Short Communication

Herbarium Record of *Vulpicida tubulosus* (Lichenized Ascomycota) from Locality Beyond Its Known Distribution Range

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Abstract

A record of *Vulpicida tubulosus* collected in 1985 in northern Poland, based on unpublished herbarium material, extends its geographic range. Based on a recent field study of the locality as well as recent and historical literature, the origin of this previous population is discussed, and notes on the distribution and ecology of the taxon in general are provided. Attempts to obtain molecular data from the specimen have failed, but the presence of contaminating fungi is discussed.

Keywords: lichenized fungi, cetraroid lichen, glacial relict, central Europe, distribution map

Introduction

The genus *Vulpicida* J.-E. Mattsson & M.J. Lai (Parmeliaceae, Ascomycota), described by Mattsson and Lai [1] and characterized in detail by Mattsson [2], comprises six species with reasonably well-known distributions and ecology [1, 3]. Recent reports resulting from a field inventory [4] as well as a revision of herbarium collections [5], however, provide new information on these lichens. This paper presents new data on *V. tubulosus* (Schaer.) J.-E. Mattsson & M.J. Lai. obtained during the revision of a collection stored in the OLTC herbarium of the Department of Mycology, Warmia and Mazury University in Olsztyn, Poland (formerly the Department of Botany, Teachers Training College). *V. tubulosus*, collected in 1985 from the Sources of the Lyna River nature reserve (Źródła rzeki Łyny) in northern Poland was found among other specimens (Fig. 1). The locality, as previously believed, extends the distribution range of the species and adds new information to our knowledge of its distribution pattern.

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Materials and Methods

The unpublished herbarium specimen of *Vulpicida tubulosus* collected in 1985 from Poland was subjected to DNA analysis. The following procedures for DNA isolation were tested: DNeasy Plant Mini Kit (Qiagen No. 69104), modified CTAB method [6], and the same procedure was preceded by an acetone extraction step in order to remove secondary metabolites from the thallus. DNA was re-suspended in 50 μl of sterile distilled water. PCR amplifications were performed using Mastercycler (Eppendorf). 50 μl of PCR reactions were prepared using 5 μl of 10x *Taq* polymerase reaction buffer, 4 μl of $MgCl_2$, 1 unit of *Taq* polymerase (Fermentas), 0.2 mM of each of the four dNTP's, 0.5 μM of each primer and 10-50 ng of DNA.

Amplification of the following markers was attempted: ITS rDNA, LSU rDNA, and mitochondrial SSU rDNA, but only an ITS rDNA fragment suitable for sequencing was obtained. mrSSU1 and mrSSU3R primers were used for mitochondrial SSU rDNA according to Zoller et al. [7] and LR7 [8], LR0R [9], and LR294 [10] primers for semi-nested PCR to amplify LSU rDNA fragment. ITS1, 5.8S and ITS2 regions were amplified with ITS1F [11] and ITS4 primers [12]. The following conditions were used for PCR: initial denaturation at 95ºC for 5 min followed by 35 cycles at 95ºC for 40 s, 54ºC for 45 s, and 72ºC for 1 min, and followed by a final elongation step at 72ºC for 10 min.

The residual oligonucleotides and dNTP's were eliminated from the 20 µl of PCR products using 10 units of Exonuclease I (Fermentas) and 2 units of Shrimp Alkaline Phosphatase (Fermentas). DNA sequencing was performed using Macrogen (Korea) service (www.macrogen.com). For sequencing, ITS1F [11] and ITS4 primers [12] were employed. The newly determined sequences were compared to the sequences available in GenBank using BLASTn search [13] in order to confirm their identity.

The Taxon

Vulpicida tubulosus (Schaer.) J.-E. Mattsson & M.J. Lai

Specimen examined: Poland. Warmia-Masuria: Olsztyn Lake District, Łyna village, Źródła rzeki Łyny Nature Reserve, on soil, 13.VII.1985, leg. K. Rubaj (OLTC L-353).

A detailed description of the species is given by Mattsson [2] and Mattsson and Lai [1]. The collected specimen exhibits morphological characteristics typical of this taxon, particularly for terricolous forms. The specimen has a subfruticose, pale yellow thallus consisting of dense cushions (c. 6 cm^2) of dorsiventral to terete, erect lobes. Apothecia, rarely observed in terricolous specimens, are absent. Pycnidia are marginal or laminal, on projections (stalked) or immersed.

Morphologically, *V. tubulosus* is similar to *V. juniperinus* (L.) J.-E. Mattsson & M.J. Lai, which has thin dorsiventral lobes with thin margins, only marginal pycnidia on projections, and is more or less intensely yellow; it is mainly corticolous (rarely terricolous), and in Europe is almost exclusively restricted to *Juniperus communis*. These two species may, however, occupy similar habitats. Another similar species is *Vulpicida tilesii* (Ach.) J.-E. Mattsson & M.J. Lai, but according to Mattsson [2] it does not occur in Europe (except a few localities in Russia). It is exclusively a terricolous species occurring on exposed, periodically wet calciferous soil. The latter species has lobes not so densely tufted as in *V. tubulosus*, dorsiventral, and usually free from each other. Moreover, pycnidia are sparse, on marginal projections. A traditional identification key for the cetrarioid species recorded in Europe based on morphological, anatomical, and chemical characters is presented in Randlane and Saag [14].

Preliminary molecular studies have showed that morphologically similar and not easily separable *V. juniperinus* and *V. tubulosus* are divided into two clearly distinguished groups in the gene trees. However, these species are mixed in both clades, appearing polyphyletic [15]. In contrast, they form one inter-mixed clade in the species tree, supporting their synonymization [16].

Ecology

Vulpicida tubulosus is a terricolous lichen growing on calcium-rich substrata, but occasionally it is recorded as an epiphyte on the bark of *Juniperus communis* [2]. In Poland it is only known from the Tatra Mountains, where it is exclusively terricolous [17], occuring on mineral soil and on plant remains over limestone and mylonite rocks, in windy places in supramontane to subnival areas [18]. It is a common component of high altitude grassland, rich in postglacial relicts [19-22]. In the Polish part of the Tatra Mts, *V. tubulosus* is mainly found in the mylonitized areas, which are centres of lichen biodiversity of the subnival belt of this region [23]. Here it is recorded at an altitude of 1,650-2,307 m.a.s.l. [23, 24], whereas in the Slovak Republic it is found at 1,400-2,328 m.a.s.l. [18].

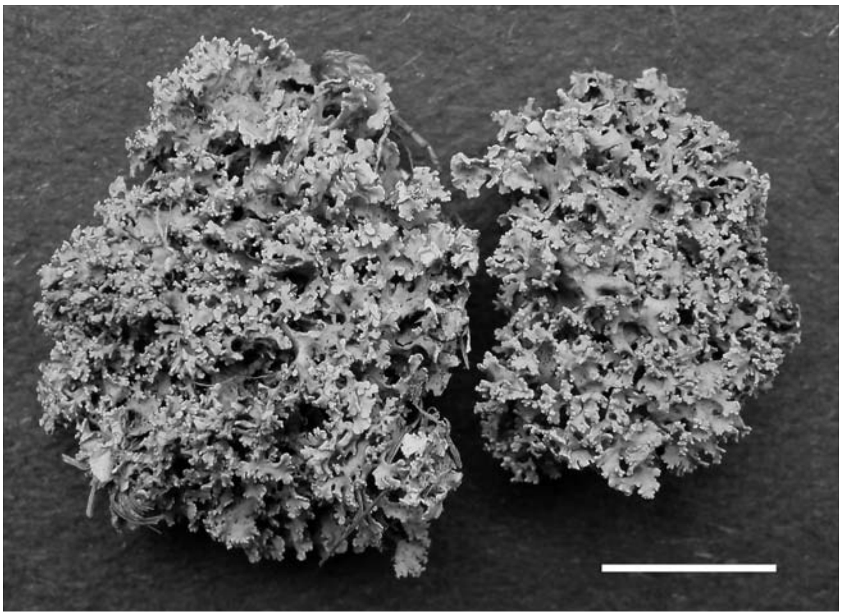

Fig. 1. The specimen of *Vulpicida tubulosus* from the Źródła rzeki Łyny Nature Reserve (OLTC L–353) (scale bar = 1 cm).

In Estonia, the closest to the Polish site under discussion (the northern locality of *V. tubulosus*), it is a component of the *Fulgensietum bracteatae* community, which occurs in treeless habitats formed on thin alvar soil that are also rich in rare lichens such as *Toninia sedifolia* (Scop.) Timdal, *Psora decipiens* (Hedw.) Hoffm., and *Flavocetraria nivalis* (L.) Kärnefelt & A. Thell [25].

The locality of *Vulpicida tubulosus* reported here is situated in northern Poland in the foreland of mesoregion (Pojezierze Olsztyńskie Lakeland), the diversified terrain of which was formed by the Vistulian (Weichselian) Glaciation (Wisconsin Glaciation). The main goal of Źródła rzeki Łyny Nature Reserve, established in 1959, is to protect rare processes of headward erosion. It covers an extensive valley with an area of 121 ha with numerous headwater systems and side ravines. The bottoms of depressions are overgrown with headwater vegetation, and alluvial valleys situated at the lower altitudes are overgrown with riparian communities with a sparse canopy of alder trees. Upland areas are overgrown with pine tree stands, for the most part planted within subcontinental mixed lime-oak-hornbeam forest (*Tilio cordatae-Carpinetum betuli*). The age of those trees is c. 120-140 years. The unique relief of the nature reserve creates a specific microclimate: the large amount of cold spring waters, as well as a topographic depression of the area and its afforestation rate, result in lower temperatures in summer and higher ones in winter, compared with the surrounding area [26].

Geographical Distribution

Vulpicida tubulosus is found only in Europe, where the species has a disjunctive boreal arctic-alpine pattern of distribution (Fig. 2). In northern Europe it has been reported in Norway, Sweden, Finland, Russia, and Estonia, but it is most abundant on the Baltic islands of Sweden and Estonia [3]. To the north, the species reaches a latitude of c. 68°N (Torne, Lappmark) in Sweden [3] and c. 69ºN (Skibotn, Troms) in Norway [4]. To the south, it grows in alpine regions of the central and southern parts of the continent (i.e. Austria, Bosnia and Herzegovina, Czech Republic, France, Germany, Italy, Montenegro, Poland, Romania, Slovenia, Slovakia, Spain, and Switzerland), reaching the Pyrenees in the south [3, 27, 28]. The taxon is red-listed in Estonia and Sweden (in both countries under the category "near threatened") [29, 30] as well as in Finland (under the category "vulnerable") [31].

However, taking into account the preliminary results of Mark et al. [15], the distribution ranges of *V. juniperinus* and *V. tilesii* should also be considered in any further biogeographical studies of *V. tubulosus*. *V. juniperinus* is distributed mainly in northern Europe (Estonia, Finland, Norway, Russia, and Sweden), as well as in isolated populations in subalpine areas of the central and southern parts of the continent; it also is reported from Asia (after [3]). *V. tilesii* is restricted to the arctic and alpine regions of Asia and North America according to Mattsson [2]; however, some specimens from alpine areas in Europe also were previously labelled as *V. tilesii* [15].

V. juniperinus and *V. tubulosus* were suggested to be almost allopatric by Mattsson [2]. However, according to Randlane and Saag [3], the distribution ranges of both species overlap in Scandinavia.

Bielczyk [32] reported the first confirmed record of *V. tubulosus* in Poland. Previously, specimens of this species were described as *Platysma juniperinum* L., *Cetraria juniperina* auct. var. *terrestris* Schaer., and *C. tilesii* Ach. [17]. However, it was also reported by Ohlert [33] from the

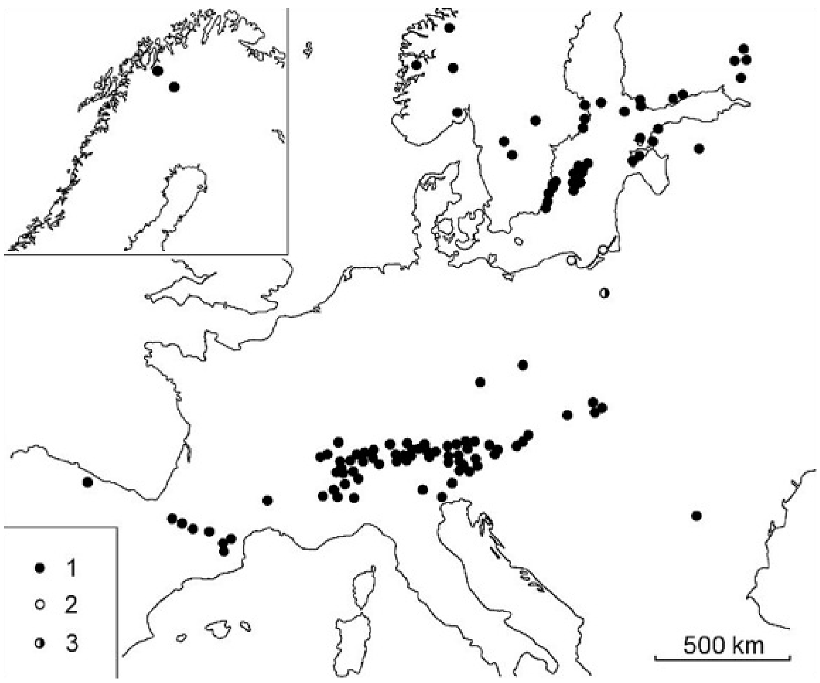

Fig. 2. European distribution of *Vulpicida tubulosus*: 1 – known localities (based on Randlane and Saag [3], Elvebakk and Bjerke [4]; complemented), 2 – historic localities reported by Ohlert [33], 3 – new locality.

surroundings of Rumia in Pomerania (northern Poland), but no herbarium material nor recently collected specimens are available to support this record (Fig. 2).

Molecular Analysis of the Specimen

In order to obtain information on the phylogeography of the species, we also attempted to obtain DNA data from the specimen reported here, but only contaminating fungi were amplified. The BLASTn searches of the sequences obtained in our study showed the highest similarity to mitosporic fungi, i.e. *Hyalodendriella* spp. and *Pleurophoma* spp.

Discussion

The exact location of the collection site of the specimen of *Vulpicida tubulosus* within the reserve is unknown. The herbarium specimen was collected and placed in a single envelope together with terrestrial lichens of the genus *Cladonia*. Since the predominant type of vegetation within the reserve is a dense forest, there are only a few sites suitable for the development of terrestrial lichens. The most likely place where the specimen of *V. tubulosus* could have been collected is a deep gorge in the southern part of the reserve; its steep sandy slopes, especially those with southern exposure, are overgrown by psammophilous and xerothermic plant communities, with a few common lichen species: *Agonimia vouauxii* (B. de Lesd.) M. Brand & Diederich, *Bacidia bagliettoana* (A. Massal. & De Not.) Jatta, *Cladonia coniocraea* (Flörke) Spreng., *C. fimbriata* (L.) Fr., *C. furcata* (Huds.) Schrad., *C. macilenta* Hoffm., *C. rei* Schaer., *Collema limosum* (Ach.) Ach, *Peltigera didactyla* (With.) J.R. Laundon, *P. rufescens* (Weiss) Humb., and *Vezdaea retigera* Poelt & Döbbeler. Exposed sand is covered with abundantly growing *Leptogium teretiusculum* (Wallr.) Arnold, a very rare and critically endangered lichen in Poland [34].

In the past, this area was probably used for extensive grazing. During the last 30 years open areas in the reserve have changed significantly as a result of spontaneous overgrowth by pine forest. In 2008-12 several attempts to find *Vulpicida tubulosus* were undertaken, but to no effect. Overgrowth by a pine forest appears to be the most probable direct cause for the extinction of the species in the reserve. The changes that took place in the natural environment of the area were confirmed by information on the extinction of several rare plants from different ecological groups, e.g.: *Pulmonaria angustifolia* L., *Potentilla alba* L., *Ranunculus polyanthemos* L., and *Petasites spurius* (Retz.) Rchb. [35]. Unfortunately, no studies have been performed that would provide at least an approximate past or present picture of the lichen biota in this area.

The origin of the *V. tubulosus* population in the reserve is uncertain. It is not clear whether the locality is a relic of the glacial period or if the diaspores of the species arrived to the site later in the Holocene. The specific microclimate conditions in the reserve favour the first hypothesis. In such a case, this locality could serve as a specific refugium, like many peatlands in this part of Europe, providing suitable conditions for many boreal species, including those regarded as glacial relicts. However, no information on the presence of other lichen species or plants of a similar character in the reserve is available [35]; furthermore, it should be noted that lichens, similarly to other organisms, reacted to the Pleistocene glaciations as suggested by the few phylogeographic studies available (for a summary see [36]). On the other hand, in the cold water of the Łyna river in the reserve a number of cold-stenothermal invertebrates, typical of the northern mountainous areas, exist [26, 37]. According to some authors [e.g. 38, 39], the main factor responsible for the decline of some terrestial arctic-alpine lichens in Western Europe was global warming.

Considering the origin of the locality of *V. tubulosus* in the Źródła rzeki Łyny Reserve, we should pay attention to the forgotten, historical records of this species reported by Ohlert [33] – the aforementioned locality in northern Poland, and the locality in the area of Svetlogorsk on the Sambia Peninsula (Kaliningrad Oblast, Russia), both of which are located outside the current disjunctive distribution range of the species. The reserve is located 420-520 km from the nearest northern locality of *V. tubulosus* in Sweden and Estonia, and 470 km from the nearest southern locality in the Tatra Mountains. In case of the historical locality in Rumia in northern Poland, the distance is only c. 250 km to the present localities of the species on the Baltic island of Öland, southeastern Sweden (Fig. 2). However, molecular studies provide many examples and evidence of gene flow and migration of lichen species over long distances [e.g. 40, 41]. Moreover, Muñoz et al. [42] showed that lichen propagules can be dispersed via global movements or high-altitude air masses, but it does not need to be in the direction of the prevailing wind [43]. Genetic studies of lichens [e.g. 44-48] suggest that effective dispersal up to several kilometers is possible, although ascospores disperse to longer distances than heavier vegetative propagules. Moreover, dispersal might not be passive, but probably is facilitated by insects and mammals or birds [e.g. 49]. Localities of *Vulpicida tubulosus* in northern Poland and the Kaliningrad region, as for example localities of the species in western Estonia, are located on the autumn migration routes of birds from the arctic regions to the southwestern part of the continent, so the possibility of the species reaching the reserve via long distance dispersal should not be excluded.

The report of Ohlert [43] of *V. tubulosus* is claimed to be unreliable by Fałtynowicz [17]. However, in light of our findings, its occurrence in the Pomeranian area should be considered as very likely. It should be noted that only a limited number of specimens were recorded in the 19th century, which demonstrate decreased vitality; thus, this population was already considered endangered. The situation of *V. tubulosus* resembles that of *Flavocetraria nivalis*, another arctic-alpine species with a similar range in Poland. *F. nivalis* is slightly more frequent in Poland, except in the mountains (Tatra Mts, Babia Góra Mt). It is known from two localities only in the lowland area – in Tuchola Forest and the Vistula Spit [17, 50, 51]. In the past 20 years, however, the lowland population of this species are in significant decline. The populations of *F. nivalis* on the Vistula Spit decreased in this period from 980 to 89 specimens [52].

Unfortunately, we failed to obtain DNA data from *Vulpicida tubulosus*. DNA isolation from lichens may be difficult due to the presence of large amounts of secondary metabolites [e.g. 53], and *V. tubulosus* contains usnic, vulpinic, and pinastric acids that may interfere with DNA. Moreover, DNA extraction from herbarium material that was stored without care for optimal DNA preservation may cause problems and, furthermore, the maximum age of specimens suitable for a standard protocol ranges from several months up to 36 years, depending on the species [54]. According to our knowledge, the specimen of *V. tubulosus* was preserved with para-dichlorobenzene, which could have affected DNA quality. However, in exceptional cases DNA can be extracted from sub-fossil material, such as lichen thalli from glacial ice after c. 1350 years, but the sequences obtained originated from contaminating fungi [55]. In our case, the sequences obtained showed the highest similarity to mitosporic fungi *Hyalodendriella* spp. and *Pleurophoma* spp. that are known to occur as endophytic fungi in plants [e.g. 56, 57]. Recently U'Ren et al. [58] showed an ecologically flexible group of symbionts that occur both as endolichenic fungi and as endophytes of mosses. However, we were unable to identify the exact source of our sequence since our approach was not dedicated to isolation of endolichenic fungi, so we have to treat it as contaminating fungus.

Conclusions

The Źródła rzeki Łyny Reserve seems to be the southernmost lowland locality of the northern-disjunction of *Vulpicida tubulosus*. Although this species probably became extinct in the area, similarly to populations from localities reported in the 19th century, its presence in this locality was confirmed from an existing herbarium specimen, which will hopefully prove valuable for further research on the migration routes of this species in the postglacial period.

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