**On the cover:**
Some rare and newly discovered *Carex* taxa from Croatia, are reported by Koompan et al. (pp. 35-47)

# ACTA BOTANICA CROATICA

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# ACTA BOTANICA CROATICA

The journal originally entitled *Acta Botanica Instituti Botanici Regalis Universitatis Zagrebensis* was founded in 1925. In 1957 its name was changed to Acta Botanica Croatica. In 1998, it became an entirely English-language journal.

The journal covers field (terrestrial and aquatic) and experimental research on plants and algae; including plant viruses and bacteria; from the subcellular level to ecosystems. Manuscripts focusing upon the lowland and karstic areas of southern Europe, karstic waters, other types of fresh water, and the Adriatic (Mediterranean) Sea are particularly welcome. More detailed information is available on the link

**http://www.abc.botanic.hr/index.php/abc/about**

The following points make Acta Botanica Croatica an attractive publishing medium: 1) article submission and publishing are free of charge, 2) manuscripts subject to international review, 3) covered by major abstracting and indexing services.

Impact Factor calculated by Journal Citation Reports:
0.7 (2024)
5-year Impact Factor: 1.2

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Editorial

# The Celebration of the first Century of *Acta Botanica Croatica* (1925–2025)

It was a privilege for the editorial team to organize the celebration of the Acta Botanica Croatica centenary last year. The Celebration was organized through a series of events organised by the Editorial Board during the autumn of 2025. Preparations for marking the journal's 100th anniversary were initiated at the annual meeting of the Editorial Board on 19 November 2024. On that occasion, an Honorary Committee was appointed, comprising Mirko Planinić (Dean of the Faculty of Science), Sandra Radić Brkanac (Head of the Department of Biology), and former Editors-in-Chief Ljudevit Ilijanić, Damir Viličić, and Branka Salopek Sondi. An Organising Committee was also established, chaired by Zrinka Ljubešić and including Mirta Tkalec, Nenad Jasprica, Sunčica Bosak, Mateja Jagić, and Sandra Vitko. This committee was responsible for designing and coordinating the programme of anniversary activities scheduled for autumn 2025 (Jasprica et al. 2025).

The jubilee was first highlighted within the programme of the 8th Croatian Botanical Symposium, held in Osijek from 3 to 6 September 2025. A poster entitled *Centennial celebration of the journal Acta Botanica Croatica* (Tkalec et al. 2025) was presented in the poster session. In addition, as part of the Evening Programme *Promotion of Croatian scientific journals*, Nenad Jasprica delivered a presentation in Croatian focusing on the journal's hundred-year history and achievements.

The main celebration took place on 25 September 2025 at the Faculty of Science, University of Zagreb. The event attracted considerable public attention and was preceded by a live segment broadcast on Croatian National Television (HTV). On the day of the ceremony, an HTV crew led by journalist Krešimir Volarević visited the Faculty and conducted interviews with Nenad Jasprica and former Editor-in-Chief Branka Salopek Sondi.

Approximately sixty guests attended the central celebration, which consisted of a formal ceremony followed by a panel discussion (Fig. 1). The programme was hosted by the Editors-in-Chief, Mirta Tkalec and Nenad Jasprica. In his opening address, Nenad Jasprica welcomed the participants and acknowledged the presence of distinguished guests, including Sandra Radić Brkanac (Head of the Department of Biology), Ivančica Ternjej (Dean of the Faculty of Science), Tomislav Josip Mlinarić (Vice-Rector for Innovation, Technology Transfer and Cooperation with the Economic Sector), representatives of the Croatian Academy of Sciences and Arts – Academicians Mirko Orlić, Goran Durn, and

**Fig. 1.** The central celebration at the Faculty of Science, University of Zagreb, led by the Editors-in-Chief, Nenad Jasprica and Mirta Tkalec (A), followed by a panel discussion (B), with active participation of the Editors-in-Chief of European botanical journals, from left to right: Gianmaria Bonari (Italy), Zoltán Botta-Dukát (Hungary), Urban Šilc (Slovenia), Marko Sabovljević (Serbia/Slovakia), and Nenad Jasprica (Croatia).

Nikola Ljubešić – and Amalija Babić, Head of the Department for the Scientific System and Technological Development at the Ministry of Science, Education and Youth. Following welcoming remarks by Sandra Radić Brkanac and Dean Ivančica Ternjej, Nenad Jasprica delivered a concise presentation outlining the historical development of *Acta Botanica Croatica* and its contribution to botanical research at both national and international levels. During the ceremony, the jubilee issue *Acta Botanica Croatica* 84(2) 2025 was officially presented. This special issue, comprising 168 pages, includes four review articles, eight original research papers, and two short communications, and opens with a Foreword by the Editors-in-Chief.

In recognition of the journal's centenary, the Editorial and Advisory Boards decided to honour individuals, institutions, and organisations that have made outstanding contributions to the journal and to botanical scientific publishing. Certificates of Appreciation were awarded to former Editors-in-Chief Ljudevit Ilijanić, Damir Viličić, Branka Salopek, and Nenad Jasprica for their leadership and long-term commitment. The certificate for Ljudevit Ilijanić was accepted on his behalf by his son-in-law, Stanko Uršić. Additional certificates recognising editorial dedication were presented to former Technical Editors-in-Chief Mirta Tkalec and Nataša Bauer, current Technical Editor-in-Chief Petra Peharec Štefanić, Quality Assurance Editor Željka Vidaković-Cifrek, and long-time language editor Graham McMaster. On behalf of the awardees, Branka Salopek addressed the audience and expressed their collective gratitude. Acknowledgement was also given to the companies Denona (Zagreb) and Laser Plus (Zagreb) for their contribution to botanical publishing. Special recognition was reserved for three institutions whose sustained financial and institutional support has been essential for maintaining the journal's continuity and quality: the University of Zagreb, the Croatian Academy of Sciences and Arts, and the Ministry of Science, Education and Youth of the Republic of Croatia. Representatives of these institutions accepted the awards and briefly addressed the audience.

The event was followed by the Panel discussion entitled "The role of scientific journals in the development of botany and strategies for enhancing their visibility and influence", moderated by Aleksandra Uhernik Đurđek, a certified incentive specialist and certified Croatian meetings and events professional. The panelist were editors-in-chief of several European botanical journals: Zoltán Botta-Dukát (*Acta Botanica Hungarica*, Hungary), Gianmaria Bonari (*Vegetation Ecology and Diversity*, Italy), Urban Šilc (*Hacquetia*, Slovenia), Marko Sabovljević (*Comprehensive Plant Biology*, Serbia), and Nenad Jasprica (*Acta Botanica Croatica*, Croatia). The panel focused on shared editorial experiences, current challenges in scholarly publishing, and strategies for increasing journal visibility and impact. Topics included reviewer availability, manuscript quality, competition with large commercial publishers, and the role of impact metrics, alongside potential solutions such as thematic issues, stronger regional collaboration, and maintaining a clear journal identity. The programme concluded with a reception held in the Faculty atrium. We would like to thank the Croatian Academy of Sciences and Arts for financially supporting the celebration.

Commemorating the 150th anniversary of the Division of Botany, Department of Biology, Faculty of Science, University of Zagreb at an event held on 24 October 2025 in the Exhibition Pavilion of the Botanical Garden, Mirta Tkalec presented a brief overview of the journal's history and anniversary activities.

At last but not least, in honour of the 100th anniversary of our journal, the Departments of Natural and Medical Sciences, together with the Faculty of Science of the University of Zagreb, organised a roundtable on the importance of Croatian scientific journals and their validation and recog-

**Fig. 2.** A roundtable on the importance of Croatian scientific journals and their validation and recognition in the national criteria for academic promotion held in the Croatian Academy of Sciences and Arts Library in Zagreb (A), moderated by academicians Vida Demarin and Goran Durn (B).

nition in the national criteria for academic promotion (Fig. 2). The event was held on November 6, 2025, in the Croatian Academy of Sciences and Arts Library in Zagreb (Ljubešić et al. in press).

Founded in 1925 by Professor Vale Vouk, Acta Botanica Croatica is among the longest-standing botanical journals in this part of Europe. Over the course of a full century, the journal has evolved alongside the discipline of botany itself, documenting scientific progress while actively contributing to it. Within its rich history, it has been particularly influential in advancing knowledge of the flora and vegetation of karstic and dry environments, with a strong focus on southern Europe. At the same time, it has continuously expanded its thematic scope, providing a forum for research in plant ecology, systematics, physiology, and, more recently, molecular biology. The journal's sustained relevance can be attributed to its commitment to high peer-review standards and its readiness to adopt innovations in scientific publishing. The celebration of the first Century of Acta Botanica Croatica served not just as an honour to history, but as a strategic look toward the future.

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Research Article

# Molecular and morpho-physiological analyses revealed inter- and intra-generic diversity of filamentous cyanobacteria from saline/alkaline soils

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**Abstract** – Cyanobacteria play important roles in soil fertility and soil productivity. Diverse filamentous cyanobacteria due to their ability to add nitrogen and organic carbon to soil and to maintain the physical properties of soil are undoubtedly one of the important groups of cyanobacteria. The present study aimed to understand the diversity of filamentous cyanobacteria (both heterocystous and non-heterocystous) obtained from saline/alkaline soils of Uttar Pradesh, India. Fuzzy cluster analyses of 47 filamentous cyanobacteria based on their cellular dimensions showed high inter-generic diversity among heterocystous cyanobacteria while in non-heterocystous cyanobacteria both inter- and intra-generic diversity was observed. The results of the present study indicated that the dimensions of vegetative cells and heterocysts can be useful for the identification and differentiation of cyanobacteria belonging to *Hapalosiphon* and *Lyngbya*. Physiological characterization also revealed a great deal of variation (5-15%) in salinity tolerance. Multiplex HIP fingerprinting revealed a higher genetic diversity in heterocystous (12-76% similarity) than in non-heterocystous cyanobacteria (13-90% similarity). Heterocystous cyanobacteria like *Aulosira laxa* O. Kirchner ex Bornet & Flahault, *Hapalosiphon* sp. and *Nostoc* sp. showed high intra-generic variability while among the non-heterocystous ones, *Lyngbya* was found to be genetically highly heterogeneous. The results of the present study highlight the importance of morpho-physiological and genetic analyses in deciphering the diversity of filamentous cyanobacteria for the understanding of their population structure, ecology and adaptations.

**Keywords:** cell size, cyanobacteria, diversity, fuzzy clustering, Highly Iterated Palindrome (HIP) fingerprinting

## Introduction

Cyanobacteria or blue green algae (BGA) are a group of oxygenic photoautotrophic prokaryotes that were responsible for the first significant increase in atmospheric oxygen (Gould et al. 2008). These diazotrophic photosynthetic microorganisms are distributed in diverse ecological habitats ranging from hot to cold springs, marine to fresh water, pristine to degraded soils, oligotrophic to hypereutrophic environments (Bhatnagar and Bhatnagar 2005, Bagul et al. 2018). Cyanobacteria are a morphologically diverse group comprising both unicellular and colonial (including filamentous) forms. Cyanobacteria are not only abundant in the soil, but also play important roles in soil fertility and soil productivity (Rossi et al. 2017). Filamentous cyanobacteria, due to their ability to add nitrogen and organic carbon to soil and to improve soil physical properties, are undoubtedly one of the important groups of cyanobacteria. Due to their filamentous nature and abundant production of polysaccharides in many species, they can significantly help in soil aggregation (Chakdar et al. 2012). It is well known that filamentous cyanobacteria are associated with biological soil crusts (BSC) the origin of which is due to entrapment of minerals and sand particles by cyanobacterial filaments (Garcia-Pichel et al. 2001). These BSCs are ecologically very important in semi-arid to arid areas as they serve as microhabitats (Zhang 2005). Filamentous cyanobacteria have also been evaluated for their potential role in salinity stress amelioration (Li et al. 2019).

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Despite their ecological significance and application for the amelioration of degraded soils, studies on the diversity of cyanobacteria from saline/alkaline/sodic soils are scanty in India. To our understanding, lack of detailed diversity studies from different habitats, routine sub-culturing based isolation and morphometry based techniques leading to the identification of a few predominant genera are some of the reasons that most of the application-based studies are centred around a few genera like *Anabaena* or *Nostoc*. In India, the area under salt-affected soils is about 6.73 million (m) ha with the states of Gujarat (2.23 m ha), Uttar Pradesh (1.37 m ha), Maharashtra (0.61 m ha), West Bengal (0.44 m ha) and Rajasthan (0.38 m ha) together accounting for almost 75% of saline and sodic soils in the country (Sharma and Singh 2015). Understanding of the genetic as well as the functional variability of the cyanobacterial population of saline sodic soils can help to establish a very broad base of genetic resources for their further exploitation. Diversity of cyanobacteria is generally studied in terms of the variabilities in cellular features (e.g. biometric characteristics of vegetative cells, heterocysts, hormogonia and akinetes; branching pattern, presence or absence of sheaths etc.) or by the use of molecular techniques. Undoubtedly, molecular analyses of diversity are more accurate and trustworthy, as morphological features are prone to environmental fluctuations (Doers and Parker 1988, Kato 1991, Chakdar and Pabbi 2012). A number of molecular tools PCR-RFLP, RAPD, finger printing based on repetitive elements have been used to study inter- and intra-generic molecular diversity of cyanobacteria (Mazel et al. 1990, Neilan et al. 2003, Prabina et al. 2005, Ezhilarasi and Anand 2010, Akoijam and Singh 2011, Chakdar and Pabbi 2012, Chakdar and Pabbi 2017).

Highly Iterated Palindrome (HIP1), a repetitive eight base sequence (5'-GCGATCGC-3'), was first identified in a cadmium-tolerant strain of *Synechococcus* PCC 6301 at the borders of a gene deletion (Gupta et al. 1993). It has been speculated that there are no comparable sequences to HIP1 in other organisms, that they are only known to occur in cyanobacteria and can also be used to fingerprint organisms (Smith et al. 1998). These repetitive sequences have been used to study several cyanobacterial taxa (Lyra et al. 2005, Prasanna et al. 2006, Selvakumar and Gopalaswamy 2008, Chakdar and Pabbi 2012, Singh et al. 2014, Shokraei et al. 2019). The conserved nature along with genome-wide distribution of the repeats and reproducibility has made them ideal tools for biodiversity studies (Selvakumar and Gopalaswamy 2008).

Looking at the insufficient information available about the diversity of filamentous cyanobacteria from saline sodic soils of India, in the present study we analyzed the diversity of 47 filamentous cyanobacteria isolated from saline/alkaline soils of eastern Uttar Pradesh, India. Here we have used HIP fingerprinting as well as fuzzy clustering based on cellular features and empirically compared the techniques to determine whether they can really be correlated.

## Material and methods

### Procurement and maintenance of cultures

Forty-seven strains of cyanobacteria isolated from saline/alkaline habitats of eastern part of Uttar Pradesh, India were procured from the National Agriculturally Important Microbial Culture Collection (NAIMCC), ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), India (On-line Suppl. Tab. 1). Cultures were maintained in chemically defined BG-11 media with and without nitrogen source for non-heterocystous and heterocystous cyanobacterial isolates respectively at 28 ± 2 °C under a light intensity of 52–55 µmol photon $m^{-2}$ $s^{-1}$ and L:D cycles of 16:8 h (Stanier et al. 1971).

### Morphometric characterization of cyanobacterial isolates

Freshly raised cyanobacterial strains were viewed under 400× and 1000× magnification under a light microscope (Olympus, Japan) (On-line Suppl. Fig. 1) to check the purity and morphological identity according to the keys provided by Desikachary (1959). The length and width of vegetative cells and heterocysts were determined by analysing the captured images using Prog Res Capture Pro 2.6 software (JENOPTIK, Germany) under 400× magnification using a fluorescent microscope (Olympus BX41, Japan). For each cyanobacteria, five vegetative cells and heterocysts (only for heterocystous cyanobacteria) of different filaments were measured. The mean values for length and width of vegetative cells and heterocysts were used for clustering.

### Physiological characterization of isolates for tolerance to salinity and alkalinity

The ability of the cyanobacterial isolates to tolerate alkalinity and salinity were checked by growing them on BG-11 $N^+/N^-$ agar with varying pH (8, 9 and 10) and salinity (5, 10 and 15% (w/v) NaCl concentrations. One mL of freshly grown cyanobacterial culture suspension was taken in sterile microcentrifuge tube (2 mL) and centrifuged at 10000 rpm for 10 min. Pellets were washed with autoclaved distilled water for 2-3 times. Sterile glass beads were added to the culture tube containing cell pellets and 1 mL of autoclaved distilled water was added to it. Cells were vortexed for 30 seconds to homogenise the filamentous cells into suspension. 40 µL of this suspension was spotted on BG-11 medium agar plates of different pH and salt concentrations. The culture plate was incubated for 10 days under conditions as described in the section Procurement and maintenance of cultures. After incubation, plates were observed for growth and results were recorded. Presence of growth was recorded as "1" and absence was recorded as "0". This binary data was further used for clustering following the procedures detailed in sub-section Genetic diversity analyses in the section Statistical analyses.

### Genomic DNA extraction

Genomic DNA was extracted from 1 mL (50–60 mg fresh biomass) of exponentially grown cyanobacterial strains by using Nucleo-pore® gDNA Fungal/Bacterial kit (Genetix Biotech Asia Pvt. Ltd) following the manufacturer's protocol with a few modifications. Quantity and purity of DNA was estimated by comparison with known standards in ethidium bromide stained 0.8% agarose (Vivantis) gel.

### Multiplex Highly Iterated Palindrome (HIP) fingerprinting

Primers

DNA samples were subjected to amplification using HIP based primers viz. HIP AT, HIP TG and HIP GC in dual combinations. The details of primers sequence used in this study are: (1) HIP AT: 5'-GCGATCGCAT-3' (2) HIP TG: 5'-GCGATCGCTG-3' and (3) HIP GC: 5'-GCGATCGCGC-3' with 60%, 70% and 80% GC content respectively.

PCR amplification

The standard, optimized PCR was performed in a total volume of 50 µL containing 25 µL of 2X Go Taq Green Master mix (Promega), dual combination of HIP primers with 10 pM each of single primer and 90 ng of template DNA (Chakdar and Pabbi 2012). Thermal cycling was achieved in a Master Cycler Gradient (peQSTAR, Germany) as described earlier by Chakdar and Pabbi (2012). PCR products were resolved along with a molecular weight marker (Promega 1 Kb DNA ladder) on 2.0% agarose gel for 6 hours (50 volts) in 1X Tris–Borate–EDTA (TBE) buffer and stained with ethidium bromide solution (1 µg/mL). These were visualized under UV light and gel photographs were captured through gel documentation system (Universal Hood II, BIO-RAD, USA) and the amplification product sizes were determined using the software FluorChem 5500 (Alfa Innotech Corporation, USA). The bands, ranging from 300 to 3000 bp, were scored for diversity analyses. The banding pattern was scored as "1" for presence and "0" for absence of a band. This binary (0, 1) matrix was used for genetic diversity analyses.

### Statistical analysis

Morphological diversity analyses

Fuzzy C-Means (FCM) clustering technique is a soft clustering algorithm proposed by Bezdek (1973, 1981). Unlike K-means clustering algorithm in which each data object is the member of only one cluster, a data object is the member of all clusters with varying degrees of fuzzy membership between 0 and 1 in FCM clustering algorithm. Hence, the data objects closer to the centers of clusters have higher degrees of membership than objects scattered in the borders of clusters. For morphological diversity analysis, FCM clustering algorithm was executed. Fitness of the clustering was measured using Dunn's fuzziness coefficient and fuzzy silhouette index. Dunn's fuzziness coefficient is a goodness-of-fit criterion for fuzzy clustering that measures how close the fuzzy solution is to the corresponding hard solution. A higher value of Dunn's fuzziness coefficient indicates good clustering. Dunn's fuzziness coefficient is generally normalized so that it varies from 0 (completely fuzzy) to 1 (hard cluster). The fuzzy silhouette index is another performance evaluation criterion of FCM clustering; it lies between 0 to 1, where 0 indicates completely fuzzy clustering and 1 indicates completely crisp or hard clustering.

Here, FCM clustering was performed for the data generated from morphometry. Before performing the FCM clustering, principal component analysis (PCA) was carried out to reduce the dimension as PCA is a dimension-reduction technique. Besides, by projecting the data into a lower-dimensional space, PCA can help eliminate noise and irrelevant features, making the underlying structure of the data more apparent for clustering. Further, reducing dimensions through PCA can help visualize the data, allowing us to assess the clustering results and the distribution of data points in a more understandable way. Separate clustering was carried out for heterocystous and non-heterocystous cyanobacteria. PCA and FCM cluster analyses were implemented using R version 3.6.3 (2020-02-29), Platform: x86_64-w64-mingw32/x64 (64-bit).

### Genetic diversity analyses

Pairwise genetic similarities among the genotypes under study were determined using Jaccard's coefficient (Jaccard 1908), $J = N_{11}/(N_{11} + N_{10} + N_{01})$, where $N_{11}$ is the number of bands present in both individuals i and j, $N_{10}$ is the number of bands present in the individual i and $N_{01}$ is the number of bands present in the individual j. Cluster analyses were carried out on similarity estimates using the unweighted pair group method with arithmetic mean (UPGMA) using NTSYS pc, version 1.80 (Shalini et al. 2008, 2009). Bootstrap value was determined using Winboot software and the confidence limit of the clustering was also checked. 1000 replicates were used for bootstrap analyses. To test the goodness of fit of a clustering to the set of RAPD data, cophenetic correlation coefficient or cophenetic value was estimated using the COPH and MXCOMP options in NTSYS pc program as described by Chakdar and Pabbi (2012). The degree of fit was interpreted subjectively as $0.9 \le r$ is very good fit, $0.8 \le r \le 0.9$ is good fit, $0.7 \le r \le 0.8$ is poor fit and $r < 0.7$ is very poor fit.

## Results

### Measurements of cellular dimensions

The mean length of the vegetative cells of heterocystous cyanobacteria ranged from 3.17 to 10.67 µm while width ranged from 3.08 to 6.05 µm. *Hapalosiphon* sp. K23 (10.67 µm) and *Nostoc* sp. K60 (3.17 µm) were recorded as having the highest and lowest cell lengths respectively. Incidentally, *Nostoc* sp. K60 also showed the lowest (3.17 µm) vegetative cell width while the highest cell width was recorded in *Hapalosiphon* sp. K76. The average length and width of heterocyst ranged from 3.87-13.12 µm and 3.67-9.69 µm respectively. The highest heterocyst cell length and width were recorded in *Hapalosiphon* sp. K28 (13.12 µm) and *Nostoc* sp. K67 (9.69 µm) respectively. On the other hand, the lowest heterocyst cell length and width were recorded in *Aulosira laxa* K53 (3.86 µm) and *Toxopsis calypsus* K49 (3.70 µm) respectively. In the case of non-heterocystous cyanobacteria, the mean cell length and width ranged from 2.87-9.95 µm and 1.9-9.0 µm respectively. *Lyngbya hieronymusii* K95 was recorded with the highest cell length (9.95 µm) and width (9.0 µm) while *Desertifilum* sp. K4 (2.87 µm) and *Phormidium* sp. K6 (2.8 µm) showed the lowest cell length and width, respectively.

### Fuzzy clustering based on cell size

For heterocystous and non-heterocystous cyanobacteria, separate clustering was carried out. Analyses of the heterocystous cyanobacteria showed 3 distinct clusters (Fig. 1A) where 78.51% variation was explained by two principal coordinates or Dimensions (Dim 1: -0.386*LV-0.502*WV-0.511*LH-**0.581*WH**; Dim 2: **0.891*LV**+0.011*WV-0.339*LH-0.303*WH). The fitness of the clustering was good as indicated by Dunn's fuzziness coefficient (0.687) and fuzzy silhouette index (0.775) which however also indicated that few of the isolates could be clustered in more than one group.

Isolates of *Hapalosiphon* spp. *viz. Hapalosiphon* sp. K23, *Hapalosiphon* sp. K28, *Hapalosiphon* sp. K32, *Hapalosiphon* sp. K50, *Hapalosiphon* sp. K62, *Hapalosiphon* sp. K76 and *Hapalosiphon* sp. K99) were distributed in Cluster 1 and 2. *Hapalosiphon* sp. K23 showed ~31% probability of being placed in Cluster 2 while *Hapalosiphon* sp. K50 showed ~26% probability of being placed in Cluster 3 (On-line Suppl. Tab. 2). All isolates of *A. laxa*, *T. calypsus*, *Scytonema* sp. and *Nostoc* spp. *viz. Nostoc* sp. K51, *Nostoc* sp. K56, *Nostoc* sp. K60 and *Nostoc* sp. K68 (except *Nostoc* sp. K67, which

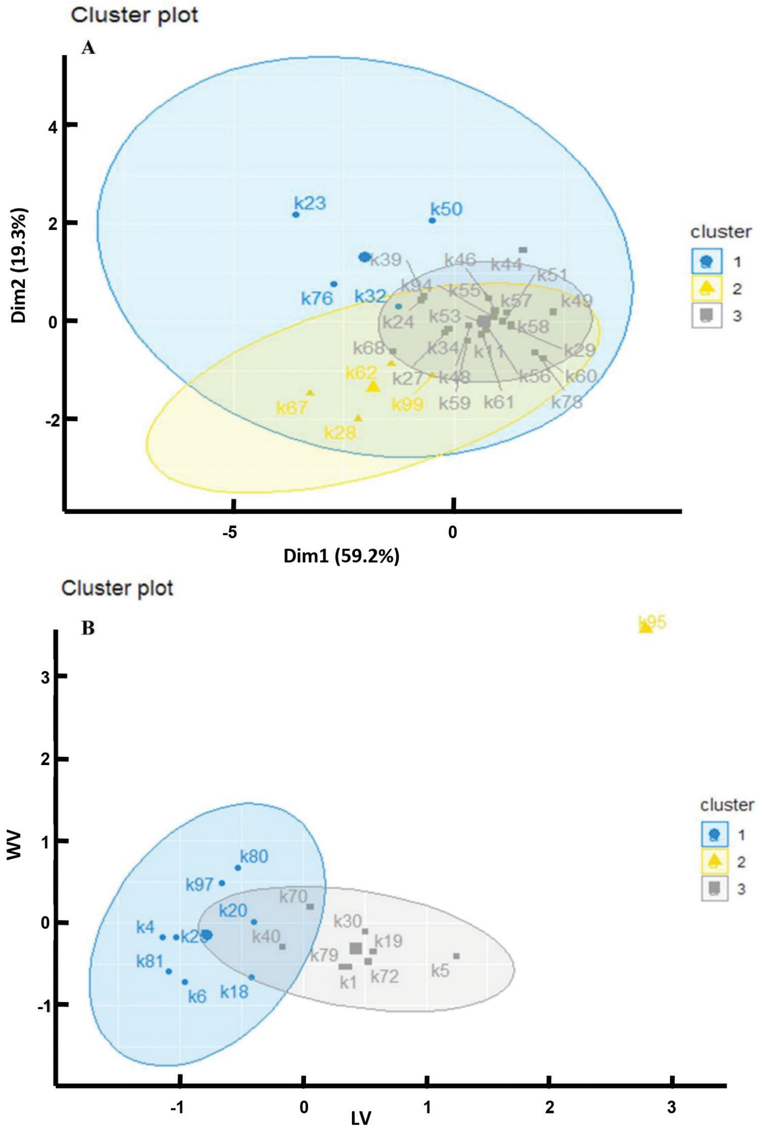


**Fig. 1.** Fuzzy cluster plot of heterocystous cyanobacteria (A) and non-heterocystous cyanobacteria (B). The fuzzy cluster plot was constructed using the cellular dimension of vegetative cells and heterocysts of heterocystous cyanobacteria. Whereas, length (LV) and width of vegetative (WV) cell were used for non-heterocystous cyanobacteria. In case of clustering of heterocystous cyanobacteria, Dimension (Dim) 1 and 2 were the most important principal coordinates explaining 59.17% and 19.33% variation. In Dim1, width of heterocyst contributed maximum variation while in dimension 2, length of vegetative cell had the major contribution.

**Tab. 1.** Final cluster prototypes for heterocystous and non-heterocystous cyanobacteria. LV – length of vegetative cell, WV – width of vegetative cell, LH – length of heterocyst, WH – width of heterocyst, NA – not applicable.

| I. Heterocystous cyanobacteria | | | | | |
|---|---|---|---|---|---|
| | LV | WV | LH | WH | Remarks |
| Cluster 1 | 7.167261 | 5.36375 | 7.61384 | 6.494746 | Large vegetative cells and medium sized heterocysts |
| Cluster 2 | 4.956879 | 4.76203 | 9.432821 | 6.941643 | Small to medium sized vegetative cells and large heterocysts |
| Cluster 3 | 4.531235 | 4.297334 | 4.943985 | 4.835359 | Small vegetative cells and small heterocysts |
| II. Non-heterocystous cyanobacteria | | | | | |
| Cluster 1 | 3.507888 | 2.794574 | NA | NA | Large vegetative cells |
| Cluster 2 | 9.937472 | 8.983922 | NA | NA | Small to medium sized vegetative cells |
| Cluster 3 | 5.76292 | 2.515487 | NA | NA | Small vegetative cells |

was placed in Cluster 2) were placed in Cluster 3. However, two isolates of *A. laxa* K24, *A. laxa* K39, *Nostoc* sp. K68 showed probabilities of being placed in Cluster 2 (On-line Suppl. Tab. 2). Isolates placed in Cluster 1 showed close proximity among themselves while isolates placed in Cluster 2 and 3 showed high variability. The results related to cluster prototype presented in Tab. 1 showed that clusters had significant variations with respect to cellular dimensions among themselves indicating high inter-generic variations as clustering was related to their generic identity.

In the case of non-heterocystous cyanobacteria also, three clusters were formed where Cluster 2 contained only one isolate *L. hieronymusii* K95 (Fig. 1B). Dunn's fuzziness coefficient (0.796) and fuzzy silhouette index (0.684) showed the good fit of the clustering; however, the fitness indices also indicated that a few of the isolates could be clustered in more than one group. Isolates of *Desertifilum* sp., *Lyngbya* sp. and *Phormidium* sp. grouped both in Cluster 1 and Cluster 3 while *Alkalinema pantanalense* K25 and isolates of *Halomicronema* sp. were placed exclusively in Cluster 1 and

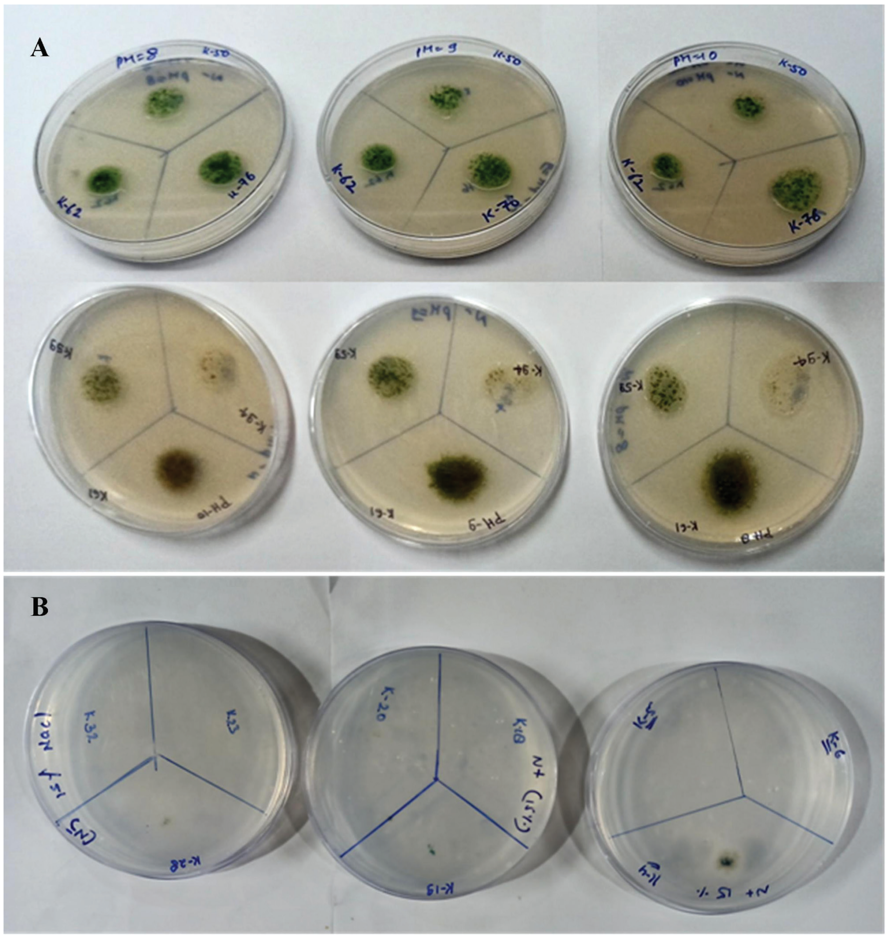


**Fig. 2.** Representative image depicting growth of heterocystous/non-heterocystous cyanobacteria on BG11 N$^+$/N$^-$ agar plate amended to different alkalinity (i.e., pH 8, 9 and 10) (A) and salinity (i.e., 5, 10 and 15% NaCl concentration) (B).

Cluster 3 respectively. *Halomicronema* sp. K70 had ~32% probability of being placed in Cluster 1 while *Desertifilum* sp. K18 had ~27% probability of being grouped in Cluster 3. *Lyngbya wollei* K80 had ~ 26% probability of being placed in both Cluster 2 and Cluster 3. Similarly, *Halomicronema* sp. K40 had 50% probability of being grouped in both Cluster 1 and Cluster 3 (On-line Suppl. Tab. 3). For non-heterocystous cyanobacteria also, clustering showed a high degree of both inter- and intra-generic variability with respect to cell dimensions (Tab. 1).

## Physiological characterization of isolates for tolerance to salinity and alkalinity

All cyanobacterial isolates except *Lyngbya aestuarii* K97 (tolerated pH 8-9) showed tolerance to pH ranging from 8-10 (Fig. 2A). However, great variation was observed in the case of salinity tolerance (Fig. 2B). Among the non-heterocystous cyanobacteria, isolates K4, K18, K19 of *Desertifilum* sp. and *Phormidium* sp. K30 showed tolerance to 15% NaCl (w/v). Only four isolates viz. *Halomicronema* sp. K79, *Desertifilum* sp. K20, *L. aestuarii* K97 and *Leptolyngbya* sp. K72 showed tolerance to 5% NaCl. Among the heterocystous cyanobacteria, *A. laxa* K24, *Hapalosiphon* sp. K28, *Hapalosiphon* sp. K62 and *Scytonema* sp. K55 showed tolerance to 15% NaCl. 16 heterocystous isolates could tolerate NaCl only to 5% while 3 isolates showed tolerance to NaCl only up to 10%.

Cluster analyses of the non-heterocystous isolates showed (Fig. 3A) three distinct clusters viz. a) high salinity & high alkalinity tolerant (04 isolates); b) Low salinity & high alkalinity tolerant (04 isolates) and c) only high alkalinity tolerant (09 isolates). On the other hand, heterocystous cyanobacteria showed (Fig. 3B) four distinct clusters viz. a) high salinity & high alkalinity tolerant (04 isolates); b) moderately high salinity & high alkalinity (03 isolates); c) low salinity & low alkalinity tolerant (09 isolates) and d) only high alkalinity tolerant (14 isolates).

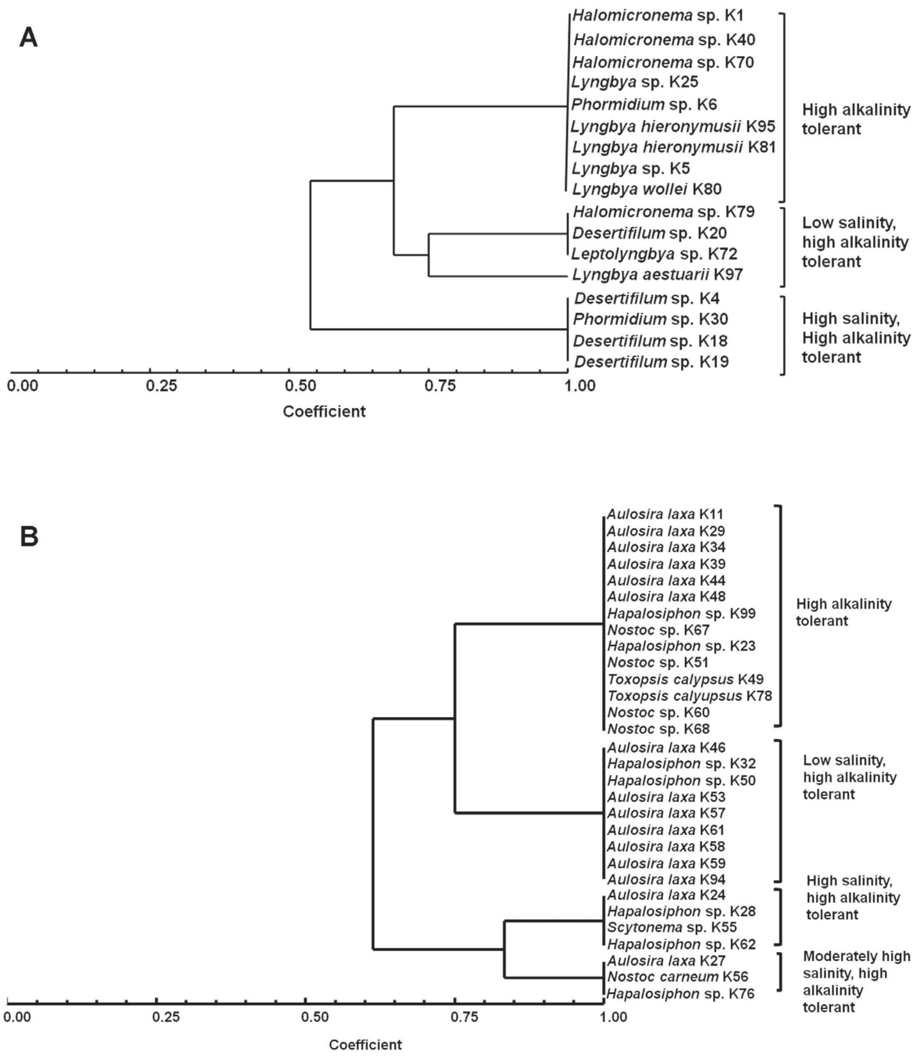


**Fig. 3.** Dendrogram representing the clustering of filamentous cyanobacteria based on their ability to tolerate variable levels of alkalinity and salinity. A – clustering of non-heterocystous cyanobacteria; B – clustering of heterocystous cyanobacteria. The heterocystous cyanobacteria were clustered in three distinct groups: high alkalinity tolerant; low salinity and high alkalinity tolerant; and high salinity and high alkalinity tolerant. The non-heterocystous cyanobacteria were clustered in four distinct groups: high alkalinity tolerant; low salinity and high alkalinity tolerant; high salinity and high alkalinity tolerant; and moderately high salinity and high alkalinity tolerant.

## HIP fingerprinting

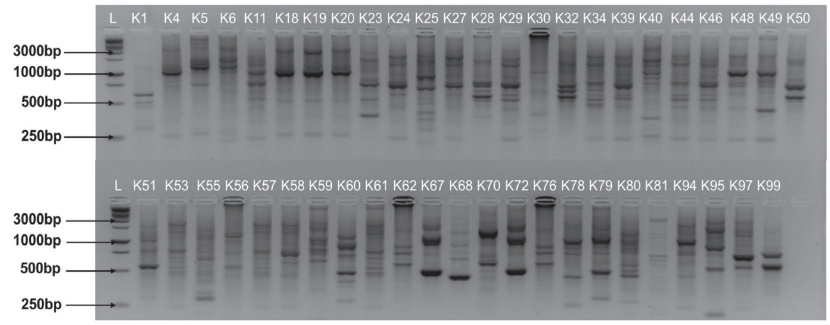
All the three primer combinations HIP AT + HIP TG; HIP AT + HIP GC and HIP TG + HIP GC showed 100% polymorphism (Tab. 2, Fig. 4A-C). HIP AT + HIP TG produced a total of 357 fragments ranging from 170-2156 bp. HIP AT + HIP GC set produced 379 fragments ranging from 157-2129 bp. 269 fragments ranging from 103 bp to 2547 bp were generated by HIP TG + HIP GC. For the purpose of scoring, only the fragments ranging from 300-3000 bp were used.

## Genetic diversity analyses

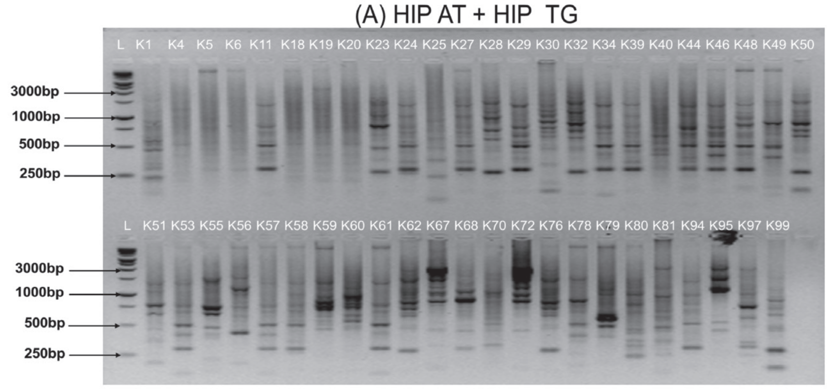
Genetic diversity analyses for heterocystous and non-heterocystous cyanobacteria were carried out separately. Genetic similarity among the 17 non-heterocystous cyanobacteria range from ~13-90% with two major clusters. The major cluster 1 comprised all isolates of *Halomicronema*, *Leptolyngbya* sp. K72, *L. wollei* K80, *L. hieronymusii* K81 and K95, *Lyngbya* sp. K5, *Phormidium* sp. K30 and *A. pantanalense* K25 (Fig. 5A). On the other hand, major cluster 2 contained all isolates of *Desertifilum*, *Phormidium* sp. K6,

**Tab. 2.** Size range of the PCR products generated by three primer combinations.

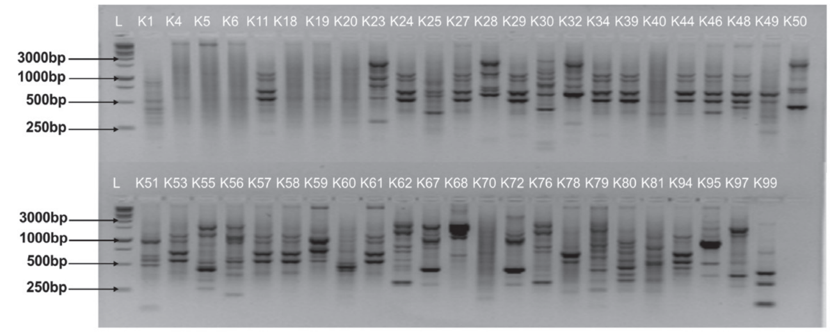
| Sl. No. | Primer combination | Total no. of fragments | % polymorphic bands | Size range (bp) |
|---|---|---|---|---|
| 1. | HIP AT + HIP TG | 357 | 100 | 170 - 2156 |
| 2. | HIP AT + HIP GC | 379 | 100 | 157 - 2129 |
| 3. | HIP TG + HIP GC | 269 | 100 | 103 - 2547 |



(A) HIP AT + HIP TG



(B) HIP AT + HIP GC



(C) HIP TG + HIP GC

**Fig. 4.** Gel photograph presenting the HIP (Highly Iterated Palindrome) element based fingerprint of the forty-seven different filamentous heterocystous and non-heterocystous cyanobacterial isolates generated using dual primer combinations: A – HIP AT + HIP TG, B – HIP AT + HIP GC, C – HIP TG + HIP GC. L: 1kb DNA ladder (Promega, G571A).

and *L. aestuarii* K97. *Desertifilum* sp. K18 and K19 showed ~90% similarity indicating that they may be different isolates of the same species. The fitness of the clustering was found to be good (r = 0.87).

Among the 30 heterocystous cyanobacteria, the genetic similarity ranged from ~12-76%. Two major clusters viz. cluster 1 with 29 cyanobacteria and cluster 2 with only *T. calypsus* K49 were observed (Fig. 5B). In the major cluster 1, all 15 isolates of *Aulosira* and 7 isolates of *Hapalosiphon* formed separate sub-clusters. On the other hand, *Nostoc* spp. showed clustering with *Hapalosiphon* (*Nostoc* sp. K51, *N. carneum* K56, and *Nostoc* sp. K68) as well as *Scytonema* sp. K55 (*Nostoc* sp. K67 and *Nostoc* sp. K60). Among the *A. laxa* isolates clustered together, the genetic similarity ranged from 26-76% while the same was found in a range of 31-57% in the case of *Hapalosiphon* sp. isolates. The degree of fitness of clustering was good (r = 0.89). The result clearly showed a very high intra-generic diversity among the studied filamentous heterocystous cyanobacteria.

In the majority of the cases the clustering of both non-heterocystous and heterocystous cyanobacteria was supported by significant bootstrap values (Fig. 5). More than 70% bootstrap values correspond to > 95% probability that true phylogeny have been found (Hillis and Bull 1993).

## Discussion

Filamentous cyanobacteria like *Nostoc*, *Anabaena*, *Phormidium*, *Microcoleus*, *Lyngbya*, *Calothrix* etc. are frequently encountered in BSC (Garcia-Pichel et al. 2001, Zhang 2005). However filamentous cyanobacteria like *Nostoc, Anabaena, Calothrix, Microcoleus, Hapalosiphon, Cylindrospermum, Scytonema, Aulosira, Phormidium* and *Oscillatoria* have been reported to be present not only in BSCs from arid and semi-arid areas but also in alkaline and saline soils (Pandey et al. 2005, Srivastava et al. 2009). The predominance of heterocystous or non-heterocystous cyanobacteria is reported to be governed by factors like pH,

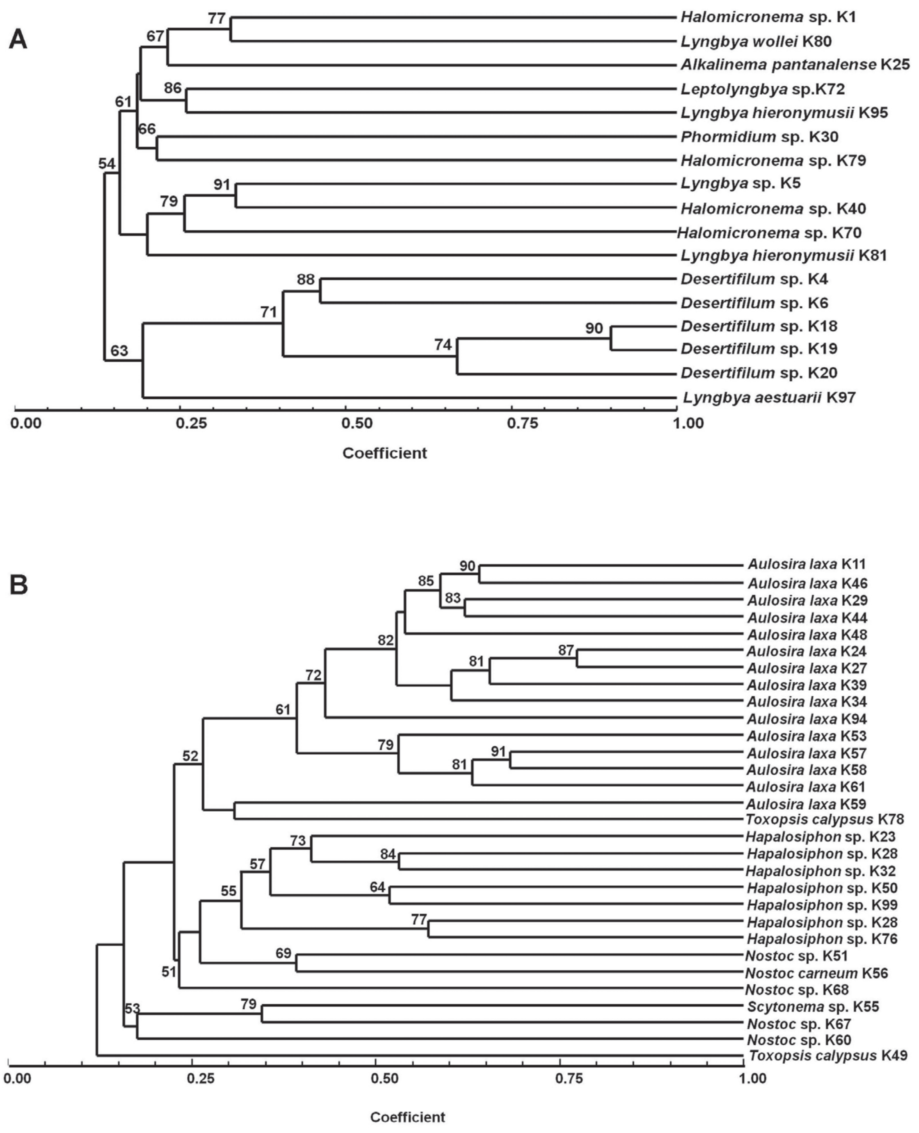


**Fig. 5.** Dendrogram depicting genetic diversity based on multiplex HIP fingerprinting: A – non-heterocystous cyanobacteria, B – heterocystous cyanobacteria. The values at the nodes are bootstrap values. Bootstrap values > 50 have been presented in the dendrograms.

salinity or nitrogen availability (Srivastava et al. 2009). Many of such filamentous cyanobacteria have been used for the amelioration of salinity stress in various crops, but it is noteworthy that the majority of these cyanobacteria were from four genera viz. *Anabaena*, *Nostoc*, *Hapalosiphon* and *Calothrix* (Li et al. 2019).

Cluster analyses of the 47 filamentous cyanobacteria based on cellular dimensions and tolerance to salinity and alkalinity showed high variability among the isolates. Among the heterocystous cyanobacteria, significant morphological variability was observed within the members of the genus *Hapalosiphon* while the other genera like *Aulosira* or *Nostoc* had limited intra-generic variability. In case of non-heterocystous cyanobacteria, inter- and intra-generic morphological variability was higher as all the clusters contained isolates belonging to different genera. Among non-heterocystous cyanobacteria, isolates of *Lyngbya* showed higher morphological variations. Initial classifications of cyanobacteria were solely based on morphology and still an important criterion for identification. However, many times their variation with environmental conditions makes them unsuitable for correct identification. Very few studies, like Mishra et al. (2015), reported that morphological attributes like trichome aggregation, heterocyst shape and akinete shape are stable features and could be used for identification. The results of the present study indicated that dimensions of vegetative cells and heterocysts can be effective for identification and differentiation of cyanobacteria belonging to *Hapalosiphon* and *Lyngbya*. This observation is in compliance with the distinction of various species of *Hapalosiphon* (like *H. welwitschii* West & G.S.West, *H. delicatulus* West & G.S.West, *H. intricatus* West & G.S.West, *H. pumilus* Kirchner ex Bornet & Flahault, etc.) and *Lyngby*a (like *L. chaetomorphae* Iyengar & Desikachary, *L. lachneri* (W.Zimmermann) Geitler, *L. infixa* Frémy, *L. baculum* Gomont, etc.) based on dimensions of filaments or trichomes (Desikachary 1959). Looking at the advances in computational techniques and the development of robust algorithms, it does not seem to be really impossible to use morphological information for understanding actual biological diversity. Heterocystous cyanobacteria showed high intrageneric variability in the case of salinity while all isolates were highly alkali-tolerant. Isolates of *Aulosira* and *Hapalosiphon* were found to have variable tolerance to salinity. However, the variability of salinity tolerance was lower in non-heterocystous than in heterocystous cyanobacteria. Growth and colonization of cyanobacteria are known to be greatly influenced by soil pH and salinity (Pandey et al. 2005, Nayak and Prasanna 2007). Cyanobacterial growth is favoured under neutral to alkaline conditions while acidic conditions may limit the growth of many cyanobacteria (Šesták 2001). In the present study, all the isolates of cyanobacteria studied could also tolerate up to pH 10. It was been reported that the metabolic trade-off between ionic balance and heterocyst formation or diazotrophy may limit the proliferation of heterocystous cyanobacteria under highly saline conditions (Vitousek et al. 2002, Berman-Frank et al. 2003). Srivastava et al. (2009) reported that low salinity favoured the presence of heterocystous cyanobacteria while highly saline soils predominantly harboured non-heterocystous cyanobacteria. Kirkwood et al. (2008) reported that heterocystous cyanobacteria could still persist under saline conditions although it might be suboptimal for growth. As observed in the present study, the varying tolerance of heterocystous cyanobacteria to salinity might be an important fitness trait to proliferate under saline/alkaline soils. Consistently with the reports of Srivastava et al. (2009), *Aulosira* turned out to be highly salinity-adapted (5-15%) heterocystous cyanobacteria. All the isolates of *Desertifilum* sp. showed higher salinity tolerance than the other non-heterocystous cyanobacteria. The salinity tolerance exhibited by the *Desertifilum* isolates in the present study are much beyond the tolerance limit (3%) of all the reported species of the genus *Desertifilum* (Dadheech et al. 2014, Cai et al. 2017).

HIP fingerprinting of heterocystous revealed higher genetic variability than in non-heterocystous cyanobacteria. Clustering of non-heterocystous cyanobacteria based on HIP fingerprinting also showed high intra- and inter-generic variability. Despite being the most commonly used molecular marker for understanding phylogeny and describing novel prokaryotic taxa, the *16S rRNA* gene often cannot resolve intra-generic diversity (Woese 1987, Tindall et al. 2010). Although *16S rRNA* gene contains informative hypervariable regions, it does not have enough divergence to resolve the differences among the closely related members of a genus (Fox et al. 1992, Drugă et al. 2013). Repetitive elements like HIP can be very effective for such purposes due to their abundance throughout the cyanobacterial genomes (Xu et al. 2018). Earlier studies showed that molecular tools like RAPD and HIP fingerprinting can effectively resolve the intra-generic diversity of cyanobacteria like *Nostoc*, *Anabaena*, *Hapalosiphon, Calothrix* etc. (Chakdar and Pabbi 2012, Shukla et al. 2013, Singh et al. 2014). In the present study, the isolates of *A. laxa*, *Hapalosiphon* sp. and *Nostoc* sp. showed distinct clustering with high intra-generic variability indicating that multiplex HIP fingerprinting could effectively differentiate among closely related members of these genera. In the case of non-heterocystous cyanobacteria like *Phormidium* and *Lyngbya 16S rRNA* genes have been reported to be insufficient for intra-generic taxonomic resolution (Marquardt and Palinska 2007, Engene et al. 2010). In the present study, *Lyngbya* was also found to be genetically highly heterogeneous and HIP fingerprinting could distinguish between two *L. hieronymusii* isolates. Furthermore, HIP fingerprinting could effectively distinguish the isolates belonging to *Halomicronema* and *Desertifilum*.

## Conclusion

The results of the present study showed the importance of morphological, physiological and genetic analyses to understand the diversity of filamentous cyanobacteria. All these analyses can supplement each other to provide a better understanding of the population structure and ecology of these cyanobacteria. The findings indicated that the variation in salinity tolerance of filamentous cyanobacteria along with their inherent alkali tolerance helped to proliferate a heterogeneous population of both heterocystous and non-heterocystous cyanobacteria in saline/alkaline soils. With great genetic and physiological diversity, such cyanobacteria can be a potential biological resource for the reclamation of such degraded soils. However, further in-depth studies are required to understand their actual genetic and physiological potential.

### Author contribution statement

H.C. contributed in conceptualization of the work. S.V. carried out the experiments and validated the study. R.V. carried out the morphometric characterization. A.B. contributed to the statistical analysis. S.V. and H.C. investigated the study and prepared the original draft. H.C., S.V., S.Y.B., N.S. and V.M. contributed in writing-reviewing the draft. H.C. and A.K.S. supervised the study and edited the final draft and gave final approval for the publication of this version. All authors read and approved the final manuscript.

### Availability of data and material

Gene sequences are available in NCBI, Cultures are available in National Agriculturally Important Microbial Culture Collection (NAIMCC) at ICAR-NBAIM, Mau, Uttar Pradesh, India and other information are available with the corresponding author.

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Research Article

# Molecular and morphological characterization of diatoms related to dense mucilage formation in Turkish coastal waters

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**Abstract** – Cryptic diatom species, despite their subtle morphological traits, play a pivotal role in harmful algal blooms (HABs) and mucilage formation, making their identification a critical challenge. This study combines molecular and morphological approaches to characterize diatom species associated with the extensive mucilage observed in Turkish coastal waters between 2020 and 2021. Among the findings, *Minutocellus polymorphus* (Hargraves & Guillard) Hasle, Stosch & Syvertsen and *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot whose molecular phylogenetic analysis was carried out by sequencing *small subunit ribosomal DNA* (*SSU rDNA*) and *internal transcribed spacer* (*ITS*), are reported for the first time in Turkish waters, expanding our understanding of local biodiversity. Additionally, *Pseudo-nitzschia pungens* (Grunow ex Cleve) G.R.Hasle was molecularly characterized for the first time in the Sea of Marmara using *large subunit ribosomal DNA* (*LSU rDNA*) and *ITS* sequences. These results underscore the indispensable role of molecular techniques in uncovering the hidden diversity of diatoms and their potential contribution to HABs and mucilage events. By linking these findings to the ongoing mucilage phenomenon, this study offers significant insights into the ecological dynamics of the Sea of Marmara and highlights the need for integrative approaches in future research.

**Keywords:** Aegean Sea, diatom, microalgae culture, molecular taxonomy, Sea of Marmara

## Introduction

Mucilage is a natural phenomenon characterized by gelatinous extracellular secretions of microalgae and bacteria (Danovaro et al. 2009, Balkis-Ozdelice et al. 2021). It can take various forms, including small flocs, strings, strips, and clouds, and has been reported in different seas over the centuries such as the Adriatic Sea and the Tyrrhenian Sea (Bianchi 1746, Gotsis-Skretas 1995). In the Sea of Marmara, large-scale mucilage events were observed for the first time in 2007 and more recently between 2020 and 2021 (Balkis et al. 2011, Balkis-Ozdelice et al. 2021). Possible causes for these events include increased nutrient input, climate change, and other anthropogenic factors, which create favorable conditions for its formation (Svetličić et al. 2011). This phenomenon has significant ecological and economic impacts, making it a critical issue for marine ecosystems.

Diatoms are among the main components of phytoplankton and play a crucial role in mucilage formation (Svetličić et al. 2011). They secrete adhesive mucilage, a type of extracellular polymeric substance (EPS), which contributes to cell stickiness and aggregation (Thornton 2002, Svetličić et al. 2011). Among these, species such as *Pseudo-nitzschia pungens* and *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C.Lewin have been identified as key contributors during previous mucilage events in the Sea of Marmara (Balkis et al. 2011, Balkis-Ozdelice et al. 2021). However, the cryptic nature of some diatom species makes it challenging to identify them based solely on morphology. This necessitates the use of molecular tools to accurately characterize and understand their role in mucilage formation.

Advances in molecular techniques, such as DNA sequencing and phylogenetic analysis, have significantly enhanced our ability to identify cryptic diatom species and elucidate their taxonomic relationships, making them particularly useful for detecting species that are morphologically indistinguishable or rare in natural samples

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(Alverson 2008). By combining molecular and morphological approaches, it is possible to gain deeper insights into the biodiversity and ecological roles of the diatoms associated with mucilage events.

The objective of this study is to investigate the molecular and morphological characteristics of diatom species linked to the recent mucilage phenomenon in the Sea of Marmara and the Aegean Sea. Specifically, we aim to identify cryptic species, understand their genetic relationships, and explore their potential contributions to mucilage formation. By doing so, we hope to provide a comprehensive understanding of the ecological dynamics driving these events and inform future management strategies.

## Material and methods

### Sampling

Mucilaginous seawater samples were collected once from five stations, including Keşan in the North Aegean Sea (September 2021, Station 5) and Burgazada (October 2021, Station 3), Heybeliada (November 2021, Station 2), Kadıköy (November 2021, Station 1), and Erdek (January 2022, Station 4) in the Sea of Marmara (Fig. 1).

Sampling was performed with a plankton net (20 µm mesh size) deployed horizontally in the coastal area. At all stations except Erdek, the net was thrown as far from the shore as possible and retrieved to collect samples from surface waters, with this process repeated 10 times. At Erdek, the sample was collected from a depth of 8 m using SCUBA diving. During the dive, no specific transect was followed; instead, the net was moved freely while maintaining a constant depth. The concentrated samples were transferred to 50 mL centrifuge tubes during the fieldwork, kept in the dark to preserve their integrity, and immediately brought to the laboratory for isolation.

### Monoalgal cell isolations and cultures

The field samples were serially diluted on 24-well plates (1:1 ratio) with enriched F/2 medium at 24 salinity (Guillard 1975). They were kept at 150 µmol photons $m^{-2}$ $s^{-1}$ (Nakamura and Umemori 1991) under a 12:12 light-dark cycle at 20 ± 2 °C until isolation (3–4 days) in the culture room. These samples were examined with an inverted microscope (Olympus CKX31), and single-cell isolations were made using a Pasteur pipette with a narrowed tip to a drop on the micro-flocculation plates. Each algal cell was transferred from one drop of filtered and autoclaved seawater to another drop of filtered and autoclaved seawater supplemented with F/2 medium until it was free from bacteria and other cells. Finally, the single cells were transferred to 96-well plates containing enriched F/2 medium and incubated in the culture room. The cells were photographed using an Olympus BX51 model transmitted LM with a Touptek XF1080B-S model digital camera attachment, and cell measurements for each species were also performed on 30 cells using this microscope. Dense cell cultures in the late-exponential phase were harvested by centrifugation (3500 g for 15 min) for subsequent DNA analysis.

For the morphological investigation of strains using scanning electron microscopy (SEM), the *Cylindrotheca* strain was transferred to a cryotube, subjected to cleaning with a 20% sulfuric acid ($H_2SO_4$) solution, and the sample was sedimented and washed with deionised water (DI) several times. Subsequently, the sample was boiled in a 50% hydrogen peroxide ($H_2O_2$) solution at 100 °C for half an hour and washed again with DI a few times. In contrast, in the *Fistulifera* strain, only a 20% sulfuric acid solution was employed to eliminate organic matter. For *Pseudo-nitzschia,* samples were digested in a 10% hydrochloric acid (HCl) solution overnight and washed with distilled water. Subsequently, the samples were boiled with 50% hydrogen

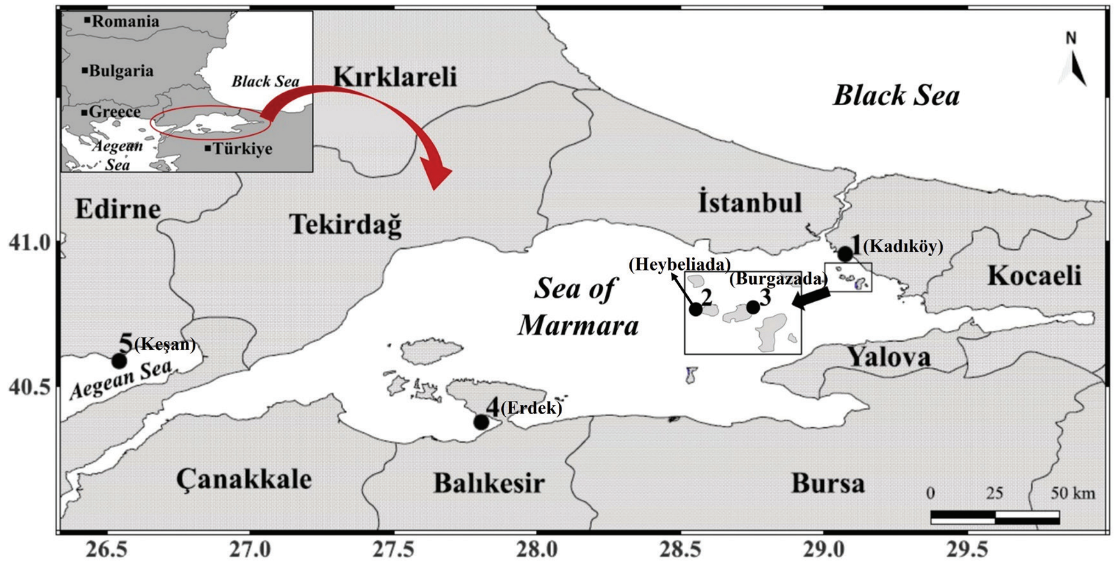

**Fig. 1.** Location of stations: Kadiköy (Station 1), Heybeliada (Station 2), Burgazada (Station 3), Erdek (Station 4), Keşan (Station 5) (QGIS, 2022).

peroxide for 4–5 hours, and the acid was removed by washing several times with distilled water. The acid-digested and acid-free samples were filtered through polycarbonate membrane filters (0.2 µm mesh size) and placed onto carbon-coated stubs. These samples were allowed to dry at room temperature and stored in a desiccator until analysis. Before analysis, they were coated with gold and examined using the FEI Versa 3D model SEM. The length and valve measurements of the species were conducted using Scanning SEM imaging analyses on 30 cells per species.

### Molecular and phylogenetic analyses

Molecular identification of the isolated cells was performed using primers D1R (Scholin et al. 1994) and D3B (Nunn et al. 1996) for the *large subunit ribosomal DNA* gene (*LSU rDNA*), EukA (Medlin et al. 1988) and 18ScomR1 (Zhang et al. 2005) for the *small subunit ribosomal DNA* (*SSU rDNA*), and LSU1R (Bowers et al. 2006) for the *internal transcribed spacer* (*ITS*). Specifically, the following genes were sequenced for the different species: *Cylindrotheca closterium* (*SSU* and *LSU rDNA*), *Fistulifera saprophila* (*SSU rDNA*), *Minutocellus polymorphus* (*SSU rDNA* and *ITS* region), and *Pseudo-nitzschia pungens (LSU rDNA* and *ITS* region). *ITS* phylogenies were drawn from one-way sequencing. Template DNA of the samples was obtained with the High Purity PCR Template Preparation Kit (Roche) using the manufacturer's protocol. Briefly, 25 µL MyTaq Red Mix 2x, 2 µL bovine serum albumin (BSA), 14 µL ddH$_2$O, 5 µL template DNA, and 2 µL of each primer was pipetted into a PCR reaction tube. *LSU* PCR (Borchhardt et al. 2021) amplification involved an initial denaturation for 4 min at 95 °C and final extension for 10 min at 72 °C, and thirty-five cycles of 30 s at 95 °C and 2 min 60 °C. *SSU* PCR (Borchhardt et al. 2021) amplification involved an initial denaturation for 2 min at 95 °C and final extension for 5 min at 72 °C, and thirty-five cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. For *ITS* PCR (Quijano-Scheggia et al. 2020) amplification, initial denaturation for 2 min at 94 °C and final extension step for 5 min at 72 °C followed by thirty-five cycles, each cycle comprising 35 s at 94 °C, 35 s at 60 °C, and 1 min at 72 °C. PCR products of all genes were verified on 1.5% agarose gel. The PCR products were sequenced using the Sanger method by BM Yazılım Danis. ve Lab. Sis. Ltd. Şti (Ankara, Türkiye).

The obtained forward and reverse gene sequences were checked in BioEdit software (Hall 1999), and the 5' and 3' ends, found to have poor reading quality, were trimmed, and made suitable for phylogenetic analysis. Similar reference sequences (only published ones) for phylogenetic analyses were determined using the NCBI BLAST database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and multiple alignments were made in the Bioedit software. Phylogenies were created using the Mega 11 (Tamura et al. 2021) multiple aligned gene sequences program. Alignments and phylogeny inferences were performed using maximum likelihood (ML) and neighbor-joining (NJ) methods. For molecular phylogenetic analyses, publicly available *LSU*, *SSU* and *ITS* rDNA diatom species sequences were retrieved from GenBank. The best models were selected based on the lowest Akaike information criterion corrected (AICc) value. The current nomenclature of the species is based on AlgaeBase (Guiry and Guiry 2024).

## Results

In the study, samples were collected from the Aegean Sea and the Sea of Marmara during the mucilage event covering the Sea of Marmara, and diatom species were investigated by establishing monoalgal cell cultures. The phylogenetic and molecular analyses revealed that three strains (HIB112101, HIB112102 and HIB112103) belonged to *Cylindrotheca closterium*, *Fistulifera saprophila* and *Minutocellus polymorphus*, respectively, and two strains to *Pseudo-nitzschia pungens* (HIB112104 and HIB112261).

### *Cylindrotheca closterium* (Ehrenberg) Reimnann & J.C.Lewin

*Cylindrotheca closterium* strain HIB112101 cells have curved, long, needle-shaped edges and two chloroplasts. They have longitudinal symmetry along the valves that were generally linear/oval-shaped. The apical axis of the HIB112101 strain ranged from 23.3 to 27.2 µm, with a transapical axis of 3.1 to 5.7 µm (Figs. 2A, 3A). HIB112101 strain was isolated from the surface waters (0.5 m) of the coastal area of Heybeliada/Istanbul.

The base pairs (bp) of the *C. closterium* strain used in the *LSU* phylogeny were 828, and in the *SSU* phylogeny 1676 bp. According to the *LSU* and *SSU* sequences in the NCBI GenBank, the DNA sequence of strain HIB112101, which

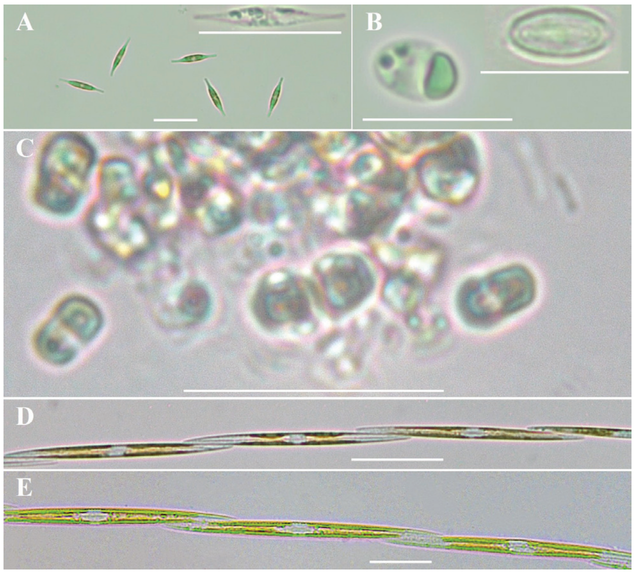

**Fig. 2.** Light microscope image of strains: A – *Cylindrotheca closterium*, B – *Fistulifera saprophila*, C – *Minutocellus polymorphus*, D, E – *Pseudo-nitzschia pungens* (D: cell of HIB112104 strain, E: cell of HIB112261 strain). Scale bars: A, C, D, E = 20 µm, B = 10 µm.

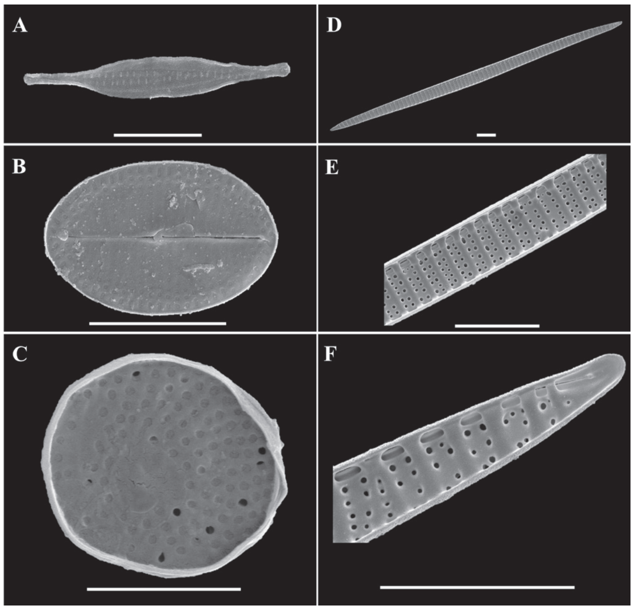

**Fig. 3.** Scanning electron microscope images of strains: A – *Cylindrotheca closterium*, B – *Fistulifera saprophila*, C – *Minutocellus polymorphus*, D-F – *Pseudo-nitzschia pungens*. Scale bars: A, B, C, E, F = 5 µm; D = 20 µm.

was determined to be *C. closterium* with high bootstrap support, was given for the first time from the Sea of Marmara. It was found to be *C. closterium* with 100% in the *LSU* phylogeny (GenBank number: OR948504, Fig. 4A, On-line Suppl. Fig. 1A) and 98% in the *SSU* phylogeny (GenBank number: PP892172, On-line Suppl. Fig. 3A). The TN93+G model was identified as the most suitable for the *SSU* analysis, while the GTR+G+I model was selected for the *LSU* analysis. In these analyses, *Nitzschia palea* and *N. microcephala* were used as outgroups.

### *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot

This species, which forms a chain colony, has two parietal chloroplasts, extending along the lateral sides of the cell. The valves were elliptical and bluntly rounded at each apex (Fig. 2B). The apical axis of *F. saprophila* strain HIB112102 ranged from 2.6 to 6.5 µm, with a transapical axis of 3.2 to 4.5 µm. The number of striae in 10 µm was 32, however, since the culture was lost, striae were counted on a single cell (Fig. 3B). The strain was isolated from the surface waters (0.5 m) of the coastal area of Keşan/Edirne (North Aegean Sea).

When the *SSU* sequences in the NCBI GenBank were analysed, HIB112102 (GenBank number: OR940683), which was determined to be 100% *F. saprophila*, was 1663 bp long, and its DNA sequence was given for the first time from the Aegean Sea (Fig. 4B, On-line Suppl. Fig. 1B). GTR+G+I was identified as the best model for *SSU*, with *Skeletonema costatum* used as an outgroup in the phylogenetic analysis.

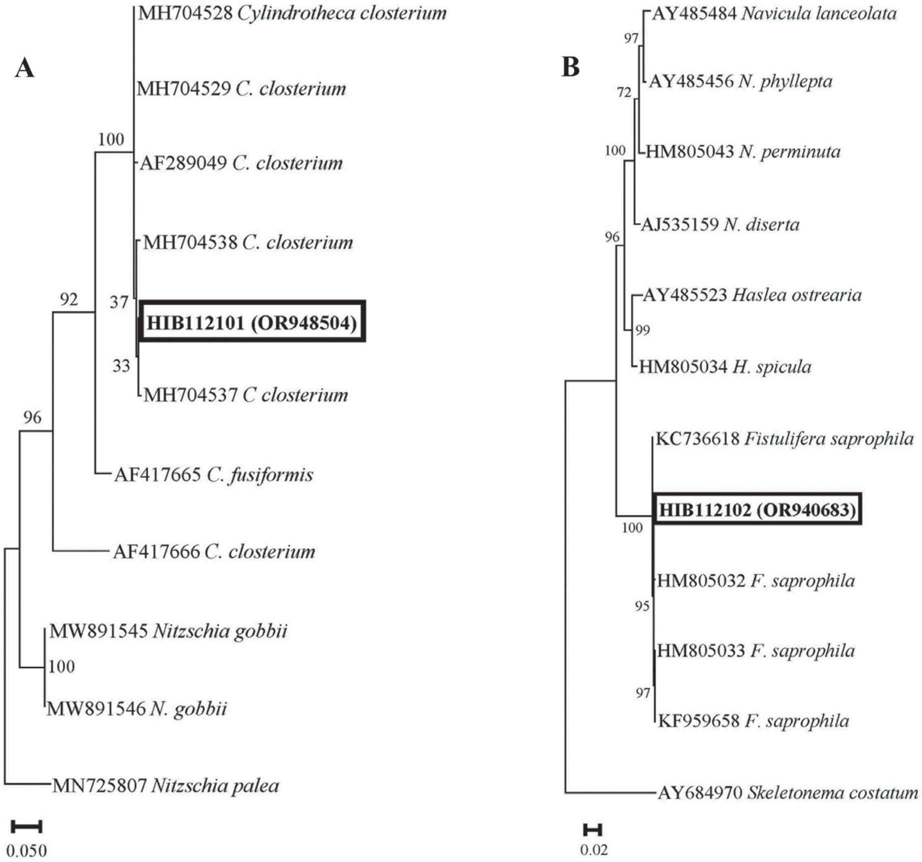

**Fig. 4.** Molecular phylogeny of *Cylindrotheca closterium* based on the *large subunit ribosomal DNA* (*LSU rDNA*) (A), *Fistulifera saprophila* based on the *small subunit ribosomal DNA* (*SSU rDNA*) (B) sequences using maximum likelihood (ML) analysis.

### *Minutocellus polymorphus* (Hargraves & Guillard) Hasle, Stosch & Syvertsen

The valves were elliptical or lanceolate and the valve surfaces had many apical pores. The nucleus of this cryptic diatom was large relative to the cell size and was located in the centre of the cell. The cells form a chain colony. The apical axis of *M. polymorphus* strain HIB112103 ranged from 4.3 to 6.1 µm, with a transapical axis of 2.9 to 4.0 µm (Figs. 2C, 3C). HIB112103 strain was isolated from the surface waters (0.5 m) of the coastal area of Kadıköy/Istanbul from the Sea of Marmara.

The strain of *M. polymorphus* used in *SSU* phylogeny has 1661 bp, while the *ITS* phylogeny sequence has 730 bp. When the *SSU* phylogeny of *Minutocellus* was analysed, strain HIB112103 (GenBank number: OR940695) showed 99% similarity with other *M. polymorphus* strains in the *SSU* phylogeny (On-line Suppl. Fig. 3B) and also showed 99% similarity in the *ITS* phylogeny (GenBank number: PP892174, Fig. 5A, On-line Suppl. Fig. 2A). *Brockmanniella brockmanni* for *ITS* and *Skeletonema costatum* for *SSU* were used as outgroups, with TN93+G as the best model for *SSU* and TN93+G+I for *ITS*.

### *Pseudo-nitzschia pungens* (Grunow ex Cleve) G.R.Hasle

Morphologically, the cells of *Pseudo-nitzschia* had narrow widths and needle-shaped cells were characterized by

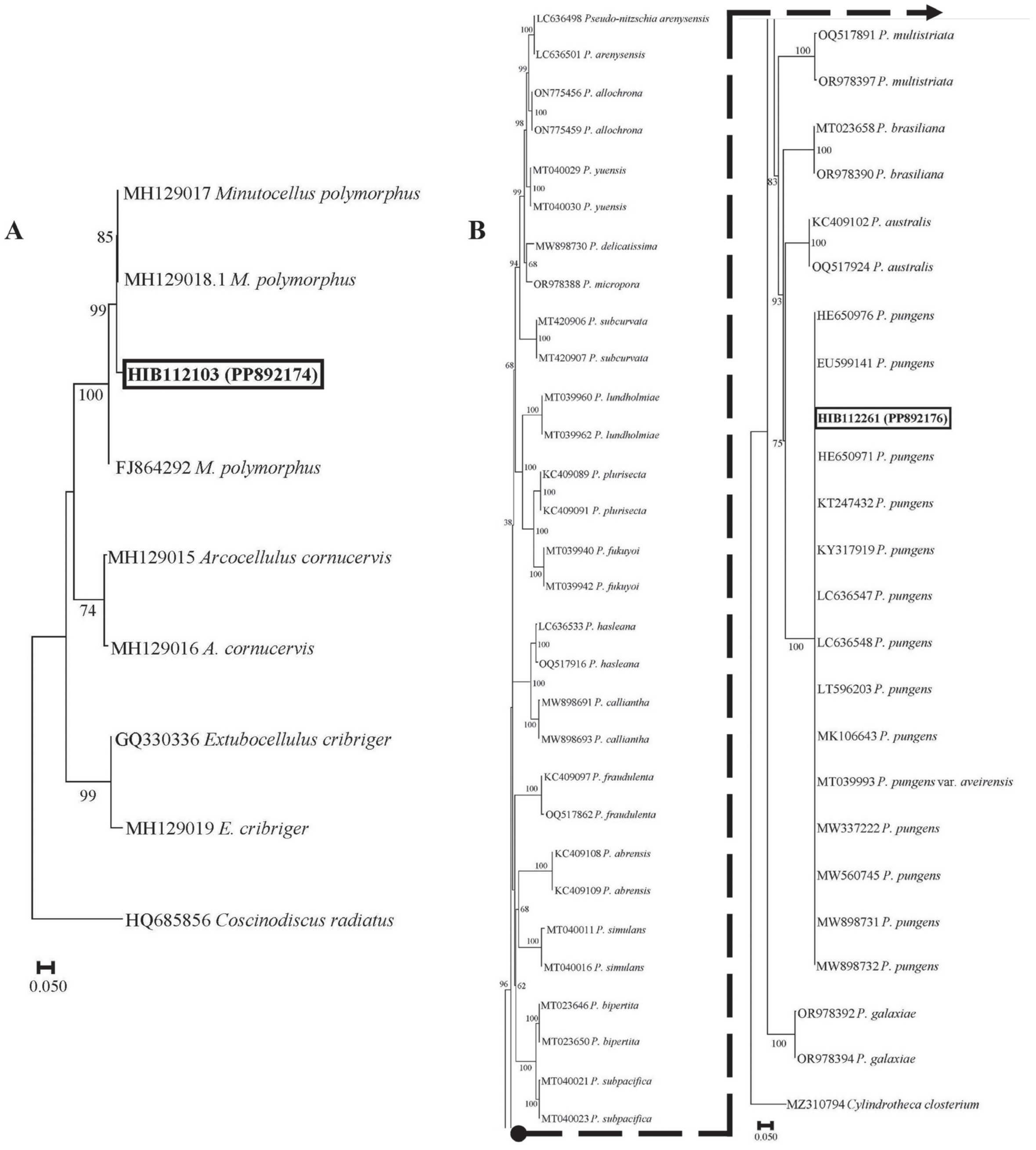

**Fig. 5.** Molecular phylogeny of *Minutocellus polymorphus* (A), *Pseudo-nitzschia pungens* (B) based on the *internal transcribed spacer* (*ITS*) sequences using maximum likelihood (ML) analysis.

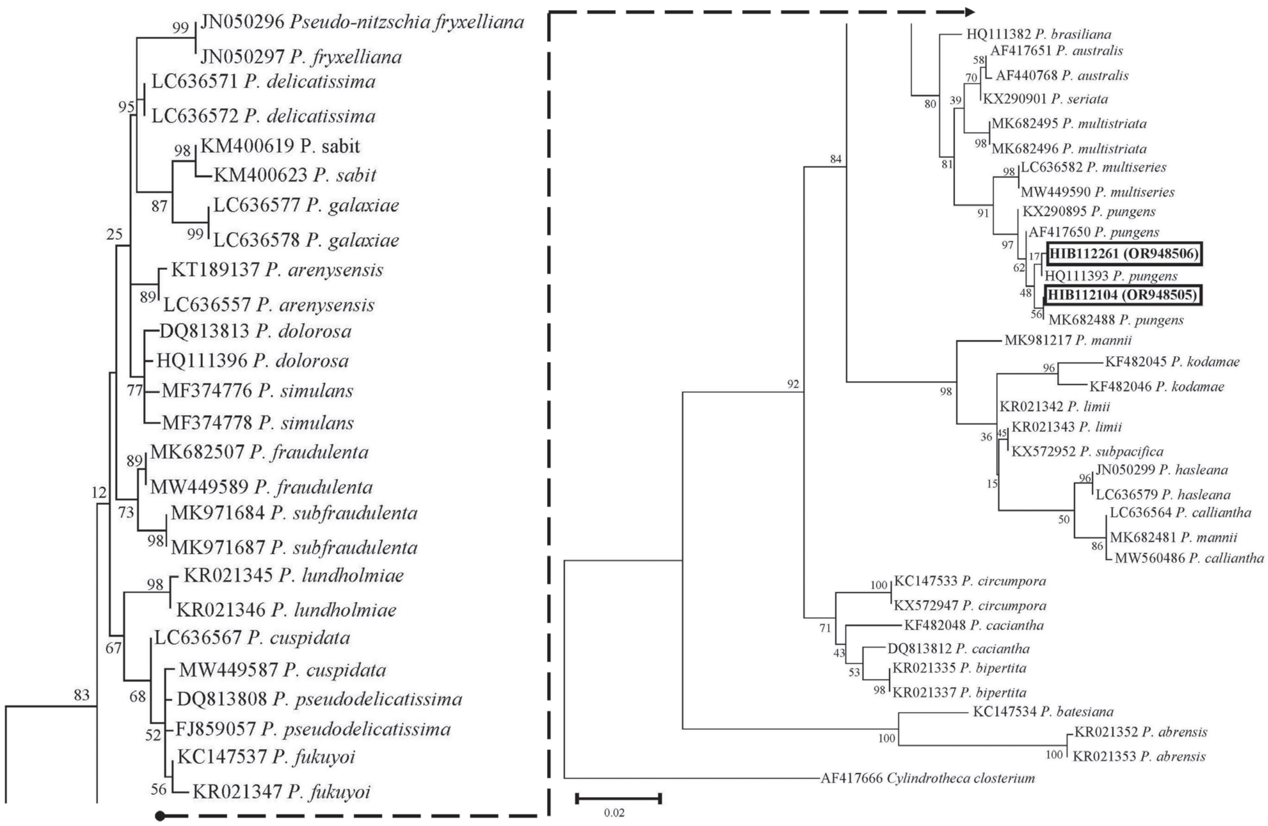

**Fig. 6.** Molecular phylogeny of *Pseudo-nitzschia pungens* based on the *large subunit ribosomal DNA* (*LSU rDNA*) sequences using maximum likelihood (ML) analysis.

their form. The central nodule was absent, and the striae were biseriate with two rows of poroids. The apical axis of *P. pungens* strain HIB112104 ranged from 76.0 to 84.4 µm, with a transapical axis of 4.9 to 6.8 µm (Fig. 2D). The number of fibulae in 10 µm was 9–11, and the number of striae in 10 µm was 10–12. Additionally, the apical axis of the HIB112261 strain varied between 68.9 and 80.2 µm, with a transapical axis of 2.9 to 6.0 µm (Fig. 2E). The number of fibulae in 10 µm was 10–12, and the number of striae in 10 µm was 11–13 (Fig. 3D–F). Also, colony-forming cells were connected to each other up to 1/5–1/6 of the cell length in the HIB112104 strain and 1/4–1/5 in the HIB112261 strain. Their chloroplasts were on two plates along the girdle. HIB112104 strain was isolated from the surface waters (0.5 m) of the coastal area of Burgazada/Istanbul (Sea of Marmara), while the HIB112262 strain was isolated from a depth of 13 m from Erdek/Balıkesir (Sea of Marmara).

Among the *P. pungens* strains used in the *LSU* phylogeny, the HIB112104 strain (GenBank number: OR948505) has 869 bp, while the HIB112261 strain (GenBank number: OR948506) has 872 bp (Fig. 6, On-line Suppl. Fig. 4). For the *ITS* phylogeny, the HIB112261 strain (GenBank number: PP892176) has 710 bp (Fig. 5B, On-line Suppl. Fig. 2B), with GTR+G+I identified as the best model for *LSU* and TN93+G+I for *ITS*, and *Cylindrotheca closterium* used as an outgroup in each phylogenetic analysis.

## Discussion

Molecular and phylogenetic analyses were conducted on monoalgal cell cultures of cryptic diatom species, which were abundant in the examined samples and may be linked to the mucilage phenomenon in Turkish coastal waters (Balkis-Ozdelice et al. 2021).

*Cylindrotheca closterium,* strain HIB112101, from Heybeliada/Istanbul showed high similarity with MH704537 and MH704538 (Stock et al. 2019) strains from Australia, MH704528 and MH704529 (Stock et al. 2019) strains from Antarctica, and AF289049 (Ben Ali et al. 2001) strain in the *LSU* phylogeny. However, no comparison could be made with these strains because no morphological description was given in the paper. The closely related strains in the other phylogeny (*SSU*) with the strain HIB112101 are MH166733 (Khaw et al. 2020) from Port Dickson (Malaysia), KY045848 (Rial et al. 2018) from Vigo Ria estuary (Spain). However, the paucity of uploaded sequences belonging to the *LSU* gene region of this genus in the NCBI database indicate that studies are rare. Molecular phylogenetic analyses show that species diversity in the genus is underrated (Li et al. 2007). Typically, the more comprehensive the sequence analysed, the more accurate the phylogeny reconstruction is considered to be (Martin et al. 1995). Therefore, the *SSU* phylogeny given in this study was drawn on 1520 bp and the ON942237 strain reported from the Sea of Marmara by

Tekdal et al. (2024) was excluded from the phylogenetic analysis due to insufficient data at 382 bp. *Cylindrotheca closterium* is a cosmopolitan species widely distributed from marine to brackish waters (Najdek et al. 2005). This may explain the high similarity of the species isolated from such different locations in the *SSU* phylogeny. This taxon is mostly found in the neritic zone and can also be benthic (Underwood and Smith 1998, Balkis-Ozdelice et al. 2021). In the water column, *C. closterium* has 2– or 3–fold smaller cell size than other diatom species, which might allow them to survive without significant biophysical damage and with certain physiological circumstances, this species can produce mucilage (Alcoverro et al. 2000, Najdek et al. 2005). *Cylindrotheca closterium*, one of the most abundant species in the Sea of Marmara and also observed in this study, has been reported to be associated with both previous (Balkis et al., 2011, Tas et al. 2020) and the most recent mucilage events (Balkis-Ozdelice et al. 2021).

*Fistulifera saprophila,* strain HIB112102, from Keşan/Edirne in the Gulf of Saros (the North Aegean Sea), was supported with 100% bootstrap on the *SSU* phylogeny. The closely related species strain KF959658 was reported from Lake Geneva (Larras et al. 2014), and strains HM805032 and HM805033 were reported from the Baltic Sea (Pniewski et al. 2010). As a result of ML and NJ analyses, it was determined that the pairwise distances between *F. saprophila* strains were below 0.05, which indicates that the strains are closely related. Since morphological descriptions of the strains were not given in previous phylogenetic studies, no comparison could be made with the findings of the current study. These strains, which are close to each other in phylogeny and reported from different habitats, may have high tolerance to environmental variables and a high probability of survival in many different environments. To reach accurate and definitive conclusions, the whole genome of the species would have to be studied. Its phylogeny could not be analyzed due to the lack of data within the publicly available NCBI nucleotide database. *Fistulifera* is a genus that includes tiny raphid diatoms that require different morphological description techniques (Berthold et al. 2020). Their frustules are not well silicified. Thus, the valve outlines are unclear, and nearly no ornamentation is visible in light microscopy, with the major difficulties in identifying this genus arising from its very small cells and easily damaged frustules (Zgrundo et al. 2013). *Fistulifera saprophila* is known as a cosmopolitan opportunistic species that thrives in eutrophic and heavily degraded environments, often polluted with industrial wastewater (Zgrundo et al. 2013, Gastineau et al. 2021). The HIB112102 strain was isolated from surface waters (0.5 m) at the Keşan/Edirne station (North Aegean Sea) and is reported here as the first record of *F. saprophila* in Türkiye's coastal seawaters. While Solak et al. (2020) previously identified this species in the Sakarya River, this study highlights its adaptability and presence in marine environments.

Another diatom strain, HIB112103, from Sea of Marmara (Kadıköy/Istanbul) was seen in the *SSU* phylogeny analysis to be 99% of *Minutocellus polymorphus*, and the pairwise distance between strains was found to be below 0.05. However, due to the lack of cell size information in previous phylogenetic studies on closely related strains, it was not possible to compare them with the strains obtained in this study. The phylogeny based on *ITS* gene sequences revealed a close relationship of strain HIB112103 with MH129017, MH129018 and FJ864292, which were obtained from the culture collection whose location and morphologic description is not provided. *Minutocellus polymorphus* is a nano-sized, cosmopolitan planktonic centric marine diatom species reported to be found as short chains or as single cells in estuaries and the oceans (Hasle et al. 1983). This species was first described as *Bellerochea polymorpha* Hargraves & Guillard, but this nomenclature has been recognized as a synonym over the years (Guiry and Guiry 2024). Although *M. polymorphus* has been previously reported from different regions in the Mediterranean Sea (Schmidt et al. 2018, Zingone et al. 2023), it was recorded from the coastal waters of Türkiye for the first time in this study. Schmidt et al. (2018) reported that this species is an endosymbiont of the foraminifera *Pararotalia calcariformata* McCulloch, which means that the distribution range of the species can be expanded with its symbionts. The HIB112103 strain was isolated and cultured from Kadıköy/Istanbul station during the intense mucilage phenomenon in the Sea of Marmara. In the previous study (Cruz and Neuer 2022) it was reported that the nano-diatom *M. polymorphus* species can form microaggregates in axenic cultures, produce transparent exopolymeric particles (TEP), and that the concentration of TEP production varies depending on the bacterial species added. However, no information was provided about its abundance in the environment, as species identification could not be performed using bright-field microscopy due to its small size in the present study.

In this study, phylogenetic analyses of two *Pseudo-nitzschia pungens* strains for the *LSU* region and one strain for the *ITS* region were conducted. Previous records of *Pseudo-nitzschia* species in the Sea of Marmara were based on cell morphology, including *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. pseudodelicatissima*, *P. pungens*, and *P. seriata* (Balkis and Tas 2016, Kaleli and Akçaalan 2021). This is the first molecular genetics study of this genus using monoalgal cultures from the Sea of Marmara. Phylogenetic analysis of the *LSU* region grouped the strains into three regions: Pacific, Adriatic, and Black Sea. The strain HQ111393 (Lim et al. 2012) showed an affinity with HIB112104. The strain HIB112261 shared similarities with *P. pungens* strain AF417650 (Lundholm et al. 2002), but no length-to-width ratio was provided for comparison. Morphologically, the Sea of Marmara strains were closest to *P. pungens* MK682488 (Turk-Dermastia et al. 2020).

*Pseudo-nitzschia* species have been observed in high abundances during mucilage events in the Sea of Marmara, particularly in 2007–2008 and 2020–2021 (Balkis et al. 2011, Tas et al. 2020, Balkis-Ozdelice et al. 2021). While some species within this genus are known for their potential toxicity, no domoic acid (DA) analyses were conducted in these studies. Similarly, in the present study, toxin analysis was not performed, preventing any assessment of DA production by the examined strains. Nonetheless, the presence of *Pseudo-nitzschia* during mucilage events highlights the need for further investigations into its ecological role and potential toxin production in the region.

In this study, *M. polymorphus* and *F. saprophila*, two small-sized and morphologically challenging species, were reported for the first time in Turkish coastal waters. Detecting such species is particularly difficult without molecular studies on monoalgal cultures, meaning they may have previously remained undetected even if they were native. However, given the high international shipping traffic in the region, the introduction of alien species through ballast waters remains a strong possibility (Öztürk and Öztürk 1996). This highlights the need for strict monitoring and regulation of ballast water discharges. Beyond taxonomic identification, the presence of these species in the region raises questions regarding their ecological roles, particularly in relation to mucilage events. The mucilage phenomenon observed in the Sea of Marmara and the Aegean Sea in recent years has been closely related to the composition and dynamics of phytoplankton communities. In particular, determining the species contributing to mucilage formation is of great importance in understanding its effects on the ecosystem. Diatoms stand out as one of the main components of mucilage due to their ability to secrete extracellular polymeric substance (EPS). Pseudo-cryptic species, which are difficult to distinguish morphologically, may play an overlooked role in mucilage formation, underscoring the importance of molecular analyses in diatom research (Alverson 2008). Determining whether species, especially *M. polymorphus* and *F. saprophila*, have high EPS production potential is a critical step in understanding the biological basis of mucilage. Providing the aspect ratios of molecularly identified strains in this study would facilitate future taxonomic comparisons and improve our understanding of species morphology. While microscopic observations remain a cost-effective and rapid approach for preliminary identification, they must be supported by genetic validation for accurate species classification. Molecular methods not only ensure precise identification but also allow for the assessment of how environmental factors influence morphological variation. Furthermore, molecular phylogenetic analyses often reveal higher species diversity than suggested by traditional morphological methods, reinforcing the need for an integrated taxonomic approach. In this context, future studies should investigate the potential relationship between the species identified in this study and mucilage formation through laboratory experiments in more detail. In particular, their EPS production levels and responses to environmental factors should be evaluated, thus clarifying their contribution to mucilage formation. The presence of cryptic species could expand our understanding of the dynamics behind mucilage formation and provide direction for future research. Therefore, long-term observations supported by molecular analyses will help us gain a more comprehensive understanding of the role these species play in the mucilage ecosystem.

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### Author contribution statement

R.S. provided the samples, performed the laboratory work, participated in phylogenetic analysis, preparation of SEM samples and the manuscript; M.B. performed the laboratory work, provided the samples, participated in phylogenetic analysis, gave final confirmation for publication as supervisor of the research; T.D. provided the samples, performed the laboratory work, participated in phylogenetic analysis, SEM sample preparation, contribution for publication; N.B.O. did morphological identification, systematic analysis, gave contribution for publication; M.S. provided the samples, SCUBA, gave contribution for publication.

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Research Article

# Genetic variability of *Linnaea borealis* – remnant of Eastern-Southeastern Alpine populations indicates a strong bottleneck and *in situ* glacial survival

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**Abstract** – In a rapidly changing environment, habitat loss and fragmentation have primarily led to a decline in the numbers of numerous plant species. We compared the genetic variability of two small, isolated populations of *Linnaea borealis* L. with three populations from the core distribution area in the boreal region. The results show three main clusters, exemplars from the boreal distribution area being represented in all three clusters. The genetic variability within the two isolated Eastern-Southeastern Alpine populations was found to be very low, while genetic variability between them was very high. In addition, a low proportion of different genotypes was revealed in the Eastern-Southeastern Alpine populations as compared to the northern population. The high genetic variability between the two isolated Eastern-Southeastern Alpine populations indicates that most likely both are glacial relict populations, probably due to consecutive bottlenecks and long-term isolation under specific environmental conditions. Glacial relicts, which tend to be associated with endangered habitats, deserve urgent attention, especially when the recent anthropogenically-driven environmental crisis is considered.

**Keywords:** disjunct distributional range, genetic variability, glacial relict, *Linnaea borealis*, microsatellites

## Introduction

The current distribution areas of plants were shaped by various environmental changes during and after the glaciation period. Rapid fluctuations in environmental conditions shifted the boundaries of colonized areas (Hewitt 1996) and caused the extreme fragmentation and disjunct distribution of many plant species (Jermakowicz et al. 2017). Plant populations that occur at the edge of their distributional range or, in the case of disjunct distribution, which is caused by habitat rarity, are prone to environmental and biotic stressors. Plants that are scattered and isolated are more susceptible to variability in climate, habitat and biogenetic factors (Kołos et al. 2015). This applies to isolated remnants of e.g. Arctic-Alpine species, resulting from shifts in their distribution range during glacial and inter-glacial periods. However, it also applies to boreal-alpine species that survived climate change during the Holocene and now only occur in a few places in Central Europe (Svenning et al. 2008, Sundberg 2014, Kołos et al. 2015, Jermakowicz et al. 2017). In recent decades, the conservation value of relict populations has been emphasized as they are adapted to stressful ecological conditions, which could be important for future distribution range shifts owing to climate change (Vogler and Reisch 2013).

In a rapidly changing environment, many plant species have become rare due to habitat loss and fragmentation and are restricted to very small, isolated populations with an increased risk of local or regional extinction (Jermakowicz et al. 2017). Apart from immediate extinction, the most severe consequence of habitat fragmentation is the loss of genetic diversity. This occurs due to random genetic drift, increased inbreeding, and reduced gene flow between populations. This leads to a loss of offspring and of population fitness and at the same time reduces adaptability to environmental changes (Willi et al. 2006).

Long distances between isolated populations further reduce pollen transfer and limit pollination (Wilcock and Neiland 2002). Many authors (Scobie and Wilcock 2009, Gaudeul et al. 2019, Kikowska et al. 2022) have reported a

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decline or even complete failure of sexual reproduction in small populations of rare plant species. All these reproduction problems are even greater in species that rely on cross-pollination (Wróblewska 2013). A typical example is the twinflower, *Linnaea borealis* L. (Caprifoliaceae), a circumboreal plant species that is already in severe decline throughout Europe (except in its boreal range in Scandinavia). However, its geographical distribution ranges across the Northern Hemisphere, occurring from Scotland and northern Europe through Russia to Siberia, northern Asia to Kamchatka and Japan, northern China and Mongolia, and from Alaska and Canada to Greenland (Alm 2006). This clonal, self-incompatible plant is a shade-tolerant, evergreen dwarf shrub. In the natural Swedish populations, *L. borealis* reproduces vegetatively, mainly by stolons, and forms large clones up to 10 m long (Eriksson 1988, Niva 2003). In Scotland, low seed production is attributed to reproductive isolation caused by the lack of availability of compatible mates within populations and limited pollen dispersal between them (Ross and Roi 1990, Wilcock and Neiland 2002, Scobie and Wilcock 2009, Wiberg et al. 2016).

In the Alps, *L. borealis* is considered a relict species with some localities in France, Switzerland, Italy (Fornaciari 1964, Hegi 1966, Wilhalm 2010), Austria and Slovenia (the southeasternmost locality) (Wraber 1963). According to the literature, there should be three almost neighbouring sites in the Eastern-Southeastern Alps (Ernet in Franz 2011): in the Austrian part of Carinthia (1) and Styria (2), and in the Slovenian part of Styria (3). However, the species is still present at two sites (Slovenian and Austrian parts of Styria, but the third site (Carinthia (AT)) has not been confirmed since 1992 (Ernet and Franz 2011). All these sites are of great importance from a biogeographical point of view and raise a number of research questions related to the survival of *L. borealis* in this disjunct southeasternmost distribution. In this respect, the *L. borealis* can be considered a model species for the study of glacial relicts in the Alps. In addition, various conservation measures have already been implemented to guard against any further loss of its habitat and genetic diversity (Wróblewska 2013, Wiberg et al. 2016).

Our aim was therefore to investigate the genetic variability within and between two isolated (but spatially close) populations of *L. borealis* from the Eastern-Southeastern Alps (Julian Alps (SI) and Gurktaler Alps (AT)) and to compare this with the genetic variability of three populations from the northern range area (Sweden, Scotland and Russia). We used nine previously described microsatellite loci (A'Hara et al. 2011) to assess genetic variability. In this way, we attempted (1) to determine the extent to which the two isolated populations are genetically distinct and (2) to answer the question of whether the *L. borealis* of the Eastern-Southeastern Alps is a postglacial colonizer or a glacial relict. Our findings will provide essential information for the successful conservation strategies for the survival of this plant species *in situ* over a longer period of time, especially from the aspect of climate change.

## Material and methods

### Study area and field methods

In our study of the *L. borealis* population, we focused on two disjunct populations in the Eastern-Southeastern Alps, while the other three populations sampled across the distribution range served as control populations, for comparison (only). The Eastern-Southeastern Alps populations were in the Julian Alps, Soteska near Nomenj (495 m a.s.l), Slovenia (SI) and the second location was Dieslingsee in the Gurktaler Alps (1850 m a.s.l.), Styria, Austria (AT). Control populations were: (1) in a mixed coniferous forest (dominated by Scots pine) just outside the town of Bollnäs in central Sweden at an elevation of about 70 m a.s.l. (SE); (2) in the Cairngorms National Park in Scotland, United Kingdom, Great Britain (GB), and (3) in the Altay Mountains along the valley of the Aktru River, Russia (RU) (Fig. 1).

The population in the Julian Alps is the southeasternmost locality of *L. borealis* in Europe (Wraber 1963). The twinflower population (Fig. 2) grows in stands of the association *Rhodothamno-Rhododendretum hirsuti* (Aichinger 1933) Br.-Bl. & Sissingh in Br.-Bl. et al. 1939 (Wraber 1963, Šilc and Čarni 2012). The location in the Gurktaler Alps is in the Styrian part of the mountain range and was first mentioned by Ernet and Franz (2011). At this location, stands with *L. borealis* belong to the association *Rhododendretum ferruginei* Rübel 1911. The two Southeast Alpine populations grow on slopes exposed to the north, where cold air emerges from under the rocks, especially in summer (Martinčič 1977).

The plants were randomly sampled at four study sites (SI, AT, SE, RU) (Fig. 1). To avoid selecting the same genet, we sampled shoots growing at least 3-5 m apart, as suggested by Wróblewska (2013). The small number of samples is correlated with study site size of the Eastern-Southeastern Alps population which it is around 0.2 ha. The shoots were stored in a cool place (4 °C) in the laboratory until DNA isolation. DNA samples from all populations were collected with permission from representative institutions. In this study, we used pre-isolated DNA samples from GB. A total of seventy-three samples of *L. borealis* were included in the study.

### DNA isolation and microsatellite analysis

Total DNA was extracted from fresh young leaves using the CTAB protocol (Doyle and Doyle 1987). To approximately 2-3 square centimetres of fresh leaf tissue, one ml of preheated (68 °C) CTAB extraction buffer [2% (w/v) CTAB, cetyltrimethylammonium bromide (Sigma, Germany), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% 2-mercaptoethanol] was added and well homogenized with a mortar and pestle and transferred to a 2 mL tube. Samples were incubated for 1.5 h at 68 °C in a water bath. After incubation, 600 μL of chloroform: isoamyl alcohol in a proportion of 24:1 were added, and the samples were thoroughly mixed. The mixtures were centrifuged at 11000 rev./min for 10 min. After centrifugation, the supernatant was trans-

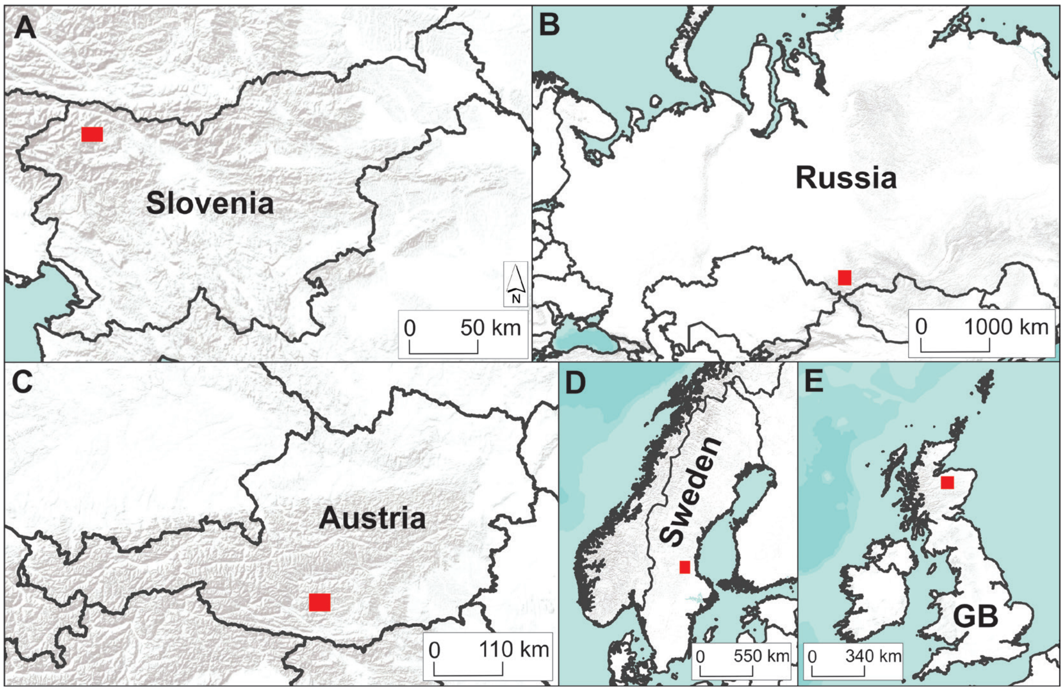

**Fig. 1.** Locations of study areas (🟥) of populations of *Linnaea borealis*. A – Soteska by Nomenj, Slovenia, B – Altay Mountains along the valley of the Aktru River, Russia, C – Dieslingsee, Austria, D – the city of Bollnäs, central Sweden, E – Great Britain.

ferred to a fresh 1.5 mL tube and the DNA was precipitated by the addition of 0.1 vol. of 3 M sodium acetate and 1 vol. of ice-cold isopropanol and kept at -20 °C for 30 min. Samples were again centrifuged at 11000 rev./min for 10 min. The pellet was washed in 70% ethanol for 20 min, air dried and rehydrated in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Two separate extractions were performed for each plant sample. The DNA concentration was estimated using a DNA fluorometer DQ 300 (Hoefer, Inc., Holliston, Massachusetts). For microsatellite analysis nine microsatellite loci developed by A'Hara et al. (2011) were used (On-line Suppl. Tab. 1). The number of primers sufficient for reliable variety identification depends on the nature and discriminating power of each primer. Cytogenetic study revealed that the number of chromosomes in cells of *L. borealis* is 2n = 32 (Packer 1964).

For the polymerase chain reaction (PCR) 5 µL multiplex PCR kit (Qiagen, Germany) 20 ng DNA, 0.5 µL of each primer (Sigma, Germany) and RNase-free water were used. PCR conditions consisted of a 15 min hot start at 94 °C; 40 cycles of denaturation at 94 °C for 30 s, annealing at 60.4 °C for 120 s and an extension step at 72 °C for 90 s. The reactions were terminated by incubation at 60 °C for 30 min. PCR was performed using a Whatman Biometra T-Gradient thermocycler (Goettingen, Germany). Capillary electrophoresis of PCR products was performed using a Beckman Coulter

**Fig. 2.** Habitat in Dieslingsee in the Gurktaler Alps, Styria, Austria (AT) (left) and *Linnaea borealis* in flower (right) (photo: N. Pipenbaher).

CEQ8000 sequencer, according to the manufacturer's instructions. Fragment size analysis was performed using the integrated software (Fragment Analyzer). A fluorescently labelled size marker (Beckman Coulter DNA Size Standard Kit 400 bp) was used as a molecular weight reference.

### Data analysis

For each microsatellite locus, the following genetic diversity parameters were assessed: the number of alleles per locus (n), allele frequencies, observed ($H_o$) and expected heterozygosity ($H_e$), and polymorphic information content (PIC) were calculated using the computer program Cervus 3.0.7 (Marshall et al. 2015). PIC is a measure of the quality of the informativeness of molecular markers. PIC values of more than 0.5 are considered highly informative for codominant markers such as SSRs (Serrote et al. 2020). In addition, we also evaluated the number of alleles present in only one genotype (private alleles). The observed ($H_o$) and expected heterozygosity ($H_e$) were also calculated for each population.

The genetic relationships among the 73 genotypes were evaluated using an unweighted Neighbor-Joining analysis with DARwin 6.0.18 software (Perrier and Jacquemoud-Collet 2005). Dissimilarities (30,000 bootstraps) were calculated using the Dice coefficient (Dice 1945), and the dendrogram was constructed.

The genetic structure of the collection was analysed using the Bayesian method performed with STRUCTURE V2.3.4 software (Pritchard et al. 2000). For each simulation, 20 independent runs were performed for each K from 1-20. A burn-in period of 100.000 followed by 750.000 MCMC (Markov Chain Monte Carlo) repetitions was performed. The Structure Harvester V0.6.94 application (Earl and Vonholdt 2012), implemented using the $\Delta$K method described by Evanno et al. (2005) determined the most relevant K value. The samples with a threshold value of 90% were assigned to a specific group and the remaining samples were classified as admixed. The Q-matrix obtained by the STRUCTURE software, complemented with the 'tidyverse' R package (Wickham et al. 2019), was used to generate the bar plots. In addition, the following parameters were calculated with the latter software: average distances (expected heterozygosity) between individuals of the same group and the divergence of allele frequencies between populations (net nucleotide distance).

To assess the proportional differences in genetic variability with and between populations (Slovenia, Austria, Sweden, Great Britain, and Russia), a PERMANOVA analysis was performed using the packages VEGAN (Oksanen et al. 2006) and LATTICE (Deepayan 2008, Sarkar 2008) in R statistical environment (R Development Core Team 2021). We operated with the Euclidian distance parameter and 999 permutations. To plot the results, an NMDS approached was performed by applying 'vegan' R package (Venables and Ripley 2002, Oksanen et al. 2006). The betadisper function and the corresponding ANOVA was used to test for differences or similarities in data dispersion with and between the study sites.

## Results

Molecular analysis: Microsatellite analysis revealed 86 alleles at 9 microsatellite loci. The number of alleles detected per locus (Tab. 1) ranged from 6 (locus D110 and D118) to 16 (locus B119), with an average of 10 alleles per locus.

**Tab. 1.** Allele sizes (bp) and allele frequencies (in parentheses) of the 73 *Linnaea borealis* genotypes at nine microsatellite loci (A5, A102, D110a, D7, D110, D118, A112, B119, C105).

| Locus | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| A5 | A102 | D110a | D7 | D110 | D118 | A112 | B119 | C105 |
| 159 (0.007) | 172 (0.007) | 220 (0.007) | 146 (0.034) | 215 (0.007) | 169 (0.260) | 223 (0.021) | 193 (0.014) | 237 (0.021) |
| 161 (0.007) | 184 (0.178) | 264 (0.007) | 156 (0.075) | 219 (0.527) | 172 (0.233) | 227 (0.164) | 196 (0.027) | 241 (0.027) |
| 163 (0.301) | 186 (0.034) | 270 (0.548) | 158 (0.274) | 221 (0.192) | 175 (0.363) | 229 (0.116) | 198 (0.014) | 243 (0.041) |
| 171 (0.322) | 192 (0.007) | 276 (0.021) | 160 (0.281) | 223 (0.123) | 178 (0.027) | 232 (0.021) | 202 (0.158) | 246 (0.082) |
| 173 (0.322) | 200 (0.103) | 278 (0.103) | 162 (0.233) | 227 (0.227) | 182 (0.110) | 235 (0.021) | 204 (0.158) | 248 (0.301) |
| 177 (0.027) | 202 (0.137) | 282 (0.274) | 16 4 (0.075) | 229 (0.041) | 184 (0.007) | 237 (0.041) | 209 (0.21) | 250 (0.110) |
| 191 (0.014) | 204 (0.048) | 284 (0.027) | 166 (0.027) | | | 239 (0.041) | 211 (0.014) | 252 (0.342) |
| | 206 (0.082) | 290 (0.007) | | | | 241 (0.116) | 214 (0.027) | 254 (0.068) |
| | 208 (0.110) | 302 (0.007) | | | | 243 (0.130) | 218 (0.082) | 263 (0.007) |
| | 210 (0.151) | | | | | 245 (0.082) | 222 (0.137) | |
| | 218 (0.103) | | | | | 249 (0.116) | 224 (0.014) | |
| | 220 (0.041) | | | | | 255 (0.027) | 226 (0.110) | |
| | | | | | | 257 (0.089) | 230 (0.007) | |
| | | | | | | 259 (0.014) | 232 (0.103) | |
| | | | | | | | 234 (0.103) | |
| | | | | | | | 238 (0.014) | |

Parameters of genetic variability were calculated for all loci (clones excluded) (Tab. 2). Observed heterozygosity ($H_o$) ranged from 0.426 (locus D110) to 0.957 (locus C105), with an average of 0.794. Expected heterozygosity ($H_e$) ranged from 0.604 (locus D110) to 0.906 (locus A112), with an average of 0.768. Differences between observed and expected heterozygosity were examined for all loci studied. The largest difference was observed at locus A5 (0.187) and the smallest at locus D7 (0.007). The average of observed (0.794) and expected (0.768) heterozygosity was quite similar. At 5 of 9 loci (A5, D110a, A112, B119, C105) the observed heterozygosity ($H_o$) was higher than the expected ($H_e$), but at four loci (A102, D7, D110 and D118) $H_o$ was lower than $H_e$. The most informative locus for this group of genotypes was A112, with a polymorphic information content (PIC) of 0.898, and the least informative locus was D110a, with a PIC of 0.553.

The parameters of genetic variability were also calculated for each of the 5 populations studied (Tab. 3). The results show the difference between the observed ($H_o$) and the expected heterozygosity ($H_e$) in all populations. The lowest value of $H_e$ (0.560) was found in population RU and the highest (0.717) in population SE. The higher expected heterozygosity in the SE population can be explained by the higher number of individuals included (14 different genotypes).

A total of 12 private alleles were found for most loci: A5 and D110 (3 private alleles), A10 (2 private alleles), D110, D118, B119 and C105 (1 private allele). No private alleles were found for loci D7 and A112. Sample AT 1 stood out from the Austrian population with three private alleles. In addition, two private alleles were found in samples SE 12 and GB 10 from Sweden and UK, while five samples had only one private allele (SE 3, SE 6, SE10, GB 2 and GB 4). The population from Sweden not only contained the highest number of genotypes with private alleles, but also the highest number of private alleles per population.

Genetic relationships: The unweighted Neighbor-Joining dendrogram calculated from the microsatellite dataset of *L. borealis* divided the material into three main clusters (Fig. 3). The first main cluster comprised five subclusters. Subcluster 1 included all samples collected in Slovenia. The second subcluster consisted only of samples from Great Britain. The third and fourth subclusters consisted of samples from Great Britain, Russia and Sweden, and the fifth subcluster contained only samples from Sweden. The second cluster was divided into two subclusters, with the first subcluster containing all samples collected in Austria and the second subcluster containing samples from Sweden. The third cluster contained only samples collected in Russia.

Although we avoided sampling the same specimens, only eight samples (out of 15) represented distinct genotypes in Slovenia and only six (out of 15) in Austria. In the Sweden population, 14 samples represented distinct genotypes, in Russia 6 out of 15, and in Great Britain 13 out of 13. Looking at the length of the horizontal branches in the dendrogram, it can be seen that, as expected, genetic variability was the highest in the boreal population and diversity was very low in the two Eastern-Southeastern Alpine populations. Surprisingly, the isolated populations from Austria and Slovenia, which are very close to each other, turned out to be completely different and showed a very high degree of variation between the populations.

**Tab. 2.** Parameters of genetic variability calculated for different microsatellite loci of the 47 (clones excluded) *Linnaea borealis* genotypes: n – number of alleles, $H_o$ – observed heterozygosity, $H_e$ – expected heterozygosity, PIC – polymorphic information content.

| Locus | n | $H_o$ | $H_e$ | PIC |
|---|---|---|---|---|
| A5 | 7 | 0.894 | 0.706 | 0.649 |
| A102 | 12 | 0.766 | 0.880 | 0.868 |
| D110a | 9 | 0.787 | 0.610 | 0.553 |
| D7 | 7 | 0.787 | 0.794 | 0.764 |
| D110 | 6 | 0.426 | 0.604 | 0.574 |
| D118 | 6 | 0.723 | 0.747 | 0.703 |
| A112 | 15 | 0.915 | 0.906 | 0.898 |
| B119 | 16 | 0.894 | 0.885 | 0.875 |
| C105 | 9 | 0.957 | 0.780 | 0.753 |
| Average | 9.667 | 0.794 | 0.768 | |

Genetic structure: the results of the Bayesian analysis revealed that the most relevant number of groups (ΔK) for the analysed data was K = 3 (On-line Suppl. Fig. 1, On-line Suppl. Tab. 2), which divided the material into three groups (Fig. 4).

Using a membership threshold value of > 90%, only 5 (7%) samples were considered admixed, while 68 (93%) samples belonged to identified groups (On-line Suppl. Tab. 3). When the distribution of groups in the material studied was observed, Group 1 (green) consisted of 13 genotypes, Group 2 of 15 genotypes and Group 3 of the highest number of genotypes (40). Group 1 contained material from Austria, Group 2 comprised genotypes from Slovenia, while Group 3 consisted of diverse material from different origins (Great Britain, Russia and Sweden). In the two admixed samples (AT 1 and AT 15) from Austria, the majority of the genome was assigned to Group 1, while the remaining samples (SE 1, SE 3 and SE 10) corresponded to Group 3. Allele

**Tab. 3.** Parameters of genetic variability calculated for five populations of the 47 *Linnaea borealis* genotypes: number of genotypes, n – number of alleles, $H_o$ – average observed heterozygosity, $H_e$ – average expected heterozygosity. AT – Austria, SI – Slovenia, SE – Sweden, GB – Great Britain, RU – Russia.

| Population | No. Genotypes | n | $H_o$ | $H_e$ |
|---|---|---|---|---|
| SI | 8 | 24 | 0.917 | 0.549 |
| AT | 6 | 26 | 0.815 | 0.497 |
| RU | 6 | 33 | 0.815 | 0.560 |
| SE | 14 | 60 | 0.698 | 0.717 |
| GB | 13 | 57 | 0.803 | 0.689 |

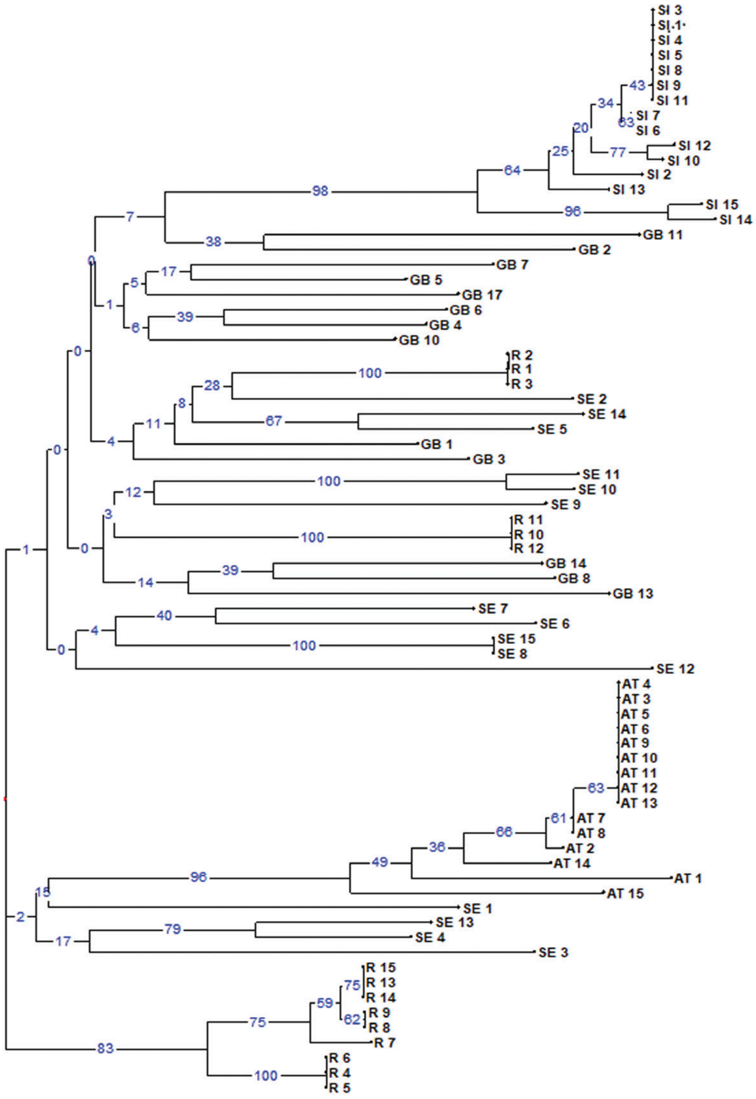

**Fig. 3.** Neighbor-Joining dendrogram describing genetic relationships among 73 *Linnaea borealis* genotypes from five locations: AT – Austria, SI – Slovenia, SE – Sweden, GB – Great Britain, RU – Russia.

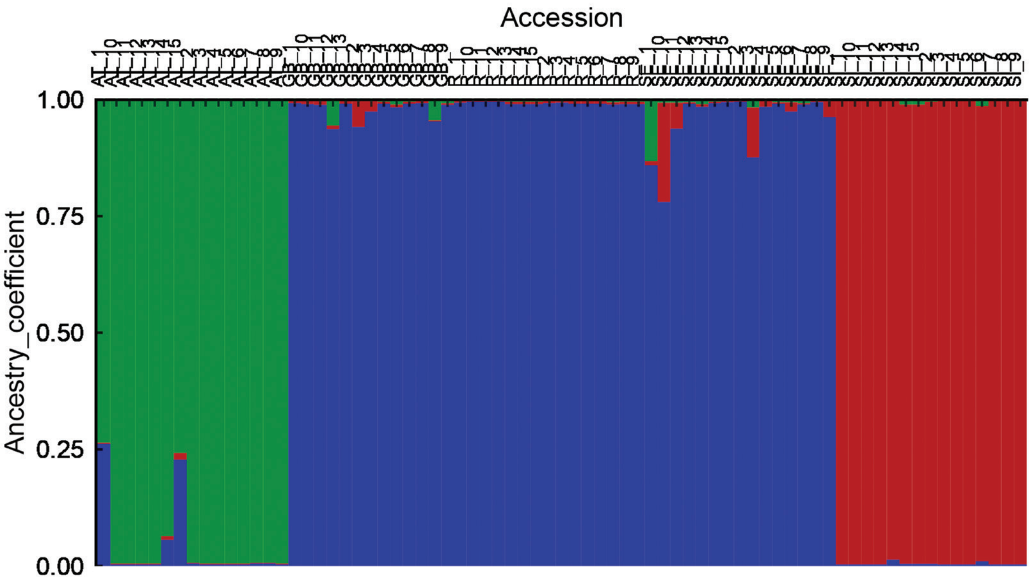

**Fig. 4.** Bar plot of Bayesian analysis results (K=3): group 1 (green), 13 genotypes from Austria; group 2 (red) 15 genotypes from Slovenia, and group 3 (blue) representing genotypes from different locations (GB, RU, and SE). Five genotypes were considered admixed.

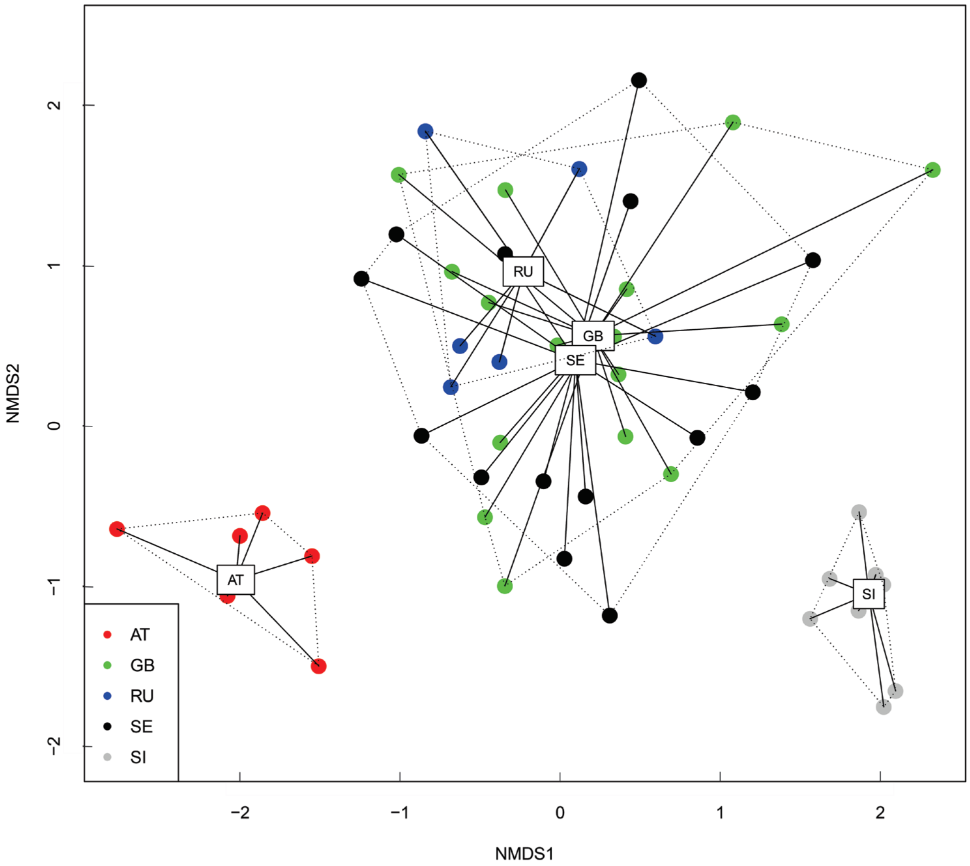

**Fig. 5.** Genetic variability with and between population at study sites. AT – Austria, SI – Slovenia, SE – Sweden, GB – Great Britain, RU – Russia.

frequency divergence between the three groups computed with STRUCTURE software revealed a relatively high proximity between Group 1 and Group 2 (0.0488), while Group 2 and Group 3 appeared more diverged (0.2134), although less than Group 1 and Group 3 (0.2547) (On-line Suppl. Tab. 4). In addition, allelic variation revealed the highest mean distances between individuals for Group 3, while the mean distances for individuals in Groups 1 and 2 were lower (On-line Suppl. Tab. 4).

The PERMANOVA analyses showed significant differences in genetic variability ($P < \alpha$, $\alpha = 0.05$) among the observed populations. However, the Euclidean distance-based NMDS plot (Fig. 5) revealed that the RU, SE and GB populations were genetically closer to each other than the AT and SI populations. This was also confirmed by the significant ($P < \alpha$, $\alpha = 0.05$) beta-dispersion test. Accordingly, genetic variability was significantly lower in the AT and SI populations. The results showed that the two observed Eastern-Southeastern Alpine populations also differ genetically (Fig. 5).

## Discussion

Analysis based on nine microsatellite loci, revealed very low genetic variability within the two isolated Eastern-Southeastern Alpine populations of *L. borealis* (SI and AT). This was to be expected as only a low number of specimens (individuals) were recorded at each location (sampling site) and therefore only a limited number of distinguishable genets were available. A low number of individuals could also be because of the small size of the remnant. In Slovenia the site of the population is around 0.2 ha, in Austria it is even smaller. However, a very high genetic variability was found between the two populations (SI and AT), even though they are, compared to other sampled populations, relatively closely positioned. This indicates a very low or no dispersal ability; nevertheless, the absence of suitable habitats should not be underestimated. This leads to the conclusion that the two spatially and reproductively isolated Eastern-Southeastern Alpine populations most likely represent glacial relicts, such a pattern having been observed in previous studies of glacial relict plants (e.g. Hensen et al. 2010, Jermakowicz et al. 2017). These researchers explained the high genetic variability between populations by consecutive bottlenecks and long-term reproductive isolation. In addition, Vogler and Reisch (2013) claimed that genetic variability may be high in the case of different populations of glacial relicts and low in the case of postglacial colonization. Low genetic diversity within the population might be also explained as a shift in mating system from outcrossing to selfing (Surina et al. 2023).

In a comprehensive study by Wróblewska (2013), a phylogeographic structure derived from plastid DNA and a moderate genome-wide diversity estimated from AFLP markers were found for *L. borealis* in Eurasia. Six haplotypes were identified in an area from Scotland to Altai and from Norway to Italy. However, Wróblewska (2013) concluded that although half of the populations studied were highly isolated, they still showed similar levels of genetic diversity across the geographic range. Furthermore, she stated that she had found no support for the hypothesis that a bottleneck and/or inbreeding. factors affecting genetic diversity, were associated with habitat fragmentation. The case of *L. borealis* clearly shows that it was not habitat fragmentation, but rapidly changed climatic conditions after the last glaciation that caused the narrow isolation of the species leading to glacial relict populations, like the one in Slovenia and Austria. Regarding the geographical distribution of Alpine populations, she only sampled in the Western Alps (Aosta, Italy; Les Allues, France) and the Central Alps (Zernez, Switzerland). However, according to Meusel and Jäger (2017), its distribution is not that rare. East of the line Innsbruck–Wipp Valley–Brenner Pass–Eisack Valley–Etsch Valley there are only scattered small populations, especially in the Hohe Tauern mountain range. From there, *L. borealis* has not been found in the rest of the Eastern Alps, with the exception of three localities on the south-eastern borders of the Alps (Ernet and Franz 2011). This could be the reason why four out of six haplotypes occur at the above-mentioned locations in the Western and Central Alps.

In any case, the warming after the end of the last glaciation led to a loss of suitable habitats, resulting in a more or less strong fragmentation of the Alpine population, which eventually led – at least in the Eastern Alps – to only a few isolated patches remaining in specific habitat conditions related to elevation, northern exposure or particular situations in terms of substrate conditions. Ernet and Franz (2011) mentioned the boulder-strewn slopes where cold air typically emerges from under the scree in summer. This is exactly the characteristic of the Austrian location in the Gurktaler Alps, but is even more pronounced at the Slovenian site, where at the extremely low elevation of only 495 m a.s.l., from holes in the rubble ice-cold air blows during the growing season. There is no direct sunlight on the steep northern slopes, which also increases soil and air humidity. According to Wraber (1963), therefore, no zonal beech forest has developed at this location, but a community dominated by *Rhododendron hirsutum* L., *Pinus sylvestris* L. and *Larix decidua* Miller.

To conclude, it is well known that the most severe negative impact of fragmentation is caused by genetic drift, increased inbreeding, and reduced gene flow among populations (Scobie and Wilcock 2009, Wiberg et al. 2016). This led to a reduced number of offspring and a reduction in population fitness (Scobie and Wilcock 2009). In the case of *L. borealis* which is a self-incompatible plant, the effect of isolation on reproductive success is even greater. For successful fertilization, self-incompatible plants like *L. borealis* require cross-pollination with a compatible mate, which does not belong to the same clone. It has been reported that in extreme cases where all remaining plants have the same alleles for genetic control of pollen-pistil interactions seed set can be reduced to zero (Demauro 1993). However, in small populations of many clonal, self-incompatible plants such as *Rubus saxatilis* L., *Aster furcatus* E.S.Burgess, *Calystegia collina* (Greene) Brummitt, *Arnica montana* L., *Ranunculus reptans* L. and *Maianthemum bifolium* (L.) F.W.Schmidt (Scobie and Wilcock 2009), the limited availability of partner plants is increasingly being identified as the cause of reproductive failure, the plants perhaps adapting to turning to selfing.

Assuming that the two isolated populations of *L. borealis* in the Eastern-Southeastern Alps are glacial relicts with considerably reduced genetic variability, further research on their viability and reproductive mating system is needed to ensure better conservation success.

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### Author contribution statement

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

### Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Research Article

# Notes on rare and newly found *Carex* taxa (Cyperaceae) in Croatia

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**Abstract** – Although the flora of Croatia is rather well known, several new *Carex* species and hybrids were able to be added during a caricological visit to Croatia and after two more years of fieldwork (2023–2024). The total number of *Carex* species is now 91; besides, there are eleven *Carex* hybrids known. During our herbarium research and revision in several European herbaria, and recent fieldwork, five new *Carex* taxa for Croatia were found: *C. distachya* subsp. *phyllostachioidea*, *C. depressa* subsp. *transsilvanica*, *C. nigra* subsp. *juncea*, *C. pairae*, and *C. secalina*, while *C. aterrima* subsp. *aterrima* was overlooked on the previous Croatian national list. In addition, eleven hybrids were found that were new for Croatia: *C.* × *alberti*, *C.* × *alsatica*, *C.* × *auroniensis*, *C.* × *leutzii*, *C.* × *oberrodensis*, *C.* × *oenensis*, *C.* × *prolixa*, *C.* × *pseudoaxillaris*, *C.* × *subviridula*, *C.* × *villacensis*, and *C.* × *xanthocarpa*. Moreover, several rare *Carex* species were confirmed at known locations and new localities were detected for multiple species. The following species were involved: *C. atrata*, *C. buekii*, *C. capillaris*, *C. cespitosa*, *C. fritschii*, *C. illegitima*, *C. lasiocarpa*, *C. pulicaris*, *C. punctata*, *C. randalpina*, *C. rupestris*, and *C. vulpinoidea*. *Carex limosa* has to be considered as extinct from Croatia. Search for the species *C. arenaria*, *C. diandra*, *C. frigida*, *C. melanostachya*, *C. pauciflora*, *C. stenophylla*, and *C. supina*, has revealed only very old herbarium specimens, but there has been no recent confirmation in the field. For *C. dioica* there was no material available, nor could it be confirmed in the field. Two hybrid names, *C.* × *rossiana* and *C.* × *villacensis*, are lectotypified here.

**Keywords**: Balkan Peninsula, biodiversity, herbarium revision, hybrids, lectotypification

## Introduction

The flora of Croatia has been studied relatively well and a lot of information can be found in the national Flora Croatica Database – FCD (Nikolić et al. 2014, Nikolić 2019, 2020a, b, c, 2024). To date, 87 species of the genus *Carex* L. (Cyperaceae) have been recorded in Croatia, with some subspecies and varieties, and two hybrids in literature (Koopman 2022). Several of these 87 *Carex* species in Croatia have been found for the first time in recent years: *C. buekii* Wimm. by Alegro and Marković (1999), *C. pulicaris* L. by Topić and Ilijanić (2001), *C. randalpina* B.Walln. by Stančić (2009), *C. punctata* Gaudin by Koopman and Topić (2011), *C. phyllostachys* C.A.Mey. by Terlević et al. (2021), and *C. vulpinoidea* Michx. by Király et al. (2021). We have been able to add in total 17 new *Carex* taxa, including eleven hybrids, to the flora of Croatia. Special attention has been paid to very rare ones.

*Carex arenaria* L., *C. frigida* All., *C. limosa* L., *C. melanostachya* M.Bieb. ex Willd., *C. pauciflora* Lightf., and *C. stenophylla* Wahlenb. are dubious in Croatia according to FCD (Nikolić 2024). According to the Red Book of Vascular Flora of Croatia (Nikolić and Topić 2005) and Topić and Stančić (2006), 16 species are threatened, of which *C. bohemica* Schreb. and *C. pulicaris* L. are critically endangered (CR); *C. davalliana* Sm., *C. divisa* Huds., *C. echinata* Murray, *C. extensa* Gooden., *C. flava* L., *C. hostiana* DC., *C. lepidocarpa* Tausch, *C. nigra* (L.) Reichard, and *C. oederi* Retz. are endangered (EN), while *C. panicea* L., *C. riparia* Curtis, *C. rostrata* Stokes, and *C. vesicaria* L. are vulnerable (VU).

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The aim of this article is to present an update of the knowledge about the genus *Carex* in Croatia, based on field investigations and herbarium revisions in 2023 and 2024.

## Material and methods

Fieldwork was carried out from May to October 2023 and during the vegetation season of 2024 (in total 54 fieldwork days), mainly in the continental lowlands of Croatia, the Mediterranean part of Croatia, as well as in the mountain area of Gorski Kotar, Mt Velebit and Mt Dinara.

Moreover, herbarium material from B, BP, CNHM, HHMR, SZUB, ZA, ZAGR, and ZAHO as well as from the virtual herbaria P, W, and WU, has been consulted and revised. Abbreviations of herbaria follow the Index Herbariorum (Thiers 2024). In total more than 3000 herbarium sheets were consulted and revised. Recently collected specimens have been deposited at ZAGR (University of Zagreb, Croatia), SZUB (University of Szczecin, Poland), and in Jac. Koopman's private herbarium (Choszczno in Poland). A complete set of herbarium specimens of new *Carex* taxa will be made accessible in ZAGR virtual herbarium according to Bogdanović et al. (2016).

The nomenclature of carices follows Koopman (2022). For nomenclatural issues the latest ICN was followed (Turland et al. 2025, Turland 2019). The Appendix presents a list of all currently known *Carex* taxa in Croatia, including hybrids.

## Results

Currently there are 91 species of *Carex* known from Croatia, the occurrence of 75 of which (82%) was confirmed with certainty during our fieldwork and study of herbarium material in several herbaria. In total, five taxa new for Croatia were found: *C. distachya* Desf. subsp. *phyllostachioidea* (Ö.Nilsson) Jac.Koopman, Więcław, Bogdanović & T.Denchev, *C. depressa* Link subsp. *transsilvanica* (Schur) K.Richt., *C. nigra* (L.) Reichard subsp. *juncea* (Fr.) Soó, *C. pairae* F.W.Schultz, and *C. secalina* Willd. ex Wahlenb. One taxon, *C. aterrima* Hoppe subsp. *aterrima*, was overlooked on the previous Croatian national list of the vascular flora and is now added. Moreover, eleven new *Carex* hybrids for Croatia were detected during our herbarium research and fieldwork (see Appendix). Below there is a discussion of the rare and new *Carex* taxa found during our fieldwork and research on herbarium material, alphabetically arranged. At present, the genus *Carex* in Croatia comprises 91 species, 26 subspecies and eleven varieties, as well as eleven hybrids with certainty.

## Discussion

### *Carex aterrima* Hoppe subsp. *aterrima*

The occurrence of *Carex aterrima* in the national Flora Croatica Database was omitted and overlooked in the recent past, although it had been mentioned in older literature (Horvat 1931:194, Degen 1936: 584, Horvat 1952: 208) from two valleys on Mt Velebit. However, this taxon has been found during our study in the herbaria of BP, ZA and ZAHO. Subsequently we carefully checked for its occurrence in potential habitats on Mt Velebit, and were able to confirm its existence at three sites (Fig. 1A).

Specimens examined: Sjeverni Velebit: Gromovača; u dolini južno Jerkovića dolca, 23 Jul 1929, *I. Horvat s.n.* (ZA 78121); Flora Croatica: Sjev. Velebit, Kukovi, 23 Jul 1926 (sub. *Carex atrata*), *I. Horvat s.n.* (ZAHO); Comit. Lika Krbava. Velebit: in dolinis ad cacumen alpis Kozjak prope pagum Stirovaca, alt circ. 1550 m, 21 Sep 1908, *J. B. Kümmerle 684* (BP); North Velebit, Nature Park Velebit, Mt Kozjak, in sinkhole, ca. 1550 m, 44°44'2.8" N, 15°1'42.3" E, 23 Jul 2024, *S. Bogdanović s.n.* (ZAGR); National Park North Velebit, in sinkhole beetwen Gromovača and Rossijeva koliba, ca. 1550 m, 6 Sep 2024, *S. Bogdanović & I. Ljubičić s.n.* (ZAGR); National Park North Velebit, in sinkhole east of Rossijeva koliba, ca. 1570 m, 44°45'55.4" N 14°59'25.0" E, 6 Sep 2024, *S. Bogdanović & I. Ljubičić s.n.* (ZAGR).

### *Carex atrata* L. subsp. *atrata*

In the Croatian flora, this rare taxon is known from a few alpine localities in Gorski Kotar and the mountains of Velebit and Plješivica (Horvat 1930, 1952, 1952–1953, Modrić Surina and Surina 2010). It occurs in the vegetation of snow-beds and in dolines (sinkholes). During our field survey, we could confirm its presence on Mt Risnjak and Mt Snježnik in Gorski Kotar, and on Zavižan on Mt Velebit (Fig. 1B).

Specimens examined: Velebit. Zavižan, Jul 1974, *I. Trinajstić s.n.* (CNHM); Lička Plješivica: Žestikovac, ca 1500 m, 17 Jul 1925, *I. Horvat s.n.* (ZA 20322); Sjeverni Velebit, Zavižan, u Modrić dolcu, u klekovini krivulja, 27 Jul 2023, *S. Bogdanović s.n.* (ZAGR); NP Risnjak, okolica Platka, Medlužine, u ponikvi u šumi, 8 Jul 2023, *S. Bogdanović & S. Ćato s.n.* (ZAGR); NP Risnjak, Snježnik-Guslica, rudine, 9 Jul 2023, *S. Bogdanović & S. Ćato s.n.* (ZAGR); Gorski Kotar, ponikva Škurina, 10 Jul 2023, *S. Bogdanović & S. Ćato s.n.* (ZAGR).

### *Carex buekii* Wimm.

The occurrence of this species in Croatia was first confirmed in April 1997 by Alegro and Marković (1999), who found the species in Gornje Prilišće and Vukova Gorica along the River Kupa in the WNW of Croatia, bordering Slovenia (see also Koopman et al. 2018). We could find this species further to the south on two sites, near Plaški and Dretulja (Karlovac County), and further to the west, near Belo (Primorje-Gorski Kotar County), also close to the River Kupa (Fig. 1C). During the revision of herbarium material in ZAGR one specimen was found from Vukovar in eastern Croatia, and in CNHM one specimen from Lika.

Specimens examined: Karlovac County, near Plaški, 45°5'28.0" N, 15°22'8.5" E, 375 m, wet meadow, *Magnocaricion*,

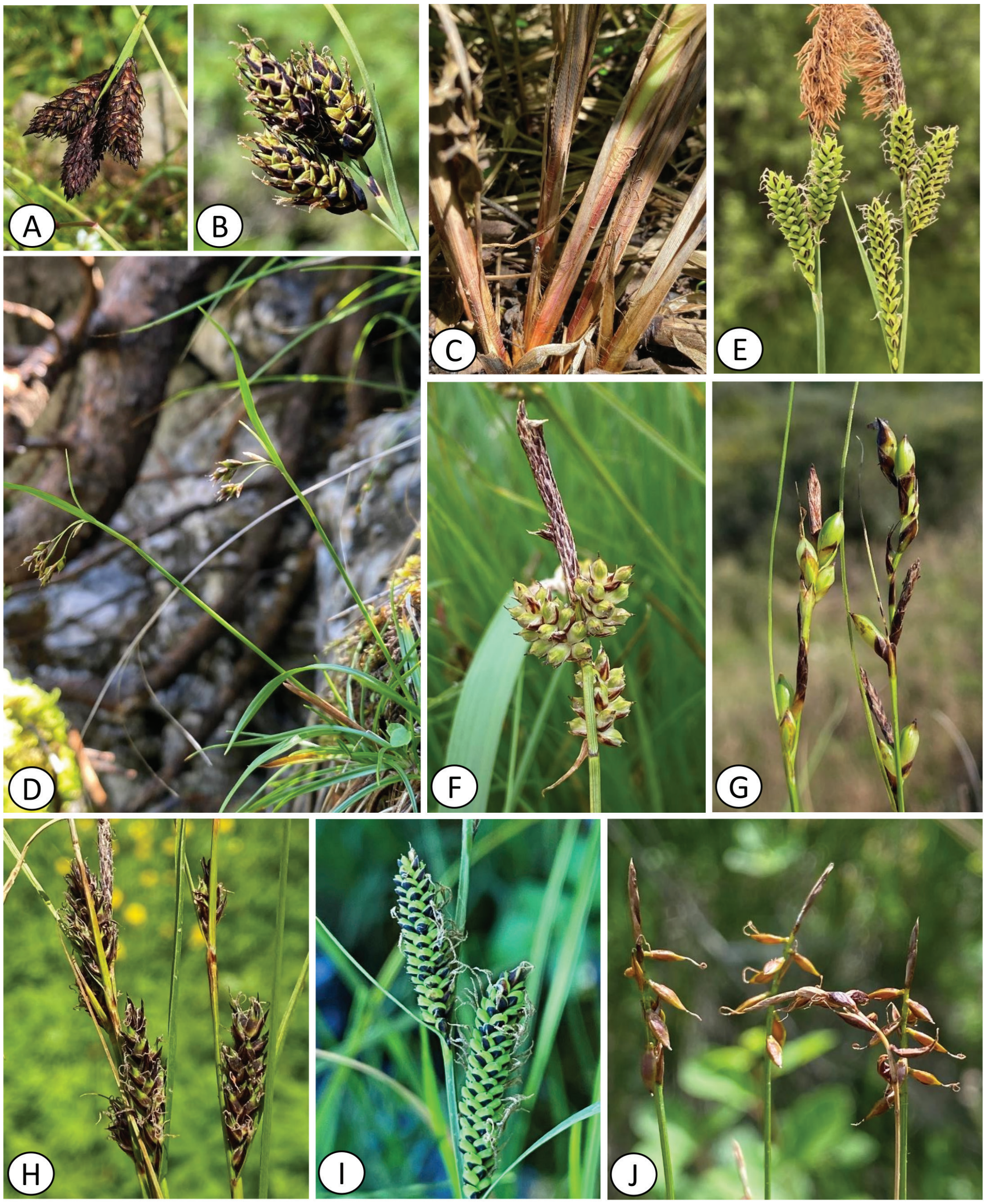

**Fig. 1.** Photos of some rare and newly found *Carex* taxa from Croatia discussed in this article. A – *C. aterrima*, B – *C. atrata*, C – *C. buekii*, D – *C. capillaris*, E – *C. cespitosa*, F – *C. fritschii*, G – *C. illegitima*, H – *C. lasiocarpa*, I – *C. nigra* subsp. *juncea*, J – *C. pulicaris* (photo: S. Bogdanović).

20 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, ZAGR); Primorje-Gorski Kotar County, 170 m E of village Belo, 45°28'30.4" N, 14°53'49.4" E, 228 m, wet meadow, 23 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, ZAGR); near Plaški, close to spring of Dretulja stream, 45°4'40.5" N 15°20'49.5" E, 21 Jun 2023, *S. Bogdanović s.n.* (ZAGR); Vukovar County, Vukovar, lokalitet Lijeva bara, vlažna livada na nagibu uz baru, 45°20'12" N, 19°0'36" E, 30 Mar 2020 (sub. *Carex elata*), *V. Mudri & I. Vitasović Kosić s.n.* (ZAGR 61036); N Croatia, Varaždinske Toplice, east of cemetery, wet meadow, 30 Jun 2023, *S. Bogdanović s.n.* (ZAGR); Značajni krajobraz, Barećeve špilje, u jarku, 44°59'35.70" N, 15°43'36.40" E, 11 May 2021, *S. Buzjak s.n.* (CNHM).

### *Carex capillaris* L. subsp. *capillaris*

In the Croatian Red Book of the Vascular Flora, *Carex capillaris* is listed as "data deficient" (Nikolić and Topić 2005). It is known from a few sites in Gorski Kotar, Vela Kapela, Klek, Lička Plješivica, and Sjeverni Velebit (Horvat 1930, 1952, Modrić Surina and Surina 2010). It is a rare species growing within the alpine belt in the vegetation of snow-beds and in valleys (sinkholes), among rocky crevices in wetlands. During our fieldwork, we confirmed it in Gorski Kotar and on Sjeverni Velebit (Fig. 1D).

Specimens examined: Sjeverni Velebit, Zavižan, u Modrić dolcu, u klekovini krivulja, 27 Jul 2023, *S. Bogdanović s.n.* (ZAGR); Gorski Kotar, ponikva Škurina, 10 Jul 2023, *S. Bogdanović & S. Ćato s.n.* (ZAGR); Gorski Kotar, Veliko Snježno, 10 Jul 2023, *S. Bogdanović & S. Ćato s.n.* (ZAGR); Velika Kapela, Bijele Stijene, na vrhu, na kamenitim blokovima, eksp. N, 25 Jun 1932, *I. Horvat s.n.* (ZAHO); Velebit, Mali Rajinac, supra Krasno, 1699 m, 26 Jul 1907, *Lj. Rossi 17.549* (ZA 15308).

### *Carex cespitosa* L.

This species was found for the first time in Croatia on Drežničko polje by J. Topić in 2009 (Nikolić 2024). Afterwards it was only mentioned for the same locality and for Rijeka but it was not confirmed by Jiménez-Mejías et al. (2014). During our survey, it was confirmed in Drežničko polje where it grows in karstic wetland (Fig. 1E).

Specimen examined: Lika, Drežničko polje, 45°9'27.7" N, 15°5'56.1" E, 21 May 2023, *S. Bogdanović s.n.* (ZA, ZAGR).

### *Carex distachya* Desf. subsp. *phyllostachioidea* (Ö.Nilsson) Jac.Koopman, Więcław, Bogdanović & T.Denchev

*Carex distachya* was split up into two varieties by Nilsson (1985), *C. distachya* var. *distachya* and *C. distachya* var. *phyllostachioidea* Ö.Nilsson, the latter only known from Asian West Türkiye and the East Aegean Islands. Koopman et al. (2025) show that this taxon has a wider occurrence in Southeast-Europe, and they raised it to subspecies level. Recent herbarium revision of *C. distachya* material has revealed the existence of this subspecies from Mt Biokovo in Croatia. From the typical subspecies it differs in having wider leaves (0.9–2.5 mm), utricles with 2 distinct veins and with a somewhat scabrid beak (see Koopman et al. 2025). This taxon occurs (at least) in Türkiye, Greece, Bulgaria, Montenegro, and Croatia. In Croatia, the westernmost part of its distribution range is involved.

Specimen examined: Kotišina, 31 May 1993, *Lj. Regula-Bevilacqua s.n.* (ZA 51309). [New for Croatia].

### *Carex depressa* Link subsp. *transsilvanica* (Schur) K. Richt.

Examination of the herbarium collection in BP revealed the existence of one herbarium sheet collected by L. Rossi from Mt Velebit, named as *Carex* × *rossiana* Degen [*C. caryophyllea* var. *trachyantha* (Dorner ex Heuff.) Nyman × *C. halleriana* Asso]. According to the protologue given by Degen (1936: 585), this hybrid is known only from the type locality in Živi Bunari on Mt Velebit. Recently it could be found neither in any other herbaria examined nor in the field. However, the herbarium specimen in BP corresponds without any doubt to Degen's prologue. Therefore, we select here as a lectotype for the name *C.* × *rossiana* the following specimen:

Lectotype (designated here): Croatia litoralis ad Živi Bunari pone pagum Stinica, 26 Apr 1913, *Lj. Rossi s.n.* (BP 243009!).

*Carex caryophyllea* var. *trachyantha* is a synonym of *C. depressa* subsp. *transsilvanica* (Koopman 2022). After careful examination of the type specimen of *C.* × *rossiana*, we noticed that the utricles were full and well developed, the shape and indumentum of utricles and female glumes belong to *C. depressa* subsp. *transsilvanica*, and therefore this hybrid name, *C.* × *rossiana*, has to be considered only a synonym and not a hybrid of that taxon. [New for Croatia].

### *Carex fritschii* Waisb.

This species grows on sandy and gravelly soils in central European oak forests of Austria, Switzerland, France, Germany, Czech Republic, Italy, Slovenia, Croatia, and Hungary (Rotreklová et. al. 2011, Koopman 2022). In Croatia, *C. fritschii* is very rare and according to the FCD only two localities are known so far (Nikolić 2024). Both localities are in the Lika region: Plaško polje and National Park Plitvička jezera, which could not be confirmed by our research. However, we found two new sites, one in Bruvno in Lika and a second one in Klana in Gorski Kotar (Fig. 1F). On the latter site *C. fritschii* grows together with *C. pilulifera* L. and forms here the hybrid *C.* × *villacensis* Kük. [*C. fritschii* × *C. pilulifera*], which is a new taxon for the Croatian flora. Determination of this hybrid is in accordance with Kükenthal (1909) and Řepka (1992). The specimens of *C. fritschii* coming from Trstenik in Gorski Kotar and deposited in NHMR herbarium, have to be attributed, after revision, to *C.* × *villacensis* rather than to *C. fritschii.*

Specimens examined: Gorski Kotar, NE of Klana, Ovčije, edge of wet grassland, 45°28'15.42" N, 14°24'10.26" E, 1 Jun 2024, *S. Bogdanović s.n.* (NHMR, ZAGR); Lika, Bruvno-Mazin, grassland along the road, 44°25'21.9" N, 15°55'12.8" E, 19 Jun 2024, *S. Bogdanović & M. Temunović s.n.* (ZAGR).

### *Carex illegitima* Ces.

The occurrence of this rare Mediterranean species in Croatia was firstly known from the island of Hvar where it was mentioned under the name *Carex pharensis* Visiani (1852: 346). According to the FCD, *C. illegitima* occurs in Croatia only on some central Adriatic islands (Brač, Šolta, Hvar, Vis, Biševo, Svetac, Korčula) and on Pelješac peninsula. This species was confirmed on the islands of Biševo and Vis. On Vis it was found at two locations, namely NW of Komiža on the west coast of the island and near Milna in the SE (Fig. 1G).

Specimens examined: Dalmatia, Island of Vis, NW of Komiža, near Sv. Blaž, 43°3'13.9" N, 16°4'47.7" E, 287 m, along the road, macchia with *Pinus halepensis* and *Erica* *multiflora*, 17 May 2023, *S. Bogdanović, H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB, ZAGR); Island of Vis, Milna-Stončica, 43°3'48.8" N, 16°14'30.1" E, 25 m, macchia along path to the sea, 17 May 2023, *S. Bogdanović, H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman); Dalmacija, otok Vis, Oključina, u šumi hrasta crnike, uz put, 27 Aug 2005, *S. Bogdanović s.n.* (ZAGR 43821); Dalmatia, Island Biševo, Potok, in *Pinus halepensis* forest, 15 m above the sea, 27 Aug 2000, *S. Bogdanović s.n.* (ZAGR 79753); o. Vis, Vis-Hum, 3 May 1964, *I. Trinajstić 6346* (CNHM); Kroatien, Dalmatien, Insel Korčula, Kuppe (mit Sender) oberhalb der Ortschaft Korčula, NE-Seite, 65 m, 42°57'31" N, 17°07'57" E, alt. 65 m, 8 Jun 2004, *Karl s.n.* (W 0284489).

### ***Carex lasiocarpa*** **Ehrh. var.** ***lasiocarpa***

In older literature, this taxon was recorded from continental lowland areas in Croatia: Mali Bukovec in Podravina, Selnica in Prekmurje, Imbrovec and Đelekovec in Slavonia, Sisak, Topolovac, Mužilovčica, and in Lonjsko polje in Posavina (Schlosser and Vukotinović 1869). At none of these localities the species could be confirmed during our investigation. These days there are only three localities in Croatia at which *C. lasiocarpa* is known and forms the well-developed community of *Caricetum lasiocarpae* Koch 1926 within bog vegetation (Šegulja 2005, Alegro and Šegota 2008, Topić and Vukelić 2009, Anonymous 2022). We were able to confirm *C. lasiocarpa* at all three known localities (Velebit, National Park Plitvička jezera and Blatuša) (Fig. 1H).

Specimens examined: Srednji Velebit, Sunđerac, vlažni travnjak uz potok (nekadašnji cret), 25 Jul 2019, *S. Buzjak s.n.* (CNHM); Flora Croatica, In fossis et aquis stagnantibus, s.d., *J.C. Schlosser s.n.* (ZA 20935); Lika, Nacionalni Park Plitvička jezera, Ljeskovačke bare, 44°50'58.88" N, 15°36'1.92" E, 5 Jun 2024, *S. Bogdanović & M. Temunović s.n.* (ZAGR); Sjeverni Velebit, Sunđerac, na cretu, 44°37'52.04" N, 15°6'41.91" E, 6 Jun 2024, *S. Bogdanović & M. Temunović s.n.* (ZAGR); Kordun, Blatuša, cret Đon močvar, 45°19'3.94" N, 15°54'27.79" E, 4 Jun 2024, *S. Bogdanović & S. Ćato s.n.* (ZAGR).

### ***Carex limosa*** **L.**

There are only two known localities of *Carex limosa* in Croatia. One is cited by Schlosser and Vukotinović (1869) in "Flora Croatica", the second is known from a single herbarium specimen in ZA herbarium that was collected from Pleternica in Slavonia by Antun B. Pavić, probably from the early 19th century. Localities of *C. limosa* have been controversial since the early 20th century, when even Horvat (1939) was dubious about its existence in Croatia. We have examined all reported old localities cited by Schlosser and Vukotinović (1869: 1187): Široko Brezje near Križevci, Sv. Leonard and Noršić Selo near Samobor, and Pleternica in Slavonia. Even after occurrences of more than 150 years at those localities *C. limosa* could not be confirmed and there were no new findings either. Such acidophilous peat bogs (*Rhynchosporion albae* Koch 1926) in which *C. limosa* usually grows do not exist anymore in Croatia or are very impoverished. Peculiarly, according to Topić (1995: 164) *C. limosa* was found within grassland vegetation in Bačinska jezera in the Neretva River Delta, but this is evidently an erroneous identification because the species that are given in the list belong to impoverished vegetation of *Isoeto-Nanojuncetea* Br.-Bl. et Tx. in Br.-Bl. et al. 1952. Analysing the extinction of fen and bog plants and their habitats in Croatia, Topić and Stančić (2006) concluded that *C. limosa* has vanished from Croatia. Therefore, we can assume that *C. limosa* is extinct in Croatia. As a boreal relict species, it is very rare in the Balkan flora; in the neighbouring countries Slovenia (Kocjan 2012, Kocjan et al. 2026), Bosnia and Herzegovina (Milanović 2017), and Serbia (Stevanović et al. 1999, Niketić and Tomović 2018) it occurs sporadically. *Carex limosa* is also extinct from the Hungarian flora (Király 2007, Bartha et al. 2015).

Specimen examined: Pleternica, s.d., *A. Pavich s.n.* (ZA 11547).

### ***Carex melanostachya*** **M.Bieb. ex Willd.**

This species is extremely rare in Croatia with a few known localities cited in the FCD (Nikolić 2024). One herbarium specimen was collected by Lj. Vukotinović in 1877 in Fužine (Gorski Kotar), two localities were known from Požega valley in Slavonia (Tomašević 1998) and Petrijevci in Baranja (photographs by J. Topić from 2011 incorporated in FCD), and more recently it has been found in Molve in Podravina on the border with Hungary (Csiky and Purger 2008). Another old record close to the Croatian border is from Bosanski Brod in Posavina (Kummer and Sendtner 1849) which is cited by Stupar et al. (2021) for the territory of Bosnia and Herzegovina. Milanović (2014) could not confirm the occurrence of *C. melanostachya* at the locality in Bosanski Brod recently. Despite an intensive search, at none of the Croatian localities *C. melanostachya* could be confirmed by us and therefore this species has to be considered at least as doubtful or possibly even extinct in Croatia.

Specimen examined: In sylvis umbrosis non rara, s.d., *J.K. Schlosser s.n.* (ZA 20942).

### ***Carex nigra*** **(L.) Reichard subsp.** ***juncea*** **(Fr.) Soó**

The *Carex nigra* complex has five subspecies of which two (*C. nigra* subsp. *nigra* and *C. nigra* subsp. *juncea*) occur in Croatia. The latter taxon was found near the village of Sunger in Gorski Kotar and represents a new taxon for Croatia (Fig. 1I). In the FCD, *C. nigra* subsp. *juncea* is listed as a synonym of *C. nigra* subsp. *nigra*, but according to Koopman (2022) and POWO (2024) these are two accepted subspecies. *Carex nigra* subsp. *juncea* differs from the typical subspecies in having the following characters: it grows caespitose (vs. rhizomatous), and it has typically shiny, light brown lowest leaf sheaths (vs. dark brown, non-shiny lowest leaf sheaths).

Specimens examined: Gorski Kotar, Sunger, on wet forest edge with *Populus alba* and *Betula pendula*, 1 Jun 2024, 45°19'28.82" N, 14°48'33.66" E, *S. Bogdanović s.n.* (ZAGR); Lika, Ličko Lešće, cret uz hotel Gacka, 44°48'46.96" N, 15°19'9.4" E, 17 Jun 2023, *S. Bogdanović s.n.* (ZAGR); Ličko Lešće, uz hotel Gacka, cret, 44°48'47" N, 15°19'10" E, 17 May 2019, *S. Buzjak s.n.* (CNMH). [New for Croatia].

### *Carex pairae* F.W.Schultz

In older Croatian literature, this species was known and cited under the name *Carex contigua* Hoppe subsp. *pairae* (F.W.Schultz) Degen, only known from a few sites on Mt Velebit (Degen 1936: 581). However, it was not on the national list in the FCD. *Carex pairae* belongs to *Carex* sect. *Phaestoglochin* Dumort., and it has to be considered a species distinct from *C. muricata*, having small (3.25–4.0 × 1.75–2.25 mm), spreading, oval to ovate utricles and obovate, acuminate, light brown female glumes (Molina et al. 2008a). *Carex pairae* is a more or less thermophilous species, usually found at lower altitudes, with a wide distribution, more frequent in southern Europe (Molina et al. 2008a).

Specimens examined: Velebit. In Carpinis fagetorum prope Alan, alt. c. 1200 m, 20 Jul 1908, *A. Degen s.n.* (BP); Süd Istrien, Pola, s.d., s.coll. (BP).

### *Carex pulicaris* L.

This species was mentioned for the first time for Croatia by Topić and Ilijanić (2001), who thought to have it found on Trstenik Moor (Gorski Kotar) in the NW of Croatia. According to Wallnöfer (2008) this concerns a misidentification of *C. davalliana* Sm. However, *C. pulicaris* has been found afterwards, at another location in Croatia (pers. comm. J. Topić, Zagreb). We found this species near Plaški near Dretulja spring (Karlovac County), in a mire, where it had been found before (Koopman and Topić 2011) (Fig. 1J).

Specimens examined: Karlovac County, surroundings of Plaški, 45°4'31.8" N, 15°20'48.4" E, 386 m, mire, 20 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman); Karlovac County, Plaški, near Dretulja spring, 45°4'26.74" N, 15°21'6.73" E, 21 Jun 2023, *S. Bogdanović s.n.* (ZAGR).

### *Carex punctata* Gaudin var. *punctata*

This taxon was found for the first time in Croatia by J. Topić, at three locations in the north of the country during 2004–2009, near the villages of Pisarovinska Bregana, Plaški, and in the Risnjak National Park (Koopman and Topić 2011). Afterwards, Glasnović et al. (2015) found this species near Pižinovac in the Neretva River Delta. We can add two new sites, one near Belo, along the River Kupa in Gorski Kotar, bordering Slovenia and a second one in Kordun, near the village Bojanjci (Fig. 2A). Recently, this species has also been discovered in the flora of Bosnia and Herzegovina by Milanović et al. (2019).

Specimens examined: Gorski Kotar, 170 m E of Belo, N side of road, 45°28'30.1" N, 14°53'49.3" E, 228 m, wet meadow, 23 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); Pisarovina, Pisarovinska Bregana, vlažna livada, 25 May 2004, *M. Vrbek s.n.* (CNHM); NP Risnjak, Bela Vodica - Španov Laz, cret, 45°24'55.68" N, 14°41'7.82" E, 16 Jul 2020, *S. Buzjak & Z. Sedlar s.n.* (CNHM); NP Risnjak, Leska, cret, 45°25'9.87" N, 14°40'35.27" E, 9 Jul 2020, *S. Buzjak s.n.* (CNHM); Gorski Kotar, Belo, wet meadow along the road, 45°28'28.4" N

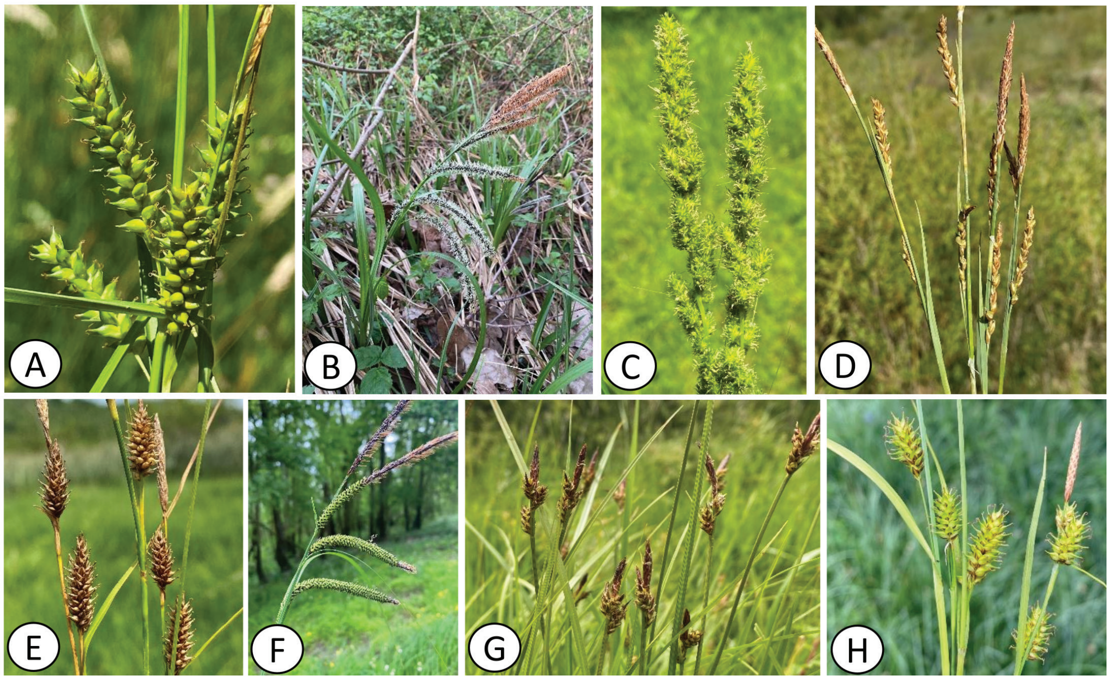

**Fig. 2.** Photos of some rare and newly found *Carex* taxa from Croatia discussed in this article. A – *C. punctata*, B – *C. randalpina*, C – *C. vulpinoidea*, D – *C.* × *alberti*, E – *C.* × *leutzii*, F – *C.* × *oenensis*, G – *C.* × *villacensis*, H – *C.* × *xanthocarpa* (photo: S. Bogdanović).

14°53'55.7" E, 30 May 2024, *S. Bogdanović s.n.* (ZAGR); Kordun, village Bojanjci near peat bog Đon močvar, along the forest path, 45°18'59.1" N, 15°54'16.73" E, 4 Jun 2024, *S. Bogdanović* & *S. Ćato s.n.* (ZAGR).

### *Carex randalpina* B.Walln.

Stančić (2009) mentioned this species for the first time for Croatia. It was found in 2006 in NW Croatia, near Zagreb, in the River Krapina valley, at four sites, near Bedekovčina, Kupljenovo, Luka, and Pojatno – Novi Dvori. We found this species near Jezerane in Lika, possibly the most southern site in Croatia, and also near Kosovići, which is just north of the Luka site by Stančić (2009), near Jurkovo Selo in Žumberak, west of Zagreb, and in Draganići (Fig. 2B).

Specimens examined: Karlovac County, Jezerane, 45°3'43.7" N, 15°10'44.3" E, 453 m, wet meadow with *Viola elatior*, 20 May 2023, *H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (Herb. Jac. Koopman); Zagreb County, Zaprešić, 45°57'2.4" N, 15°44'49.4" E, 166 m, wet meadow along road in village, roadside mown, 21 May 2023, *H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (SZUB); Zagreb County, Zaprešić, 45°57'0.1" N, 15°47'9.8" E, 154 m, 21 May 2023, *H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (SZUB); Petrovina, stream Stupanj, 45°41'9.5" N, 15°33'49.1" E, 200 m, *Caricetum randalpinae* at open place in *Alnetum* forest, 23 May 2023, *S. Bogdanović, H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); Zagreb Country, Jurkovo Selo, 45°42'24.1" N, 15°28'6.4" E, 200 m, meadow along stream, *S. Bogdanović, H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (SZUB); Žumberak, Potok-Potok, vlažna livada, 300 m.n.v., 15 May 2003, *M. Vrbek* & *S. Buzjak s.n.* (CNHM); Karlovac County, Draganići gas station, Ribnjaci Draganići, along the channel, 45°33'30.6" N, 15°36'35.1" E, 7 Apr 2024, *S. Bogdanović* & *S. Ćato s.n.* (ZAGR); Žumberak, Bukovac Svetojanski, wet meadow along stream Žumberačka reka, 45°42'17.48" N, 15°35'10.79" E, 9 May 2024, *S. Bogdanović* & *I. Ljubičić s.n.* (ZAGR); Karlovac County, Draganići gas station, Ribnjaci Draganići, along the channel, 45°33'30.6" N, 15°36'35.1" E, 12 May 2024, *S. Bogdanović* & *S. Ćato s.n.* (ZAGR).

### *Carex rupestris* All. subsp. *rupestris*

This is one of the rarest *Carex* taxa in Croatia. It is known from a single locality within the Bijele i Samarske stijene Strict Reserve on Vela Kapela. It was recorded by Horvat (1952) and for a long period it could not be confirmed. Recently we have confirmed its occurrence on the summit plateau of Bijele stijene on Vela Kapela. This species is also very rare in the rest of the Dinaric Mountains on the western Balkan Peninsula, where it is known only from Mt Notranjski Snežnik in Slovenia (Wraber 1966), from Mt Kamešnica in Bosnia and Herzegovina, and from Mt Durmitor in Montenegro (Horvat 1952).

Specimen examined: Vela Kapela, Bijele i Samarske stijene, plateau on the summit of Bijele stijene, 45°13'9.39" N, 14°58'32.89" E, 13 Oct 2023, *S. Bogdanović* & *M. Temunović s.n.* (ZAGR).

### *Carex secalina* Willd. ex Wahlenb. var. *secalina*

During herbarium revision of *Carex hordeistichos* Vill. in ZA we found one herbarium sheet with *C. secalina*, a new species for Croatia. In historical literature of the Croatian flora, *C. secalina* was cited as a synonym of *C. hordeistichos* and several continental localities were cited, among them Toplike, today known as Varaždinske Toplice (Schlosser and Vukotinović 1869: 1195). We were not able to confirm the occurrence of *C. secalina* on the cited localities, probably because of extensive usage and changes of agricultural land in the continental part of Croatia. Therefore, this species is presumably extinct in Croatia, while the current occurrence of *C. hordeistichos* in Croatia is at least doubtful.

Specimen examined: In arenosis humidis Toplike et alibi, s.d., *J. C. Schlosser s.n.* (ZA 24921). [New for Croatia].

### *Carex vulpinoidea* Michx.

This is an introduced species, native to N America, and known from just one site in Croatia, east of the village of Belo, close to the River Kupa in Gorski Kotar (Király et al. 2021), where it was confirmed during our fieldwork (Fig. 2C). Three clumps of this species were found. It was also mentioned by Király et al. (2021) west of Belo, but there we could not find any specimen.

Specimen examined: Gorski Kotar, just E of Belo, N side of road, 45°28'26.4" N, 14°53'55" E, 230 m, roadside, 23 May 2023, *H. Więcław, J.M. Kocjan* & *Jac. Koopman s.n.* (Herb. Jac. Koopman); Gorski Kotar, Belo, along the road, 45°28'26.1" N 14°53'54.7" E, 30 May 2024, *S. Bogdanović s.n.* (ZAGR).

## New *Carex* hybrids for Croatia

During herbarium research and fieldwork, we found eleven *Carex* hybrids new for Croatia. The distribution in Europe given for each hybrid below is according to Koopman (2022), and Croatia can thus be added at each hybrid. In the cited References more information about these hybrids is given.

### *Carex* × *alberti* H.Lév. [*C. flacca* × *C. panicea*] (Fig. 2D)

Distribution: Europe: Czech Republic, France, Germany, Italy, Netherlands, Poland, Slovakia, Switzerland, Ukraine.

Reference: Koopman et al. (2022).

Specimen examined: Velebit, Baške Oštarije, on Ljubica bog, 44°31'40.4" N 15°11'12.1" E, 10 May 2024, *S. Bogdanović* & *S. Ćato s.n.* (ZAGR).

### *Carex* × *alsatica* Zahn [*C. demissa* × *C. flava*]

Distribution: Europe: Belgium, Czech Republic, Finland, France, Germany, Italy, Latvia, Netherlands, Norway, Poland, Slovakia, Spain, Sweden, Switzerland, United Kingdom.

References: Jermy et al. (2007), Więcław (2014), Więcław and Wilhelm (2014), Koopman et al. (2022).

Specimens examined: Gorski Kotar, just E of Belo, N side of road, 228 m, wet meadow, 45°28'30.1" N, 14°53'49.3" E, 23 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); In pratis humidis vallis Dobra ad Jalsa pone Karlovac, 26 May 1888, *Lj. Rossi 5650* (ZA 7269); In pratis humidis ad Borl pone Karlovac, 9 Jun 1887, *Lj. Rossi 5652* (ZA 7270).

**_Carex_ × _auroniensis_ L.C.Lamb. [_C. acuta_ × _C. acutiformis_]**

Distribution: Europe: Belarus, Czech Republic, France, Germany, Italy, Latvia, Lithuania, Sweden, United Kingdom.

This hybrid grows often together with both parental species.

Reference: Koopman et al. (2022).

Specimen examined: Primorje-Gorski Kotar County, after Crni Lug, wet meadow, 691 m, 45°24'40.9" N, 14°41'38.5" E, 24 May 2023, *J.M. Kocjan, A. Jakob, H. Więcław & Jac. Koopman s.n.* (Herb. Jac. Koopman, ZAGR).

**_Carex_ × _leutzii_ Kneuck. [_C. hostiana_ × _C. lepidocarpa_] (Fig. 2E)**

Distribution: Europe: Belarus, Belgium, Czech Republic, Estonia, Finland, Germany, Italy, Latvia, Lithuania, Norway, Poland, Slovakia, Slovenia, Sweden, Switzerland, United Kingdom.

References: Jermy et al. (2007), Więcław and Koopman (2013), Więcław (2014).

Specimens examined: Lika, near Plaški, 45°4'31.8" N, 15°20'48.4" E, 386 m, mire, 20 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); Flora croatica, In pratis humidis hic inde, *J.C. Schlosser s.n.* (ZA 7267); Karlovac County, Plaški, near Dretulja spring, 45°4'31.8" N, 15°20'48.4" E, 21 Jun 2023, *S. Bogdanović s.n.* (ZAGR).

**_Carex_ × _oberrodensis_ B.Walln. [_C. elata_ × _C. randalpina_]**

Distribution: Europe: Austria, Italy.

This hybrid was only mentioned, but not found, during investigation of *C. randalpina* communities in the River Krapina valley by Stančić (2009). However, we could confirm its current occurrence in Croatia from one site.

Reference: Wallnöfer (1993).

Specimen examined: Karlovac County, Draganići gas station, Ribnjaci Draganići, along the channel, 45°33'30.6" N, 15°36'35.1" E, 7 Apr 2024, *S. Bogdanović & S. Ćato s.n.* (ZAGR).

**_Carex_ × _oenensis_ A.Neumann ex B.Walln. [_C. acuta_ × _C. randalpina_] (Fig. 2F)**

Distribution: Europe: Austria, Germany, Italy, Slovenia.

Reference: Wallnöfer (1993).

Specimens examined: Nature Park Lonjsko polje, N of Mužilovčica, wet meadow along the forest edge, 45°25'47.6" N, 16°41'31.4" E, 23 Apr 2024, *S. Bogdanović & M. Temunović s.n.* (ZAGR); Žumberak, Bukovac Svetojanski, wet meadow along stream Žumberačka reka, 45°42'17.48" N, 15°35'10.79" E, 9 May 2024, *S. Bogdanović & I. Ljubičić s.n.* (ZAGR).

**_Carex_ × _prolixa_ Fr. [_C. acuta_ × _C. elata_]**

Distribution: widespread in Europe: Austria, Belarus, Belgium, Bosnia and Herzegovina, Czech Republic, Estonia, Finland, France, Germany, Italy, Latvia, Lithuania, Montenegro, Netherlands, Poland, Russia, Slovakia, Slovenia, Sweden, United Kingdom.

References: Jermy et al. (2007), Koopman et al. (2022).

Specimen examined: Lika, Ličko Lešće, near Hotel Gacka, margin of bog, 44°48'47.5" N, 15°19'07.4" E, 17 Jun 2023, *S. Bogdanović s.n.* (ZAGR).

**_Carex_ × _pseudoaxillaris_ K. Richt. [_C. otrubae_ × _C. remota_]**

Distribution: Europe: Austria, Belgium, France, Germany, Italy, Ireland, Netherlands, Poland, Romania, Slovakia, United Kingdom.

*Carex* × *pseudoaxillaris* was found in Croatia only at one site, in two clumps, together with its parental species.

References: Jermy et al. (2007), Koopman et al. (2023).

Specimen examined: Posavina, Turopoljski Lug, *Quercus robur* wood, broadleaved forest border, in ditch along road, 45°38'12.2" N, 16°12'29.3" E, 125 m, 21 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, ZAGR).

**_Carex_ × _subviridula_ Fernald [_C. flava_ × _C. oederi_]**

Distribution: widespread in Europe: Austria, Belarus, Belgium, Bulgaria, Estonia, Finland, France, Germany, Latvia, Lithuania, Norway, Poland, Romania, Russia, Sweden, Switzerland.

This hybrid grows often together with both parental species.

References: Więcław (2014), Więcław and Wilhelm (2014).

Specimen examined: Flora Croatica: Lika, Gospić, Divoselo, 16 Jun 1961, *I. Horvat s.n.* (ZAHO).

**_Carex_ × _villacensis_ Kük. [_C. fritschii_ × _C. pilulifera_]**

Distribution: Europe: Austria, Czech Republic.

This is a very rare hybrid in the European flora, known so far only from Austria and the Czech Republic. This taxon has been found for the first time in Croatia in Gorski Kotar (Fig. 2G). *Carex* × *villacensis* was described by Kükenthal (1929: 35) from Villach, a town in Carinthia in S Austria. After herbarium research on the type material, deposited in several herbaria (B, BP, BR, P, MW, NY, and WU), we noticed that the name *C.* × *villacensis* had not been typified yet. Therefore, we designate here a lectotype from B herbarium that is in accordance with the description provided in the protologue by Kükenthal (1929):

Lectotype (designated here): AUSTRIA. Villach, in pratis silvae prope Lind, 9 Jun 1928, *G. Kükenthal s.n.* (B!).

References: Kükenthal (1929), Řepka (1992).

Specimens examined: Liburnijski krš, Gorski Kotar, Trstenik, ombrotrofni cret u sukcesiji, 11 Jul 2007, 45°29'22.7" N, 14°27'19.4" E, *B. Berković & Ž. Modrić s.n.* (NHMR 521); Liburnijski krš, Gorski Kotar, Trstenik, travnjak, 25 Jun 2007, 45°29'29.6" N, 14°27'14.8" E, *Ž. Modrić s.n.* (NHMR 518); Liburnijski krš, Gorski Kotar, Trstenik, ombrotrofni cret u sukcesiji, 11 Jul 2007, 45°29'21.1" N, 14°27'18.9" E, *B. Berković & Ž. Modrić s.n.* (NHMR 515); Gorski Kotar, NE of Klana, Ovčije, edge of wet grassland, 45°28'15.42" N, 14°24'10.26" E, 1 Jun 2024, *S. Bogdanović s.n.* (NHMR, ZAGR); Gorski Kotar, NE of Klana, Gumance, grassland, 45°30'23.58" N, 14°25'33.1" E, 1 Jun 2024, *S. Bogdanović s.n.* (ZAGR).

***Carex* × *xanthocarpa* Degl. [*C. flava* × *C. hostiana*] (Fig. 2H)**

Distribution: widespread in Europe: Austria, Belgium, Czech Republic, Estonia, Finland, France, Germany, Italy, Latvia, Lithuania, Netherlands, Norway, Poland, Romania, Russia, Slovakia, Sweden, Switzerland, United Kingdom.

References: Jermy et al. (2007), Więcław and Koopman (2013), Więcław (2014), Koopman et al. (2022).

Specimens examined: Lika-Senj County, NW of Korenica, 44°46'33.3" N, 15°40'54.5" E, 685 m, mire, 19 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); Karlovac County, Jezero, SE of Plaški, 45°5'47.5" N, 15°21'15.6" E, 380 m, wet meadow with *Scilla litardierei*, 20 May 2023, *H. Więcław, J.M. Kocjan & Jac. Koopman s.n.* (Herb. Jac. Koopman, SZUB); Karlovac County, Plaški, near Dretulja spring, 45°4'42.4" N 15°20'42.6" E, 21 Jun 2023, *S. Bogdanović s.n.* (ZAGR).

## Conclusions

Currently the genus *Carex* in the Croatian flora comprises 91 species with certainty, including 26 subspecies and eleven varieties, as well as eleven hybrids. Of these 91 species 75 were confirmed by our research, as well as all the eleven hybrids. Three species are represented by two subspecies: *Carex distachya* (*C. distachya* subsp. *distachya* and *C. distachya* subsp. *phyllostachioidea*), *Carex flacca* (*C. flacca* subsp. *flacca* and *C. flacca* subsp. *erythrostachys*), and *C. nigra* (*C. nigra* subsp. *nigra* and *C. nigra* subsp. *juncea*). All the other lower taxa concern the nominotypical ones. In total, six new *Carex* taxa for Croatia were found during our research: *C. aterrima* subsp. *aterrima*, *C. distachya* subsp. *phyllostachioidea*, *C. depressa* subsp. *transsilvanica*, *C. nigra* subsp. *juncea*, *C. pairae*, and *C. secalina*, as well as eleven new *Carex* hybrids.

Only some very old herbarium specimens of the following seven species, *C. arenaria*, *C. diandra*, *C. frigida*, *C. melanostachya*, *C. pauciflora*, *C. stenophylla*, and *C. supina*, have been found; in the case of *C. frigida* there is only one old herbarium sheet, without any precise location and year of collection. It was not possible recently to confirm in the field the occurrence of these seven species in Croatia. In herbaria there was no *C. dioica* material. We therefore believe that the eight species listed above should be considered dubious for the current flora of Croatia. Their status, historical and chorological notes in Croatia will be published in a separate article. Although older herbarium specimens of *C. bohemica* were found in ZA herbarium, the species was recorded recently in the eastern part of Croatia by Topić (1989), Csiky and Purger (2008), and Rožac et al. (2018). The situation regarding *C. disticha* is very similar, with, only one old herbarium specimen being found, confirmed recently for the Island of Krk by Starmühler (2004, 2006) and by Rottensteiner (2024). *Carex limosa* seems to be extinct in Croatia, as the only available collection dates back to the 19th century. Finally, *C. egorovae* Molina Gonz., Acedo & Llamas from the *C. divulsa* aggregate, is mentioned from the National Park Paklenica on Mt Velebit in Croatia (WU 0044091!) by Molina et al. (2008b). All our newly collected material from the National Park Paklenica and Mt Velebit clearly belongs to *C. leersii*. All revised herbarium material in B, BP, CNHM, ZA, ZAGR and ZAHO belongs without any doubt to *C. leersii*, too. Further attention has to be paid to *C. egorovae* during future fieldwork.

Koopman (2022) mentioned two *Carex* hybrids for Croatia, *C.* × *rossiana*, erroneously as *C. caryophyllea* × *C. halleriana*, and *C. halleriana* × *C. michelii*. The former turned out to be *C. depressa* subsp. *transsilvanica*, see above, while the latter was actually found just on the other side of the border in Romania. Therefore, counted in the current *Carex* flora of Croatia are the eleven hybrids discussed above.

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## Appendix: The genus *Carex* in Croatia

List of all currently known *Carex* taxa in Croatia, including hybrids. Taxa in bold were confirmed during fieldwork research (May–October 2023, April–August 2024). Underlined taxa were only confirmed through herbarium research. H NEW – taxa found in herbarium research in 2023–2024; F NEW – taxa found during fieldwork research (May–October 2023, April–August 2024); D? – doubtful taxa, as herbarium material is missing and the taxon could not be found during our fieldwork; D!? – doubtful taxa, as no location is given in the herbarium collection(s); not found during our fieldwork either; † – extinct from the flora.

1. ***C. acuta***
2. ***C. acutiformis***
3. ***C. alba***
4. ***C. appropinquata***
5. *C. arenaria*
6. ***C. aterrima***
    - **6.1. *C. aterrima*** subsp. ***aterrima***, F NEW
7. ***C. atrata***
    - **7.1. *C. atrata*** subsp. ***atrata***
8. *C. bohemica*
9. ***C. brachystachys***
10. ***C. brizoides***
11. ***C. buekii***
12. ***C. canescens***
    - **12.1. *C. canescens*** subsp. ***canescens***
13. ***C. capillaris***
    - **13.1. *C. capillaris*** subsp. ***capillaris***
14. ***C. caryophyllea***
    - **14.1. *C. caryophyllea*** var. ***caryophyllea***
15. ***C. cespitosa***
    - **15.1. *C. cespitosa*** var. ***cespitosa***
16. ***C. davalliana***
17. ***C. demissa***
    - **17.1. *C. demissa*** subsp. ***demissa***
18. ***C. depauperata***
19. *C. depressa*
    - 19.1. *C. depressa* subsp. *transsilvanica*, H NEW
20. *C. diandra*
21. ***C. digitata***
22. *C. dioica*, D?
23. ***C. distachya***
    - **23.1. *C. distachya*** subsp. ***distachya***
    - **23.2. *C. distachya*** subsp. ***phyllostachioidea,*** H NEW
24. ***C. distans***
    - **24.1. *C. distans*** subsp. ***distans,***
25. *C. disticha*
26. ***C. divisa***
27. ***C. divulsa***
28. ***C. echinata***
    - **28.1. *C. echinata*** subsp. ***echinata***
29. *C. egorovae*
30. ***C. elata***
    - **30.1. *C. elata*** subsp. ***elata***
31. ***C. elongata***
32. ***C. ericetorum***
33. ***C. extensa***
34. ***C. ferruginea***
35. ***C. firma***
36. ***C. flacca***
    - **36.1. *C. flacca*** subsp. ***flacca***
    - **36.2. *C. flacca*** subsp. ***erythrostachys***
37. ***C. flava***
38. *C. frigida*, D!?
39. ***C. fritschii***
40. ***C. halleriana***
41. ***C. hirta***
42. *C. hordeistichos*
43. ***C. hostiana***
44. ***C. humilis***
    - **44.1. *C. humilis*** var. ***humilis***
45. ***C. illegitima***
46. ***C. kitaibeliana***
    - **46.1. *C. kitaibeliana*** var. ***kitaibeliana***
47. ***C. lasiocarpa***
    - **47.1. *C. lasiocarpa*** var. ***lasiocarpa***
48. ***C. leersii***
49. ***C. lepidocarpa***
    - **49.1. *C. lepidocarpa*** subsp. ***lepidocarpa***
50. ***C. leporina***
51. *C. limosa*, †

52. ***C. liparocarpos***
    **52.1.** ***C. liparocarpos*** subsp. ***liparocarpos***
53. *C. melanostachya*, D!?
54. ***C. michelii***
55. ***C. montana***
56. ***C. mucronata***
57. ***C. muricata***
    **57.1.** ***C. muricata*** subsp. ***muricata***
58. ***C. nigra***
    **58.1.** ***C. nigra*** subsp. ***juncea***, F NEW
    **58.2.** ***C. nigra*** subsp. ***nigra***
59. ***C. oederi***
    **59.1.** ***C. oederi*** var. ***oederi***
60. ***C. ornithopoda***
    **60.1.** ***C. ornithopoda*** subsp. ***ornithopoda***
61. ***C. otrubae***
62. *C. pairae*, H NEW
63. ***C. pallescens***
64. ***C. panicea***
65. ***C. paniculata***
    **65.1.** ***C. paniculata*** subsp. ***paniculata***
66. *C. pauciflora*
67. ***C. pendula***
68. ***C. phyllostachys***
69. ***C. pilosa***
70. ***C. pilulifera***
    **70.1.** ***C. pilulifera*** subsp. ***pilulifera***
71. ***C. praecox***
72. ***C. pseudocyperus***
    **72.1.** ***C. pseudocyperus*** var. ***pseudocyperus***
73. ***C. pulicaris***
74. ***C. punctata***
    **74.1.** ***C. punctata*** var. ***punctata***
75. ***C. randalpina***
76. ***C. remota***
    **76.1.** ***C. remota*** subsp. ***remota***
77. ***C. riparia***
78. ***C. rostrata***
    **78.1.** ***C. rostrata*** var. ***rostrata***
79. ***C. rupestris***
    **79.1.** ***C. rupestris*** subsp. ***rupestris***
80. *C. secalina*
    80.1. *C. secalina* var. *secalina*, H NEW
81. ***C. sempervirens***
82. ***C. spicata***
    **82.1.** ***C. spicata*** subsp. ***spicata***
83. *C. stenophylla*
    **83.1.** *C. stenophylla* subsp. *stenophylla*
84. ***C. strigosa***
85. *C. supina*
    **85.1.** *C. supina* var. *supina*
86. ***C. sylvatica***
    **86.1.** ***C. sylvatica*** subsp. ***sylvatica***
87. ***C. tomentosa***
88. ***C. umbrosa***
    **88.1.** ***C. umbrosa*** subsp. ***umbrosa***
89. ***C. vesicaria***
90. ***C. vulpina***
91. ***C. vulpinoidea***, [Introduced]
92. ***C. × alberti*** [***C. flacca × C. panicea***], F NEW
93. ***C. × alsatica*** [***C. demissa × C. flava***], F NEW
94. ***C. × auroniensis*** [***C. acuta × C. acutiformis***], F NEW
95. ***C. × leutzii*** [***C. hostiana × C. lepidocarpa***], F NEW
96. ***C. × oberrodensis*** [***C. elata × C. randalpina***], F NEW
97. ***C. × oenensis*** [***C. acuta × C. randalpina***], F NEW
98. ***C. × prolixa*** [***C. acuta × C. elata***], F NEW
99. ***C. × pseudoaxillaris*** [***C. otrubae × C. remota***], F NEW
100. *C. × subviridula* [*C. flava × C. oederi*], H NEW
101. ***C. × villacensis*** [***C. fritschii × C. pilulifera***], F NEW
102. ***C. × xanthocarpa*** [***C. flava × C. hostiana***], F NEW

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Research Article

# The North American weed *Amaranthus tuberculatus* (Amaranthaceae) new to Portugal: previously overlooked or spreading rapidly?

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**Abstract** – The dioecious species *Amaranthus tuberculatus* (Moq.) J.D.Sauer, native to North America and a major weed of agricultural fields and riparian habitats, was discovered on the banks of the Tagus (Tejo) River in the late summer of 2024, apparently for the first time in Portugal. The species occurs here in exceptionally large numbers, with probably tens of thousands of plants, and in numerous locations on the sandy and gravelly banks of the river in the Ribatejo area, roughly between Azambuja and Azinhaga, over a distance of about 60 km. It may also occur further upstream, where no prospecting has been carried out but where suitable habitats are also available. Considering its current distribution and the number of plants observed, the species has either been present in the region for a long time but has been overlooked (although no older observations could be found on e.g. GBIF or iNaturalist, not even under other amaranth species names), or it is indeed a recent introduction that is spreading very rapidly. However, it can certainly be considered an invasive species. Here, the current naturalized range of the species in Europe and the Mediterranean area is critically reevaluated. Although the species is known from relatively many countries, it is currently only naturalized with certainty in Israel, Italy, Croatia, Serbia and now Portugal. The species is described and illustrated and a local distribution map is presented; notes on its ecology are also given.

**Keywords:** amaranth, dioecious species, invasive plants, naturalization, riparian habitats, Tagus River

## Introduction

*Amaranthus* L. (Amaranthaceae) accommodates 65–70 species that are mostly confined to the tropics, subtropics and warm-temperate regions of the world. Approximately half of the species are native to the Americas (Mosyakin and Robertson 2003, Iamonico 2015) while the rest are native to Africa, Asia and Europe (e.g. Hassan et al. 2022, Raus 2022). The genus is economically important because quite a few species are used for various purposes, e.g. as ornamentals, for medicinal purposes or as crop plants (pseudocereals, vegetables) (Das 2016). On the other hand, the genus includes numerous agricultural weeds that are harmful to various crops (very numerous references, e.g. Bayón 2022). Both monoecious and dioecious species occur, but the distribution of the latter is limited to North America. For sexual reproduction, the dioecious species require both male and female individuals in their introduced range; nevertheless, several of these dioecious species have proven to be very troublesome weeds, in particular *A. palmeri* S.Watson (e.g. Torra et al. 2020, Sukhorukov et al. 2021) and *A. tuberculatus* (Moq.) J.D.Sauer (incl. *A. rudis* J.D.Sauer). The latter species, also referred to as the waterhemp complex, has been known as a major weed pest in corn and soybean fields in the U.S.A. (Steckel 2007) but is increasingly recorded as a weed elsewhere in the world as well. Whereas the species is mainly harmful to agricultural crops in its area of origin, in Europe and the Mediterranean area it mostly occurs in more natural dynamic habitats, particularly on exposed riverbanks (Soldano 1982, Rimac et al. 2020). It is now listed on the EPPO A2 List of pests recommended for regulation as quarantine pests in the European Union (EPPO 2024).

In Portugal, the genus *Amaranthus* is possibly insufficiently known. According to Carretero (1990) the following species have been reliably recorded there: the probably native species *Amaranthus blitum* L. and *A. graecizans* L. and the introduced species *A. albus* L., *A. blitoides* S. Watson, *A. deflexus* L., *A. hybridus* L., *A. hypochondriacus* L., *A. muricatus* (Moq.) Hieron., *A. powellii* S.Watson, *A. retroflexus* L. and *A. viridis* L. Domingues de Almeida and Freitas (2006) subsequently added two further non-native

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species: *A. caudatus* L. and *A. cruentus* L., while a record of *A. crispus* (Lesp. & Thévenau) J.M.Coult. & S.Watson (Euro+Med Plantbase 2025) requires confirmation. No dioecious species are known.

During recent fieldwork in the Tagus valley in the Ribatejo area, in the late summer of 2024, dioecious plants greatly similar to plants that the author knew as *A. tuberculatus* from Northern Italy were found. Further research confirmed that they indeed belonged to that species. Although the species was extremely abundant, these seem to represent the first records in the wild in Portugal.

## Material and methods

The distribution data presented in this article are the result of fieldwork carried out by the author between 1st and 15th September 2024. On that occasion, numerous localities were explored in the wide Tagus River valley (Fig. 1), in the former Portuguese provinces of Estremadura and Ribatejo, especially in coastal and lowland areas. The main focus was on riparian and other damp habitats, such as rice fields. Anthropogenic, often urban habitats (such as roadsides, parks, etc.), agricultural fields, etc. were also explored.

The species was identified using relevant literature sources, including Sauer (1955), Aellen (1959), Pratt and Clark (2001), Mosyakin and Robertson (2003) and Iamonico (2015). Voucher specimens were collected in several of the populations and these were deposited in the herbarium of Meise Botanic Garden, Belgium (BR), with some duplicates in the herbarium of the University of Valencia (VAL). Specimens deposited in BR will in due time become available online at https://www.botanicalcollections.be. In addition, numerous photos were taken in several of the discovered localities. All records (including the photographs) were registered on the observation.org online platform (https://observation.org/), data which were subsequently also included in GBIF (Global Biodiversity Information Facility; https://www.gbif.org/).

To determine whether or not the species was known from Portugal, numerous relevant sources were consulted, e.g. Carretero (1990), Domingues de Almeida (1999, 2018, 2024) and Domingues de Almeida and Freitas (2006, 2012), as well as online sources such as iNaturalist (https://www.inaturalist.org/), GBIF, observation.org and Flora-On (https://flora-on.pt/).

The nomenclature in this paper follows Plants of the World Online (POWO 2024), which means that *A. rudis* is considered a synonym of *A. tuberculatus* (see also: Pratt and Clark 2001 and Mosyakin and Robertson 2003).

## Results

### Species description

*Amaranthus tuberculatus* (Fig. 2) is an annual, dioecious herb with erect, branched stems (or simple in smaller individuals), typically ranging from 1 to 2 meters in height (occasionally up to 3 m). Leaves have petioles measuring one-quarter to one-half the length of the blade. The blade shape varies from ovate or obovate proximally, and oblong, elliptic, or narrowly lanceolate distally, measuring 1.5–15 × 0.5–3 cm. The base is cuneate, margins are entire, and the apex ranges from obtuse or rounded to acute. Inflorescences are terminal, forming linear spikes or panicles that may occasionally be interrupted. Pistillate flowers feature bracts 1–2 mm long, with absent or rudimentary tepals (1–3 mm). Style branches are more or less erect, and stigmas number three. Staminate flowers have bracts with an inconspicuous to prominent midrib, 1–2 mm long, with an acuminate to short-subulate apex. There are five tepals, sometimes with prominent midribs excurrent as rigid spines, measuring 2–3 mm, with apex shapes varying from obtuse to acute, acuminate, or indistinctly mucronulate. Stamens also number

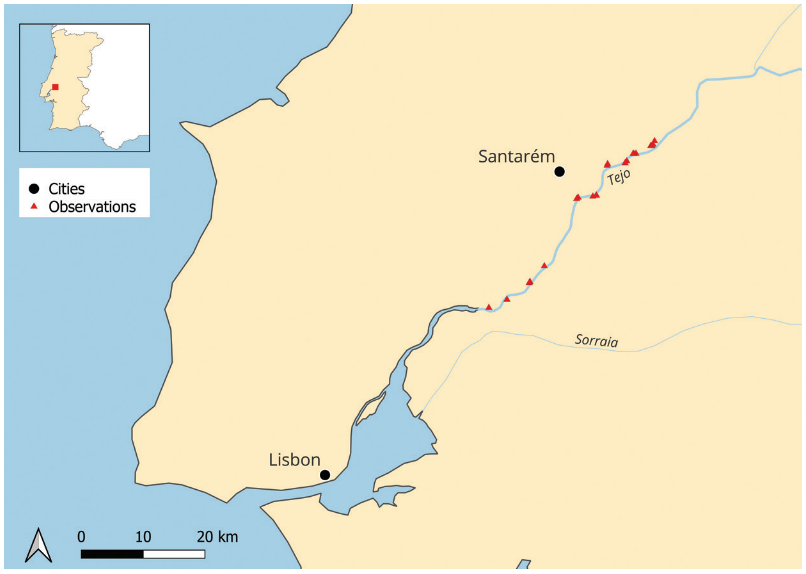

**Fig. 1.** Distribution of *Amaranthus tuberculatus* along the Tagus River in Portugal.

Fig. 2. *Amaranthus tuberculatus* on the banks of the Tagus River in Santa Iria da Ribeira de Santarém, 3rd September 2024. As is often the case, the species is accompanied by *Persicaria pensylvanica*, another American invasive weed (**a**). Female (left) and male (right) individuals in Pombalinho, 11th September 2024 (**b**). In Almeirim, 3rd September 2024. The morphological variation of the species is great, especially regarding the colour and size of the plants, the branching pattern of the inflorescence, etc. (**c**).

five. Utricles are dark to reddish-brown, unribbed, and vary from obovoid to subglobose, 1.5–2 mm in size, with thin, nearly smooth to irregularly rugose surfaces. They may be indehiscent, irregularly dehiscent, or regularly dehiscent. Seeds are dark reddish-brown to dark brown, shiny, and measure 0.7–1 mm in diameter (adapted from Mosyakin and Robertson 2003).

## Native and critical revision of the secondary distribution

*Amaranthus tuberculatus* is native to North America. The original area may have been limited to the region from the north of Missouri and Tennessee to the Great Lakes. As a major weed of agricultural fields and other disturbed habitats, it is now introduced in many parts of North America far outside its original range (Mosyakin and Robertson 2003). During the 20th century, the species was unintentionally introduced into other continents. It has been naturalized in Japan since 1959 (Egawa and Koyama 2023), potentially representing the oldest naturalized populations outside of America. The species was also repeatedly introduced into Europe and the Mediterranean area, with its first recorded introduction occurring in Austria in 1949 (Aellen 1959). Meanwhile, the species has been observed in numerous European countries: Austria, Belgium, Bosnia and Herzegovina, Croatia, Czech Republic, Denmark, Finland, Germany, Great Britain, Israel/Palestine, Italy, Jordan, the Netherlands, Romania, Russia, Spain, Sweden, Syria and Ukraine (Euro+Med Plantbase 2025). In addition, *A. tuberculatus* is also known from Switzerland (EPPO 2020). It should be noted, however, that in the vast majority of these countries, the species is an ephemeral alien that depends on the repetitive supply of seeds, especially in port areas. Although the EPPO Pest Risk Analysis is recent (EPPO 2020), its status in some countries needs to be reevaluated. First of all, the species is now also known from Croatia and Serbia, where it is naturalized in the Sava River valley (Rimac et al. 2020, Tabašević et al. 2020).

As this river forms the border between Croatia and Bosnia and Herzegovina, *A. tuberculatus* is in all probability naturalized in the latter country as well, recently having been reported (from another region) as a casual alien (Maslo et al. 2020). In some countries, the species was reported by EPPO (2020) as being in the process of naturalization, but this needs qualification. In Spain, the species was only seen once, in 2011, near grain silos in Palos de la Frontera (Sánchez Gullón and Verloove 2013). No mention was made of possible naturalization by the authors and, at least for now, the species is considered a casual alien in Spain. In the Netherlands, *A. tuberculatus* has occasionally been recorded on sandbars of the Meuse and Waal Rivers, which are a very suitable habitat for the species. However, based on current knowledge, only female plants have been discovered so far, with no seeds observed. In addition, the most recent sighting dates back to 2018 (https://waarneming.nl/). Given the frequency of surveys in the area, this suggests that the species has not (yet) managed to establish a permanent presence in the Netherlands. For Israel, on the contrary, the species was reported as being only locally established by EPPO (2020). However, the species has made rapid progress in recent years and has spread over a large part of the territory, both along rivers and roadsides and in agricultural crops. In addition, herbicide-resistant populations have also been detected (Roth et al. 2023). The status of the species in Romania is unclear according to EPPO (2020). The species is known from the port of Constanta, where it only temporarily persisted, and the delta of the Danube River. Although the latter location provides suitable habitats, it has not been observed there in recent years (comm. C. Sîrbu, November 2024).

In conclusion, based on the available data, *A. tuberculatus* is currently only established in four Mediterranean countries – Israel, Italy, Serbia and Croatia. The oldest established populations are from **Israel** where the species has been known since 1970 (Danin and Liston 1986), first from exposed lake margins in the Upper Jordan area. As mentioned, the species has now become a widespread and difficult-to-control weed. The status of the species in the neighboring countries of Jordan and Syria is unclear; the species may also have become naturalized there, but this needs to be confirmed. In **Italy**, the species has been known since 1975 from riparian habitats, first from the Po River (Soldano 1982) but in the intervening years the species has spread to other river systems in the northern half of Italy. It is now considered invasive in four regions (Lombardy, Emilia-Romagna, Veneto and Marche) and naturalized or casual in four others (Piemonte, Friuli Venezia Giulia, Tuscany and Trentino-Alto Adige) (Iamonico 2015). *A. tuberculatus* was more recently discovered along the banks of the Sava River, first in **Serbia** in 2016 (Tabašević et al. 2020) and later in **Croatia** in 2019 (Rimac et al. 2020). Considering its wide distribution in both countries, the species has at least become naturalized there, not to say invasive.

The species is here reported for the first time in the wild from **Portugal**. These are apparently the first naturalized/invasive populations in Europe outside the Mediterranean area (Serbia has no Mediterranean coastline but is biogeographically and climatologically part of it). Some previous Portuguese findings are documented on GBIF but these relate to plants that were formerly cultivated in the Botanical Garden of Coimbra. *A. tuberculatus* was discovered on 2nd September 2024 on sandbanks of the Tagus River upstream of Porto de Muge (Valada). During the following days, more targeted searches were carried out for the species, both upstream and downstream of this location. It should be noted that downstream, exposed sand and mud banks (and therefore suitable habitats) are hardly present. Large parts of the river are also inaccessible or barely accessible. Nevertheless, *A. tuberculatus* was found at numerous locations, roughly between Azambuja and Azinhaga, over a distance of approximately 60 km (Fig. 1). The further upstream, the more numerous the species was, especially from Santarém onwards. Near Azinhaga, it also grew abundantly along the lower stretch of the Almonda River, a tributary of the Tagus, near its confluence with the Tagus. It is very likely that *A. tuberculatus* also occurs further upstream, but no prospections were carried out there. An overview of records is available as Supplementary Material (On-line Suppl. Tab. 1).

**The following herbarium collections were made:**

1. Valada, Porto de Muge, Rio Tejo, upstream of Ponte Rainha Dona Amélia, sand bank of the river, about 15 individuals, a very invasive but not previously recognized weed on the banks of the Tejo (male individual in this collection), 02.09.2024, *F. Verloove* 15221 (BR0000027058992V, dupl. VAL);
2. Chamusca, Vale de Cavalos, Rio Tejo, sand bank of the river, male individual in this collection, 03.09.2024, *F. Verloove* 15227 (BR 0000027058978V);
3. Chamusca, Vale de Cavalos, Rio Tejo, sand bank of the river, very common, female individual in this collection, 03.09.2024, *F. Verloove* 15228 (BR 0000027058954V);
4. Almeirim, Rio Tejo near sand extraction company, sand bank of the river, very common, male individual in this collection, 03.09.2024, *F. Verloove* 15231 (BR0000027058930V);
5. Almeirim, Rio Tejo near sand extraction company, sand bank of the river, very common, female individual in this collection, 03.09.2024, *F. Verloove* 15232 (BR0000027058947V);
6. Azinhaga, Rio Almonda near its junction with Rio Tejo, dried out river bed, very common, 11.09.2024, *F. Verloove* 15257 (BR 0000027058770V).

## Habitat and ecology

In its natural range, *A. tuberculatus* is found in various types of wet habitats, such as margins of rivers, ponds, marshes, lakes and creeks as well as in disturbed habitats, such as agricultural fields, roadsides and railroads (Mosyakin and Robertson 2003). It is mainly in agricultural crops and in other disturbed habitats that the species has become a major weed, both in its native area and far beyond.

In the newly discovered secondary range in Portugal, *A. tuberculatus* is nearly exclusively found on the sand and gravel riverbanks (Fig. 2a), in particular of the Tagus and Almonda Rivers. It is striking that, in most localities, the species is completely absent outside the actual riparian zone, despite its massive occurrence along the banks. Although the Tagus is bordered by vast agricultural areas with a lot of maize, a crop in which the species occurs as a noxious weed in the U.S.A. (e.g. Vyn et al. 2006, Soltani et al. 2009, Hamberg et al. 2024), it was not observed in any of these fields. Only in the vicinity of Azinhaga was the species seen, and then sporadically along the field roads, a few hundred meters away from the river.

On the sand and gravel bars, *A. tuberculatus* was found in several plant associations, ranging from annual dwarf communities on sand near the waterline (*Nanocyperion* Koch 1926 alliance), to associations of slightly taller annual species, further from the waterline and usually developed on organo-mineral sediments (*Bidention tripartitae* Nordhagen ex Klika et Hadač 1944 alliance). It also occurs on the edges and in clearings of the alluvial forest that is mostly composed of willows (*Salicion albae* Soó 1951 alliance). All these plant communities are characterized by the abundant presence of other non-native species, such as *Cyperus esculentus* L., *Dysphania ambrosioides* (L.) Mosyakin & Clemants, *D. pumilio* (R.Br.) Mosyakin & Clemants, *Eclipta prostrata* (L.) L., *Eragrostis virescens* J.Presl, *Lindernia dubia* (L.) Pennell, *Mollugo verticillata* L., *Panicum dichotomiflorum* Michx., *Persicaria pensylvanica* (L.) M.Gómez, *Setaria parviflora* (Poir.) Kerguélen, *Solanum chenopodioides* Lam., etc. Native species that are found in the company of *A. tuberculatus* are, among others, *Cyperus fuscus* L., *C. michelianus* (L.) Link, *Eragrostis pilosa* (L.) P.Beauv. and *Glinus lotoides* L.

## Discussion

During recent fieldwork, the massive presence of the North American weed *A. tuberculatus* was noted on the banks of the Tagus River in Portugal. Tens of thousands of individuals of this dioecious species, both male and seed-bearing female plants (Fig. 2b), were observed on the exposed sand and mud banks, over a distance of approximately 60 km. The plants were almost exclusively observed in the immediate vicinity of the river. Only near Azinhaga, where the species is also abundant along the tributary Almonda, was it also found along field roads, at a distance of about half a kilometer from the river. Although much of the valley is intensively farmed (a lot being devoted to maize) and many of these crops are severely affected by weeds, including several amaranth species, *A. tuberculatus* was not observed in any fields. This is remarkable because the species is known to have been introduced into Europe as a contaminant in American cereals or oilseeds. Such grain or oil seed aliens usually end up in fields *via* animal manure (i.e. undigested seeds or fruits; compare with Pleasant and Schlather 1994 or Larney and Blackshaw 2003). The presence of quite a few weeds in the local corn fields can probably be explained in this way, but this does not apply to *A. tuberculatus*.

The species' introduction pathway thus can only be speculated upon. There are several grain transhipment locations along the Tagus, including a Cargill mill, where cereals and oilseeds from America are unloaded, but these are located far downstream from the sites of *A. tuberculatus*. In the vicinity of these factories, grain aliens were found during our recent fieldwork, such as *Amaranthus palmeri*, another quite invasive dioecious amaranth not previously recorded in Portugal, as well as *Persicaria pensylvanica*. It is rather unlikely that the presence of *A. tuberculatus* further upstream is related to this industry. It is nevertheless interesting that the species, which is a fairly typical American soybean alien, was found along with other soybean aliens on the banks of the Tagus, such as the previously mentioned *Persicaria pensylvanica* but also *Physalis angulata* L., another American weed that had not previously been found in Portugal (Verloove and Alves, in press). Interestingly, *A. tuberculatus* was once cultivated in the Coimbra Botanical Garden (GBIF), at least between 1938 and 1953. It is unclear whether this is still the case, but probably not (comm. J. Domingues de Almeida, January 2025). However, given the distance between Coimbra and the Tagus River, about 150 km, and the absence of the species in the area in between, it is probably unlikely that the origin of the plants should be sought there. Two further possible vectors of introduction are probably the most plausible. The species could have been introduced by migratory birds from (relatively) nearby regions where the species is naturalized, most likely Italy. The recent appearance of the species in Croatia is also explained in this way (Rimac et al. 2020). Alternatively, international fishing tourism along the river, including the provision of fish food, could also be a potential source of introduction. The Po and Tagus Rivers, known for their (invasive) catfish populations, attract specialized catfish anglers who may inadvertently introduce seeds through their equipment (compare with Nagy et al. 2025). However, how exactly the species arrived on the banks of the Tagus will remain a mystery.

In any case, if *A. tuberculatus* reaches and becomes established in the agricultural fields along the Tagus River, it could become a troublesome weed. The species is a very strong, fast-growing and therefore competitive weed (despite its annual life form, it can easily reach 3 m in height) (Mosyakin and Robertson 2003). Furthermore, the species is resistant to several herbicides (Schultz et al. 2015, Shergill et al. 2018, Roth et al. 2023). It is unclear what impact the species currently has in the invaded areas. As mentioned before, it is primarily a potentially aggressive weed in agricultural areas, while the negative impact of the species in (semi-)natural environments, such as riparian habitats, remains unclear. Given the unusually large populations in which the species occurs, a logical consequence of the massive seed production and the vigor of the species, it occupies large areas. It outcompetes much less robust native species from periodically exposed shores with stable, mesotrophic sediments with pioneer or ephemeral vegetation, such as

*Cyperus michelianus*, *Glinus lotoides*, etc. Additionally, a large proportion of other species growing on the sand and mud bars of the Tagus River are also exotic, posing a threat to native flora and vegetation.

Finally, questions can be raised regarding such a massive presence of *A. tuberculatus* along the Tagus River. Perhaps the species has been present there for a long time but remained unnoticed, as a result of confusion with other amaranths? Or is it perhaps a recent, rapidly expanding introduction? To answer these questions, online biodiversity databases such as GBIF, iNaturalist or observation.org were consulted, specifically for the region where *A. tuberculatus* was found. Although the valley is visited regularly by naturalists, no previous observations of the species have been detected, neither under the name *A. tuberculatus* nor under other amaranth species names (several other amaranths have nevertheless been documented with photographs in recent years from the banks of the Tagus River). Either the species has not been noticed until now (despite its size and the fact that it is clearly different from the other amaranths present on the banks; Fig. 2), which therefore seems rather unlikely, or it is indeed a relatively recently introduced, very rapidly spreading neophyte. Considering how the species has spread over large parts of northern Italy in just a few decades, especially along river systems, the species' future expansion in Portugal seems almost inevitable.

## Acknowledgements

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Research Article

# First records of the invasive weed of Union Concern *Cenchrus setaceus* (Poaceae) in Crete (Greece)

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**Abstract** – *Cenchrus setaceus* (Forssk.) Morrone has been included in the list of invasive alien species of Union Concern (the EU list) since 2017. This means that the species can no longer be imported, cultivated, transported, commercialized, planted, or intentionally released into the environment in the whole of the European Union. Despite this, the species was recently found in the wild in Crete. The species was observed in 2024 along the route between the cities of Heraklion and Agios Nikolaos, i.e. over a distance of more than 60 kilometers. Research into the presence of the species in Crete revealed that it was also known to occur on the western side of the island (Chania area) from 2020 onwards. Given the relative extent of the species' local distribution area, it appears that the species is either a recent, very rapidly expanding weed, or that it has been overlooked for several years. It is especially alarming that the species is currently still being planted in new green areas, both public and private, and is therefore apparently still available for sale in the local horticultural trade, despite EU regulations. Details of these first records, including a distribution map and new illustrations, are presented.

**Keywords:** European Union, horticultural trade, new data, *Pennisetum setaceum*, weeds

## Introduction

*Cenchrus setaceus* (Forssk.) Morrone (for a long time called *Pennisetum setaceum* (Forssk.) Chiov. and better known under the latter name) is a grass species with its main natural distribution in North and East Africa, Arabia and West Asia. It grows on dry stony soils and in rock crevices (Phillips 1995). It is cespitose, 40-150 cm tall, with convolute, scabrid, long and narrow leaves (blades are 20-65 cm long and 2-3.5 mm wide). Its inflorescence is a narrow and compact panicle up to 32 cm long, erect or more often arching and pinkish colored (Wipff 2003) (Fig. 1).

The species is a graceful perennial grass that until recently was often used in ornamental horticulture in the warm-temperate regions of the world (usually called "Fountain grass"). The spikelets, which detach very easily at maturity, are covered with numerous hairs and bristles, which promote wind dispersal over long distances. As a result, the species very easily escapes from wherever it is planted, after which it can become established in suitable habitats. In recent decades, the species has succeeded in establishing itself permanently in large parts of North and South America, southern Africa, Australia and southern Europe, to such an extent that it increasingly forms monotypic vegetation, especially in habitats and climates similar to those from which it came (e.g. CABI 2024). It is therefore considered very invasive in most areas in which it has been able to naturalize. This certainly also applies to the European Union, where the species has long been a major problem, especially in the Canary Islands. It is one of the most invasive species there (González-Rodríguez et al. 2010) and its potential area is likely to increase under future climatic scenarios (Da Re et al. 2020) and doubtlessly also under increasing anthropogenic pressure. The species is also known in the EU from Cyprus, France, Italy (incl. Sardinia and Sicily), Malta, Portugal (incl. Madeira; Cabral et al. 2020, Verloove et al. 2024) and Spain, almost without exception as an invasive species (EPPO 2025). Precisely because of its invasive characteristics, the species was included in 2017 in the list of Invasive Alien Species of Union Concern (the Union list). This means that the species must no longer be imported, cultivated, transported, commercialized, planted, or intentionally released into the environment in the whole of the European Union (Regulation 1143/2014 on Invasive Alien Species). In the European Union, Mediterranean coastal areas and islands are of particular concern and need to be prioritized

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**Fig. 1.** General habit of *Cenchrus setaceus* in Chersonisos, island of Crete, Greece, in May 2024 (A) and detail of inflorescence of *Cenchrus setaceus* in Chersonisos, island of Crete, Greece, in May 2024 (B) (photos: F. Verloove).

for monitoring (Brundu 2017). In Greece, *C. setaceus* was, surprisingly, completely unknown until now, with the exception of a recent find on the island of Milos where the species was considered to be "unlikely to persist outside cultivation" (Biel and Tan 2021). Arianoutsou et al. (2023) mentioned, without further details, that the species was found as a casual escape from cultivation, probably referring to the recent record from Milos.

In that context and with this background, the recent discovery of numerous populations of *C. setaceus* in Crete, whose presence has already been established in a significant area, deserves particular attention. This article lists the locations, including georeferenced coordinates, which will allow local authorities to eradicate the species. A number of other aspects are also discussed, including the future dispersal and establishment opportunities of the species in Crete and the species' local supply routes.

## Material and methods

The distribution data presented in this article are the result of fieldwork carried out by the author between 24th May and 7th June 2024. On this occasion, numerous localities were explored in the central northern part of Crete, i.e. in the area between Heraklion and Agios Nikolaos, especially along the coast and in the lowland areas. The main focus was on riparian and anthropogenic, often urban habitats (such as roadsides, parks, etc.), agricultural land, etc.

The species was identified using relevant literature sources, including Clayton and Renvoize (1982), Phillips (1995), Wipff (2003) and Fish et al. (2015).

Voucher specimens were collected in some of the populations and these were deposited in the herbarium of Meise Botanic Garden, Belgium (BR). Specimens deposited in BR will soon be made available online at https://www.botanicalcollections.be/#/en/home. In addition, photos were taken in several of the localities identified. Moreover, all records (including photographs) were registered on the observation.org online platform (https://observation.org/), data which were subsequently also included in GBIF (Global Biodiversity Information Facility; https://www.gbif.org/).

The presence or absence of the species in Crete has been verified in various recent online databases and literature references: Arianoutsou et al. (2010), Dimopoulos et al. (2013, 2016), Arianoutsou et al. (2023), the Flora of Greece Web (2024), and Muer et al. (2024).

The nomenclature in this paper follows Plants of the World Online (POWO 2024).

## Results

Below there is a chronological detailed overview of the recently discovered growth sites (see also Fig. 2).

1. Chersonisos, Dimokratias Street (35.3189 N, 25.3891 E), 24th May 2024. A single huge flowering individual at the foot of a fence next to the archaeological site. Herbarium: *F. Verloove* 15043 [BR 0000027060377V];
2. Chersonisos, harbor (35.3226 N, 25.3851 E), 25th May 2024. A single flowering individual in front of the Cine Creta Maris building;
3. Chersonisos, near the entrance of the Convention Center Mikis Theodorakis (35.3231 N, 25.3840 E), 25th May 2024. About ten huge flowering individuals at the foot of a stone wall;
4. Chersonisos, Nikos Kazantzakis Street (35.3192 N, 25.3854 E), 26th May 2024. Two flowering individuals along the road, on the verge of an olive grove;

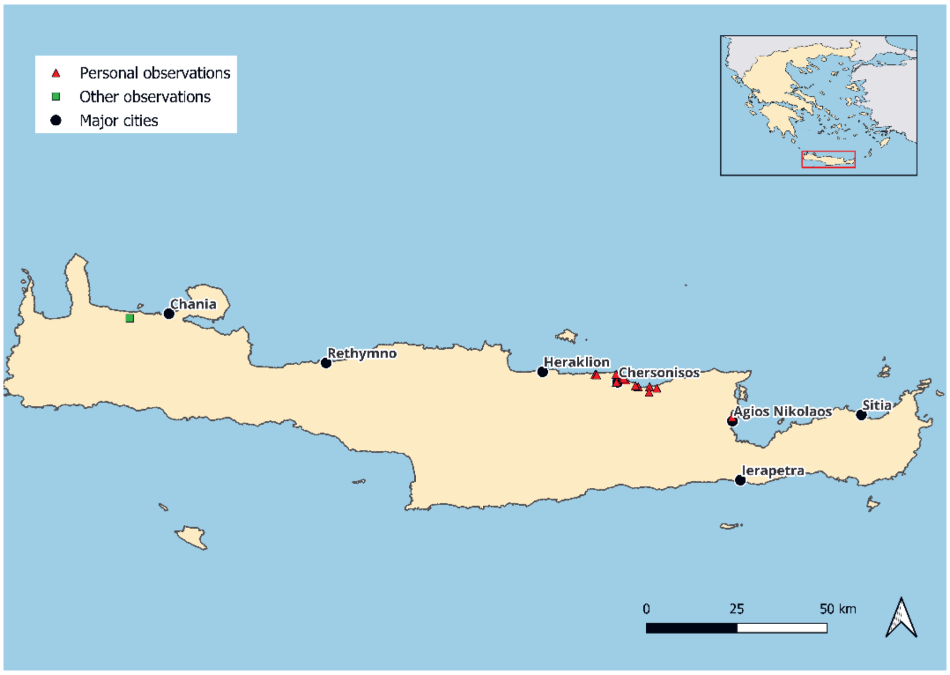

**Fig. 2.** Distribution of *Cenchrus setaceus* in Crete, Greece: records from 2024 (red triangles) and first record from 2020 (green box).

5. Stalída, Agiou Ioanni Street (35.2961 N, 25.4241 E), 26th May 2024. A flowering individual in a joint of the asphalt, along the sidewalk. The species obviously escaped from a nearby, recent ornamental plantation;
6. Chersonisos, old village, near to the Church of Agios Dimitrios (35.3075 N, 25.3693 E), 26th May 2024. A single flowering individual on the side of the road, under a crash barrier;
7. Stalída, Agiou Ioanni Street near to Resort Beach Hotel (35.2929 N, 25.4323 E), 27th May 2024. A single flowering individual;
8. Agios Nikolaos, Ammoudi, in front of the Lato Hotel (35.2022 N, 25.7150 E), 28th May 2024. A single flowering individual at the foot of a brick wall;
9. Chersonisos, old village, near the entrance of the National Road 90 (35.3066 N, 25.3664 E), 29th May 2024. Several dozen flowering and non-flowering individuals on the side of the road, extending over about a hundred meters;
10. Malia, in front of the Pyrgos Beach Hotel (35.2934 N, 25.4666 E), 29th May 2024. Two flowering individuals on rough ground;
11. Chersonisos, Agkisaras (35.3309 N, 25.3658 E), in front of Caldera Theros Villas, 30th May 2024. Scattered individuals along the road;
12. Malia, National Road 90 (35.2768 N, 25.4652 E), 31st May 2024. About a dozen flowering specimens on the side of the road, under the guardrail;
13. Malia, secondary road near to the National Road 90 (35.2894 N, 25.4887 E), 31st May 2024. Two flowering individuals on the verge of olive groves;
14. Chersonisos, Agia Pelagia, behind Michalis Apartments (35.3136 N, 25.3948 E), 1st June 2024. A single individual on rough land behind the hotel;
15. Chersonisos, near Dimokratias Street (35.3186 N, 25.3888 E), 3rd June 2024. Six flowering individuals;
16. Kato Gouves, Nikos Kazantzakis Street (35.3295 N, 25.3024 E), 4th June 2024. One flowering individual along the side of the road;
17. Kato Gouves, near Villa Maria Apartments (35.3324 N, 25.3046 E), 4th June 2024. One flowering individual along the side of the road, bordering a small stream;
18. Kato Gouves (35.3292 N, 25.3074 E), 6th June 2024. At least a dozen flowering and non-flowering individuals on the side of the road.

In addition to these 18 registered observations, *C. setaceus* was occasionally observed, from a passing car, along the N90 main road between Heraklion and Agios Nikolaos. The species is distributed in small populations along the guardrail between these two cities, which are more than 60 kilometers apart.

While investigating the presence of this species in Crete, it was discovered that *C. setaceus* is also known from the western side of the island (Platanias, Chania, 35°30' N, 23°54' E, comm. Christoforos Chiladakis via Kit Tan). This may be the very first record of the species in Crete.

## Discussion

During recent fieldwork, in the spring of 2024, the presence of *C. setaceus* was established for the first time in Crete. As far as is known, the very first find on the island dates from September 2020. There are a number of reasons to believe that the situation is precarious, apart from the fact that the species has long been known for its highly invasive behavior in the Mediterranean, where islands are particularly vulnerable to species invasion (Brundu 2017).

From the native range (Phillips 1995) it can be deduced that the species naturally occurs in a hot desert climate (Bwh climate type according to the Köppen-Geiger classification; Kottek et al. 2006). Remarkably, *C. setaceus* mainly behaves as an invasive species under a Csa climate type (hot-summer Mediterranean climate), characterized by hot and dry summers and mild winters (Deputy Direction of Nature 2016). This climate type predominates on the largest Mediterranean islands where the species is an increasing problem, for example in Sardinia, Sicily and Malta (Pasta et al. 2010, Mifsud 2022). In the Canary Islands, *C. setaceus* is very invasive in both the Bwh and Csa climate types. With the exception of the higher parts, almost all of Crete falls under a Csa climate type, potentially putting the entire island at risk.

Most of the current populations are relatively isolated and close to the coast (Fig. 2), often close to, and in some cases even directly associated with, human settlements and tourist infrastructure (where the species has clearly escaped from recently developed green areas). However, it is worrying that the species also occurs in several places along or very close to the main road connecting Heraklion with Agios Nikolaos (the National Road 90), an important connecting road with a lot of traffic. The species regularly occurs in small populations over a distance of more than 60 kilometers. Because *C. setaceus* produces large numbers of wind-dispersed seeds, its spread could be rapid, further enhanced by passing cars and trucks. In addition, the NR 90 passes through relatively unspoilt nature, with open rocky habitats, that are very suitable for the species. The almost completed construction of a new motorway connecting the north (exactly near Chersonisos, where the largest concentration of populations are located) and south of the island may soon facilitate the further spread of the species to the south of Crete. It should also be noted that only a relatively limited part of Crete was inventoried in 2024. There is a real (and even high) chance that other, undiscovered, populations occur elsewhere on the island, particularly in the Chania area, from where the species has been known since 2020.

In this context, a possible confusion with *C. orientalis* (Rich.) Morrone, a species native to Greece (floristic regions of Sterea Ellas and Western Aegean Islands, Web 2024), must also be considered. This species is very similar to *C. setaceus* and could be confused with it. Its midculm leaves are much wider and flat (3-11 mm wide *vs* 2-3.5 mm wide and inrolled) and the lower florets of the spikelets are staminate (*vs* usually sterile) (Wipff 2003). Records from Greece of *C. orientalis* may therefore hide observations of *C. setaceus*. It should be noted, however, that the species is apparently poorly documented in Greece, making data difficult to verify. No records of *C. orientalis* from Greece can be found on GBIF (https://www.gbif.org/) or iNaturalist (https://www.inaturalist.org/), and a search of some European herbaria (incl. online herbaria) (e.g. B, BR, G, L, P, WU) yielded no results either.

Finally, it is very disturbing that *C. setaceus* is currently still being planted in public green spaces and private gardens in Crete, despite EU regulations. At several of the above sites, the species had clearly escaped from nearby, recently established plantations (as was the case in Milos, the only other site from which the species is known in Greece; Biel and Tan 2021). This means that *C. setaceus* is still being offered for sale in the local ornamental plant trade and therefore the main source of supply for the species therefore still exists.

Although alarming, the situation may still be manageable: there are already a few dozen growing sites, but these often only contain few plants, which can easily be eradicated. For this purpose, the exact locations of the populations are presented in this paper. More importantly, however, the EU-imposed ban on the sale of the species must be enforced with immediate effect.

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Research Article

# A phytosociological description of a new plant association of the *Saturejion subspicatae* alliance in southern Croatia (SE Europe)

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**Abstract** – This paper presents a phytosociological study of grassland communities in the transition zone between the Mediterranean and Temperate regions in the interior of southern Croatia. Thirty-two vegetation relevés were carried out according to the phytosociological approach of Braun-Blanquet. A new association belonging to the *Saturejion subspicatae* alliance and characterised by hemicryptophytic dominance, namely *Saturejo subspicatae-Scabiosetum delminianae*, including three subassociations – *typicum*, *jurinetosum mollis* and *dalmatocytisetosum dalmatici* is described and discussed from a floristic and ecological point of view.

**Keywords**: Dinaric karst, *Festuco-Brometea*, new syntaxon, phytosociology, syntaxonomy, vegetation

## Introduction

Grasslands are one of the world's most important ecosystems, covering almost a third of the Earth's terrestrial surface (Bengtsson et al. 2019, and references therein). Both natural and semi-natural grasslands are of great importance due to their biodiversity and their ability to provide ecosystem services (e.g. Richter et al. 2021, Pergola et al. 2024). Unfortunately, grasslands have declined globally over the last century (Egoh et al. 2016), mainly due to conversion to cropland for the production of animal feed and, conversely, lack of management and abandonment of land (Queiroz et al. 2014). In addition, the biodiversity of grassland ecosystems is undergoing significant upheaval due to the extent of climate change and the overexploitation of natural resources, including in protected areas.

In Croatia, dry grassland habitats are an extremely interesting and diverse element of the vegetation landscape of the hilly and montane areas of the Dinaric Karst. The region is recognised as part of the World Natural Heritage and is valued for its exceptional biodiversity and high level of endemism due to palaeoclimatic and geological events that have shaped the region over time (Nikolić et al. 2015). The very rich species pool and the diverse land use patterns over thousands of years have contributed to the high degree of coenological diversification (cf. Trinajstić 2008).

However, there are still many gaps in the cenological knowledge of calcareous rocky grasslands on shallow soils that lie between the Mediterranean and the Temperate zone, i.e. extending into the interior of southern Croatia. In fact, phytosociological knowledge of the study area (especially the Dalmatian hinterland) has been significantly neglected over the last 40 years (e.g. Trinajstić and Pavletić 1988, Milović et al. 2020).

In this paper, new phytosociological data, the ecological context and the phytosociological classification of grasslands where endemic species occur are presented and discussed. In particular, we have formalised the proposal of a new phytosociological association of grasslands dominated by *Scabiosa delminiana* Abadžić and *Dalmatocytisus dalmaticus* (Vis.) Trinajstić, two species that have a limited distribution in Croatia (Milović et al. 2022, 2025). This research aims to identify suitable habitats and vegetation types to enable population monitoring, facilitate the discov-

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ery of new occurrences, support conservation efforts and contribute to ecological restoration in areas where this is necessary.

## Material and methods

### Study area

This study covers the area between the village of Udbina (839 m a.s.l.) in the north and the town of Sinj (326 m a.s.l.) and the neighbouring area in the south (Fig. 1). This area belongs to the long-term carbonate deposits of the platform, usually referred to as "karst Dinarides" and more precisely classified as megafacies of "limestones with chert" (Tišljar et al. 2002). In the morphologically lower parts of the areas, the karstic poljes and dolines, evaporites and associated sediments represent the oldest deposits. In Sinj, on the other hand, dolomites alternate with evaporites alongside calcareous marls and clayey limestones, or they can be found as intercalations in limestones (Šušnjara and Sakač 1988, Šušnjara et al. 1992).

In Udbina and the neighbouring karstic fields, various soil types can be found – from medium-deep eutric Cambisols to Rendzina, Luvisols, colluvial and other soils on Quaternary and glaciofluvial deposits (Bogunović et al. 2001). The most widespread soils in Sinj and the surrounding area are brown soils (Calcocambisol) and black soils (Calcomelanosol) on limestone and dolomite (Bogunović et al. 1996).

In the Udbina region, the mean annual air temperature is 8.7 °C and the mean annual precipitation is 1365 mm (data for the period 1971–2000 from the nearest meteorological station Gospić; Zaninović et al. 2008). In Sinj, the mean annual air temperature is 12.9 °C and the mean annual precipitation is 1147 mm (data for the period 1981–2010 from the Croatian Meteorological and Hydrological Service). According to Köppen's climate classification (Köppen and Geiger 1954, Sträßser 1998), the entire study area has a temperate humid climate with warm summers or a Cfb climate type (Šegota and Filipčić 2003: 35).

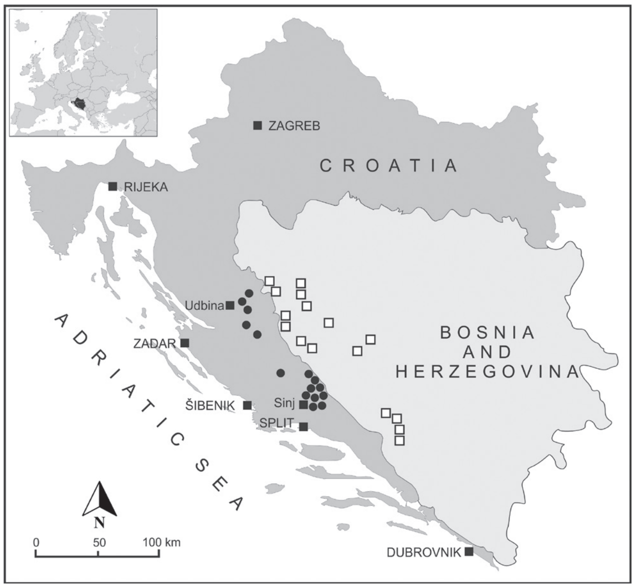

**Fig. 1.** Geographical distribution of thirty-two relevés with *Scabiosa delminiana* collected in southern Croatia (●). The distribution range of *Scabiosa delminiana* in Bosnia and Herzegovina (□) is also shown (*sensu* Abadžić 2007).

According to the assumed biome typology and the occurrence of thermotypes in each of the climatic zones (see Loidi et al. 2022), the area belongs to the temperate ombro-estival ecozone, the temperate deciduous forest biome and the temperate deciduous forest subbiome. The deciduous vegetation is present in the lower altitudes and belongs to the thermophilic deciduous oak forests of the *Quercetea pubescentis* vegetation class, while mesophilic deciduous and mixed forests of the *Carpino-Fagetea sylvaticae* class occur in the higher belts (Horvat 1961-1962, Nikolić et al. 2010). On the other hand, the study area also includes pastures and stony grasslands of the order *Scorzoneretalia villosae*, i.e. grasslands on shallow, calcareous soils at lower altitudes of the *Chrysopogono grylli-Koelerion splendentis* (Terzi et al. 2024) and at higher altitudes of the *Scorzonerion villosae* and *Saturejion subspicatae* alliances (see Terzi 2011, 2015 for details).

Today, the area around Udbina (Krbava field, Podlapača) is recognised as one of the Important Plant Areas (IPAs) in Croatia due to the high structural diversity of its vegetation (for details, see Nikolić et al. 2010). This region is also part of the Croatian NATURA 2000 ecological network (site code HR2000632, among others) (Official Gazette 2019). Similarly, Sinj and its surroundings are included in an IPA that is also part of the NATURA 2000 network (site code HR2001313, which includes the middle reaches of the Cetina River and the Hrvace and Sinj fields). In this study, the vegetation is also sampled on Kamešnica mountain near Sinj, which is part of the Dinara Nature Park.

Human impact has been manifested over the last 30 years in the form of depopulation. The reduced intensity of grazing on grasslands has generally led to natural succession into scrub and forest, while fires continue to occur frequently.

### Vegetation sampling and data analysis

The field data comprise thirty-two vegetation samples (relevés) and were collected from August 2020 to August 2021. The relevés were conducted according to the Braun-Blanquet approach (Westhoff and van der Maarel 1980) on plot sizes of 25–60 m$^2$, with a mode value of 30 m$^2$. The main physical characteristics of the habitat (e.g. slope, exposure, altitude) were also recorded.

The taxonomic nomenclature follows the portal *Flora Croatica Database* (Nikolić 2025). The biological form was verified in the field and denoted according to the categories given in Pignatti et al. (2017-2019), which are based on the classification of Raunkiaer (1934). Unweighted average ecological indicator values (EIVs) were used to interpret the ecological conditions of the vegetation types studied

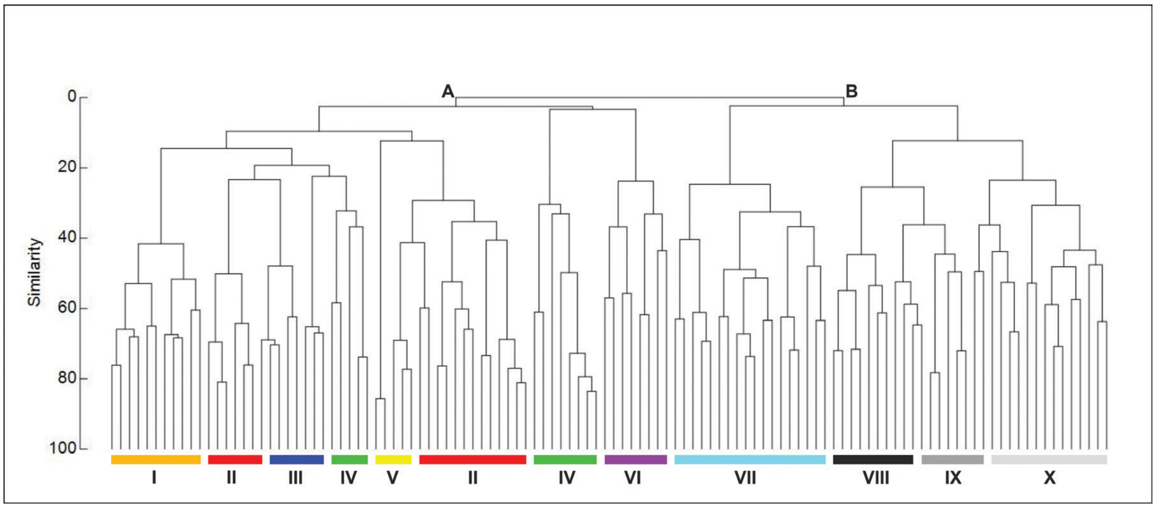

**Fig. 2.** Dendrogram of the hierarchical cluster analysis based on the Bray-Curtis similarity distance and Ward's minimum variance method for the data matrix of 346 taxa × 114 relevés. I = *Minuartio-Genistetum pulchellae* (Mosor Mt.), II = *Carex humilis-Centaurea rupestris* (incl. *Carici-Centaureetum rupestris* (Medvjeđak, Biokovo and Kozjak Mts.), III = *Bromo-Seslerietum tenuifoliae* (Krk Island, Biokovo Mt.), IV = *Saturejo subspicatae-Caricetum humilis* (Velebit Mt., Gorski Kotar, Tomislavgrad – BA), V = *Stipo-Caricetum humilis* (Krk Island), VI = *Astragalo-Seslerietum robustae* (Biokovo Mt.), VII = *Genisto-Globularietum bellidifoliae* (Lovćen Mt.), VIII = *Saturejo subspicatae-Scabiosetum delminianae typicum* (this study), IX = *Saturejo subspicatae-Scabiosetum delminianae jurinetosum mollis* (this study), X = *Saturejo subspicatae-Scabiosetum delminianae dalmatocytisetosum dalmatici* (this study). See On-line Suppl. Tab. 1 for the sources of the phytosociological relevés included in the data set.

(Pignatti et al. 2005, 2017-2019), calculated in JUICE 7.0. The syntaxonomic nomenclature follows the EuroVegChecklist, EVC (Mucina et al. 2016, Škvorc et al. 2017) and FloraVeg. EU (Chytrý et al. 2024). Nomenclature decisions follow the fourth edition of the International Code of Phytosociological Nomenclature (ICPN) (Theurillat et al. 2021).

The vegetation was classified using numerical methods. We created a matrix with the aim of comparing the relevés collected in this study with those previously published and originally assigned to the *Saturejion subspicatae* alliance (Trinajstić 2008; On-line Suppl. Tab. 1). The matrix consists of 346 taxa × 114 samples (relevés). It is important to emphasise that the aim of this work was not to revise the alliance (see for example Terzi et al. 2024), but to tentatively position the newly described communities within the *Saturejion subspicatae* alliance. The taxon scores originally recorded according to the Braun-Blanquet scale were replaced by the 1–9 ordinal values (van der Maarel 1979, Westhoff and van der Maarel 1980) prior to the numerical analyses.

An agglomerative hierarchical clustering algorithm based on Bray-Curtis similarity and the Ward method for determination of group linkages was used (McCune and Mefford 2016). Differences between the groups obtained during classification were tested by analysis of similarities (ANOSIM). Cluster analyses were performed using the PRIMERv7 software packages (Clarke and Gorley 2015). To visualise the floristic and ecological relationships among clusters of relevés, nonmetric multidimensional scaling (NMDS) was applied using the Bray-Curtis similarity matrix. NMDS with passive projection of environmental variables was performed using the R package 'vegan' (https://cran.r-project.org/ web/packages/vegan) operated through the JUICE software (Tichý 2002).

## Results

From the total of 114 relevés (rels.), the dendrogram obtained from the hierarchical clustering analysis of the data matrix makes it possible to distinguish two main groups (A, B) of clusters (Fig. 2). The first cluster (A) primarily consists of relevés originally assigned to various communities of the *Saturejion subspicatae* alliance (On-line Suppl. Tab. 1). These relevés cover an area extending from the island of Krk and Gorski Kotar in the north to Mount Biokovo in the south at an altitude between 370 and 1550 m a.s.l. The second cluster (B) includes relevés from four relatively similar groups: (i) *Genisto-Globularietum bellidifoliae* from Mount Lovćen, Montenegro, altitudes between 650 and 1275 m a.s.l. (Fig. 2, cluster VII), and (ii) the thirty-two relevés collected in this study (Fig. 2, clusters VIII, IX and X), covering altitudes between 330 and 1171 m a.s.l. The latter clusters are proposed here as a new plant association, *Saturejo subspicatae-Scabiosetum delminianae* with three subassociations: (i) *typicum*, (ii) *jurinetosum mollis*, and (iii) *dalmatocytisetosum dalmatici* (Fig. 2, clusters VIII, IX, X).

In the dendrogram, some relevés belonging to the same communities (II and IV, i.e., *Carex humilis-Centaurea rupestris* and *Saturejo subspicatae-Caricetum humilis*) and are placed in different clusters, primarily reflecting the geographical origin of the relevés (On-line Suppl. Tab. 1). This issue needs to be addressed in the context of a revision of the *Saturejion subspicatae* alliance and falls beyond the aims of this study.

The NMDS (Fig. 3) resulted in a two-axis solution with a final stress of 0.224, which indicates a fairly good ordination. The relevés originally classified in the *Saturejion subspicatae* are mainly located in the left part of the diagram. All three

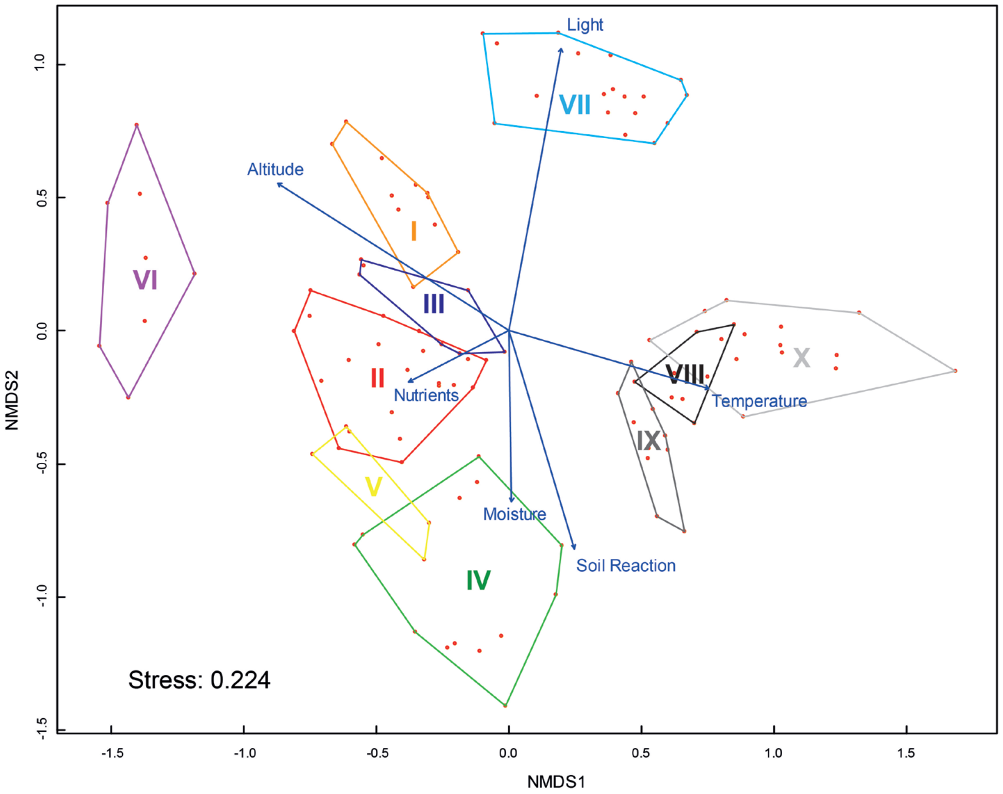

**Fig. 3.** Ordination of the relevés classified into the ten communities based on non-metric multidimensional (NMDS) of a Bray-Curtis similarity matrix. The community acronyms correspond to Fig. 2.

subassociations proposed here (clusters VIII-X) are clearly separated from each other in the right part of the diagram (Fig. 3). These stands occupy warmer habitats with shallow soils, mainly based on dolomite. The closely related association *Genisto-Globularietum bellidifoliae* (cluster VII) showed higher light values with stands located on the skeleton-rich soils, often on the southern slopes of Lovćen Mt.

Based on these results and according to the phytosociological table, a new association *Saturejo subspicatae-Scabiosetum delminianae*, including three subassociations: *typicum*, *jurinetosum mollis*, and *dalmatocytisetosum dalmatici*, was proposed and discussed here (On-line Suppl. Tab. 2). Their holotype relevés are listed in the Appendix. Photos of typical stands of subassociations are provided in On-line Suppl. Fig. 1.

The analysis of plant life forms showed that the association, including all three subassociations, was dominated by hemicryptophytes (49-63%) followed by chamaephytes (20-28%) and phanerophytes (7-17%), whereas therophytes and geophytes contributed the least (2-5%) (On-line Suppl. Tab. 3). These plant communities can be classified as in the following syntaxonomic scheme:

*Festuco-Brometea* Braun-Blanquet & Tuxen ex Klika & Hadač 1944
    *Scorzoneretalia villosae* Kovačević 1959
        *Saturejion subspicatae* Tomić-Stanković 1970
            *Saturejo subspicatae-Scabiosetum delminianae* ass. nov. hoc loco
                *S.s.-S.d. typicum* subass. nov. hoc loco
                *S.s.-S.d. jurinetosum mollis* subass. nov. hoc loco
                *S.s.-S.d. dalmatocytisetosum dalmatici* subass. nov. hoc loco

## Discussion

The study identified plant communities dominated by *Scabiosa delminiana* forming grasslands in the transitional zone between the Mediterranean and Temperate regions in the interior of southern Croatia.

*Scabiosa delminiana* was first described by Abadžić (2007) from karstic fields (*polje*) and dolomitic areas in western Bosnia and Herzegovina, where it occurs in a northwest-southeast belt roughly following the Croatia-Bosnia and Herzegovina border (Fig. 1). In earlier literature (e.g., Ritter-Studnička 1967, 1972), the species was attributed to *S. canscens* Waldst. & Kit. However, all previously recorded localities were later confirmed to correspond to *S. delminiana* (Abadžić 2007). According to Nikolić (2025), the species is considered endemic to Croatia, and this study documents the westernmost limit of its entire distribution range.

The grassland vegetation dominated by *S. delminiana* is proposed here as a new association, *Saturejo subspicatae-Scabiosetum delminianae* (On-line Suppl. Tab. 2). According to a total of 32 vegetation relevés, this association is characterized by a relatively high floristic richness, comprising 130 taxa overall. The number of species per relevé ranged from 17 to 38, with an average of 26.8. Similar levels of species richness have been reported for other associations within the *Saturejion subspicatae* alliance, such as *Carici-Centaureetum rupestris* (Trinajstić and Pavletić 1988, 1990). In the present study, the high species richness may be attributed to the relatively broad geographic scope of the sampling area, as well as to specific orographic conditions that allow Mediterranean climatic influences to penetrate areas otherwise characterized by different climatic regimes. The association occurs across an altitudinal range from 330 to 1171 m a.s.l., most frequently on northern and eastern exposures, on slopes ranging from 0° (flat) to 40°. Vegetation cover ranges from 70% to 100%, with full coverage (100%) being most commonly observed.

The taxa *S. delminiana*, *Globularia cordifolia* L. subsp. *bellidifolia* (Ten.) Wettst., and *Satureja subspicata* Bartl. ex Vis. are considered constant and dominant within the proposed association. The association includes eight taxa characteristic of the *Saturejion subspicatae* alliance and 22 taxa of the *Scorzoneretalia villosae* order. In total, 53 taxa are attributed to the *Festuco-Brometea* class. Additionally, 40 companions were recorded, originating from 14 different vegetation classes. Among the companions, the majority belong to the *Quercetea pubescentis* (13 taxa) and *Ononido-Rosmarinetea* (7 taxa) classes. Notably, the association includes a relatively high proportion (12.3%) of endemic taxa in Croatia (*sensu* Nikolić et al. 2015), such as *Edraianthus tenuifolius* (Waldst. & Kit.) A.DC., *Onosma echioides* (L.) L. subsp. *dalmatica* (Scheele) Peruzzi & N.G. Passal., and *Seseli montanum* L. subsp. *tommasinii* (Rchb. f.) Arcang., etc.

In our analysis (Figs. 2, 3), *Saturejo subspicatae--Scabiosetum delminianae* shows a close relationship to relevés of *Genisto-Globularietum bellidifoliae*, reported from the dolomitic area of Mount Lovćen, Montenegro (Tomić-Stanković 1970), primarily due to the presence of numerous dolomitic taxa. Furthermore, *S. delminiana* (originally attributed to *S. canescens*) has been documented as a characteristic species of two associations – *Saponario--Scabiosetum canescentis* and *Festuco-Linetum flavi* – described from karstic fields and surrounding dolomitic areas in western Bosnia and Herzegovina (Ritter-Studnička 1967, 1972; see also Fig. 1). Unfortunately, *Saponario--Scabiosetum canescentis* was only briefly described, lacking a supporting phytosociological table, which limits its comparability with our dataset. Floristically and ecologically, *Saturejo subspicatae-Scabiosetum delminianae* differs notably from *Festuco-Linetum flavi.* While our proposed association clearly fits within the *Saturejion subspicatae* alliance, the syntaxonomic position of the Bosnian-Herzegovinian associations has been subject to varying interpretations. According to the EuroVegChecklist (EVC; Mucina et al. 2016, Terzi et al. 2024), these associations should be subordinated to the *Chrysopogono grylli-Koelerion splendentis* alliance.

Our results support the distinction and formal proposal of three subassociations: *typicum*, *jurinetosum mollis*, and *dalmatocytisetosum dalmatici* (On-line Suppl. Tab. 2; see Appendix for holotype relevés). In addition to differences in differential taxa, the subassociations also exhibit variation in some ecological conditions, particularly with respect to altitudinal distribution. The *typicum* subassociation occurs at higher elevations, ranging from 655 to 1171 m a.s.l. In contrast, the *jurinetosum mollis* subassociation, distinguished by the presence of *Jurinea mollis*, is primarily found on lower slopes at altitudes below 400 m a.s.l. The subassociation *dalmatocytisetosum dalmatici*, dominated by *Dalmatocytisus dalmaticus* and sharing many species with *jurinetosum mollis*, is typically located on north- or northeast-facing slopes between 330 and 410 m a.s.l. *Dalmatocytisus dalmaticus* is a Croatian stenoendemic species, classified as 'Endangered' and strictly protected by Croatian law (Official Gazette 2013, Milović et al. 2022). Its distribution is restricted to a predominantly dolomitic area of approximately 1.5 km² near the town of Sinj in southern Croatia (Nikolić et al. 2015). Notably, within the subassociation *dalmatocytisetosum dalmatici*, *Scabiosa delminiana* reaches the southwesternmost limit of its entire distribution range.

At several localities within the study area, the encroachment of planted black pine (*Pinus nigra* J.F.Arnold) forests onto grasslands is already evident. As a result, beyond forest edges and clearings, *D. dalmaticus* occupies a significant portion of the herb layer within *P. nigra* stands, growing on shallow soils derived from the erosion of soft, porous calcareous marls (Šušnjara et al. 1992). This indicates a certain level of shade tolerance in *D. dalmaticus*. However, it is hypothesized that further forest development and the consequent closure of the canopy could substantially decrease light availability in the understory, thereby threatening the long-term survival of this species.

In other parts of the area, the grasslands show clear signs of succession toward shrubland and forest, primarily dominated by *Quercus pubescens* Willd. and *Ostrya carpinifolia* Scop. This successional trend likely accounts for the notable representation of companion species from the *Quercetea pubescentis* class. Milović et al. (2022) highlighted the significant anthropogenic pressures in the area inhabited by *D. dalmaticus*, including habitat reduction, fragmentation, and ruderalization, primarily resulting from settlement expansion, quarrying, and surface construction activities. These findings underscore the urgent need to develop and implement targeted conservation strategies to safeguard both *D. dalmaticus* and its associated grassland communities.

## Conclusion

This study introduces a new grassland association, *Saturejo subspicatae-Scabiosetum delminianae*, comprising three subassociations, thereby contributing to the understanding of Croatian syntaxonomic diversity – an important indicator of overall ecological diversity. The association is characterized by high floristic richness and a notable presence of endemic species. These findings highlight the urgent need for targeted conservation measures, including succession management and the reduction of anthropogenic pressures, to preserve these valuable grassland habitats and ensure the long-term protection of regional biodiversity. Future research should focus on comprehensive phytosociological surveys and ecological analyses to further explore the complex ecosystems of the Dinaric karst and promote their sustainable management.

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### Author contribution statement

MP – planned the research and sampled vegetation. NJ – led the writing with contributions of ŽŠ and MP. MM, JK – identified plant specimens and revised herbarium material. NJ, ŽŠ – made the statistical analysis. NJ, ŽŠ, MP – critically revised the manuscript. MP and NJ – share the first authorship. All authors have read and agreed to the published version of the manuscript.

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# Appendix

### Holotypes of the new plant communities.

***Saturejo subspicatae-Scabiosetum delminianae typicum*** [locality: Croatia, Zadar County, Gračac Municipality, the Cerovac Bruvanjski karstic field; holotypus: On-line Suppl. Tab. 2, relevé no. 3; altitude 785 m a.s.l.; aspect E; slope 10°; plot size 40 $m^2$; vegetation cover 100%; date 19.9.2020; coordinate: 44.431028 N, 15.926356 E]: *Scabiosa delminiana*, 4; *Globularia cordifolia* subsp. *bellidifolia*, +; *Satureja subspicata*, 1; *Plantago holosteum*, +; *Teucrium montanum*, +; *Eryngium amethystinum*, +; *Seseli montanum* subsp. *tommasinii*, +; *Dorycnium germanicum*, 1; *Inula ensifolia*, +; *Plantago media*, +; *Betonica officinalis*, +; *Pseudolysimachion barrelieri*, +; *Carex humilis*, 1; *Asperula purpurea*, +; *Linum tenuifolium*, +; *Fumana procumbens*, +; *Asperula aristata* subsp. *scabra*, +; *Cirsium acaulon*, +; *Sanguisorba minor* subsp. *muricata*, +; *Carlina vulgaris*, +; *Hieracium hoppeanum* subsp. *testimoniale*, +; *Koeleria macrantha*, +; *Bromus erectus*, 1; *Centaurea weldeniana*, +; *Teucrium chamaedrys*, +; *Prunella laciniata*, +; *Carlina acaulis*, +; *Helleborus multifidus*, +; *Thesium divaricatum*, +; *Genista sylvestris* subsp. *sylvestris*, +; *Inula hirta*, +; *Euphrasia salisburgensis*, +; *Cuscuta europaea*, +; *Knautia* sp., +.

***Saturejo subspicatae-Scabiosetum delminianae jurinetosum mollis*** [locality: Croatia, Split-Dalmatia County, the town of Sinj, Petrada hill; holotypus: On-line Suppl. Tab. 2, relevé no. 16; altitude 360 m a.s.l.; slope 0°; plot size 60 $m^2$; vegetation cover 100%; date 27.9.2020; coordinate: 43.731461 N, 16.650800 E]: *Scabiosa delminiana*, 4; *Globularia cordifolia* subsp. *bellidifolia*, 2; *Satureja subspicata*, 1; *Edraianthus tenuifolius*, +; *Jurinea mollis*, +; *Teucrium montanum*, +; *Eryngium amethystinum*, +; *Seseli montanum* subsp. *tommasinii*, +; *Koeleria splendens*, +; *Linum alpinum* subsp.

*julicum*, +; *Betonica officinalis*, +; *Stachys recta*, +; *Potentilla australis*, +; *Carex humilis*, +; *Asperula purpurea*, +; *Linum tenuifolium*, +; *Fumana procumbens*, +; *Cirsium acaulon*, +; *Sanguisorba minor* subsp. *muricata*, +; *Hieracium hoppeanum* subsp. *testimoniale*, +; *Ononis antiquorum*, +; *Bromus erectus*, 1; *Helianthemum nummularium* subsp. *obscurum*, +; *Artemisia alba*, +; *Echinops ritro* subsp. *ruthenicus*, +; *Onosma echioides* subsp. *dalmatica*, +; *Ruta graveolens*, +; *Cotinus coggygria* juv., +; *Rhamnus intermedia*, +; *Thesium divaricatum*, +; *Hieracium heterogynum*, +; *Pinus nigra* juv., +.

***Saturejo subspicatae-Scabiosetum delminianae dalmatocytisetosum dalmatici*** [locality: Croatia, Split-Dalmatia County, the town of Sinj, Planica hill; holotypus: On-line Suppl. Tab. 2, relevé no. 32; 330 m a.s.l.; aspect W; slope 40°; plot size 50 m$^2$; vegetation cover 95%; date 12.8.2021; coordinate: 43.727598 N, 16.691710 E]: *Dalmatocytisus dalmaticus*, 4; *Globularia cordifolia* subsp. *bellidifolia*, 2; *Satureja subspicata*, 1; *Teucrium montanum*, +; *Koeleria splendens*, +; *Eryngium amethystinum*, +; *Dorycnium germanicum*, +; *Asperula purpurea*, +; *Asperula aristata* subsp. *scabra*,+; *Carex humilis*, +; *Echinops ritro* subsp. *ruthenicus*, +; *Anthericum ramosum*,+; *Thymus longicaulis*, +; *Prunella laciniata*, +; *Fraxinus ornus* juv., +; *Quercus pubescens* juv., +; *Cotinus coggygria*, +; *Genista sylvestris* subsp. *sylvestris*, +; *Hieracium heterogynum*, +.

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Research Article

# Croceous glands are oil secretory cavities in fruits of *Senega longicaulis* (Polygalaceae)

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**Abstract** – Croceous glands are yellowish glands that occur exclusively in some species of *Senega* Spach (Polygalaceae). These glands are especially conspicuous in reproductive organs, such as the fruits, but their structure, origin, and secretion are still uncertain. The objective of this study is to determine the nature of the croceous glands in *Senega* from an analysis of the fruits of *Senega longicaulis* (Kunth) J.F.B. Pastore. Flowers and fruits at different developmental stages were analysed through light microscopy and scanning electron microscopy to investigate the origin, structure, and composition of the secretion of the glands. Croceous glands of the pericarp are cavities that originate early, while still in the ovary, through a schizolysigenous process. In addition, new glands are formed *de novo* during fruit development, and the persistent calyx observed surrounding the fruit, also has such cavities. The secretory tissue is constituted by a single layer of epithelial cells, lining a spheroidal lumen that stores the secretion. The exudate is composed of essential oils, indicating functions related to protection against herbivory and microbial attacks. Our results partially diverge from previous reports, and further comprehensive analyses of Polygalaceae glands are essential to understand the nature of croceous glands and the evolution of defence strategies within the family.

**Keywords:** cavities, defence, ovary, Polygalaceae, schizolysigeny, *Senega longicaulis*, terpenes

## Introduction

Croceous glands are conspicuous yellowish glands observed in the vegetative and reproductive organs of some Neotropical species of Polygalaceae (Chodat 1893). Their occurrence has taxonomic importance and has already been used to delimit a section of *Polygala* – *Timutua* DC. (Pastore and Harley 2009, Pastore 2018). Recently, the Neotropical species of *Polygala* were segregated to the genus *Senega* Spach, now the only genus in which the croceous glands occur, being reported for the new sections *Cruciatae* J.F.B. Pastore & J.R. Abbott, *Galioidae* (Chodat) J.F.B. Pastore, *Incarcanatae* (Chodat) J.F.B. Pastore & J.R. Abbott and *Trichospermae* (Chodat) J.F.B. Pastore & J.R. Abbott (Lüdtke et al. 2013, Pastore 2018, Pastore et al. 2023). The genus *Senega* comprises 229 chiefly Neotropical species (Pastore et al. 2023).

The nature and function of croceous glands have intrigued researchers since they were first described, but they have only recently been analysed for the first time in *Senega adenophora* (DC.) J.F.B. Pastore (= *Polygala adenophora*) by Jorge et al. (2024), who observed that these glands are cavities and ducts present in both vegetative and reproductive organs. The secretory spaces (cavities and ducts) are widely used in the characterisation of numerous other genera within Fabales (Teixeira and Rocha 2009, Milani et al. 2012, Duarte-Almeida et al. 2015, Mendes et al. 2019). Despite the taxonomic potential of these glands for Polygalaceae (Chodat 1893, Holm 1929, Metcalfe and Chalk 1950, Aguiar-Dias and Cardoso-Gustavson 2011, Jorge et al. 2024), there is little information in the literature about them.

Internal glands, such as idioblasts, laticifers, cavities and ducts, generally have a defensive function (Fahn 1979, Costa

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et al. 2021, Tölke et al. 2021, 2022, Freitas et al. 2024). However, analysis of the structure of the gland alone is not sufficient to identify it, as its function depends, at least in part, on the composition of its secretion (Fahn 1979, Demarco 2023). In this context, to determine the nature of croceous glands, we selected *Senega longicaulis* (Kunth) J.F.B. Pastore, a species in which these glands had previously been observed in the fruits (pers. obs.). This species belongs to *Trichospermae*, a section characterised by the presence of bilocular capsule-type fruits with a persistent calyx, in which croceous glands are present in both the perianth and pericarp (Pastore et al. 2023).

The glands of Polygalaceae may provide data on potential functions, insights into ecological relationships, as well as anatomical novelties with taxonomic and evolutionary significance. Therefore, this study aimed to understand (1) what type of gland croceous glands correspond to, (2) the origin and anatomical structure of these glands, and (3) the composition and potential function of their secretion.

## Material and methods

### Plant material

Flowers and fruits of *Senega longicaulis* (Kunth) J.F.B. Pastore at different developmental stages were collected in Marapanim (Pará, Brazil), and voucher specimens were deposited in the MG Herbarium of the Museu Paraense Emílio Goeldi (MG 248141). The analysis of reproductive organs was based on previous observations of croceous glands in this species.

### Structural and ontogenetic analyses

Ovaries with the persistent calyx and samples of developing and mature fruits were isolated, fixed in formalin–acetic acid–alcohol (FAA) solution for 24 h (Johansen 1940) and buffered neutral formalin in 0.1 M sodium phosphate buffer (pH 7.0) for 48 h (Lillie 1965). Part of the material was dehydrated through a tertiary butyl alcohol series (Johansen 1940), embedded in Paraplast® (Leica Microsystems Inc., Heidelberg, Germany) and serially sectioned on a Leica RM2245 rotary microtome at 11 µm thickness. The sections were stained with astra blue and safranin O (Gerlach 1984), and the slides were mounted with Canada balsam. The other part of the fixed material was dehydrated in an ethanol series, embedded in Leica Historesin®, and serially sectioned at 5 µm thickness. The sections were stained with toluidine blue pH 4.8 (O'Brien et al. 1964). Observations and photographic register were performed using a Leica DM6 B light microscope.

### Analysis of secretion composition

The composition of the croceous gland secretion was investigated using the histochemical tests: Sudan black B for lipids (Pearse 1985), Nile blue for acidic and neutral lipids (Cain 1947), Nadi reagent for terpenoids (essential oils and resin; David and Carde 1964), periodic acid–Schiff's (PAS) reaction for polysaccharides (Jensen 1962), ruthenium red for acidic mucilage (Gregory and Baas 1989), tannic acid and ferric chloride for mucilage (Pizzolato 1977), Lugol's reagent for starch (Johansen 1940), fixation in ferrous sulphate–formalin for phenolic compounds (Johansen 1940), and Dragendorff's reagent for alkaloids (Svedsen and Verpoorte 1983). Standard control procedures were carried out according to Demarco (2023).

### Micromorphological analysis

For the micromorphological study, the FAA-fixed samples were dehydrated in a graded ethanol series, dried by the critical point method, mounted on aluminium stub, and sputter-coated with gold, with subsequent observation in a Tescan Mira3 scanning electron microscope (Tescan, Brno, Czech Republic).

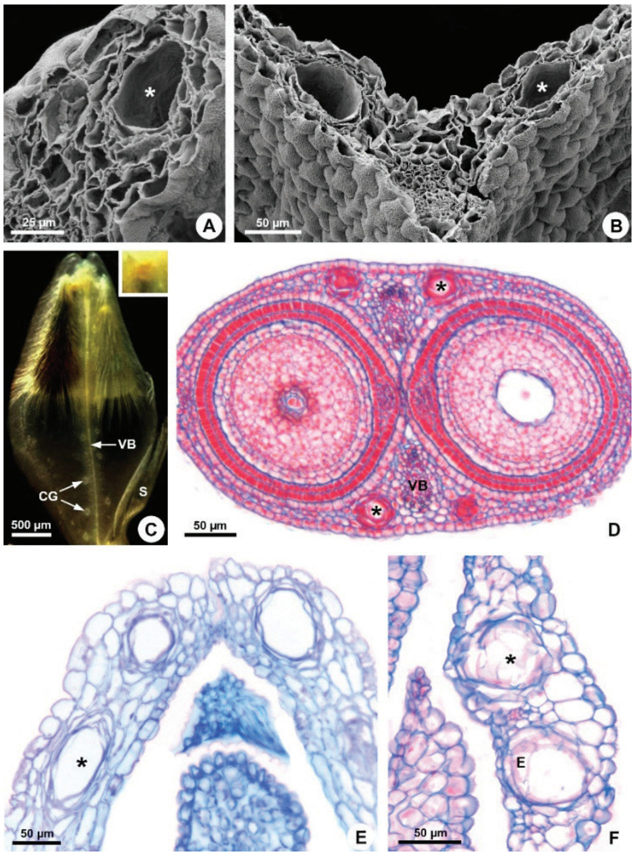

**Fig. 1.** Croceous glands (secretory cavities) in fruits (pericarp) and calyx of *Senega longicaulis*. Ellipsoidal cavity in the pericarp close to the septum (A), secretory cavities in the sepal near the vascular bundle (B), croceous glands forming two rows along with the pericarp vascular bundle, extending from the top to the base of the fruit (C); note the yellow secretion within the croceous gland (inset) and the presence of a persistent calyx with the fruit. Secretory cavities in the ovary near the synlateral vascular bundles (D), longitudinal rows of secretory cavities in the pericarp (E), secretory cavities in the sepal (F); note the presence of epithelium: CG – croceous gland, E – epithelium, S – sepal, VB – vascular bundle, asterisk – secretory cavity. Longitudinal sections (A, E). Transversal sections (B, D, F). Translucent fruit after fixation (C). Scanning electron microscopy (A-B). Stereomicroscopy (C). Light microscopy (D-F).

## Results

The yellowish croceous glands of *Senega longicaulis* occur in the pericarp (Fig. 1A, C) and in the persistent calyx (Fig. 1B) that surrounds the fruit (Fig. 1C). These glands are cavities arranged in longitudinal rows following the main vascular bundles (Fig. 1C-F). Their shape varies from spherical to ellipsoidal (Fig. 1A-F), and, in the latter case, the major axis never exceeds twice the length of the minor axis (Fig. 1A). Although the cavities are aligned and close to each other, they are not adjacent and never merge (Fig. 1C, E).

### Pericarp

In the pericarp, the cavities occur only in the septum and are arranged in four rows, close to the synlateral/synventral vascular bundles (Fig. 1C-D), extending from the top to the base of the fruit (Fig. 1C). These cavities are formed early in the ovary (Fig. 2A-J), but new cavities are originated *de novo* during fruit development, increasing the number and extension of the cavity rows along with the fruit expansion (Fig. 2K). In the ovary, they originate from the ground meristem (Fig. 2A) and are observed in the ovarian mesophyll (Fig. 1D), whereas in the pericarp (Fig. 1E) the new cavities are formed by meristematic cells of the mesocarp.

### Calyx

The cavities found in the persistent calyx are located in the mesophyll and are arranged in rows along the sepals, near the midrib (Fig. 1F). As the cavities formed during floral development, these are also originated from ground meristem. There is no formation of new cavities during fruiting.

### Structure and ontogeny

The cavities in the calyx, ovary, and pericarp are similar, formed by a uniseriate secretory epithelium composed of globose secretory cells during the secretory phase (Fig. 1F, Fig. 2J), which become flattened in the post-secretory phase (Fig. 2K).

The ontogeny of the cavities begins with a meristemoid (Fig. 2A), a cell rich in cytoplasm that expands, becoming much larger than the others. Successive divisions of the meristemoid form a cluster of cells (Fig. 2B), and periclinal divisions (in relation to the location of the future lumen) form a peripheral layer that will give rise to the epithelium (Fig. 2C). The central cell remains much larger than those that surround it. After multiple divisions (Fig. 2D-F), the cluster assumes a spherical or ellipsoidal shape, and the cells split away (schizogeny), leaving the large central cells more or less loose in the centre (Fig. 2G). The surrounding cells, which will become the secretory epithelium, continue to move away, and large vacuoles are observed in the central cells, marking the beginning of the programmed cell death (Fig. 2H). Subsequently, the vacuole ruptures, and the walls of the central cells dissolve, causing the complete lysis of these cells, which disintegrate (lysigeny), originating the cavity lumen (Fig. 2I).

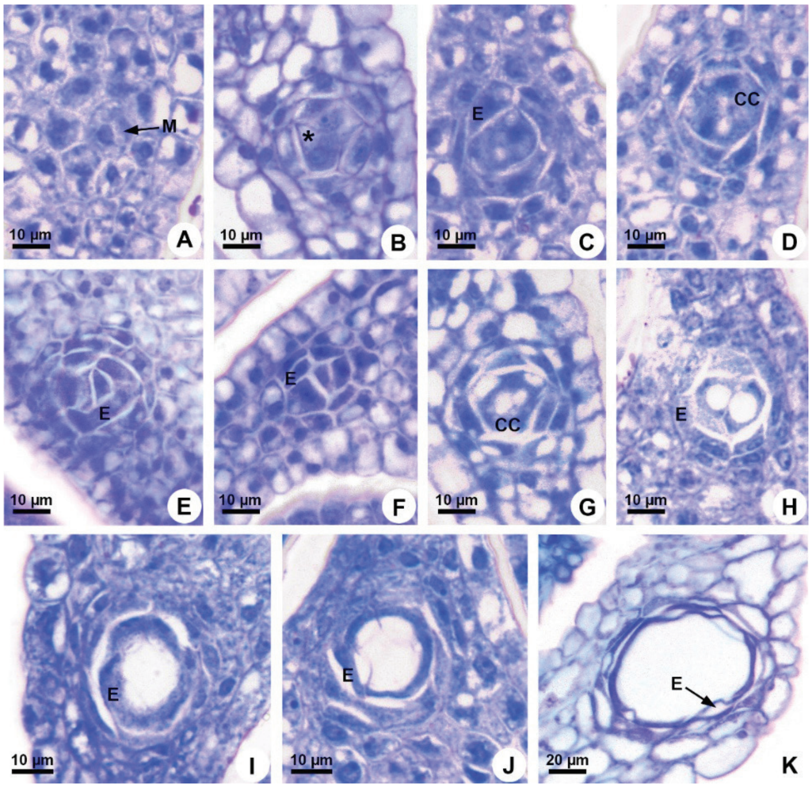

**Fig. 2.** Ontogeny of the croceous gland (secretory cavity) of *Senega longicaulis*. Meristemoid, *i.e.*, initial cell of the cavity (A), first division of the meristemoid (asterisk) (B), periclinal division giving rise to the layer that will originate the epithelium (C), subsequent cell divisions forming a cluster of meristematic cells (D-F), vacuolation of the central cells; beginning of the programmed cell death (G), separation (schizogeny) of the epithelium from the central cells, which are dying (H), epithelium in secretory activity after the lysis of the central cells (I-J). Epithelium in post-secretory phase (K), Ovary (A-J) and pericarp (K). M – meristemoid, CC – central cells, E – epithelium.

Then, the epithelial cells start their secretory activity (Fig. 2J), releasing the secretion into the lumen.

Since cavities are also formed during fruit development, their origin is asynchronous, and cavities at different developmental stages can be observed from the ovary to the mature fruit.

### Secretion composition

The cavities are oleiferous (Tab. 1, Fig. 3).

Histochemical analysis revealed the exclusive presence of lipids in the secretion (Fig. 3A-B), identified as essential oils (Fig. 3C), *i.e.*, volatile terpenes.

stele corners of the stem (Holm 1929, Metcalfe and Chalk 1950). In addition, mucilaginous ducts have been observed in the same position in the stems of *Senega poaya* (as *Polygala angulata*; Aguiar-Dias and Cardoso-Gustavson 2011). Although these glands are not croceous, they demonstrate that secretory spaces are present in other species of the genus, and are distinguished by the composition of the secretion.

Internal secretory structures typically occur throughout the entire plant. On the other hand, when their occurrence is restricted to specific locations, this peculiar distribution usually has taxonomic potential, as previously noted for idioblasts and laticifers (Metcalfe and Chalk 1950, Demarco et al. 2013), and/or implications regarding their origin and function, as reported for ducts (Costa et al. 2021, Tölke et al. 2021). In *S. longicaulis*, we observed that the cavities of the ovary and pericarp occur only in the septum, forming four rows adjacent to the synventral bundles, and in the sepals only near the midrib. No croceous glands were observed in any other part of the fruit or calyx. This distribution is similar to that reported for the croceous glands in the same organs of *S. adenophora* (Jorge et al. 2024) and might have taxonomic and/or systematic significance, particularly considering that croceous glands are restricted to the genus *Senega*. Further studies are needed to evaluate the croceous glands present in vegetative organs as well (Jorge et al. 2024).

The co-occurrence of secretory cavities and ducts has already been described in leaves and stems of some North American species of *Polygala* (Holm 1929, Metcalfe and

**Tab. 1.** Histochemical tests performed on the croceous glands (secretory cavities) of fruits of *Senega longicaulis* (+ = present; - = absent).

| Histochemical test | Target substance | Croceous gland |
|---|---|---|
| Sudan Black B | lipids | + |
| Nile blue | acidic and neutral lipids | + |
| Nadi reagent | essential oils and resin | + |
| PAS reaction | polysaccharides | – |
| Ruthenium red | acidic mucilage | – |
| Tannic acid and ferric chloride | mucilage | – |
| Lugol's reagent | starch | – |
| Ferrous sulphate-formalin | phenolic compounds | – |
| Dragendorff's reagent | alkaloids | – |

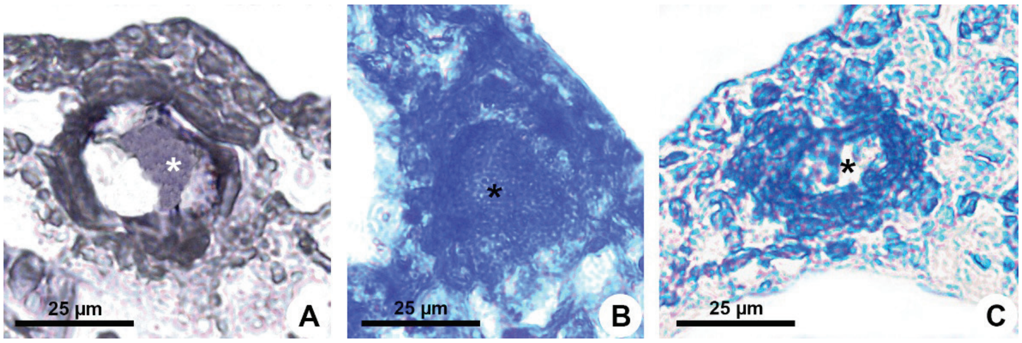

**Fig. 3.** Histochemical analysis of the croceous gland (secretory cavity) of *Senega longicaulis*. Lipids detected using Sudan black B (A), lipids stained with Nile blue (B), essential oils identified using Nadi reagent (C). Asterisk - secretion detected in the croceous gland lumen.

## Discussion

Our study revealed that the croceous glands of *Senega longicaulis* are oil cavities whose yellow colour, typical of these glands, is due to their lipid secretion. This result partially diverges from previous findings for *Senega adenophora*, in which croceous glands can be either ducts or oil cavities (Jorge et al. 2024). Despite this, the nature of the croceous glands has been elucidated and they can be considered, in general terms, secretory spaces (*sensu* Fahn 1979), a generic term that encompasses both types of epithelial glands.

In Polygalaceae, cavities have been described in the leaves (mesophyll) of *Senega paniculata* (L.) J.F.B. Pastore & J.R. Abbott (= *Polygala paniculata* L.; Aguiar-Dias et al. 2012). Cortical oil ducts were also reported in some species of North American *Polygala*, which are almost always located in the

Chalk 1950), and many other reports are found for Asteraceae, Boraginaceae, Combretaceae, Hypericaceae, Malvaceae, Polygalaceae, Rutaceae, Salicaceae, among others (Metcalfe and Chalk 1950, Aguiar-Dias and Cardoso-Gustavson 2011, Aguiar-Dias et al. 2012, Fernandes et al. 2018, Garcia et al. 2020, Tölke et al. 2022). Furthermore, cavities and ducts have been described as occurring side by side within the same organ in some species (Fernandes et al. 2018, Garcia et al. 2020, Tölke et al. 2022, Jorge et al. 2024). Considering that in many of these reports both structures have the same origin and secrete the same exudate, further studies are needed to verify the mode of formation of these epithelial glands. In Malvaceae, it was discovered that the ducts originate from the coalescence of aligned and contiguous cavities (Garcia et al. 2020), similarly to the findings of Jorge et al. (2024) for *S. adenophora*.

Cavities and ducts are histologically and ontogenetically similar. The only difference between these two glands lies in their three-dimensional shape, which is more or less spherical in cavities and elongated in ducts (Fahn 1979). It is not yet possible to establish whether there is an anatomical pattern for the croceous glands of Polygalaceae since multiseriate epithelial cavities have been observed in *S. adenophora* (Jorge et al. 2024), whilst we found uniseriate epithelium cavities in *S. longicaulis*. However, there does seem to be an ontogenetic pattern. In both species studied so far, croceous glands are formed from the ground meristem in the same locations and have a lumen formed by a schizolysigenous process, in which the separation of cells occurs first and then the central cells disintegrate through programmed cell death. However, this developmental process does not always occur in this sequence. In the ducts of *Kielmeyera* Mart. & Zucc., the schizolysigenous formation of the lumen begins with the lysis of the central cells of the rosette, forming an initial space, followed by the separation of the remaining cells. This mixed lumen formation involves cytoskeletal rearrangement and various coordinated subcellular changes during programmed cell death (Costa and Demarco 2024).

The composition of the secretion of croceous glands and its mode of release into the lumen in *S. longicaulis* are also consistent with those observed in *S. adenophora* (Jorge et al. 2024), which seems to indicate a pattern for this type of gland within the family. In both species, the mode of release is merocrine, and the epithelium can be observed in mature glands in the post-secretory phase.

Based on the current data, the yellowish secretion, typical of croceous glands, is composed of volatile oils (terpenes). Similar oil cavities, visualised as pellucid dots, are characteristic of some families, such as Rutaceae and Myrtaceae. In both families, the cavities occur in vegetative organs, flowers and fruits, and their volatile oils are composed of terpenes with the role of defence (eventually, phenolic compounds; Metcalfe and Chalk 1950, Lange 2015, Paschoalini et al. 2022, Tölke et al. 2022, Ladd 2024). As described for those two families, the structure, distribution, and composition of the secretion of croceous glands indicate a protective function in *Senega*.

Volatile oils, produced by internal glands and released only when the tissue is disrupted, act in defence against herbivores and microorganisms (Waterman 1992, Bennett and Wallsgrove 1994, Lange 2015, Tölke et al. 2022). There are many examples of plant terpenes involved in resistance to insects and pathogens, showing an insecticidal effect due to their action as antifeedants (or deterrents), toxins, or as modifiers of insect development, and as inhibitors of fungal proliferation (Bennett and Wallsgrove 1994, and references therein). The presence of croceous glands in the pericarp and persistent calyx, which are largely formed in the flower, helps to protect the developing fruit and ensures the perpetuation of the species.

Our study revealed that croceous glands are secretory spaces that produce volatile terpenes with a probable protective function in *Senega*. Structurally, these glands are cavities in *S. longicaulis*, and the divergence in their typology, compared to other species of the genus, suggests the presence of transitional structures between cavities and ducts. Their specific distribution in organs has potential taxonomic and evolutionary value, but further studies are needed to assess the mode of formation and the systematic importance of croceous glands for Polygalaceae.

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## Author contribution statement

L.K.C.L.: data curation, formal analysis, investigation, methodology, writing – original draft. A.C.A.A.D.: conceptualization, data curation, formal analysis, funding acquisition, project administration, supervision, writing – original draft, and writing, review, and editing. J.F.B.P.: writing, review, and editing. D.D.: conceptualization, data curation, formal analysis, supervision, writing – original draft, and writing, review, and editing. All authors contributed to the article and approved the submitted version.

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Research Article

# The allelopathic effects of exogenous pyrogallol on antioxidant metabolism and leaf gas exchange in arsenic-stressed maize (*Zea mays* L.) seedlings

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**Abstract –** Pyrogallol (PG) is a polyphenol naturally occurring in the leaves and fruits of various plants and is widely utilized as an active component in pharmaceuticals. Although the allelopathic activities of phenolic compounds are well-documented, the allelopathic effects of pyrogallol under heavy metal stress remain poorly understood. This study investigated the effects of PG on oxidative stress indicators, enzymatic and non-enzymatic antioxidant responses, and leaf gas exchange parameters in maize (*Zea mays* L.) seedlings under arsenic (As) stress. The combined treatment with PG and As led to a significant 5-fold increase in arsenic accumulation compared to treatment with As alone. This application also caused excessive oxidative stress, which exceeded the antioxidant system's capacity. Although the application of PG or As alone enhanced activity of antioxidant enzymes, their combined application suppressed these enzymes, reducing total antioxidant capacity. Similarly, the combination of PG and As caused a significant decline in photosynthetic performance, further disrupting redox balance and physiological stability. These findings reveal the synergistic toxicity of PG and As, which severely impair plant metabolism. In As-contaminated soils, phenolic compounds like PG may intensify oxidative stress, influencing plant physiology, depending on the concentration. This study underscores the importance of careful management of phenolic compounds in agricultural systems exposed to heavy metal pollution.

**Keywords:** antioxidant capacity, heavy metals, oxidative stress, phenolic compounds, photosynthesis

## Introduction

Arsenic (As) is a toxic heavy metal and a significant global concern, particularly due to its increasing prevalence in water pollution across many countries (Abedi and Mojiri 2020, Bali and Sidhu 2021). The mobilization of As is strongly influenced by geochemical, microbial, and anthropogenic activities. Plants absorb As ions from the soil in the forms of arsenate (As V) or arsenite (As III). This uptake leads to elevated levels of reactive oxygen species (ROS) in plants, including superoxide radicals, singlet oxygen, hydroxyl radicals, and hydrogen peroxide ($H_2O_2$), which induce oxidative stress and damage plant cells (Tripathi et al. 2012, Nahar et al. 2022). As a result, cellular macromolecules are compromised, and essential physiological processes like photosynthesis are inhibited, ultimately reducing plant growth and productivity (Khan et al. 2021, Asgher et al. 2022). Studies indicate that plants grown in arsenic-contaminated soils exhibit heightened sensitivity to this toxic element, leading to developmental disruptions and impairing their antioxidant defense systems (Srivastava et al. 2016, Asgher et al. 2021, Bali and Sidhu 2021).

Exogenous applications of phenolic acids such as salicylic acid (SA), gallic acid (GA), and caffeic acid (CfA) effectively mitigate the adverse effects of heavy metal (HM) stress in plants. For instance, foliar SA application enhances tolerance to heavy metal stress by enhancing antioxidant defenses, reducing ROS levels, and limiting metal accumulation in *Solanum tuberosum* L. under cadmium (Cd) stress and in *Melissa officinalis* L. under nickel stress (Ni) (Soltani Maivan et al. 2017). In sunflower seeds, GA pretreatment reversed Cd-induced effects, including elevated $H_2O_2$ levels, lipid peroxidation, and antioxidant enzyme activity, while restoring thiols, chlorophyll, and lipid content (Saidi et al. 2021). Similarly, CfA acts as a precursor for ferulic acid and melatonin, with its key enzyme, caffeic acid O-methyltransferase (COMT), showing increased expression in *Medicago sativa* L. and *Brassica napus* L. under copper (Cu), Cd, and aluminum (Al) stress (Vega et al. 2022). Additionally, phenolic acids such as catechol, benzoic acid, p-hydroxybenzoic acid,

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GA, SA, syringic acid, vanillic acid, protocatechuic acid, and gentisic acid have been confirmed to exhibit allelopathic activity (Gulzar et al. 2016). Allelopathy, first defined by the German scientist Molish (1938), refers to the interactions among plants including microorganisms. This definition was later expanded by Rice (1984) to describe allelopathy as the beneficial or detrimental effects of one organism on another through the release of organic compounds into the environment (Cheng and Cheng 2015). These organic compounds, known as allelochemicals, are crucial in natural ecosystems. Allelopathy is a widespread phenomenon that plays a vital role in understanding plant community structures, species distributions, successional dynamics, coevolutionary relationships, and biological invasions (Kalisz et al. 2021). Phenolic allelochemicals are synthesized through the shikimate pathway and serve as defensive agents against pathogens while acting as signaling molecules in plant-pathogen interactions (Misra et al. 2023). Phenolic compounds, which are classified into categories such as flavonoids, tannins, coumarins, lignans, quinones, stilbenes and curcuminoids (Kisiriko et al. 2021), are particularly notable for their strong allelopathic effects (John and Sarada 2012).

Pyrogallol (1, 2, 3-trihydroxybenzene, PG), an important phenolic acid, is naturally found in various organisms, including fruit and vegetables. Numerous studies have documented its allelopathic activity. For instance, *Myriophyllum spicatum* L. produces PG acid, which, along with ellagic acid, GA, and catechin, inhibits the growth of the cyanobacterium *Microcystis aeruginosa* (Nakai et al. 2000). PG applied in high concentrations (e.g., 1 and 2 g $L^{-1}$) has also been shown to inhibit seed germination and seedling growth in *Lolium perenne* L. (Sang et al. 2024), highlighting its allelochemical potential. However, the role of PG in stress tolerance and its effects on plants under stress conditions remain largely unexplored. To overcome this knowledge deficiency, the following research questions arise: (i) What is the role of PG as a phenolic allelochemical in modulating plant responses to As-induced stress? (ii) Does PG, under arsenic stress conditions, influence antioxidant defense mechanisms and photosynthetic efficiency in plants? (iii) How does the allelopathic activity of PG interact with arsenic stress to affect plant physiological and biochemical processes? For this reason, this study comprehensively investigated the effects of PG and As applications, both individually and in combination, on maize seedlings. Gas exchange parameters, including photosynthetic rate ($P_n$), stomatal conductance ($g_s$), transpiration (E), and intercellular carbon dioxide ($C_i$) levels, were measured to evaluate the extent of photosynthetic impairment. Oxidative stress levels were assessed through thiobarbituric acid reactive substances (TBARS) and $H_2O_2$ accumulation, while the antioxidant defense system was analyzed both enzymatically including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and non-enzymatically (total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC). Additionally, As uptake was determined by inductively coupled plasma mass spectrometry (ICP-MS). This study provides the first detailed evidence of the destructive impacts of PG on photosynthesis and antioxidant status under As stress, offering new insights into its allelopathic role.

## Material and methods

### Plant material and treatments

*Zea mays* L. (cultivar, ADA 523) seeds were sourced from Sakarya Maize Research Institute. The seedlings were cultivated in a growth chamber for 21 days under controlled conditions: relative humidity of 60–65%, light intensity of 250 µmol $m^{-2}$ $s^{-1}$ (Demiralay 2022) a temperature of 25 ± 2 °C, and a photoperiod of 16 h light/8 h dark. When the seedlings reached the three-leaf stage, their above-ground parts were excised to ensure uniform and efficient uptake of PG by the maize plants. Following this step, the seedlings were placed in glass tubes (16 × 100 mm) containing distilled water (DW) for 1 h to reduce stress-induced damage. Pyrogallol concentration was set at 5 mM (0.63 g $L^{-1}$) according to previous findings that higher concentrations, such as 1 and 2 g $L^{-1}$, exhibit strong inhibitory effects on germination and root growth due to their allelopathic properties, while lower concentrations, such as 0.25 – 0.50 g $L^{-1}$, have moderate effects (Sang et al. 2024). The selected concentration aims to strike a balance between avoiding excessive toxicity and allowing measurable impacts on plant physiology. We selected 100 µM as the As stress concentration as Ghosh et al. (2016) clearly demonstrated that this concentration triggers a pronounced oxidative stress response in maize seedlings. The experimental design included four treatment groups: (i) Control group with no treatment: seedlings exposed to DW for 18 h, (ii) As-stressed group: pre-treated with DW for 6 h, followed by exposure to 100 µM As for 12 h, (iii) PG-stressed group: pre-treated with 5 mM PG for 6 h, followed by DW treatment for 12 h and (iv) PG+As group: pre-treated with 5 mM PG for 6 h, followed by treatment with 100 µM As for 12 h.

### Determination of As content

For the simultaneous multielement detection of As contents in maize seedlings, an Agilent 7900 model inductively coupled plasma mass spectrometer (ICP-MS) was employed. First, 0.1 g of each sample was digested using 8 mL of 65% (v/v) $HNO_3$ and 1 mL of 35% (v/v) $H_2O_2$ in polytetrafluoroethylene vessels. These vessels were then placed in a microwave oven (Anton Paar Microwave Reaction System), with the following conditions: heating to 185 °C for 20 min, maintaining a constant temperature for 15 min, and cooling to 60 °C for 21 min. Each sample was subsequently diluted with 50 mL of DW. This prepared solution was then analyzed for As content using an ICP-MS device equipped with a concentric nebulizer, a quartz torch with a quartz injector tube, and a cyclonic spray chamber. On-line Suppl. Tab. 1 displays displays the ICP-MS working conditions.

### Determination of oxidative stress parameters

The amount of lipid peroxidation was measured in terms of TBARS content (Heath and Packer 1968). Leaf sample (0.1 g) was homogenized in 0.1% trichloroacetic acid (TCA), centrifuged, and 1 mL of supernatant was mixed with 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 95 °C for 30 min, cooled, and absorbance was measured at 532 nm with nonspecific absorbance at 600 nm subtracted. TBARS content was calculated using a molar absorption coefficient of 155 $mM^{-1}$ $cm^{-1}$.

The $H_2O_2$ assay was performed according to the method described by Velikova et al. (2000). Leaf tissue (0.1 g) was homogenized in 0.1% TCA and centrifuged. From the resulting supernatant, 1 mL was mixed with 10 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (KI). The absorbance was measured at 390 nm. The $H_2O_2$ content was quantified using a standard curve prepared with known $H_2O_2$ concentrations ranging from 0 to 100 µM.

### Determination of enzymatic antioxidant activities

The plant leaf sample (0.5 g) was ground in liquid nitrogen and homogenized in 5 mL of extraction buffer (50 mM $K_2HPO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7 and 0.1% polyvinylpolypyrrolidone (PVPP)). The homogenate was centrifuged at 20000 g for 20 min at 4 °C, and the resulting supernatant was used for enzymatic activity analysis.

The SOD (EC 1.15.1.1) activity was determined using the method described by Beauchamp and Fridovich (1971). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 µM nitroblue tetrazolium (NBT), 50 µL enzyme extract, and 2 µM riboflavin. An enzyme-free mixture (blank) was also prepared as a control. Upon illumination, riboflavin generated superoxide anions that reacted with NBT to produce formazan, a blue-colored complex. The reduction in formazan formation was proportional to the SOD content, and a 50% decrease in formazan formation was defined as one unit of SOD activity. The reaction was initiated and terminated by turning the light on and off. The mixture was exposed to white light at an intensity of 375 µmol $m^{-2}$ $s^{-1}$ for 10 min, after which absorbance at 560 nm was recorded. SOD activity was calculated using the following formula:

(Absorbance of blank - Absorbance of sample)/Absorbance of blank/(%50 × reaction mixture volume).

The CAT (EC 1.11.1.6) activity was determined according to Aebi (1984). 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 30 mM $H_2O_2$, and 20 µL enzyme extract was measured at 240 nm for 5 min. The activity was calculated using an extinction coefficient of 39.4 $mM^{-1}$ $cm^{-1}$ for $H_2O_2$.

The APX (EC 1.11.1.11) activity was measured based on the decrease in absorbance at 290 nm (Nakano and Asada 1987). The assay was performed using a 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 250 µM ascorbate (ASC), 5 mM $H_2O_2$, and 20 µL enzyme extract. The activity was calculated using an extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$ for ASC at 290 nm.

Protein content was determined in antioxidant enzyme extracts according to the method of Bradford (1976). A standard curve was generated using bovine serum albumin (BSA) standards in the range of 0-100 µg $ml^{-1}$. The absorbance of the complex formed between Coomassie Brilliant Blue G-250 dye and the protein was measured at 595 nm. Protein concentration was calculated in mg and used to express enzymatic activities.

### Determination of antioxidant capacity

The powdered dry maize leaf sample 1 g was mixed with methanol (20 mL) and incubated at 25 ± 1 °C for 24 h to ensure optimal extraction of polar phytochemicals. The mixture was then passed through Whatman No. 1 filter paper to remove particulate matter. The filtrate was concentrated under reduced pressure using a rotary evaporator set at 40 °C until a semi-solid extract was obtained. The obtained crude extracts were stored at −20 °C in amber vials until further use. Antioxidant properties of the extracts were evaluated through the determination of total phenolic and flavonoid contents. In addition, their reducing capacity was assessed using FRAP and CUPRAC assays. To identify and quantify specific phytochemical constituents, high-performance liquid chromatography (HPLC) analysis was performed.

### Determination of gas exchange parameters

The LI-6800 Portable Photosynthesis System (LI-COR Biosciences, Inc., Lincoln, NE, USA) was used to measure the $P_n$, E, $g_s$, and $C_i$ in *Zea mays*. Measurements were taken from the uppermost fully expanded third leaf of five plants per group, with ten readings recorded at 5-second intervals. Conditions included a light intensity of 250 µmol $m^{-2}$ $s^{-1}$ (Demiralay 2022), 25 °C block and leaf temperatures, and 60% relative humidity. $CO_2$ level was set to 400 µmol $mol^{-1}$ and allowed to equilibrate for 30 min before measurement.

### Statistical analysis

All experiments were conducted in five replicates. The results were calculated using the SPSS (version 27 for IBM SPSS Statistic) program with One-way ANOVA, and significant differences were determined by Duncan's multiple range tests, with $P < 0.05$ considered significant.

## Results

### As uptake

The As uptake did not differ significantly between the control and PG application. However, the As uptake was significantly increased compared to control ($P < 0.05$) with the application of As alone, showing a 1.5-fold increase. Meanwhile, the PG+As application significantly increased

($P < 0.05$) As uptake by 5-fold compared to the application of As alone (Tab. 1).

**Tab. 1.** Effects of exogenous pyrogallol (PG) and arsenic (As) alone or in combination (PG+As), on As uptake in maize seedlings. Results are shown as means ± standard errors of five replicates. Means followed by different letters are significantly different according to Duncan's test at $P < 0.05$.

| Treatment groups | As content (μg g$^{-1}$) |
|---|---|
| Control | 152.6 ± 22$^{c}$ |
| 5 mM PG | 163.1 ± 10$^{c}$ |
| 100 μM As | 237.5 ± 54$^{b}$ |
| PG+As | 1193 ± 76$^{a}$ |

### Oxidative stress parameters

The TBARS content significantly increased ($P < 0.05$) with the application of PG or As, alone and the PG+As combination compared to the control group (Fig. 1A). The TBARS content with the application of PG or As alone was 1.3-fold higher than in the control group. Furthermore, the PG+As combination increased the TBARS content by 1.8-fold compared to the application of As alone.

The $H_2O_2$ content significantly increased ($P < 0.05$) with PG or As alone compared to the control (Fig. 1B), showing a 2.5-fold increase with the application of PG alone and a 2.3-fold increase with the application of As alone. Consistent with the lipid peroxidation results, the PG+As combination significantly increased ($P < 0.05$) the $H_2O_2$ content 1.5-fold compared to the application of As alone.

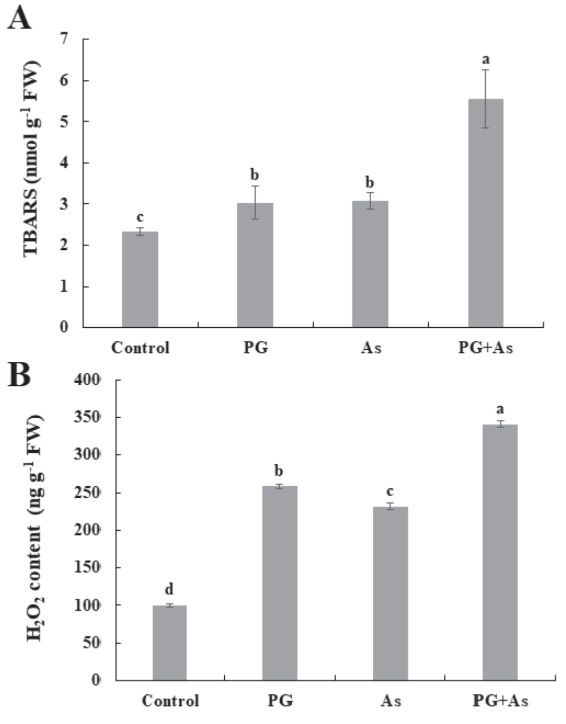

**Fig. 1.** Effects of exogenous pyrogallol (5 mM PG) and arsenic (100 μM As) alone or in combination (PG+As), on oxidative stress of maize seedlings: A – thiobarbituric acid reactive substances (TBARS) and B – hydrogen peroxide ($H_2O_2$) contents. Results are shown as means ± standard errors of five replicates. The difference between the means marked with the different letters on the columns is significant ($P < 0.05$).

### Activities of antioxidant enzymes

SOD, CAT and APX activities significantly changed ($P < 0.05$) with the application of PG or As alone and PG+As combination. SOD activity increased by 2.9-fold and 1.9-fold with the application of PG or As alone, respectively (Fig. 2A). However, the PG+As combination significantly decreased ($P < 0.05$) SOD activity 1.4-fold, 4.1-fold, and 2.7-fold, respectively, compared to the application of control or PG, or As alone.

CAT activity significantly increased ($P < 0.05$) with the application of PG or As alone, resulting in 2.4-fold and 2.2-fold increases, respectively, compared to the control (Fig. 2B). However, the PG+As combination caused a significant decrease ($P < 0.05$), reducing CAT activity 1.9-fold and 1.7-fold compared to the application of PG or As alone.

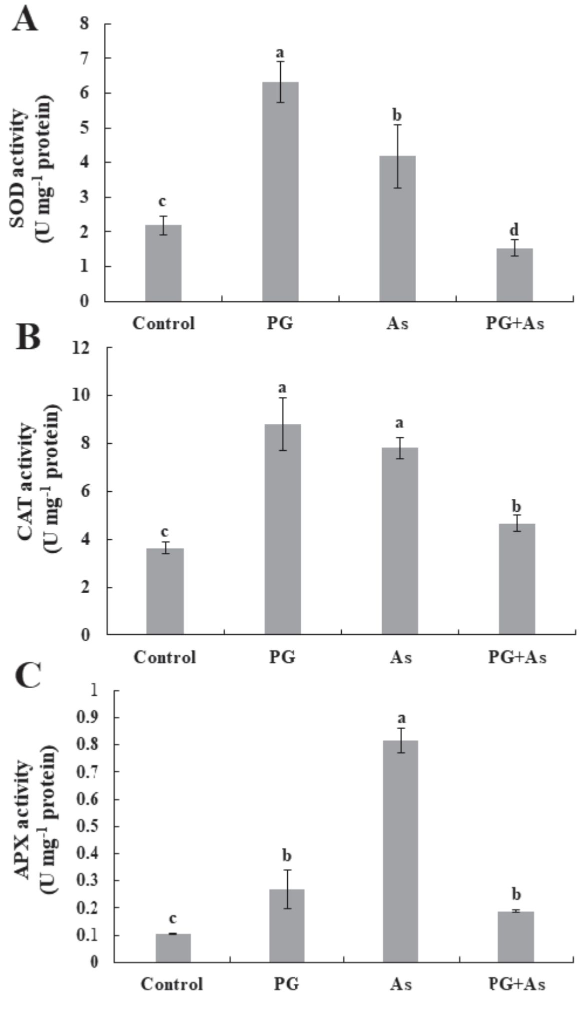

**Fig. 2.** Effects of exogenous pyrogallol (5 mM PG) and arsenic (100 μM As) alone, or in combination (PG+As) on enzyme activities of maize seedlings: A - superoxide dismutase (SOD), B - catalase (CAT), C - ascorbate peroxidase (APX). Results are shown as means ± standard errors of five replicates. The difference between the means marked with the different letters on the columns is significant ($P < 0.05$).

APX activity significantly increased ($P < 0.05$) with the application of PG or As alone, increasing 2.6-fold and 7.9-fold, respectively, compared to the control (Fig. 2C). However, the application of PG alone and PG+As combination caused a 3-fold and 4.3-fold significant reduction ($P < 0.05$) in APX activity compared to the application of As alone.

## Antioxidant capacity

TPC significantly increased ($P < 0.05$) with the application of PG or As alone, showing a 1.4-fold increase, compared to the control. However, the PG+As combination caused a significant decrease ($P < 0.05$), reducing the TPC 1.3-fold, 1.8-fold, and 1.8-fold, compared to the application control or PG or As alone, respectively (Tab. 2).

There was no statistically significant difference in TFC between the application of control, PG, or As alone. However, the PG+As combination significantly decreased ($P < 0.05$) the TFC, 1.6-fold, compared to the application of As alone (Tab. 2).

The FRAP values significantly increased ($P < 0.05$) in the application of PG or As alone, with both increasing 1-fold, compared to the control. However, the PG+As combination significantly decreased ($P < 0.05$) the FRAP value, resulting in 1.4-fold, 1.5-fold and 1.5-fold reductions compared to the application of control or PG or As alone, respectively (Tab. 2).

The CUPRAC values significantly increased ($P < 0.05$) with the application of PG or As alone, reducing the CUPRAC 1.3-fold and 1.2-fold compared to the control. However, the PG+As combination significantly decreased ($P < 0.05$) the CUPRAC value, resulting in 1.9-fold, 2.5-fold and 2-fold decreases compared to the application of control or PG or As alone, respectively (Tab. 2).

## Gas exchange parameters

Gas exchange parameters, including $P_n$, E, $C_i$, and $g_s$, significantly declined ($P < 0.05$) with the application of PG

**Tab. 2.** Effects of exogenous pyrogallol (PG) and arsenic (As) alone or in combination (PG+As) on total phenolics content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) in maize seedlings. Results are shown as means ± standard errors of five replicates. Means followed by different letters are significantly different according to Duncan's test at $P < 0.05$.

| Treatments | Total phenolics content (mg GAE $g^{-1}$ FW) | Total flavonoid content (mg QE $g^{-1}$ FW) | FRAP (µmol Fe $g^{-1}$ FW) | CUPRAC (mmol TEAC $g^{-1}$ FW) |
|---|---|---|---|---|
| Control | 3.36 ± 0.3$^{b}$ | 6.41 ± 0.25$^{a}$ | 4.72 ± 0.05$^{b}$ | 0.04 ± 0.006$^{c}$ |
| 5 mM PG | 4.70 ± 0.3$^{a}$ | 6.43 ± 0.7$^{a}$ | 4.93 ± 0.05$^{a}$ | 0.06 ± 0.0003$^{a}$ |
| 100 µM As | 4.60 ± 0.5$^{a}$ | 6.55 ± 0.17$^{a}$ | 4.88 ± 0.02$^{a}$ | 0.05 ± 0.001$^{b}$ |
| PG+As | 2.58 ± 0.09$^{c}$ | 4.11 ± 0.08$^{b}$ | 3.30 ± 0.09$^{c}$ | 0.02 ± 0.001$^{d}$ |

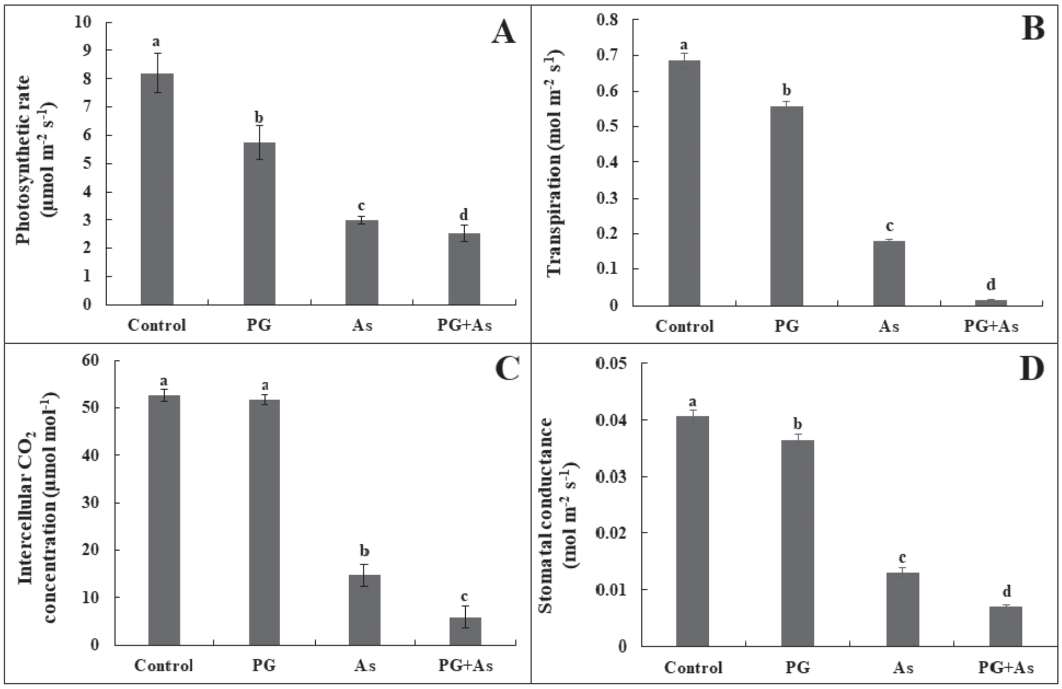

**Fig. 3.** Effects of exogenous pyrogallol (5 mM PG) and arsenic (100 µM As) alone, or in combination (PG+As) on photosynthetic gas exchange parameters of maize seedlings: A – photosynthetic rate ($P_n$), B – transpiration rate (E), C – intercellular $CO_2$ concentration ($C_i$), D – stomatal conductance ($g_s$). Results are shown as means ± standard errors of five replicates. The difference between the means marked with the different letters on the columns is significant ($P < 0.05$).

or As alone compared to the control. However, there was no statistically significant difference in the $C_i$ value between the control and PG applications. In the PG application, the decreases were 1.4-fold, 1.2-fold, 1-fold, and 1.1-fold for $P_n$, E, $C_i$, and $g_s$, respectively. The As application significantly decreased these parameters ($P < 0.05$), resulting in 2.7-fold, 3.8-fold, 3.6-fold, and 3.1-fold reductions for $P_n$, E, $C_i$, and $g_s$, respectively. Furthermore, the PG+As combination significantly decreased ($P < 0.05$) all gas exchange parameters compared to the control, the application of PG, or As alone (Fig. 3). For example, compared to the control, the PG+As combination significantly decreased ($P < 0.05$) $P_n$, E, $C_i$, and $g_s$ values 3.3-fold, 47.5-fold, 9-fold, and 5.8-fold, respectively.

Compared to the application of PG alone, the PG+As combination significantly decreased ($P < 0.05$) the $P_n$, E, $C_i$, and $g_s$ values 2.3-fold, 38.7-fold, 8.8-fold, and 5.2-fold, respectively. Finally, compared to the application of As alone, the PG+As combination significantly decreased ($P < 0.05$) the $P_n$, E, $C_i$, and $g^s$ values 1.2-fold, 1.3-fold, 2.5-fold, and 1.9-fold, respectively (Fig. 3).

## Discussion

Allelochemicals possess a remarkable ability to disrupt plant physiological and biochemical processes, acting as both stressors and regulators in plant systems (Soares et al. 2012). Their toxic effects are highly dependent on key variables such as concentration, plant developmental stage, and environmental conditions (Won et al. 2013). By inducing oxidative stress, these compounds significantly inhibit plant growth and development (Bich and Kato-Noguchi 2012). This highlights the complicated and dynamic nature of plant responses to allelochemical-induced stress, presenting both challenges and opportunities for understanding plant-environment interactions.

Phenolic compounds have emerged as powerful modulators of plant stress responses, owing to their pronounced allelopathic activities and multifunctional roles in enhancing stress tolerance. With over 48,000 structurally diverse molecules, polyphenols are synthesized via the shikimic acid pathway and serve as crucial secondary metabolites in plants (Stiller et al. 2021). Their redox properties allow them to function as potent antioxidants, electron donors, and radical scavengers, positioning them as central agents in mitigating oxidative stress and sustaining plant health under adverse conditions (Zagoskina et al. 2023). Especially polyphenols are recognized for their strong antioxidant properties, primarily driven by their redox potential, which allows them to function as electron donors, singlet oxygen quenchers, and radical scavengers. While these properties highlight their protective roles in plants, phenolic compounds also exhibit notable allelopathic effects in plant-plant and plant-environment interactions (Pisoschi and Pop 2015). Specifically, phenolic acids such as GA, p-hydroxybenzoic acid, CfA, cinnamic acid, syringic acid, ferulic acid, SA, and vanillic acid are known to play complex roles in regulating plant responses to abiotic stress (Mughal et al. 2024).

Many studies have highlighted PG's allelopathic activity, including its inhibitory effects on seed germination (Sang et al. 2024), specific enzyme activities, and microbial growth (Yan et al. 2010, Li et al. 2010). However, the specific mechanisms linking PG to its regulatory role in stress tolerance remain unclear. To address this knowledge gap, this study investigated the effects of PG on maize seedlings exposed to As stress. The primary aim was to determine whether PG contributes to enhanced stress tolerance and to elucidate its potential mechanisms. By focusing on the interaction between PG and As, this research has sought to provide a deeper understanding of PG's allelopathic and stress-regulatory properties.

Our findings revealed that a 5 mM PG application resulted in a dramatic 5-fold increase in As accumulation in maize tissues compared to As alone (Tab. 1). This substantial increase is indicative of severe metabolic disruptions, consistent with earlier studies that highlight As's harmful effects on plant productivity (Wu et al. 2011). The allelopathic activity of PG, previously reported to delay germination and retard development in *Lolium perenne* (Sang et al. 2024) likely aggravates developmental toxicity under As stress. The underlying mechanism of this interaction likely originates from pyrogallol's auto-oxidation, leading to an increased production of $H_2O_2$ and ROS (Inui et al. 2004, Upadhyay et al. 2010). The production of ROS damages the membrane, increasing its permeability and enabling more efficient As uptake. Moreover, the catalytic interactions with metal ions amplify oxidative stress, forming a toxic feedback loop that severely damages plant cells and physiological balance.

Phenolic compounds are potent inducers of oxidative stress in plants. For example, cinnamic acid has been shown to amplify oxidative damage in cucumber by inhibiting antioxidant enzymes, leading to excessive ROS production and cellular damage (Ye et al. 2006, Chai et al. 2013). Similarly, PG, despite its beneficial properties, induces oxidative stress through the generation of free radicals, which is the central mechanism of its toxicity (Upadhyay et al. 2010). Our results demonstrated that high concentrations of PG (5 mM) significantly elevated $H_2O_2$ and TBARS levels compared to application of PG or As alone, indicating that the PG+As combination triggers an excessive ROS burst (Fig. 1). This is consistent with the previous research findings that high concentrations of PG and related organic acids, such as syringic acid and vanillic acid, increased TBARS levels in *Pinus koraiensis* Sieb. et Zucc. and *L. perenne* L. seedlings (Liang et al. 2021, Sang et al., 2024). The synergistic toxicity of PG+As likely aggravates lipid peroxidation, further damaging cellular structures and inducing oxidative stress in maize seedlings.

The sensitivity of protective enzymes to allelochemicals is highly variable, depending on their concentration and plant species, often leading to disruptions in the precise balance between antioxidant defenses and ROS (Araniti et al. 2018, Šoln et al. 2022). Phenolic compounds, including PG, exhibit strong inhibitory effects on key enzyme activities, interfering with metabolic pathways and amplifying oxidative stress. For instance, chlorogenic acid, CfA, and cinnamic acid derivatives target critical enzymes such as phosphorylase and ATPase, while tannic acid inhibits essential antioxidant enzymes like CAT and APX (Rice 1979, Batish et al. 2008). Our study revealed that while 5 mM PG application increased the activities of antioxidant enzymes such as SOD, CAT, and APX in maize seedlings, its combination with As resulted in a marked suppression of these enzymes (Fig. 2). This suppression highlights the toxic synergism of PG+As, which overwhelms the antioxidant defense system, leaving the plant vulnerable to oxidative damage.

Phenolic compounds source their antioxidant capacity from their remarkable ability to neutralize ROS (Zheng and Wang 2001). Notably, this potential can be amplified through synergistic interactions with other phytochemicals, further enhancing their protective effects (Plaza et al. 2011). Our findings revealed that while PG or As applied alone significantly elevated the TPC, FRAP, and CUPRAC values in maize seedlings, the PG+As combination caused a dramatic reduction in these antioxidant parameters. This indicates that 5 mM PG application partially activates the antioxidant defense system, helping to maintain redox balance and mitigate oxidative damage. However, the PG+As combination created a toxic synergistic effect by drastically increasing ROS levels, overwhelming the plant's antioxidant capacity and causing severe suppression of its defense mechanisms (Tab. 2). These results are consistent with the findings of Tian and Li (2018), who demonstrated that phenolic compounds under stress conditions diminished antioxidant capacity in maize seedlings, reinforcing the potential risks associated with their overuse.

Photosynthesis is an indispensable physiological process vital for plant growth and survival. However, As toxicity severely compromises this process by damaging chloroplast structure, disrupting the synthesis of photosynthetic pigments, and significantly impairing the activity of photosystem I (PSI) and photosystem II (PSII). This disruption has been well documented, with As stress being shown to inhibit chlorophyll biosynthesis in various plants, including *Zea mays* L., *Trifolium pratense* L., and *Lactuca sativa* L. (Suneja 2014, Emamverdian et al. 2015, Nabi et al. 2019). Beyond As stress, phenolic allelochemicals, such as grandinol, homograndinol, CfA, coumaric acid, ferulic acid, cinnamic acid, and vanillic acid, have also demonstrated severe inhibitory effects on photosynthesis. These compounds reduce chlorophyll production and significantly lower the photosynthetic rate, ultimately stunting plant growth (Patterson 1981, Yoshida et al. 1988). In our study, both PG and As independently caused substantial reductions in key photosynthetic parameters, including $P_n$, E, $C_i$, and $g_s$. Remarkably, their combined application exacerbated these effects, inducing a dramatic decline in gas exchange (Fig. 3). This highlights the synergistic toxicity of PG and As, which not only disrupts core photosynthetic processes but also imposes severe metabolic constraints on maize seedlings. These findings are consistent with those of Patterson (1981), who observed that 10-30 μmol $L^{-1}$ concentrations of phenolic acids like CfA, coumaric, ferulic, cinnamic, and vanillic acids suppressed photosynthesis and reduced chlorophyll content in soybean. The PG+As combined likely suppressed the antioxidant defense system while disrupting stomatal regulation, exacerbating water imbalance and photosynthetic inefficiency.

PG dramatically disrupts cellular redox balance under As stress, significantly impairing the fundamental antioxidant defense system and consequently triggering excessive production of ROS, intense TBARS, and cellular damage. Moreover, the potent allelopathic activity of PG synergistically interacts with As to markedly increase As uptake, leading to striking declines in critical photosynthetic parameters such as $P_n$, $g_s$, E, and $C_i$. This synergistic interaction clearly demonstrates that PG not only severely compromises the defense mechanisms against As stress but also undermines the overall physiological and biochemical integrity of the plants.

## Conclusion

In sum, our results reveal a toxic synergy between PG and As in maize seedlings. While PG alone induces modest activation of antioxidant defenses, its combination with As leads to an increase in As accumulation up to fivefold accompanied by a burst of ROS and severe lipid peroxidation. This dual effect results in disruption of cellular redox homeostasis, significant suppression of key antioxidant enzymes, and impairment of photosynthetic efficiency, ultimately compromising both cellular integrity and metabolic function. These findings illustrate the complex, double-edged nature of phenolic allelochemicals, which can act both as mitigators and as amplifiers of stress depending on environmental conditions. This study highlights the need to carefully manage phenolic compounds in ecosystems exposed to HM pollution and the importance of precise agricultural practices to ensure sustainable crop productivity and environmental health.

## Author contribution statement

Altuntaş Cansu, Aksu Kalmuk Nurşen and Gümrükçüoğlu Abidin designed the present study and have supervised this work. Altuntaş Cansu, Aksu Kalmuk Nurşen and Gümrükçüoğlu Abidin performed the experiments. The analysis and interpretation of the results were carried out by Altuntaş Cansu, Aksu Kalmuk Nurşen and Gümrükçüoğlu Abidin. The drafting of the manuscript was carried out by Altuntaş Cansu with the assistance of Aksu Kalmuk Nurşen and Gümrükçüoğlu Abidin. All the authors contributed and reviewed the results and approved the final manuscript.

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Short Communication

# First record of *Spiraea hypericifolia* subsp. *hypericifolia* (Rosaceae) in Kosovo

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**Abstract** – This study reports the first record of *Spiraea hypericifolia* subsp. *hypericifolia* in Kosovo, found on Mt Golesh at 740 m a.s.l. The taxon forms a dense population on serpentine substrates, occupying a specific ecological niche. Due to its limited distribution and specific habitat requirements, it faces a high risk of extinction. A preliminary conservation status assessment suggests it is Vulnerable (VU) D2 according to IUCN criteria. Additional field surveys and habitat monitoring are needed to protect this population against environmental and anthropogenic pressures.

**Keywords:** central Kosovo, IUCN, plant ecology, plant taxonomy, serpentine

## Introduction

The genus *Spiraea* L. (Rosaceae) is well-known for its ornamental values and ecological importance. Different species have demonstrated notable biological activities, including anti-inflammatory, antioxidant, antiviral, antibacterial, antifungal and anticancer effects, through the study of their extracts and individual compounds (Kostikova and Petrova 2021, Kostikova et al. 2022). Comprising approximately 80 to 100 species, it is widely distributed across the temperate regions of the Northern Hemisphere, from North America to East Asia and extending into Europe (Potter et al. 2007, Roloff and Bärtels 2008). Taxa within this genus are characterized by their diverse morphological traits and adaptability to various environmental conditions, which have allowed them to occupy a range of habitats from forests to open grasslands (Kubitzki 2004).

In Europe, *Spiraea* is known to have seven native species with eight subspecies: *S. cana* Waldst. & Kit., *S. chamaedryfolia* L., *S. crenata* L. [*S. crenata* subsp. *crenata*, *S. crenata* subsp. *parvifolia* (Pau) Romo], *S. decumbens* W.D.J.Koch, [*S. decumbens* subsp. *decumbens*, *S. decumbens* subsp. *tomentosa* (Poech) Dostál], *S. hypericifolia* L. [*S. hypericifolia* subsp. *hypericifolia*, *S. hypericifolia* subsp. *obovata* (Willd.) H. Huber], *S. media* Schmidt [*S. media* Schmidt subsp. *media*, S. *media* subsp. *polonica* (Błocki) Dostál] and *S. salicifolia* L. (Kurtto 2009). The only species of the genus mentioned for Kosovo thus far is *S. crenata*, reported from the Gorge of Prizren (Jovanović 1972). Here we report for the first time *Spiraea hypericifolia* subsp. *hypericifolia* in Kosovo, found during fieldwork on Mt Golesh (central Kosovo).

The major part of *Spiraea hypericifolia*'s geographical range lies in Eurasia, with a native range extending from southwestern Europe to central and southwestern Asia (Conti and Bartolucci 2023). It is regarded as one of the most evolutionarily developed species within its genus (Yu et al. 2018). The native range of *S. hypericifolia* subsp. *hypericifolia* in Europe includes central Italy and Bulgaria, while its distribution extends eastward to Siberia and the western and central Himalayas (POWO 2024). The discovery of *S. hypericifolia* subsp. *hypericifolia* in Kosovo marks a significant contribution to the botanical knowledge of the region, as it extends the known distribution range of this taxon further west within the Balkans. This record not only highlights the ecological significance of Kosovo as a refuge for species with broad Eurasian distributions but also provides important insights into the biogeographical patterns and evolutionary history of the species in question in the context of the Balkan Peninsula.

## Material and methods

The current study is based on field observations on Mt Golesh during 2024. Mt Golesh (22.2 km$^2$) lies in the central part of Kosovo and belongs to the central mountain chain of the Carraleva Mountains (Marković 1990). It separates the region of Drenica from the Kosovo plain (Pllana 2015), and its highest peak is 1019 m above sea level (a.s.l), making it the highest mountain system in the central part of Kosovo (Çavolli 1997). Approximately 70% of this mountainous area is situated at altitudes ranging from 600 to 980 meters above sea level, while the upper regions exceed 980 m a.s.l. Golesh is recognized as a massif primarily composed of

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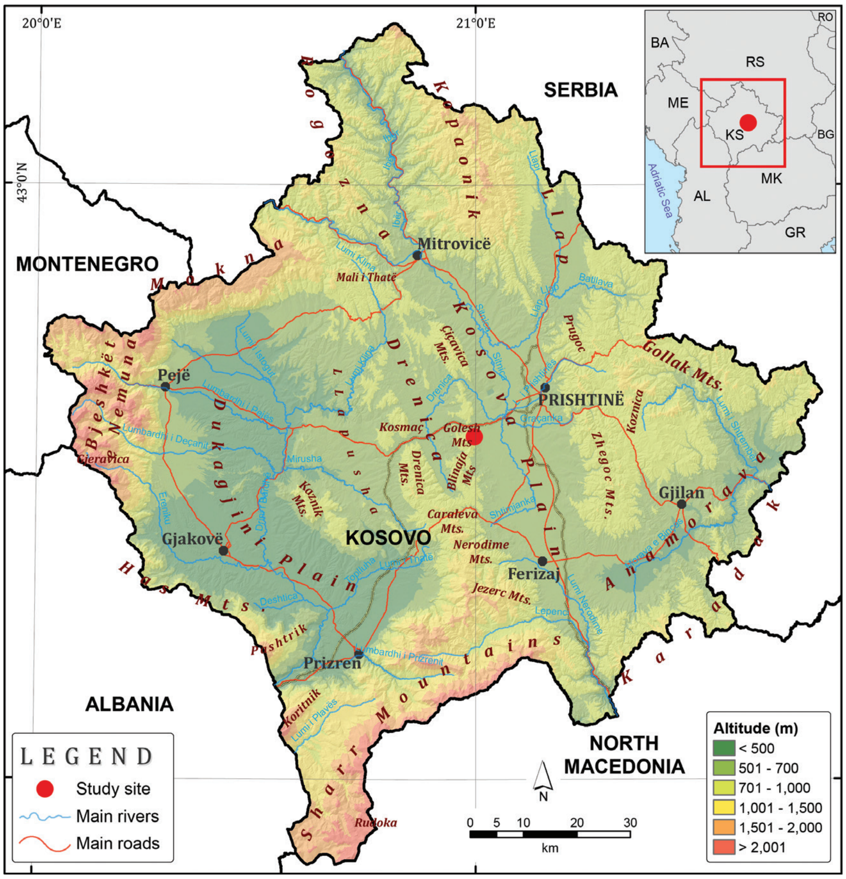

**Fig. 1.** Map of the study area on Mt Golesh, central Kosovo (circled in red).

harzburgite rock, which consists mainly of two minerals: olivine and low-calcium pyroxene, and features a high percentage of ultramafic (serpentinite) bedrock (Labus 1973). This composition creates a challenging environment for plant growth and development due to chemical, physical, and biotic factors, including drought, the presence of heavy metals, and limited sources of essential nutrients (Herath et al. 2014). From a pedological perspective, the surveyed area consists of loamy deluvium, brownized red soil on compact limestone, reddish-brown loamy soil on red sediments, and moderately deep brown soil on metamorphic rocks (Pavićević et al. 1974). Climatically, the studied site is characterized by harsh, cold winters and hot, dry summers, with an average annual temperature of 9.9 °C, typical of a continental climate (Pllana, 2015). Significant parts of Mt Golesh are legally protected in Kosovo (Nature Monument – IUCN Category III) due to the presence of natural habitats of the Balkan endemic plant species *Forsythia europaea* Degen & Bald (AMMK 2019). The habitat in which *Spiraea hypericifolia* subsp. *hypericifolia* has been recorded lies within this protected area. However, it is situated at the edge of Harilaq Castle, a popular destination for visitors, and thus may be subject to potential negative impacts from tourism.

In addition to the plant taxa of interest, accompanying plant species were also recorded. The nomenclature of the taxa follows the Euro+Med Plantbase Checklist (Kurtto 2009). The collected plant specimens were deposited in the herbarium of the Faculty of Mathematics and Natural Sciences at the University of Prishtina.

## Results and discussion

On May 12, 2024, *Spiraea hypericifolia* subsp. *hypericifolia* was found for the first time in Kosovo. The species was growing on the southern slopes of Mt Golesh, at an altitude of 740 meters a.s.l. (Fig. 1). Geographical coordinates: 42.577758 N, 20.996615 E. Coll./Det. Naim Berisha, herbarium code: 00002010.

It was found on the dry, sun-exposed southern slopes of the mountain, over serpentine substrates (Fig. 2). It formed a small population occupying an area of 430 m$^2$. It was recorded growing in the vicinity of an old oak (*Quercus pubescens* Willd.) forest, on dry grassland with a cover of 90%, along with the following accompanying species: *Alyssum montanum* group, *Chrysopogon gryllus* (L.) Trin., *Trigonella esculenta* Willd., *Goniolimon tataricum* (L.) Boiss. subsp. *tataricum*, *Orlaya grandiflora* (L.) Hoffm., *Xeranthemum annuum* L., *Bupleurum flavicans* Boiss. et Heldr., *Astragalus onobrychis* L., *Teucrium chamaedrys* L., etc.

During the visit to the mountain, only this single, rather dense population was recorded. The subspecies was not found in other parts of the mountain with a similar ecology, nor has it previously been reported in Kosovo. It was also not mentioned in a publication on the flora of Mt Golesh itself (Krasniqi et al. 2019). This may be because the population of this shrub closely resembles blackthorn (*Prunus spinosa* L.) from a distance and has thus been overlooked. It is difficult to determine whether this subspecies population is introduced or native. However, local people reported

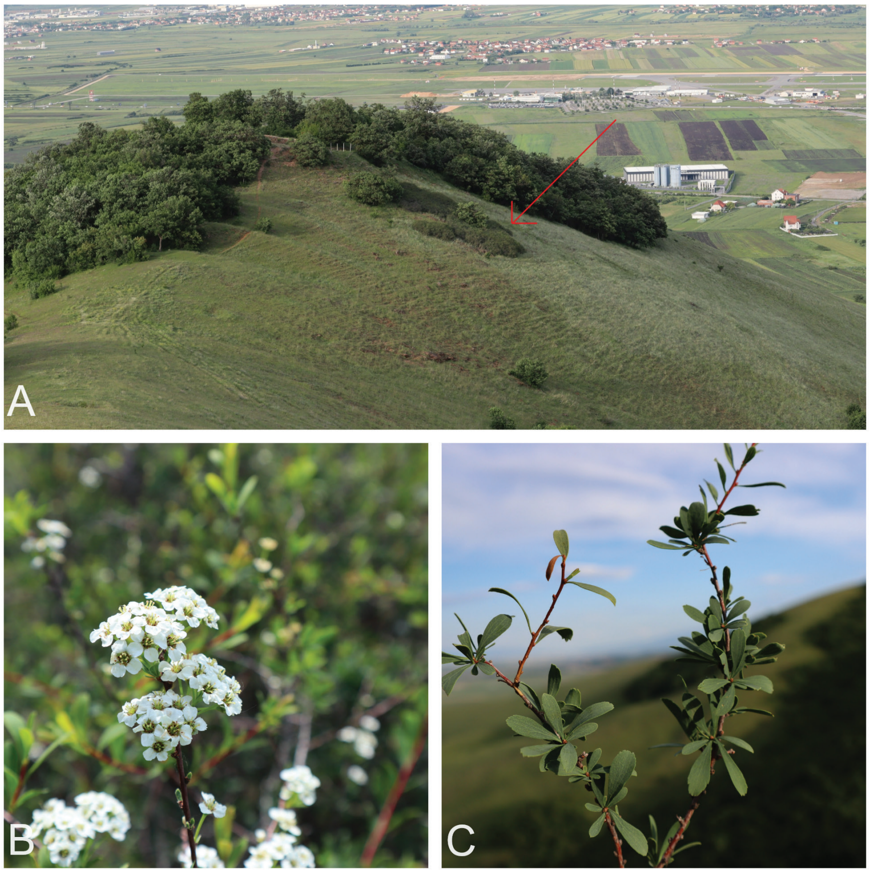

**Fig. 2.** *Spiraea hypericifolia* L. subsp. *hypericifolia* in Kosovo, Mt Golesh, central Kosovo. A – habitat and location on the mountain (indicated with red arrow), B – flowers, C – stem with leaves (photo: N. Berisha).

that the plant has always been present there, in the same area, extending to those same boundaries, and is particularly known for providing shelter to rabbits.

In a broader European context, this population of *Spiraea hypericifolia* subsp. *hypericifolia* recorded in central Kosovo is located between the reported populations in southern Bulgaria (Jordanov, 1973) and those of central Italy (Conti and Bartolucci 2023). However, the database for reporting the distribution of plant taxa in Europe (Kurtto 2009) appears to be outdated, as it includes only Bulgaria and other Eastern European countries and regions, Moldova, Ukraine, Belarus and European Russia.

In Europe, taxa of the genus *Spiraea* are commonly found in a variety of habitats including mountainous regions, riverbanks, and meadows. These species contribute significantly to local biodiversity and provide important ecological functions such as habitat for pollinators and erosion control. *S. hypericifolia* subsp. *hypericifolia* is known to thrive in arid hemicryptophytic grasslands and shrublands, where it usually forms monophytic shrubs at elevations typically ranging from 670 to 900 m a.s.l. (Dostàl 2010, Conti and Bartolucci 2023). In the Caucasus region, this subspecies is documented in volcanic rocky habitats and at higher altitudes (1290 m a.s.l.) compared to its occurrences in Europe. For example, in Georgia, *S. hypericifolia* subsp. *hypericifolia* was recorded by K. Reiner (Herbarium GJO – Ref.: 28.201), in bushy and volcanic rocky environments.

*Spiraea hypericifolia* subsp. *hypericifolia* is a shrub taxon that exhibits a highly restricted distribution within Kosovo, being known from only a single locality with an occupied area of merely 430 m$^2$. Additionally, its presence in the wider region is limited, with isolated populations in central Italy and Bulgaria.

According to the IUCN Red List criteria (IUCN 2024), several criteria are considered in determining the threat level to a plant taxon. Those include: Extent of occurrence (EOO), Area of occupancy (AOO) as well as Population size and trends. Since this is the first report of the occurrence of *S. hypericifolia* subsp. *hypericifolia* in Kosovo, the following data on the site and surrounding area have been taken into consideration. The taxon covers an area of 430 m$^2$, which means its AOO and EOO are both 4 km$^2$. During monitoring at its location, the population was estimated at fewer than 250 mature individuals. Combined with the fact that it is known to occur in only one locality within Kosovo, *S. hypericifolia* subsp. *hypericifolia* faces a high risk of extinction in the wild.

Based on IUCN criteria and the current situation of *S. hypericifolia* subsp. *hypericifolia* in Kosovo, a preliminary conservation assessment suggests that the taxon should be classified as Vulnerable (VU) D2. The taxon's EOO is less than 5000 km$^2$ due to its very restricted range, and its AOO is only 430 m$^2$, well below the threshold of 500 km$^2$. With an estimated population size of fewer than 250 mature individuals, the species is found in fewer than five locations, making it susceptible to stochastic events, environmental changes, and anthropogenic pressures. However, as there is insufficient evidence of population decline, trends, or extreme fluctuations to meet the full criteria for a higher threat category (CR or EN) under Criterion B, the most appropriate classification based on the available data is Vulnerable (VU) D2.

These factors underscore the urgent need for conservation measures to protect the remaining habitat and ensure the survival of *S. hypericifolia* subsp. *hypericifolia* in Kosovo and the broader region. We currently lack data on the habitat situation of *S. hypericifolia* subsp. *hypericifolia*, specifically whether its habitat is stable, declining, or expanding. Local people have noted that, to their knowledge, the shrub has always been present to the same extent. However, it is essential to monitor its habitat in the coming years to determine whether its extent and quality are stable or declining.

In my opinion, conducting additional field surveys is essential to accurately determine the distribution and ecology of the genus *Spiraea* in general, and specifically, the reported taxon *Spiraea hypericifolia* subsp. *hypericifolia* in Kosovo. Furthermore, it is crucial to monitor this taxon and officially assign a national conservation status to the population described here.

## Acknowledgements

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Short Communication

# First records of three *Hericium* species (Basidiomycota) in Kosovo

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**Abstract** – This study presents the first report of three *Hericium* species in Kosovo: *Hericium cirrhatum* (Pers.) Nikol., *H. coralloides* (Scop.) Pers. and *H. erinaceus* (Bull.) Pers. These species were found in *Fagus sylvatica* (beech) and *Quercus pubescens* (oak) forests in the central-eastern part of the country.

**Keywords**: fungal diversity, mycobiota, new records, Kosovo

## Introduction

*Hericium* species known for their cream-colored fruiting bodies with tooth–like hymenophoral structures and monomitic hyphal system (Stalpers 1996), belong to the *Hericiaceae* family (Russulales, Basidiomycota) and are closely related to *Laxitextum* Lentz, which has stereoid basidiomes (Larsson 2007). The species usually grow on dead standing and fallen trunks but can also colonize living trees (Kirk et al. 2008, Bernicchia and Gorjón 2010). Some of them are used in traditional food and medicine, notably *Hericium erinaceus* (Bull.) Pers. in China (Jumbam et al. 2019).

While various organism groups in Kosovo have been extensively documented, such as vascular flora (Millaku et al. 2013), the national forest inventory (Tomter et al. 2013), Red Book of Fauna (Ibrahimi et al. 2018), endangered and endemic plants (Berisha et al. 2020) and both plants and spiders (Berisha and Geci 2023), the fungi biota was less studied until recent research (Ramshaj et al. 2021, 2022). This study constitutes  the first report of the presence of three *Hericium* species in Kosovo: *Hericium cirrhatum* (Pers.) Nikol., *H. coralloides* (Scop.) Pers. and *H. erinaceus.*

## Materials and methods

The study is based on field research in three districts in Kosovo (Suhareka, Lipjan and Prishtina) from 2017 to 2022 (Fig. 1).

The fruiting bodies of *Hericium* species were collected from different locations during several field expeditions over the years. The collected species was deposited in the

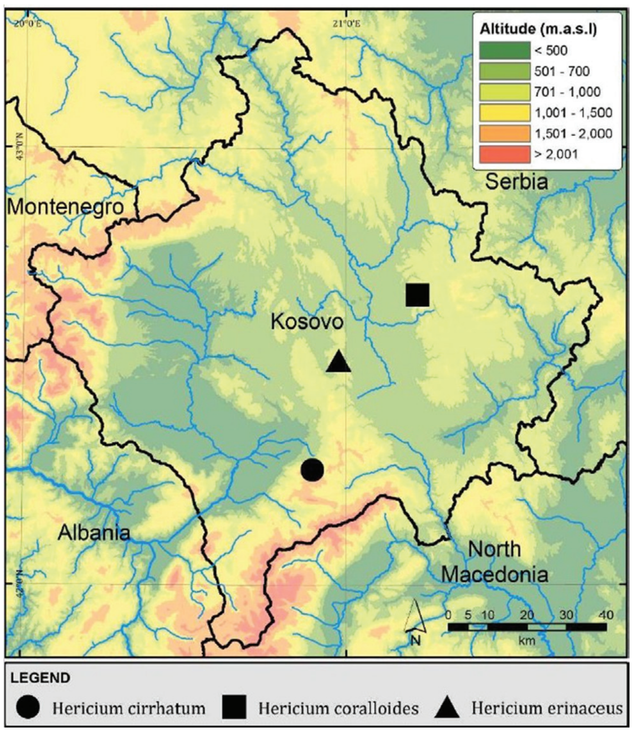

**Fig. 1.** Locations of newly recorded species in Kosovo.

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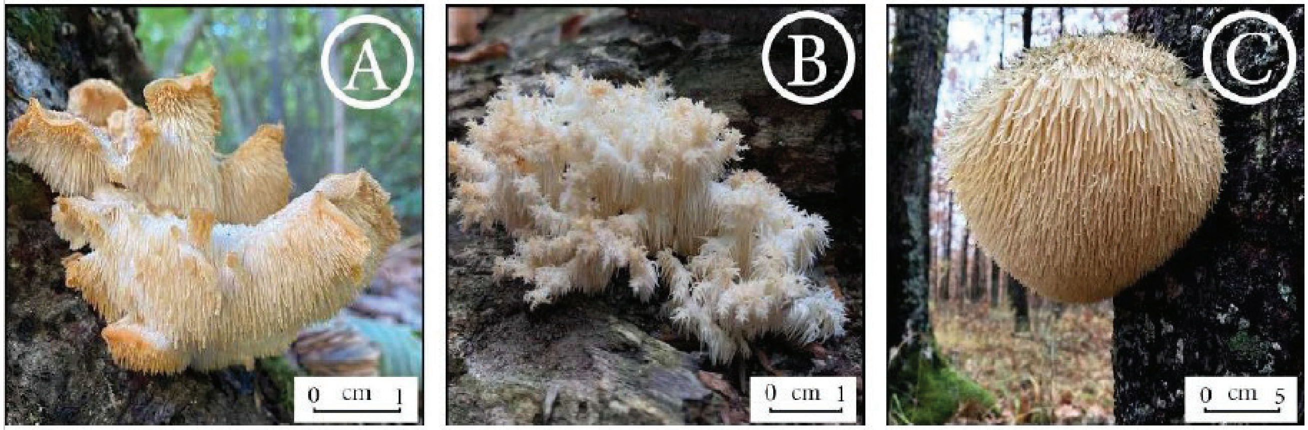

**Fig. 2.** Species of *Hericium* recorded for the first time in Kosovo. A – *Hericium cirrhatum*, B – *H. coralloides*, C – *H. erinaceus* (photos *in situ*: A, B – Q. Ramshaj, C – B. Fetiu).

Herbarium of the Faculty of Mathematics and Natural Sciences of the University of Prishtina. Nomenclature of the taxa follows Fungorum database.

## Results and discussion

Three species of *Hericium* were recorded for the first time in Kosovo. The descriptions of their locations are given below.

*Hericium cirrhatum* (Pers.) Nikol.

The species was found on 24th of September 2022 in the southeastern part of Kosovo (42°15'53.5" N, 20°53'41.5" E), in Sharri National Park, which is a part of Suhareka town area, at 1090 m a.s.l., in beech forest, on a growing tree of *Fagus sylvatica* L. (Figs. 1, 2A). The reference specimen RQPC 2022/613 was collected by Q. Ramshaj and identified by Q. Ramshaj, S. Tofilovska and M. Karadelev. *Hericium cirrhatum* is a rare species that mainly colonizes dead trees, especially cut logs, but is rarely found twice on the same substratum (Marren and Dickson 2000, Boddy and Wald 2003). It is listed as Under Assessment in the Fungal Global Red List (https://redlist.info/iucn). Additionally, it is included in the list of strictly protected species in Serbia (www.fungiserbia.com). It is a vulnerable species in Denmark, an endangered species in the Franche–Comté region of France (Stoltze and Pihl 1998, Sugny et al. 2013).

*Hericium coralloides* (Scop.) Pers.

On 1st of November 2019, the species was recorded in Gërmia Park nature reserve (42°15'53.5" N, 20°53'41.5" E), located northeast of Prishtina, at an altitude of 850 m a.s.l. It was found on a living *Fagus sylvatica* L. tree in a beech forest (Figs. 1, 2B). The reference specimen, RQPC 2019/308, was collected by Q. Ramshaj and B. Fetiu, with identification confirmed by Q. Ramshaj and M. Karadelev. *Hericium coralloides* usually grows on European ash (*Fraxinus excelsior* L.) and various species of *Quercus*, *Acer* and *Betula* (Crockatt 2008). The species has a different conservation status in Europe. It is considered a near threatened species in North Macedonia, Armenia, Denmark, Bulgaria and Estonia (Nanagulyan 1997, Stoltze and Pihl 1998, Gyosheva et al. 2006, Karadelev and Rusevska 2013, Saar et al. 2019). In Croatia, it is categorized as an endangered species, while in Serbia it is strictly protected (Tkalčec et al. 2008, Ivančević et al. 2012).

*Hericium erinaceus* (Bull.) Pers.

The species was found on 21st of September 2022 within the boundaries of the Blinaja Protected Landscape Area located 15 kilometers west of the town of Lipjan, in the east-central part of Kosovo (42°40'21.1" N, 21°12'42.9" E) (Figs. 1, 2C). It was recorded at an elevation of 740 m a.s.l., on a living tree of pubescent oak (*Quercus pubescens* Willd.). The specimen, as RQPC 2022/607, was collected by B. Fetiu and identified by Q. Ramshaj and M. Karadelev. *Hericium erinaceus* is rarely found in Eastern Europe and mainly grows on the trunks of dead trees in temperate beech and oak forests. Due to the gradual disappearance of its habitats, this species is included on the red lists of 15 European countries (Bohlin and Gràcia 2004, Govaerts et al. 2011). It is considered an endangered species in Albania, Bulgaria and Croatia (Gyosheva et al. 2006, Tkalčec et al. 2008, Karadelev 2014) while in Armenia, the Czech Republic, Romania, North Macedonia and Alsace (France) it is classified as rare or vulnerable (Nanagulyan 1997, Tănase and Pop 2005, Holec and Beran 2006, Muller et al. 2014, Karadelev et al. 2021). In Serbia the species is categorized as strictly protected (Ivančević et al. 2012) while in Slovakia it is listed as a species of lower risk (Lizoň 2001).

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Short Communication

# First record of *Lindernia dubia* (Linderniaceae) in Georgia, and note on *L. procumbens* in the South Caucasus

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**Abstract** – *Lindernia dubia* (L.) Pennell, a species of American origin, is reported for the first time from the South Caucasus. In 2023, a relatively large population of *L. dubia* was found in the Chorokhi River floodplain in the Autonomous Republic of Adjara, Georgia, the South Caucasus. The species is considered to be intensively spreading around temperate and subtropical regions worldwide, commonly occupying disturbed wetland habitats. The new record further expands the introduced range of the species in the Caucasus, where it was first reported from rice-growing regions of the Krasnodar Territory in 2017.

**Keywords**: Adjara, alien species, Caucasus, neophyte, wetland annuals

## Introduction

The Georgian vascular flora includes approximately 4275 species from 1048 genera, including around 460 taxa of alien origin (Davlianidze et al. 2018), and there is ample evidence that there is a steady increase in the number of alien species. West Georgia, with its developed port infrastructure along the Black Sea coast, long-standing cultivation of various subtropical crops, and extensive landscape gardening, has long been regarded as a gateway for alien plant species into the Caucasus, particularly those found in aquatic and wetland habitats.

In this report, *Lindernia dubia* (L.) Pennell (Linderniaceae, formerly Scrophulariaceae) is recorded for the first time in the South Caucasus from Georgia. The species, native to the Americas (Lewis 2000), has been rapidly spreading across Europe, South, and East Asia in recent decades (Casper and Krausch 1981, Šumberová et al. 2012, Krishnasamy and Arumugam 2015, Schmotzer 2015, Hrivnák et al. 2016, etc.). This species commonly occupies disturbed wet habitats and is a noxious weed in rice-growing regions. The new record marks the second *Lindernia* All. species in the South Caucasus flora, where only the palearctic *L. procumbens* (Krock.) Philcox has previously been reported (as *L. pyxidaria* All. ex L.; Grossheim 1967). The discovery of *L. dubia* further extends the introduced range of the species, filling the gap between its European and Asian distributions.

The study aims to provide new data on the distribution of both *Lindernia* species in the South Caucasus. The morphology of *L. dubia* is briefly described based on newly collected herbarium material.

## Material and methods

Field studies focusing on wetland flora were conducted in 2023 in the Chorokhi River floodplain, near the Black Sea coast, where a relatively large population of the alien *Lindernia* species was discovered. This area is part of the Kakhaberi Lowland, a small alluvial plain in the southernmost part of the Kolkheti Lowlands, bordered by the foothills of the Lesser Caucasus. It is formed by the solid sediments deposited by the Chorokhi River, with wetland vegetation largely confined to swampy areas south of the delta. The Kakhaberi Lowland is densely populated, being part of the Batumi and Khelvachauri municipalities of Adjara, with numerous disturbed or anthropogenically created habitats.

Specimens were collected by hand and pressed for herbarium storage. Measurements of quantitative features (N = 10) were taken from herbarium material. To further

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investigate *Lindernia* distribution, specimens from the BATU, TBI, TGM, and BAK herbaria were examined. The herbarium vouchers were deposited in BATU, IBIW, and LE.

## Results and discussion

*Lindernia dubia* (L.) Pennell 1935, Monogr. Acad. Nat. Sci. Philadelphia 1: 141. ≡ *Gratiola dubia* L. 1753, Sp. Pl.: 17. ≡ *Ilysanthes dubia* (L.) Barnhart 1899, Bull. Torrey Bot. Club 26: 376 (Fig. 1): Georgia / Sakartvelo, Autonomous Republic of Adjara, Khelvachauri municipality, near Batumi, the Chorokhi River floodplain, quarry, 41.58935° N, 41.60387° E, 7 m a.s.l., in shallow water and on muddy substrate, *Schoenoplectus–Lindernia* community, coll.: Vishnyakov, Aug 5, 2023, https://www.inaturalist.org/observations/176780187 (IBIW, BATU); ibid., Aug 28, 2023, https://www.inaturalist.org/observations/180643998 (IBIW, LE); ibid., Sept 21, 2023, https://www.inaturalist.org/observations/184639935 (IBIW, LE).

Glabrous plants with mostly erect stems rooting at lower nodes, 3–30 cm in height. Leaves opposite, sessile, 5 veined; ovate to ovate-linear, with slightly dentate margins, 9–20 × 5–10 mm. Flowers solitary, axillary; pedicels shorter, equal or slightly longer than the subtending leaves. Calyx deeply 5-lobed; sepals narrow, linear, 3.5–5 mm. Corolla zygomorphic, tubular, 7–8.2 mm long, white in color, with blue to violet tint; lower lip distinctly 3-lobed. Stamens in two pairs, one posterior fertile and one anterior rudimentary. Capsules ovate, tapering towards the apex, 4–5.5 mm long, containing numerous, dust-like, yellowish seeds less than 0.5 mm long.

The morphology of the collected plants closely matched published descriptions (Casper and Krausch 1981, Lewis 2000, Aybeke 2016). *L. dubia* differs from the reported *L. procumbens* by having mostly chasmogamous flowers, a rudimentary pair of stamens, a longer corolla, shorter pedicels, and toothed leaves (at least the upper ones), which have 5 veins (Škondrić et al. 2023). In the absence of flowers, the species can be distinguished by the morphology of the capsules (Molnár et al. 2000). Additionally, *L. dubia* typically branches in the middle and upper parts, unlike *L. procumbens*, which branches at the base (Zelenskaya 2020).

The *Lindernia* population was found in a shallow quarry, created a year earlier on the left bank of the Chorokhi River, which is no longer in use. The locality was revisited multiple times since spring (May), when the quarry was completely filled with rainwater, and pioneer communities of charophytes and pondweeds had developed. The *Lindernia* plants were first observed in early August as the water receded. By that time, the species had formed the lower tier of a sparse *Schoenoplectus triqueter* (L.) Palla community in the shallowest parts. By September, the quarry had dried up and was fully occupied by the *Lindernia* community, which also included various wetland annuals, with a prominent role being taken by Cyperaceae species: *Cyperus difformis* L., *C. flavescens* L., and *Fimbristylis bisumbellata* (Forssk.) Bubani. A few isolated plants were also observed along the bank of the Chorokhi channel below the quarry in September 2023: https://www.inaturalist.org/observations/184639702.

This is the first record of the species in Georgia and the South Caucasus. The nearest known localities are in the Krasnodar Territory, Russia, almost 500 km northwest of Adjara, where the species has become a common weed in rice fields of the Kuban River valley since 2017 (Zelenskaya 2020). Other nearby localities belong to southwest Romania (Neacșu et al. 2021), northwest Turkey (Aybeke 2016), and India (Krishnasamy and Arumugam 2015). It was demonstrated that the recent range expansion of this species is linked to human activity, particularly fish farming and

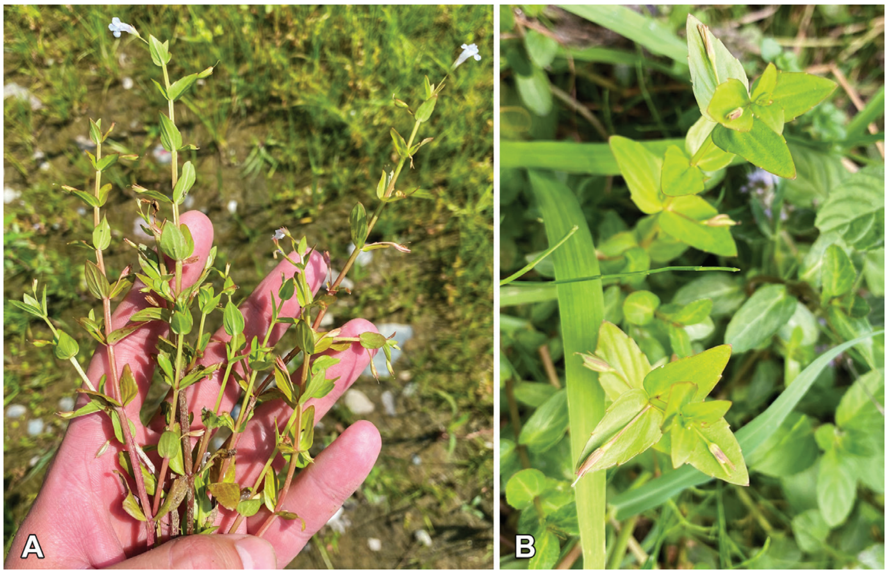

**Fig. 1.** *Lindernia dubia* (L.) Pennell from Georgia: A – group of plants collected for herbarium vouchers from the quarry Aug 5, 2023, note veins clearly visible from the abaxial side of leaves; B – plants from the Chorokhi channel observed Sept 21, 2023, note toothed upper leaves and short axillary pedicels.

shipping, as well as to hydrochory and avichory (Tzonev and Šumberová 2004, Šumberová et al. 2012, Schmotzer 2015). In the case of the Georgian locality, the exact mode of introduction remains unclear. It is possible that the species invaded the region only recently, as its distribution in the Chorokhi floodplain appears limited. However, due to the species' known invasive potential and high likelihood of further spread from the already invaded area, monitoring of the *L. dubia* population is essential. The quarry is frequented by humans and livestock, and *L. dubia* seeds can easily be transported by mud particles on rubber boots and hooves. Spring floods of the Chorokhi River may also facilitate seed dispersal.

The new locality is part of the region dominated by Colchis flora, which includes several endemic species restricted to wetland habitats. Among them, *Rhamphicarpa medwedewii* Albov was found growing alongside *L. dubia*, though it was represented by only a few scattered plants (IBIW). The invasion of *L. dubia* could pose a threat to this rare species of low abundance.

Herbarium search confirmed that the genus *Lindernia* was previously represented in the South Caucasus by a single species, *L. procumbens*. Notably, the most recent collection from the region dates back to 1917 (for Azerbaijani specimens). In Georgia, the species was last collected in 1910: Russian Lazistan, swampy bank of the Malyi Chorokh, coll.: G. Woronow no. 2679, Aug 27, 1910, TGM 40879!, TGM 40885!. Woronow's plants originate from the same area as *L. dubia*, i.e., south of the Chorokhi River delta in the Kakhaberi Lowland. However, no *L. procumbens* plants were found in this area in 2023. One possible explanation is that the species has gone unobserved for a long time or may be extinct. A decline in localities of this species has been observed in Europe, making it locally threatened. The invasion of *L. dubia* also presents a competitive disadvantage for the species (Šumberová et al. 2012, Schmotzer 2015). Another possibility is that *L. procumbens* only emerges from the soil seed bank during favorable conditions that occur intermittently (cf. Kaplan et al. 2016).

The findings of this study highlight the need for targeted searches for new localities of both *Lindernia* species in order to resolve the issue of *L. dubia* invasiveness and the current status of *L. procumbens* in the South Caucasus.

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# Celebration of the 150th Anniversary of the Division of Botany, Department of Biology, Faculty of Science, University of Zagreb

The jubilee celebration marking the 150th anniversary of the Division of Botany at the Department of Biology, Faculty of Science, University of Zagreb, held in 2025 brought together representatives of numerous academic and related institutions. A commemorative ceremony took place in the Exhibition Pavilion of the Botanical Garden of the Faculty of Science, University of Zagreb, on Friday, 24 October 2025, honouring one and a half centuries of continuous teaching and scientific activity in botany at the University (Fig. 1A). As part of the University's long tradition of higher education, which dates back more than three centuries, the Division of Botany has played a central role in the development of botanical teaching and botanical literacy in Croatia.

The anniversary programme highlighted the historical significance of the appointment of Bohuslav pl. Jiruš, on 13 November 1875, as the first professor of botany at the then Faculty of Philosophy, which marked the beginning of organised academic work in botany and the establishment of the Department of Botany and Physiology, headed by Prof. Jiruš from 1877. From that moment onwards, botany acquired a formal position within the academic community of the University of Zagreb. Prof. Jiruš delivered his first lecture on 24 April 1876, on plant anatomy, histology and general morphology, and since then the tradition of teaching botanical subjects by members of the Division has remained unbroken to the present day. The Division has undergone several changes in name and organisational structure during its long history, but has functioned as part of the Department of Biology and the Faculty of Science since 1946. The celebration thus served both as a commemoration of this foundational moment and as an opportunity to reflect on the 150 years of development of the Division.

The guests were welcomed by the Dean of the Faculty of Science, Prof. Ivančica Ternjej, and the Head of the Department of Biology, Assoc. Prof. Zoran Marčić (Fig. 1B). The programme was moderated by Assoc. Prof. Sunčica Bosak, current Head of the Division. A historical overview was presented by Prof. Antun Alegro, Assoc. Prof. Sunčica Bosak, Prof. Gordana Rusak and Prof. Božena Mitić, highlighting key milestones in the Division's development. The celebration included a musical performance by the Faculty of Science Choir "Cantus naturae", conducted by Kristijan Hiršel. The choir performed two carefully selected pieces ("Cetina" and "Neka cijeli ovaj svijet") that symbolically connected aquatic and terrestrial ecosystems and underscored the links between nature, landscape and botanical science. In doing so, the artistic component complemented the historical and scientific content of the event and further underscored the role of the Division of Botany in integrating diverse aspects of the natural sciences into a coherent view of the living world. As a gesture of appreciation to participants in the jubilee event, the Division prepared symbolic gifts, including lavender balms produced by the phytochemistry group and small pieces of jewellery created by artist Julijana Rodić Ozimec in the form of endemic Croatian plant species (*Campanula tommasiniana* and *Asplenium hybridum*), thereby underscoring the central role of plant diversity in the identity and work of the Division of Botany.

Several short presentations showcased the scientific, educational and institutional activities associated with the Division. A significant part of the Division's mission is embodied in the collections and databases that it maintains and develops, including Herbarium Croaticum, the Flora Croatica Database and the Croatian National Diatom Collection, important national and international resources for taxonomic, floristic and ecological research. Dr. Nina Vuković presented the Herbarium Croaticum collection, emphasising its role as a central national repository of plant biodiversity, while Dr. Vedran Šegota spoke about the Flora Croatica Database, an online platform that integrates data on Croatian flora and supports research and teaching.

The Faculty of Science is also the home institution of the journal Acta Botanica Croatica, a long-standing scientific periodical that this year celebrates one hundred years of uninterrupted publication and plays a key role in the dissemination of botanical research. Prof. Mirta Tkalec presented the role of Acta Botanica Croatica in the work of scientists and teachers at the Division of Botany, underlining its long tradition and importance. The journal originated in 1925 as the official journal of the Division, under the title *Acta Botanica Instituti Botanici Regalis Universitatis Zagrebensis*, founded by Prof. Vale Vouk, then Head of the Division.

**Fig. 1.** Members of Division of Botany together with the guests at the ceremonial event in front of Exhibition Pavillion in Botanical Garden, Zagreb (A), Chair of Department of Biology Zoran Marčić, Assoc. Prof. addressing the audience, at its left is the portrait of Antun Heinz, founder of Botanical garden and second chair of the Division of Botany, the visual identity and the logo of the event is shown at the screen (B) (photos: Renata Horvat)

As the final lecture, Dr. Sanja Kovačić presented the Botanical Garden of the Faculty of Science, a key university facility for teaching, research and public outreach in botany, and highlighted its long-standing institutional links with the Division and its staff. Established in 1889 by Prof. Antun Heinz, who at that time was both the founder of the Botanical Garden and Head of the Department of Botany and Physiology, the Garden was organizationally united with the Department and its successors until 2013, when it became a separate unit while remaining closely integrated with the activities of the Division of Botany.

Historically, the Division was first housed in premises belonging to the Gornjogradska gimnazija high school and even then maintained a plant collection of more than 10,000 specimens. From these modest rooms it was moved to the main University building, today the Rectorate at Trg Republike Hrvatske 14, and later to the building in Marulićev trg 20. Today, the Division of Botany comprises 19 members in scientific–teaching grades, from assistant professors to full professors, as well as three professors emeriti. In total, including collaborators, the Division has around 40 members, some of whom are also partially appointed at the Department level. Over time, its organisational structure and name have changed, and certain subunits have branched off for various reasons, but since 2013, the Division has existed in its current form as an independent organisational unit within the Department of Biology. Today it is geographically distributed across three locations in Zagreb – in buildings within the Botanical Garden, at Marulićev trg 20 and at Rooseveltov trg 6 – all of which are currently at different stages of post-earthquake reconstruction. This spatial dispersion has historically posed organisational challenges, but it also reflects the breadth of teaching and research activities carried out by the Division.

Teaching in various branches of botany has been delivered continuously for 150 years. Field courses, often organised in collaboration with zoologists, have formed an integral part of the curriculum from its earliest days and are closely linked to experimental exercises and laboratory work. At present, staff of the Division participate in the delivery of several dozen courses and as many as 13 field classes. Through this sustained teaching activity, the Division has been crucial in educating future teachers of natural science subjects and in promoting botanical literacy more broadly. The contribution of the Division to higher education is also evident in the impressive array of textbooks and professional literature produced by its members, covering topics from plant anatomy and morphology to systematics, evolution, geobotany, plant physiology and marine phytoplankton.

Research at the Division encompasses all major fields of botany and a wide array of taxa, from algae and bryophytes to vascular plants, and addresses processes at cellular, organismal, community and ecosystem levels. These diverse activities are united by a shared focus on photosynthesis as a fundamental process defining plant life, a focus that is reflected in the visual identity of the Division, which incorporates the chlorophyll molecule as a central motif. In addition, a stylised chequered fritillary, conceived as a fictional representative species, has been adopted as a unifying symbol that underscores the common orientation towards plant life despite the diversity of research interests among staff members.

Members of the Division are actively involved in professional societies, in the organisation of scientific and expert meetings and serve on editorial boards of journals and society publications. The anniversary celebration highlighted this broader engagement, which contributes to the visibility of Croatian botany and strengthens collaboration with related institutions.

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Department of Biology
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# Professor Ivan Regula – on the occasion of his ninetieth birthday

In the middle of January 2026, I was glad to be able to wish Professor Ivan Regula a happy 90th birthday. Professor Regula spent his entire professional career at the Division of Botany, Faculty of Science in Zagreb, teaching plant physiology to many generations of students. At the end of 2025, we celebrated the 150th anniversary of continuous botany teaching in Zagreb, marking the historical origins of today's Division of Botany. The time spent with Professor Regula at that celebration brought back fond memories of the days when his presence at the Division was part of everyday life.

Professor Ivan Regula was born on 18 January, 1936 in Gornja Stubica, in Hrvatsko Zagorje, a region well known for its natural beauty and predominantly green landscape. It is therefore not surprising that plant biology became the main focus of his professional life. After attending elementary school in Gornja Stubica and high school in Zagreb, he enrolled at the Faculty of Science, University of Zagreb. In 1962, he graduated in biology with the thesis "Light and $CO_2$ compensation points of Mediterranean plants cultivated in the Zagreb Botanical Garden" under the supervision of Professor Zlatko Pavletić. He obtained his master's degree in 1967, with a thesis entitled "Indolic compounds in nettle (*Urtica dioica* L.)" under the supervision of Professor Dina Keglević, founder and head of the Tracer Laboratory at the Ruđer Bošković Institute. Professor Regula continued his research on indolic compounds under the guidance of Professor Zvonimir Devidé, the distinguished Croatian biologist and botanist, and Dr. Sergije Kveder, senior scientist at the Ruđer Bošković Institute. In 1978 Professor Regula defended his doctoral thesis entitled "5-hydroxyindolic compounds in plants".

Professor Ivan Regula began his professional career in 1963 as an assistant at the Division of Botany, Department of Biology, Faculty of Science, University of Zagreb. From his first days of working at the faculty, he participated in teaching, with an emphasis on laboratory classes in Plant Physiology. He particularly enjoyed introducing new methods and ideas and helping students acquire skills in experimental techniques, thus preparing them for independent laboratory work. Professor Ivan Regula was appointed assistant professor in 1982, promoted to associate professor in 1991, and became a full professor in 1999. In addition to Plant Physiology, a mandatory course in the biology study programme, Professor Regula introduced the elective course Selected Chapters in Plant Physiology for students with a special interest in plant biology. In postgraduate studies, Professor Regula taught the course Physiology of Seed Germination. As a professor of Plant Physiology, he was also involved for several years at the Faculty of Natural Science, Mathematics and Education, University of Split, and at the Faculty of Education, J. J. Strossmayer University of Osijek, contributing to the education of future biology teachers. His extensive teaching experience was incorporated into several biology textbooks for elementary and secondary schools. In 1995, he and co-author Professor K. D. Dubravec published the university textbook Plant Physiology for students of biology and agriculture. Professor Regula supervised many students working on their bachelor's, master's or doctoral theses. He was not only an expert in plant physiology but also an excellent botanist. Together with his wife, Professor Ljerka Regula-Bevilacqua, a botanist and long-term head of the Botanical Garden of the Faculty of Science, he devoted considerable time to studying plants in their natural habitats and took part in professional visits to many botanical gardens abroad. This field experience provided numerous interesting examples for his Plant Physiology lectures, which became popular even among students who were not particularly interested in plant biology. Over the years, Professor Ivan Regula undertook numerous duties at the Department of Biology, Faculty of Science. From 1992 to 1995, he was head of the Department of Biology, and from 1993 to 1999, he served as head of the Division of Botany.

In his research, Professor Regula focused on the occurrence and physiological function of biogenic amines in plants. He mainly studied 5-hydroxyindolic compounds, which are not very common in plants, examining their distribution across different plant species, presence in plant tissues, possible roles, precursors, and metabolites. He paid particular attention to the occurrence and role of 5-hydroxytryptamine (serotonin) in plants. He succeeded in adapting reagents normally used for detecting indoles on chromatograms for the histochemical localisation of serotonin in plant tissues and cells. He thus established that serotonin is present in the vacuoles of the lower leaf epidermis, in the protein bodies of walnut seeds, in the stinging hairs of nettle, and in the mesocarp tissue of tomato fruit. Professor Regula has actively participated in many research projects and was the principal investigator for the projects "The role of selected plant hormones in plant development" and "The effect of chemicals on physiological processes in plants" funded by the Ministry of Science, Education, and Sports of the Republic of Croatia. The results of his research have been published in more than 30 articles in national and international journals and proceedings. He has also presented the results of his work at many international and national scientific conferences. During his professional career, Professor Regula collaborated with both domestic and foreign research institutions. He visited, for example, the Institut für Botanik in Zürich, Switzerland; the Botany School in Cambridge, the Jodrell Laboratory in Kew, United Kingdom; Institut Botaniki – odjel Fizjologii róslin Uniwersytet Wroclawski, Poland; the Department of Forest Genetics and Plant Physiology University of Umeå, Sweden.

In addition, Professor Regula participated enthusiastically in the popularisation of biology and plant physiology. As a member of the state committee "Znanost mladima" (1973–1985), Professor Regula attended meetings of junior biologists for many years, motivating young people to take an interest in science. Even after his retirement, he was always willing to take part in various popularisation activities, such as public lectures, radio and television broadcasts, and the writing of articles for "Priroda", a magazine for the popularisation of natural history.

Professor Regula was an active member of numerous national and international professional societies, including the Croatian Biological Society, the Croatian Ecological Society, the Croatian Society of Natural History, the Federation of European Societies of Plant Biology (FESPB) and the International Association for Danube Research (IAD). His activities in the Croatian Society of Plant Physiologists are particularly notable. He was one of the founders of the society in 1977 and served as president from 1990 to 1992. He is now an honorary member and remains involved in the activities of the society, which is now called the Croatian Society of Plant Biologists. He was also the Croatian representative for FESPB for many years and as a member of organising committee he participated in the organisation of the 6th FESPB Congress held in Split in 1988. In 1992, Professor Regula was elected as an active member of the New York Academy of Sciences.

I met Professor Ivan Regula in the third year of my molecular biology studies at the Faculty of Science in Zagreb, and a few years later I joined his team as an assistant. He was always willing to help young collaborators by answering questions and offering advice drawn from his extensive experience in education, research, and departmental administration. I am glad to have had the opportunity to learn from Professor Regula, not only about plant physiology, but also about teaching, including how to motivate students and, most importantly, how to maintain professional enthusiasm even in challenging situations. With particular kindness, tolerance, and understanding, Professor Regula encouraged junior collaborators to pursue their own ideas and to develop their individual professional identities. Even after his retirement in October 2006, Professor Regula remained interested in the work and life of our research group. We have turned our meetings at work into coffee breaks at a patisserie, and with wishes for good health and fortune on the occasion of the Professor's 90th birthday, we are looking forward to continuing this pleasant custom in the new decade of his life.

Professor Željka Vidaković-Cifrek
University of Zagreb, Faculty of Science,
Department of Biology, Division of Botany

# INSTRUCTIONS FOR AUTHORS

The interest of the journal is field (terrestrial and aquatic) and experimental botany including plant viruses, bacteria, archaea, algae and fungi, from subcellular level to the ecosystem level with a geographic focus on karstic areas of the southern Europe and the Adriatic Sea (Mediterranean).

The journal welcomes manuscripts for publication in the following categories: research papers, short communications, book reviews, social news and announcements. Review articles are accepted on editor invitation only.

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## Type of contribution

RESEARCH PAPER

A research paper is a fully documented report of original research. The manuscript should be divided into Introduction, Material and methods, Results, Discussion, Acknowledgements, References (**maximum 40**), Table and figure captions, Tables, Figures. There may be up to **12 single-spaced typewritten pages**, excluding figures and tables. There may be up to **8 tables and/or figures in total** per manuscript. Additional figures and/or tables can be published online only as supplementary materials. All tables and figures should be cited in the text properly (Fig. 1, 2, …, On-line Suppl. Tab. or Fig.). Exceptionally, in papers dealing with conceptual and theoretical bases, especially botanical and phytosociological nomenclature, Results and Discussion can be combined into one section.

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The metric system should be used throughout the manuscript. If required, equivalent values in other systems may be placed in parentheses immediately after the metric value.

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Colangelo, E. P., Guerinot, M. L., 2006: Put the metal to the petal: metal uptake and transport throughout plants. Current Opinion in Plant Biology 9(3), 322–330. https://doi.org/10.1016/j.pbi.2006.03.015

Books:

Horvat, I., Glavaš, V., Ellenberg, H., 1974: Vegetation Sudosteuropas. Geobotanica selecta 4. Gustav Fischer Verlag, Stuttgart.

Chapter in a book:

Broadwater, S. T., Scott, J., 1994: Ultrastructure of unicellular red algae. In: Seckbach, J. (ed.), Evolutionary pathways and enigmatic algae: *Cyanidium caldarium* (Rhodophyta) and related cells, 215–230. Kluwer Academic Publishers, Amsterdam.

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