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Alive and kicking, or, living on borrowed time? – Microsatellite diversity in natural populations of the endangered *Ulmus minor* Mill. sensu latissimo from Croatia

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Abstract – The main objective of this research was to assess the genetic diversity of 5 natural field elm populations in Croatia. The study results suggest that the observed populations are characterized by a satisfactory amount of heterozygosity, and that the impact of the Dutch elm disease on the amount of genetic diversity in the sampled populations is currently negligible. However, one population displayed a significant excess of heterozygosity, implying a genetic bottleneck. The existence of a very clear genetic differentiation between the continental and the Mediterranean populations of *Ulmus minor* in Croatia was noticed.

Keywords: bottleneck event, endangered species, genetic diversity, natural populations, nuclear microsatellites, *Ulmus minor*

Introduction

The majority of contemporary studies of elms are based on the need to preserve their genetic diversity and to resolve the complex situation in their classification through research into genetic variability and morphological analyses (Heimler et al. 1990, Jeffers 1999, Nielsen and Kjær 2010). The reduction in genetic diversity of European elms is largely due to the anthropogenic-induced destruction of habitat, introduction of new elm species and spontaneous hybridization with ornamental species (*U. pumila* L.). Likewise, the extremely high susceptibility of elms of the *Ulmus* Heybr. section to the *Ophiostoma novo-ulmi* Brasier pathogenic fungus, played an important role in the catastrophic wilting of adult elm trees through Europe (Collin et al. 2000). The Dutch elm disease (DED) caused by the fungus was first observed in France and the Netherlands in around 1910 and rapidly spread worldwide afterwards (Spierenburg 1921, Buisman 1928).

The European field elm (*Ulmus minor* Mill. sensu latissimo) is a noble hardwood which, together with the European white elm (*Ulmus laevis* Pall.) and the wych elm (*Ulmus glabra* Huds.), belongs to the European segment of the *Ulmus* L. genus. *U. minor* s.l. is present in most of Europe except in northern areas, and also appears along the Mediterranean coast, on most of the islands of the Mediterranean Sea, as well as in Northern Africa, Asia Minor, Caucasus and Transcaucasia (Richens 1976). The natural populations of the field elm are characterized by a very wide ecological tolerance, and therefore it occupies a wide range of habitats, from floodplain forests to dry Mediterranean forest communities (Richens 1983, Namvar and Spethmann 1985). The taxonomic status of the field elm in Europe could be viewed in two, mutually completely exclusive, ways. The first, proposed by Melville (1975, 1978) implies the existence of a larger number of small species (microspecies treatment). The second, suggested by Richens (1968, 1980) affirms the existence of only one collective species of the field elm on the European continent, *U. minor* Mill. sensu latissimo, as adopted in this paper.

In contemporary research on field elm genetic diversity, classical sampling methods, due to the small number of adult trees as a result of DED, have not been used by a majority of researchers. Therefore, in recent studies, "a popu-

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lation" consists of set of trees, originating from the same geographic region and the researched samples include a mixture of trees derived from clonal plantations, *ex situ* collections as well as DED tolerant clones and trees from parks and hedges (Machon et al. 1997, Cogolludo-Agustín et al. 2000). Hence, the heterogeneity of sampled material is extremely great and yet the genetic diversity and structure of natural elm populations remains unknown. There is no dispute about the indigenous nature of the field elm in Croatia. This taxon occupies a well-defined ecological niche, with the possibility of hybridization with the wych elm reduced to a minimum. Indeed, the only issue in Croatia is the problem of assessing the variability of the taxon.

The field elm appears in the continental and the Mediterranean part of Croatia, where the ecological conditions of the distribution range are very different, which is manifested by the high variability of morphological traits (Zebec 2010). Pure stands of the field elm are also frequent, particularly in anthropogenic habitats, as well as on abandoned farmland and pastures. A clear separation of the continental and the Mediterranean populations of the field elm according to leaf morphology was stated by Zebec (2010), who conducted the first extensive research on the entire area of this species' spread in Croatia.

As microsatellite markers for the field elm were not developed until 2004, most of the research on genetic diversity to date has been conducted using isozymes (Machon et al. 1995, Cogolludo-Agustín et al. 2000), allozymes (Machon et al. 1997), RAPD technique and ISSRs (Coleman et al. 2000, Goodall-Copestake et al. 2005) and cpDNA PCR-RFLP markers (Collin et al. 2004). Microsatellites found their application in the resolving of the taxonomic status of *U. procera* Salisb. (Gil et al. 2004) and recently in assessing whether the field elm is or is not indigenous to the Balearic Islands (Fuentes-Utrilla et al. 2014).

The aim of this research was to assess the level of genetic diversity between and within the field elm populations in Croatia, and thus determine the degree of the negative impact of DED on the biodiversity of the species. There are several advantages of performing this research in Croatia. Firstly – since the Balkan region is considered a glacial refugium, Croatia by its geographical position remains a place of presumed high biodiversity. Secondly – Croatia combines aspects of both the continental and the Mediterranean area of elm distribution. Thirdly – in the southern area of its distribution (including Croatia) the field elm favors sexual reproduction and since there were a sufficient number of individuals for the research to be performed, we defined the term "population" in this study in the standard biological way.

Material and methods

Plant material

The quantification of genetic diversity of the field elm was conducted by an analysis of genotypes of 96 individuals from 5 populations (Đurđevac, Zagreb, Pula, Nin, Neretva). The populations were carefully selected, in order to enable, in concert with their geographic location and climatic adaptation, precise insight into the genetic variability of the field elm in Croatia (Fig. 1). Taxonomical identification of sampled plant material was confirmed by Dr. Zlatko Liber, a plant taxonomist of the Department of Botany of the University of Zagreb and voucher specimens of plants (IDs 37109 – 37113) have been deposited with the Herbarium Croaticum (ZA), University of Zagreb, Croatia (On-line Suppl. Tab. 1).

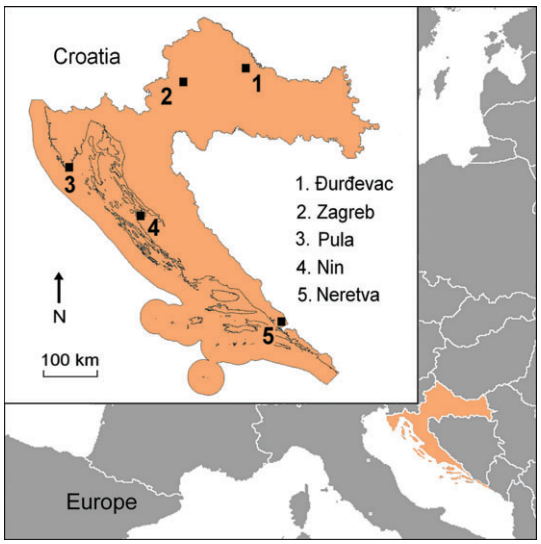

Fig. 1. Geographical position of sampled populations. Populations are indicated by following numbers: 1 – Đurđevac; 2 – Zagreb; 3 – Pula; 4 – Nin; 5 – Neretva.

DNA extraction, PCR amplification and microsatellite genotyping

Total genomic DNA was extracted from silica-dried leaf terminal buds using a DNeasy® Plant Mini Kit (Qiagen®) according to the manufacturer's instructions. For this procedure 25 mg of dry tissue of single plants was used. The quality and concentration of the DNAs were checked by electrophoresis in 0.8% (w/v) agarose gel and additionally by Qubit 2.0® Fluorometer using Quant-iT™ dsDNA BR Assay Kit (Invitrogen®).

Five SSR markers were used in the study (Whiteley et al. 2003, Collada et al. 2004): *Ulm2* (*AY300797*), *Ulm8* (*AY300800*), *Ulmi1–21* (*AY520827*), *Ulmi1–98* (*AY520829*) and *Ulmi1–165* (*AY520830*) as presented in Tab. 1. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems®). The PCR conditions described by Collada et al. (2004) were used for the PCR amplifications of three microsatellite loci *Ulmi1–21* (*AY520827*), *Ulmi1–98* (*AY520829*) and *Ulmi1–165* (*AY520830*), whereas the amplification protocol proposed by Whiteley et al. (2003) was used for microsatellite loci *Ulm2* (*AY300797*) and *Ulm8* (*AY300800*), respectively. Amplification products were separated and detected on an automated sequencer (ABI PRISM 3130 DNA sequencer, Applied Biosystems, Foster City, CA, USA) at Macrogen Inc. (Korea). Fragment sizes were determined using the program GENEMAPPER® 4.0 (Applied Biosystems® 2005).

Tab. 1. Source, repeat motifs, size ranges, number of alleles (Na), observed (Ho) and expected heterozygosity (He) and polymorphic information content (PIC) for five microsatellite loci used in five *Ulmus* populations (n = 96).

Source	Locus	Repeat motif	Size range (bp)	N_a	H_o	H_e	PIC
Whiteley et al. (2003)	Ulm2 (AY300797)	$(CAG)_8$	98–110	8	0.625	0.662	0.610
	Ulm8 (AY300800)	$(GCT)_{12}CTT(GCT)_2$	cca. 180	10	0.472	0.487	0.468
Collada et al. (2004)	Ulmi1–21 (AY520827)	$(CT)_{10}$	204–220	11	0.634	0.736	0.699
	Ulmi1–98 (AY520829)	$(CT)_6N_{14}(CT)_7$	124–156	7	0.462	0.533	0.507
	Ulmi1–165 (AY520830)	$(CT)_9$	128–166	18	0.777	0.871	0.858
	Mean			10.80	0.594	0.658	0.628
	Min			7	0.462	0.487	0.468
	Max			18	0.777	0.871	0.858

Data analysis

By means of descriptive statistical analysis, performed on original microsatellite data matrix using POWERMARKER 3.23 software (Liu 2002), the total number of alleles per locus (N_a), the observed heterozygosity (H_o), the expected heterozygosity or gene diversity (H_e) and the polymorphism information content (PIC) for each microsatellite locus as well as the average number of alleles N_{al}, H_o, H_e in each population across loci were calculated. In order to assess allelic richness (N_{ar}) and to estimate the significance of genetic differentiation (F_{ST}) between population pairs we used the FSTAT 2.9.3.2 program package (Goudet 1995).

GENEPOP 3.4 (Raymond and Rousset 1995) was used to test genotypic frequencies for each locus in each population for conformance to Hardy-Weinberg (HW) expectations as well as for calculation of the inbreeding coefficient (f) for each locus in each population following Weir and Cockerham (1984). The probability test was based on the Markov chain method (Guo and Thompson 1992, Rousset and Raymond 1995) using 10,000 de-memorization steps, 100 batches and 5,000 iterations per batch. Sequential Bonferroni adjustments (Holm 1979, Rice 1989) were applied to correct for the effect of multiple tests using SAS 8.02.

The program BOTTLENECK 1.2.02 (Cornuet and Luikart 1996, Piry et al. 1999) was used to test for evidence of recent bottleneck events on the basis of this theoretical expectation. The gene diversity observed (H_e) was compared to the gene diversity expected at mutation-drift equilibrium (H_{eq}) and calculated from the observed number of alleles under different mutation models: infinite allele model – IAM (Kimura and Crow 1964), stepwise mutation model – SMM (Ohta and Kimura 1973) and an intermediate two-phase model – TPM (Di Rienzo et al. 1994). The TPM model was applied assuming 30% – TPM 1 (Pascual et al. 2001, Hoelzel et al. 2002, Kuehn et al. 2003) and 5% multistep changes – TPM 2 (Piry et al. 1999). Based on the number of loci in our dataset, the Wilcoxon sign-rank test (Luikart et al. 1998) was chosen for the statistical analysis of heterozygote excess or deficiency as recommended by Piry et al. (1999). With the intention of depicting genetic relationships among elm trees visually, a factorial correspondence analysis (FCA) was carried out using GENETIX 4.05 (Belkhir et al. 2004). Finally, pairwise Nei's standard genetic distances (Nei 1972) were calculated and an unrooted phylogenetic tree was constructed using a neighbor-joining algorithm (Saitou and Nei 1987) with 10,000 bootstraps (Felsenstein 1985) over microsatellite loci as implemented in the PHYLIP 3.6b software package (Felsenstein 2004).

Results

Microsatellite diversity

In all, 54 alleles were detected by surveying 96 *Ulmus* trees from 5 populations using 5 SSR markers. The number of alleles per SSR marker ranged from seven (Ulmi1–98) to eighteen (Ulmi1–165) with an average of 10.80 alleles per locus. PIC values varied for a single locus from 0.468 to 0.858, with an average value of 0.628, by which it can be concluded that all microsatellite loci scored in this research, displayed a sufficient level of polymorphism. Overall observed heterozygosity values (per marker) ranged from 0.462 to 0.777, with a mean value of 0.594. The results showed some elevated levels of expected heterozygosity compared to those of observed heterozygosity, with values ranging from 0.487 to 0.871 and a mean value of 0.658 (Tab. 1).

Intrapopulation diversity and HW equilibrium

The mean number of alleles per locus in the populations ranged from 3.2 (Nin) to 7.4 (Đurđevac). The number of alleles per locus independent of sample size (the allelic richness) ranged from 3.06 (Nin) to 6.80 (Đurđevac). The observed heterozygosity per population varied from 0.530 (Zagreb) to 0.709 (Pula) and the expected heterozygosity per population varied from 0.418 (Nin) to 0.642 (Pula). Expected heterozygosity values were higher than observed heterozygosity values in the Đurđevac and Zagreb populations or vice versa in the Pula and Nin populations. The Neretva population had similar values of H_o and H_e (Tab. 2).

Significant deviations from HW equilibrium using the inbreeding coefficient (f) were found for the Pula population at most loci except Ulm8 and also for the Nin population but only at loci Ulm2 and Ulmi1–165. The inbreeding coefficient values were negative in the discussed cases, indicating excess of heterozygotes in a population compared to HW equilibrium, while other populations exhibited no significant deviations from HW equilibrium (Tab. 3).

Tab. 2. Population, sample size and genetic variability estimates based on data from five microsatellite loci in five *Ulmus* populations. No – number of populations; n – sample size; N_a – mean number of alleles; N_{ar} – number of alleles per locus independent of sample size (allelic richness); observed (H_o) and expected heterozygosity (H_e).

No.	Population	n	N_a	N_{ar}	H_o	H_e
1	Đurđevac	20	7.4	6.80	0.566	0.619
2	Zagreb	20	6.8	6.21	0.530	0.626
3	Pula	20	3.8	3.74	0.709	0.642
4	Nin	19	3.2	3.06	0.626	0.418
5	Neretva	17	4.2	4.12	0.547	0.561

Tab. 3. Inbreeding coefficients (f) across five microsatellite loci in five *Ulmus* populations. Significant deviations from Hardy-Weinberg equilibrium after sequential Bonferroni corrections: ** – significance at the 1% nominal level; * – significance at the 5% nominal level; ns – non-significant values. No – number of populations; [a] – monomorphic locus.

No.	Population	Ulm2	Ulm8	Ulmi 1–21	Ulmi 1–98	Ulmi 1–165
1	Đurđevac	0.177 ^{ns}	–0.166 ^{ns}	–0.072 ^{ns}	0.215 ^{ns}	0.306 ^{ns}
2	Zagreb	0.166 ^{ns}	–0.048 ^{ns}	0.263 ^{ns}	0.230 ^{ns}	0.125 ^{ns}
3	Pula	–0.045 **	0.111 ^{ns}	0.209 **	–0.596 **	–0.099 *
4	Nin	–0.615 **	–0.245 ^{ns}	–0.397 ^{ns}	– ^{a}	–0.581 *
5	Neretva	0.491 ^{ns}	–0.141 ^{ns}	0.032 ^{ns}	–0.067 ^{ns}	0.017 ^{ns}

Test for mutation-drift equilibrium at all observed polymorphic loci under four mutation models in five *Ulmus* populations revealed a recent reduction in effective population size (genetic bottleneck) only in the Pula population. Accordingly, the Pula population displayed significant ($p < 0.05$) gene diversity excess ($H_e > H_{eq}$) on average across all 5 polymorphic loci under IAM and TPM1, or across 4 polymorphic loci under TPM2 and SMM, respectively. The Đurđevac population showed signs of population expansion regarding significant ($p < 0.05$) gene diversity deficiency ($H_e < H_{eq}$) on average across all polymorphic loci under mutation models TPM1, TPM2 and SMM. Other populations did not show statistically significant departures from optimal heterozygosity level, assumed by mutation-drift equilibrium (Tab. 4).

Tab. 4. Number of loci showing heterozygosity deficiency / heterozygosity excess. Test for mutation-drift equilibrium at polymorphic loci in five *Ulmus* populations under four mutation models: infinite allele model (IAM), two phase model assuming 30% multistep changes (TPM1), two phase model assuming 5% multistep changes (TPM2), stepwise mutation model (SMM). * – significant ($p < 0.05$) gene diversity deficiency ($H_e < H_{eq}$) on average across all polymorphic loci using Wilcoxon's test – sign of population expansion. [#] – significant ($p < 0.05$) gene diversity excess ($H_e > H_{eq}$) on average across all polymorphic loci using Wilcoxon's test – sign of population bottleneck.

No.	Population	Mutation model			
		IAM	TPM1	TPM2	SMM
1	Đurđevac	3 / 2	5*/ 0	5*/ 0	5*/ 0
2	Zagreb	1 / 4	2 / 3	3 / 2	3 / 2
3	Pula	0 / 5 $^{\#}$	0 / 5 $^{\#}$	1 / 4 $^{\#}$	1 / 4 $^{\#}$
4	Nin	2 / 2	2 / 2	3 / 1	3 / 1
5	Neretva	2 / 3	2 / 3	3 / 2	3 / 2

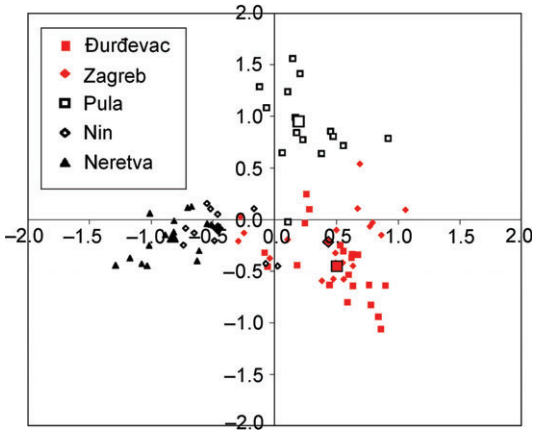

Fig. 2. Factorial correspondence analysis (FCA) of 96 *Ulmus* trees belonging to five populations. Each individual genotype is indicated by a small sign, while the population barycenters are represented by larger ones. Populations are depicted by following symbols: Đurđevac – filled squares; Zagreb – filled rhombs; Pula – unfilled squares; Nin – unfilled rhombs; Neretva – filled triangles.

Genetic differentiation among populations

The results of FCA are graphically presented by Fig. 2, which delineates the projection of the individuals and population barycenters on the plane defined by the first two FCA axes, thus providing a better insight into genetic relationship between studied populations. The first two axes accounted for 35.32% and 31.68% of the total inertia, respectively. Clear differentiation between continental (Đurđevac, Zagreb) and Mediterranean (Nin, Neretva) populations was notable along the first axis, whilst the Pula population located centrally with a barycenter closer to the continental group. There was some minor overlap in positioning of trees from the Đurđevac and Zagreb populations as well as Nin and Neretva. Poor differentiation between continental (Đurđevac, Zagreb) and Mediterranean (Nin, Neretva) populations was evident along the second FCA axis, whereas the Pula population occupied a discrete, distinct area of multivariate space.

By means of Nei's standard genetic distance (D_{NEI72}) and index of genetic differentiation (F_{ST}) values, a similar pattern of differentiation among observed populations was expressed (Tab. 5). The greatest D_{NEI72} values were detected between Pula and Neretva (0.409), whilst the lowest values were observed between Zagreb and Đurđevac (0.106). Intriguingly, lower D_{NEI72} values were more remarkable between the Pula and Zagreb populations (0.278) than between those of Pula and Nin (0.371). Relatively small genetic distance was determined among the Mediterranean populations of Nin and the Neretva; likewise D_{NEI72} values

Tab. 5. Nei's standard genetic distance (above diagonal) and pairwise F_{ST} values (below diagonal) between five *Ulmus* populations. Pairwise significance after sequential Bonferroni corrections: ** – significance at the 1% nominal level, * – significance at the 5% nominal level, ns – non-significant values. No – number of populations.

No.	Population	1	2	3	4	5
1	Đurđevac		0.106	0.293	0.208	0.312
2	Zagreb	0.029^{ns}		0.278	0.332	0.406
3	Pula	0.105^{**}	0.098^{**}		0.371	0.409
4	Nin	0.140^{**}	0.195^{**}	0.210^{**}		0.134
5	Neretva	0.130^{**}	0.160^{**}	0.158^{**}	0.102^{**}	

among continental populations of Zagreb and Đurđevac were also low. Correspondingly to the calculated D_{NEI72} values for the 5 studied populations, it is evident that the distances between all population pairs within the same region were less than the genetic distance between pairs of populations belonging to different regions (continental/Mediterranean). Statistically significant F_{ST} values were present between most population pairs, excluding Đurđevac and Zagreb. Calculated significant values ranged from 0.098 between Zagreb and Pula to 0.210 concerning Nin and Pula. According to the values of F_{ST}, pronounced genetic differentiation among populations belonging to different regions was apparent. Interestingly, only Pula stands out from this trend of population division (Tab. 5).

The unrooted neighbor-joining tree visually elucidates the pattern of genetic differentiation between the observed populations, suggesting the existence of a clear genetic differentiation between continental and Mediterranean populations of the field elm in Croatia (Fig. 3). The above mentioned differentiation is supported by high bootstrap values, resulting in grouping of populations based on an eco-geographical principle. Bootstrap support value of 68% confirmed the separation between continental (Đurđevac, Zagreb) and Mediterranean (Nin, Neretva) populations, whilst the separation among geographically southern (Nin, Neretva) and northern (Zagreb, Đurđevac, Pula) populations was verified by even higher bootstrap support values of 99% (Fig. 3).

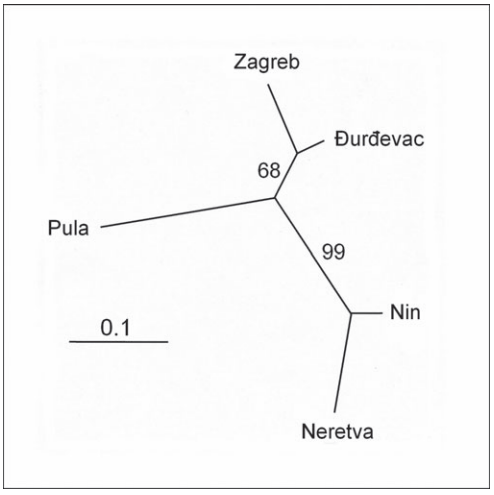

Fig. 3. Unrooted neighbor-joining tree based on Nei's standard genetic distance between five *Ulmus* populations. Numbers above branches indicate bootstrap support percentage over 50% in 10,000 pseudoreplicates.

Discussion

This research revealed abundant allelic variation over five loci and high overall genetic diversity in natural field elm populations in Croatia. The presented results are congruent with those obtained by studies of allozymes (Machon et al. 1997), isozymes (Machon et al. 1995, Cogolludo et al. 2000), RAPDs and ISSRs (Coleman et al. 2000, Goodall-Copestake et al. 2005) and SSRs (Fuentes-Utrilla et al. 2014). Although studies of the field elm on the European level suggest a high degree of intrapopulation genetic diversity, they also suggest conclusions concerning the small genetic differentiation between populations belonging to different geographic regions. As each individual author uses a different system of molecular markers and defines the term "population" differently, one should exercise a great deal of caution when comparing results. Therefore, comparison of results obtained in this study with results from above mentioned studies in Europe, is possible only for the purpose of orientation.

The values of descriptive statistics parameters (N_a, N_{ar}, H_o, H_e) suggest a native status of the field elm in Croatia (Tab. 2). In continental populations (Đurđevac, Zagreb), higher values of N_{al} are present, as well as of N_{ar} in relation to Mediterranean populations (Pula, Nin, Neretva). The Đurđevac, Zagreb and Neretva populations did not deviate significantly from HW equilibrium, unlike the Pula and Nin populations, where an excess of heterozygotes was found for specific loci (Tab. 3). As elm populations in Croatia do not demonstrate a statistically significant deviation from HW equilibrium, it could be claimed that the danger of the appearance of inbreeding within observed populations is relatively small. The Pula population somewhat deviates from the obtained results, which unequivocally suggest the separation of continental from Mediterranean populations. The reasons for the deviations of the Pula population from HW equilibrium, as well as for a significant excess of heterozygosity should be sought in the recent bottleneck event (Tab. 4).

According to Cornuet and Luikart (1996), a recently bottlenecked population is expected to show fewer rare alleles, as they are lost most quickly. Allelic diversity is reduced faster than heterozygosity during a bottleneck, because rare alleles are lost rapidly and have little effect on heterozygosity, thus producing a transient excess in heterozygosity relative to that expected in a population of constant size with the same number of alleles. Namely, the Pula locality is situated in the area of a former pure elm forest which was turned into an agro-biotope by intensive felling and clearing of land, which has a direct and negative impact on the gene diversity of this population. On the other hand, a lack of heterozygosity was found in the Đurđevac population, which thus shows signs of expansion. The causes of

population expansion can be various. As this is a larger forest complex, a stand in which economic measures are implemented, there is a possibility of a recent introduction of new individuals. The reasons for this could be to stimulate the natural renewal of the field elm in that locality or improve the economic quality by forcing the planting of plus trees. If a population has been severely attacked by DED and consequently been under the influence of a genetic bottleneck, it will strive to establish as soon as possible a new, lower mutation-drift equilibrium by the process of expansion.

Although the incidence of DED in Croatia is high, the parameters of genetic diversity of observed populations do not deviate significantly from the ideal state of HW equilibrium. Consequently, one could conclude that the disease has not had a significant impact on the degree of genetic diversity, i.e. on the stability of genetic resources of the field elm in Croatia. However, if we take into account the fact that genetic diversity in natural populations of forest trees is very slowly decreasing, as well as the presence of a very small number of adult trees of the field elm in the researched area, concern about the stability of this species' genofund acquires a new dimension.

This study, conducted in Croatia, revealed a high genetic diversity of the field elm at the intrapopulation level, but also discovered a significant differentiation at the interpopulation level. Although the separation of populations at such a high level could generate certain taxonomic implications, the final decision about the taxonomic validity of hypothetical entities i.e. conservation units within the *U. minor* s. l. complex in Croatia is possible solely if this issue is resolved on the European level. In such research, a systematic study of fructification, modalities of dispersion and the monitoring of phenological phenomena over a certain time period would be necessary.

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