JOURNAL OF FOREST SCIENCE, *60*, 2014 (12): 511–518

Assessment of genetic variability in autochthonous elm populations using ISSR markers

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ABSTRACT: Genetic diversity between 110 individuals from 6 elm populations (*Ulmus minor*, *U. glabra* and *U. laevis*) was determined using ISSR markers. Altogether 73 ISSR markers were evaluated with the average rate of polymorphic bands of 99.1%, which indicates high genetic diversity between the populations/species. The higher genetic diversity was revealed particularly in the population of *U. glabra* and this result was supported by the analysis of genetic diversity and differentiation of elm populations. Molecular analyses of ISSR markers allowed to assess the extent of genetic variability of native elm populations and characterize the levels of their genetic diversity and differentiation. Their further use can be seen in conservation and management activities.

Keywords: *Ulmus minor*; *U. glabra*; *U. laevis*; genetic diversity and differentiation; molecular markers

Elms are a group of tree species native to the temperate zone, treasured as a source of high quality wood but also highly valued as an important element of garden/urban and landscape architecture. Due to the good stress tolerance to environmental conditions such as air pollution, soil salinity, drought and floods elm is perfectly suited to urban plantings and due to the rich root system it belongs to the amelioration and stabilizing tree species. The importance of elms in maintaining genetic diversity of European forests is still considered a priority. Two international projects provide the co-ordination of conservation and assessment of the elm genetic resources in a European perspective: GENRES 78 project, carried out in 1997–2001 with the financial support of the European 32 Commission (EC), was focused on the *ex situ* conservation of elms, and The European Forest Genetic Resources Programme (EUFORGEN) promoting 34 conservation and sustainable utilisation of forest genetic resources in Europe, defined, along 35 with other forest tree species, conservation strategies for the European species of elms (Collin et al. 2004).

Elms (*Ulmus minor*, *U. glabra* and *U. laevis*) belong to native tree species in the Czech Republic and in forest communities they have always represented a significant component of biocoenologial stability. Since the 1930's, the elm population of *U. minor* and *U. glabra* has suffered significant losses in their occurrence and abundance due to an attack of the fungal disease known as *Dutch elm disease* (DED). A similar trend has been evident throughout the European continent, resulting in the fragmentation of populations, reducing the frequency of elm in the environment and significant reduction of genetic variability (Cox et al. 2013).

Under the influence of the rapidly spreading disease *U. minor* almost disappeared, while *U. laevis* has been affected to a lesser extent only (Collin 2002). In spite of the decline, *U. glabra* is still the most numerously occurring elm species in Central Europe. If we want not only to monitor the present state but also to predict the future development of the remnant populations of elms and realize their rescue, it will be necessary to obtain information about their genetic

Supported by Ministry of Agriculture of the Czech Republic, Project No. QI92A247, and by the Grant Agency of the University of South Bohemia in České Budějovice, Project No. GAJU 063/2013/Z, and by EU Education for Competitiveness Operational Programme, Project No. CZ.1.07/2.3.00/30.0006.

variability. The reason is that the development of the natural population depends on the diversity of the genomes of individuals that created this population. Genetic diversity in forest trees, and also in elms, was studied by the analysis of isoenzymes (Ivanek et al. 2005), or more precisely by DNA approach (Wiegrefe et al. 1993; Whiteley et al. 2003; Cox et al. 2012).

The main aim of this study was to assess intra- and inter-population variability of selected autochthonous elm populations and to compare the extent of genetic variation in all three elm species using ISSR markers. Determination of *Ulmus* species based on ISSR fingerprints, verification of stability and resolution capability of ISSR markers were another aims of this study.

MATERIAL AND METHODS

Plant material. One hundred and ten supposed genotypes of elm were collected during 2011–2013 in six selected Czech autochthonous populations of *Ulmus minor*, *U. glabra* and *U. laevis* (the list of samples and description of sites is given in Table 1). Selected populations are considered to be "core populations" in terms of conservation of the elm gene pool in the Czech Republic, and based on historical data and forest maps they are considered to be autochthonous. Selected populations were represented by up to dozen individual trees, individuals were selected randomly and all individuals were recorded. The sample size of approximately 20 individuals per populations adequately represents the population, and even exceeds the size of the analysed set of individuals used in studies where ISSR markers were used (López-Aljorna et al. 2007; Cox et al. 2012; Nelson et al. 2014).

DNA isolation. Template DNA was extracted from fresh or frozen material using the DNeasy Plant Mini Kit (QIAGEN). DNA was quantified in a Qbit fluorometer using the Qbit dsDNA BR assay kit (Invitrogen Corporation, Carlsbad, CA) and stored at –20°C.

ISSR analysis. After initial screening of a wide range of 30 ISSR primers from the University of British Columbia, three primers generating the stable and repeatable pattern of markers were selected for ISSR analyses: UBC 807–$[AG]_8T$, UBC 810–$[GA]_8T$, and 880–$[GGAGA]_3$. PCR amplification reactions were carried out in the total volume of 10 µl containing 1× PPP Master Mix (Top-Bio), 12.5 pmol of primer, 1× BSA and 50 ng template DNA. Amplifications were performed using the following programme: pre-denaturation for 2 min at 95°C, 40 cycles of 20 s at 93°C, 60 s annealing (UBC 807 55°C; UBC 810 48°C; UBC 880 51°C), and 20 s at 72°C, finally, 6 min at 72°C. PCR products were subjected to electrophoresis on 2% agarose gel in 1× TBE buffer containing ethidium bromide at 40 V for 20 min followed by 280 min at 80 V. Photographs of the gels were taken with UV transillumination using a Canon imaging system.

Band scoring and data analysis. All analyses were performed in duplicate, and only samples with the same pattern of ISSR markers in both replicates were scored. Molecular data were analysed using digital image analysis and fingerprint patterns were transformed into a binary character matrix with 1 for presence or 0 for absence of a band at a particular position in a lane. Genetic distance matrices were generated using Nei and Li (1979) metrics. This similarity matrix was employed to construct a dendrogram by the unweighted pair group method with arithmetical av-

Table 1. Set of analysed elm samples

Category	Location	Size of population (ha and % of species representation)	Number of analysed individuals
Ulmus laevis			
UL_Br01-20	Břežanské údolí (49°57.277'N; 14°24.769'E)	0.35 ha, 5%	20
UL_L01-19	Lanžhot (48°41.091'N;16°56.098'E)	10 ha, 1%	19
Ulmus minor			
UM_HL01-19	Lanžhot (48°41.259'N; 16°56.352'E)	300 ha, 1%	19
UM_CS01-20	České středohoří (50°31.891'N; 14°5.584'E)	0,5 ha, 50%	20
Ulmus glabra			
UG_J01-20	Jeseníky (50°8.236'N; 17°2.165'E)	17 ha, 5%	20
UG_LZ01-12	Lužické hory (50°50.538'N; 14°36.26'E)	150 ha, 5%	12

minimal distance between two sampled individuals was 50–100 m, in the case of a small local population (Břežanské údolí, České středohoří) 10–15 m

erages (UPGMA) using MVSP 3.1 software package (Kovach Computing Services, Pentraeth, UK) and to perform neighbour-joining and principal coordinates analysis (PCO) using DARwin 5.0.158 software package (CIRAD, F). Genetic diversity and differentiation parameters were calculated using Structure 2.3.4 software package (Pritchard et al. 2000). To determine the most likely number of clusters we followed the approach of Evanno et al. (2005) and Kolář et al. (2012).

RESULTS AND DISCUSSION

ISSRs were chosen as molecular markers for their ability to detect genetic diversity, and also as a method not requiring the prior knowledge of DNA sequences, a method easy to operate (Esselman et al. 1999). Unlike the previously widely used RAPD markers, ISSR markers are reported as a well reproducible and repeatable technique (Goulão, Oliveira 2001). Another advantage is that AFLP and ISSR are not influenced by low reproducibility and repeatability, which are the problems typical of the formerly widely used multilocus RAPD analysis (Bagley et al. 2001).

Three ISSR primers produced a total of 73 bands and 99.1% of these bands were polymorphic. From the obtained pattern of ISSR fingerprints of individual supposed genotypes/samples, it was possible to identify clearly all analysed species as well as all analysed populations, which is demonstrated from outputs of N-J (neighbour-joining analysis) (Fig. 1) and PCO analyses (Fig. 2). These results of the ISSR analyses demonstrate (a) suitability of ISSRs for determination of elm species, which should be valuable in the case of determination of unclear samples and (b) noticeable extent of intrapopulation genetic variability in natural populations and clear differentiation between assessed populations (Fig. 1a). This fact is particularly important with regard to the definition of the territory occupied by the autochthonous population and is important for the transfer of seeds/seedlings (protection and proper management of natural populations to prevent genetic erosion of these populations). Differentiation between populations is not the same in all three species, and although two analysed populations of *U. laevis* exhibited high similarity, still it was possible to distinguish between them and only marginal overlapping was recorded, as is shown in Fig. 1b. Based on the analysis of ISSR markers in 6 pilot populations it was possible to assign individuals/populations to the particular species and differentiation on a species level is absolutely clear and indisputable (Fig. 1b and 2). The significant distinction between the analysed species can help in managing genetic resources, where in some cases the assignation to species is not clear.

Detailed assessment of genetic diversity and differentiation of the analysed elm populations/species identified six groups (Fig. 3); colour bars of these outputs from STRUCTURE represent six "genomes" (types of genomic constitutions) and bars with mixed colours indicate the presumable composition ("structure") of analysed individuals. These calculations as well as outputs are very similar to the approach of non-hierarchical K-means clustering applied by Cox et al. (2012) and both calculations are based on the approach of Evanno et al. (2005) to select the most likely number of groups using intergroup inertia as a proxy of clustering accuracy. All samples for both populations of *U. laevis* formed one group. These results correspond with results of N-J and PCO analyses where lower variation was recorded and both populations were clustered closely. Populations of *U. laevis* analysed in our study exhibited a very restricted extent of genetic variability and both populations belong to the same group. Contrary to these patterns of high genetic uniformity of analysed Czech populations of *U. laevis* Cox et al. (2012) reported the highly variable composition of Dutch, Belgian and French *U. laevis* populations. Uniformity was found only in very small populations. Two groups corresponding to two populations were recorded in *U. minor*. Some variation is evident mainly in three samples (79–80 = UM_CS08-10). This variation may be explained by possible hybridisation with *U. glabra* occurring also in this area. Natural hybridisation between these two species was recorded by Petrokas and Baliuckas (2012) but hybrid individuals in a contact zone were studied and determined only on the basis of phenotypical data. The highest variability was recorded in *U. glabra* especially in a population from the Jeseníky Mts. Results of assessment of genetic diversity and differentiation of elm populations support previous outputs showing the clear distinction of three analysed taxa. And also in this case the highest variability in populations of *U. glabra* was recorded. In comparison with the study of Ivanek et al. (2005) on the basis of ISSR markers it was possible to reliably differentiate all three analysed species and distinguish not only between populations but also between individuals. The molecular approach then provides better resolution ability than the analysis of isoenzymes.

Results of analyses of molecular markers show a relatively high range of genetic variability. Although the investigated populations are isolated, geographically limited and sparsely abundant, the elm gene pool in

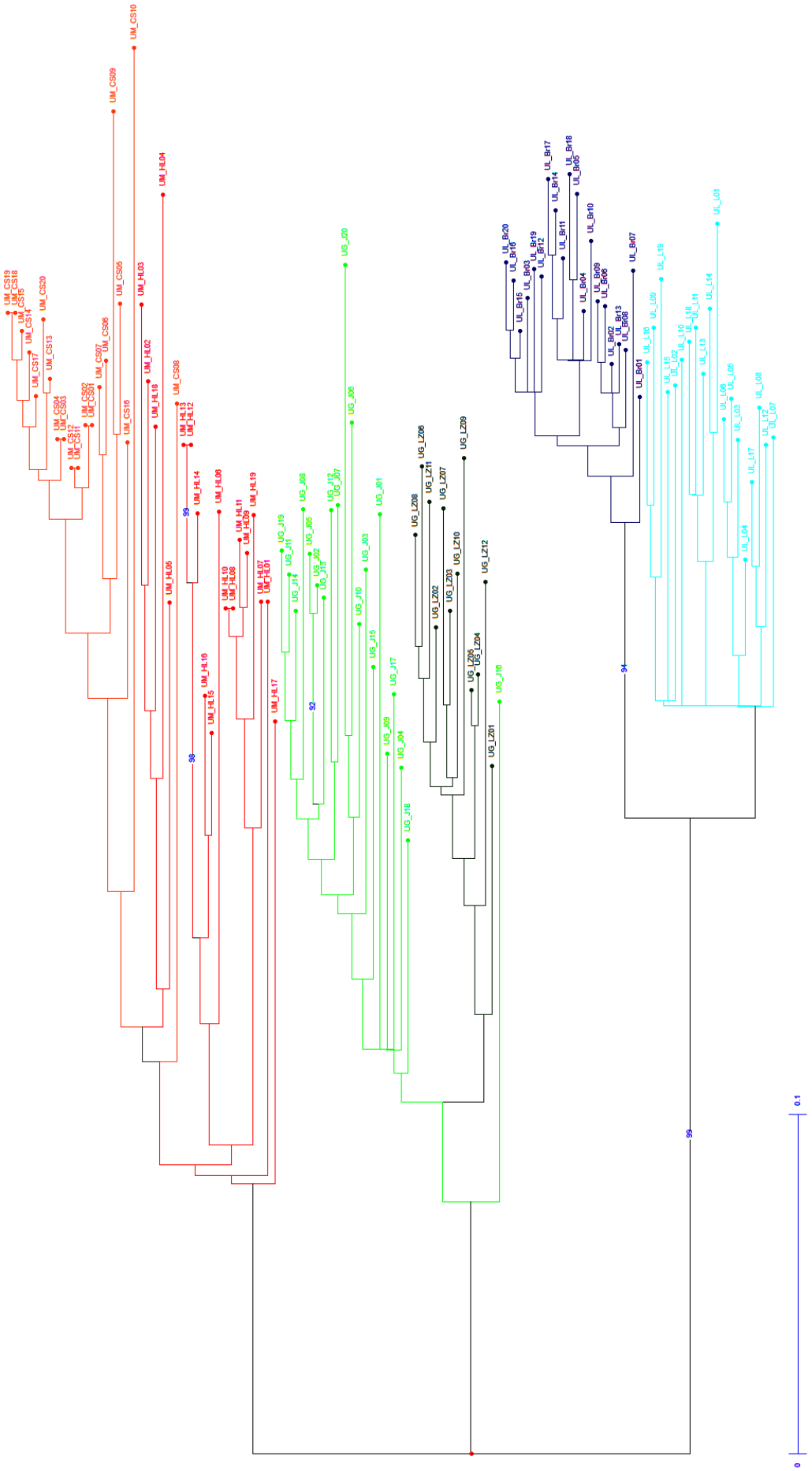

Fig. 1a. Results of the neighbour-joining analysis of ISSR markers in elm populations (DARwin, horizontal tree, bootstrap analysis); population codes correspond to Table 1; analysis of ISSR markers generated by using UBC 807, UBC 810 and UBC 880 primers

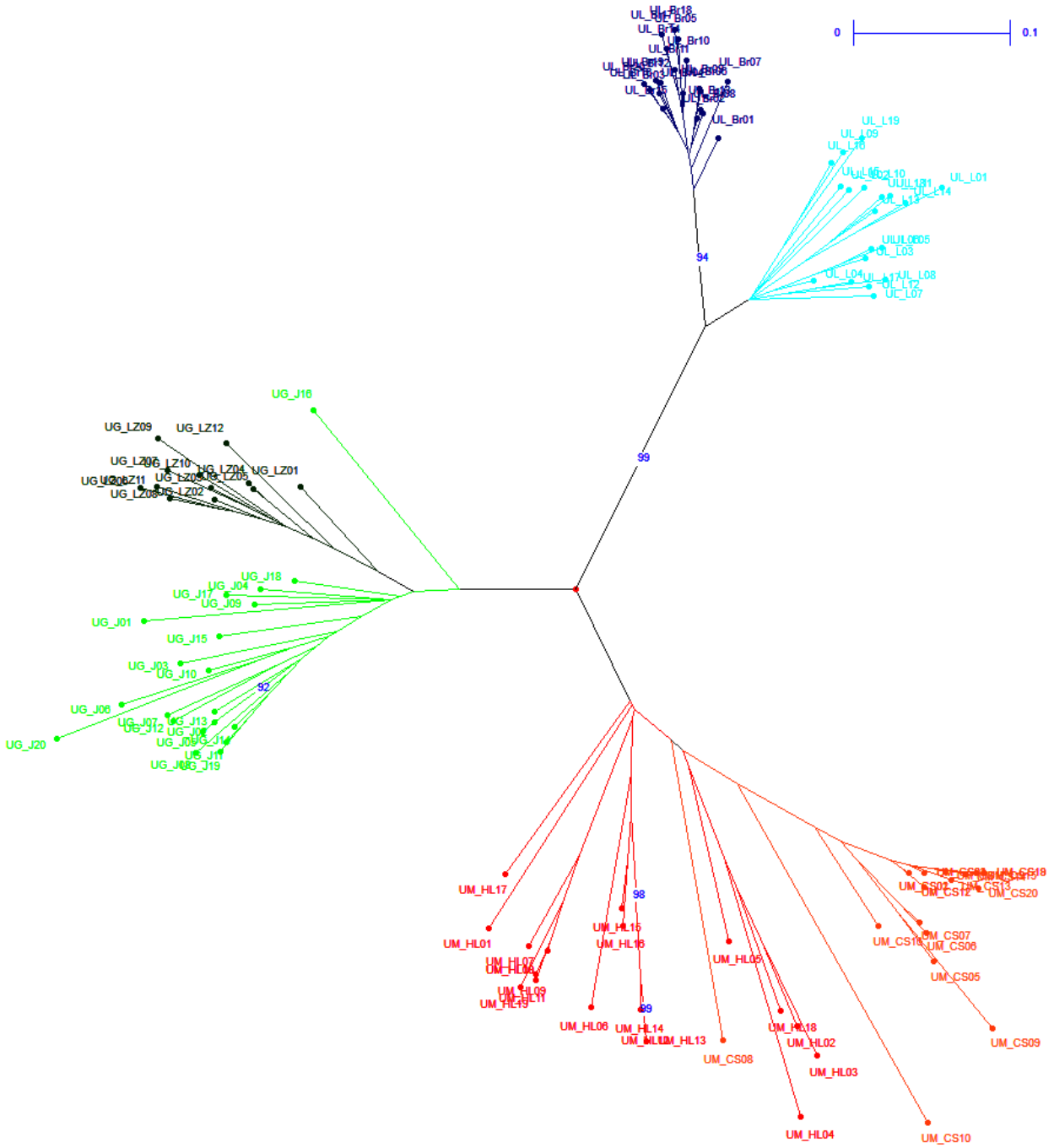

Fig. 1b. Results of the neighbour-joining analysis of ISSR markers in clear differentiation between the three analysed species (DARwin, radial tree, bootstrap analysis); population codes correspond to Table 1; analysis of ISSR markers generated by using UBC 807, UBC 810 and UBC 880 primers

the Czech Republic is not homogeneous and strongly restricted at all. The higher genetic diversity was expressed in particular individuals and population of *U. glabra*. This phenomenon can be explained by the fact that it is a sexually reproducing tree species, while individuals of *U. minor* reproduce mainly asexually. Populations of *U. glabra* are generally more common and naturally relatively large. Populations of *U. minor* are then often limited to a few individuals, often reproducing vegetatively. *U. laevis* exhibits the lower level of genetic diversity than the other species. The reason can be seen in possible effects of genetic drift, which is caused by the radical reduction of population sizes associated with human activities: historical deforestation, habitat fragmentation and loss. A similar situation was described by Machon et al. (1995) in the French population of elms and more recently by Cox et al. (2012). Also Pooler and Townsend (2005) concluded that the molecular method is a useful tool for determining genetic distances between species and the distinction between clones and hybrids. Knowledge of the extent of genetic variabil-

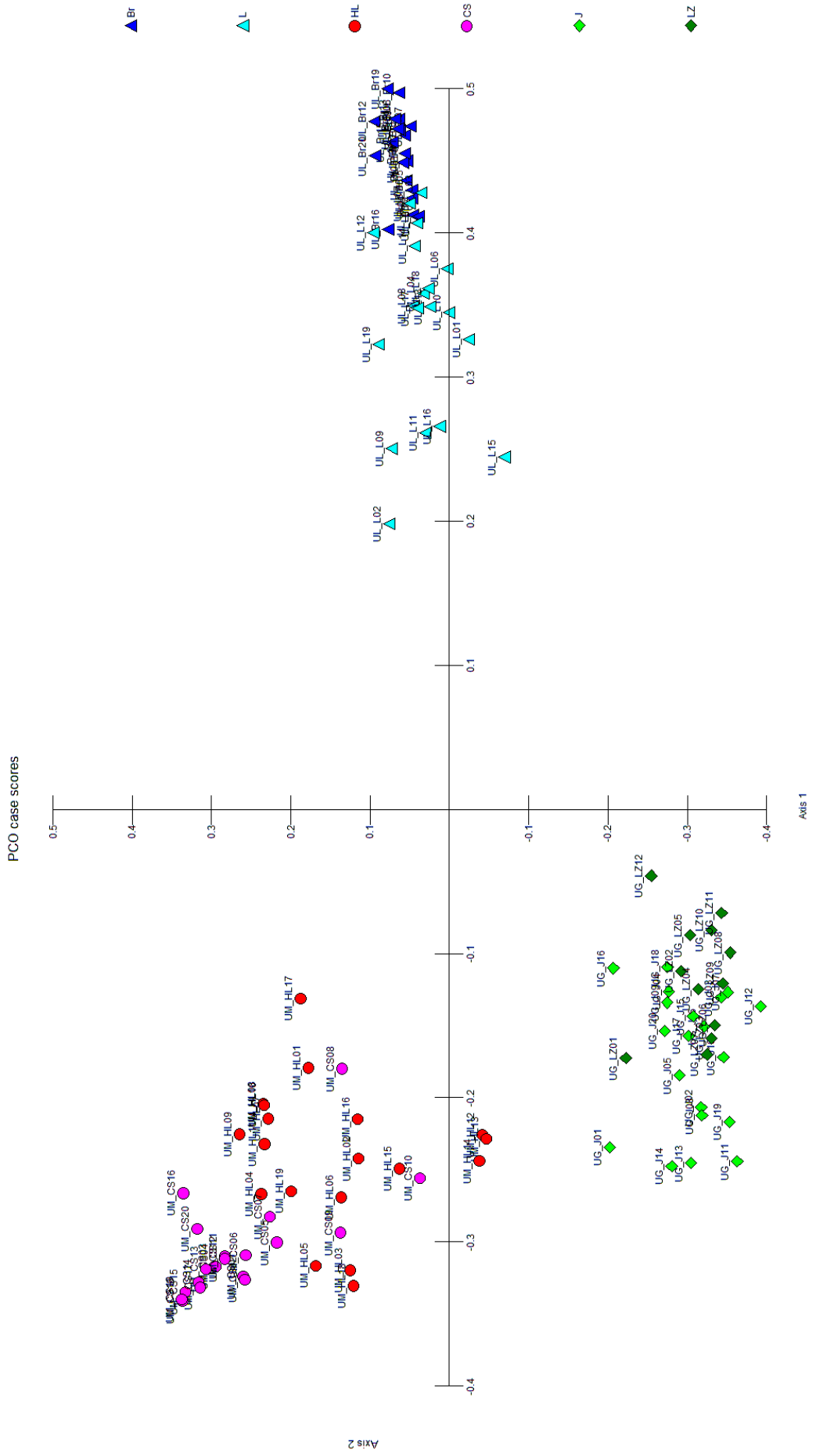

Fig. 2. Principal coordinate analysis (PCO) of ISSR markers in six elm populations and clear differentiation between the three analysed species; population codes correspond to Table 1; PCO analysis was calculated using MVSP software (data without standardisation, all axes extracted, scatter plot based on the first two axes); analysis of ISSR markers generated by using UBC 807, UBC 810 and UBC 880 primers

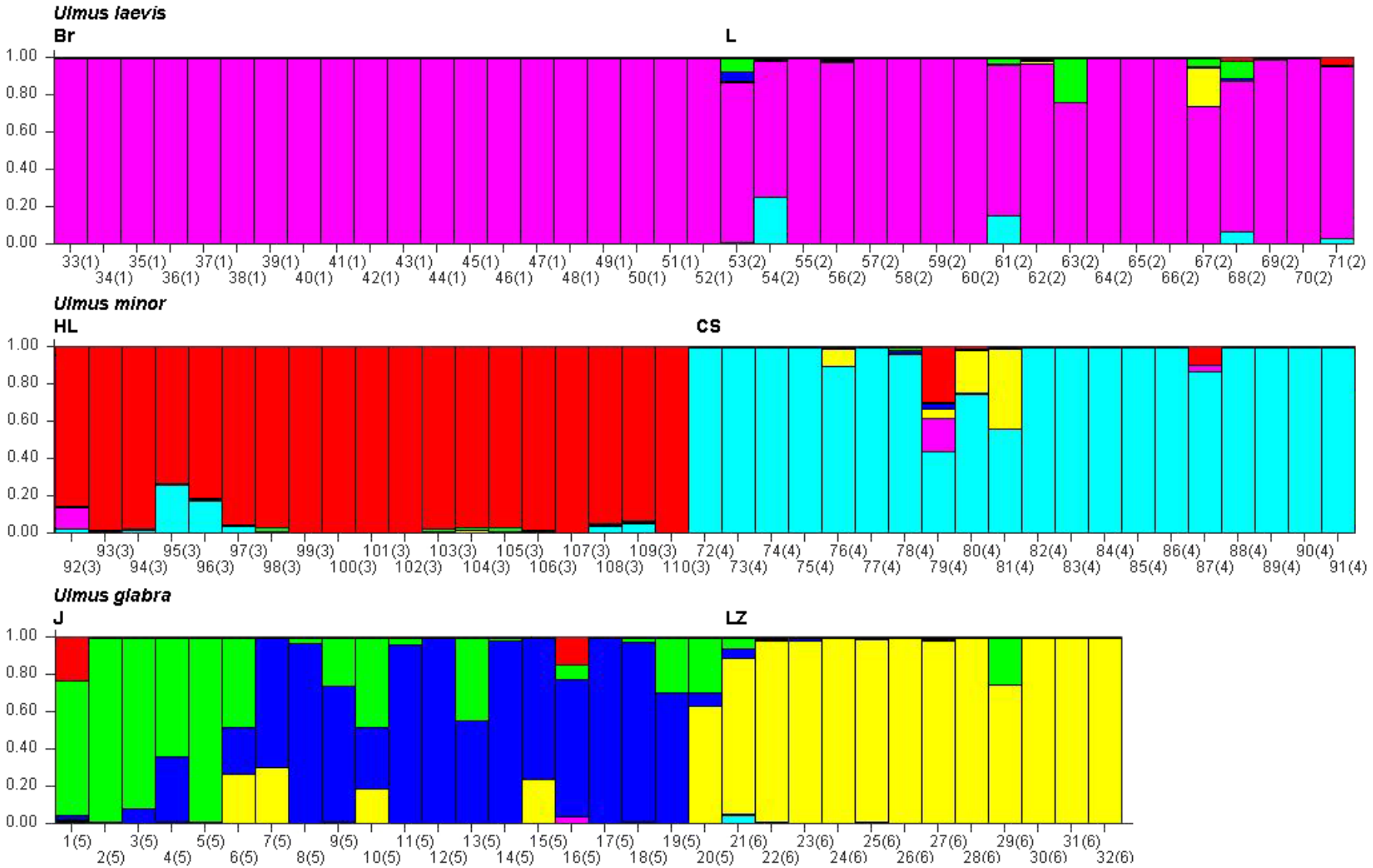

Fig. 3. Results of the analysis of genetic diversity and differentiation of elm populations using Structure software package (Admixture Model, Allele Frequencies Correlated, K = 6, Length of Burn-in Period: 100,000); letters indicate populations; No. of samples is located below the corresponding colour bar

ity and genetic diversity of analysed populations is important also for effective management of the elm germplasm and effective conservation of core populations. As was mentioned above, molecular marker based analyses can reveal the actual extent of genetic variability and specify the autochthonous population, and also define the area occupied by this specific population. This fact can play an important role in the issue of the seed/seedling transfer and maintaining genetic uniqueness of specific population. The knowledge that we gained through the analysis of ISSR markers in Czech populations of elms will be used to characterize elm genotypes in established clonal seed orchards and archives, and for the selection of suitable genotypes for the extension of elm gene banks of explants.

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Received for publication November 8, 2014
Accepted after corrections October 29, 2014

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