Consistency of phenolic profiles with taxonomic distribution and adaptation of birch species (*Betula* L.) to environmental conditions

Artur F. Likhanov,[1,2*] Roman D. Vasylyshyn,[1] Yurii M. Marchuk,[1] Oleksandr M. Kurdyuk,[1] Hanna Yu. Honchar,[2] Oleksandr V. Borysov,[3,4] Svitlana Yu. Bilous,[1,5] Borys Ye. Yakubenko,[1] Yahya Al Naggar [6,7]

[1]Department of Botany, Dendrology and Forest Tree Breeding, Education and Research Institute of Forestry and Landscape-Park Management, National University of Life and Environmental Sciences of Ukraine, 03041, Kyiv, 2 Henerala Rodimtseva st., Ukraine
[2]Institute for Evolutionary Ecology NAS of Ukraine, 03143, Kyiv, 37 Lebedeva st., Ukraine
[3]Institute of Organic Chemistry, National Academy of Sciences of Ukraine, 02660, Kyiv, 5 Murmanska st., Ukraine
[4]Enamine Ltd. 02094, Kyiv, 78 Chervonotkatska st., Ukraine
[5]Forestry Department, Weihenstephan-Triesdorf University of Applied Science, Hans-Carl-von-Carlowitz-Platz 3, 85354 Freising, Germany
[6]Zoology Department, Faculty of Science, Tanta University, Tanta 31527, Egypt
[7]Department of Community Ecology, UFZ-Helmholtz Centre for Environmental Research, Theodor-Lieser-Str. 4, 06120 Halle (Saale), Germany

**Corresponding author:* likhanov.bio@gmail.com, ORCID iD: 0000-0001-6580-7241

Abstract

The phenolic compounds in the leaves of 12 species of birch trees of the subgenera *Neurobetula*, *Betulenta*, and *Betula* were biochemically profiled using HPTLC (De Jong, 1993). The duration of the vegetation period was found to be significantly related to the content of total phenols (r = 0.74) and flavonoids in leaves (r = 0.65). The correlations for *Neurobetula* plants were 0.86 and

0.91, respectively. The relationship between the duration of the growing season and the concentration of phenolic compounds in *Betula* plants was inverse (r = -0.84). A cluster analysis of phytochemical profiles revealed that the studied birch species form groups that coincide with the subgenera proposed by De Jong (1993) due to an affinity with the qualitative composition of phenolic compounds. A multiple correlation analysis confirmed the relationship between the qualitative composition of phenolic compounds and the morphological characteristics of the leaves. The results of phytochemical profiling revealed that the qualitative composition of polyphenols in the leaves of 12 birch species is quite specific, allowing the use of individual compounds as additional differential biochemical characters in identifying species and hybrids and studying their potential role in plant adaptation to habitat conditions.

Keywords: *Betula*, chemosystematics, introduction, flavonoids, tannins

Introduction

The plants of the genus *Betula* L. play an important role in the flora of the temperate forest zone, as well as the Boreal and Sub-arctic zones (Furlow, 1990). Birches have a significant morphological polymorphism. This is due to the level of ploidy, as well as interspecific hybridization and plant variability under the influence of environmental factors. The ability of *Betula* plants to cross and spontaneously polyploidize with a relatively small number of species-specific morphological features makes identification and development of the nomenclature system at the section level difficult; as a result, the genus *Betula* remains taxonomically difficult (Li et al., 2007; Salojärvi et al., 2017). There are currently multiple viewpoints on the number of taxa and the integration of species into distinct sections or clades. (Winkler, 1904; De Jong, 1993; Skvortsov, 2002; Ashburner, McAllister, 2013). According to genetic analysis of polymorphism of sequences of internal transcribed spacers (ITSs) of ribosomal genes, most *Betula* species form

a common clade (Wang et al., 2016; Tarieiev et al., 2021). However, the phylogenetic relationships based on ITSs between the four sections of the subgenus *Betula* remain controversial and uncertain. The *Costatae* (Regel) Koehne section is closely related to the *Betula* section (Wang et al., 2016). The *Betula* section is thought to have separated from *Costatae* during the evolution process. This is demonstrated by the presence of fertile hybrids and an incomplete reproductive barrier (Parkhomenko, 2011), particularly between *Betula pubescens* Ehrh. and *Betula ermanii* Cham., and between *Betula pendula* Roth and *Betula ermanii* (Johnsson). Hybridization and adaptive introgression are also common in the subgenus *Betula* (Thorsson et al., 2010). This is especially important in terms of hybrid distribution, naturalization, and invasion of new territories (Wang et al., 2016), including after their introduction.

The origin of the plant determines the qualitative composition of SMs (Deepak et al., 2018). Their synthesis is closely linked to the provision of essential plant functions. The concentration of phenols in the plant body, for example, is determined by the available resources required to balance plant growth and SM synthesis. It can also vary significantly under stressful conditions depending on environmental factors (Winkel-Shirley, 2002; Mattson, 2005; Churilov et al., 2020). Plants from southern origin have a higher concentration of highly hydrophobic flavonoids in their leaves. The high adaptability and morpho-physiological plasticity of most *Betula* species and hybrids is due to the composition of secondary metabolites (SMs), which includes phenolic compounds (Lattanzio, 2013). This highlights the significance of chemotypes as plant adaptation reserves. The phytochemical profiles of closely related taxa's SMs indicate the presence or absence of specific biochemical features (phenes), which are also important in chemophenetics, species ecology, and the formation of individual chemoraces. At the same time, M. Wink (2003) claims that the individual inconsistency of SM profiles means that the value of phytochemical features for taxonomy, like traditional morphological markers, is open to

interpretation. At the population level, the flavonoid content of the leaves can also be quite variable. Simultaneously, trees with high chemical similarity have been discovered among polymorphs within a single population (Stark et al., 2008). Differential expression of the corresponding genes, recombination of features during crossing, hybridization, diploid-tetraploid introgression, and natural polyploidization of plants explain the differences in SM profiles in different species of birches (Thomson et al., 2015; Zohren et al., 2016; Wang et al., 2021).

The absence of individual flavonoids or changes in their total amount can be attributed to the plant's ploidy. However, there are differing perspectives on the effect of ploidy on plant productivity and stability (Patrushev, Minkevich, 2008). In *Betula* species with high levels of ploidy, a general pattern of increasing genome size dispersion (1Cx) is found, possibly due to deletions (Buggs et al., 2012) or an increase in the number of retrotransposons in the genome (Bennetzen et al., 2005; Piegu et al., 2006). Polyploids have advantages when it comes to adapting to stressors and moisture deficiency (Li et al., 1996; Balcar, 2001). Polyploidization, particularly allopolyploidization, can slow development due to the relatively large size of the genome (Lavergne et al., 2010), a lack of nitrogen and phosphorus (Knight et al., 2005), and low temperatures to which cells with more chromosomes are sensitive (Grime and Mowforth, 1982). The latter assumption is supported to some extent by the fact that the most common in Eurasia are low-ploidy birches of the subgenus *Betula* (*B. pendula*, *B. nana* L., and *B. glandulosa* Michx.) (Wang et al., 2016). Thus, the Asian white birch (Parkhomenko, 2011), which is usually diploid (2n = 28) (Keinanen, 1999), is the most cold-resistant of the birches of Eastern Siberia. Meanwhile, low-invasive the subgenus *Aspera* species with high ploidy (*Betula insignis* Franch., *B. megrelica* Sosn., *B. globispica* Shirai, and *B. fargesii* Franch.) have narrow ranges, have been attributed to their slow growth (Wang et al., 2016). These polyploid birches are found in areas dominated by relict species. Their small populations could be remnants of relict flora that once

inhabited much larger habitats (Wang et al., 2016). The reference genome of *Betula pendula* was sequenced, and it revealed several duplicate genes involved in plant responses to the environment. Their appearance is the result of ongoing tandem duplication processes rather than polyploidy. Such duplicates have recently been extensively studied in terms of organism adaptations at the inter-population level (Salojärvi et al., 2017).

Flavonoids are highly active compounds that have regulatory, protective, and adaptive properties in plants (Agati et al., 2007; Charles et al., 2010; Likhanov et al., 2019; Thitz et al., 2020). *Betula*'s main phenolic compounds are flavonoid glycosides, myricetin, and quercetin derivatives (Pawlowska, 1983). There are various data on qualitative and quantitative indicators of phenolic compound content in birch tree vegetative organs. According to Riipi (2002), the concentration of soluble proanthocyanidins in *B. pendula* leaves increases during the growing season, while the concentration of cell wall-associated galotannins and flavonoid glycosides decreases after leaf growth. Other researchers report a large variation in total phenol content in birch (*B. pendula*) leaves during the growing season (Stark et al., 2008). Furthermore, the synthesis of phenolic compounds in silver birch leaves is affected by the duration and intensity of UV radiation exposure (Tegelberg et al., 2001; Keski-Saari et al., 2005). Secondary metabolite profiles in silver birch populations are sensitive markers of oxidative stress in the presence of elevated ozone levels in the air (Kontunen-Soppela et al., 2007). Additionally, some broad trends have emerged: variability in the content of secondary metabolites on the surface of silver birch leaves is primarily determined by plant genotypes, but their qualitative composition is also influenced by geographical origin (Deepak et al., 2018). Taken together, quantitative and qualitative analysis of secondary metabolites may be useful for development of a nomenclatural system for the genus *Betula*.

The aim of this study (i) is to identify species and new hybrids of the close subgenera *Neurobetula*, *Betulenta*, and *Betula* using phytochemical profiles, as well as (ii) to identify potentially important markers of ecological plasticity in plants. This goal is especially important given that the studied species are grown in cultivation at botanical gardens, where they can hybridize spontaneously.

Materials and methods

Samples and data collection

Twenty species of birches from various natural habitats, belonging to two evolutionarily and genetically related sections with incomplete reproductive barriers, were chosen to study the composition of the SMs from the collection of the arboretum of the Department of Dendrology and Park Studies of the M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine:

- Subgenus *Neurobetula*: *Betula davurica* Pall.; *B. ermanii*; *B. schmidtii* Regel; *B. costata* Trautv.; *B. raddeana* Trautv.;

- Subgenus *Betula*: *B. pendula*; *B. platyphylla* Sukaczev; *B. pubescens*; *B. oycoviensis* Besser; *B. papyrifera* Marshall; *B. szechuanica* (C. K. Schneid.) C.-A. Jansson;

- Subgenus *Betulenta: B. grossa* Siebold & Zuss (table 1).

The vast majority of the species chosen were collected in the Far East, Primorsky Krai, Central and Eastern Europe, and the Caucasus. The paper birch (*Betula papyrifera*) is a representative of North American flora, and the silver birch (*B. pendula*) and downy birch (*B. pubescens*) are the two most common species of the subgenus *Betula* in Eurasia.

Dr. Parkhomenko identified *Betula* species in the arboretum using classical morphological characteristics and compared them to herbarium specimens from the Komarov Botanical Institute

of the Russian Academy of Sciences (Parkhomenko, 2011). The ploidy of birch species is given after Pawlowska (1983) and Keinanen et al. (1999).

Methods of sample collection:

For phytochemical studies of the phenolic complex of the leaves, three reproductive trees (n = 3) up to 50 years old were used. Leaves (n = 10) were collected from the lower part of the crown at a height of 2.0-2.5 m in June (2017 and 2018).

The leaf samples were ground after being dried at 37°C until constant weight. The dry powder that resulted was sieved through a No. 40 sieve (425 µm). To determine the total phenolic content in the dry leaves, 1 g of each sample received 10 mL (1/10) of 80% methanol. 70% ethanol was used to extract flavonoids. For 24 hours, the extraction was carried out at 20°C. The extracted samples were centrifuged at 8000 g for 10 minutes before being analyzed. Prior to phytochemical analysis, the samples were stored in a freezer (-20 °C).

Determination of the phenolic content in leaves

The total content of phenolic compounds (Ph) in the leaves was determined using UV–Vis spectrophotometry (Optizen Pop, South Korea) by means of Folin-Ciocalteu's phenol reagent (Singleton et al., 1999). Briefly, 100 µL of extract was mixed with 500 µl of Folin-Ciocalteu's reagent (10 fold diluted) and kept for 3 min at 23 °C. Later, 400 µL of 1M sodium carbonate solution (Na_2CO_3) was added to the reaction mixture, and kept for 2 hours in the thermostat at 23 °C. The absorbance was measured at 760 nm. A calibration curve ($R^2 = 0.999$) was performed using gallic acid (0–100 µg mL^{-1}).

Determination of total flavonoid content

The total flavonoid (Fl) content in the leaves was determined using the Romanian Pharmacopoea (2005) with some modifications for spectrophotometry. To 100 µL of aqueous ethyl alcohol (70%) extract (1/10), 200 µL of a 0.1 M solution of aluminum chloride ($AlCl_3$), 300

μL of 1 M sodium acetate (CH_3COONa), and 400 μL of bi-distilled water were added. After 15 min incubation, the reaction mixture was measured at =419 nm. The calibration curve (R^2 = 0.998) was performed using quercetin (Sigma, Germany).

Determination of chlorophyll a and b and carotenoids

In methanol extracts, the concentration of plastid pigments in the leaves was determined. The quantitative content of chlorophyll (C_a and C_b) and carotenoids ($C_{(x+c)}$) was calculated by UV–Vis spectrophotometry using the following formulas (Wrolstad et al., 2005):

$$C_a\ (mg/mL) = 16.72A_{665.2} - 9.16A_{652.4}$$

$$C_b\ (mg/mL) = 34.09A_{652.4} - 15.28A_{665.2}$$

$$C_{(x+c)}\ (mg/mL) = (1000A_{700} - 1.63C_a - 104.96C_b) / 221$$

Spectrophotometric analyses of pigments, phenolic compounds, and flavonoids in plant leaves were carried out in four biological samples.

Investigation of the phenolic complex by high-performance thin layer chromatography. Biochemical profiling of vegetative organs of birch plants was performed by HPTLC on silica gel G60 (Merck) plates. The general phenolic compounds and flavonoids were separated using the following solvent systems: ethyl methyl ketone/ethyl acetate/methanol/water (v / v / v/ v — 30: 20: 5: 5); ethyl acetate/formic acid/acetic acid/water (v / v / v /v — 100: 11: 11: 25).

The standard (quercetin, rutin and chlorogenic acid) solutions (3.0 μL of each at a concentration of 1 mg mL^{-1}) were applied to the plates. The derivatization was performed with a 0.5% NP reagent (1.0 g diphenylborinic acid 2-aminoethyl ester dissolved in 200 mL of ethyl acetate) and 1% PEG 400 (polyethylene glycol), followed by heating (5 min at 105°C). The detection of phenolic substances on the chromatogram was carried out in UV at 366 nm. The retention factor (Rf) of individual compounds was determined photodensitometrically using the

software Sorbfil TLC ver. 2.3.0.2994 (JSC Sorbopolymer). The Rf value is equal to the distance travelled by the individual compound divided by the distance travelled by the mobile phase front.

Morphometric analysis of Betula spp. leaves

The study included 300 leaves from 12 birch species (n=25 leaves from each species). The leaves were pressed, dried, and scanned with the Epson Perfection V33 Scanner (at 600 dpi resolution in JPEG format). For morphometric analysis, we used the length of the petiole (Pl), the area of the leaf blade (Ar), the perimeter of the leaf blade (Pr), the width of the leaf blade (W), the length of the petiole with length of the leaf blade (Lp), the number of pairs of veins (V), the length of the leaf blade (L), the ratio of the length and width of the leaf blade (L / W), the ratio of the perimeter of the leaf blade. These measurements were performed with the computing software ImageJ 1.52u (Wayne Rasband (NIH), USA).

Statistical data processing. The difference between *Betula* species in plastid pigments, total phenols and flavonoids was assessed using the Kruskal–Wallis test. Dunn's post hoc test was used to compare the pigment and flavonoid content of *Betula* species. The control plant was *Betula pendula*, which is native to Ukraine. The statistical tests were run in R 4.1.0 (R Core Team, 2021). The XLSTAT program (Addinsoft Inc., USA, 2010) was used to perform the cluster and principal component analyses. The correlation analysis (Pearson correlation coefficient) was used to investigate the effect of flavonoids and phenols on the morphometric measurements of the leaf blade. This analysis was performed in R 4.1.0 (R Core Team, 2021). Correlations were plotted using the package “Corrplot” (0.92) (Wei et al., 2021).

Results

Plant phenols are multifunctional metabolites that have a variety of adaptive functions. The rate of accumulation of secondary metabolism products in the assimilation organs is directly proportional to photosynthesis activity. The vast majority of *Neurobetula* and *Betula* species are

photophilous plants. The extremely shade-tolerant *Betula costata* and the shade-tolerant species *B. grossa* and *B. ermanii* are the exceptions. The highest concentration of green pigments was found in the leaves of the very photophilous *Betula pendula*, *B. papyrifera*, and *B. szechuanica*, as well as the photophilous *B. oycoiensis*, *B. schmidtii*, and *B. raddeana*. However, the chlorophyll content of *B. pubescens* leaves was relatively low (Table 2).

Carotenoids were found in the highest concentrations in the leaves of the introduced species *Betula schmidtii* and *B. costata*, which are drought-resistant. The chlorophyll a/b ratio was relatively constant across the species studied. It had a higher value in the leaves of *Betula szechuanica*, *B. oycoiensis*, and *B. davurica*, where the relative amount of chlorophyll b was significantly lower. These species had the highest ratios of total chlorophylls to carotenoids (5.45, 4.27, and 4.40, respectively). *Betula costata* had the lowest ratio (2.90), owing to its high carotenoid content. In general, there is no clear relationship between the content and ratio of photosynthetic pigments in the leaves and the light requirements of the birch species studied. The amount of phenols and flavonoids in the leaves contributed to distinguishing the subgenera (Table 3). Total phenol content was highest in the leaves of the subgenus *Neurobetula*. Flavonoid synthesis was more active in plants of the subgenus *Betula*, which is thought to have originated from the *Neurobetula*. This is demonstrated by the flavonoids-to-total-phenols ratio (Fl/Ph). This indicator's informative value lies in determining the priority of individual metabolic pathways in the plant body that are involved in the implementation of the plant's adaptive strategy.

According to the results of the analysis of variance with pairwise comparison, the difference in the content of total phenolic compounds in the leaves of *Betula pendula* and the studied species was significant for *Betula schmidtii*, *B. costata*, *B. ermanii* from the subgenus *Neurobetula*, and *B. grossa* from the subgenus *Betulenta*. Interestingly, the flavonoid content of

B. pendula leaves was significantly higher than that of the polyploid species *Betula pubescens* and the closely related *B. papyrifera* from the subgenus *Betula*.

The inverse relationship ($r = –0.91$, $p < 0.05$) between the ratio of the number of flavonoids in the leaves to the total content of phenols and the ploidy of the plants brought interesting results. A significant correlation was observed between the duration of vegetation period, the content of total phenols ($r = 0.74$, $p < 0.05$) and flavonoids ($r = 0.65$, $p < 0.05$) in the leaves. The levels of correlation between the duration of the growing season and the content of total phenols and flavonoids in the leaves of *Neurobetula* birches were 0.86 and 0.91, respectively. This is significantly higher than for plants in the subgenus *Betula*.

Only the duration of the vegetation season and the concentration of phenolic compounds were found to have a reliable relationship in *Betula* plants. In contrast to plants in the subgenus *Neurobetula*, this relationship was negative ($r = -0.84$). This diametrically opposed relationship between phenological and phytochemical parameters in representatives of different sections necessitates a thorough examination and additional research. The observed effect could be related to plant ploidy because the ratio of flavonoids to phenolic compounds decreased significantly in polyploid birch species (all the *Neurobetula* species studied). The phytochemical profiling of phenolic compounds revealed that the qualitative composition of flavonoids, coumarins, and oxycinnamic acid conjugates is quite specific in the leaves of the studied birch species. This enables the compounds to be used for species identification as well as research into the potential role of individual compounds (phenes) in the processes of adaptation to habitat conditions. The species of the subgenus *Betula* have very similar biochemical profiles (Fig. 1). Three flavonoids, for example ($Rf \sim 0.58; 0.63; 0.73$) were discovered in six species in this section. The presence of 13 flavonoids in the leaves of the subgenus *Betula* confirms the findings of other researchers (Keinanen et al., 1999).

The phytochemical profiles of representatives of Far Eastern natural flora (*Betula szechuanica*, *B. schmidtii*, and *B. costata*) revealed a significant amount of flavonols with Rf values ranging from 0.73 to 0.81. Significant amounts of rutin (quercetin-3-O-rutinoside) were discovered in the leaves of *Betula davurica* and *B. raddeana*. A medium polar flavonol (Rf ~ 0.63) was discovered in eight of the birch species studied. The biochemical profile of *Betula ermanii* leaves revealed flavonoids with Rf ~ 0.38-0.63. At the same time, *B. ermanii* was distinguished from other species by the presence of four products that fluoresced bright blue (after processing the chromatogram with NP-reagent and UV, λ = 366 nm). A phytochemical profiling cluster analysis confirmed that the birch species form groups based on the composition of phenolic compounds, which mostly correspond to the system proposed by de Jong, 1993 (Fig. 2). The species of the subgenus *Betula* are divided into three subclusters (Ia, Ib, and Ic) in the first cluster. *Betula papyrifera* and *B. pubescens* are members of Subcluster Ia. *Betula pendula* and *B. oycoviensis* are both members of Subcluster Ib. Six common phenes were discovered in their chromatographic profiles. This discovery is explained by the fact that *Betula oycoviensis* is a hybrid of *B. pendula* and *B. szaferi* Jent. -Szaf. ex Stasz. R. Linda. There were no significant morphological or genetic differences between *Betula pendula* and *B. oycoviensis* (Linda et al., 2020), so *B.* x *oycoviensis* is proposed as *B. pendula* var. *oycoviensis*. This hybrid is currently found primarily in the south of Poland, the Czech Republic, and the north-east of Hungary, but its range is gradually shrinking. *Betula platyphylla* and *B. szechuanica* are combined in the subcluster Ic. The taxa's ecobiomorphological similarity confirms their extraordinary affinity. According to modern classification, the latter species is *Betula platyphylla*, with the synonymous name *B. platyphylla* var. *szechuanica* (Miq.) H. Hara. In terms of the complex of phenolic compounds (nine flavonoids and chlorogenic acid), these species are the closest among all the plants of the genus *Betula* used in this study.

The second cluster combines the highly phenolic species of *Betula schmidtii* and *B. costata*. These are representatives of the flora of the Far East and Northwest China. They are commonly found in dry oak groves alongside *Quercus mongolica* Fisch. ex Ledeb and have extremely strong wood.

In the third cluster, four species were combined: photophilous *Betula davurica* and *B. raddeana*, which have similar phenolic compounds profile, as well as shade-tolerant *B. ermanii* and *B. grossa*. The latter two are very similar morphologically and ecologically, although *Betula grossa* is more thermophilic than the Erman's birch.

A PCA (principal component analysis) of biochemical profiles confirmed the close relationship between the complex of flavonoids and oxycinnamic acid conjugates (especially the chlorogenic and neochlorogenic acids) and the ecological characteristics of birch species. These phytochemical phenes are linked to plant ploidy, resistance to low temperatures, and moisture deficiency (Fig. 3).

Individual compounds in the flavonoid complex of the genus *Betula* are highly informative markers of biochemical variability within the subgenus. Six of the isolated flavonoids (Rf ~ 0.49; 0.57; 0.63; 0.73; 0.87; 0.95) were found to be synthesized in the leaves of *Betula plants*. In the dimensional plane of the principal components, they form a group that includes *Betula pendula*, *B. szechuanica*, *B. platyphylla*, *B. oycoviensis*, and *B. pubescens*. *B. papyrifera*, an introduced species, is located somewhat separately in that dimensional plane with the greatest distance along PC2 and has a biochemical profile like *B. pubescens*.

Betula schmidtii and *B. costata* are the closest species to the subgenus *Betula* in the PC1 and PC2 dimension planes. The phytochemical profiles of *Betula davurica* and *B. raddeana* contributed the most to the total dispersion of PC1 and are the most distant from the subgenus *Betula* along the axis of this component.

The phytochemical profiles of plants were used to analyze species within three subgenera, and the results generally agreed with the general clustering. Five species from the subgenus *Neurobetula*, one from the subgenus *Betulenta*, and six from the subgenus *Betula* were grouped into clusters in pairs (Fig. 4, b, d). Rutin and chlorogenic acid were detected in the profiles of *Betula davurica* and *B. raddeana*, and these species differed in phenolic compounds with Rf ~ 0.23 and 0.92, respectively (Fig. 4, a). *Betula ermanii* had a flavonoid marker with Rf ~ 0.40, and *B. grossa* had a flavonoid marker with Rf ~ 0.67. The first cluster, which included *Betula schmidtii* and *B. costata*, had two flavonoids with Rf ~ 0.73 and 0.81 as markers. This group is related to two species in the subgenus *Betula*: *B. pendula* and *B. platyphylla*, both of which have these flavonoids in their biochemical profiles.

Adaptive reserves of *Neurobetula* birch species support their growth in mountainous conditions. Species in clusters II and III (Fig. 4, b) represent the flora of mountain forests in North America, as well as mountain systems in the Caucasus and East Asia. As previously stated, phenolic compounds that actively absorb light in the UV spectrum play an important role in plant resistance to increased insolation (Keski-Saari et al., 2005; Zhang et al., 2011).

Multiple correlation analysis between the qualitative composition of phenolic compounds and the morphological characteristics of the leaves of the studied species also suggests the existence of such relationships. The results of the analysis confirmed significant positive relationships between the presence of phenolic substance with Rf ~ 0.20 and the perimeter of the leaf blade, the number of veins ($r = 0.71$, $p = 0.01$), and the ratio of the perimeter to the area of the leaf blade ($r = 0.71$, $p = 0.003$) (Fig. 5). The petiole length was negatively correlated with the phenolic substance; Rf ~ 0.92 ($r = -0.66$, $p = 0.019$). For rutin (Rf ~ 0.43), this correlation coefficient was higher ($r = -0.81$, $p = 0.002$). Furthermore, a positive correlation was found between a flavonoid (Rf ~ 0.78) and the area and perimeter of the leaf blade ($r = 0.68$, $p = 0.02$; r

= 0.63, p = 0.03, respectively). A negative correlation was found between the ratio of leaf blade length to width and the ratio of flavonoids to total phenols (r = – 0.75, p = 0.05). This suggests that the content of flavonoids in the total pool of phenolic compounds decreases in species with more elongated leaf blades. Flavonoids contributed the most to this relationship, with Rf values of ~ 0.63, 0.57, and 0.49 (r = – 0.70, p = 0.01; r = – 0.67, p = 0.02; and r = – 0.85, p = 0.001, respectively).

Discussion

Studies of plants from different geographical origins but growing under similar conditions, such as in botanical gardens, are of great interest in understanding the role of individual metabolites and their complexes in the adaptation strategies of species within sections or subgenera. In order to understand the secondary synthesis of birch, it is necessary to first understand the composition and ratios of the pigment complex and its components, because the secondary synthesis is dependent on its condition and functionality. Deepak et al. (2020) found that chlorophyll content was the only leaf trait that differed by plant provenance when studying the reflection properties of birch leaves in the visible/near-infrared spectrum. The total content and ratio of chlorophyll *a* and *b* in the leaves of 12 birch species were found to be relatively balanced in this study. *Betula szechuanica*, *B. oycoiensis*, and *B. davurica* had higher chlorophyll ratios despite having significantly lower chlorophyll *b* content in their leaves. We were unable to establish a clear relationship between the content and ratio of photosynthetic pigments in leaves and the light regime requirements of the plants among the birch species studied. This could imply that the quantitative and qualitative composition of plastid pigments in birch leaves is a rather plastic trait that varies according to plant sensitivity to environmental conditions.

The quantitative and qualitative analysis of phenolic compounds in the leaves of 12 birch species revealed two major plant groups, which are shown in pairs in Fig. 3. According to De

Jong (1993), the subgenus *Neurobetula* is the most closely related to the subgenus *Betulenta*. In the principal component coordinates (PC) spatial plane, *Betula schmidtii* and *B. costata* are closest to the subgenus *Betula*. The profiles of flavonoids and other phenolic substances in the leaves of *Betula schmidtii* and *B. pendula* were found to be largely consistent in this study. This is consistent with Keinänen's (1999) findings, as well as the composition of secondary stem metabolites (Julkunen-Tiitto et al., 1996). This is supports the suggestion that this member of the subgenus *Neurobetula* is more closely related to the subgenus *Betula* than to *Betulenta* species (Keinänen et al., 1999). However, the results of network analysis based on ITS (internal transcribed spacer) do not support the species' relationship. *Betula schmidtii* differs from other birch species in terms of ITS2 secondary structure, and it should be classified as a separate subgenus, according to Bina et al. (2016).

Birch trees of the subgenera *Neurobetula* and *Betulenta* were classified into clusters II and III (Fig. 4b). In terms of dendrogram dissimilarity, *Betula grossa* (also known as *B. ulmifolia* Siebold & Zucc.) of the subgenus *Betulenta* is ecomorphologically closest to *B. ermanii* (the subgenus *Neurobetula*), which is also known as *B. ulmifolia* var. *grandulossa* (H.J.P. Winkl). *Betula ermanii*, like *B. grossa*, is found in eastern Siberia, Primorskii Territory, the Amur region, the Korean peninsula, and the Japanese islands of Hokkaido, Shikoku, and Honshu, where it grows in mixed forests. The spread of *Betula grossa* has spread to several Japanese islands (Kyushu, Shikoku, and Honshu). *Betula grossa* is most likely the result of allopolyploidization between members of the subgenera *Betula* and *Asperae*, according to genetic analyses (Wang et al., 2016). The results of genetic analysis using AFLP markers indicate that *Betula costata*, *Betula ermanii*, and *B. davurica* should be assigned to the subgenus *Betula* (Schenk et al., 2008). The existence of hybrids between these species and the species of the subgenus *Betula*, according to the authors, confirms the Asian origin of these species.

With the exception of *Betula raddeana*, the amount of total phenols in this section was significantly higher, reaching 10% of the leaf dry weight (Table 3). This backs up previous findings about the relatively high content of gallotannins in young birch leaves (Osipov et al., 1997). Birch phenols are primarily composed of tannins, both condensed (proanthocyanidins) and hydrolyzable (gallotannins and ellagitannins). Tannins perform numerous ecophysiological functions (Kraus et al., 2003). They play a key role in plant resistance to increased insolation (Tegelberg et al., 2001). Tannins actively absorb UV light and protect assimilating organs from damage. This characteristic promotes plant growth on stony soils, hillsides, and high in the mountains. Plants have thus been subjected to natural selection for this trait for a long time. Obviously, the "high-phenolic" individuals have gained an advantage, and the additive genetic variability for this trait has decreased. The qualitative composition of phenolic substances in birch leaves may also be influenced by clinal variability, such as increased content of more hydrophobic flavonoids in plants of southern origin (Deepak et al., 2018). This could explain why species of the subgenus *Neurobetula*, which was introduced into central Ukraine, accumulate more phenols in their leaves than native species. This isn't always good for the plants. The activity of oxidases, which regulate hormonal status and are involved in lignin synthesis, is significantly affected by phenolic compounds (Mierziak et al. 2014). Tannin synthesis is generally negatively correlated with tree growth rate (Stevens et al., 2007). This explains the insufficient maturation of young shoots in birch trees, their later entry into winter dormancy, and, as a result, winter damage caused by low temperatures. Thus, the high polyphenol content of *Betula schmidtii* and *B. costata* leaves has had a negative impact on the plants. The findings support the hypothesis that the composition of phenolic compounds, including those found in *Betula pendula*, is genetically controlled (Klaper et al., 2001; Laitinen et al., 2005; Deepak et al., 2018).

Tannins are antioxidants that protect plants from excessive light energy. They have antifeedant properties and can inhibit pathogen development (Saleem et al., 2010). At various stages of leaf development, different tannin classes have been found to provide protection against phytophages. For example, in *Betula pubescens* subsp. *czerepanovii* (Orlova) Hämet-Ahti, the high content of gallotannins and ellagitannins is related to the low fitness of young leaves to herbivorous *Lepidoptera*. Proanthocyanidins perform this function in mature leaves (Henriksson et al., 2003). Tannins, on the other hand, do not appear to harm phytophages (Kopper et al., 2002; Kraus et al., 2003; Barbehenn, R., & Constabel, C., 2011). Plants can withstand insect damage if it is not catastrophic and does not occur over a long period of time. As a result, tannins in birch leaves play a role other than protecting plants from pest damage.

What, then, is the role of tannins aside from protection against harmful factors? The importance of tannins in providing essential nutrients to plants is well known (Northup et al., 1998; Madrich, M., & Lindroth, R., 2015). Changes in the concentration of condensed tannins have been shown to be closely related to nitrogen recovery by plants after insect-induced defoliation. Birch trees from the subgenus *Neurobetula* had the highest phenolic content in our studies. These polyploid species are found primarily in mountainous areas. Only *Betula papyrifera* had a relatively high phenolic content among the subgenus *Betula* birch species. This species' hexaploids (2n = 84) are common in the Rocky Mountains and northwestern Canada (Li, 1996). As a result, there is a link between birch trees' ability to root and grow on rocky slopes in the mountains and their high tannin content. The amount of organic matter on stony substrates in the mountains, on mountain slopes, and in mountain river valleys is insignificant when compared to forested gentle hillsides in valleys. This has an impact on overall biodiversity, trophic group structure, and the number of soil microbes. Tannin-producing plants are related to mycorrhizal fungi, which can grow in polyphenol-rich environments (Joanisse et al., 2009). Micromycetes

associated with *Betula ermani*, for example, vary in diversity and species composition along an altitudinal gradient (Osono, T., & Hirose, D., 2009). Under these conditions, plants' ability to extract nutrients and trace elements from the mineral part of substrates on their own is critical, and they have it. Polyphenols can form complexes with metals and participate in chemical weathering processes, as previously demonstrated (Cruz et al., 2000; Kraal et al., 2006). Most metal ions can be chelated by hydrolysed tannins containing several o-dihydroxy and trihydroxy aromatic rings. Tannins, as a result, have an effect on the mobility and bioavailability of trace elements in soil (Tiarks et al., 1989). Mineral weathering provides rock nutrients to microorganisms and plants. The impulse supply of significant amounts of aromatic compounds from fresh leaf litter temporarily increases the rate of microbial decomposition of soil organic matter (priming effect). Thus, the type of chemical weathering and its congruence are influenced by soluble organic matter (Fang et al., 2023). This property is especially important for birch trees growing in poor stony soils because the plants are deficient in important macro- and micronutrients.

An important feature of the birch trees of the subgenus *Neurobetula* is their ability to accumulate and release significant amounts of tannins into the environment, which have several important functions for growing in mountain systems: protection against ultraviolet radiation, which is especially important for the young organs (leaves, stems, and flowers), protection against low and high temperatures, and providing mineral nutrients to plants and soil microorganisms.

The subgenus *Betula* members are widespread on the Eurasian plains. They grow in moist, rich soils. Compared to the subgenus *Neurobetula*, trees of the subgenus *Betula* had 1.8–2.5 times fewer phenolic compounds in their leaves, and their phenolic compound class ratio (F1/Ph) was shifted towards flavonoids (Table 2). We discovered positive correlations between the levels of

individual flavonoids in the leaves (Fig. 5). The degree of consistency in their composition was greater than that of the phenolic acids. Previously, Deepak et al. (2018) described such consistency in the synthesis and deposition of various groups of secondary metabolites on the surface of silver birch leaves. Flavonoids accumulate primarily in the leaf epidermis of vegetative organs (Deepak et al., 2018). Their synthesis is malleable and responsive to environmental cues. The amount of flavonoids in leaves increases in response to increased UV exposure (Kanazawa et al., 2012). They effectively shield vulnerable young plant tissues from UV radiation. Flavonoids can sensitize photoactive molecules and receive and transmit light energy (Sisa et al., 2010). Some flavonoids interact with protein ATP-binding sites (Arrighi et al., 2006). These polyphenols inhibit the activity of membrane NADPH oxidase, which is involved in the formation of superoxide anion radicals (Hodnick et al., 1994). The presence and position of hydroxyl groups in the aromatic A and B rings determines flavonoids' ability to neutralize free radicals in cells and protect membrane phospholipids from peroxidation (Heim et al., 2002). In comparison to kaempferol, the higher antioxidant activity of myricetin found in many birch species (Pawlowska, 1983) is explained by the greater number of hydroxyl groups in the aromatic B ring (Arora et al, 1998). Flavonoid molecules' chemical structure allows them to be incorporated and distributed in the lipid phase of cell membranes. As a result, they have an effect on their selective properties, act as ionophores, and are stable at low temperatures. As a result, flavonoids have the functional ability to increase plant cold and frost resistance (Kaplan, 2004; Korn et al., 2008). This is especially important for *Betula* species that grow in areas with frequent thaws and frosts.

Flavonoids (including quercetin and its glycosides) are known to play a role in auxin transport by regulating specific transport proteins (Murphy et al., 2000; Brown, 2001; Peer et al., 2004; Taylor, Grotewold, 2005; Santelia, 2008). In *Arabidopsis*, a mutation that reduces

480 flavonoid synthesis increases auxin transport activity significantly. This results in phenotypic
481 changes and structural abnormalities in the plant (Buer et al., 2009). The morphological
482 characteristics of species rich in dihydroxyflavonoids or monohydroxyflavonoids differ
483 (Mathesius, 2001; Potters et al., 2009; Mierziak et al., 2014). These and other flavonoid functions
484 help to explain their role in plant adaptation at the cellular and tissue levels. As a result, there is
485 reason to look for a link between flavonols and the shape of leaves and other organs, which is
486 used to identify birch species in particular. The multiple correlation analyses presented above
487 confirm the relationship between individual phenolic compounds, flavonoids/total phenols ratio,
488 and leaf morphometric parameters (Fig. 5).

489 Thus, the previously established taxonomic system based on a complex of ecological and
490 biomorphological characters (bark structure, leaf blade shape, fruit structure, etc.) is confirmed at
491 the level of biochemical phenes in this study. The relatively high flavonoid content of the leaves
492 of the birch subgenus *Betula* may attest to the evolution of a new, more perfect, adaptive strategy
493 aimed at maintaining homeostasis through the development of a system of complex biochemical
494 regulation of metabolism. This is realized at the ecosystem level through the active interaction of
495 plants with endophytic and soil microorganisms via flavonoids (Dixon and Steele, 1999). The
496 wide range of the white birch, which covers almost the entire territory of Eurasia and North
497 America, attests to the efficacy of this strategy.

498 **Conclusions**

499 The analysis of phenolic compounds in native and introduced birch species of the
500 subgenera *Betula* and *Neurobetula* confirms the hypothesis that there is a close relationship
501 between phenolic compounds, morphogenesis, and plant adaptive abilities. Except for *Betula*
502 *papyrifera* (0.05), the ratio of flavonoids to total phenols (Fl/Ph) in the leaves of the subgenus
503 *Betula* trees was relatively high (0.11–0.18). This index was lower in plants of the subgenera

504 *Neurobetula* (0.04–0.10) and *Betulenta* (0.03), indicating subgeneric differences in polyphenol
505 synthesis priorities. Given flavonoids' multifunctional role in redox reactions, auxin transport,
506 and the enzymatic activity of individual metabolic pathways, as well as plant interactions with
507 soil microorganisms, there is reason to believe that an increase in the proportion of flavonoids in
508 birch leaves of the subgenus *Betula* is adaptive, allowing plants to expand their area of growth in
509 today's climatic conditions. At the same time, the increased total phenol content in the leaves of
510 *Neurobetula* birches may be indicative of their adaptation to the low nutrient conditions typical of
511 the high mountains.
512
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525 Data available within the article and supplementary files.
526
527

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Table 1. Location of the original birch planting material for the arboretum of the M.M. Gryshko National Botanic Garden (Kyiv, Ukraine)

Species	Distribution	Country	Year
	Subgenus *Betula*		
B. pendula	The Botanical Garden of NULES, Kyiv	Ukraine	1948
B. platyphylla	State Natural Biosphere Reserve "Kedrovaya Pad", Primorsky Krai	Russia	1949
B. szechuanica	Yelizovsky District, Kamchatka Krai	Russia	1944
B. oycoiensis	Warsaw	Poland	1950
B. pubescens	Rivne region	Ukraine	1980
B. papyrifera	Ottawa	Canada	1949
	Subgenus *Neurobetula*		
B. schmidtii	State Natural Biosphere Reserve "Kedrovaya Pad", Primorsky Krai,	Russia	1949
B. costata	Primorsky Krai	Russia	1950
B. ermanii	Headwaters of the Kamchatka river	Russia	1949
B. davurica	State Natural Biosphere Reserve "Kedrovaya Pad", Primorsky Krai	Russia	1948
B. raddeana	O.V. Fomin Botanical Garden of Taras Shevchenko National University, Kyiv	Ukraine	1950
	Subgenus *Betulenta*		
B. grossa	Kornik	Poland	1950

Table 2. Content of plastid pigments (mg·g^{-1} of dried mass) and their ratio in the leaves of plants of the genus *Betula* (x ± SE, n = 4)

Species	*Chl$_a$	Chl$_b$	Chl$_{a/b}$	Chl$_a$ + Chl$_b$	Kr
			Subgenus *Betula*		
B. pendula	4.41 ± 0.14	1.39 ± 0.06	3.16 ± 0.08	5.80 ± 0.19	1.46 ± 0.05
B. platyphylla	**3.37 ± 0.18** [b]	**0.94 ± 0.01** [b]	3.57 ± 0.16	**4.32 ± 0.19** [b]	1.32 ± 0.02
B. oycoiensis	4.45 ± 0.21	1.07 ± 0.04	**4.18 ± 0.21** [b]	5.51 ± 0.23	1.29 ± 0.02
B. pubescens	**2.99 ± 0.15** [c]	**0.76 ± 0.07** [c]	**3.97 ± 0.27** [a]	**3.76 ± 0.21** [c]	**1.00 ± 0.04** [b]
B. szechuanica	4.02 ± 0.17	**0.94 ± 0.02** [b]	**4.29 ± 0.14** [b]	4.96 ± 0.18	**0.91 ± 0.03** [b]
B. papyrifera	4.29 ± 0.20	1.31 ± 0.02	3.27 ± 0.20	5.60 ± 0.18	1.48 ± 0.02
			Subgenus *Neurobetula*		
B. schmidtii	4.42 ± 0.20	1.43 ± 0.03	3.09 ± 0.15	5.84 ± 0.19	1.76 ± 0.04
B. costata	3.82 ± 0.19	1.17 ± 0.03	3.26 ± 0.20	4.98 ± 0.19	1.72 ± 0.03
B. ermanii	**3.23 ± 0.16** [b]	1.05 ± 0.04	3.06 ± 0.04	**4.29 ± 0.20** [a]	**0.98 ± 0.04** [b]
B. davurica	**3.69 ± 0.14** [a]	**0.96 ± 0.06** [b]	**3.89 ± 0.33** [a]	**4.65 ± 0.14** [a]	**1.13 ± 0.02** [a]
B. raddeana	4.41 ± 0.20	1.36 ± 0.04	3.24 ± 0.05	5.76 ± 0.24	1.31 ± 0.03
			Subgenus *Betulenta*		
B. grossa	**3.60 ± 0.12** [a]	**0.97 ± 0.05** [a]	3.73 ± 0.31	**4.57 ± 0.10** [a]	1.15 ± 0.02

* Chl$_a$; Chl$_b$ — chlorophylls *a* and *b*, respectively; Kr — carotenoids; the Dunn's post hoc test for comparisons with *B. pendula,* the pairwise differences are statistically significant: [a] – p-values ≤ 0.05, [b] – p-values ≤ 0.01, [c] – p-values ≤ 0.001

Table 3. The content of total phenols, flavonoids (mg·g^{-1} of dried mass) and their ratio in the leaves of plants of the genus *Betula* (x ± SE, n = 4)

Species	2n*	Ph	Fl	Fl/Ph
		Subgenus *Betula*		
B. pendula	28	81.2 ± 1.26	14.3 ± 0.32	0.18
B. platyphylla	28	87.5 ± 1.39	9.5 ± 0.41	0.11
B. oycoiensis	28	78.0 ± 1.74	9.6 ± 0.45	0.12
B. pubescens	56	58.6 ± 1.36	**6.3 ± 0.33** [a]	0.11
B. szechuanica	28	84.9 ± 1.71	14.2 ± 0.71	0.16
B. papyrifera	56, 70, 84	117.1 ± 1.56	**5.8 ± 0.30** [a]	**0.05** [c]
		Subgenus *Neurobetula*		
B. schmidtii	28	**199.3 ± 1.72** [b]	19.7 ± 0.71	0.10
B. costata	28	**256.8 ± 3.93** [b]	23.0 ± 0.38	**0.09** [a]
B. ermanii	56,112	**139.3 ± 2.44** [a]	7.5 ± 0.31	**0.05** [b]
B. davurica	56, 84, 112	119.1 ± 1.02	**4.7 ± 0.30** [b]	**0.04** [c]
B. raddeana	84	61.9 ± 1.14	**3.8 ± 0.28** [b]	**0.06** [b]
		Subgenus *Betulenta*		
B. grossa	84	**139.7 ± 2.03** [a]	**3.5 ± 0.28** [c]	**0.03** [c]

Ph — phenolic compounds, Fl — flavonoids, Fl / Ph — the ratio of flavonoids to total phenols; Dunn's post hoc test for comparisons with *B. pendula,* the pairwise differences are statistically significant: [a] — p-values ≤ 0.05, [b] — p-values ≤ 0.01, [c] — p-values ≤ 0.001; * plant ploidy according to Pawlowska, 1983; Li et al., 1996; Keinanen et al., 1999; Wang et al., 2016

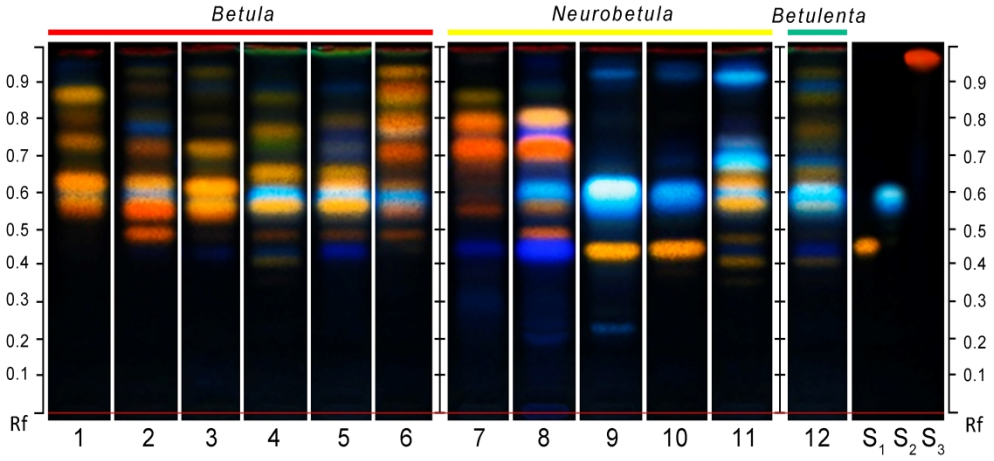

Fig. 1. Chromatographic profiles of leaf extracts of plants of the genus Betula
Figure legends: 1 – Betula pendula; 2 – B. platyphylla; 3 – B. oycoviensis; 4 – B. papyrifera; 5 – B. pubescens; 6 – B. szechuanica; 7 – B. schmidtii; 8 – B. costata; 9 – B. davurica; 10 – B. raddeana; 11 – B. ermanii; 12 – B. grossa; S1 – rutin, S2 – chlorogenic acid, S3 – quercetin

303x138mm (300 x 300 DPI)

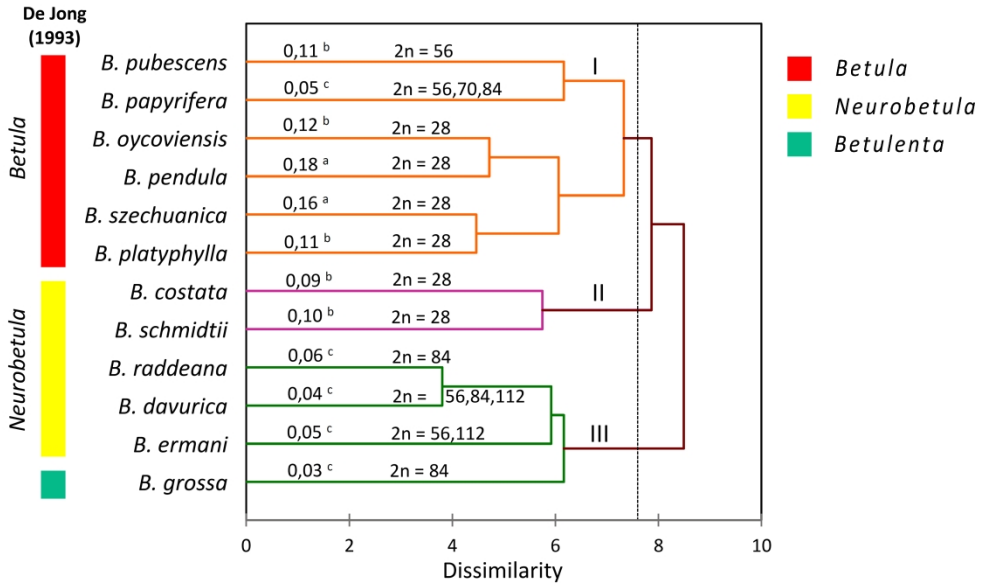

Fig. 2. Dendrogram of affinity of species of the genus Betula by the qualitative composition of phenolic compounds in the leaves and their position in subgenera by De Jong (1993)
Figure legends: a (0.15-0.21), b (0.08-0.14), c (0.01-0.07) – high, medium and low ratio of flavonoids to total phenols in the leaf, respectively; (species ploidy according to Pawlowska, 1983; Keinanen et al., 1999; Wang et al., 2016)

369x220mm (300 x 300 DPI)

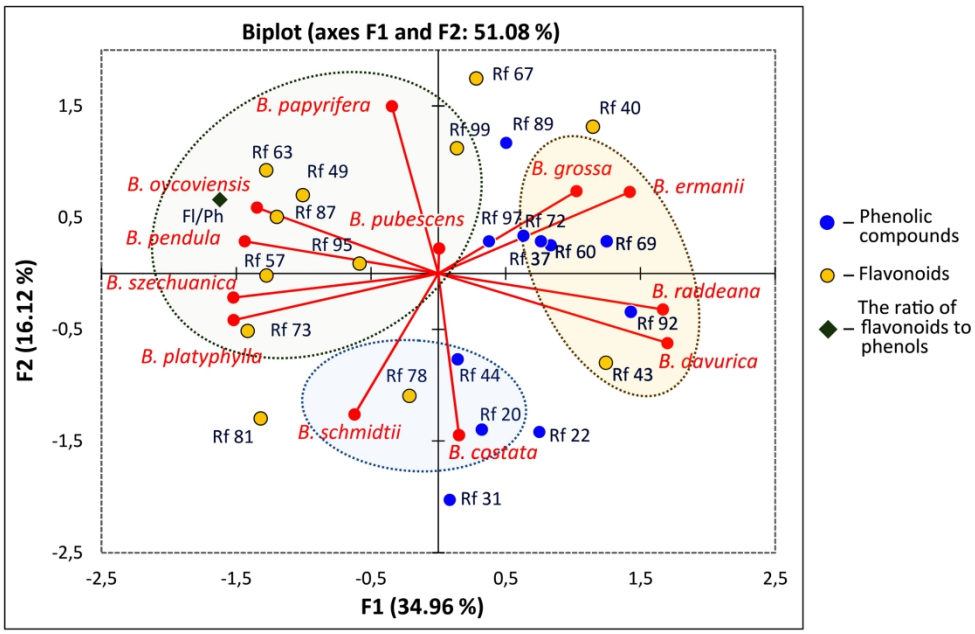

Fig. 3. The results of principal component analysis on the polyphenol profiles of birch leaves of the subgenera Neurobetula, Betulenta and Betula

199x129mm (300 x 300 DPI)

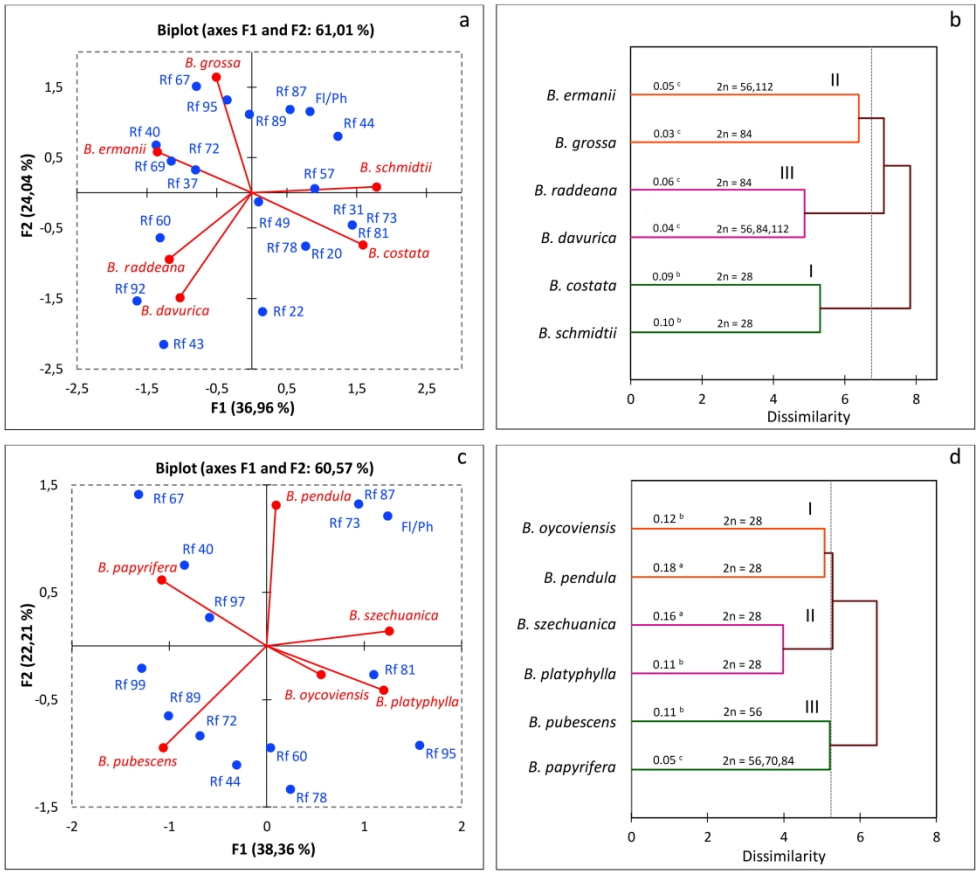

Fig. 4. Distribution of birch species in the subgenera Neurobetula, Betulenta (a, b) and Betula (c, d) by coordinates of principal components (PCA) and in clusters according to the results of analysis of the qualitative composition of phenolic compounds in the leaves

299x267mm (300 x 300 DPI)

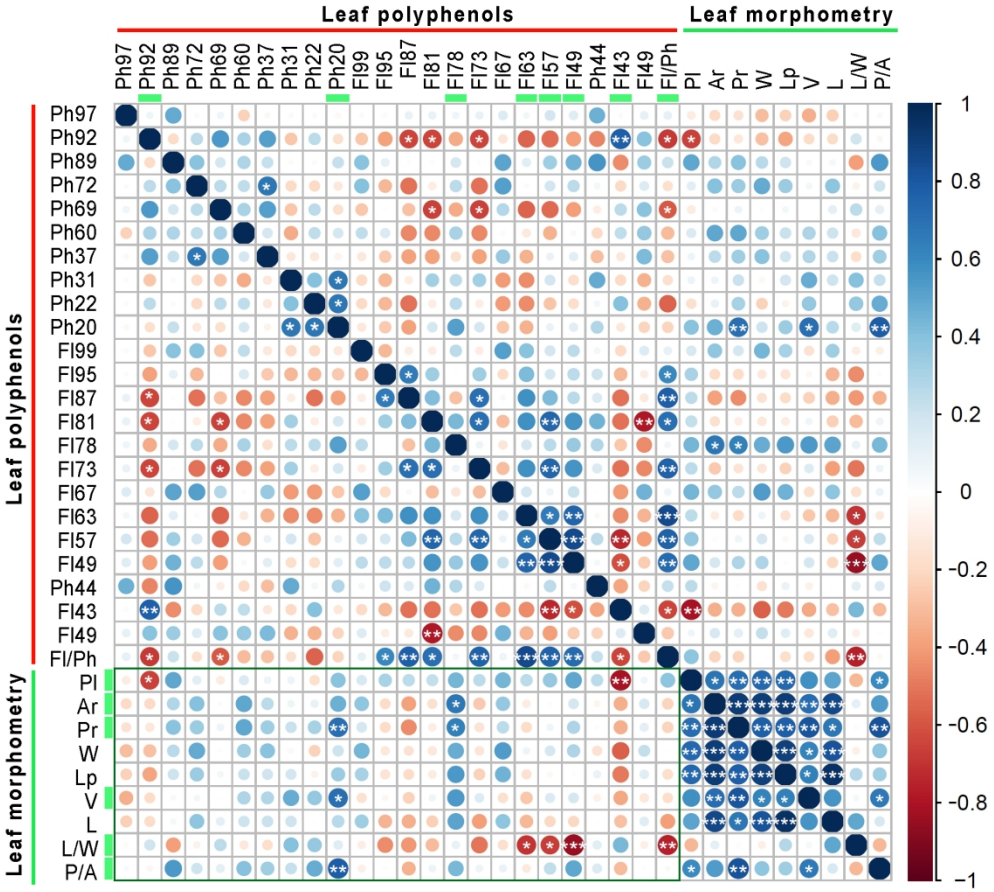

Fig. 5. Correlation matrix of phytochemical and morphological phens of birch leaves of the subgenera Neurobetula, Betulenta and Betula

Figure legends: Ph — phenolic compound, Fl — flavonoid, Fl / Ph — the ratio of the content of flavonoids to total phenols; Pl — the length of the petiole, Ar — the area of the leaf blade, Pr — the perimeter of the leaf blade, W — the width of the leaf blade, Lp — the length of the petiole with length of the leaf blade, V — the number of pairs of veins, L — the length of the leaf blade, L / W — the ratio of the length and width of the leaf blade, P / A — the ratio of the perimeter of the leaf blade and square root of leaf area; * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$

178x160mm (300 x 300 DPI)