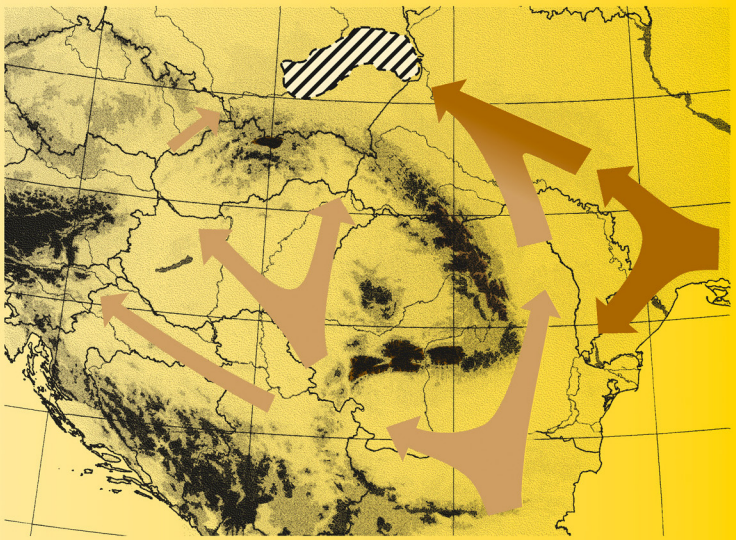
POLISH BOTANICAL STUDIES

30

2014

Phylogeography of Pontic-Pannonian species in Central Europe

ELŻBIETA CIEŚLAK



POLISH ACADEMY OF SCIENCES
W. SZAFER INSTITUTE OF BOTANY

P O L I S H B O T A N I C A L S T U D I E S

Vol. 30 **2014**

Polish Botanical Studies is a companion series of *Polish Botanical Journal* publishing monographs and more comprehensive papers longer than 50 printed pages covering various aspects of vegetation science, plant and fungi biodiversity, biosystematics (including plant anatomy, cytology and embryology), phytogeography, evolution and ecology. The series appears at irregular intervals.

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http://www.botany.pl/ibwyd/pol-b-j/pol-b-j.htm

This work was funded by the Ministry of Science and Higher Education* (*2007–2011*, *grant no. N N304 154633*), *National Science Centre* (2011–2014, *grant no. N N304 300940*), *and in part through the statutory fund of the W. Szafer Institute of Botany of the Polish Academy of Sciences

Published, sold and distributed by

W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Kraków, Poland
Tel./Fax: +48 12 4241731; e-mail: wydawnictwa@botany.pl; http://www.botany.pl

Typesetting and page layout: Marian WYSOCKI *Cover design*: Maciej PIERZCHAŁA

Issued: 28 January 2014 **ISBN: 978-83-62975-18-1**
Printed in Poland: Drukarnia Kolejowa Kraków sp. z o.o., Forteczna 20A, 32-086 Węgrzce **ISSN: 0867-0730**

Polish Botanical Studies 30: 1–53, 2014

PHYLOGEOGRAPHY OF PONTIC-PANNONIAN SPECIES IN CENTRAL EUROPE

ELŻBIETA CIEŚLAK

Abstract. This phylogeographical study concentrates on five species representing the Pontic-Pannonian subelement of the Polish flora: *Carlina onopordifolia* Besser *ex* Szafer, *Cirsium pannonnicum*, *Inula ensifolia* (L. fil.) Link, *Linum flavum* L. and *Linum hirsutum* L. Material was collected from populations in the following geographical regions of Central and Eastern Europe: the Wyżyna Małopolska upland (Poland); Wyżyna Lubelska upland (including Volhynian Polissya and the western part of the Volhynian Upland, Poland); the Podolian Upland (Ukraine); the southern (Hungary, Romania) and northwestern (Czech Republic, Slovakia, Austria) parts of the Panonnian area; the Balkan Peninsula (Bulgaria) and the northern Adriatic coast (Italy, Slovenia). The aim of the study was to verify hypotheses regarding migration routes, the time of migration of these species to southern Poland and more broadly to Central Europe, and the historical role of eastern and southern Poland in these processes. The 1434 samples collected in this work were analyzed after amplified fragment length polymorphism genotyping. Genetic variation was analyzed on the level of populations, population groups from specific geographical areas, and all sampled populations per species. The level of genetic variation was determined based on Nei's gene diversity index, Shannon's diversity index, frequency-downweighted marker values, and the number of polymorphic, private and discriminating bands. To test for isolation by distance between populations, the correlations between pairwise F_{ST} and geographical distances, were examined with the Mantel test. The relationships between individuals for each species were analyzed based on principal coordinate analysis, neighbor-joining, molecular variance and Bayesian analysis. Analysis of the genetic variation of this selected group of steppe species showed it to be at similar levels in all the studied populations, and revealed location-dependent differences in the distribution of genetic lineages in the populations. Examination of individual migration routes of the five species from the south to the north of Central Europe, including the uplands of southern Poland, indicated that the main migration route ran westward along the northern side of the Carpathian Mts. The analysis did not support the existence of a direct route from the south via the Moravian Gate and/or passes and valleys of the Carpathians as a major pathway of northward migration. The divergence of genetic lineages identified in the study suggests that the populations from the Wyżyna Małopolska upland had an independent history and are older than those from the Wyżyna Lubelska upland, and indicates more than one migration wave of the steppe element in the southern uplands of Poland. Thus the populations from the Wyżyna Małopolska upland may represent remnants of a more ancient migration wave which may have arrived immediately after the Sanian 1, Sanian 2 glaciation, when the steppe element could penetrate southern Poland. For these species it may be the only migration wave that reached the Wyżyna Małopolska upland. They would then be relicts of the Pleistocene glacial period in the Wyżyna Małopolska upland. The range of the subsequent migration wave(s) in the late glacial and/or postglacial period would have been limited to the Wyżyna Lubelska upland.

Key words: *Carlina onopordifolia*, *Cirsium pannonnicum*, *Inula ensifolia*, *Linum flavum*, *Linum hirsutum*, xerothermic species, southeastern Poland, Central Europe, genetic variability, phylogeography, migration routes, time of migrations, AFLP

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CONTENTS

Introduction

The origin of floras and the history of species distributions are key subjects for botanical studies. Such work examines the diachronic diversity of plant life, identifies historical processes and determines the conditions influencing contemporary range patterns (Szafer 1977a; Kornaś & Medwecka-Kornaś 2002). Especially interesting and still underexplored is the history of species associated with xerothermic habitats in Central Europe, where they form one of the most valuable elements of biodiversity. Depending on the range type, their regional centers of occurrence and diversity cover the Black Sea steppe area, the Hungarian Plain, the Mediterranean Sea region and/or Central Asia. In Central Europe, xerothermic species occur extrazonally as island populations (Kozłowska 1931; Medwecka-Kornaś & Kornaś 1977). This group is often described as 'steppe species' broadly defined (Kozłowska 1923). The group is further divided by type of geographical distribution pattern: Sub-Mediterranean, Irano-Turanian and Pontic-Pannonian (Pawłowska 1977). Five species belonging to the last subgroup are investigated here. Their extrazonal localities in Central Europe and (exceptionally) in the southern part of Northwestern Europe, distinguished by a specific combination of orographic, soil and microclimatic conditions, form a characteristic, repeated pattern (Motyka 1946, 1947; Fijałkowski & Izdebski 1957; Medwecka-Kornaś & Kornaś 1977).

It is still unresolved whether the present island-type range of steppe species in Central Europe results from natural historical factors and thus is relict or whether it is a relatively recent pattern that may have been affected variously by anthropogenic factors. In the latter case their migration and establishment may have been promoted by, for example, agriculture (especially pasturing) leading to deforestation of carbon-rich soils, most pronounced since the Holocene climatic optimum. Persistent human-induced disturbances of the natural habitat have created conditions favoring the development of steppe plant formations (Medwecka-Kornaś & Kornaś 1977). Thus, numerous secondary anthropogenic localities are recorded in addition to natural ones in many regions. Often the origin of localities and current species distributions is due to anthropopression. Reconstruction of the historical aspects of a locality frequently is very difficult or impossible. The colonization of Central Europe, especially its northern areas after Pleistocene glaciations, has been the subject of wide-ranging discussion (Pott 1995; Hewitt 1996; Bredenkamp *et al*. 2002). Few data on the range history of steppe plants are available for treatment. Migration routes could be traced using extant localities of these species. Their current distribution is an indicator of site availability and the adaptive potential of the species. There are valuable studies which have attempted to reconstruct the plant cover of different historical periods and to make a combined spatial and temporal analysis based on paleobotanical and paleoclimatic data (Tarasov *et al*. 2000; Kuneš *et al.* 2008). Unfortunately the palynological data and macrofossils of steppe species are scarce and available for only a few genera such as *Stipa*, *Artemisia* or *Festuca* (Kozłowska 1926; Szafer 1946; Środoń 1977). These are associated with the northern steppes of Eurasia, the 'cold steppe' (Kulczyński

1927; Szafer 1946, 1977b; Środoń 1952), and do not correspond to the contemporary types of the majority of Central-European 'steppe' grasslands. Paleobotanical data for steppe species of the Pontic or Pannonian regions in Europe are not available. For Pontic-Pannonian species there is a need for research that can help verify hypotheses on their migration routes, the time of these migrations from southern to northern Central Europe, and the range and periodicity of these migrations.

VEGETATION OF XEROTHERMIC GRASSLANDS IN POLAND

In Poland, xerothermic vegetation develops mostly in regions showing the highest degree of climatic continentalism, producing suitable orographic and soil conditions. The main areas of its occurrence include the Wyżyna Lubelska upland, Wyżyna Małopolska upland, Wyżyna Śląska upland, the vicinity of Przemyśl, the lower Vistula and the lower Oder valleys, and the Toruń-Eberswalde spillway (Medwecka-Kornaś & Kornaś 1977). Patches of xerothermic grasslands of the class *Festuco-Brometea* occur at scattered island localities on exceptionally warm, dry and sun-exposed sites. They chiefly colonize small areas facing south, southwest and southeast on the edges of river valleys and spillways, on rock outcrops and anthropogenically deforested slopes of moraine and upland hills. They range widely in type, including pioneer communities with sparse cover and cluster structure, with grasses dominant (communities of the alliance *Festuco-Stipion*); low, species-rich grasslands and tall, abundant mesophilic communities with a high contribution of perennial dicotyledons (communities of the alliance *Cirsio-Brachypodion pinnati*) (Dziubałtowski 1916; Kozłowska 1923, 1928; Fijałkowski & Izdebski 1957; Medwecka-Kornaś & Kornaś 1977). Along with habitat factors such as climatic conditions in the region, landscape structure or the lie of the land, the glaciations during the Quaternary period in Northern Europe had a considerable influence on the species diversity of these communities in Poland (Środoń 1977; Szafer 1977b; Dybova-Jachowicz & Sadowska 2003; Mojski 2005).

The closest areas of natural (zonal) steppe flora occur in Eastern Europe; thus the main direction of steppe species migration to Poland has been assumed to be northwards from the east and southeast (Szafer 1977b). The probable migration routes were (*i*) from the Pontic region and Podolia through Transnistria (Trans-Dniester region) and along the northern edge of the Carpathian Mts, (*ii*) from the Pannonian Plain through the Moravian Gate or passes of the Beskid Niski Mts, and (*iii*) from the northwest from Thuringia and the North German Plain, the Toruń-Eberswalde spillway. The availability of suitable habitats in particular regions in Poland was an important factor influencing the history of migrations once the species arrived in Poland. Due to a variety of factors affecting the migration of steppe species, elements originating from different ages may co-occur on the country scale (southern vs northern Poland) and also the regional scale (different fragments of the uplands belt in southern Poland) (Medwecka-Kornaś & Kornaś 1977; Szafer 1977b).

HISTORY OF RESEARCH ON THE VEGETATION OF POLAND'S XEROTHERMIC GRASSLANDS

Generations of botanists have taken a keen interest in the history of xerothermic steppe communities in northern regions of Central Europe and have pondered the origin of the Pontic-Pannonian subelements in these communities. Since the early 20th century they have published hypotheses on the migration routes of xerothermic species to Poland and more broadly to Central Europe. Regional floristic studies have contributed much to this discussion (Dziubałtowski 1916, 1923; Szafer 1918; Kozłowska 1923, 1928; Gajewski 1931, 1932; Medwecka-Kornaś 1952; Fijałkowski & Izdebski 1957; Tacik 1959; Ceynowa 1968; Fijałkowski 1969, 1972; Szwagrzyk 1987; Babczyńska-Sendek 2005; Ratyńska & Waldon 2010; Towpasz 2011 and references therein). The taxonomy of selected species of xerothermic grassland communities has also received attention (Kozłowska 1926; Ceynowa-Giełdon 1976). A number of important studies have considered the location of refuge areas of steppe vegetation on the northern edges of the Eastern Carpathians in Podolia, Pokuttya, eastern

Volhynia and Opillya, often subsumed in the collective term 'Podolian-Volhynian refuge', and in Moravia (Paczoski 1900; Raciborski 1916; Szafer 1918, 1923, 1926, 1930, 1977b; Koczwara 1925, 1934; Kozłowska 1930, 1931; Gajewski 1932, 1934, 1937; Kulczyński & Motyka 1936; Medwecka-Kornaś 1958; Medwecka-Kornaś & Kornaś 1977). Other work covers zonal steppe communities in Central Europe (Kulczyński 1927; Gajewski 1934; Koczwara 1934, 1946; Motyka 1946; Szafer 1946). Also discussed in the literature are the potential routes and time of migration of steppe species from Southeastern Europe, and the role played in these migrations by the Moravian Gate (Kozłowska 1923, 1931; Szafer 1926; Šmarda 1946, 1956, 1963; Medwecka-Kornaś 1958; Ceynowa-Giełdon 1976; Medwecka-Kornaś & Kornaś 1977; Pawłowska 1977; Babczyńska-Sendek 2005) and the valleys of rivers such as the Poprad, Dunajec and Cisa (Pawłowski 1925; Kornaś 1955; Cyunel 1959; Tacik 1959; Ceynowa-Giełdon 1976; Babczyńska-Sendek 2005). Authors have proposed a number of routes for migrations of 'steppe' species to the present territory of Poland after the last glacial period. The following have been considered the most important (as reviewed by, e.g., Medwecka-Kornaś & Kornaś 1977; Paul 2010):

1. the 'Podolian route' from the southeast: from Podolia along the northern edge of the Carpathians;

2. the 'Moravian route' from the south: from the Pannonian Basin across the low-lying parts of the Beskid Niski Mts and/or Moravian Gate;

3. from the west: from the Prealps and southern Germany along the northern edges of the Sudeten Mts;

4. the 'Brandenburg-Pomeranian route' from the northwest: from the North German Plain across the Toruń-Eberswalde spillway;

5. from the northeast along the lakeland moraine belt.

Some of these studies have explored historical aspects of the development of grassland steppe communities in Poland. Phytosociological works may be of special value, as they can identify the dynamics of change in communities. This can help determine the actual role of a variety of factors, for example, factors related to succession processes, or human activities that could affect the formation of communities of steppe species outside the steppe zone.

The advent of molecular research techniques has spurred renewed interest in historical biogeography. They can be used to verify hypotheses unverifiable by traditional forms of data analysis. Molecular phylogeography reconstructs the historical processes and the dynamics of these processes that shaped contemporary species ranges and the spatial distribution of individual genetic lineages (cf. Taberlet *et al*. 1998; Avise 2000a, b). Analyses of the genetic variation and/or diversity of species (especially within species) as a function of their geographical distribution are used to establish the genetic correlations between populations in time and space.

Molecular techniques have not been used extensively to examine the history of the xerothermic flora of Central Europe. The few phylogeographical studies done on, for example, *Stipa capillata* (Hensen *et al*. 2009; Wagner *et al*. 2011) and *Iris aphylla* (Wróblewska & Brzosko 2006; Wróblewska 2008; Wróblewska *et al*. 2010) present the genetic structure of single species. Broader studies are needed to determine the range development of steppe species outside their climax zone. The species of the Pontic-Pannonian subelement are a separate group, differing in habitat preference, climatic requirements and geographical range from species characteristic of cold steppe (associated with loess areas of southern Poland), so their migration histories need to be differentiated. The historical relationships between different parts of the range can be elucidated by genetic structure analyses that treat the cold steppe and Pontic-Pannonian species separately. Such studies will refresh the debate about the range development history of these elements in Central Europe. In particular, they can help us understand the historical role of regions east and south of Poland in the migration of species to southern Poland, where the greatest number of localities of species of the Pontic-Pannonian subelement are recorded at present. Those regions are thought to have been refuge areas of this group.

Populations of steppe species have been steadily decreasing in abundance in Poland and across Europe. The urgent need to protect their localities makes genetic studies especially important. To plan effective protection of this important and interesting element of Central Europe's plant cover we need information from studies of the genetic structure and variation of selected model taxa, as the genetic resources of a species are critical to its ecological plasticity and evolutionary potential (Haig 1998; Moritz & Faith 1998).

AIMS AND OBJECTIVES

This phylogeographical study examined five model species representing the Pontic-Pannonian subelement of the Polish flora: *Carlina onopordifolia* Besser *ex* Szafer, *Cirsium pannonicum* (L. fil.) Link, *Inula ensifolia* L., *Linum flavum* L. and *Linum hirsutum* L.

The main aim of the study was to verify hypotheses regarding how and when these species of the Pontic-Pannonian element migrated to Poland and more broadly to Central Europe. Their genetic variation and structure were analyzed in order to establish the genetic relationships between populations of these species. The most important tasks were these:

1. to determine the range of intra- and interpopulation genetic variation and to establish the relationships between individual island-like fragments of steppe species localities in Central Europe;

2. to estimate the similarity of the patterns of genetic structure and genetic variation of five species having similar ranges and geographical distributions;

3. to determine whether this group of Pontic-Pannonian species shows similar histories of range development in the northern part of Central Europe;

4. to suggest factors possibly affecting the range of Pontic-Pannonian species in the northern part of Central Europe;

5. to verify earlier hypotheses on the direction, routes and time of migrations of steppe species to southern Poland.

MATERIAL

THE STUDY SPECIES

The species investigated in this work represent the Pontic-Pannonian geographical subelement and have a homogenous distribution pattern in Poland, where they reach the northern or northwestern limit of their continuous range (Zając & Zając 2009). They are recorded only in the southeastern part of the country, on the Wyżyna Małopolska upland (342), Wyżyna Lubelska upland (343.1), Volhynian Polissya (Polesie Wołyńskie, 845.3) and Wyżyna Wołyńska upland (851.1) (division and numbering of regions according to Kondracki 2002), where they form a group of species characteristic of the association *Inuletum ensifoliae* Kozł. (alliance *Cirsio-Brachypodion pinnati*, Matuszkiewicz 2005).

Species nomenclature follows Mirek *et al*. (2002). For aggregate species only taxa occurring in Poland were included in the analyses. The geographical ranges of species presented below are given after Meusel *et al*. (1978) and Meusel and Jäger (1992).

Carlina onopordifolia Besser *ex* Szafer (Asteraceae)

Almost stemless perennial with ground leaf rosette, reaching *ca* 15 cm in height. Flowering individuals have a shortened stem ending in a single flower head. Flowers in anthodium uniform, hermaphrodite, tubular and yellowish. Fruits are pubescent achenes with pappus 20–25 mm long. Plants reproduce by seeds, semelparous (individuals die after flowering) (Jasiewicz 1972). A hemicryptophyte. Chromosome number $2n = 20$ (Czapik 1959). *Carlina onopordifolia* does not exhibit taxonomic diversity. The species range comprises isolated localities in the Central European and Pontic provinces (Jasiewicz 1972; Zaveruha 1981; Meusel & Jäger 1992) (Figs 1 & 2).

Cirsium pannonicum (L. fil.) Link (Asteraceae)

Perennial. Stem straight, erect and unbranched, reaching *ca* 120 cm, sometimes ending in several elongated small 1-anthodium branches. Anthodia covered with imbricate squamulae. Flowers in anthodia tubular, purple. Leaves oblong, lanceolate, undivided, with shallowly denticulate margins. Fruits are achenes, with pinnate branched bristles of pappus. Reproduces by seeds (Sychowa 1971). Chromosome number $2n = 34$ (Czapik 1974). The continuous range of the species covers the Central European – sub-Mediterranean – (NE) Balkan – Pontic – Pannonian region and isolated localities in the

Fig. 1. *Carlina onopordifolia* Besser *ex* Szafer in Wały Reserve (Wyżyna Małopolska upland, Poland). Photo E. & J. Cieślak.

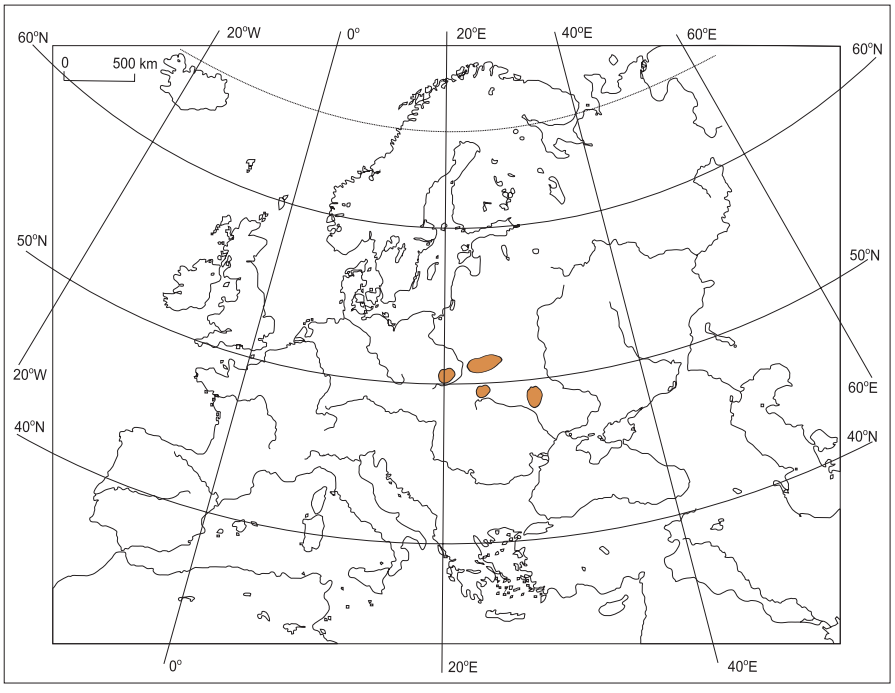

Fig. 2. Range of *Carlina onopordifolia* Besser *ex* Szafer (after Meusel & Jäger 1992).

Fig. 3. *Cirsium pannonicum* (L. fil.) Link in Zolochyiv (Podolian Upland, Ukraine). Photo E. & J. Cieślak.

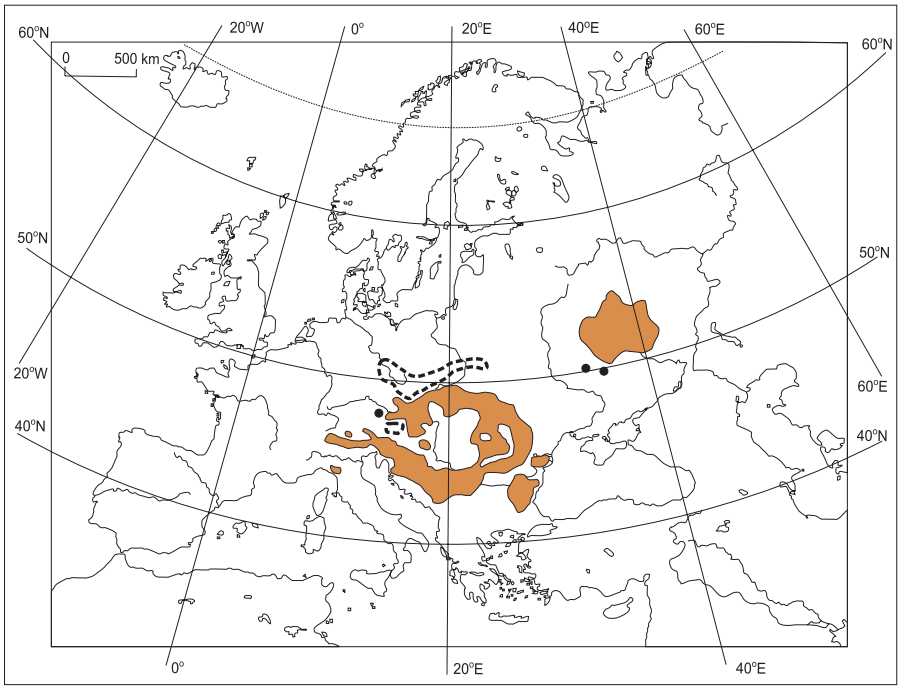

Fig. 4. Range of *Cirsium pannonicum* (L. fil.) Link (after Meusel & Jäger 1992).

central part of the East European Plain (Werner 1976; Meusel & Jäger 1992) (Figs 3 & 4).

Inula ensifolia L. (Asteraceae)

Perennial. Stem erect or ascending, usually unbranched and ending with one anthodium, reaching 30 cm. Stem leaves linear or linear lanceolate, arranged alternately. Flowers uvular, ligulate in center, golden yellow. Fruits are achenes with a pappus. It is a hemicryptophyte and usually reproduces by seeds (Zarzycki 1971). Chromosome number 2n = 16 (Bauer 1959). Lower-rank taxa are not distinguished within the species. Its range comprises the (NE) sub-Mediterranean – Central European – Pontic – Pannonian – (N) Balkan region and isolated localities in Gotland (Tutin 1976; Meusel & Jäger 1992) (Figs 5 & 6).

Linum flavum L. (Linaceae)

Perennial. Stem erect, mostly unbranched apart from inflorescence, reaching *ca* 40 cm. Stem leaves spathulate, arranged alternately. Large flowers at top of stem, with short pedicel, collected in cymose inflorescences, petals golden yellow. Fruits are capsules. Hemicryptophyte, reproduces by seeds (Pawłowska 1959; Kaźmierczakowa 1991). Chromosome number 2n = 30 (Izmaiłow 1988). *L. flavum* is characterized by high morphological variability. Lower-rank taxa within the species are distinguished especially in the Pontic (Black Sea) part of its range. Its general range is continuous and comprises the (E) sub-Mediterranean – (NE) Balkan – Pontic – Pannonian – East European region (Ockendon & Walters 1968; Meusel *et al*. 1978) (Figs 7 & 8).

Linum hirsutum L. (Linaceae)

Perennial. Stem single, erect, branched at top, reaching *ca* 70 cm. Upper stem leaves lanceolate, lower oblong, arranged alternately, sessile, without stipules. Flowers large, with short pedicel, collected in cymose inflorescences, mostly 2 or more dichasia, petals lavender-blue. Fruits are spherical capsules. Hemicryptophyte, reproduces by seeds. (Pawłowska 1959). Chromosome number 2n = 16 (Izmaiłow 1987). Intraspecific morphological diversity in *L. hirsutum* is low. Lower-rank taxa are reported only from the Pannonian range. The species range comprises the (NE) sub-Mediterranean – Balkan – Pontic-Pannonian – (W) Turanian region (Ockendon & Walters 1968; Meusel *et al*. 1978) (Figs 9 & 10).

COLLECTION OF MATERIAL

Material was collected in Central and Eastern Europe during three vegetative seasons from June to September in 2008–2010, from populations in the following geographical regions: the Wyżyna Małopolska upland (MU); the Wyżyna Lubelska upland including Volhynian Polissya and the western part of the Volhynian Upland (Poland) (LU); the Podolian Upland (Podil's'ka vysochyna upland, Ukraine) (PU); the southern part of the Panonnian area (Hungary, Romania) (PS); the northwestern part of the Panonnian area (Czech Republic, Slovakia, Austria) (PW); the Balkans (Bulgaria) (BP) and the coast of the northern Adriatic (Italy, Slovenia) (PA) (Fig. 11). Samples of each species were taken from the following geographical regions:

Carlina onopordifolia – Wyżyna Małopolska upland, Wyżyna Lubelska upland together with Volhynian Polissya and the Podolian Upland (populations DAB and RAC from the Wyżyna Małopolska upland are introduced populations; Kaźmierczakowa 2003) (Fig. 12, Table 1);

Cirsium pannonicum – Wyżyna Małopolska upland, Wyżyna Lubelska upland, Podolian Upland, northwestern and southern parts of Pannonian area (see Fig. 15, Table 5);

Inula ensifolia – Wyżyna Małopolska upland, Wyżyna Lubelska upland, Podolian Upland, northwestern and southern parts of Pannonian area, Balkans and coast of northern Adriatic region (see Fig. 18, Table 9);

Linum flavum – Wyżyna Małopolska upland, Wyżyna Lubelska upland, Podolian Upland, northwestern and southern Pannonian area (see Fig. 21, Table 13);

Linum hirsutum – Wyżyna Małopolska upland, Podolian Upland, northwestern and southern Pannonian area (see Fig. 24, Table 17).

Plants were sampled randomly from each population. A single sample consisted of a fragment of a well-developed stem leaf. Each fragment was placed in a plastic container with silica gel upon collection. The samples in silica gel were stored at room temperature until DNA isolation. The same collection practices were applied across populations to allow comparisons of genetic structure.

Vouchers were collected from the majority of the sampled populations from a given area for each species and are deposited in the herbarium of the Institute of Botany, Polish Academy of Sciences (KRAM). The number of populations and specimens sampled are as follows: *Carlina onopordifolia* – 16 populations, 298 specimens; *Cirsium pannonicum* – 17 populations, 247 specimens; *Inula ensifolia* – 30 populations, 417 specimens; *Linum flavum* – 21 populations, 288 specimens; *Linum hirsutum* – 14 populations, 184 specimens. A total 1434 specimens of all species were analyzed.

Fig. 5. *Inula ensifolia* L. in Biała Góra Reserve (Wyżyna Małopolska upland, Poland). Photo B. Binkiewicz.

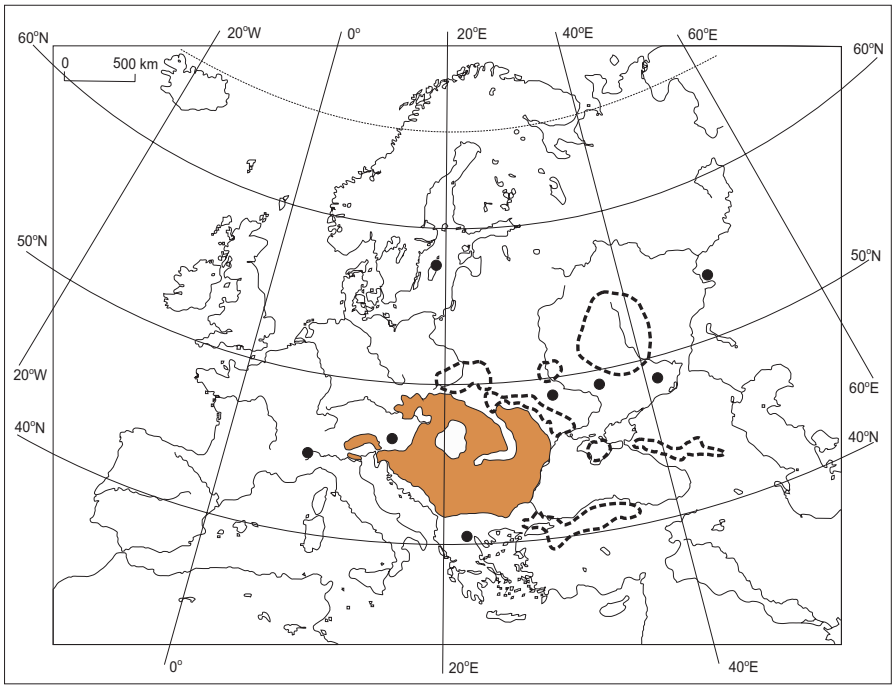

Fig. 6. Range of *Inula ensifolia* L. (after Meusel & Jäger 1992).

Fig. 7. *Linum flavum* L. in Biała Góra Reserve (Wyżyna Małopolska upland, Poland). Photo Z. Szeląg.

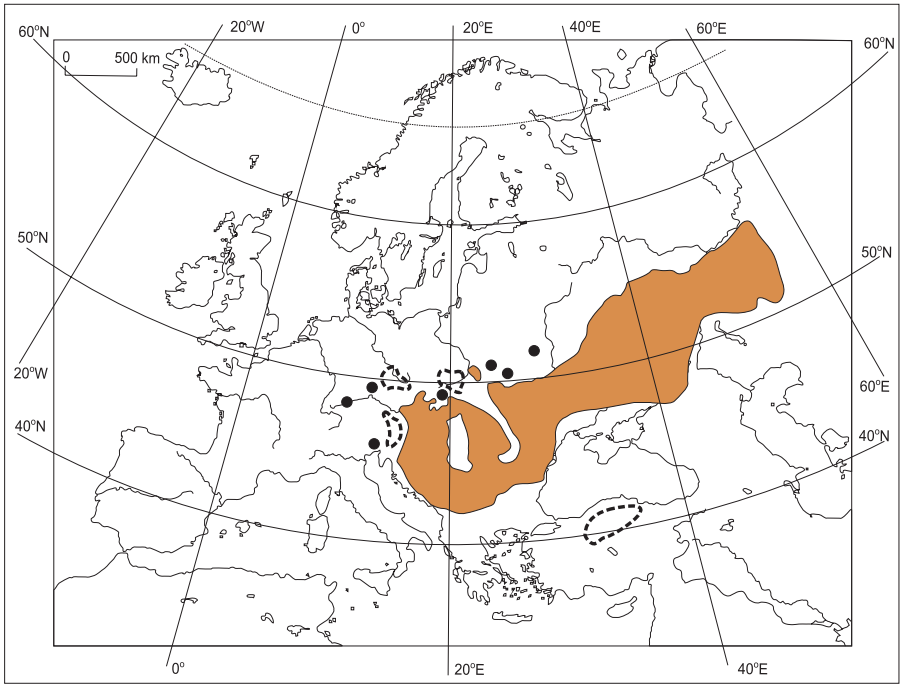

Fig. 8. Range of *Linum flavum* L. (after Meusel *et al*. 1978).

Fig. 9. *Linum hirsutum* L. in Wały Reserve (Wyżyna Małopolska upland, Poland). Photo Z. Szeląg.

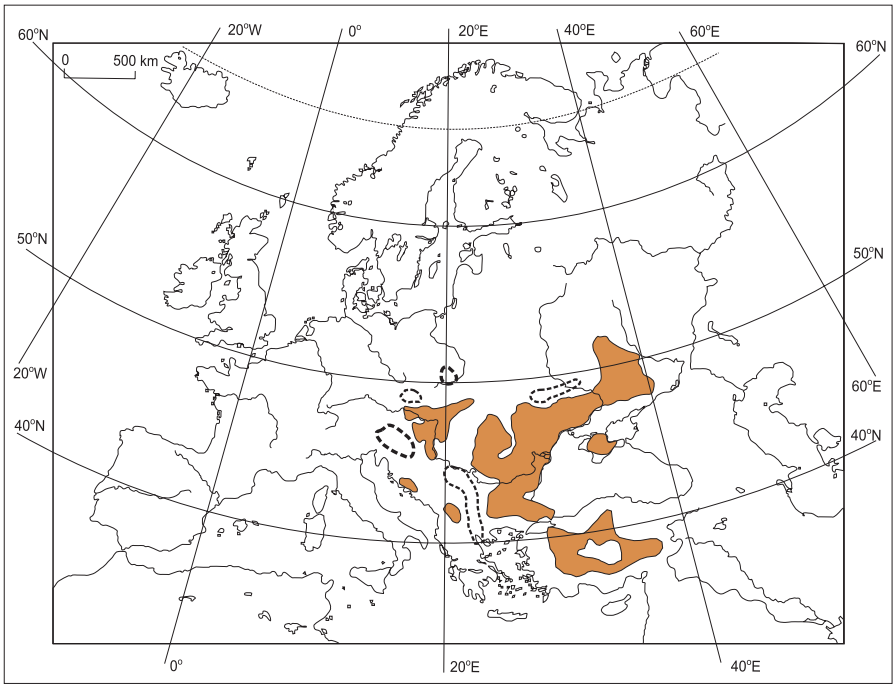

Fig. 10. Range of *Linum hirsutum* L. (after Meusel *et al*. 1978).

Fig. 11. Study area. Note: SE Polish Uplands (brown area) include the Wyżyna Małopolska and Wyżyna Lubelska uplands (for details see Material).

Methods

Molecular analysis

Amplified fragment length polymorphism (AFLP) was analyzed to determine genetic variation. The total genetic variation in populations can be estimated accurately by this method; it usually provides a number of polymorphic markers of great value for biogeographical and population analyses. As these markers are almost exclusively neutral (Weising *et al.* 2005), it can be assumed that inferences about the historical accumulation of genetic variation in individual lines are not significantly disturbed by adaptational variation. At present AFLP is the best method for analyzing total genomic variation (genomic fingerprinting), providing high resolution and having the advantage that the analyzed DNA regions do not have to be known a priori. AFLP markers have been used successfully to analyze the genetic variation of high-mountain species in European mountains (e.g., Schönswetter *et al.* 2005; Ronikier *et al.* 2008; Ronikier 2011 and references therein).

DNA extraction. Total DNA was extracted from approximately 10–15 mg of dried leaf tissue using the DNeasy Plant Mini Kit system (Qiagen), according to the protocol recommended by the manufacturer. The quality of genomic DNA was checked on 1% agarose gels.

AFLP procedure. In the first step, method reproducibility and primer sets for each species were tested on two samples from five populations of each species. The tests used duplicates of each individual and 14 primer pairs. Sets with reproducibility over 95% and even and distinct bands were used for further analyses.

AFLP analysis generally followed the procedure described by Vos *et al*. (1995) as modified by Ronikier *et al*. (2008). Genomic DNA was digested with *Eco*RI and *Mse*I restriction enzymes (New England Biolabs Inc.). Then double-strand adapters were ligated to *Eco*RI and *Mse*I specific ends by T4 DNA Ligase (Roche Diag-

nostics). Products of digestion/ligation were checked by electrophoresis on 1.5% agarose gels and subsequently diluted 1:10 with sterile deionized water. Preselective amplification was performed using primers with single selective nucleotides: *Eco*RI+A and *Mse*I+C. The products were diluted 1:20 with sterile deionized water. Selective amplifications were performed using *Eco*RI and *Mse*I primers with three selective nucleotides:

Carlina onopordifolia: *Eco*RI-AAG/*Mse*I-CTG, *Eco*RI-AGC/*Mse*I-CTA, *Eco*RI-AGT/*Mse*I-CAC

Cirsium pannonicum: *Eco*RI-ACA/*Mse*I-CGT, *Eco*RI-AGC/*Mse*I-CTA, *Eco*RI-ATC/*Mse*I-CAG

Inula ensifolia: *Eco*RI-AAG/*Mse*I-CGC, *Eco*RI-AGT/*Mse*I-CAC, *Eco*RI-ATG/*Mse*I-CGC

Linum flavum: *Eco*RI-AAG/*Mse*I-CGC, *Eco*RI-AGT/*Mse*I-CGT, *Eco*RI-ATG/*Mse*I-CAC

Linum hirsutum: *Eco*RI-ACA/*Mse*I-CGT, *Eco*RI-AGC/*Mse*I-CAT, *Eco*RI-ATG/*Mse*I-CAA.

The *Eco*RI primers were 5'-fluorescence-labelled (6-FAM). The fluorescence-labelled selective amplification products were diluted 1:20 with sterile deionized water and separated on POP-7 polymer with GeneScan-500 (ROX) internal size standard on an ABI Prism 3130 automated sequencer (Applied Biosystems).

Genotyping reproducibility was tested (Bonin *et al*. 2004) by including intra- and interplate duplicates of *ca* 5% samples of each species (analyzed independently of the first AFLP step). Reproducibility tests were conducted during the primer tests and during analyses of the entire material.

DATA ANALYSIS

The AFLP profiles were analyzed with Genographer v1.6.0 (J. Benham, Montana State University, 1998–2001, http://hordeum.oscs.montana.edu/genographer), which was applied to read fragments in the 50–500 bp range and to record the results as a binary 0/1 matrix.

Statistical analyses employed band-based and allele frequency-based methods (Bonin *et al.* 2007). Genetic variation was analyzed on the levels of population, groups of populations within geographical areas, and all sampled populations per species. Separate analyses were also performed for two geographical groups within Poland (Wyżyna Małopolska upland vs Wyżyna Lubelska upland).

The number of polymorphic bands as a percentage of all bands ($\%_{poly}$) was calculated. Private bands (M_p) (present in at least 75% of individuals in a population) and discriminating bands (M_d) (occurring in all individuals in a population and totally absent in other samples; Cieślak *et al.* 2007) were identified. The same principle was followed for the parameters calculated for geographical regions (M_{pR} – present in at least 75% of individuals in a region; M_{dR} – occurring in all individuals in a region and totally absent in other regions).

The level of genetic variation was determined based on Nei's gene diversity index (M_H) (Nei 1978) and the Shannon index (M_S) (Lewontin 1972) calculated using POPGENE v1.32 (Yeh *et al.* 1997). As a measure of divergence and to identify long-term isolation, frequency-down-weighted marker values (M_{DW}) (Schönswetter & Tribsch 2005) were calculated for all AFLP data sets using R-script, AFLPdat (Ehrich 2006). Index values were not calculated for populations of five or fewer individuals. The significance of differences in genetic diversity parameters between groups of populations was assessed with the Mann-Whitney U-test in STATISTICA v5.0.

To test for isolation by distance among populations, independence between pairwise F_{ST} (Wright 1978; Excoffier *et al*. 1992) and geographical distances was examined using the Mantel test (Mantel 1967) with 10,000 permutations in Mantel Nonparametric Test Calculator v2.0 (Liedloff 1999). The relationships between individuals for each species were analyzed based on a Nei and Li (1979) distance matrix tested by principal coordinate analysis (PCoA) and neighbor-joining (NJ) (Saitou & Nei 1987) with a bootstrap procedure (10,000 replicates) to estimate support for the branches. PCoA and NJ analyses were computed with FAMD v1.108 beta (Schlüter and Harris 2006).

Historical gene flow was estimated roughly at $M_{Nm} = 0.25(1-F_{ST})/F_{ST}$ (Slatkin & Barton 1989) using POPGENE v1.21 (Yeh *et al.* 1997); F index-F_{ST} was calculated according to Wright (1978).

Analysis of molecular variance (AMOVA) was based on groups defined a priori in a hierarchical system (population and geographical region). It was based on pairwise square Euclidean distance among molecular phenotypes. Significance levels were determined by 1023 permutations. AMOVA and F index (depending on the level of analysis: F_{ST}, F_{SC} and F_{CT}) values were calculated using ARLEQUIN v3.5 (Excoffier & Lischer 2010).

Analyses based on a Bayesian algorithm with individuals as basic analytical units were performed using STRUCTURE v2.2 (Pritchard *et al.* 2000; Falush *et al.* 2007) and TESS v2.3 (Chen *et al.* 2007).

STRUCTURE implements a Bayesian analysis to assign individuals to a predefined number of clusters on the basis of probabilistic analysis of the multilocus genotypes. AFLP data sets were converted with the AFLPdat script (Ehrich 2006) employing the option

for dominant markers (Falush *et al.* 2007). The analyses used an admixture model, 10^6 iterations and a burn-in of 2×10^5 iterations. The admixture model using correlated allele frequencies was the preferred model as it most accurately assigns individuals to closely related groups (Falush *et al.* 2003), but it tends to overestimate the number of groups (K), so I also made analyses using the admixture model with uncorrelated allele frequencies (Pritchard *et al.* 2000). Ten replicates were analyzed for each K from $K=1$ to $K=10$. The numbers of groups for each species were chosen after the STRUCTURE and output files were analyzed in R software (R Development Core Team, 2004) using the Structure.sum script (D. Ehrich; available from http://www.nhm.uio.no/ncb). This script visualizes the estimated likelihood of each run, the similarity coefficient between runs (Rosenberg *et al.* 2002), and Evanno's ΔK (Evanno *et al.* 2005). In the first place the ΔK measure was used as proposed by Evanno *et al.* (2005). The most appropriate number of clusters was determined from lnP(D) values and estimates of posterior probabilities provided in STRUCTURE outputs, examined as a function of increasing K. STRUCTURE 2.2 analysis was done on the Bioportal at the University of Oslo (http://www.bioportal.uio.no).

TESS (Chen *et al.* 2007) implements a Bayesian clustering algorithm for spatial population genetic analyses. It can do both individual geographical assignment and admixture analysis. It is designed to seek genetic discontinuities in continuous populations and estimate spatially varying individual admixture proportions. Given individual geographical locations, the program builds a network structure which describes the geographical relationships between individuals. TESS delivers genetic displays of geographical cluster assignments or admixture proportions (depending on the model used) and textual output of the admixture Q matrix (Chen *et al.* 2007; Durand *et al.* 2009; François *et al.* 2006).

For each species the analyses were done for all populations collected in the study area. Calculations were based on the admixture model (Durand *et al.* 2009) separately for two different values of the influence parameter ι ($\iota=0.60$ and $\iota=0.99$) and for the parameter value freely adjusted by the program. Parameter ι values were lower than 0.2 in the latter case, regardless of the species and number of clusters. No significant effect of ι on the results of individual analyses was observed (data not shown). As the parameter describes the weight attached to geographical coordinates, its relatively low value computed by the program demonstrates that the clusters calculated in the analyses are based mostly on the genetic distances of the populations and less based on geographical distances.

The number of clusters K is always smaller or equals the maximum value assigned by the operator (usually K_{MAX} stands for the total number of populations). A low K value is used in the calculations initially, and then increased while checking the value of DIC (deviance information criterion), which is a measure of the quality of the model's description of diversity. The lower the DIC, the better the problem was modelled. The DIC values will be lowest for the greatest possible K; however, analysis of the rate at which DIC changes with the increase of K sometimes shows when a further increase in the number of clusters only slightly improves the adjustment value. That K value is often accepted as 'K-effective', or K_{ef}. The K_{ef} values were high in the analyses described below, due to continuous adjustment of the quality of the fit as the number of clusters increased. The results obtained using such high K_{ef} values are characterized by a high degree of model adjustment to the data. In most cases, however, the accuracy and the level of detail of the results are not justified by the accuracy of the data acquisition method (AFLP) and are an artificial, often numerical, effect. In these cases the choice of K_{ef} must be made arbitrarily based on the results and the operator's experience. In practice, the reliability of conclusions to be formulated based on them has been used as the criterion to select K_{ef}. This is reflected in the presentation for different K values, including those smaller than K_{ef}. In all cases the choice of K greater than K_{ef} leads to a cluster structure contrary to the geographical distribution of natural migration barriers (mountains, seas) and very low probability that individuals belong only to one cluster, causing the divisions within a group of individuals to disappear.

Calculations were performed for 7000 cycles and were repeated 60 times for each K. The six cycles with the lowest DIC were selected for further analyses. CLUMPP was used to analyze the assignment of individuals to clusters based on the six cycles selected (Jakobsson & Rosenberg 2007). Spatial interpolation of the probabilities of assignment to clusters for each point of the geographical area was performed. The krigging procedure implemented in R software was used.

Since the division into groups (based on calculations for all collected material and for Polish populations only) for each discussed species by STRUCTURE and by TESS was similar (also for ΔK in STRUCTURE corresponding to K_{ef} in TESS), only TESS results (including the final divisions for all populations) are presented in the figures.

Results

Below are the results of analyses of genetic variation at several levels (population, geographical area, species), described separately for each taxon.

Carlina onopordifolia

A total of 298 *Carlina onopordifolia* individuals were analyzed (Table 1). Altogether 142 AFLP markers were recorded, of which 13 (9.15%) were polymorphic. The parameter values describing genetic diversity (Nei's gene diversity index, Shannon index) were very low for *C. onopordifolia* in the study area: $M_H = 0.02$ (SD = 0.09) and $M_S = 0.03$ (SD = 0.13). The coefficient of gene flow M_{Nm} was 0.03. The Mantel test showed a statistically significant correlation between genetic and geographical distance ($r = 0.41$, for $p = 0.05$, $Z = 6423.61$ and $g = 3.59$).

Very low within-population genetic variation was recorded in the polymorphism analysis. The number of polymorphic bands in populations of *C. onopordifolia* ranged from zero (BOG, DAB, PAS, RAC, ZMU, Poland; UGL, Ukraine) to two (PIN, STW, Poland; ULG, Ukraine). Single polymorphic bands were detected in populations RZE, SWR, WAL, ROG, ZUR (Poland), URA and UTR (Ukraine). There were also discriminating bands in these populations. Other private bands were not recorded in any of the populations (Table 2).

The minimum value of Nei's gene diversity index and the Shannon index was 0 (in populations displaying no polymorphic bands). The maximum value of Nei's gene diversity index was $M_H = 0.0061$ (ULG, Ukraine); the mean for all populations was $M_H = 0.12$ (SD = 0.02). The maximum value of the Shannon index was $M_S = 0.0088$ (ULG, Ukraine); the mean for all populations was $M_S = 0.17$ (SD = 0.03). Coefficient M_{DW} of the frequency of rare markers in populations ranged from 2.85 (ROG, Poland) to 30.28 (WAL, Poland); the mean for all populations was 8.75 (SD = 7.15) (Table 2).

Three discriminating bands were recorded in the polymorphism analysis of population groups (see Materials), including one for the populations from the Wyżyna Małopolska upland and one for those from the Podolian Upland. A further discriminating band was detected for the population group from the western part of the Podolian Upland. Private bands were not found for any group. There were no significant differences in the values of individual parameters between geographical groups. None of the parameters differed significantly between the population groups from Poland (Wyżyna Małopolska and Wyżyna Lubelska uplands) (Table 3).

Two genetic groups are distinguished in the NJ dendrogram. One is formed by populations from the Wyżyna Małopolska upland and the other by populations from the Wyżyna Lubelska upland and Podolian Upland. The first group does not show internal variation. The second shows population subgroups corresponding to the Wyżyna Lubelska upland and eastern Podolian Upland on the one hand and the western Podolian Upland on the other.

PCoA results (Fig. 13A) also show a low degree of within-population variation of *C. onopordifolia*. Axes 1 and 2 account for 87.72% and 4.65% of the variation, respectively. Along the first axis there is a clear division into two groups. The first group is limited to populations from the Wyżyna Małopolska upland (left side of graph), and the second to populations from the Wyżyna Lubelska upland and Podolian Upland. Within the second group the populations from the Podolian Upland are separated from those in the Wyżyna Lubelska upland along the second axis. Along the third axis (explaining 4.29% of the variation) the western Podolian Upland group is separated from the eastern Podolian Upland group and from the Wyżyna Lubelska upland. The main points of this analysis are that the Wyżyna Małopolska upland populations are genetically differentiated from the others studied, and that the Wyżyna Lubelska upland populations show greater genetic similarity to those from the eastern Podolian Upland than to those from the neighboring western part of it.

In separate PCoA of the Polish populations of *Carlina onopordifolia* (Fig. 13B) (Axes 1 and 2, accounting for 13.52% and 8.08% of the variation,

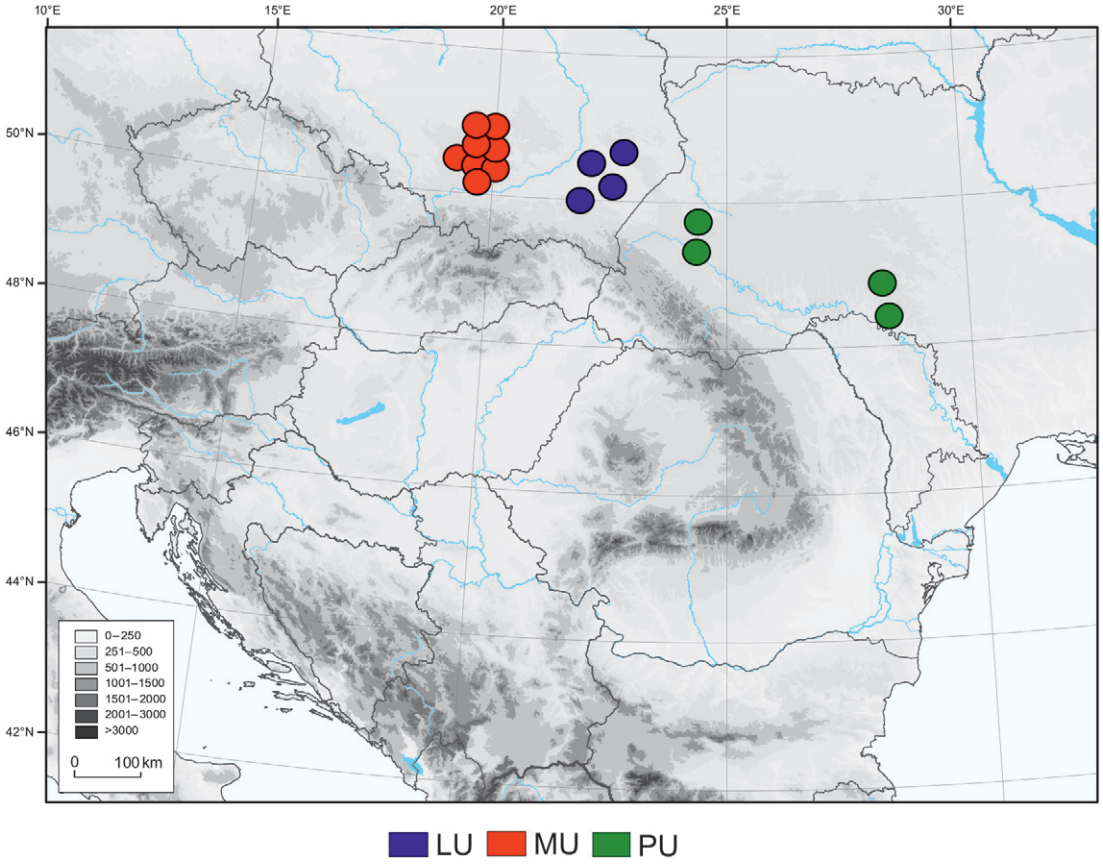

Fig. 12. Localities of the populations of *Carlina onopordifolia* Besser *ex* Szafer. Localities are divided into geographical groups, details in Table 1. LU – Wyżyna Lubelska upland; MU – Wyżyna Małopolska upland; PU – Podolian Upland.

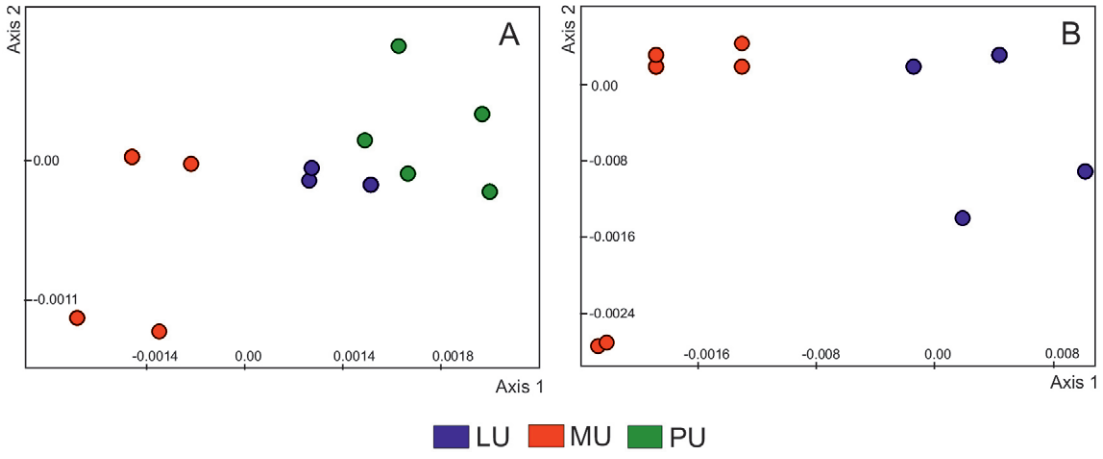

Fig. 13. PCoA scatterplot of *Carlina onopordifolia* Besser *ex* Szafer individuals based on Nei and Li genetic distances of AFLP data. A – all studied populations, B – Polish populations. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PU – Podolian Upland.

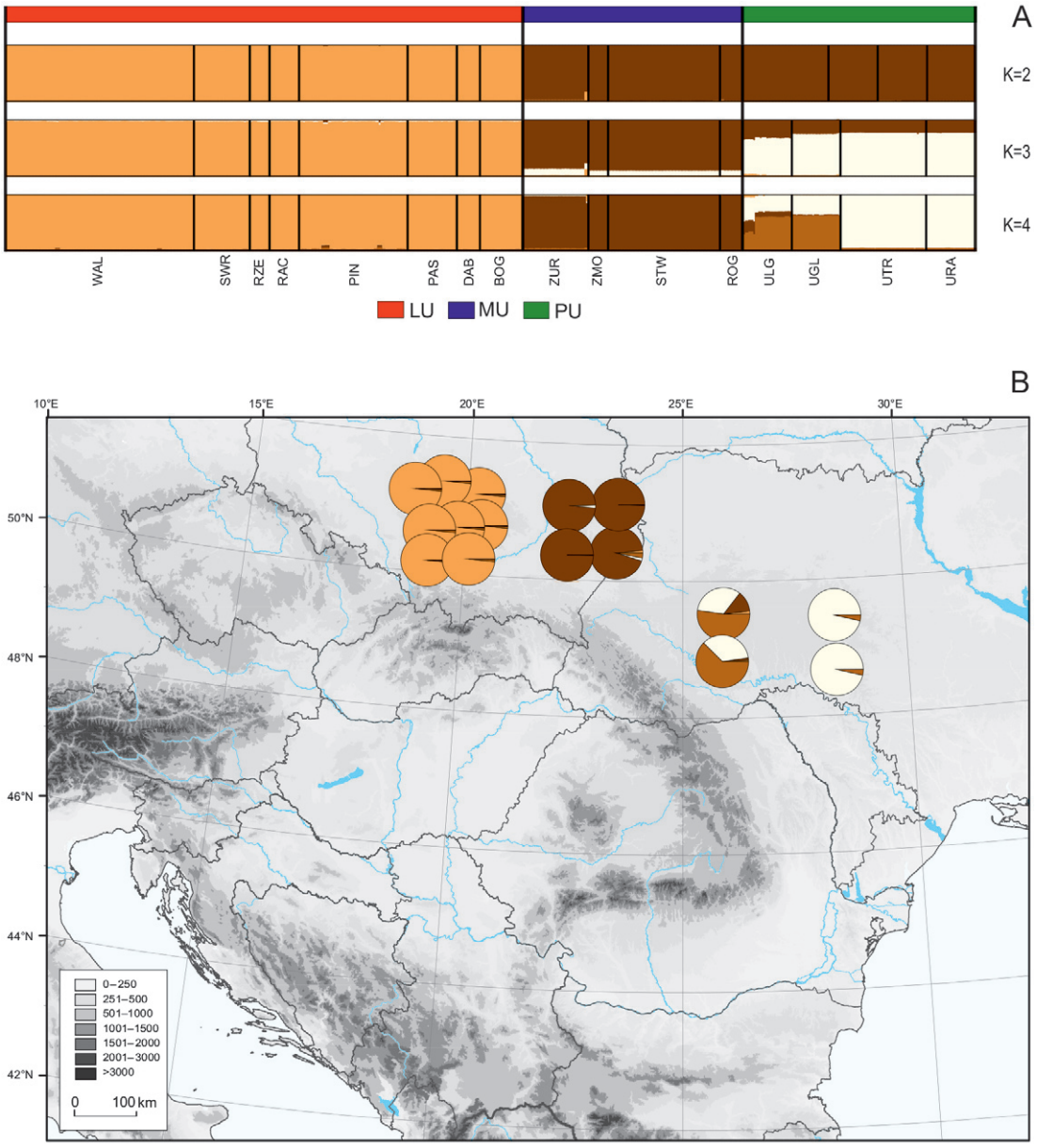

Fig. 14. Results of Bayesian analysis (TESS software) for *Carlina onopordifolia* Besser *ex* Szafer. A – bar graphs of individuals for K = 2, K = 3, K = 4; populations are separated by vertical lines. B – pie charts showing the proportions of the clusters present within the populations, detected by the Bayesian analysis for K = 4. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PU – Podolian Upland. For population codes see Table 1.

Table 1. Origin of the plant material of *Carlina onopordifolia* Besser *ex* Szafer, used in the present study. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PU – Podolian Upland (for details see Material). Material collected by the author if not marked otherwise.

Population code	Geographical group	Locality	Latitude (N)/ Longitude (E)
URA	PU	Ukraine, Podolian Upland, Romashkovje	48°15′/29°18′
UTR	PU	Ukraine, Podolian Upland, S of Chechel'nik	48°12′/29°20′
ULG	PU	Ukraine, Podolian Upland, W of Zolochyiv	49°48′/24°43′
UGL	PU	Ukraine, Podolian Upland, WSW of Berezhani	49°24′/24°49′
ROG	LU	Poland, Wyżyna Lubelska upland, Grabowiec, Rogów Reserve	50°48′/23°31′
ZUR	LU	Poland, Wyżyna Wołyńska upland, Machnowska Góra Reserve	51°22′/23°34′
ZMU	LU	Poland, Polesie Wołyńskie lowland, Żmudź	51°00′/23°40′
STW	LU	Poland, Polesie Wołyńskie lowland, NWof Chełm, Stawska Góra hill	51°12′/23°24′
RZE	MU	Poland, Wyżyna Małopolska upland, Rzeżuśnia	50°20′/19°59′
PIN	MU	Poland, Wyżyna Małopolska upland, Pińczów	50°32′/20°31′
WAL	MU	Poland, Wyżyna Małopolska upland, Wały Reserve	50°20′/20°13′
DAB	MU	Poland, Wyżyna Małopolska upland, Dąbie-Klonów Reserve	50°20′/20°10′
SWR	MU	Poland, Wyżyna Małopolska upland, Skowronno Reserve	50°32′/20°29′
PAS	MU	Poland, Wyżyna Małopolska upland, Pasturka	50°30′/20°33′
BOG	MU	Poland, Wyżyna Małopolska upland, Bogucice	50°30′/20°34′
RAC	MU	Poland, Wyżyna Małopolska upland, Racławice	50°19′/20°14′

Table 2. Parameters of AFLP genetic diversity for populations of *Carlina onopordifolia* Besser *ex* Szafer. N – number of individuals in population, $\%_{poly}$ – percentage of polymorphic bands, $M_{d/p}$ – discriminating/private bands in population, M_H – mean (±SD) Nei's genetic diversity, M_S – mean (±SD) Shannon genetic index, M_{DW} – frequency down-weighted marker values. For population codes see Table 1.

Population code	N	$\%_{poly}$	$M_{d/p}$	M_H (±SD)	M_S (±SD)	M_{DW}
URA	15	0.71	1/0	0.0009 (±0.01)	0.0018 (±0.02)	6.56
UTR	26	0.71	1/0	0.0013 (±0.02)	0.0023 (±0.03)	11.82
ULG	15	1.43	2/0	0.0061 (±0.05)	0.0088 (±0.07)	7.31
UGL	15	0	0/0	0	0	–
ROG	7	0.71	1/0	0.0010 (±0.01)	0.0019 (±0.02)	2.85
ZUR	20	0.71	1/0	0.0004 (±0.01)	0.0008 (±0.01)	8.52
ZMU	6	0	0/0	0	0	–
STW	34	1.43	2/0	0.0008 (±0.01)	0.0019 (±0.02)	15,38
RZE	9	0.71	1/0	0.0035 (±0.04)	0.0048 (±0.05)	3.05
PIN	33	1.43	2/0	0.0050 (±0.04)	0.0076 (±0.06)	16.77
WAL	57	0.71	1/0	0.0016 (±0.02)	0.0028 (±0.03)	30.28
DAB	7	0	0/0	0	0	–
SWR	17	0.71	1/0	0.0004 (±0.01)	0.0010 (±0.01)	7.69
PAS	15	0	0/0	0	0	–
BOG	13	0	0/0	0	0	–
RAC	9	0	0/0	0	0	–

Table 3. Parameters of AFLP genetic diversity for geographical groups of *Carlina onopordifolia* Besser *ex* Szafer. N – number of individuals in group, $\%_{poly}$ – percentage of polymorphic bands, $M_{dR/pR}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_{Nm} – value of gene flow.

Geographical group	N	$\%_{poly}$	$M_{dR/pR}$	M_H (±SD)	M_S (±SD)	M_{Nm}
Podolian Upland (PU)	71	1.43	1/0	0.001 (±0.04)	0.007 (±0.056)	1.34
Wyżna Lubelska upland (LU)	67	2.14	0/0	0.001 (±0.05)	0.002 (±0.012)	15.64
Wyżna Małopolska upland (MU)	157	1.43	1/0	0.02 (±0.19)	0.004 (±0.035)	0.95

respectively) the individuals cluster according to their geographical location, indicating significant genetic differentiation of the populations from the two regional distribution areas: the Wyżyna Małopolska and Wyżyna Lubelska uplands.

Analysis of molecular variance (AMOVA) of *C. onopordifolia* at the level of populations shows that interpopulation variation makes a much greater contribution than intrapopulation variation: 93.10% and 6.90%, respectively ($F_{ST} = 0.93$, $p < 0.001$). Variation between geographical units dominates over interpopulation variation in the range of these units as well as over intrapopulation variation in them (obtained from hierarchical AMOVA; see Table 4 for details).

In Bayesian analysis in TESS (Fig. 14) for *C. onopordifolia* the population group from the Wyżyna Małopolska upland also was genetically differentiated from the other populations (Wyżyna Lubelska upland and Podolian Upland) in the total data set for $K = 2$. For $K = 3$ the populations from the Wyżyna Małopolska upland also form a genetically homogeneous group separate from the Wyżyna Lubelska upland and Podolian Upland. Two genetic pools aggregate in the latter group. The first consists mainly of populations from the Wyżyna Lubelska upland and the second consists of populations from the Podolian Upland. For $K = 4$ an additional division is observed within the Podolian Upland populations (western vs southeastern parts

Table 4. Analyses of molecular variance (AMOVA) based on AFLP markers for *Carlina onopordifolia* Besser *ex* Szafer including different hierarchical levels and geographical groups (significance tests – 1023 permutations). d.f. – degrees of freedom, SS – sum of squares, F index – Fixation index, $p < 0.001$.

Source of variation	d.f.	SS	Variance component	Percentage of variation	F index
Among all populations	15	393.608	1.46657	93.10	
Within populations	279	30.331	0.10871	6.90	F_{ST} 0.93099
Total	294	423.939	1.57528	–	
Polish populations *vs* other populations	1	174.584	1.43915	59.13	F_{CT} 0.59130
Among populations within groups	14	219.024	0.88599	36.40	F_{SC} 0.89071
Within populations	279	30.331	0.10871	4.47	F_{ST} 0.95533
Total	294	423.939	2.43385	–	
Wyżyna Małopolska *vs* Wyżyna Lubelska	1	180.254	1.90936	94.27	F_{CT} 0.94265
Among populations within groups	10	6.033	0.03113	1.54	F_{SC} 0.26801
Within populations	212	18.026	0.08503	4.20	F_{ST} 0.95802
Total	223	204.312	2.02552		
Among geographical groups	2	354.838	1.93297	87.36	F_{CT} 0.87358
Among populations within groups	13	38.770	0.17101	7.73	F_{SC} 0.61136
Within populations	279	30.331	0.10871	4.91	F_{ST} 0.95087
Total	294	423.939	2.21269	–	

of the area). The populations from the Wyżyna Lubelska upland show greater genetic similarity with populations in the southeastern than with the western Podolian Upland. Further analyses for successive K did not further refine the spatial structure of the species. In STRUCTURE the division into two groups was similar for K = 2 (indicated as optimal based on the ΔK parameter), with the Wyżyna Małopolska upland populations clearly differentiated from the others (Wyżyna Lubelska upland and Podolian Upland). For K = 2, K = 3 and K = 4 the division into population groups was the same in STRUCTURE and TESS. $K_{ef} = 4$ was finally accepted based on the simulations in TESS (Fig. 14).

In the analysis of the Polish populations (Wyżyna Małopolska upland vs Wyżyna Lubelska upland) two separate and internally homogeneous groups of populations form for K = 2 (data not shown). For K = 3 the contributions of two separate genetic pools are observed in the Wyżyna Lubelska upland populations, while those of the Wyżyna Małopolska upland remain a separate group. Further divisions were not statistically significant.

Cirsium pannonicum

I analyzed 247 individuals of *Cirsium pannonicum* (Table 5), recording 164 bands in the polymorphism analysis, 118 of which were polymorphic (72.4%). Nei's gene diversity index is $M_H = 0.12$ (SD = 0.02) and the Shannon index is $M_S = 0.19$ (SD = 0.03) for *C. pannonicum* in the study area. The Mantel test showed a significant correlation between genetic diversity and geographical distance (r = 0.59, for p = 0.05, Z = 5722.31 and g = 6.946). The gene flow coefficient for the populations is $M_{Nm} = 1.00$.

The number of polymorphic bands ranged from 54 (33.13%; BYC, Poland) to 76 (46.63%; CBK, Czech Republic), with 64 (39.16%) polymorphic bands per population on average (SD = 10.02). Discriminating bands were not recorded for any of the populations of *C. pannonicum*. Twenty-one private bands were recorded for 11 populations; only one such band was recorded in populations BYC, HTA and FEF (Poland), UKG (Ukraine), CBS (Czech Republic), RCJ (Romania) and 4, the highest number of private bands, in UCZ (Ukraine) and CBK (Czech Republic). The genetic diversity index values were lowest for BRO (Poland): $M_H = 0.11$ and $M_S = 0.16$. The highest Nei's index value was $M_H = 0.16$ (ULG, Ukraine), and the mean for the populations was $M_H = 0.13$ (SD = 0.02). The highest Shannon index value was $M_S = 0.25$ (CBK, Czech Republic), and the mean for the populations was $M_S = 0.19$ (SD = 0.03). M_{DW} varied greatly, ranging from 5.03 (ULG, Ukraine) to 29.88 (BRO, Poland; CBK, Czech Republic), with a mean of 11.87 (SD = 6.38) (Table 6).

No discriminating or private bands were recorded for the geographical groups of *C. pannonicum* (see Materials). The number of polymorphic bands and the genetic diversity indices were lowest for the southern part of the Pannonian region group and highest for the Wyżyna Małoposka upland group, but the differences in these parameters between regions were not significant. None of the parameters differed significantly between the population groups from Poland (Wyżyna Małopolska and Wyżyna Lubelska uplands) (Table 7).

The PCoA ordination (Fig. 16A) shows high genetic variation of *C. pannonicum*. However, the ordination along axis 1, which accounts for 13.52% of the variation, corresponds with the north-south gradient. Axis 2 accounts for 8.08% of the variation. Individuals of the southern Pannonian region are concentrated in the right-hand portion of the southern group on the plot, and individuals from the Małopolska upland are at the opposite end of axis 1 from the northern group. The Wyżyna Małopolska upland population group is clearly separated from the Wyżyna Lubelska upland group. Also, individuals from the Wyżyna Lubelska upland show greater genetic similarity to those from the Podolian Upland than to those from the Wyżyna Małopolska upland.

In separate PCoA of Polish individuals of *C. pannonicum* (Fig. 16B), axis 1 accounts for 17.46% and axis 2 for 10.46% of variation; the samples from the Wyżyna Małopolska upland and the Wyżyna Lubelska upland form separate clusters, supporting the genetic divergence of the populations from these two regions.

Table 5. Origin of plant material of *Cirsium pannonicum* (L. fil.) Link., used in the present study. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part (for details see Material). Material collected by the author if not marked otherwise.

Population code	Geographical group	Locality	Latitude (N)/ Longitude (E)
ULG	PU	Ukraine, Podolian Upland, W of Zolochyiv	49°48′/24°43′
UCZ	PU	Ukraine, Podolian Upland, E of Rogatin	49°24′/24°40′
UKG	PU	Ukraine, Podolian Upland, Kamenna Gora hill	49°47′/25°03′
BYC	LU	Poland, Wyżyna Lubelska upland, Bychawa, *leg. A. Cwener*	51°01′/22°31′
BRO	LU	Poland, Wyżyna Lubelska upland, Broczówka, *leg. A. Cwener*	50°52′/23°22′
NFL	LU	Poland, Wyżyna Lubelska upland, Nowy Folwark, *leg. A. Cwener*	50°57′/23°29′
HTA	LU	Poland, Wyżyna Lubelska upland, Huta Tarnawacka, *leg. A. Cwener*	50°32′/23°27′
OSG	MU	Poland, Wyżyna Małopolska upland, Pęczelice	50°26′/20°47′
POL	MU	Poland, Wyżyna Małopolska upland, W of Młodzawy Duże	50°46′/20°50′
WAL	MU	Poland, Wyżyna Małopolska upland, near Dosłońce village	50°20′/20°13′
FEF	MU	Poland, Wyżyna Małopolska upland, Feflówka	50°21′/20°09′
TUN	MU	Poland, Wyżyna Małopolska upland, Tunel (part of Uniejów-Rędziny village)	50°26′/19°58′
DAB	MU	Poland, Wyżyna Małopolska upland, Klonów	50°20′/20°10′
CBS	PW	Czech Republic, České středohoří Mountains, NE Litoměřice; near Skalice	50°33′/14°08′
CBK	PW	Czech Republic, Bélé Karpaty Mountains, E of Veselí n./Moravou	48°53′/17°31′
RCT	PS	Romania, Podişului Transilvaniei plateau, Cheile Turzii (Turda Gorges)	46°33′/23°40′
RCJ	PS	Romania, Podişului Transilvaniei plateau, N of Cluj Napoca, Fânaţele Clujului	46°50′/23°38′

Table 6. Parameters of AFLP genetic diversity for populations of *Cirsium pannonicum* (L. fil.) Link. N – number of individuals in population; $\%_{poly}$ – percentage of polymorphic bands; $M_{d/p}$ – discriminating/private bands in population; M_H – mean (±SD) Nei's genetic diversity; M_S – mean (±SD) Shannon genetic index; M_{DW} – frequency down-weighted marker values. For population codes see Table 5.

Population code	N	$\%_{poly}$	$M_{d/p}$	M_H (±SD)	M_S (±SD)	M_{DW}
ULG	9	44.79	0/0	0.16 (±0.19)	0.16 (±0.27)	5.03
UCZ	15	38.04	0/4	0.13 (±0.18)	0.19 (±0.27)	10.73
UKG	10	36.81	0/1	0.12 (±0.18)	0.18 (±0.26)	6.55
BYC	15	33.13	0/1	0.10 (±0.18)	0.17 (±0.26)	14.83
BRO	15	36.81	0/0	0.11 (±0.16)	0.16 (±0.24)	29.88
NFL	27	44.79	0/0	0.14 (±0.18)	0.21 (±0.27)	18.98
HTA	14	39.88	0/1	0.12 (±0.18)	0.19 (±0.26)	9.93
OSG	15	42.95	0/2	0.14 (±0.18)	0.21 (±0.27)	13.45
POL	21	38.04	0/3	0.13 (±0.18)	0.19 (±0.27)	17.04
WAL	14	40.49	0/0	0.11 (±0.17)	0.18 (±0.24)	6.69
FEF	15	38.65	0/1	0.13 (±0.18)	0.19 (±0.27)	13.87
TUN	13	35.58	0/0	0.11 (±0.18)	0.17 (±0.26)	8.02
DAB	15	41.72	0/2	0.13 (±0.18)	0.19 (±0.26)	10.14
CBS	15	42.33	0/1	0.14 (±0.19)	0.21 (±0.27)	14.82
CBK	15	46.63	0/4	0.15 (±0.20)	0.25 (±0.29)	29.88
RCT	4	–	–	–	–	–
RCJ	15	44.79	0/1	0.13 (±0.17)	0.21 (±0.26)	10.96

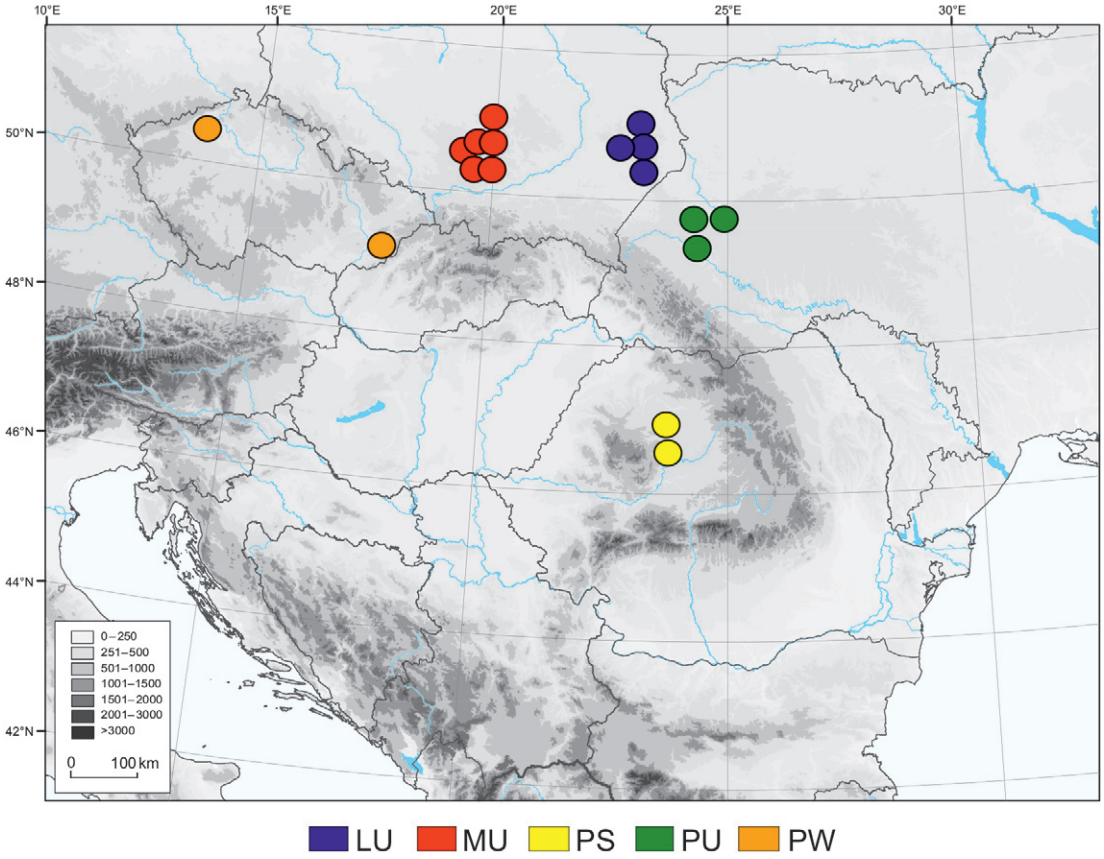

Fig. 15. Localities of the populations of *Cirsium pannonicum* (L. fil.) Link. Localities are divided into geographical groups, details in Table 5. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

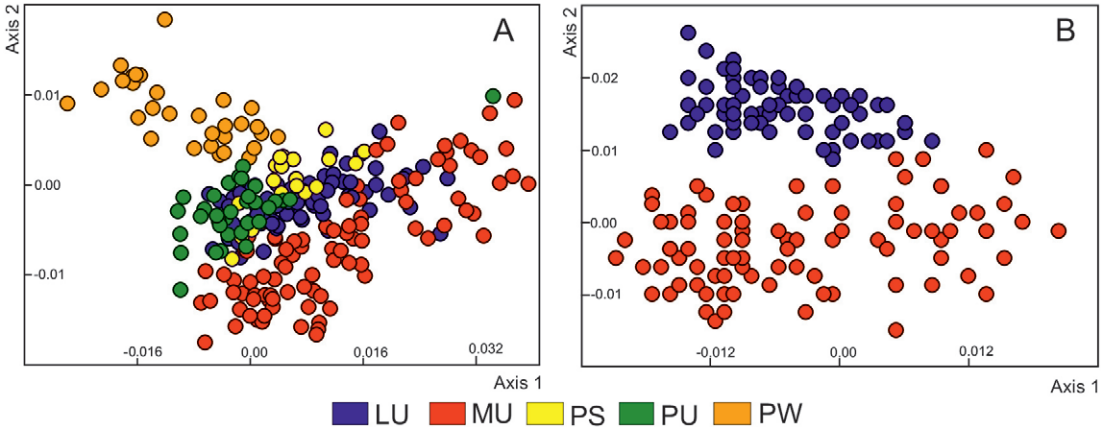

Fig. 16. PCoA scatterplot of *Cirsium pannonicum* (L. fil.) Link. individuals based on Nei and Li genetic distances of AFLP data. A – all studied populations, B – Polish populations. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

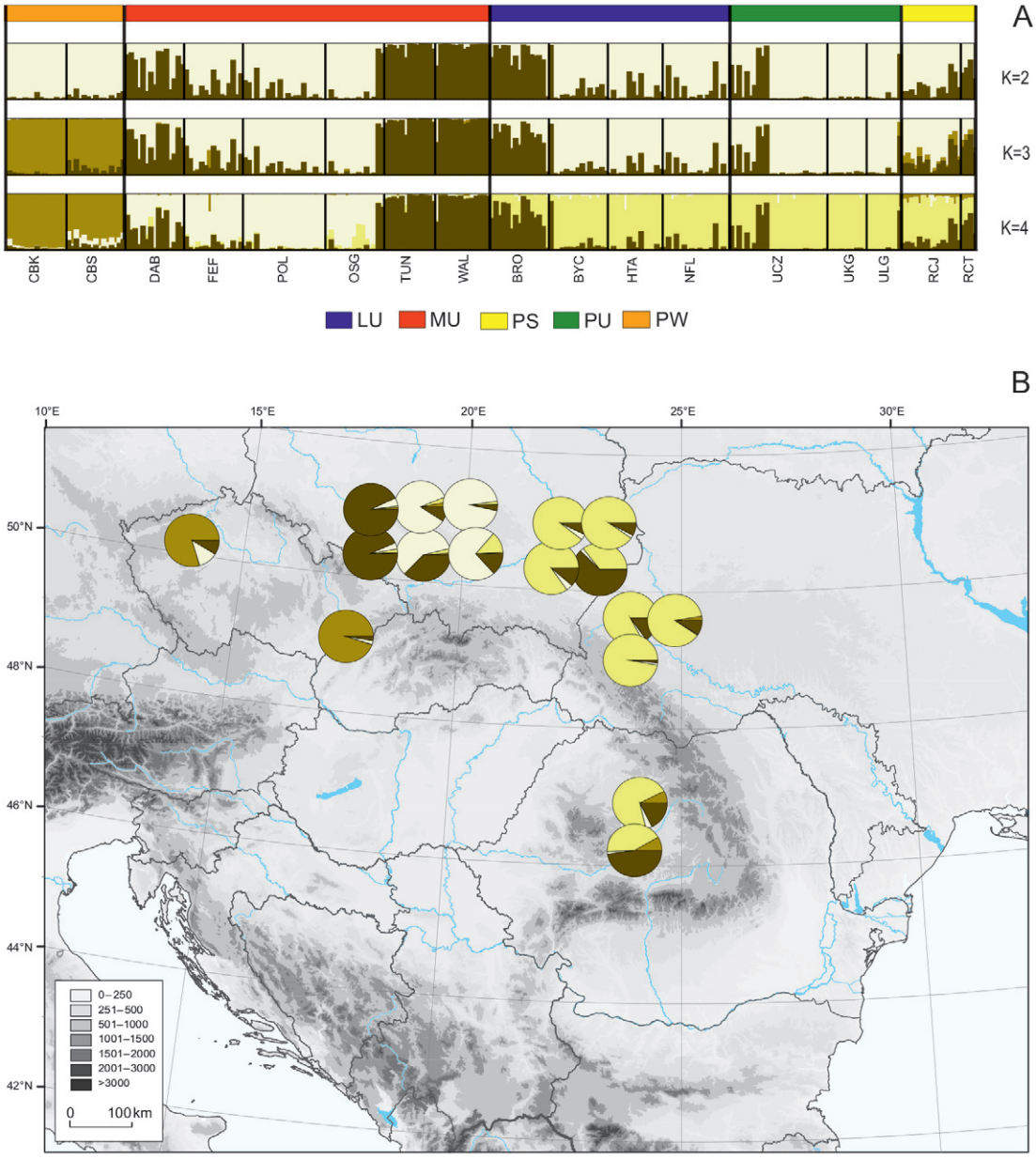

Fig. 17. Results of Bayesian analysis (TESS software) for *Cirsium pannonicum* (L. fil.) Link. A – bar graphs of individuals for K = 2, K = 3, K = 4; populations are separated by vertical lines. B – pie charts showing the proportions of the clusters present within the populations, detected by the Bayesian analysis for K = 4. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part. For population codes see Table 5.

Table 7. Parameters of AFLP genetic diversity for geographical groups of *Cirsium pannonicum* (L. fil.) Link. N – number of individuals in group, $\%_{poly}$ – percentage of polymorphic bands, $M_{dR/pR}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_{Nm} – value of gene flow.

Geographical group	N	$\%_{poly}$	$M_{dR/pR}$	M_H (±SD)	M_S (±SD)	M_{Nm}
Podolian Upland (PU)	34	55.21	0/0	0.16 (±0.17)	0.24 (±0.25)	3.89
Wyżna Lubelska upland (LU)	71	59.51	0/0	0.14 (±0.17)	0.23 (±0.25)	2.66
Wyżna Małopolska upland (MU)	93	64.42	0/0	0.18 (±0.18)	0.27 (±0.26)	0.98
Pannonian region, NW part (PW)	30	56.44	0/0	0.18 (±0.19)	0.27 (±0.28)	2.69
Pannonian region, S part (PS)	19	46.63	0/0	0.13 (±0.17)	0.21 (±0.26)	2.94

The NJ dendrogram displayed no well-defined clusters and the bootstrap support was very low (< 30%), confirming high genetic variation and lack of strong variation in the data set.

AMOVA at the level of *Cirsium pannonicum* populations showed the dominance of intrapopulation (68.60%) over interpopulation variation (31.40%) ($F_{ST} = 0.31$, $p < 0.001$). The same pattern held in hierarchical analysis with division into five geographical groups. The negative value of the variance component between the groups from Poland and the other studied populations (–0.54%) indicates the absence of genetic structure at this level of analysis ($F_{CT} = –0.005$, $p < 0.5$). AMOVA results are given in Table 8.

In Bayesian analysis of the whole data set in TESS (Fig. 17) only the *C. pannonicum* populations of the northwestern part of the Pannonian region formed one genetic pool at $K = 2$. These populations are genetically separated from all the

Table 8. Analyses of molecular variance (AMOVA) based on AFLP markers for *Cirsium pannonicum* (L. fil.) Link. including different hierarchical levels and geographical groups (significance tests – 1023 permutations). d.f. – degrees of freedom, SS – sum of squares, F index – Fixation index, $p < 0.001$ if not marked otherwise.

Source of variation	d.f.	SS	Variance component	Percentage of variation	F index p
Among populations	17	1358.975	5.06421	31.40	
Within populations	229	2533.770	11.06450	68.60	F_{ST} 0.31399
Total	246	3892.745	16.12871	–	
Polish populations *vs* other populations	1	73.726	–0.08198	–0.54	F_{CT} –0.00538 $p < 0.5$
Among populations within groups	14	992.834	4.54211	29.80	F_{SC} 0.29640
Within populations	201	2167.237	10.78227	70.74	F_{ST} 0.29261
Total	216	3233.797	15.24240	–	
Wyżyna Małopolska *vs* Wyżyna Lubelska	1	125.580	0.97845	6.42	F_{SC} 0.23538 $p < 0.02$
Among populations within groups	8	413.863	3.35906	22.03	F_{CT} 0.06416
Within populations	119	1298.526	10.91198	71.56	F_{ST} 0.28444
Total	128	1837.969	15.24949	–	
Among geographical groups	3	345.325	0.53088	3.27	F_{CT} 0.03270 $p < 0.04$
Among populations within groups	14	1013.650	4.64083	28.58	F_{SC} 0.29549
Within populations	229	2533.770	11.06450	68.15	F_{ST} 0.31853
Total	246	3892.745	16.23621	–	

rest at K = 3. The remaining populations from the Wyżyna Małopolska upland, the Wyżyna Lubelska upland, the Podolian Upland and the other populations from the Pannonian region were characterized by admixture of two widely distributed genetic pools. Here, populations TUN and WAL (Wyżyna Małopolska upland) show the dominant contribution of one of these genetic pools, and the populations from the Podolian Upland show dominance of the other pool. The other populations show relatively even contributions of both genetic pools. For K = 4, besides the population group from the Czech Republic and the group of the northwestern Pannonian region, the population group from the Wyżyna Małopolska upland splits into two groups with distinctive gene pools (populations WAL and TUN; popopulations DAB, FEF, POL and OSG).

The fourth genetic group consists of the populations from the Wyżyna Lubelska upland, Podolian Upland and the southern part of the Pannonian region, but BRO from the Wyżyna Lubelska upland additionally displays a significant admixture of one of the neighboring groups from the Wyżyna Małopolska upland.

In the Bayesian analysis in TESS, K = 2 was shown to be the best division based on the ΔK parameter. It was also the best division in STRUCTURE, based on the same parameter. For K = 3 and K = 4 most individuals were also assigned to one specific group with a probability of 0.8 or more, and some populations displayed admixture. The delimitation of genetic groups for these K values was identical in STRUCTURE and TESS, and $K_{ef} = 4$ was finally accepted based on simulations in TESS (Fig. 17).

The *Cirsium pannonicum* population groups from the Wyżyna Małopolska upland and Wyżyna Lubelska upland form two genetically separate groups in Bayesian analysis of the Polish populations in TESS for K = 2 (data not shown). For K = 3 there is an additional division within the populations from the Wyżyna Małopolska upland. Populations WAL and TUN form an independent group, as do DAB and FEF, with characteristic genetic pools differing from the populations in the Wyżyna Lubelska upland. Further divisions were not statistically significant.

INULA ENSIFOLIA

I analyzed 417 individuals of *Inula ensifolia* (Table 9) detecting 178 AFLP fragments, 152 (88.2%) of which were polymorphic. Nei's gene diversity index is $M_H = 0.21$ (SD = 0.17) and the Shannon index is $M_S = 0.32$ (SD = 0.24) for *I. ensifolia* in the study area, and the gene flow coefficient is $M_{Nm} = 1.5$. The Mantel test indicated a significant correlation between the genetic diversity and geographical distance of individual populations (r = 0.26, for p = 0.05, Z = 25705 and g = 4.99).

The number of polymorphic bands ranged from 53 (29.78%; NHB, Austria) to 131 (73.6%; DAB, WAL, Poland), with an average 96.58 (54.84%) bands per population (SD = 30.76). No discriminating bands were detected for any of the populations. Eleven private bands were recorded for six populations of *I. ensifolia*; population RBD (Romania) yielded the greatest number of these bands (3), and populations USU (Ukraine) and NHB (Austria) the least (1). Populations EHK (Austria), RBD (Romania) and SBA (Slovenia) had the lowest Nei's gene diversity index (0.12) and Shannon index (0.16), and population DAB (Poland) the highest (0.22 and 0.34, respectively); the mean for the populations was 0.16 (SD = 0.04) and 0.25 (SD = 0.07), respectively. The M_{DW} coefficient ranged from 2.00 (EHK, Austria) to 19.52 (DAB, Poland), with a mean of 6.41 (SD = 4.43) (Table 10).

The number of polymorphic bands and genetic diversity indices were lowest for the population group from the North Adriatic, which is the southwestern range limit of *Inula ensifolia* in Europe (see Materials). The highest number was recorded for populations from the Wyżyna Małopolska upland, Podolian Upland and Pannonian region.

None of the parameters differed significantly between the population groups from Poland (Wyżyna Małopolska and Wyżyna Lubelska uplands) (Table 11). In the total data set, M_H and M_S differed significantly between the Polish population group and the population group from the northwestern part of the Pannonian region (U = 0, Z = 2.569, p < 0.01) as well as the North Adriatic

Table 9. Origin of plant material of *Inula ensifolia* L. used in the present study. BP – Balkan Peninsula, LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PA – Adriatic area, N part, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part (for details see Material). Material collected by the author if not marked otherwise.

Population code	Geographical group	Locality	Latitude (N)/ Longitude (E)
USU	PU	Ukraine, Podolian Upland, E of Ust'e; on N bank of Dnyistjer river	48°33′/26°41′
UCZ	PU	Ukraine, Podolian Upland, E of Rogatin	49°24′/24°40′
ULG	PU	Ukraine, Podolian Upland, W of Zolochyiv	49°48′/24°43′
MAJ	LU	Poland, Wyżyna Lubelska upland, NE of Tomaszów Lubelski; Biała Góra hill	50°28′/23°28′
ROG	LU	Poland, Wyżyna Lubelska upland, SW of Grabowiec	50°48′/23°31′
BRO	LU	Poland, Wyżyna Lubelska upland, NNW of Skierbieszów	50°52′/23°22′
NFL	LU	Poland, Wyżyna Lubelska upland, Nowy Folwark, *leg. A. Cwener*	50°57′/23°29′
DAB	MU	Poland, Wyżyna Małopolska upland, Klonów	50°20′/20°10′
WAL	MU	Poland, Wyżyna Małopolska upland, Dosłońce	50°20′/20°13′
FEF	MU	Poland, Wyżyna Małopolska upland, Feflówka	50°21′/20°09′
PRZ	MU	Poland, Wyżyna Małopolska upland, Prześlin	50°22′/20°43′
SKG	MU	Poland, Wyżyna Małopolska upland, Skotniki Górne Reserve	50°25′/20°38′
TUN	MU	Poland, Wyżyna Małopolska upland, N of Miechów; Biała Góra hill	50°26′/19°58′
PIN	MU	Poland, Wyżyna Małopolska upland, Pińczów	50°32′/20°31′
CZN	PW	Czech Republic, Morava Region, S of Čižov	48°51′/15°35′
CBK	PW	Czech Republic, Bélé Karpaty Mountains, E of Veselí n./Moravou	48°53′/17°31′
SDK	PW	Slovakia, Malé Karpaty Mountains, Devínska Kobyla	48°11′/16°59′
EHK	PW	Austria, Niederösterreich (Hundsheimer Berge), *leg. W. Paul & P. Kiełtyk*	48°07′/16°56′
BSB	PW	Austria, Niederösterreich, Wiener Berge (Eichenkogel), S of Mödling, *leg. P. Kiełtyk*	48°03′/16°17′
NHB	PW	Austria, Niederösterreich, S of Bad Sauerbrunn, *leg. W. Paul*	47°45′/16°21′
WDK	PS	Hungary, Dunántúli-középhegység Mountain, Gerecse-hegység; Kálvária hill, *leg. K. Dobolyi*	47°42′/18°43′
WKS	PS	Hungary, Dunántúli-középhegység Mountain, Pilisszentiván, Kis-Szénás hill, *leg. K. Dobolyi*	47°36′/18°51′
WIH	PS	Hungary, Dunántúli-középhegység Mountain, Pilisszentiván, Iváni hill, *leg. K. Dobolyi*	47°36′/18°53′
WBU	PS	Hungary, Dunántúli-középhegység Mountain, near Budaőrs, Odvas hill, *leg. K. Dobolyi*	47°28′/18°58′
RCJ	PS	Romania, Podişului Transilvaniei plateau, W Cluj-Napoca	46°45′/23°32′
RCI	PS	Romania, Munţii Cindrel Mountains, Cheile Cibinului hill	45°40′/23°54′
RBD	PS	Romania, Banat Mountains, Berzasce, on bank of Dunaj, *leg. F. Karhulec*	44°38′/21°54′
BDR	BP	Bulgaria, Čepăn Mountains, Dragoman	42°56′/22°58′
IRS	PA	Italy, Kras [Monti de Carso], SE of Trieste, Val Rosandra	45°37′/13°52′
SBA	PA	Slovenia, Istrian Peninsula, between Abitanti and Gadin	45°27′/13°15′

region ($U = 0$, $Z = -2.171$, $p < 0.02$). M_{DW} differed significantly between the Polish population group and the population from the Balkans (Bulgaria) ($U = 0$, $Z = 2.171$, $p < 0.02$).

The PCoA ordination shows high genetic variation of *I. ensifolia* in the studied part of the range (Fig 19A). Axes 1 and 2 account for 7.97% and 4.35% of the variation, respectively. Individuals cluster along the first axis without a clear hiatus but displaying a gradient corresponding to the west-east distribution of populations, with populations from the northern coast of the Adriatic on the

Table 10. Parameters of AFLP genetic diversity for populations of *Inula ensifolia* L. N – number of individuals in population, $\%_{poly}$ – percentage of polymorphic bands, $M_{d/p}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_S – mean (±SD) Shannon genetic index, M_{DW} – frequency down-weighted marker values. For population codes see Table 9.

Population code	N	% poly	$M_{d/p}$	M_H (±SD)	M_S (±SD)	M_{DW}
USU	15	69.66	0/1	0.21 (±0.19)	0.32 (±0.27)	7.03
UCZ	14	61.24	0/2	0.19 (±0.19)	0.29 (±0.28)	6.42
ULG	14	67.98	0/0	0.21 (±0.18)	0.31 (±0.26)	7.42
MAJ	9	53.93	0/0	0.18 (±0.19)	0.27 (±0.28)	4.02
ROG	20	67.98	0/0	0.18 (±0.18)	0.29 (±0.25)	8.38
BRO	22	64.04	0/0	0.17 (±0.18)	0.27 (±0.26)	10.59
NFL	13	57.31	0/0	0.18 (±0.19)	0.27 (±0.27)	4.78
DAB	22	73.60	0/0	0.22 (±0.17)	0.34 (±0.25)	19.52
WAL	23	73.60	0/0	0.21 (±0.17)	0.32 (±0.24)	14.38
FEF	22	70.22	0/0	0.19 (±0.17)	0.31 (±0.25)	11.43
PRZ	13	53.93	0/0	0.16 (±0.18)	0.25 (±0.27)	4.84
SKG	22	66.85	0/0	0.19 (±0.18)	0.31 (±0.26)	8.08
TUN	23	69.66	0/0	0.18 (±0.18)	0.29 (±0.25)	12.95
PIN	20	66.29	0/0	0.19 (±0.18)	0.29 (±0.26)	7.79
CZN	20	67.98	0/0	0.21 (±0.18)	0.31 (±0.26)	13.17
CBK	15	66.29	0/0	0.22 (±0.19)	0.33 (±0.27)	7.84
SDK	15	65.73	0/0	0.19(±0.18)	0.31 (±0.26)	6.06
EHK	5	30.35	0/0	0.12 (±0.17)	0.16 (±0.26)	2.00
BSB	8	41.57	0/2	0.14 (±0.18)	0.21 (±0.27)	2.34
NHB	5	29.78	0/1	0.18 (±0.18)	0.23 (±0.27)	4.78
WDK	10	52.25	0/0	0.16 (±0.18)	0.29 (±0.26)	3.69
WKS	10	58.99	0/0	0.18 (±0.18)	0.27 (±0.27)	4.97
WIH	10	52.25	0/0	0.16 (±0.18)	0.24 (±0.27)	4.84
WBU	15	65.17	0/0	0.19 (±0.18)	0.29 (±0.25)	6.38
RCJ	14	61.24	0/0	0.18 (±0.18)	0.27 (±0.26)	5.57
RCI	10	54.49	0/0	0.17 (±0.19)	0.26 (±0.27)	3.27
RBD	6	34.84	0/3	0.12 (±0.17)	0.16 (±0.27)	2.43
BDR	3	–	–	–	–	–
IRS	4	–	–	–	–	–
SBA	15	46.07	0/2	0.12 (±0.18)	0.16 (±0.26)	5.05

Table 11. Parameters of AFLP genetic diversity for geographical groups of *Inula ensifolia* L. N – number of individuals in group, $\%_{poly}$ – percentage of polymorphic bands, $M_{dR/pR}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_{Nm} – value of gene flow.

Geographical group	N	% poly	$M_{dR/pR}$	M_H (±SD)	M_S (±SD)	M_{Nm}
Podolian Upland (PU)	45	82.58	0/0	0.22 (±0.19)	0.35 (±0.25)	1.21
Wyżna Lubelska upland (LU)	64	80.90	0/0	0.21 (±0.17)	0.32 (±0.24)	3.46
Wyżna Małopolska upland (MU)	145	83.71	0/0	0.21 (±0.16)	0.34 (±0.23)	4.31
Pannonian region, S part (PS)	78	81.46	0/0	0.20 (±0.16)	0.32 (±0.23)	1.72
Pannonian region, NW part (PW)	68	82.58	0/0	0.22 (±0.17)	0.35 (±0.25)	2.07
Adriatic area, N part (PA)	19	50.00	0/0	0.14 (±0.18)	0.22 (±0.26)	2.77
Balkan Peninsula (BP)	3	71.91	0/0	0.14 (±0.18)	0.25 (±0.25)	1.47

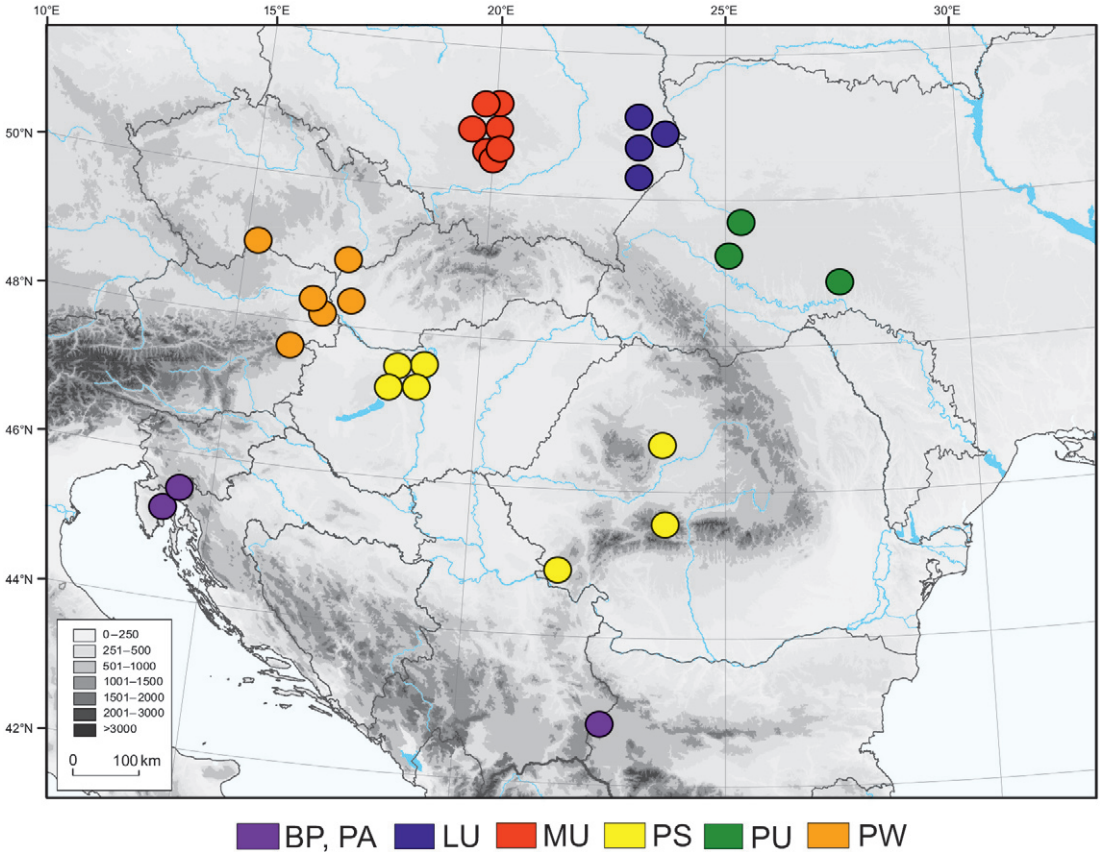

Fig. 18. Localities of the populations of *Inula ensifolia* L. Localities are divided into geographical groups; details in Table 9. BP & PA – Balkan Peninsula and N part of Adriatic area, LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

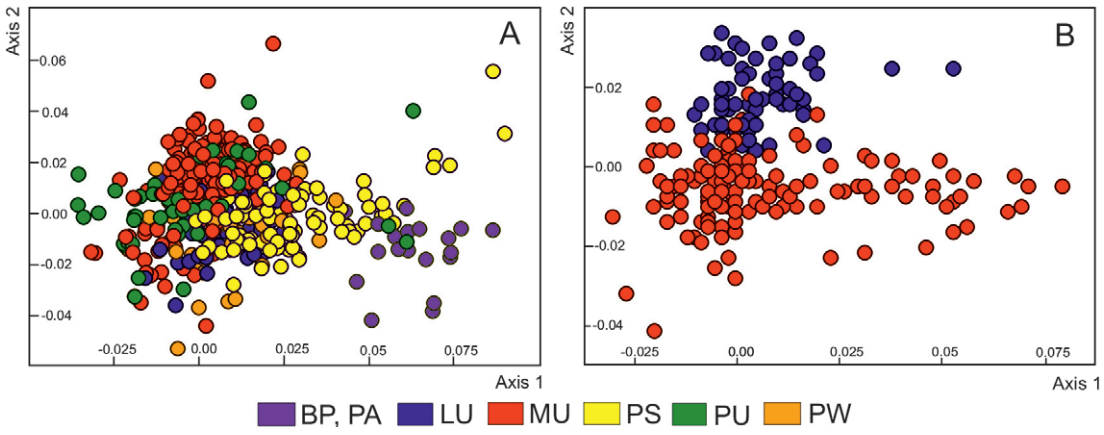

Fig. 19. PCoA scatterplot of *Inula ensifolia* L. individuals based on Nei and Li genetic distances of AFLP data. A – all studied populations, B – Polish populations. BP & PA – Balkan Peninsula and N part of Adriatic area, LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

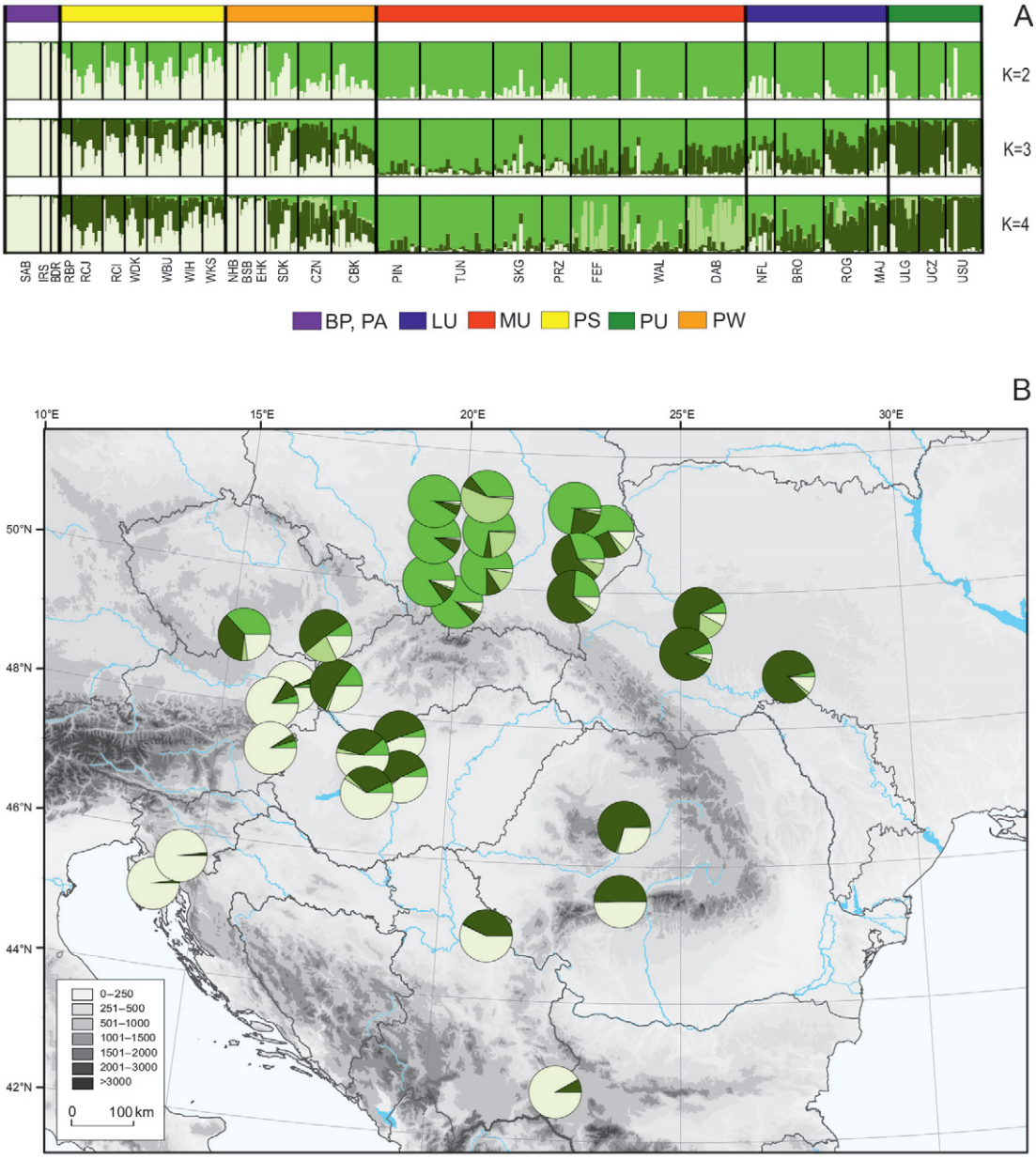

Fig. 20. Results of Bayesian analysis (TESS software) for *Inula ensifolia* L.. A – bar graphs of individuals for K = 2, K = 3, K = 4; populations are separated by vertical lines. B – pie charts showing the proportions of the clusters present within the populations, detected by the Bayesian analysis for K = 4. BP & PA – Balkan Peninsula and N part of Adriatic area, LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part. For population codes see Table 9.

Table 12. Analyses of molecular variance (AMOVA) based on AFLP markers for *Inula ensifolia* L. including different hierarchical levels and geographical groups (significance tests – 1023 permutations). d.f. – degrees of freedom, SS – sum of squares, F index – Fixation index, $p < 0.001$.

Source of variation	d.f.	SS	Variance component	Percentage of variation	F index
Among populations	30	2170.793	3.82896	17.22	
Within populations	410	7549.257	18.41282	82.78	F_{ST} 0.17215
Total	440	9720.050	22.24178	–	
Polish populations *vs* other populations	1	256.582	0.80638	3.56	F_{CT} 0.03564
Among populations within groups	29	1914.211	3.40924	15.07	F_{SC} 0.15623
Within populations	410	7549.257	18.41282	81.37	F_{ST} 0.18630
Total	440	9720.050	22.62845	–	
Wyżyna Małopolska *vs* Wyżyna Lubelska	1	140.928	0.83339	3.81	F_{CT} 0.03812
Among populations within groups	9	594.642	2.26729	10.37	F_{SC} 0.10783
Within populations	220	4127.226	18.76012	85.82	F_{ST} 0.14184
Total	230	4862.797	21.86080	–	
Among geographical groups	5	821.843	1.57415	6.98	F_{CT} 0.6980
Among populations within groups	25	1348.949	2.56690	11.38	F_{SC} 0.12235
Within populations	410	7549.257	18.41282	81.64	F_{ST} 0.18361
Total	440	9720.050	22.55387	–	

right side of the plot and those from the Podolian Upland on the left side of the plot. The population group from the Wyżyna Małopolska upland diverges from the other populations along axis 3, which accounts for 4.18% of the variation. The spatial arrangement of individuals on the PCoA plot indicates a geographically concordant division within the Polish populations of *I. ensifolia* into the group from the Wyżyna Małopolska and Wyżyna Lubelska uplands and the greater genetic similarity of the Wyżyna Lubelska upland populations to the Podolian Upland and Pannonian populations than to those from the Wyżyna Małopolska upland.

In separate PCoA of Polish populations, individuals of *I. ensifolia* from the two regions (Wyżyna Małopolska and Wyżyna Lubelska uplands) form genetically separate groups along axes 1 and 2, which respectively account for 13.52% and 8.08% of the variation (Fig. 19B).

Neighbor-joining indicated high interpopulation variation and the absence of intrapopulation genetic differentiation of *I. ensifolia*. This is confirmed by very low bootstrap support not exceeding 30%.

AMOVA showed the contribution of intrapopulation variation (82.78%) to be much greater than that of interpopulation variation (17.22%) in all the populations of *I. ensifolia* taken together ($F_{ST} = 0.17$, $p < 0.001$). Taken as a group, the populations from Poland did not show significant variation from the rest of the data set. Intrapopulation variation remained dominant in AMOVA of seven groups corresponding to geographical location, indicating very low genetic differentiation of *I. ensifolia* in the study area. All AMOVA results are compared in Table 12.

In Bayesian analysis of all populations of *I. ensifolia* in TESS (Fig. 20), a population group from the northern coast of the Adriatic and the Balkans forms a homogeneous genetic pool for $K = 2$. For $K = 3$ the population group of the northern coast of the Adriatic and the Balkans is still separated. A large contribution of its genetic pool is detected in populations from the Pannonian region and occasionally in those from the Wyżyna Małopolska upland, Wyżyna Lubelska upland and Podolian Upland. The dominant part of the genetic pool of the Wyżyna Małopolska upland populations

contributes to a small extent to the populations from the Wyżyna Lubelska upland and the northern part of the Pannonian region. For K = 3, the population group from the Wyżyna Małopolska upland forms a relatively homogeneous and separate genetic pool. Another genotype which dominates in the Podolian Upland populations occurs also in those from the Wyżyna Lubelska upland and the Pannonian region, but makes only a small contribution to those in the Wyżyna Małopolska upland.

For K = 4 the genetic structure inferred is the same as for K = 3 except for the appearance of an additional group comprising two populations from the Wyżyna Małopolska upland. The results indicate the absence of clearly genetically homogeneous population groups of *Inula ensifolia* and a high level of admixture across geographical regions. Internally the population groups from the northern coast of the Adriatic and the Balkans as well as from the Wyżyna Małopolska upland were the most genetically homogeneous. The Wyżyna Małopolska upland and Wyżyna Lubelska upland population groups show lower genetic similarity between populations. The Wyżyna Lubelska upland populations are genetically similar to those from the Podolian Upland, and these to populations from the Pannonian region.

In STRUCTURE the ΔK parameter indicated K = 2 as the most appropriate number of groups. For this K value the main division is between the populations from the Balkans and the northern coast of the Adriatic and those from the remaining areas. For K = 3 and K = 4 most individuals were assigned to a particular group with a probability of 0.8 or more. The genetic groups for these K values were the same in STRUCTURE and TESS. $K_{ef} = 4$ was finally accepted based on the simulations in TESS (Fig. 20).

In a separate TESS analysis of the Polish populations of *I. ensifolia*, individuals from the Wyżyna Małopolska and Wyżyna Lubelska uplands form separate and internally homogeneous groups for K = 2 (not shown). An additional homogeneous group comprising populations DAB and FEF from the Wyżyna Małopolska upland is separated at K = 3. Here a small admixture of genetic pools characterizing the Wyżyna Małopolska upland populations is additionally present in the Wyżyna Lubelska upland population group. Further divisions were not statistically significant.

Linum flavum

I analyzed 288 specimens of *Linum flavum* (Table 13), recording 207 bands, 161 (77.8%) of which were polymorphic. Nei's gene diversity index is $M_H = 0.21$ (SD = 0.17) and the Shannon index is $M_S = 0.32$ (SD = 0.24) for *L. flavum* in the study area. The Mantel test showed significant correlations between genetic diversity and geographical distance for all populations (r = 0.4, for p = 0.05, Z = 10787.48, and g = 5.73). The gene flow coefficient is $M_{Nm} = 1.11$.

The number of polymorphic bands in individual populations ranged from 75 (36.23%; ULG, Ukraine) to 117 (56.52%; RCJ, Romania), with 90 bands (43.7%) per population on average (SD = 16.72). Discriminating bands were not recorded for any of the populations. Twenty-nine private bands were detected for 12 populations, the highest number (6) of them from population UZM (Ukraine) and the lowest number (1) from populations FEF, MAJ and ROG (Poland). Population TUN (Poland) had the lowest Nei's gene diversity index ($M_H = 0.12$) and Shannon index ($M_S = 0.19$), and population RCJ (Romania) had the highest ($M_H = 0.18$, $M_S = 0.27$). The means for the populations were $M_H = 0.14$ (SD = 0.02) and $M_S = 0.22$ (SD = 0.03). The M_{DW} coefficient ranged widely from 3.05 (ULG, Ukraine) to 20.65 (UKK, Ukraine), with a mean of 11.12 (SD = 5.01) (Table 14).

No discriminating bands and no private bands were detected for the predefined geographical groups (see Materials). The number of polymorphic bands and genetic diversity indices were lowest for the Wyżyna Małopolska upland populations and highest for the Podolian Upland populations. No parameters differed significantly between the two Polish population groups (Wyżyna Małopolska and Wyżyna Lubelska uplands) (Table 15). Nei's gene diversity index differed significantly between the populations from Poland as a whole and those from the southern Pannonian (U = 2, Z = 2.13, p < 0.03). The M_{DW} index differed significantly between the

Table 13. Origin of plant material of *Linum flavum* L. used in the present study. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part (for details see Material). Material collected by the author if not marked otherwise.

Population code	Geographical group	Locality	Latitude (N)/ Longitude (E)
UCZ	PU	Ukraine, Podolian Upland, E of Rogatin	49°24′/24°40′
UCG	PU	Ukraine, Podolian Upland, SE of Burshtin	49°13′/24°41′
UZM	PU	Ukraine, Podolian Upland, Storozhinets', *leg. O. Optasyuk*	48°15′/25°54′
UKK	PU	Ukraine, Podolian Upland, N of Kam'yanets'-Podyils'kij	48°48′/26°38′
ULG	PU	Ukraine, Podolian Upland, W of Zolochyiv	49°48′/24°43′
ZMU	LU	Poland, Wyżyna Lubelska upland, Źmudź	51°00′/23°40′
ROG	LU	Poland, Wyżyna Lubelska upland, SW of Grabowiec	50°47′/23°31′
ORL	LU	Poland, Wyżyna Lubelska upland, Orłów Murowany-Kolonia, *leg. A. Cwener*	50°54′/23°14′
WIR	LU	Poland, Wyżyna Lubelska upland, Wirkowice, *leg. A. Cwener*	50°52′/23°03′
MAJ	LU	Poland, Wyżyna Lubelska upland, Majdan	50°28′/23°28′
BGG	LU	Poland, Wyżyna Lubelska upland, NE of Tomaszów Lubelski, Biała Góra hill	50°28′/23°28′
TUN	MU	Poland, Wyżyna Małopolska upland, N of Miechów, Biała Góra hill	50°26′/19°58′
FEF	MU	Poland, Wyżyna Małopolska upland, Feflówka	50°21′/20°09′
PRA	MU	Poland, Wyżyna Małopolska upland, Prandocin-Iły	50°16′/20°04′
CBS	PW	Czech Republic, České středohoří Mountains, Skalice	50°33′/14°08′
CBK	PW	Czech Republic, Bílé Karpaty Mountains, E of Veselí n./Moravou	48°53′/17°31′
SDK	PW	Slovakia, Malé Karpaty Mountains, Devínska Kobyla	48°11′/16°59′
SVH	PW	Slovakia, Malé Karpaty Mountains, Vrchná hora hill, *leg. S. Spaniel & Mereďa*	48°16′/17°03′
ARO	PW	Austria, Niederösterreich, near Marzer Kogel, *leg. W. Paul & P. Kiełtyk*	47°43′/16°27′
WNP	PS	Hungary, Mezőföld lowland, Németkér; Puputeve-hát hill, *leg. K. Dobolyi*	46°44′/18°49′
RCJ	PS	Romania, Podişului Transilvaniei plateau, W Cluj-Napoca	46°45′/23°32′

Pannonian region group and the Podolian Upland group in ($U = 2$, $Z = 1.94$, $p < 0.05$) (Table 15).

The PCoA ordination shows high variability of *Linum flavum* in the studied part of its range (Fig. 22A). Axes 1 and 2 account for 10.11% and 5.30% of the variation, respectively. Two groups are separated along the first axis, the first formed by individuals from the Pannonian region and the second by those from the Podolian Upland, the Wyżyna Małopolska upland and Wyżyna Lubelska upland (left side of plot). In the latter group the populations from the Wyżyna Małopolska upland are separated from the Wyżyna Lubelska upland and Podolian Upland populations along axis 2.

In separate PCoA of the Polish populations (Fig. 22B), individuals of *L. flavum* from the Wyżyna Małopolska and Wyżyna Lubelska uplands form two separate groups along axes 1 (13.52% of variation) and 2 (8.08% of variation).

NJ showed weak differentiation into subgroups, with very low bootstrap support.

AMOVA demonstrated the dominant contribution of intra- over interpopulation variation ($F_{ST} = 0.31$, $p < 0.001$). When the population group from Poland was separated against the rest, the variance component was negative (–1.38%), suggesting the absence of genetic structure at this hierarchical level ($F_{CT} = -0.01$, $p < 0.9$). When the groups were subdivided according to the predefined geographical regions, the contribution of intrapopulation variation was considerably higher than that of variation between groups or between populations within groups. All AMOVA results are compared in Table 16.

Table 14. Parameters of AFLP genetic diversity for populations of *Linum flavum* L. N – number of individuals in population, $\%_{poly}$ – percentage of polymorphic bands, $M_{d/p}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_S – mean (±SD) Shannon genetic index, M_{DW} – frequency down-weighted marker values. For population codes see Table 13.

Population code	N	$\%_{poly}$	$M_{d/p}$	M_H (±SD)	M_S (±SD)	M_{DW}
UCZ	15	49.28	0/0	0.15 (±0.18)	0.32 (±0.26)	8.41
UCG	15	53.62	0/0	0.16 (±0.18)	0.25 (±0.27)	9.72
UZM	10	44.44	0/6	0.14 (±0.18)	0.21 (±0.26)	9.88
UKK	23	55.56	0/0	0.17 (±0.19)	0.26 (±0.27)	20.65
ULG	5	36.23	0/0	0.14 (±0.19)	0.21 (±0.28)	3.05
ZMU	21	43.96	0/0	0.14 (±0.19)	0.21 (±0.27)	12.00
ROG	15	45.98	0/1	0.16 (±0.20)	0.24 (±0.28)	12.5
ORL	12	38.65	0/2	0.13 (±0.19)	0.19 (±0.27)	17.84
WIR	10	36.71	0/3	0.13 (±0.19)	0.19 (±0.27)	6.27
MAJ	22	43.96	0/1	0.15 (±0.19)	0.22 (±0.27)	17.84
BGG	11	38.16	0/0	0.13 (±0.18)	0.19 (±0.26)	5.98
TUN	17	43.96	0/0	0.12 (±0.18)	0.19 (±0.25)	13.93
FEF	17	47.34	0/1	0.15 (±0.19)	0.23 (±0.27)	10.55
PRA	10	38.16	0/3	0.14 (±0.20)	0.21 (±0.28)	8.44
CBS	15	45.89	0/2	0.15 (±0.19)	0.23 (±0.27)	15.61
CBK	15	46.38	0/2	0.15 (±0.19)	0.23 (±0.27)	14.38
SDK	3	18.38	0/0	–	–	–
SVH	15	44.44	0/2	0.15 (±0.19)	0.22 (±0.27)	13.93
ARO	10	45.41	0/3	0.14 (±0.19)	0.22 (±0.27)	13.61
WNP	10	45.41	0/3	0.14 (±0.18)	0.22 (±0.27)	8.66
RCJ	17	56.52	0/0	0.18 (±0.20)	0.27 (±0.28)	20.34

Table 15. Parameters of AFLP genetic diversity for geographical groups of *Linum flavum* L. N – number of individuals in group, $\%_{poly}$ – percentage of polymorphic bands, $M_{dR/pR}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_{Nm} – value of gene flow.

Geographical group	N	$\%_{poly}$	$M_{dR/pR}$	M_H (±SD)	M_S (±SD)	M_{Nm}
Podolian Upland (PU)	68	71.50	0/0	0.19 (±0.18)	0.29 (±0.25)	2.12
Wyżna Lubelska upland (LU)	69	65.7	0/0	0.18 (±0.19)	0.28 (±0.26)	1.57
Wyżna Małopolska upland (MU)	66	63.77	0/0	0.18 (±0.18)	0.28 (±0.26)	1.85
Pannonian region, NW part (PW)	58	68.8	0/0	0.21 (±0.19)	0.31 (±0.27)	0.99
Pannonian region, S part (PS)	27	68.50	0/0	0.19 (±0.19)	0.29 (±0.27)	2.809

In Bayesian analysis of all *L. flavum* populations in TESS (Fig. 23), the Pannonian region was separated from the remaining areas for K = 2. A small admixture of the Pannonian genetic pool was also detected in the Podolian Upland populations. For K = 3, the Pannonian region group was maintained and the Wyżyna Małopolska upland was separated from the Wyżyna Lubelska upland and Podolian Upland, which together formed a third group. Each of these groups was relatively homogeneous internally. A small contribution of the genetic pool characteristic of the Wyżyna Małopolska upland population was present in the Wyżyna Lubelska upland and Podolian Upland populations. A small share of the genetic pool characteristic of the Pannonian populations was present in Podolian Upland populations. For K = 4 the population group from the Czech Republic was separated from other Pannonian populations. The genetic pool formed by the remaining part of

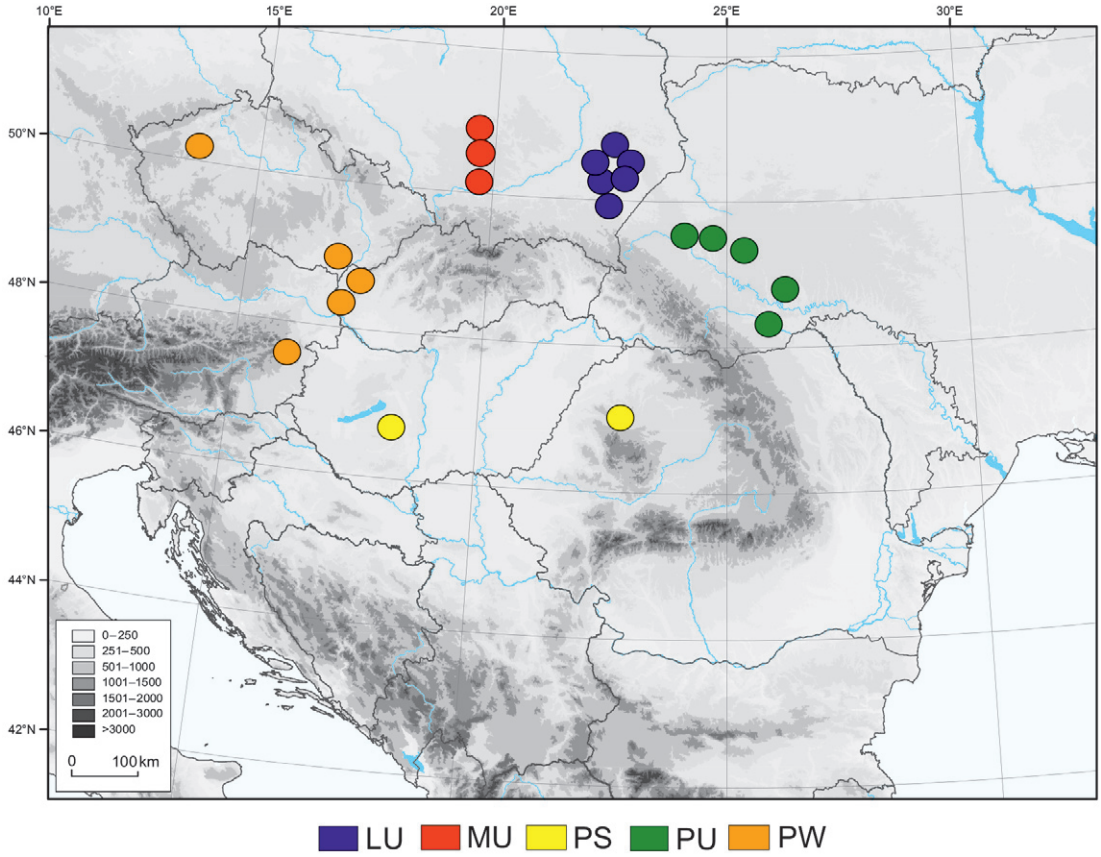

Fig. 21. Localities of the populations of *Linum flavum* L. Localities are divided into geographical groups; details in Table 13. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

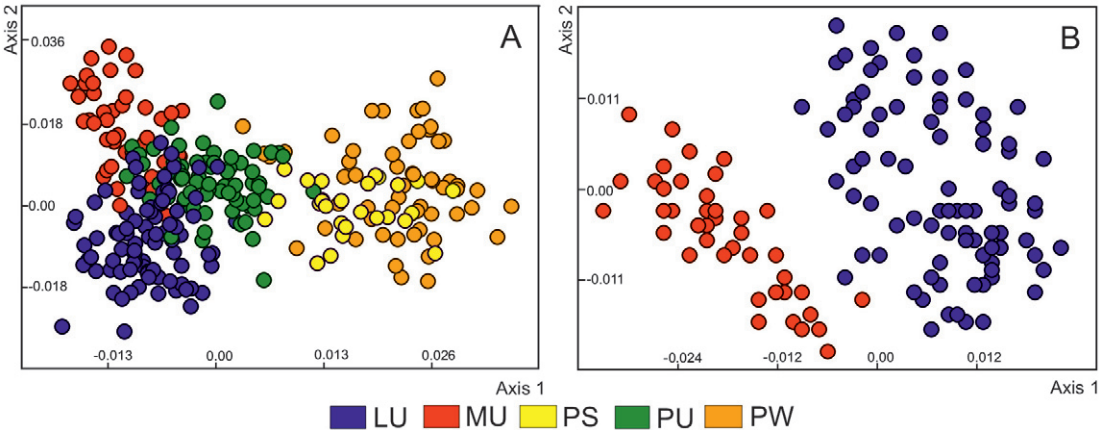

Fig. 22. PCoA scatterplot of *Linum flavum* L. individuals based on Nei and Li genetic distances of AFLP data. A – all studied populations, B – Polish populations. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part.

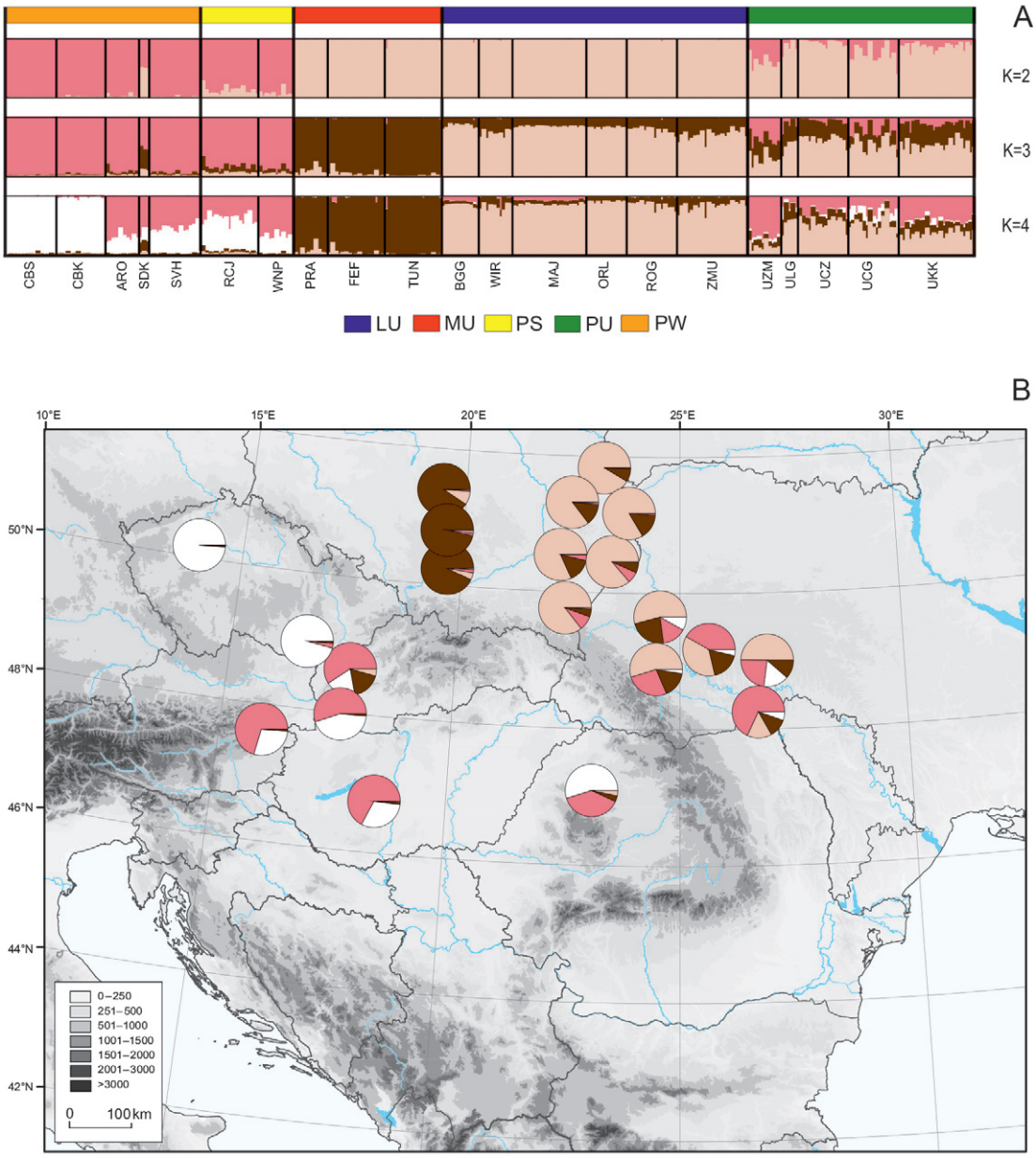

Fig. 23. Results of Bayesian analysis (TESS software) for *Linum flavum* L. A – bar graphs of individuals for K = 2, K = 3, K = 4. Populations are separated by vertical lines. B – pie charts showing the proportions of the clusters present within the populations, detected by the Bayesian analysis for K = 4. LU – Wyżyna Lubelska upland, MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PU – Podolian Upland, PW – Pannonian region, NW part. For population codes see Table 13.

Table 16. Analyses of molecular variance (AMOVA) based on AFLP markers for *Linum flavum* L. including different hierarchical levels and geographical groups (significance tests – 1023 permutations). d.f. – degrees of freedom, SS – sum of squares, F index – Fixation index, $p < 0.001$ if not marked otherwise.

Source of variation	d.f.	SS	Variance component	Percentage of variation	F index p
Among populations	20	2330.793	7.35565	31.06	
Within populations	267	4359.197	16.32658	68.94	F_{ST} 0.31060
Total	287	6689.990	23.68223	–	
Polish *vs* other populations	1	86.017	–0.32564	–1.38	F_{CT} –0.01384 $p < 0.9$
Among populations within groups	19	2244.776	7.52754	31.99	F_{SC} 0.31557
Within population	267	4359.197	16.32658	69.39	F_{ST} 0.30609
Total	287	6689.990	23.52847	–	
Wyżyna Małopolska *vs* Wyżyna Lubelska	1	145.925	0.00267	10.01	F_{CT} 0.10008 $p < 0.01$
Among populations within groups	7	1155.301	8.70127	23.42	F_{SC} 0. 26019
Within populations	126	2232.560	16.06158	66.58	F_{ST} 0. 33423
Total	134	3533.785	24.76552	–	
Among geographical groups	4	842.017	1.86322	7.76	F_{CT} 0.07764
Among populations within groups	16	1488.776	5.80974	24.21	F_{SC} 0.26245
Within populations	267	4359.197	16.32658	68.03	F_{ST} 0.31971
Total	287	6689.990	23.68223	–	

the Pannonian region and some Podolian Upland populations made a contribution to other populations from this area. The population group from the Wyżyna Małopolska upland formed a genetically separate group and made a minor contribution to populations from the Wyżyna Lubelska upland and Podolian Upland. The genetic pool dominating in the Wyżyna Lubelska upland group was also present in populations from the Podolian Upland.

For the STRUCTURE data set, the ΔK parameter indicated $K = 2$ as the most appropriate number of groups. For $K = 3$ and $K = 4$ most individuals were assigned to a specific group with a probability of 0.9 or more, and some populations were mixed. The groups formed for successive K values in STRUCTURE and TESS were identical, but genetic homogeneity was greater for each population group in STRUCTURE than in TESS. $K_{ef} = 4$ was finally accepted based on simulations in TESS (Fig. 23).

The Wyżyna Małopolska upland and Wyżyna Lubelska upland population groups formed separate internally homogeneous groups in Bayesian analysis of the Polish populations in TESS for $K = 2$ (data not shown). For $K = 3$ the Wyżyna Małopolska upland populations formed a genetically homogeneous group, and two subgroups were separated within the Wyżyna Lubelska upland: populations BGG and MAJ vs ORL, ROG, WIK and ZMU. Further divisions were not statistically significant.

Linum hirsutum

I analyzed 184 individuals of *Linum hirsutum* (Table 17) and recorded 253 bands in the polymorphism analysis, including 164 polymorphic bands (64.8%). The genetic diversity of *L. hirsutum* in the study area is low: Nei's gene diversity index is $M_H = 0.19$ (SD = 0.18) and the Shannon index is $M_S = 0.30$ (SD = 0.26). The gene flow coefficient is $M_{Nm} = 0.77$. The Mantel test showed a significant

Table 17. Origin of plant material of *Linum hirsutum* L. used in the present study. MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PW – Pannonian region, NW part (for details see Material). Material collected by the author if not marked otherwise.

Abbreviation	Geographical group	Locality	Latitude (N)/ Longitude (E)
URA	PU	Ukraine, Podolian Upland, Romashkovje	48°14′/29°19′
UTR	PU	Ukraine, Podolian Upland, S of Chechel'nik	48°12′/29°20′
UMO	PU	Ukraine, Podolian Upland, N of Mogilyiv Podyil's'kij	48°29′/27°48′
UZD	PU	Ukraine, Podolian Upland, Ust'e; on N bank of Dnyistjer river	48°33′/26°41′
WAL	MU	Poland, Wyżyna Małopolska upland, Dosłońce	50°20′/20°13′
FEF	MU	Poland, Wyżyna Małopolska upland, Feflówka	50°21′/20°09′
SKO	MU	Poland, Wyżyna Małopolska upland, Skorocice Reserve	50°25′/20°40′
PIN	MU	Poland, Wyżyna Małopolska upland, Pińczów	50°32′/20°31′
CKZ	PW	Czech republik, Bílé Karpaty Mountains, Veselí n./Moravou	49°01′/17°34′
SDK	PW	Slovakia, Malé Karpaty Mountains, Devínska Kobyla	48°11′/16°59′
ARO	PW	Austria, Niederösterreich, Marzer Kogel	47°43′/16°27′
WNK	PS	Hungary, Dunántúli-középhegység Mountains, Nagykovácsi Szénás, hill	47°35′/18°52′
WDK	PS	Hungary, Dunántúli-középhegység Mountains, Gerecse-hegység; Kálvária hill, *leg. K. Dobolyi*	47°42′/18°43′
WNP	PS	Hungary, Mezőföld lowland, Puputeve-hát, hill, *leg. K. Dobolyi*	46°44′/18°49′

Table 18. Parameters of AFLP genetic diversity for populations of *Linum hirsutum* L. N – number of individuals in population, $\%_{poly}$ – percentage of polymorphic bands, $M_{d/p}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_S – mean (±SD) Shannon genetic index, M_{DW} – frequency down-weighted marker values. For population codes see Table 17.

Population code	N	$\%_{poly}$	$M_{d/p}$	M_H (±SD)	M_S (±SD)	M_{DW}
URA	11	32.81	0/7	0.11 (±0.11)	0.17 (±0.25)	19.84
UTR	12	34.39	0/7	0.12 (±0.18)	0.18 (±0.26)	23.29
UMO	15	30.43	0/4	0.10 (±0.17)	0.19 (±0.25)	29.18
UZD	14	37.15	0/4	0.13 (±0.18)	0.19 (±0.26)	33.03
WAL	24	47.04	0/1	0.15 (±0.18)	0.23 (±0.27)	38.61
FEF	16	26.48	0/0	0.09 (±0.17)	0.14 (±0.25)	34.03
SKO	11	31,23	0/0	0.11 (±0.17)	0.16 (±0.25)	14.51
PIN	21	42.29	0/1	0.14 (±0.19)	0.22 (±0.26)	33.63
CKZ	13	36.76	0/3	0.13 (±0.18)	0.19 (±0.26)	18.05
SDK	14	38.74	0/2	0.13 (±0.18)	0.19 (±0.26)	21.96
ARO	10	33.20	0/7	0.12 (±0.18)	0.17 (±0.25)	18.91
WNK	7	29.64	0/5	0.11 (±0.17)	0.15 (±0.25)	10.12
WDK	6	24.90	0/5	0.10 (±0.17)	0.14 (±0.25)	10.51
WNP	10	29.64	0/1	0.12 (±0.18)	0.18 (±0.18)	14.43

correlation between genetic diversity and geographical distance ($r = 0.41$, $p < 0.05$, $Z = 6423.61$ and $g = 3.59$).

The number of polymorphic bands ranged from 63 (24.9%; WDK, Hungary) to 119 (47%; WAL, Poland), and the population mean was 87 (34.4%; SD = 15.09). Discriminating bands were not detected in the populations. Forty-seven private bands were recorded in 12 populations, the highest number (7) in populations URA, UTR (Ukraine),

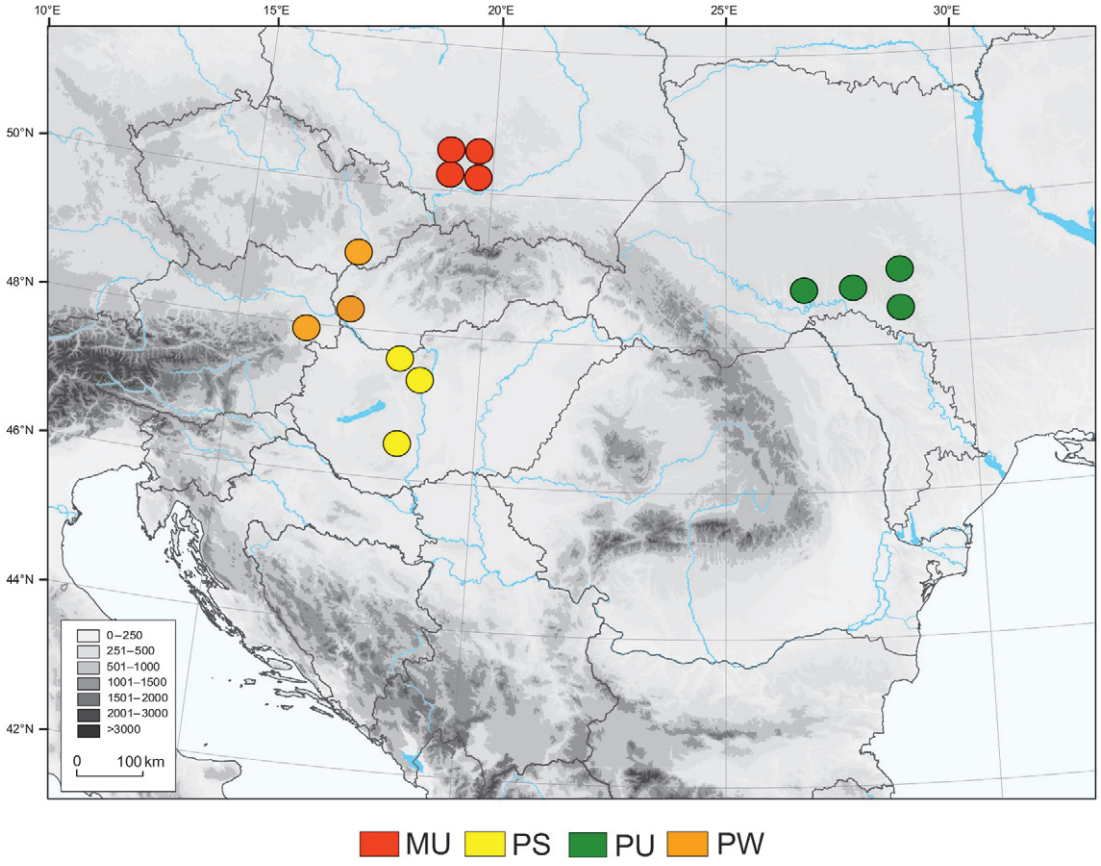

Fig. 24. Localities of the populations of *Linum hirsutum* L.; details in Table 17. MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PW – Pannonian region, NW part.

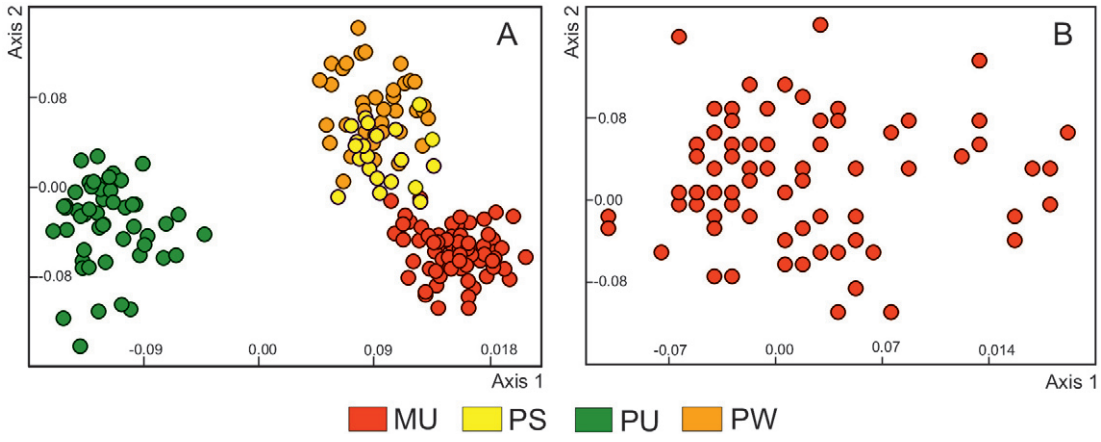

Fig. 25. PCoA scatterplot of *Linum hirsutum* L. individuals based on Nei and Li genetic distances of AFLP data. A – all studied populations, B – Polish populations. MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PW – Pannonian region, NW part.

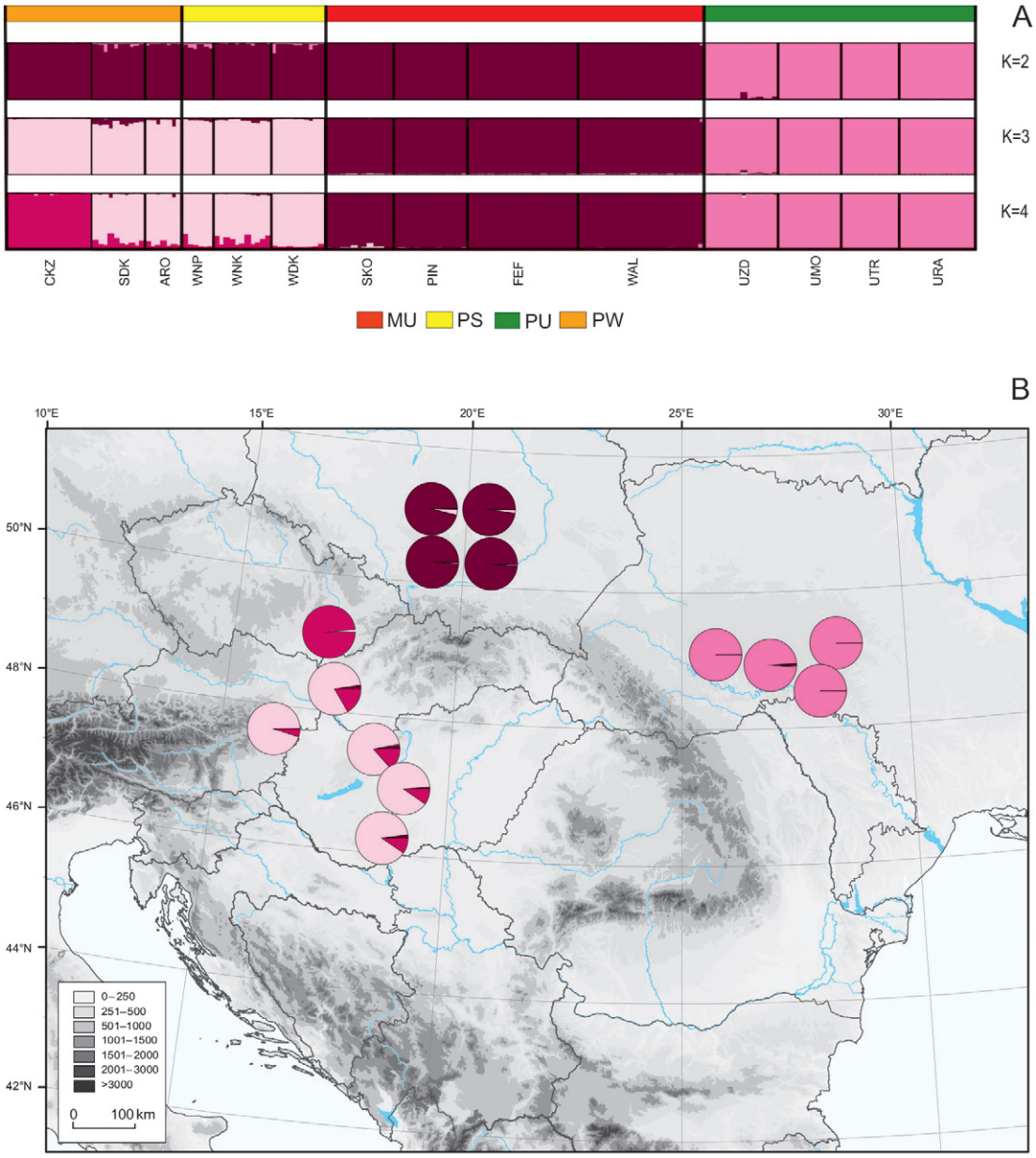

Fig. 26. Results of Bayesian analysis (TESS software) for *Linum hirsutum* L. A – bar graphs of individuals for K = 2, K = 3, K = 4. Populations are separated by vertical lines. B – pie charts showing the proportions of the clusters present within the populations, detected by the Bayesian analysis for K = 4. MU – Wyżyna Małopolska upland, PS – Pannonian region, S part, PW – Pannonian region, NW part. For population codes see Table 17.

Table 19. Parameters of AFLP genetic diversity for geographical groups of *Linum hirsutum* L. N – number of individuals in group, $\%_{poly}$ – percentage of polymorphic bands, $M_{dR/pR}$ – discriminating/private bands, M_H – mean (±SD) Nei's genetic diversity, M_{Nm} – value of gene flow.

Geographical group	N	$\%_{poly}$	$M_{dR/pR}$	M_H (±SD)	M_S (±SD)	M_{Nm}
Podolian Upland (PU)	52	49.80	1/2	0.15 (±0.18)	0.24 (±0.27)	1.59
Wyżna Małopolska upland (MU)	72	55.34	0/2	0.17 (±0.19)	0.26 (±0.276)	1.494
Pannonian region, NW part (PW)	37	52.57	0/3	0.17 (±0.19)	0.25 (±0.27)	1.41
Pannonian region, S part (PS)	23	47.43	0/1	0.15 (±0.19)	0.23 (±0.27)	1.31

Table 20. Analyses of molecular variance (AMOVA) based on AFLP markers for *Linum hirsutum* L. including different hierarchical levels and geographical groups (significance tests – 1023 permutations). d.f. – degrees of freedom, SS – sum of squares, F index – Fixation index, $p < 0.001$ if not marked otherwise.

Source of variation	d.f.	SS	Variance component	Percentage of variation	F index p
Among all populations	13	1979.731	10.43672	38.78	
Within populations	170	2801.399	16.47882	61.22	F_{ST} 0.38776
Total	183	4781.130	26.91553	–	
Polish populations *vs* other populations	1	477.164	3.65843	12.76	F_{CT} 0.12757 $p < 0.02$
Among populations within groups	12	1502.567	8.54109	29.78	F_{SC} 0.34137
Within populations	170	2801.399	16.47882	57.46	F_{ST} 0.42539
Total	183	4781.130	28.67834	–	
Wyżyna Małopolska					
Among populations	3	203.834	2.81117	13.21	
Within populations	68	886.083	18.46282	86.79	F_{ST} 0.13214
Total	71	1459.306	21.27399	–	
Among geographical groups	3	1093.648	6.35849	22.42	F_{CT} 0.22423
Among populations within groups	10	886.083	5.51964	19.46	F_{SC} 0.25091
Within populations	170	2801.399	16.47882	58.11	F_{ST} 0.41888
Total	183	4781.130	28.35695	–	

ARO (Austria), and (5) in WDK and WNK (Hungary); only one private band was found in populations PIN, WAL (Poland) and WNP (Hungary). Nei's gene diversity index ranged from 0.09 (FEF, Poland) to 0.15 (WAL, Poland), with a mean of 0.12 (SD = 0.018), and the Shannon index ranged from 0.14 (FEF, Poland and WDH Hungary) to 0.23 (WAL, Poland), with a mean of 0.17 (SD = 0.03). The M_{DW} coefficient varied greatly and ranged between 10.12 (WNK, Hungary) and 38.61 (WAL, Poland), with a mean for the populations of 22.86 (SD = 9.35) (Table 18).

In the polymorphism analysis of predefined geographical groups (see Materials) only one discriminating band for the Podolian Upland populations was detected. Two private bands were identified for the Wyżyna Małopolska upland and Podolian Upland, three for the northwestern part of the Pannonian region, and one for the southern part of the Pannonian region. The highest within-region variation of Nei's gene diversity index, Shannon index and number of characteristic bands was found for the Wyżyna Małopolska upland group (Table 19).

Only the M_{DW} index differed significantly between the population group from Poland and the population group from the southern Pannonian region ($U = 0$, $Z = 2.12$, $p < 0.03$). It also differed significantly between the southern Pannonian populations and those in the Podolian Upland ($U = 0$, $Z = 2.12$, $p < 0.03$) and the northwestern part of the Pannonian region ($U = 0$, $Z = -1.96$, $p < 0.05$).

Three genetic groups can be delimited based on the NJ dendrogram. The populations from the Podolian Upland form a strongly divergent cluster (99% bootstrap support). There is a further division between the Wyżyna Małopolska upland populations and the Pannonian region populations but with lower bootstrap support (data not shown).

The PCoA ordination of *Linum hirsutum* confirms the results of cluster analysis (Fig. 25A, B). Axes 1 and 2 account for 24.26% and 7.03% of the variation, respectively. Along the first axis the Podolian Upland populations (left side of plot) are clearly separated from the other populations. In the other group, the Wyżyna Małopolska upland populations are separated from the Pannonian region along axis 2 but without an evident hiatus. The arrangement shows that the Wyżyna Małopolska upland populations are more similar to those in Southern Europe than to those from eastern areas, represented here by the Podolian Upland.

AMOVA at the level of *L. hirsutum* populations demonstrated the dominance of intra- over inter-population variation: 61.22% and 38.78%, respectively ($F_{ST} = 0.38$, $p > 0.001$). Separate analysis of the Polish populations and hierarchical analysis of geographical regions also indicated a high share of within-population variation. In each case AMOVA demonstrated much greater intrapopulation variation than between groups and between populations within groups (Table 20).

In Bayesian analysis of the whole data set in TESS for $K = 2$, the population group from the Podolian Upland was separated from other populations of *L. hirsutum* in Central Europe (Fig. 26). For $K = 3$ the three groups segregated populations geographically (Podolian Upland, Wyżyna Małopolska upland and Pannonian region). This division shows that the populations differ genetically between regions.

Bayesian analysis in STRUCTURE yielded the same division for $K = 2$ and $K = 3$. ΔK had the highest value at $K = 2$, but $K = 3$ was chosen as the most appropriate number of AFLP groups. Individuals were assigned to groups with a probability of 0.9. $K_{ef} = 3$ was finally accepted based on simulations in TESS (Fig. 26).

In a separate Bayesian analysis of the Polish populations of *Linum hirsutum* (only from the Wyżyna Małopolska upland) in TESS, populations FEF and WAL were distinguished from PIN and SKO for $K = 2$ (data not shown). Further divisions were not significant.

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These analyses of the genetic variation and diversity of all the studied species indicate genetic divergence between the populations from two Polish regions – the Wyżyna Małopolska upland and the Wyżyna Lubelska upland – as well as between the Polish populations and those in areas east and south of them. Regardless of the species, the Wyżyna Lubelska upland populations are more similar to those from the Podolian Upland than to those from the Pannonian region or the Balkans. In the Pannonian population group, the populations from the Czech Republic (northwestern part of Pannonian region) are distinguished from the other populations of this area (Slovakia, Hungary, Romania) by having greater homogeneity and a contribution from a different genetic pool. Still, the Pannonian group shows greater genetic similarity between its populations than with those of the Podolian upland. This structure suggests a genetic division between populations on the northern and southern sides of the Carpathian Mts and Sudeten Mts.

Discussion

Genetic variation of steppe species in Central Europe

In this study the genetic structure of five steppe species was analyzed: *Carlina onopordifolia*, *Cirsium pannonicum, Inula ensifolia*, *Linum hirsutum* and *L. flavum*. The level of genetic variation was

uniform and comparable across all the populations of each species. Statistically significant differences between parameters describing intrapopulation variation were not found. Genetic variation was high for four species (*Cirsium pannonicum*, *Inula ensifolia*, *Linum flavum*, *L. hirsutum*) and very low for *Carlina onopordifolia*. Molecular variance analysis confirmed the high genetic variation in the four species. Intrapopulation variation ranged between 61.22% and 82.78% among those four species and dominated over interpopulation variation, which ranged from 17.22% to 38.78%. The high level of variation within populations was confirmed by low F_{ST} values ranging between 0.17 and 0.38. Intrapopulation variation in *Carlina onopordifolia* was low at only 6.90%, and F_{ST} was very high at 0.93.

FACTORS DETERMINING THE LEVEL OF GENETIC VARIATION IN STEPPE SPECIES

The genetic variation of a species results from a variety of factors, some related to the evolutionary history of a species and some related to its biology: the breeding system, life cycle length, and ecology. Low genetic variation is frequently recorded in long-lived perennial life forms in which cross-fertilization is not observed (selfing, apomictic species) and whose range is small. High genetic variation is usually noted in short-lived perennial life forms that have a generative breeding system (or in which mixed mating occurs) and a wide geographical range (Hamrik & Godt 1996; Nybom 2004). In this study I found high intrapopulation variation in four species: *Cirsium pannonicum, Inula ensifolia*, *Linum flavum* and *L. hirsutum*. These species are short-lived and have continuous ranges (in particular, their peripheral island localities in Central Europe are not very distant from the range center) and either a generative or mixed breeding system (Kaźmierczakowa 1991; Ockendon & Walters 1968; Werner 1976; Tutin 1976). Intrapopulation variation was low in *Carlina onopordifolia*, which is a semelparous apomictic perennial with a small range (Poznańska 1991; Poznańska & Spiss 1985). The level of genetic variation I found in the five steppe species corresponds with their biological traits as described in the literature (Nybom 2004 and references therein).

The high and uniform level of intrapopulation variation shows that the species are rather genetically homogeneous. Extrazonal Polish populations on the northern or northwestern limit of the range are not genetically depauperate in relation to populations closer to the central parts of their ranges. This distribution of genetic variation suggests the absence of genetic processes such as genetic drift and/or inbreeding which would lead to reduced genetic variation and/or increased interpopulation diversity in populations on the range periphery (Eckert *et al*. 2008). It is thought that a species with a high level of variation, related to greater genetic plasticity, can better adjust to environmental changes (Hamrick 1982; Booy *et al*. 2000; Gitzendanner & Soltis 2000). The high level of genetic variation in four of the five species may be the main factor in their adaptation to changing habitats, mostly induced by anthropopression. *Carlina onopordifolia* is a different case, as it had very low intrapopulation variation. Meusel and Kästner (1994) believe that apomixis observed in this taxon is the main factor facilitating its colonization and persistence at new sites. Apomixis may allow *C. onopordifolia* to persist at its present localities despite the transformations taking place in its environment. In apomixis the most important factor is thought to be uniparental reproduction, enabling single individuals to colonize new areas and ensuring reproduction without the need for pollinators (Hörandl *et al*. 2008; Hörandl 2011).

Note that two different types of reproduction – generative and apomictic, generating two different levels of genetic variation – could give the corresponding species similarly effective opportunities to migrate to new areas.

Although the level of genetic variation in the populations was homogeneous, PCoA and Bayesian analysis showed genetic differentiation between geographical regions. Especially in PCoA, individuals of the Wyżyna Małopolska upland populations formed a compact subgroup, relatively homogeneous as compared to populations from other regions (Figs 13, 16, 19, 22, 25). The Mantel test confirmed the correlations between geographical

and genetic distance for each species, indicating that these species represent the genetic pattern of isolation by distance (Ellstrand & Elam 1993). The relationship in which variation increases with distance is generated first of all by limited gene flow. Genetic differentiation of a population results mainly from physical isolation such as presented by a variety of topographic barriers (mountains, rivers) or by distance between populations. In the latter case the range of dispersion of both pollen and seeds limits cross-fertilization despite the lack of a physical barrier.

Bayesian analysis for $K = 3$ and/or $K = 4$ (Figs 14, 17, 20, 23, 26) shows that populations north and south of the mountain barrier formed by the Sudeten Mts and Carpathian Mts do not share genetic structure. *Carlina onopordifolia*, which occurs only in the Pontic part, is the obvious exception. This may indicate that the Sudeten-Carpathian arc may have been and still is a physical barrier limiting gene flow in this group of species.

The pattern of genetic variation observed in *Cirsium pannonicum, Inula ensifolia*, *Linum flavum* and *L. hirsutum*, showing high variation within and low differentiation between populations, can also result from historical events affected by, for example, the rate or the manner in which these species spread (cf. Van Rossum & Prentice 2004; Duminil *et al*. 2007). The distribution of intra- and interpopulation genetic diversity may also reflect gene flow between populations in the past when distance did not present a barrier because the populations occurred in higher abundance. A uniform level of variability without genetic differentiation over a large area indicates that the species migrated in a broad wave (step by step) (Hewitt 1999; Austerlitz *et al*. 2000; Helm *et al*. 2006; Šmídová *et al*. 2011). An even distribution of genetic variation can suggest that phenomena such as the founder effect (loss of alleles and increased homozygosity observed in newly formed populations due to their low abundance; Hewitt 1999) did not play a role in shaping the genetic structure.

In *Carlina onopordifolia*, where intrapopulation genetic variation was low, the main factor determining the level of genetic variation is attributable to its biological traits. Its high interpopulation differentiation may be either the result of biological characteristics or the effect of historical events that affected the speed and the manner in which it spread.

STEPPE SPECIES IN THE UPLANDS OF SOUTHERN POLAND: PROBABLE MIGRATION ROUTES AND DIRECTIONS

The history of migration of steppe plants to southern Poland is closely correlated with changes in the flora of Central Europe during dynamic climatic transformations in the Quaternary period. Favorable periods of climate, habitat availability and the absence of natural barriers such as forests (Late and Upper Pleistocene) promoted the spread of steppe plants from Southern Europe as well as Eurasian sites (Środoń 1977).

The analysis of the genetic structure and variation of *Carlina onopordifolia*, *Cirsium pannonicum*, *Inula ensifolia*, *Linum flavum* and *Linum hirsutum* showed that while the level of genetic variation in all the populations was uniform, the distribution of genetic pools in populations varied depending on their geographical location. Two main patterns of genetic structure were identified based on the spatial distribution of genetic lineages (Bayesian analysis results, Figs 14, 17, 20, 23, 26).

The first type was observed in *Linum hirsutum*. In this case, the genetic homogeneity of the populations from northern and southern parts of the Carpathians indicates that the migration pathway to the Wyżyna Małopolska upland probably was only from Pannonia through the Moravian Gate and/or across the Carpathian Mts (e.g., passes or river valleys).

The genetic separateness of the Podolian Upland from the Wyżyna Małopolska upland and the Pannonian region corresponds to the distribution pattern of this species (Meusel *et al*. 1978), in which the Pannonian and Podolian parts of the range are separated by the Carpathians.

The second pattern was observed in the four other species: *Carlina onopordifolia*, *Cirsium pannonicum*, *Inula ensifolia* and *Linum flavum*. Here, Wyżyna Małopolska upland populations were

related to Podolian Upland populations through Wyżyna Lubelska upland populations, as genetic pools from both the Wyżyna Małopolska upland and Podolian Upland were detected in the Wyżyna Lubelska upland. Only the *Carlina onopordifolia* populations from the Wyżyna Małopolska upland were clearly divergent genetically from the Wyżyna Lubelska upland and Podolian Upland, as the main genetic split in this species (K = 2). In the other species the Wyżyna Małopolska upland populations differed genetically from the other regions in further analyses (K = 3 or K = 4).

In all species (except *Carlina onopordifolia* – Pontic element absent) no genetic pool characteristic of northern Pannonian populations was detected in Wyżyna Małopolska upland populations. Admixtures of genetic groups dominating in the Wyżyna Małopolska upland, Podolian Upland and the south of the Pannonian region were found in the Wyżyna Lubelska upland populations.

These results suggest that, irrespective of their genetic structure, the studied steppe species migrated most probably from the south and southeast to the northern part of Central Europe, including the uplands of southern Poland. The south-north migration probably followed different routes: a direct path from the south northwards, mainly across the Carpathian Mts, and a second one occasionally through the Moravian Gate. The routes ran from the south of Europe first towards the northeast and then northwest via the Podolian Upland along the north flank of the Carpathian Mts.

The probable direct migration route from the south northwards through the Carpathian arc is represented by only one species, *Linum hirsutum*. Apparently it did not migrate through the Moravian Gate but crossed the Carpathians along river valleys or passes of the Beskid Niski Mts, as suggested by the distribution of its localities in the area (Pawłowski 1925; Tacik 1959; Szafer 1977b). The north flank of the Carpathian Mts probably was the main migration route of the four other species: *Carlina onopordifolia*, *Cirsium pannonicum*, *Inula ensifolia* and *Linum flavum*. The pathway ran from the south through the Bessarabian Gate and the Podolian Upland belt to Poland. My genetic data did not support a direct route through the depression between the Sudeten Mts and Carpathian Mts (Moravian Gate), regarded up to now as a very important route especially for the Wyżyna Małopolska upland (Szafer 1926, 1977b; Medwecka-Kornaś & Kornaś 1977). Nor did the analyses of these species support migration to Poland from the west via the foreland of the Ore Mts and Sudeten Mts.

No immediate migration barriers obstructed the route from the east to southern Poland; the Sudeten-Carpathian mountain arc posed a natural obstacle to direct migration from southern regions. Consequently, migrations towards the northwest on both sides of the Carpathian arc occurred independently. This is confirmed by the distribution of genetic lineages and their dissimilarity on the northern and southern flanks of the arc. The dissimilarity is particularly evident between the populations from the Wyżyna Małopolska upland and the northern part of the Pannonian region (except *Linum hirsutum*) (Bayesian analysis for K = 4). Pawłowski (1925) long ago referred to the Carpathian arc as an important barrier to migration of steppe vegetation from the south of Europe to Poland.

Molecular analyses suggest that the main migration route of the four species to the southern uplands of Poland was from the east. This is consistent with Szafer's (1946) view that the route from the east and southeast is the most important one for steppe vegetation, based on the number of species that apparently migrated this way as well as the absence of barriers. As the analyses did not confirm that the five species migrated directly from the south through the Moravian Gate and through the lower parts of the Carpathians (passes in Beskid Niski Mts and/or along river valleys only in the case of *Linum hirsutum*), this route may have played a considerably less important role than previously believed (e.g., Szafer 1926, 1946, 1977b; Medwecka-Kornaś & Kornaś 1977). The spatial distribution of genetic groups across populations indicates that steppe species migration also proceeded mostly westwards along Poland's southern upland belt from the Wyżyna Lubelska upland to the Wyżyna Małopolska upland. The current distribution of *Linum hirsutum*, which occurs only

in the Wyżyna Małopolska upland, might suggest that it did not follow that route, but we cannot rule out the possibility that in earlier glacial periods after the largest glaciation it could also have migrated westwards to Poland. A historical locality in the Wyżyna Lubelska upland (Fijałkowski 1954) holds such a possibility, but the literature data on the locality was never confirmed in the field and herbarium vouchers were not found, so for now the record is not taken as reliable.

TIME OF MIGRATION OF STEPPE SPECIES TO SOUTHERN POLAND

The arrival time of the studied species in the uplands of southern Poland has been a matter of debate. According to Szafer (1977), the history of steppe association formation in the upland belt from Silesia to the Vistula valley, including the Wyżyna Małopolska upland, goes back to the late glacial period and the Holocene. The majority of steppe flora taxa in Roztocze and the Wyżyna Lubelska upland would have arrived in the last glacial period and the early postglacial period (Szafer 1977b). Some components of the contemporary northern European steppe flora may have come even before the last glacial period (Szafer 1946; Pawłowska 1977) but after 'the biggest glaciation in the geographical area of Poland' (Szafer 1946). The temporal ranges presented in this section (e.g., biggest or younger glaciations, L3 or L4) are cited after their authors. Based on the currently accepted stratigraphy of the Pleistocene in Poland, the 'L3 glacial period' or 'the biggest glaciation in the geographical area of Poland' coincided with the Mindel glaciation, currently known as Sanian 1, Sanian 2 glaciation (Lindner & Marks 2008). Glacial periods younger than that coincide with the Riss glacial period, equivalent to the Odranian, Wartanian glaciation, while the youngest glacial period in Poland, the Vistulian, corresponds with the Würm (Kulczyński 1927; Lindner & Marks 2008). Researchers agree on the factors regarded as most favorable for the migration of steppe species to the north of Central Europe. These include environmental factors and species-specific traits. It is generally believed that the cold glacial periods of the Quaternary encouraged migration in this group. The most important factors (after Szafer 1918, 1946) are these: (*i*) steppe species' resistance to low winter temperatures and summer droughts, (*ii*) the absence of deciduous forests which would have hindered the free and rapid spread of these heliophilous plants, (*iii*) decreased competition due to the general impoverishment of the flora during glacial periods, and (*iv*) the presence of wide, treeless valleys with dry, bare slopes, together with the presence of loess areas which were particularly convenient migration routes for this element to migrate westward from the east.

According to Dziubałtowski (1916), xerophyte species (term he used interchangeably with 'steppe species') migrated to the Wyżyna Małopolska upland during the interglacial after the preceding 'biggest glaciation in the geographical area of Poland' (Sanian 1, Sanian 2 glaciation), promoted by the climate and the availability of sites. Also according to Kozłowska (1923), the first penetration of species associated with the *Inuletum ensifoliae* association into the Wyżyna Małopolska upland may have taken place after 'the L3 glaciation had receded' (Sanian 1, Sanian 2) at *ca* 440 ka or 'with the advance of L4' (Odranian, Wartanian glaciation). Szafer (1923, 1930) believed that *Carlina onopordifolia* belongs to one of the first groups that colonized the Wyżyna Małopolska upland and that it arrived in the interglacial period to the area of an 'older glaciation [Sanian 1, Sanian 2 glaciation] that was not covered with a younger glaciation' (Odranian, Wartanian glaciation). He also stated that its migration westwards took place by two routes; the first led to the Wyżyna Małopolska upland and other to central Opillya (western part of Podolian Upland).

Kozłowska (1923) believed that the formation of loess areas south of the ice sheet edge was the main factor in the migration of steppe species to the Wyżyna Małopolska upland during L4 (Odranian, Wartanian glaciation) at *ca* 130 ka. Based on a comparison of forest formations in southern Poland, she suggested that the density of forest areas on loess generally depended on the thickness of the loess layer. Forests developed where the loess layer was shallow, while the terrain over

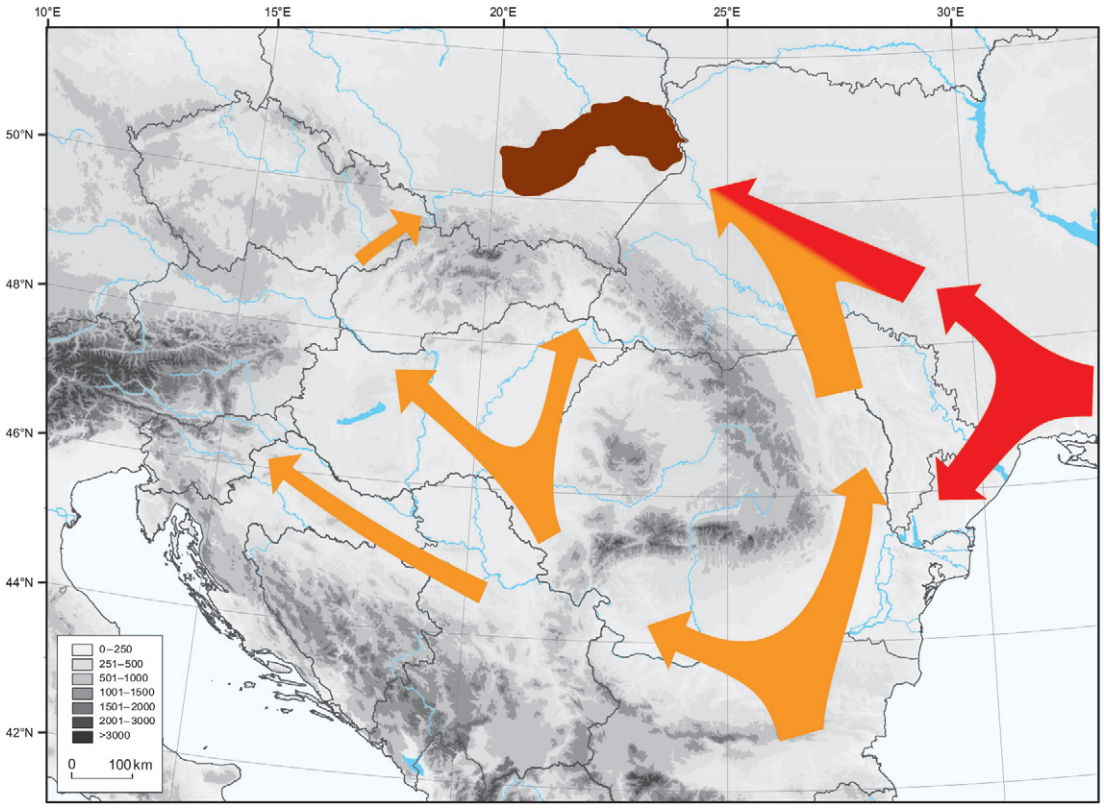

Fig. 27. Probable directions and routes of the migrations of *Carlina onopordifolia* Besser *ex* Szafer, *Cirsium pannonicum* (L. fil.) Link., *Inula ensifolia* L., *Linum flavum* L. and *L. hirsutum* L. into southeastern Polish uplands (brown area), based on results presented in the paper and literature: southeastern route from Pontic region (red arrows) and the southern route from the Pannonian Basin and the Balkans (orange arrows).

a thicker layer had no forest. Isolated unforested areas probably were the only sites on which heliophilous steppe species could occur and survive further interglacial periods (which promoted the development of forests) after L4 (Odranian, Wartanian glaciation). She also stated that the uneven distribution of forest on loess in southern Poland enabled later migration of the steppe element to the Wyżyna Małopolska upland in the subboreal period of the Holocene (Kozłowska 1928). Data permitting a test of the correlation between loess layer thickness and the degree of forestation are not available, however.

My study showed that the populations of each of the five species in the Małopolska part of Poland's southern upland belt clearly diverge genetically from those of other regions (Bayesian analysis). The divergence of the Wyżyna Małopolska upland populations was strongest for *Carlina onopordifolia*. Generally, the genetic pool characteristic for populations from the Małopolska part of the study area contributes only a slight admixture to the Wyżyna Lubelska upland populations and is not shared with other regions.

The pattern of genetic structure recorded in this study (genetic homogeneity of populations and their dissimilarity from populations in other areas) seems to correspond to an earlier, possibly the most important colonization of the Wyżyna Małopolska upland, which may have occurred between the L3 (Sanian 1, Sanian 2 glaciation) and L4 (Odranian, Wartanian glaciation). A later migration or migrations may have taken place during the late glacial period and/or the postglacial period as suggested

by Kozłowska (1926, 1928) and Szafer (1930) from the east or southeast, but may have reached and covered only the Wyżyna Lubelska upland.

Two results suggest that the populations of the Wyżyna Małopolska upland are of older origin than those of the Wyżyna Lubelska upland: the genetic lineages of the Wyżyna Małopolska upland species were characteristic of that upland only; and there were evident genetic connections between the populations of the Wyżyna Lubelska upland (including the Podolian Upland belt) and the southern Pannonian region. The populations from the Wyżyna Małopolska upland would then be a relict of a migration wave that likely took place after Sanian 1, Sanian 2 glaciation, when the steppe element could penetrate southern Poland (Szafer 1923, 1946; Kozłowska 1926; Pawłowska 1977). This may have been the only migration wave of the study species that reached the Wyżyna Małopolska upland. In such a scenario the present populations of these species would be relicts of the glacial period in the Wyżyna Małopolska upland. The western part of the range of a subsequent migration wave (or waves) of the steppe element in southern Poland was most probably limited to the Wyżyna Lubelska upland. Possibly the present steppe species in the Wyżyna Lubelska upland are elements of a migration in the late glacial or postglacial period from the east or southeast; this would be consistent with other authors' views (Kozłowska 1928; Szafer 1946; Pawłowska 1977). Unfortunately the precise time and number of migration waves cannot be identified from the pattern of genetic structure in the study species. Nor do the data (uniform level of genetic variation across the studied area) clearly indicate where these species had refugia in the studied parts of Europe. However, the repeatedly inferred connection with the east suggests that the most important refugial areas were east of the present Polish border.

Conclusions

My analysis of genetic variation in five selected steppe species showed a uniform level of the genetic diversity/variability of all the populations of *Carlina onopordifolia*, *Cirsium pannonicum*, *Inula ensifolia*, *Linum flavum* and *Linum hirsutum*. It also revealed location-dependent differences in the distribution of genetic lineages in the populations.

My examination of the individual migration routes of the five species from the south to the north of Central Europe, including the uplands of southern Poland, suggests that the main migration route ran from the east or southeast westwards on the north side of the Carpathian Mts (Fig. 27). The present data indicate that a direct route from the south via the Moravian Gate and/or passes and valleys of the Carpathians did not play a major role in northward migration. The divergence of genetic lineages identified in the study may also mean that the Wyżyna Małopolska upland populations are older than and have a history independent of the Wyżyna Lubelska upland populations, and that more than one migration wave of the steppe element occurred in the southern Polish uplands. The Wyżyna Małopolska upland populations may be remnants of a more ancient migration wave which arrived immediately after the Sanian 1, Sanian 2 glaciation, when the steppe element could penetrate southern Poland. For these five species it may be the only migration wave that reached the Wyżyna Małopolska upland. This would make these populations relicts of the glacial period in the Wyżyna Małopolska upland. The range of a subsequent migration wave or waves in the late glacial and/or postglacial period would be limited to the Wyżyna Lubelska upland.

Acknowledgements. I am indebted to Professor Zbigniew Mirek for inspiring me to approach this interesting subject and for encouragement and support during the course of the study. I am grateful to Professor Zbigniew Szeląg and Dr. Michal Ronikier for reviewing the manuscript and providing valuable comments and constructive suggestions. I thank Professor Zbigniew Mirek, Professor Róża Kaźmierczakowa and Dr. Wojciech Paul for advice, criticism and helpful discussions on the results of the survey, and also for critically reading and commenting on the manuscript. I thank Professor Dorota Nalepka, Professor Agnieszka Wacnik and Dr. Renata Stachowicz-Rybka for valuable discussions on the Quaternary history of the Polish flora. I am grateful to Dr. Anna Cwener, Dr. Konstantin Dobolyi, Dr. Piotr Kiełtyk, Professor František

Krahulec, Dr. Stanislav Španiel and Dr. Pavol Mereďa for collecting samples and to Dr. Ivana Jongepierová and Dr. Alexander A. Kagalo for advice on selection of the localities and for helpful collaboration during field studies. I thank Dr. Jakub Cieślak and Katarzyna Cieślak for their company and for very useful assistance during my field trips. All my colleagues and friends who were a source of support or contributed to this work in any way are greatly acknowledged. I especially thank Jolanta Urbanik for technical comments and assistance with drafting the figures and Dr. Jan J. Wójcicki for editorial support and collaboration. Collection and storage of samples of the species in Poland was done under permits from the regional Offices of Environmental Protection in Kielce, Kraków and Lublin. This work was funded by the National Science Centre (Poland) through grants no. N 304 15 46 33 and no. N 304 3000940 (partialy), and by the W. Szafer Institute of Botany, Polish Academy of Sciences, through its statutory fund.

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Received 10 December 2012