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Cladonia trapezuntica (Cladoniaceae, lichenized Ascomycota): a robust morphotype of *Pycnothelia papillaria*, a taxonomic study with conservational survey

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Abstract: The long-neglected lichen described as *Cladonia trapezuntica* was rediscovered at its type locality in Turkey thanks to historical maps available at the Natural History Museum in Vienna. Molecular, morphological, and chemical analyses were applied to determine whether *C. trapezuntica* is a distinct species or rather falls within the phenotypic variation of *Pycnothelia papillaria*. Although most of the podetia appear to be unusual for *P. papillaria*, the molecular evidence indicates that *C. trapezuntica* should be considered as a synonym of *P. papillaria*, and the cushion-like growth form, taller podetia with irregular to dichotomously or trichotomously branching pattern, and scarce primary thallus should be recognized as morphological variability. Consequently, the southeastern distribution limit of *P. papillaria* is extended to Turkey. This species has not been found elsewhere in Turkey; therefore, it is considered as critically endangered in the country.

Key words: Giresun, iron oxide, ITS, IUCN, lectotypification, phylogeny, Turkey

1. Introduction

Cladonia trapezuntica J. Steiner was described from northeastern Turkey (Steiner, 1909) and is known only from the type locality. Its specimens were collected in 1907 by the Austrian botanist Handel-Mazzetti on mosses near Eseli village, which was evacuated after World War I; prior to the war, Eseli was a mining village associated with iron production. Today, this village is neither found on current maps nor known by the local population.

After more than 100 years, during fieldwork in 2008, the first author found the lichen at its type locality in an open area near a forest. The specimens are characterized by very dense cushions built up of turgid, branched, and robust podetia. In terms of morphology, the closest genus appears to be *Pycnothelia* Dufour; on the other hand, such large cushions and tall podetia have not been reported in the genus *Pycnothelia*. The weaker specimens without broad cushions of *C. trapezuntica* at the type locality resemble a morph called *P. papillaria* "f. *molariformis*", which is a synonym of *Pycnothelia papillaria* Dufour.

Fresh specimens of *C. trapezuntica* were collected later in 2011 and shown to several colleagues during the IAL7 Symposium in Bangkok in 2012. Although the name *C. trapezuntica* was already synonymized with *Pycnothelia papillaria* (Laundon, 1986; Ahti, 2000), the majority of lichenologists assumed these fresh specimens to represent a species different from *P. papillaria*, due to its growth form. Therefore, the specimens were subjected to detailed examinations in order to determine their taxonomical status.

2. Materials and methods

2.1. Study area, the type locality of *C. trapezuntica*

The type locality "Eseli" is a currently unpopulated place located near Karabörk village of the province of Giresun. Since the local population was evacuated after World War I, it is not included in any recent maps, and for this reason, neither the type locality nor the topotype specimens were recognized prior to this study. This place has been defined on the basis of the historical maps at the Natural History Museum in Vienna, and 2 years later, this locality was discovered during a field study. Today, only remnants of a few houses and a church exist, and 2 houses are inhabited outside of, but near, Eseli. The Eseli mine has been inactive since World War I; however, the rubble that originated from the mine is still deposited in front of the entrance in the form of a huge hill (Figures 1a and 1b). Although the

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Figure 1. The robust morphological form of *Pycnothelia papillaria* (described by J. Steiner (1909) as *Cladonia trapezuntica*). **a–b.** Views on rubble at the type locality of *Cladonia trapezuntica*. **c.** A cushion built up of well-developed, long, and branched podetia (this stage was dominant in the study area). Part of the thallus was removed from the cushion to show its initial structure. Primary thallus has disappeared (only remnants can be seen when visible). **d.** Conidiomata on the tips of podetia. Some broken podetia with hollow central cylinders are visible. **e.** Well-developed cushions (this stage was dominant in the study area). **f.** Early development stage was rarely seen in the locality. Here, the primary thallus and short and globose podetia are visible. **g.** Another early development stage with denser short subglobose podetia (this stage was rarely observed in the study area). **h.** Young thallus; primary thallus is still visible, with prolonged and rarely branched podetia (this stage was rarely observed in the study area).

species epithet refers to the city of Trabzon, this locality is about 100 km away from that town, but it belonged to the sanjak of Trabzon during the Ottoman Empire.

The open, bare soil surface on this rubble and the rocks on it compose the substrate of *C. trapezuntica*. This lichen is not in heavy competition with other lichens, but it is limited by mosses and grassland plants. Therefore, deeper soil patches are overgrown by mosses and grasses, while the shallow soil patches are suitable substrate for the lichen. A few short *Vaccinium* shrubs also occur in this area. The grassland is surrounded by dense and high *Rhododendron* shrubs that inhibit epigeic lichen vegetation. These heathlands merge into forests. Like typical *P. papillaria*, specimens at the Eseli locality grow on poor soils on rocks in open areas near forest.

The climate is predominantly oceanic. The winters are cool (7.1–11.5 °C); summers are fairly warm (15.5–23.3 °C), usually very cloudy, and without a dry season. The precipitation is high (with an annual average of 1265 mm/year) and rather evenly dispersed throughout the year. On the other hand, daily mean minimum and maximum relative humidity values are 67.0% and 78.4% with an annual average of 73.1%, respectively (Turkish State Meteorological Service Office, 2012). Under the Köppen climate classification, the zone belongs to the Cfb category, which generally refers to a region without a dry season and with a hot summer.

2.2. Determination of lichen specimens

Morphological characters were studied on type material of *Cladonia trapezuntica* deposited in the herbaria W and WU in Vienna, and on freshly collected specimens using a CARL ZEISS Stemi 2000-C dissecting microscope.

Secondary metabolites were analyzed by high-performance thin-layer chromatography (HPTLC) in accordance with Arup et al. (1993), using a CAMAG Nanomat 4 sample applicator and a CAMAG horizontal development chamber.

2.3. DNA extraction, PCR amplification, and sequencing

The molecular study was based on fresh material collected from the type locality of *C. trapezuntica* in 2011. DNA was extracted according to Aras and Cansaran (2006). Primers for amplification were ITS1F and ITS4. PCR amplifications for sequence analysis were performed in a 50-µL volume containing 30 ng of genomic DNA, 5 µL of 10X reaction buffer, 2.5 mM $MgCl_2$, 0.4 µL of dNTPs (10 µM), 0.2 µM of each of the primers (25 pmol/mL), and 1 U of Taq polymerase (Fermentas). The thermal cycling for PCR comprised incubation at 94 °C for 3 min and 35 cycles, each with 94 °C for 30 s, 52 °C for 1 min, and extension of 90 s at 72 °C. After the last cycle, the temperature was maintained at 72 °C for 8 min for the final extension step. The amplified PCR products were purified using a Beckman Coulter Genomel Lab DTCS Quick Start Kit according to the manufacturer's instructions. PCR products and DNA markers (100 bp, Fermentas) were analyzed by electrophoresis in 1.2% agarose gel (AppliChem) containing 0.5 µg/mL ethidium bromide for 2 h at 100 V. Sequencing reactions were purified using the Beckman Coulter Agencourt Clean SEQ Kit. PCR products were sequenced with a 3100 Genetic Analyzer (Applied Biosystems HITACHI) by the cycle sequencing method, using a dye terminator cycle sequencing kit (Amersham Pharmacia) according to the manufacturer's protocol.

2.4. Sequence alignment and phylogenetic analyses

Sequence data of the internal transcribed spacer (ITS) of the nuclear rDNA gene cluster obtained from topotype specimens of *C. trapezuntica* were included in the phylogenetic analysis, along with 27 specimens of *Carassea*, *Cladonia*, and *Pycnothelia* species available in GenBank (Table 1), especially those taxa that have recently been shown to be fairly close to the genus *Pycnothelia* (Stenroos et al., 2002) or are believed to be close to *C. trapezuntica*. *Notocladonia cochleata* (Müll. Arg.) S. Hammer was selected as an outgroup species. The alignment of sequences was performed manually, as gaps were few and easily interpreted. Maximum parsimony (MP), neighbor-joining (NJ), minimum evolution (ME), and UPGMA analyses were performed using MEGA 5 software. Bootstrapping was performed based on 1000 replicates with random sequence additions. To test for potential conflict, parsimony bootstrap analyses were performed on each individual dataset, and 75% bootstrap consensus trees were examined for conflict. Homoplasy levels were assessed by calculating the consistency index (CI), retention index (RI), and rescaled consistency (RC) index from each parsimony search. Bayesian analyses were also performed for the most probable reconciliations and to estimate the probability of any reconciliation given the observed gene tree.

2.5. Determination of mineral composition of the substrate

To determine mineral composition of the rubble, rubble samples were crushed in a mortar and then analyzed using X-ray diffractometry (XRD) at the Center for Materials Research of the İzmir Institute of Technology, İzmir, Turkey (Erginal et al., 2009).

2.6. Search strategy and evaluation of the IUCN category

The lichen was first examined around the iron mine in Eseli. Thereafter, 3 other iron mines not far from Eseli were also searched for this lichen in 2008. In 2011, the type locality was visited again to study the ecology of this species as well as to examine the condition of the population in nature, according to IUCN guidelines (2010). After 2011, further potential habitats for the species, i.e. open areas with iron-rich stony rubble at high altitudes in an oceanic climate,

Table 1. Sequences used in the phylogenetic analyses; the newly produced one bolded and the others downloaded from GenBank.

Species	Country, collection no., and herbarium	GenBank accession no.
Carassea connexa	Brazil, Minas Gerais, Stenroos 5024 (TUR)	AF453270
Cladonia bellidiflora	Finland, Stenroos 5112 (TUR)	AF453700
Cladonia corymbescens [1]	Bhutan, Søchting 9206 (H)	AF455239
Cladonia corymbescens [2]	New Caledonia, Dennetière 0045 (TUR)	AF455235
Cladonia diversa	United Kingdom, EDNA09-02387 (E)	FR799160
Cladonia farinacea [1]	Chile, Prov. Magallanes, Feuerer 60101 (TUR)	AF455215
Cladonia farinacea [2]	Canada, Nova Scotia, Ahti 57238 (H)	AF455216
Cladonia furcata	USA, Georgia, Ahti 58283 (TUR)	AF455220
Cladonia multiformis	USA, Nova Scotia, Ahti 57065 (H)	AF455213
Cladonia pocillum	Canada, Manitoba, Normore 1059 (SB)	DQ530205
Cladonia pyxidata [1]	United Kingdom, EDNA09-02403 (E)	FR799170
Cladonia pyxidata [2]	United Kingdom, EDNA09-02409 (E)	FR799154
Cladonia rangiformis [1]	Sweden, Stenroos 5125 (TUR)	AF455172
Cladonia rangiformis [2]	Faeroe Islands, Stenroos 4692 (H)	AF455234
Cladonia robbinsii	USA, North Carolina, Ahti 56696 (H)	AF455167
Cladonia scabriuscula [1]	Canada, Newfoundland, Ahti 56969 (H)	AF455217
Cladonia scabriuscula [2]	China, Koponen et al. 54509 (H)	AF455218
Cladonia scabriuscula [3]	Chile, Feuerer 60212 (TUR)	AF455219
Cladonia signata [1]	Brazil, Stenroos 4955 (TUR)	AF455233
Cladonia signata [2]	Guyana, Stenroos 4876 (TUR)	AF457901
Cladonia subcervicornis	Norway, Tønsberg 26971 (BG)	AF517922
Cladonia subulata	United Kingdom, EDNA09-02367 (RGBE)	FR799174
Cladonia trapezuntica	Turkey, Şenkardeşler 4291 (hb. A. Şenkardeşler)	KC603901
Cladonia turgida	Finland, Jääskeläinen s.n. (TUR)	AF455203
Cladonia wainioi	Canada, Newfoundland, Ahti & Scott 56960 (H)	AF455204
Notocladonia cochleata	New Caledonia, Dennetière 53 (TUR)	AF453267
Pycnothelia papillaria [1]	Canada, Nova Scotia, Ahti 57067 (H)	AF453271
Pycnothelia papillaria [2]	AFTOL-ID 1377	HQ650595

were targeted during the fieldwork of projects in Turkey concerned with other genera, such as *Usnea* Adans. and *Cladonia* P. Browne.

3. Results

3.1. Morphological and chemical characteristics

Pycnothelia papillaria **Dufour**, *Ann. Gén. Sci. Phys.* **8**: 46 (1821).

Syn. ***Cladonia trapezuntica*** **J. Steiner**

Ann. Naturhist. Mus. Wien **23**: 112, figure 1 (1909); type: [Turkey] Asia Minor, districtus (Sandschack) Trapezunti, in ditione vici Eseli prope oppidum Goerele (Elehu) [Görele], supra vicum *Rhododendris*, substrato argillo-cretaceo, alt. ca. 720 m, 20.VII.1907, *Handel-Mazzetti* No. 908 (lectotype, designated here: WU without number!, isolectotype: W1908-06196!).

New topotypes: Giresun: ca. 3 km SE of Aydınlar village, in the unpopulated Eseli village, close to the mine entry, alt. 720 m a.s.l., on soil on siliceous substrate, 9 Aug 2011, *A. Şenkardeşler* 4291 (BP, H, herb. A. Şenkardesler).

The specimens collected from the type locality (Figures 1c–1e) form cushions 30 cm in diameter built up mainly of tall podetia. Primary thallus is scarce and only visible on young specimens with weakly developed and unbranched podetia, crustose, granulose to verruculose, and greenish gray (Figures 1f–1h). Podetia strongly developed, up to 50 mm tall and up to 1 mm thick, turgid, irregularly to dichotomously or trichotomously branched, forming very dense cushions; only weakly developed (or young) thalli with visible primary thallus have short subglobose podetia (Figures 1f–1g); greenish gray, but yellowish in herbarium specimens, sometimes blackened at the base, surface uneven, shiny when wet; axils closed. Podetial wall fragile when dry, 150–180 µm; outer layer corticoid, ca. 20–40 µm thick; outer medulla 35–50 µm, inner medulla 50–70 µm thick. Central axis hollow (Figure 1d). Conidiomata only at tips of podetia (Figure 1d). No ascomata observed.

These specimens have a unique habit with similarities to typical *Pycnothelia papillaria*, but differ in several characters: 1) the cushion-like growth form of the thallus, 2) the rather scarce primary thallus (it is usually continuous in *P. papillaria*), and 3) branched, tall podetia with irregular to dichotomous or trichotomous branching pattern (Table 2).

The secondary chemistry of the material essentially corresponds to that of *P. papillaria*. Our specimens contain atranorin and chloroatranorin. Although secondary metabolites such as protolichesterinic acid, lichesterinic acid, and more rarely squamatic acid, as well as 2 unidentified compounds, have been reported as additional metabolites for *P. papillaria*, none of them have been detected in our freshly collected topotype specimens. Small amounts of the lichesterinic acids are also difficult to identify with TLC alone.

3.2. Molecular studies

According to DNA extraction results, the concentrations of DNA of *C. trapezuntica* ranged approximately from 475.7 to 933.5 ng/µL at 260 nm/280 nm ratios. Purity of DNA ranged between 0.89 and 1.98.

The new sequence of this species was aligned with sequences obtained from GenBank as listed in Table 1. A matrix of 636 unambiguously aligned nucleotide position characters was produced; 337 characters in the alignment were constant, 293 variable but parsimony uninformative, and 198 parsimony informative.

The search resulted in 9 equally parsimonious trees of 180 steps in length (CI = 0.756, RI = 9.932, RC = 0.704). The 9 most parsimonious trees were generated by aligning the sequence data with allied groups. Dendrograms were obtained according to different phylogenetic methods, such as NJ, ME, MP, and UPGMA. The trees yielded similar topology, showing only slight rearrangements within the groups. Since the topologies of the MP, maximum likelihood, and Monte Carlo chains-based Bayesian analysis did not show any strongly supported conflicts, only the tree of ME analysis is shown (Figure 2). The ME analysis produced a single best-scoring tree with a likelihood score of 1550.96588, and the RA × ML search resulted in 1 tree with a final ME optimization likelihood of –1584.557787.

According to the cladogram, *C. trapezuntica* is close to *P. papillaria* with 0.004 distance index, while the genera *Cladia* and *Cladonia* are located on distinct subclades. The *Cladonia trapezuntica* sequence groups relatively weakly with one of *P. papillaria* with 62% bootstrap support. This group is surrounded by the second *P. papillaria* sequence as a sister group in paraphyletic manner and with strong bootstrap support of 100%. This result supports the statement that there is no genetic distinctness between *C. trapezuntica* and *P. papillaria*, at least on the level of ITS sequences. Moreover, *C. trapezuntica* is nested outside the rest of *Cladonia* species, which proves, on the other hand, its genetic distinctness from the rest of the genus. *Notocladonia cochleata*, which was used as an outgroup, was distant from all studied samples with a 0.184–0.232 genetic distance index.

Table 2. Morphological and chemical differences between the robust and typical forms.

	The robust form from Turkey (formerly *C. trapezuntica*)	The typical form
Habit	Cushions from a few cm to 30 cm diameter built up by tall and irregularly branched podetia, with scarce primary thallus	Usually with dominating perennial primary thallus, with short, simple to few irregularly branched podetia
Primary thallus	Occasional, only present in very young specimens, crustose, granulose to verruculose, gray	Persistent, crustose, granulose to verruculose, gray
Podetia	Up to 50 mm tall, to 1 mm thick, greenish gray, only young podetia are subglobose, soon becoming turgid and irregularly branched, forming very dense cushions	Usually 3–7 mm tall, 0.5–1.5(–2) mm thick, gray, erect, tooth-like, at first subglobose, then becoming clavate, turgid, irregularly digitate
Conidiomata	Only at tips of podetia	At tips of young podetia or on primary granules
Chemistry	Atranorin, chloroatranorin	Atranorin, chloroatranorin, ±protolichesterinic acid, ±lichesterinic acid, ±squamatic acid, and 2 unidentified compounds

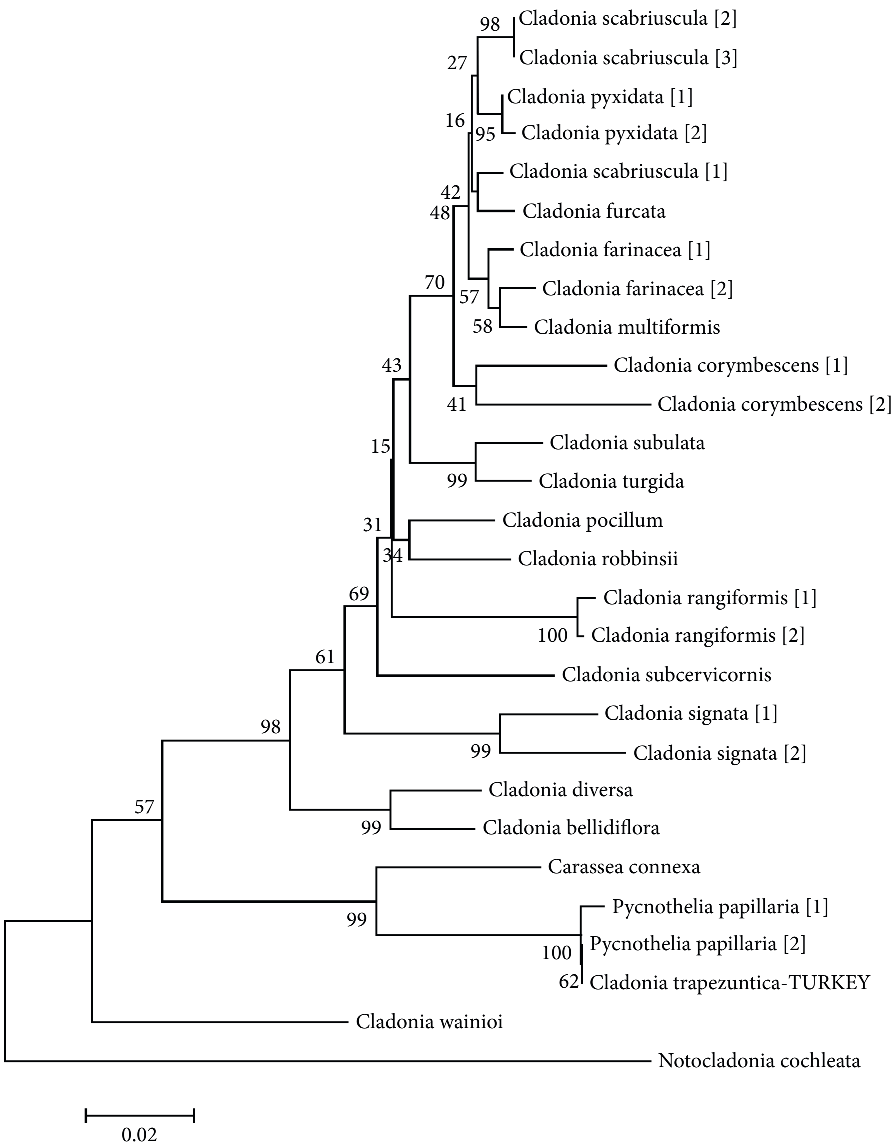

Figure 2. Phylogenetic relations of some *Cladonia* species occurring in Anatolia and other additional *Cladonia*, *Carassea*, *Notocladonia*, and *Pycnothelia* species according to ME analysis.

3.3. Habitat and evaluation of the IUCN category

Typical individuals of *P. papillaria* were not found in Turkey, and the robust morphological form was discovered only at the Eseli locality.

According to the results of XRD, the substrate is richest in iron oxide, followed by calcium phosphate hydroxide, calcium carbonate, and barium aluminum silicate.

The margins of the rubble limit the distribution of the lichen population, which covers an area of less than 0.1 km^2. However, hundreds of individuals occur in the cushions.

Other mine entrances in the region are located inside forests, are occupied by grasses, or have steep slopes suitable for erosion without providing a possibility for growth of any lichen.

Our results clearly show that *P. papillaria* must be considered and managed as a threatened species in Turkey. This lichen appears to be critically endangered (CR) based on several criteria (IUCN, 2010): B1 (extent of occurrence is estimated to be less than 100 km^2, and it is known to exist at only a single location), B2 (area of occupancy is estimated to be less than 10 km^2, and it is known to exist at only a single location), and C2 (at least 90% of mature individuals are in 1 subpopulation) criteria (IUCN, 2010). *Pycnothelia papillaria* is a species that is not easily overlooked; for this reason, we do not expect a wider geographical distribution of this species in Turkey.

4. Discussion

Altogether, more than 750 papers and conference articles concerning lichens collected from Turkey have been published to date (John, 1992, 1995; Çobanoğlu, 2011b; Şenkardeşler, 2011, 2012). The literature data include several MSc and PhD theses on lichen biota of northeastern Turkey (e.g., Cevahir, 1992; Aslan and Öztürk, 1994; Yazıcı, 1995, 1999; Aslan, 2000; Cansaran-Duman and Yurdakulol, 2007) and some other major studies carried out in this part of the country in the current century (Yazıcı and Aslan, 2002; Aslan et al., 2002; Aslan and Yazıcı, 2003, 2006; John and Breuss, 2004; Kınalıoğlu and Engin, 2004; Kınalıoğlu, 2005, 2006; Çobanoğlu, 2011a; Kınalıoğlu and Aptroot, 2011; Osyczka et al,. 2011; Yazıcı et al., 2011). In spite of these detailed field studies, neither *C. trapezuntica* nor *P. papillaria* have ever been recorded in Turkey.

In contrast to the morphological traits, our phylogenetic analysis clearly indicates that *C. trapezuntica* is related to *P. papillaria*. Thus, *C. trapezuntica* is regarded here as a robust morphotype of *P. papillaria*. Moreover, the first development stages of *C. trapezuntica* (Figures 1f–1h) demonstrate a similar habit to that of *P. papillaria*: primary thallus is dominant and podetia are inconspicuous, subglobose, and unbranched. This further indicates that *C. trapezuntica* and *P. papillaria* represent the same species.

Very robust, tall (to 3 cm), slender, and branched morphs do not only occur in Turkey; they are also quite common along the southern fringe of distribution of *P. papillaria* in the United States, e.g., on granitic outcrops in the states of Alabama, South Carolina, and Virginia, as observed by the last author (specimens in H). Since the descriptions of *Pycnothelia* do not indicate such robust morphs, an addendum that shows a larger variability than thought before is now required.

Pycnothelia papillaria has been recognized as an amphi-Atlantic taxon (Galloway, 2008) in the northern hemisphere. However, it also occurs in temperate South America (Ahti, 2000) and has also recently been found in Alaska and on the Asian side of the Bering Strait (Dillman et al., 2012). Since *C. trapezuntica* was synonymized with *P. papillaria*, the southeastern border of the distribution area of the species in Eurasia is now extended to Turkey. Perhaps the robust morphological form occurring at the Eseli locality represents an outpost of *P. papillaria* in Eurasia. This agrees with the well-known fact that in the marginal zone of the distribution area of some species, individuals are exposed to environmental stress, which can influence their metabolism, sexual reproduction, or morphological development, favoring extremities or aberrant individuals (Kärnefelt, 1979).

We classified this species as critically endangered in Turkey. Recently, *P. papillaria* was listed as extinct, critically endangered, endangered, or vulnerable in Austria (outside the Alps) (Türk and Hafellner, 1999), the Czech Republic (Liška et al., 2008), Estonia (Randlane et al., 2008), Latvia (Piterans and Vimba, 1996), Poland (Cieśliński et al., 2006), Slovakia (Pišút et al., 2001), and Wales (Woods, 2010).

The main question on the occurrence of this species in Turkey might be the availability of suitable habitat: it grows only on rubble rich in iron oxide in open areas with an oceanic climate. It seems that the main risk for this species is the removal or destruction of its habitat; the locality in Eseli has not been affected by either for over 100 years, and it is not located near settlements, construction, or agriculture. This place constitutes an important refuge for the lichen in Turkey, and this site should be declared a protected area by the local administration.

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