

Preliminary investigation of the simple thalloid genus *Aneura* (Marchantiophyta) in Bulgaria

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Received: November 15, 2024 ▷ Accepted: December 10, 2024

Abstract. *Aneura pinguis* (Marchantiophyta) has extensively been investigated in Europe. It has been shown that under that name, a large complex of cryptic species exists, which for now, are only identified based on genetic markers. Here, we report the results of a preliminary investigation of the genetic composition of *Aneura pinguis* in Bulgaria. Six cryptic species have been genetically identified as occurring in Bulgaria and more are expected to be found.

Key words: *Aneura pinguis*, barcoding, cryptic species, liverworts

Citation: Gospodinov, G. & Natcheva, R. 2024. Preliminary investigation of the simple thalloid genus *Aneura* (Marchantiophyta) in Bulgaria. – Phytologia Balcanica, 30(3): 339-344. – ISSN 1310-7771 (print), 1314-0027 (online).

Introduction

Liverworts are generally divided into two main groups: complex thalloid liverworts (Marchantiopsida), which produce differentiated tissues, and simple thalloid and leafy liverworts (Jungermanniopsida), which do not produce differentiated tissues. Genus *Aneura* Dumort. belongs to the simple thalloid order Metzgeriales, family Aneuraceae (Schuster 1984). Within that order, Aneuraceae is the most species rich family, with over 300 taxa listed in TROPICOS (Preußing & al. 2010). Being anatomically and morphologically simple, the species among the Aneuraceae exhibit overlapping morphological traits, which makes the proper species delimitation questionable.

In the last twenty years, the taxonomic arrangement and geographic distribution of the genus *Aneura* have been discussed in a growing number of studies (Bączkiewicz & Buczkowska 2005; Buczkowska & Bączkiewicz 2006; Wickett & Goffinet 2008; Sérgio & Garcia 2009; Preußing & al. 2010; Bączkiewicz & Buczkowska 2016; Bączkiewicz & al. 2017; Myszczyński & al. 2017; Anantaprayoon & al. 2023; Long & al. 2023; Söderström & al. 2023) based on different datasets, such as morphology, genetic markers and ecology. Despite the relatively extensive research on the genus, a conclusive and well-resolved taxonomic treatment has not been established. Only three accepted taxa occur in Europe: *Aneura pinguis* (L.) Dumort., *A. mirabilis* (Malmb.) Wickett & Goffinet, and *A. latissima*

Spruce (Gradstein 2013; Long & al. 2023). The tropical *Aneura maxima* (Schiffn.) Steph. has been excluded as occurring in Europe (Forrest 2015; Paton 2022; Long & al. 2023).

Despite being taxonomically poor in Europe, *Aneura* shows remarkable genetic diversity and habitat versatility, growing at altitudes from the sea level, to the high-mountain bogs. Based on isozyme (Bączkiewicz & Buczkowska 2005) and DNA sequence data (Bączkiewicz & Buczkowska 2016; Bączkiewicz & al. 2017; Myszczyński & al. 2017), it has been shown that *Aneura pinguis* s.l. is a genetically heterogenic group of cryptic species, provisionally named from A to J. Due to the lack of conclusive morphological characters, a formal taxonomic rank has not been assigned to any of them.

In this work, we present results from the research on the genetic composition of Bulgarian populations of *Aneura* s.l. This is also the first attempt for a detailed genetic investigation of a Bulgarian liverwort species.

Materials and methods

Thirty-two samples were collected from various sites across Bulgaria. The fresh samples were fixed in silica gel, until further processing. ITS1-5.8S-ITS2 regions were sequenced, following Bączkiewicz (2017). Specimens were deposited in SOM and the access to the sequences is available at Barcode of Life Data (BOLD) Systems v4 - <https://v4.boldsystems.org/index.php> (Table. 1). The obtained chromatograms of the DNA sequences were analyzed and edited with MEGA 11.0.13 (Tamura & al. 2021), and consensus sequences were generated with PhyDe v0.9971 (Müller & al. 2010). Alignment was generated using the algorithm of MUSCLE (Edgar 2004), with *Lobati-riccardia lobata* (Schiffn.) Furuki (PGBMA3877-20) as an outgroup, obtained from the BOLDSYSTEMS database (<https://boldsystems.org>), and the available at NCBI (<https://www.ncbi.nlm.nih.gov>) sequences of the Polish genotypes (KY705484, KY705512, KY705469, KY705411, KY705524, KY705527, KY705509, KY705505, KY705506, KY705500,

KY705465, KY705452, KY705436, KY705486). The final sequences were with total length of 725 nucleotides. A Maximum Likelihood Tree (Fig. 1) was generated using IQ-TREE multicore version 2.0.7 (Minh & al. 2020) for Linux 64-bit, with bootstrap analysis of 1000 pseudoreplicates and a GTR + G model. The generated tree was visualized and edited with FigTree Version 1.4.4.

Results and discussion

Nine haplotypes were identified in the Bulgarian populations of *A. pinguis* s.l. (Fig. 1). Of these, six have been grouped with the already known haplotypes in Europe (Bączkiewicz & al. 2017). The most numerous clade is the one of “haplotype F”, which is considered to be *A. pinguis* s.str. and is the most widespread in Europe (Long & al. 2023). The clade named “*Aneura* sp. 1” grouped the samples, which were identified as *A. maxima* in Bączkiewicz & al. (2017) but were later revised in the work of Söderström & al. (2023) as a complex undescribed group of species, where also groups *A. latissima* (Long & al. 2023). It is worth mentioning that coexistence of more than one haplotype seems possible, since *Aneura* grows abundantly in various habitats, displaying pioneer behavior. Some of the populations were comprised of thalli with very well-developed rhizoids, while others, like those growing among *Sphagnum* or other mosses, seemed not to produce any, or just a few. It is not clear, if this is in response to the environment, or it is a species specific trait, and extensive cultivation experiments are needed to clarify the morphological variation in different conditions.

Conclusion

Aneura pinguis s.str. is here confirmed as occurring in Bulgaria, based on the nuclear barcode markers ITS1-5.8S-ITS2. Six cryptic species are found, belonging to already described genetic cryptic species in Europe. The presence of the formerly reported as

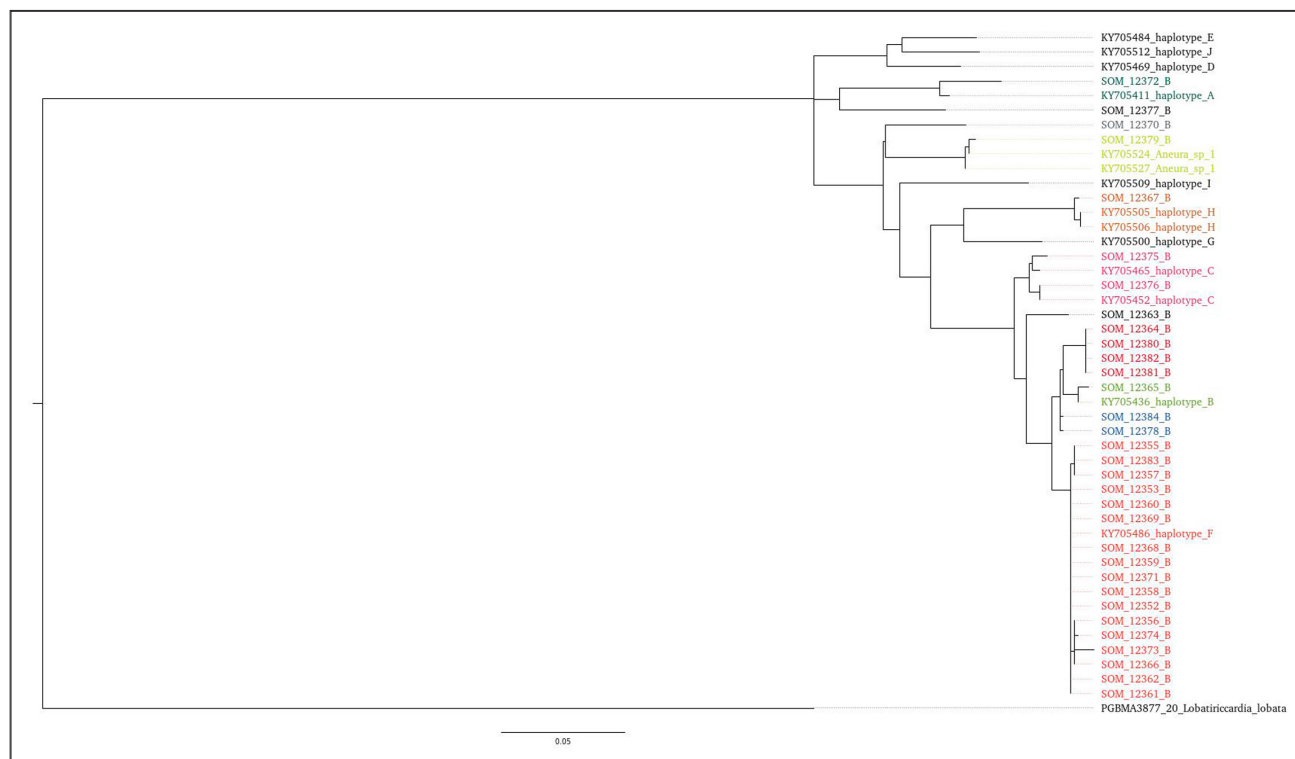
Table 1. Herbarium numbers and BOLD Database numbers with geographic coordinates, and elevation.

Herbarium number	BOLD database number	Latitude	Longitude	Elevation
12352	SOM 12352-B	42.534779	23.579602	923.16
12353	SOM 12353-B	42.534779	23.579602	923.16
12355	SOM 12355-B	42.552345	23.59608	852
12356	SOM 12356-B	42.552345	23.59608	852
12357	SOM 12357-B	42.548314	23.60344	819.27
12358	SOM 12358-B	42.548314	23.60344	819.27
12359	SOM 12359-B	42.530668	23.582248	933.56
12360	SOM 12360-B	42.530815	23.580977	935.18
12361	SOM 12361-B	42.52908	23.577648	939.88
12362	SOM 12362-B	42.525923	23.58404	937.21
12363	SOM 12363-B	43.222679	23.377412	303
12364	SOM 12364-B	42.252442	23.446521	1201.4
12365	SOM 12365-B	41.772487	23.54997	1257.31
12366	SOM 12366-B	41.861812	23.395402	1012.44
12367	SOM 12367-B	41.877091	23.359793	1103.3
12368	SOM 12368-B	42.59046	23.48362	930
12369	SOM 12369-B	41.592755	24.439293	1237
12370	SOM 12370-B	41.591332	24.442228	1320
12371	SOM 12371-B	41.59219	24.44117	1250
12372	SOM 12372-B	41.590927	24.442009	1332
12373	SOM 12373-B	41.59067	24.43982	1370
12374	SOM 12374-B	42.08394	23.610507	1992
12375	SOM 12375-B	42.101696	23.590628	2186.57
12376	SOM 12376-B	42.564603	23.593948	880.97
12377	SOM 12377-B	42.989741	23.100325	727.24
12378	SOM 12378-B	42.62375	22.462809	1591.67
12379	SOM 12379-B	42.227018	23.374725	1504.66
12380	SOM 12380-B	42.229814	23.376333	1437.87
12381	SOM 12381-B	42.230674	23.376662	1434.38
12382	SOM 12382-B	42.229814	23.376333	1437.87
12383	SOM 12383-B	41.85997	23.393398	1022.05
12384	SOM 12384-B	42.218997	23.321199	2103

A. maxima genotype is also confirmed (Ellis & al. 2021). Three more clades have been identified from single samples as new clades, as compared to the already established ones. Our results indicate considerable haplotype diversity within the *Aneura pinguis* complex. Since our sample number is relatively lim-

ited, a larger number of genotypes may be expected to be found in Bulgaria, both with broader occurrence in Europe as well as local haplotypes. Discriminative morphological or ecological features cannot be established for proper species delimitation, the different genotypes can be identified only by genetic markers.

Fig. 1. A Maximum Likelihood Tree showing the arrangement of the Bulgarian *Aneura* samples in relation to the already described cryptic species.



Acknowledgements. This work was supported by the Bulgarian National Science Fund, project “Cryptic species in Bulgarian flora - molecular species delimitation in the *Aneura pinguis* complex” (KП-06-H21/15 19.12.2018).

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