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Short Communication

A molecular re-evaluation of *Parmelia encryptata* with notes on its distribution

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DNA barcoding using the internal transcribed spacer region (nuITS rDNA) is a widely used method in the identification of fungi (Schoch *et al.* 2012). However, sequence-based identification using databases such as GenBank should be treated with caution, as previously pointed out by Nilsson *et al.* (2006) who reported that *c.* 20% of all fungal sequences deposited there may be incorrectly annotated at species level. This may be especially difficult in the case of genera in which cryptic species have been described, and for which the proportion of mislabelled records may be even higher. Although BLAST searches facilitate quick determination of taxa based on DNA sequences, the recognition of genetically similar species may be challenging (e.g. Lücking *et al.* 2020*a*, *b*; Moncada *et al.* 2020).

Forests are one of the most important ecosystems and provide habitat for more than half of terrestrial biodiversity (Jaroszewicz *et al.* 2019). They are also under increasing threat from direct human activity (e.g. logging, expansion of invasive species) and climate change. Natural forests are vanishing along with often unique and rare species of various groups of associated organisms and the rich biodiversity (e.g. Sylvester *et al.* 2017; Łubek *et al.* 2018). Białowieża Forest is one such forest ecosystem. This large forest complex is one of the best preserved primeval forests in Central-Eastern Europe (Jaroszewicz *et al.* 2019) and is characterized by the rich lichen biota, including a significant number of relict and rare lichens, among them rare and endangered parmelioid taxa and some recently described species (e.g. Cieśliński *et al.* 1996; Motiejūnaitė *et al.* 2004; Guzow-Krzemińska *et al.* 2016, 2017, 2018; Ertz *et al.* 2018*a*, *b*; Łubek *et al.* 2018, 2020). *Parmelia encryptata* A. Crespo *et al.* (*Parmeliaceae*, Ascomycota) was described as a cryptic species found in the Iberian Peninsula and Ireland, morphologically indistinguishable from *P. sulcata* Taylor (Molina *et al.* 2011). This species has not been reported again since it was described; however, during the examination of nuITS rDNA sequences of *Parmelia* species deposited in GenBank, we discovered three sequences that were similar to those of *P. encryptata* but were misidentified as *P. sulcata*. This unexpected discovery encouraged us to re-examine all sequences of the *P. sulcata* group, including the most similar species, *P. barrenoae* Divakar *et al.* and *P. sulcata*. The aim of this paper is to present new records of *P. encryptata*, re-evaluate molecular data and discuss its possible forest relict character.

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Materials and Methods

We obtained new nuITS rDNA sequences from three specimens of *P. sulcata* from Estonia, Italy and Poland and one sample of *P. barrenoae* from Italy, which were subjected to a BLAST search (Altschul *et al.* 1990) in order to check their identity. DNA was extracted and amplified using the protocols described in Ossowska *et al.* (2018, 2019). New sequences have been deposited in GenBank (see Supplementary Material Table S1, available online).

We downloaded all nuITS rDNA sequences of sorediate species of the *P. sulcata* group from GenBank (Supplementary Material Table S1). For the phylogenetic analyses the alignment was reduced to representative sequences of *P. sulcata* originating from all available countries, together with all sequences of *P. barrenoae* and *P. encryptata*; we used a sequence of *P. saxatilis* (L.) Ach. (KU845667) as an outgroup (Ossowska *et al.* 2018). The newly generated sequences and selected representatives of *Parmelia* spp. were automatically aligned using MAFFT (Multiple Alignment using Fast Fourier Transform; Katoh *et al.* 2002) as implemented in UGENE (Okonechnikov *et al.* 2012), followed by elimination of terminal ends. The final alignment consisted of 99 nuITS rDNA sequences and 525 characters.

We used PartitionFinder 2 (Lanfear *et al.* 2016) implemented on the CIPRES Science Gateway (Miller *et al.* 2010) to determine the best substitution model for each partition under the Akaike Information Criterion (AIC) and greedy search algorithm (Lanfear *et al.* 2012). Three different models were found for partitions: SYM + G for ITS1, TRNEF + I for 5.8S and K80 + G for ITS2 regions.

A Bayesian analysis was carried out using the Markov chain Monte Carlo (MCMC) method, in MrBayes v.3.2.6 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) on the CIPRES Web Portal (Miller *et al.* 2010) with the above-mentioned substitution models. Two parallel MCMC runs were performed, each using four independent chains and 10 million generations, sampling every 1000th tree. Posterior Probabilities (PP) were determined by calculating a majority-rule consensus tree after discarding the initial 25% of trees of each chain as burn-in.

A maximum likelihood (ML) analysis was performed using RAxML-HPC2 v.8.2.10 (Stamatakis 2014) with 1000 ML bootstrap iterations (BS) and the GTRGAMMAI model for both analyses. Phylogenetic trees were visualized using FigTree v.1.4.2 (Rambaut 2012) and modified in Inkscape (https://inkscape.org/).

In addition, separate alignments for *P. encryptata* only and sequences belonging to the *P. encryptata* and *P. sulcata* clades were performed and analyzed to define intra- and interspecific variation.

Results and Discussion

The nuITS rDNA matrix consisted of 98 samples representing five *Parmelia* species of the *P. sulcata* group and one sequence of *P. saxatilis* (outgroup) (Supplementary Material Table S1, available online). The *Parmelia sulcata* group also includes sequences of *P. squarrosa* Hale and *P. fertilis* Müll. Arg., although these taxa are morphologically similar to the isidiate species of the *P. saxatilis* group. The phylogenetic position of these species has been discussed, for example in Molina *et al.* (2004, 2017), Divakar *et al.* (2005) and Ossowska *et al.* (2018). The RAxML tree did not contradict the Bayesian tree topology for the strongly supported branches and only the latter is shown with posterior probabilities (PP) and bootstrap support values (BS) (see Supplementary Material Fig. S1, available online); PP ≥ 0.95 and BS ≥ 70 were considered to be significant and are shown above the branches.

The phylogenetic analyses showed that two sequences (MN387037 and MN387038) previously labelled as *P. sulcata* from Białowieża Forest in Poland (Singh *et al.* 2019) clustered with *P. encryptata* in a well-supported clade including the sequence of the type (Supplementary Material Fig. S1). An additional sequence was obtained from a specimen from Switzerland (MN654571), previously identified as *P. sulcata* by Mark *et al.* (2020) but occurring in the same clade as *P. encryptata* (Supplementary Material Fig. S1); however, we have not examined this specimen. Furthermore, the single newly obtained sequence of *P. barrenoae* (UGDA L-26612, MW793515) represents the first record of this species from Italy confirmed by molecular methods. An additional clade formed by three sequences (EU788032, EU788033 and AY036981), all labelled as *P. sulcata* in GenBank but supported outside the *P. sulcata* clade, might represent an additional undescribed cryptic species (labelled as *P.* aff. *sulcata* in Supplementary Material Fig. S1). However, since we did not examine reference material, we refrain from describing a new species. These sequences were also used in a phylogenetic analysis by Molina *et al.* (2011) and defined as clade B2.

Parmelia encryptata is morphologically and chemically identical to *P. sulcata* but genetically different (Molina *et al.* 2011), and despite similarities, these species are not closely related (Molina *et al.* 2017). Both *P. encryptata* and *P. sulcata* have adnate to loosely adnate thalli, lobes that are sublinear with a greyish upper surface with brown tips and laminal and marginal pseudocyphellae, rhizines simple to squarrose, and soralia that are laminal (Molina *et al.* 2011). When examining the specimens of *P. encryptata* from Poland, we found that rhizines were predominantly simple, and squarrose ones appeared only in the central parts of the thalli, whereas in *P. sulcata* they are usually squarrose in all thallus parts. The type of rhizine is an important feature that distinguishes another morphologically similar species, *P. barrenoae*, from *P. sulcata* s. str. (Divakar *et al.* 2005; Barreno & Herrera-Campos 2009; Hodkinson *et al.* 2010). Therefore, the abundance of squarrose in proportion to simple rhizines might be a diagnostic feature which is worth considering in the identification of *P. encryptata*. However, we examined only two samples of the species, which were very young, thus more material needs to be studied to evaluate whether this character is diagnostic. Another feature that separates *P. sulcata* from *P. barrenoae* is the ontogeny and abundance of the soralia (Hodkinson *et al.* 2010; Ossowska & Kukwa 2016); however, in the case of *P. encryptata*, we have not observed any differences in comparison to *P. sulcata*.

Molina *et al.* (2011) provided the following diagnosis of *P. encryptata*: 'Similis *Parmeliis sulcatis* sed differt in intron group I, et sequencis ACATAAGCTCGC in [gene ITS 1] at positions 113–124 in alignment'. They defined four autapomorphic nucleotides in ITS 1 that distinguish *P. encryptata* from *P. sulcata*. However, based on a wider sampling including all sequences of *P. sulcata* and *P. encryptata* available in GenBank, we re-evaluated these data. Of the nucleotides mentioned in the *P. encryptata* description by Molina *et al.* (2011) in position 113 of their alignment (position 112 of sequence AY579456 obtained from the type material), we observed A, T or deletion in *P. encryptata* specimens (Fig. 1A) while in *P. sulcata* nucleotide T or deletion may occur; thus it cannot be used to distinguish these species. Therefore, based on analysis of all available sequences from GenBank, we propose here that six nucleotide positions distinguish *P. encryptata* from *P. sulcata* (Fig. 1B), of which three were previously reported as autapomorphic nucleotides by Molina *et al.* (2011): 118 (equivalent to position 119 in Molina *et al.* (2011)), 120 (equivalent to position 122, which, however, was mislabelled in Molina *et al.* (2011) as according to their fig. 3 it should be position 121) and 123 (equivalent to 124 in Molina *et al.* (2011)). Most of these differences were observed within ITS1, except position 495 which was located in ITS2 (Fig. 1B). The sequences of *P. encryptata* from Poland and Switzerland (MN387038, MN387037 and MN654571) do not differ from the sequence of the type specimen (Fig. 1A), except for a single deletion in MN387037 in position 505; however, both sequences from the Polish specimens contain some missing data at the 3′-ends of the nuITS rDNA sequence.

This study confirms that sequence-based identification using databases such as GenBank should be treated with caution, as previously pointed out by numerous researchers (e.g. Nilsson *et al.* 2006; Lücking *et al.* 2020*a*). Such databases facilitate quick determination of taxa based on DNA sequences, but are of limited use since the identification using a simple blast search of a single marker may bias the recognition of genetically similar species (e.g. Lücking *et al.* 2020*b*; Moncada *et al.* 2020). Furthermore, numerous records are mislabelled in GenBank, as recently reported by Hofstetter *et al.* (2019). This seems to be the case with *P. encryptata*, for which three sequences were incorrectly identified as *P. sulcata* using a blast search by Singh *et al.* (2019) and Mark *et al.* (2020). This probably resulted from comparisons to sequences of *P. encryptata* from specimens used for the original description of *P. encryptata* by Molina *et al.* (2011) which had incorrect names in GenBank (e.g. AY579449, EU788036 and EU788037 were labelled as *P. sulcata*). Nevertheless, the ITS marker can still be used as an efficient barcode in the identification of these two and other *Parmelia* species as it differentiates most species included in the genus (Divakar *et al.* 2016). In all cases, identification using blast searches requires careful examination of the results or should be

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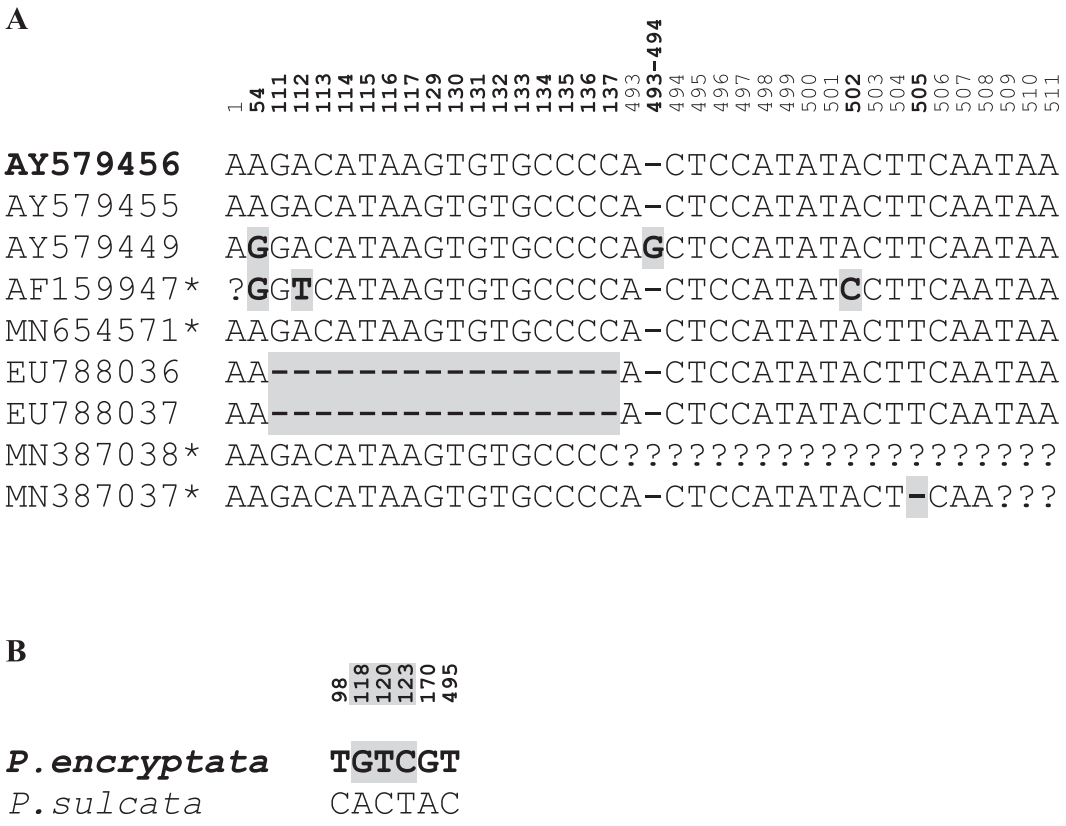

Fig. 1. A, part of the alignment of *Parmelia encryptata* nuITS rDNA showing variable positions (marked in bold and grey) including missing data. Missing nucleotides are marked with '?'. AY579456 originating from the type specimen is treated as the reference and terminal ends of the remaining sequences were trimmed to the length of the reference. Sequences labelled as *P. sulcata* in GenBank and not analyzed in Molina *et al.* (2011) but representing *P. encryptata* are marked with *. B, positions distinguishing *P. encryptata* from *P. sulcata* based on multiple alignment of all available sequences. Positions are numbered according to the reference nuITS rDNA sequence of type *P. encryptata* AY579456. Autapomorphic nucleotides described in Molina *et al.* (2011) and confirmed in this study are marked in grey.

supplemented with phylogenetic analysis, including reference material (e.g. RefSeq or Types), which is more reliable in recognizing the identity of sequenced specimens.

Molina *et al.* (2011) described *P. encryptata* as being characterized by the presence of the group I intron. Unfortunately, this intron sequence is not available from the type material in GenBank since it was removed from the analysis in Molina *et al.* (2011), and the original sequence is trimmed. However, another sequence is available for comparison (DQ084168 originating from specimen MAF-Lich 9902 and representing *P. encryptata*). We analyzed sequences of *P. encryptata* from Białowieża Forest and found that in both specimens, partial sequences of the same intron are identical to sequence DQ084168.

The presence of *P. encryptata* in Poland is an unexpected discovery due to the distance from previously known localities. It has so far been confirmed only from the Białowieża Forest, the large and well-preserved temperate forest complex in Central-Eastern Europe which hosts a rich biodiversity of numerous rare and endangered species (e.g. Cieśliński *et al.* 1996; Motiejūnaitė *et al.* 2004; Łubek *et al.* 2018, 2020). Other locations of *P. encryptata* are also known from large forest complexes such as the Killarney National Park in Ireland, the slope of the Iberian Mountain (Molina *et al.* 2011) and the Swiss Alps (Mark *et al.* 2020); however, we do not know their exact locations and local environmental conditions. Large forest complexes, such as Białowieża Forest, maintain internal microclimatic stability (e.g. humidity and daytime temperature), ensuring the occurrence and continuity of many lichens, including those classified as 'indicators of primeval forests' (Łubek *et al.* 2018). Taking into account the rarity of the species and its occurrence in the areas mentioned above, we hypothesize that *P. encryptata* might be a forest relict species, maintained in isolated populations in large forest ecosystems in Europe. However, it cannot be ruled out that the species requires only sheltered and humid conditions available in such ecosystems and may be found in other forests not necessarily of primeval character. Nevertheless, the species seems to be rare as, of the 44 sequences of *P. sulcata* s. lat. from Poland used in this study (not all of them included in the phylogenetic analyses), eight were from Białowieża Forest, and only two sequences represented *P. encryptata*, both originating from Białowieża Forest (Supplementary Material Fig. S1). The recently described *P. rojoi* A. Crespo *et al.* has also been considered a hypothetically relict lichen but, in contrast to *P. encryptata*, it did not extend its range after Pleistocene glaciations and remained in refugia in Spain (Crespo *et al.* 2020).

In contrast to *P. encryptata*, *P. sulcata* s. str. is widely distributed with a much wider habitat amplitude and grows frequently on roadside trees, rocks, nitrogen-rich habitats and managed forest (Hawksworth *et al.* 2008, 2011; Thell *et al.* 2011; Tsurykau *et al.* 2019). We analyzed over 120 sequences of the *P. sulcata* group (not all of them included in the phylogenetic analyses) from various habitats in Europe and found no additional records of *P. encryptata*, showing that the latter species may be a rare lichen restricted to certain types of habitats.

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The new records of *P. encryptata* suggest that a certain degree of caution and critical evaluation should be exercised during *Parmelia* species identification, as rare species may be accidentally overlooked. *Parmelia encryptata* is a morphologically cryptic species that can only be identified by molecular techniques. This approach is also recommended for other *Parmelia* species (Divakar *et al.* 2016; Corsie *et al.* 2019). Our assumptions about the shape and abundance of the rhizines and the ecological requirements can be a good basis for further detailed research. Nevertheless, at this point there are too few comparative specimens and localities of the species to draw any far-reaching conclusions.

Specimen examined of Parmelia barrenoae. **Italy:** *Valle d'Aosta*: Pila, alt. 2200 m, 45°40′17″N, 7°19′07″E, on *Picea* sp., 22 viii 2019, *R. Szymczyk* s. n. (UGDA L-26612).

Specimens examined of Parmelia encryptata. **Poland:** *Bielska Plain*: Białowieża Primeval Forest, S of Czerlonka, forest section np. 469C, 52°41′16″N, 23°43′02″E, *Tilio-Carpinetum* with old pines, on *Ulmus scabra*, 2016, *M. Kukwa* 17942 & *A. Łubek* (UGDA L-24994); *ibid*., Białowieski National Park, forest section no. 256, *Tilio-Carpinteum*, on *Carpinus betulus*, 2015, *M. Kukwa* 17163 & *A. Łubek* (UGDA L-25048).

Specimens examined of Parmelia sulcata. **Estonia:** *Saare County*: Muhu Island, Nõmmküla alvar, 58°40′03.90″N, 23°12′21.38″E, saxicolous, 2019, *M. Kukwa* 20482 (UGDA).—**Italy:** *Valle d' Aosta*: Courmayeur, 45°48′58″N, 6°57′25″E, alt. 1300 m, roadside trees, on *Fraxinus* sp., 21 ix 2019, *R. Szymczyk* s. n. (UGDA L-26630).—**Poland:** *Western Bieszczady*: Czarne forest division, forest section no. 123 g, 49°18′50″N, 22°14′18″E, alt. 654 m, saxicolous, 9 x 2019, *R. Szymczyk* s. n. (UGDA L-32558).

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