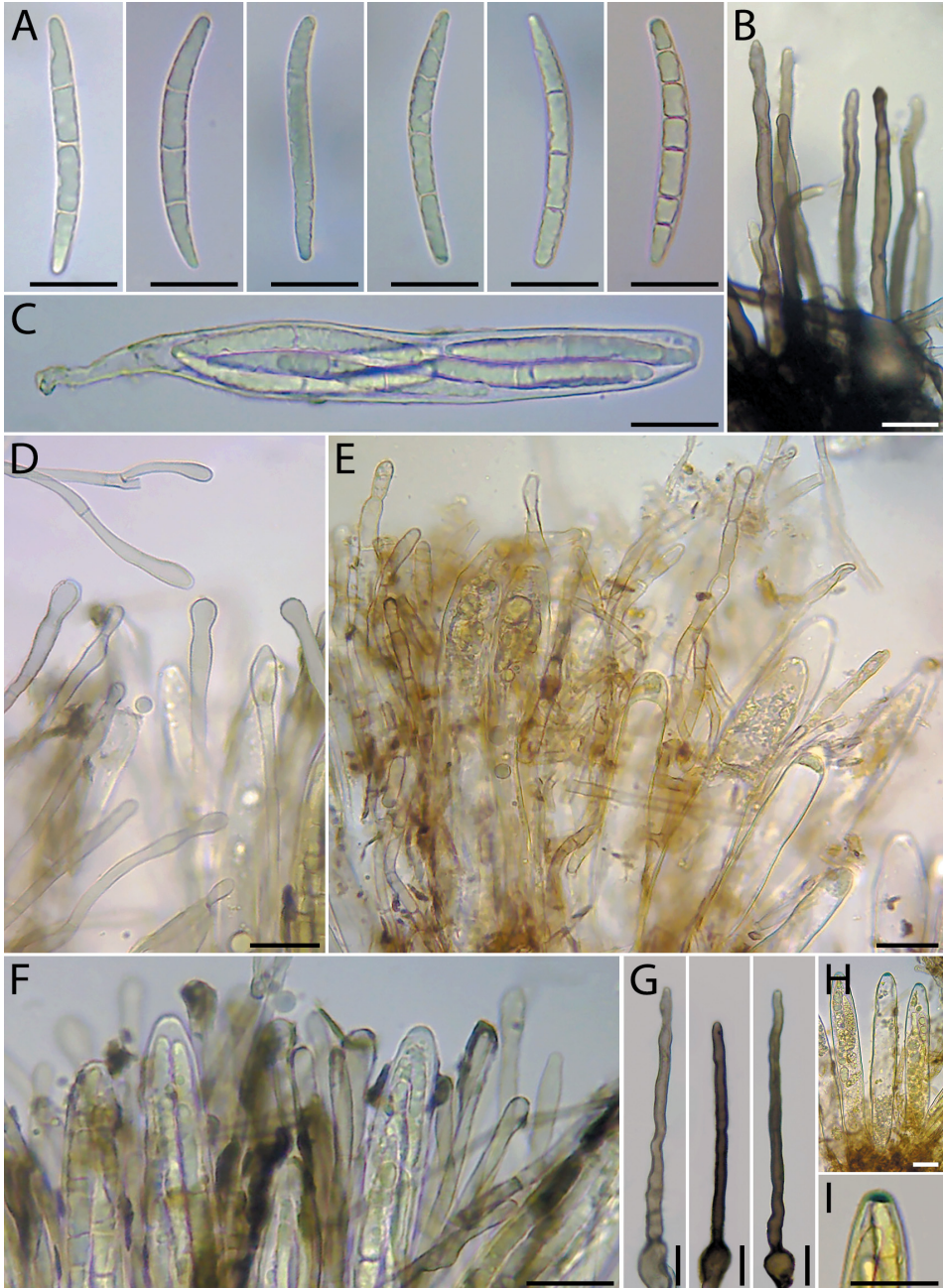
Asci clavate to broadly clavate, (154)160–179(200) × (19)22–23(25) μm in fresh material and (152)155–165(170) × (16)17–18(20) μm in rehydrated material, Q = (7.2)8.1–9.5(12) and Q = 6.8–9(9.5) respectively, 8-spored with euamyloid apical pore blueing in Melzer’s reagent. Ascospores elongate-clavate, tapering to the ends, sometimes slightly curved, (50)70–80(85) × (5)6–7.5(8) μm in fresh material and (47)60–72(82) × 5–6 μm in rehydrated material, Q = (8.7)9–12(14) and Q = (7.6)8.7–11.1(13.3) respectively, hyaline, then browning with age, 0–6-septate (mainly 3–4-septate). Ascoconidia not observed. Paraphyses cylindrical, sparsely septate, 2–3 in diameter, straight or slightly curved with an inflated apex, variously shaped, hyaline in basal part and light brown in apical part which is immersed in a brown amorphous matrix visible in 5% KOH as dark spots on the paraphysis surface. Apical cells of paraphyses often inflated and compressed or pyriform, sometimes simply cylindrical, (10)15–48(55) × (3)4.5–9(11) μm. Stipe surface scaly, composed of tufts of brown, septate, setose hairs, (80)95–130(148) μm in length.



**Fig. 3.** Sporophores of *Hemileucoglossum pusillum* in situ, among *Tomentypnum nitens* and *Hamatocaulis vernicosus*, Řeka NR, calcareous fen, 19 September 2021, leg. V. Kučera (SAV F-11789). Photo F. Fuljer.



**Fig. 4.** Sporophores of *Hemileucoglossum pusillum* in situ, Řeka NR, calcareous fen, 19 September 2021, leg. V. Kučera (SAV F-11789). Photo F. Fuljer.



**Fig. 5.** Micromorphological characters of *Hemileucoglossum pusillum* (SAV F-11789, rehydrated material): **A** – free mature ascospores, **B**, **G** – setose hairs, **C** – mature ascus with ascospores, **D** – upper parts of paraphyses, **E**, **F** – hymenium, **H**, **I** – apical pore (A–G in KOH, H, I in Melzer's reagent). Scale bars = 20 µm. Photos V. Kučera.



**Material studied**

Czech Republic. Krucemburk, Řeka Nature Reserve, calcareous fen (*Caricion davallianae* alliance) near eastern bank of the Doubrava River, 49°39'59.7" N, 15°51'12.0" E, alt. 553 m, among brown mosses (*Tomentypnum nitens*, *Hamatocaulis vernicosus*, *Scorpidium cossonii*, etc.), 13 October 2015, leg. K. Čížková (BRNU 680005). – Ibidem, 2 September 2021, leg. D. Dvořák, collection code DD 210902-15 (BRNU 680006). – Ibidem, 19 September 2021, leg. V. Kučera (SAV F-11789).

DISCUSSION

**Morphology**

Despite phylogenetic evidence supporting *H. pusillum* as closely related to *H. alveolatum* (Crous et al. 2017, Crous et al. 2020, Fadnes et al. 2021, this study), *H. pusillum* is morphologically more similar to *H. littorale* because of its 3–4-septate hyaline ascospores with an overlapping range of variability in size. However, on average, *H. littorale* has smaller ascospores, 50–66 × 4–6 μm (Rostrup 1892, Kers et Carlsson 1996, Kučera et Fedosova 2017) vs 50–80 × 5.5–7.5 μm in *H. pusillum* (Crous et al. 2017, Fadnes et al. 2021, this study). Moreover, *H. littorale* has a notable network-like structure on the stipe formed by long, branched, dark brown hyphae (Fadnes et al. 2021).

The collections of *H. pusillum* from the Czech Republic are morphologically more similar to the collection from Norway than to the other ones. The sporophores are taller (up to 60 mm) and more robust than the type and Norwegian collections (up to 35 mm tall). Microscopically, our material fits well with previous descriptions, only the asci and ascospores are slightly longer but in the range of the described variability. Phylogenetic analyses clearly confirmed the identification of the species *H. pusillum*.

**Ecology**

The biota of Řeka NR, where all currently known Czech collections of *H. pusillum* were made, is well explored in terms of vegetation and flora of vascular plants and bryophytes (e.g. Štechová et Manukjanová 2015, Peterka 2016). The site where *H. pusillum* was found to occur is the most valuable part of the locality in terms of biodiversity and belongs to the best-preserved calcareous fens of the *Caricion davallianae* alliance in the region. It is one of the richest Czech locations of the moss *Hamatocaulis vernicosus*, protected under the EU Habitats Directive (Annex II). Also another species protected on the European level, the snail *Vertigo geyeri*, has the largest European population here (Schenkova et Horsák 2013). Some interesting mycological finds in recent years [for example, *Arrhenia lobata* (Pers.) Kühner et Lamoure ex Redhead, *Bovista paludosa* Lév. and *Entoloma mougeotii* (Fr.) Hesler] affirm the high value of the site.

The composition of vascular plants and mosses is documented by a phytosociological relevé made at the site of *H. pusillum* by Peterka (2016): vascular plants – *Eleocharis quinqueflora* 2a, *Menyanthes trifoliata* 2a, *Molinia caerulea* agg. 2a, *Valeriana dioica* 2a, *Carex lepidocarpa* 1, *Carex panicea* 1, *Carex rostrata* 1, *Succisa pratensis* 1, *Alnus incana* juv. +, *Briza media* +, *Carex dioica* +, *Carex nigra* +, *Cirsium palustre* +, *Equisetum palustre* +, *Juncus articulatus* +, *Linum catharticum* +, *Parnassia palustris* +, *Potentilla erecta* +, *Galium uliginosum* r, *Picea abies* juv. r, *Salix pentandra* juv. r, *Viola palustris* r; mosses – *Scorpidium cossonii* 4, *Campylium stellatum* 2b, *Hamatocaulis vernicosus* 2a, *Bryum pseudo-triquetrum* 1, *Philonotis calcarea* 1, *Calliargon giganteum* +, *Cratoneuron commutatum* +, *Plagiomnium* sp. +, *Tomentypnum nitens* + [values of abundance are given according to the modified Braun-Blanquet scaling system (Moravec 1994)].

When compared to habitat conditions of other known localities, the Slovakian site Rojkov NR is very similar, being one of the best preserved and oldest calcareous fens in the country (Hájková et al. 2015). The type locality (Močiar NR; at only some 600 m from Rojkov) is also strongly calcareous, however sporophores of *H. pusillum* did not grow here on peat, but on gravelly soil with some fen-specialised plants such as *Triglochin palustre*, *Trichophorum pumilum* and *Centaurium littorale* subsp. *uliginosum* (Crous et al. 2017). In Norway, *H. pusillum* is known from one locality in semi-natural grassland on limestone in the south-western part of the country, accompanied by many grassland fungi. It was observed there repeatedly at a single spot among mosses on a north-facing slope near the seashore. The site is probably the richest in Norway in terms of grassland fungus diversity (Fadnes et al. 2021). Most of the Spanish collections originate from sandy river shores with *Chamaecyparis lawsoniana* trees and undergrowth of *Carex pendula* (Arauzo et Iglesias 2014, as *H. littorale*). To sum up, the ecological niche of *H. pusillum* seems to be rather wide, although it seems to have some preference for well-preserved grassland or fen sites.

The morphologically similar *H. littorale* was formerly presented as specific to oligotrophic lake shores with *Littorella uniflora* (Kers et Carlsson 1996). Later, however, it was documented from a fen with *Phragmites* sp., *Eriophorum* sp., and *Salix* sp. in Slovakia (Kučera et Fedosova 2017). Hence, the requirements of the two discussed earth tongue species seem to overlap slightly, as both *H. pusillum* and *H. littorale* may occur in calcareous fens or open shore vegetation on calcareous bedrock.

There is some variation in the phenology of *H. pusillum*. At Řeka NR, fresh sporophores were collected in September and October, Spanish material was collected from early July to November, Norwegian material in September, and at the type locality in November. Although *H. pusillum* was observed in Norway and Spain for several consecutive years, the species was not observed at Řeka NR in 2022 and 2023 despite serious effort made during repeated visits.

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