

Theses of doctoral (Ph.D.) dissertation



**THE ACTUAL AND HISTORICAL BIOGEOGRAPHY
OF *SYRINGA JOSIKAEA* JACQ. FIL. EX RCHB.**

Bertalan Lendvay

Doctoral School of Horticultural Science

Corvinus University of Budapest

Department of Genetics and Plant Breeding

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Name: Doctoral School of Horticultural Science

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Head of Ph.D School: Prof. Dr. Magdolna Tóth
Doctor of the Hungarian Academy of Sciences, D.Sc.
Corvinus University of Budapest,
Faculty of Horticultural Science,
Department of Fruit Sciences

Supervisors: Dr. Mária Höhn
Associate Professor, C.Sc.
Corvinus University of Budapest,
Faculty of Horticultural Science,
Department of Botany and Soroksár Botanical Garden

Dr. Andrzej Pedryc
Professor, D.Sc.
Corvinus University of Budapest,
Faculty of Horticultural Science,
Department of Genetics and Plant Breeding

The applicant met the requirement of the Ph.D. regulations of the Corvinus University of Budapest and the thesis is accepted for the defense process.

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Dr. Magdolna Tóth

.....
Dr. Mária Höhn

.....
Dr. Andrzej Pedryc

Head of Ph.D. School

Supervisors

INTRODUCTION

Syringa josikaea Jacq. fil. ex Rchb. is an endemic shrub species of the Apuseni Mountains (Romania) and the Ukrainian Carpathians. Following the recognition that *Syringa* species most similar to *S. josikaea* are found in East Asia, this narrow endemic has attracted the attention of botanists and has been considered a relict species for over a century; Pax (1898) was the first to interpret *S. josikaea* as a preglacial relict. His hypothesis steadily gained acceptance, and was supported by Middle Miocene and Quaternary fossils recovered from Central Europe. Assessment of relict species' historical biogeography may significantly complement our knowledge on the vegetation history of an area. Theories based on the distribution of species or few fossils may be confirmed or rejected by results of molecular genetic studies. In the past decades the development of molecular techniques gave new opportunities to reveal historical biogeography; in the case of *S. josikaea* it became possible to assess its divergence time from related species and its correlate the divergence time to geologic events.

Syringa josikaea is confined to a few localities in the Ukrainian Carpathians and the Apuseni Mountains. Due to its rarity *S. josikaea* is legally protected in both the Ukraine and Romania, and is listed in several international red lists. The accurate knowledge of the distribution of a species as rare as *S. josikaea* is essential for the establishment of its effective conservation strategy. However, the last comprehensive summary of its distribution was published by Fekete and Blattny (1913) until recently, although a systematic reassessment of its populations has been completed only in the Ukrainian Carpathians (Kohut 2013). The reassessment of the distribution of *S. josikaea* in the Apuseni Mountains was an urgent task in times when the anthropogenic affects on the forest ecosystems are increasing in the Apuseni Mountains.

Significant reproductive isolation may occur among disjunctly distributed and small populations; therefore the survival of the species may depend on the viability of single isolated populations. An assessment of the genetic diversity of *S. josikaea* populations and the distances among them may reveal the connectivity among populations. Our study may lead to important conservation implications, which can contribute to the establishment of an appropriate conservation strategy for the species.

DNA samples covering the entire distribution range and identification of variable DNA sites are inevitable for phylogeographic and conservation genetic studies.

AIMS

1. Assessment of the recent distribution of *Syringa josikaea* in the Apuseni Mountains (Romania) and of the size and vulnerability of its populations.
2. Development and characterization of microsatellite markers for *Syringa josikaea*, and identification of DNA sequences with intraspecific variability.
3. Determination of the divergence time of *Syringa josikaea* from its relatives based on molecular clock analysis.
4. Assessment of the intra-specific phylogeography of *Syringa josikaea*.
5. Estimation of the genetic relationship among populations of *Syringa josikaea* and assessment of the genetic diversity of the small populations.

MATERIALS AND METHODS

Assessment of the distribution of *Syringa josikaea* in the Apuseni Mountains, Romania

I reviewed the scientific literature pertaining to the distribution of *S. josikaea* in the Apuseni Mountains and revised its specimens deposited in herbariums dating back to the description of the species. I conducted a field survey covering all sites in the Apuseni Mountains where *S. josikaea* populations have ever been mentioned. I assessed the size of the populations, their habitat and recorded the local features possibly threatening them. Populations of *S. josikaea* were assessed along altogether 13 valleys. The GPS coordinates of the individuals or edges of contiguous clumps of individuals were recorded.

Sample collection

Population sampling covered all known 25 locations of *S. josikaea* with a representative sample collected from each population. We avoided re-sampling clones.

Identification of intra-specific variable DNA loci, development and characterization of nuclear microsatellite markers

Sequences of 21 plastid and nuclear genomic loci were obtained from individuals representing the distribution area of *S. josikaea* in order to test their variability. Of the 21 loci only two, nuclear ribosomal ITS and ETS, proved to be variable and were sequenced for altogether 62 individuals from all populations with four samples from the larger and two or one samples from the smaller populations.

I tested the applicability of twelve markers developed for related species. Two of these markers were developed by de la Rosa et al. (2002) for *Olea europaea* L., while the other ten were developed by Kodama et al. (2008) for *Ligustrum ovalifolium* Hassk. I attempted to optimize PCR reactions to amplify these loci; size-variability of the successfully amplified loci was tested on specimens from two distinct ranges of the distribution area of *S. josikaea*.

The microsatellite markers of the related species were complemented with microsatellite markers newly developed for *S. josikaea*. The marker development was based on the combination of the microsatellite-enriched library techniques of Bloor et al. (2001) and Zane et al. (2002). These protocols work as following: genomic DNA is fragmented, the microsatellite-containing DNA fragments are isolated, sequenced and PCR primers are designed on the flanking regions of the microsatellite sequences. The amplification and size-variability tests of the developed markers were similar as for the markers developed for the related species.

I calculated the variability of the successfully amplified and variable markers and tested their power to discriminate among individual DNA samples.

Calculation of the divergence time of *Syringa josikaea* with molecular clock analysis

I calculated the divergence time of *S. josikaea* from its relatives with a fossil calibrated phylogenetic tree. The analysis was based on nuclear ribosomal ETS and ITS sequences, which were aligned independently, and their phylogenetic congruence was tested. The substitution models best fitting the two loci were identified and Markov-chain Monte Carlo simulation was performed with BEAST 1.7.5 (Drummond et al. 2012) software. Based on the heterogeneity of the substitution rates an uncorrelated lognormal relaxed clock was set. For calibrating the molecular clock the oldest known *Fraxinus* fossil (Call and Dilcher 1992) was used. With this I determined the upper age of the divergence of the *Fraxinus* and *Syringa* genera as 40 million years.

Sequence variability among the populations of *Syringa josikaea*

The variable ETS and ITS sequences were collapsed to ribotypes (ribosomal genotypes). The ribotypes of the two most closely related *Syringa* species, *S. villosa* and *S. wolfii* ribotypes were included in the analysis as outgroup. Based on the ribotypes a minimum-spanning haplotype tree was constructed with Arlequin 3.5 (Excoffier and Lischer 2010) and HapStar (Teacher and Griffiths 2011) software.

Microsatellite data analysis

The individuals of the *S. josikaea* populations were assigned to genetic groups with a bayesian nonhierarchical clustering method implemented in STRUCTURE 2.3.4 (Hubisz et al. 2009) software. The cluster number best fitting the data was determined with STRUCTURE HARVESTER (Earl and vonHoldt 2012). Based on the genetic characteristics revealed by STRUCTURE geographic barriers separating the populations were identified with Barrier 2.2 (Manni et al. 2004) software. The allele number, number of unique alleles and allele diversity (uHe) was calculated for the Apuseni Mountains and the Ukrainian Carpathians. The correlation between genetic and geographic distances was calculated with a Mantel-test. These analyses were performed in GenAlEx 6.5 (Peakall and Mouse 2012) software.

RESULTS AND DISCUSSION

Assesment of the recent distribution of *Syringa josikaea* in the Apuseni mountains, Romania

I found seven populations and planted specimens from an eighth, possibly extinct population. The populations are generally small; in four cases comprise only a few specimens. Several populations are threatened by logging and road constructions have caused the severe damage in the populations of *S. josikaea* in the past decades.

The divergence time of *Syringa josikaea*

Syringa josikaea forms a highly supported clade together with *S. villosa* and *S. wolfii* (Fig. 1). I revealed that the divergence time of *S. josikaea* from the species *S. villosa* and *S. wolfii* is 1,88 million years (confidence interval: 0.30-4.04 million years 95 % highest posterior density confidence interval, HPD). This means that *S. josikaea* diverged from its East-Asian relatives, *S. villosa* and *S. wolfii*, during the Pliocene or the early Pleistocene. The most likely time of divergence, 1.88 million years, corresponds to the early Pleistocene when a large number of the Tercier species got extinct in Europe (Willis and Niklas 2004; Postigo-Mijarra et al. 2010). Thus, we can assume that a common ancestor of *S. josikaea*, *S. villosa* and *S. wolfii* had a common Eurasian distribution area, which has disrupted during the early Pleistocene due to climate cooling and aridification, and has gone extinct from the majority of its distribution area.

Variability of DNA sequences among the populations of *Syringa josikaea*

Of the 18 loci tested for variation (together 13739 bp), of which several have yielded variation at low taxonomic level in other, only two, ITS and ETS, showed variation within *S. josikaea*. The three variable positions of the concatenated ITS and ETS sequences were collapsed into four ribotypes. The minimum-spanning network of the ribotypes was star-like, with the most ancient ribotype in the center (Fig. 2).

In the Ukrainian Carpathians all four, while in the Apuseni Mountains three ribotypes were found, with the most ancient ribotype (labeled A in Fig. 2) present in both areas. Altogether the three variable nucleotides from the 13739 bp sequence mean extremely low sequence variability. The very low sequence variation is likely to reflect an ancient bottleneck in the evolutionary history of the species which may have occurred either during its origin or at a later time as the result of glacial range contraction. The minimum-spanning tree of the four ribotypes was star-like, which generally is considered typical for geologically young evolutionary events (O'Corry-Crowe et al. 1997).

Test and development of microsatellite markers

I tested amplificability and variability of 12 microsatellite markers that were designed for related species. Only one of these (Lig15) was easy to amplify and proved to be variable at the same time on the samples of *S. josikaea*. I designed additional three highly variable microsatellite markers for *S. josikaea* (their primers: Syr4, F:5'-GGTGTGTCCAAAAATTCTG-3' R:5'-TCGAACCATTGCTTATTTCAAC-3'; Syr5, F:5'-TTGGTCTAATGCTGGACACAA-3' R:5'-CAATGCGGGCAAATATTCAC-3'; Syr9; F:5'-GGAAATCTGCTTGTTGATACC-3' R:5'-CACACACACAGAGAGAGAG-3'). These four markers together assigned individual multilocus genotypes for 98 % of the *S. josikaea* samples. These markers may be applied in genetic surveys of cultivars and hybrids of *S. josikaea*, as well.

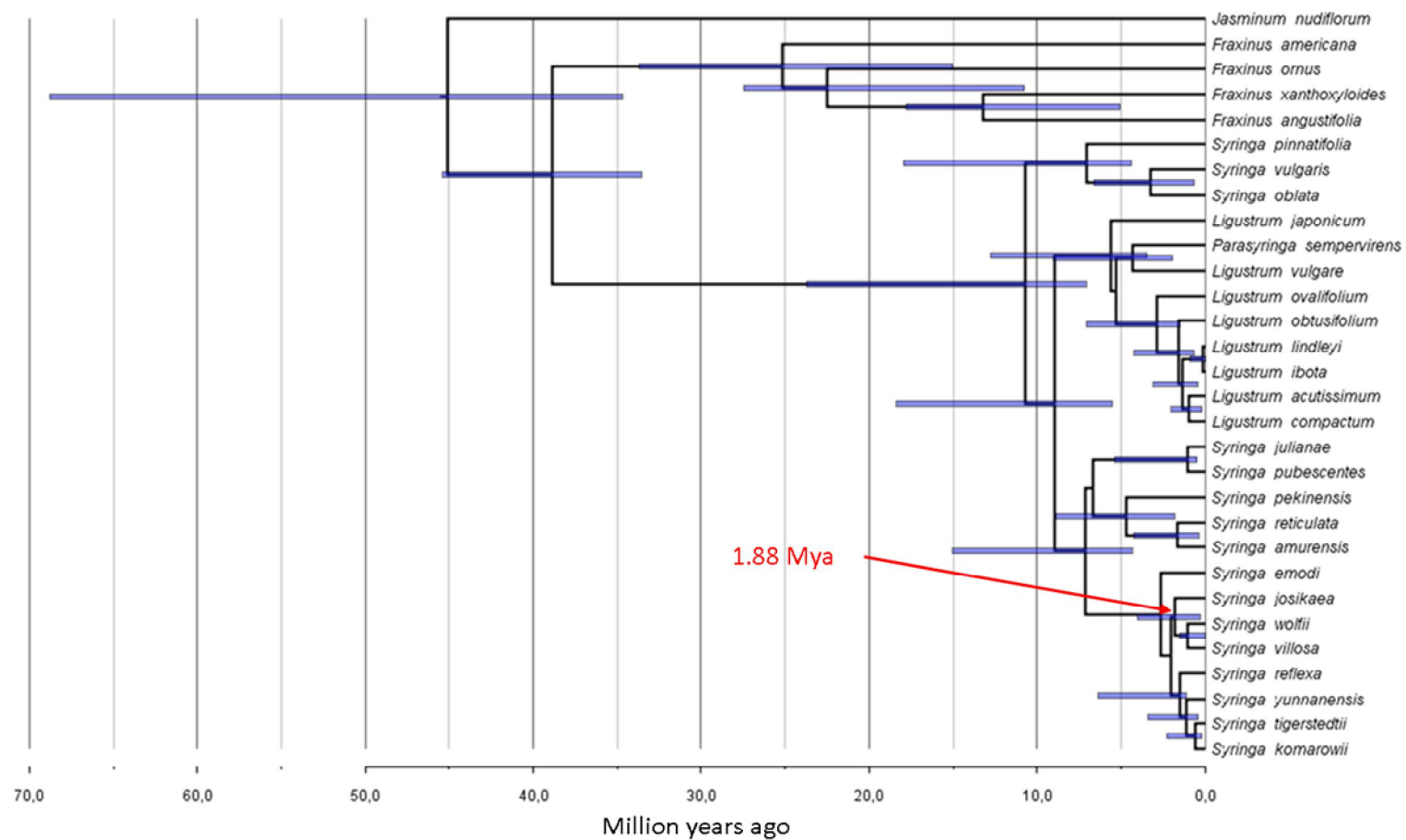


Fig. 1 Molecular clock chronogram of the genus *Syringa* based on nuclear ribosomal ITS and ETS sequence data created with BEAST 1.7.5 (Drummond et al. 2012). 95% posterior density distribution of the nodes are given with blue bars for clades supported with >80% posterior probability. The horizontal axis shows the divergence time of the lineages in million years. Arrow highlights the divergence between *Syringa josikaea* and the clade of *Syringa villosa*/*Syringa wolfii*.

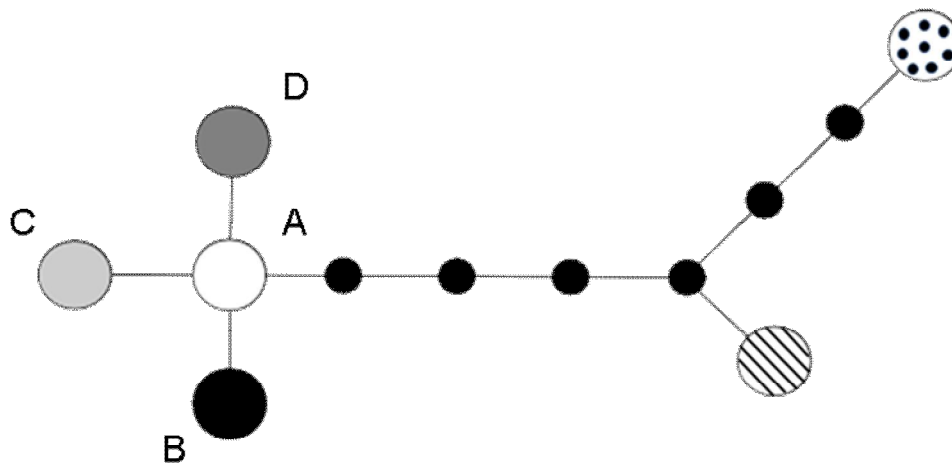


Fig. 2 Minimum-spanning tree of the ribotypes found in *Syringa josikaea* based on nuclear ribosomal ITS and ETS sequences of 62 individuals. The four *S. josikaea* ribotypes (A-D) are presented with circles in grayscale; outgroup ribotypes of *Syringa villosa* and *Syringa wolfii* are presented by striped and dotted circles, respectively; hypothetical ribotypes are indicated by smaller filled circles. The ribotype network was created from ribotype differences in Arlequin 3.5 (Excoffier and Lischer 2010) and visualized with HapStar (Teacher and Griffiths 2011).

Microsatellite results

The Bayesian genetic clustering with STRUCTURE revealed pronounced genetic differentiation between the *S. josikaea* populations from the Apuseni Mountains and the Ukrainian Carpathians. The number of genetic groups with highest probability was five. At five genetic groups two genetic clusters were characteristic to the Apuseni Mountains, while three other genetic clusters were found in the Ukrainian Carpathians (Fig. 3). Correlation between genetic and geographic distances implies limited gene flow in the Apuseni Mountains. In contrast, the correlation between genetic and geographic distances was negligible in the Ukrainian Carpathians. Microsatellite allele number and diversity was higher in the Apuseni Mountains compared to the Ukrainian Carpathians, which result is surprising as the sample size of the Apuseni Mountains was significantly lower. The genetic differences of the *S. josikaea* populations from the Apuseni Mountains and the Ukrainian Carpathians highlight the importance of conserving natural *S. josikaea* populations at both parts of the range.

Irrespective of our effort to avoid sampling clones, our analyses revealed that about one quarter of our samples originated from an already sampled clone, implying strong clonal reproduction of the species and substantial spatial extent of clones. This has important

consequence from a conservation perspective, as the number of genetic individuals present in a population can be highly overestimated when counting physical individuals.

The microsatellite results suggest that *S. josikaea* populations of the Apuseni Mountains and Ukrainian Carpathians belong to two distinct gene pools. These two areas may have been separated since the last glacial maximum or their separation may be of a postglacial origin from one glacial refugial area.

NEW SCIENTIFIC RESULTS

1. I have assessed the present distribution of *Syringa josikaea* in the Apuseni Mountains. Among the populations reported in the literature, eight were found to exist, several have gone extinct and several of the earlier reports have been found to be either indirect or to obviously originate from misunderstanding or mistranslation of geographical names. *Syringa josikaea* populations in the Apuseni Mountains are generally small, and are endangered by anthropogenic disturbance, predominantly road constructions and logging.
2. I have assessed the divergence time between *Syringa josikaea* and its closest relatives using a fossil calibrated dated phylogeny. The divergence time between *Syringa josikaea* and *Syringa villosa/Syringa wolfii* was estimated to 1.88 (0.30-4.04 HPD) million years. This time corresponds to the period of Early Pleistocene extinctions, when many Tertiary plant taxa went extinct in Europe.
3. The low intra-specific sequence variation in *Syringa josikaea* is likely to reflect an ancient bottleneck in the evolutionary history of the species which may have occurred either during its origin or at a later time as the result of glacial range contraction.
4. Analysis of DNA sequence variability has shown that the disjunct distribution area of *S. josikaea* in the Apuseni Mountains and Ukrainian Carpathians may have evolved through the disruption of a former common distribution area.
5. I have developed and characterized nuclear microsatellite markers, which are suitable for genetic analyses of *Syringa josikaea* populations.
6. Microsatellite markers identified two geographically distinct gene pools in the Apuseni Mountains, while in the Ukrainian Carpathians genetic groups are mixed among geographic areas.

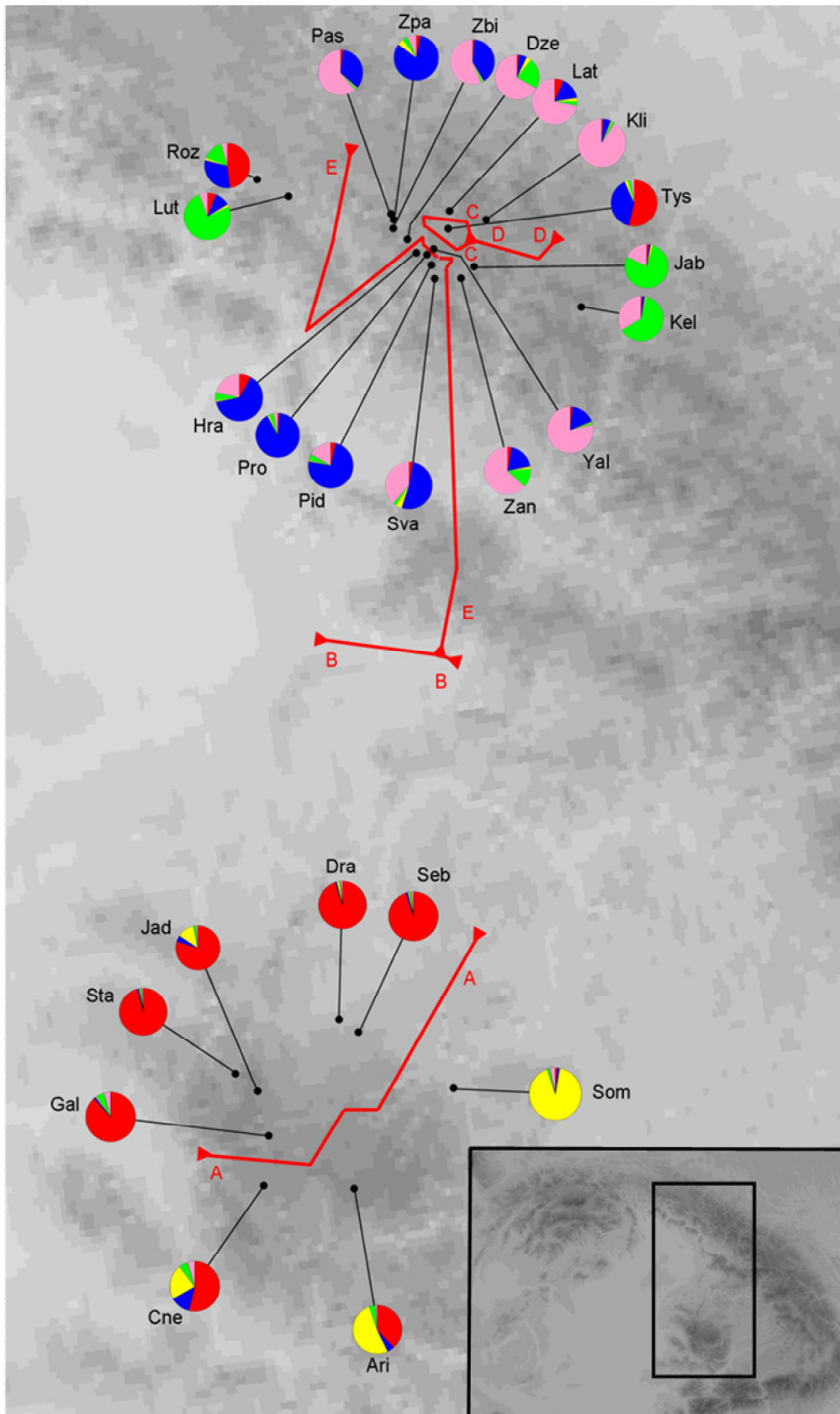


Fig. 3 Mean population assignment of the *Syringa josikaea* populations to genetic clusters in STRUCTURE 2.3.4 (Hubisz et al. 2009), and geographic barriers (A-E) calculated with Barrier 2.2 (Manni et al. 2004). The inset map shows the location of the populations in the Carpathians.

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