Tree Genetics & Genomes (2017) 13:18
DOI 10.1007/s11295-017-1107-2

ORIGINAL ARTICLE

Genetic diversity of the sweet chestnut (*Castanea sativa* Mill.) in Central Europe and the western part of the Balkan Peninsula and evidence of marron genotype introgression into wild populations

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Received: 21 March 2016 / Revised: 2 January 2017 / Accepted: 10 January 2017

Abstract The sweet chestnut (*Castanea sativa* Mill.) is a widely spread and important multipurpose tree species in the Mediterranean area, which has played an important role in human history. Natural events, such as glaciations, and human influence played significant roles in the distribution and genetic makeup of the sweet chestnut. In order to better understand how natural and human-mediated past events affected the current genetic diversity and structure of the sweet chestnut, we analysed populations from Central Europe and the western part of the Balkan Peninsula, utilizing ten polymorphic nuclear microsatellite markers. The study revealed the existence of three genetically and, to a large extent, geographically distinct and well-defined groups of sweet chestnut populations. Two not entirely separated groups of populations were detected in the northern part of the studied area and one in the southern. Our results indicate that the genetic structure of sweet chestnut populations in Central Europe and the western part of the Balkan Peninsula is the result of both natural colonization events and significant and lengthy human impact. Furthermore, it has been proven that the gene flow between cultivated/grafted trees' and wild chestnut stands can influence their genetic structure. However, our results reveal that cultivated-to-wild introgression in the sweet chestnut is dependent on the close proximity of chestnut orchards and naturally occurring populations.

Keywords Sweet chestnut · Genetic variability · Population structure · Introgression · Microsatellites

Communicated by A. Kremer

Electronic supplementary material The online version of this article (doi:10.1007/s11295-017-1107-2) contains supplementary material, which is available to authorized users.

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Introduction

The *Castanea* Mill. genus, Fagaceae family, encompasses seven economically and ecologically significant tree species, widely spread in the temperate forest zone of the northern hemisphere (Johnson 1988; Lang et al. 2007). The only native species of the *Castanea* genus in Europe, the sweet chestnut (*Castanea sativa* Mill.), is a widely spread and important multipurpose tree in the Mediterranean area, used for its wood, fruit, honey, and tannin. It is also a valuable species in ecosystems and landscapes. In addition to the grapevine and olive, the sweet chestnut is one of the oldest cultivated species, influenced by man to such an extent that it is practically impossible to trace its natural distribution (Conedera et al. 2004). One of the most widely accepted theories is that the sweet chestnut is originally from Asia Minor, where it was initially domesticated and from where, under human influence, it was

Published online: 19 January 2017

transferred, first to Ancient Greece and then to the Apennine Peninsula and other parts of Europe (Zohary and Hopf 1988; Villani et al. 1991, 1992, 1994, 1999). However, more recent research based on fossil remains and pollen analyses (Krebs et al. 2004), as well as microsatellite data (Martín et al. 2010a, 2012; Mattioni et al. 2013), supports the hypotheses that either the populations from Italy and Spain could have originated from a common refugium in western Europe or they could have originated from human-mediated transplanting of plant material or a combination of the two. According to Krebs et al. (2004), the main macroregions with scattered environmentally favourable microhabitats suitable for the sweet chestnut during the main glacial events were an area around the southern coast of the Black Sea, the Apennines on the Italian Peninsula, the hills of the Prealps in north-eastern Italy with a possible extension to Slovenia, central French Alps in eastern France, southern Galicia and the Cantabrian coast in the Iberian Peninsula with a possible extension to French Basque Country, the Balkan Peninsula with one centre in southern Greece and another one in Macedonia and south-western Bulgaria and the hilly region along the Mediterranean coast of Syria and Lebanon.

In addition to natural events, such as glaciations, human influence on the distribution, genetic diversity and structure of sweet chestnut populations was inevitable (Mattioni et al. 2008, 2013; Martín et al. 2012; Lusini et al. 2014). The distribution of the cpDNA haplotypes revealed low spatial genetic structure, which Fineschi et al. (2000) explained by the strong human impact on this species. Judging from the pollen data, the first real expansion of the chestnut under the human influence occurred 3700 years ago in the area encompassing present-day Turkey, north-eastern Greece and south-eastern Bulgaria (van Zeist et al. 1975; van Zeist and Bottema 1991). Based on pollen data, archaeology and historical and classical literature, Conedera et al. (2004) attempted to reconstruct the history of chestnut cultivation in Europe. Although it was believed that for the most part the ancient Greeks and Romans were responsible for the expansion of the sweet chestnut in Europe, using a multidisciplinary approach, Conedera et al. (2004) showed that the greatest interest in chestnut cultivation occurred in the Middle Ages. They pointed out that the Romans may have introduced the idea of cultivating and using the sweet chestnut and, occasionally, even introduced the species in certain areas. However, there is no evidence of systematic planting of the sweet chestnut during the Roman times.

In the last few centuries, humans have influenced the genetic diversity and structure of sweet chestnut populations through propagation and transplanting of plant material, intensive cultivation of grafted plants, silvicultural practices and fragmentation of populations as a consequence of changes in land use (Mattioni et al. 2008). Mattioni et al. (2008) evaluated the effect of stand management type (naturalized stands, managed coppice and grafted fruit orchards) on the genetic diversity of sweet chestnut populations and revealed that long-term management techniques may influence the genetic diversity of populations. In the case of the sweet chestnut, the introgression of grafted trees from the orchards into natural populations cannot be disregarded. Most of the countries in which the sweet chestnut is grown have their own traditional varieties, obtained through long and hard work, i.e. selection over several centuries (Fernández-López and Pereira 1993; Conedera et al. 1994; Hennion 2009; Bounous 2009; Bouffier and Maurer 2009; Gomes-Laranjo et al. 2009; Pereira-Lorenzo et al. 2001a, b, 2009; Martín et al. 2007, 2010b, c; Soylu et al. 2009). The best and most widely known cultivars from the standpoint of nut quality are those of the marron type. Marrons are cultivars (varieties) of the sweet chestnut with the best quality tasty, large fruits of oblong shape and with a small scar, light brown in colour, with slightly protruding, longitudinal dark stripes, easy to peel and rarely having double seeds. Marron in Croatia (known as 'Lovran Marron') was planted on private estates on the eastern slopes of the Učka mountain, near the town of Lovran, where the oldest plantations are several hundred years old (Idžojtić et al. 2012). The trees from wild chestnut populations in those areas grow either very close to or intermixed with grafted cultivated units. Marron trees in those areas are several hundred years old, while those from natural populations are at least 70 years old. In addition, in these forest/orchard mixed populations, the so-called 'Marušnjak trees' (local name) can be found, denoting sweet chestnut trees with fruits similar to marrons (Poljak et al. 2016). Marušnjak trees are probably grown from the Lovran Marron seeds, and as they are not grafted, they can be easily distinguished from the locally cultivated marron trees.

In Mediterranean countries such as Italy, France, Spain and Portugal, with a long tradition of cultivating chestnut varieties, genetic diversity was studied using different markers (Pereira-Lorenzo et al. 1996, 2006; Goulão et al. 2001; Ramos-Cabrer and Pereira-Lorenzo 2005; Martín et al. 2009, 2010c, 2016; Torello Marinoni et al. 2013) but without insights into the gene flow between wild and cultivated populations (Pereira-Lorenzo et al. 2010). Even though the genetic diversity of chestnut varieties Europe-wide is well documented, no evidence of cultivated-to-wild gene flow has been provided thus far.

The main objective of this study was to assess the genetic diversity and structure of wild and cultivated sweet chestnut populations in the as of yet poorly researched part of its natural distribution. Of particular interest here are wild populations from the western part of the Balkan Peninsula, which are believed to have been one of the most important refugia for the European flora during the ice ages (Huntley and Birks 1983; Taberlet et al. 1998; Petit et al. 2002; Krebs et al. 2004; Heuertz et al. 2004a, b, 2006; Magri et al. 2006; Liepelt et al. 2009; Cornille et al. 2013; Temunović et al.

2012, 2013; Havrdová et al. 2015). In addition, we analysed whether there is gene flow between cultivated and wild sweet chestnut populations and what is the origin of so-called Marušnjak trees.

Materials and methods

Plant material

A total of 327 sweet chestnut trees were sampled in 15 wild populations from Central Europe and the western part of the Balkan Peninsula as well as 26 individuals belonging to the Lovran Marron cultivar (Fig. 1, Table S1). From the population P01 in the south-east to the population P12 in the north-west, samples of all known populations were collected. In Bosnia and Herzegovina and Montenegro, except in the most western part, as well as along the eastern Adriatic coast, there are no recorded wild and cultivated populations of the sweet chestnut. The minimum distance between individual trees was at least 50 m, to avoid the sampling of close relatives.

DNA extraction and SSR analysis

Total genomic DNA was extracted from fresh leaf tissue using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was determined with NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA). SSR amplification reactions were performed in a total volume of 20 μL containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 5 μM forward and reverse primers, 0.5 U *Taq*HS polymerase (TaKaRa Bio Inc., Shiga, Japan) and 10 ng of template DNA. Ten microsatellite loci were studied: CsCAT01, CsCAT02, CsCAT03, CsCAT04, CsCAT06, CsCAT14, CsCAT16, CsCAT17 (Marinoni et al. 2003), EMCs15 (Buck

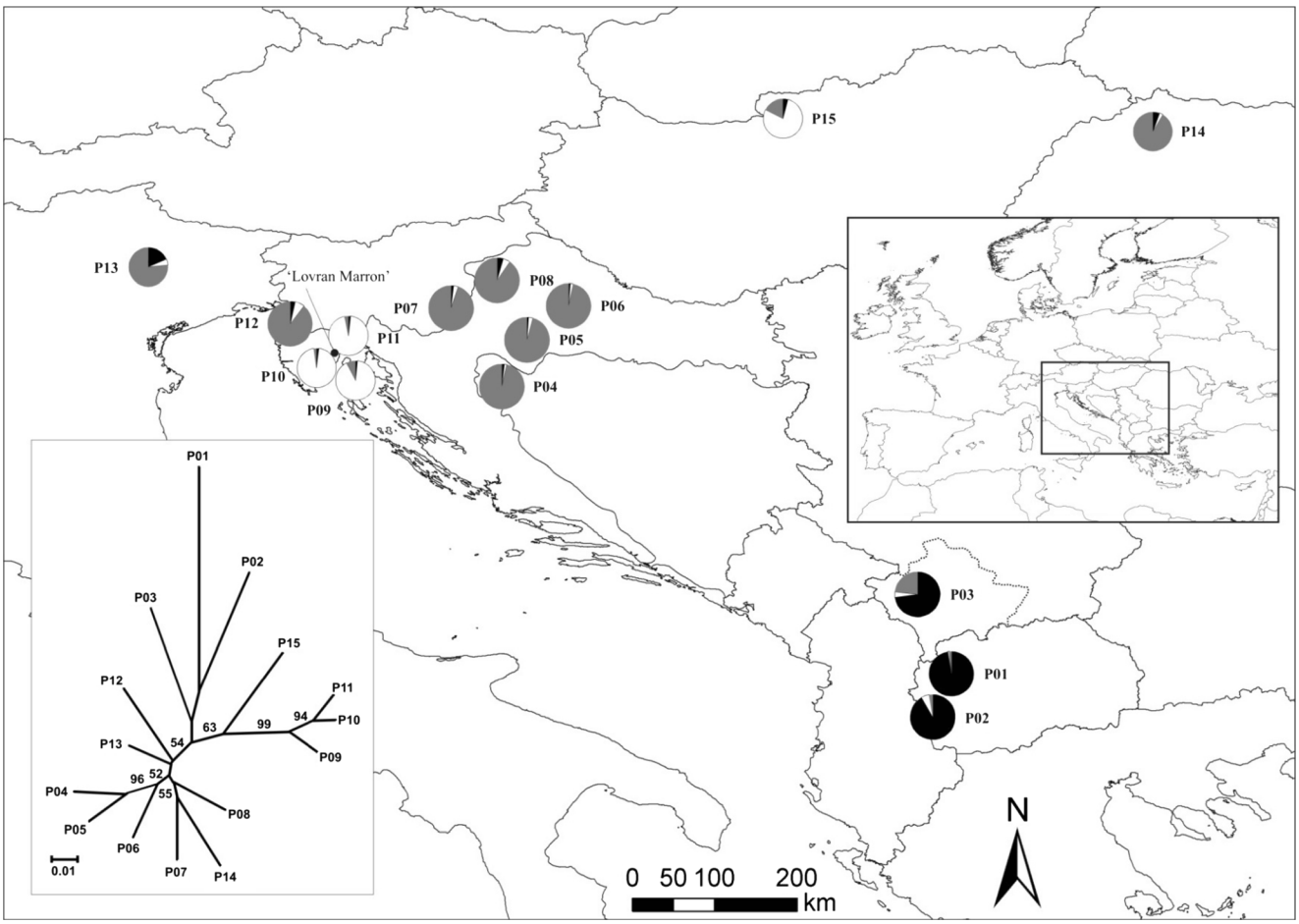

Fig. 1 Geographical distribution of the 16 sampled *Castanea sativa* populations. Bayesian analysis of the population structure of 15 wild populations using the software STRUCTURE assuming most probably $K = 3$ (the proportions of the ancestry of each population in each of the defined genetic clusters are colour-coded/genetic cluster A—*black*, genetic cluster B—*grey* and genetic cluster C—*white*) and unrooted *neighbour-joining* tree based on pairwise Cavalli-Sforza and Edwards' chord distances between 15 wild *C. sativa* populations (*numbers above the branches* indicate bootstrap support percentage over 50% in 10,000 pseudoreplicates)

et al. 2003) and OAL (Gobbin et al. 2007), using fluorescently labelled forward PCR primers. Amplification was performed in the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA). After the initial denaturation at 94 °C for 2 min, 35 three-step cycles followed: denaturation at 94 °C for 30 s; annealing for 45 s using different annealing temperatures (Ta) for each primer pair, as suggested by Marinoni et al. (2003), Buck et al. (2003) and Gobbin et al. (2007); and elongation at 72 °C for 90 s, after which a final elongation step at 72 °C for 8 min was added. The amplification products were run on an ABI3730XL analyser (Applied Biosystems, Foster City, California, USA). The resulting chromatograms were analysed for allele sizes using the GeneMapper 4.0 software (Applied Biosystems, Foster City, California, USA).

Data analysis

The polymorphism information content (*PIC*; Botstein et al. 1980) of each microsatellite marker was calculated by the PowerMarker 3.23 (Liu 2002) software. GENEPOP 4.0 (Raymond and Rousset 1995) was used to estimate population genetic parameters (the average number of alleles per locus, N_{av}; observed heterozygosity, H_O; expected heterozygosity, H_E; inbreeding coefficient, F_{IS}) and to test population genotypic frequencies for conformance to Hardy–Weinberg (HW) expectations. Sequential Bonferroni adjustments (Holm 1979; Rice 1989) were applied to correct for the effect of multiple tests using SAS 9.1 (SAS Institute 2004).

We used the programme Micro-Checker (van Oosterhout et al. 2004) to check for potential problems related to allele dropout and presence of null alleles. Estimates of null allele frequencies based on the expectation–maximization algorithm (Dempster et al. 1977) were then calculated using FreeNA (Chapuis and Estoup 2007).

Allelic richness, N_{ar}, as the measure of the number of alleles per locus independent of sample size and F_{ST}, and their respective *P* values, as the measures for genetic differentiation between all pairs of populations, were calculated by FSTAT 2.9.3.2 (Goudet 1995, 2002).

The programme BOTTLENECK 1.2.02 (Cornuet and Luikart 1996; Piry et al. 1999) was used to test for evidence of recent bottleneck events. The observed gene diversity (H_o) was compared to the expected gene diversity at mutation–drift equilibrium (H_{EQ}) and calculated from the observed number of alleles under the two-phase model (TPM). The TPM model was applied assuming 30% multistep changes and a variance of 30 (Pascual et al. 2001). Based on the number of loci in our dataset, the Wilcoxon signed-rank test (Luikart et al. 1998) was chosen for the statistical analysis of heterozygote excess or deficiency as recommended by Piry et al. (1999).

The analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed using Arlequin 3.0 (Excoffier et al. 2005). AMOVA was used to partition the total microsatellite diversity among and within populations. The variance components were tested statistically by non-parametric randomization tests using 10,000 permutations.

Pairwise Cavalli-Sforza and Edwards' chord distances (Cavalli-Sforza and Edwards 1967) were calculated, and the cluster analysis was performed using the Fitch-Margoliash algorithm with 1000 bootstraps (Felsenstein 1985) over microsatellite loci as implemented in SEQBOOT, GENDIST, FITCH, and CONSENSE programmes of the PHYLIP 3.6b (Felsenstein 1993).

A model-based clustering method was applied to infer genetic structure and define the number of clusters using the software STRUCTURE 2.3.3 (Pritchard et al. 2000). Given a value for the number of clusters, this method assigns individual genotypes from the entire sample to clusters so that linkage disequilibrium (LD) is maximally explained. The first dataset included all populations, while in the second, cultivated/naturalized populations were omitted. Ten runs per each cluster (*K*) ranging from 1 to 11 were carried out at the Isabella computer cluster at the University of Zagreb (Croatia), University Computing Centre (SRCE). Each run consisted of a burn-in period of 200,000 steps followed by 10^6 Markov chain Monte Carlo (MCMC) replicates assuming an admixture model and correlated allele frequencies. The choice of the most likely number of clusters (*K*) was carried out by comparing the average estimates of the likelihood of the data, $ln[Pr(X|K)]$, for each value of *K* (Pritchard et al. 2000), as well as by calculating an ad hoc statistic ΔK, based on the rate of change in the log probability of data between successive *K* values as described by Evanno et al. (2005) and implemented in Structure-sum-2011 (Ehrich et al. 2007).

After STRUCTURE analysis, AMOVA was also used to partition the total microsatellite diversity between the proposed STRUCTURE clusters, among populations within the clusters and within populations.

In order to determine whether the wild chestnut populations show signs of introgression from the Lovran Marron, relatedness between the marron and each wild chestnut tree was estimated. Relatedness (*r*) is defined as the expected fraction of alleles that are shared identical by descent (Blouin 2003). Thus, relatedness is the genetic similarity between two individuals, relative to that between random individuals from a reference population (Pamilo 1990). Relatedness coefficients range from −1 to 1, with positive values signifying two individuals sharing more alleles that were identical by descent than expected by chance. Expected relatedness coefficients are $r = 0.50$ between first-order relatives (e.g. parent–offspring and full sibling pairs), $r = 0.25$ between second-order relatives (e.g. half-siblings) and $r = 0.00$ for pairs of unrelated individuals. We calculated several relatedness estimators based on different method of moments approaches [Queller and Goodnight's (1989), Lynch and

Ritland's (1999) and Wang's (2002)], as well as by the maximum-likelihood method of Konovalov and Heg (2008), using the KINGROUP v2 software (Konovalov et al. 2004). All pairwise correlations between relatedness estimators were strong, ranging from 0.91 (Wang's vs. Konavolov and Heg's) to 0.96 (Lynch and Ritland's vs. Konovalov and Heg's). Thus, we present only the results of Konovalov and Heg's relatedness coefficient, as maximum-likelihood estimates generally exhibit lower standard errors and are less biased than traditional estimators based on the method of moments approach (Milligan 2003). Relatedness estimates were tested for significance by generating a null distribution (Belkhir et al. 2002) using the resampling procedure of Guo and Thompson (1992). Another approach in kinship analysis is to test hypotheses of pedigree relationships between pairs of individuals by calculating the likelihood ratio that a pair of genotypes fits a particular hypothesised relationship (Goodnight and Queller 1999). In this way, we tested the hypothesis that alleles shared by the marron and each wild chestnut tree were identical by descent (IBD) as a consequence of the primary hypothesis (kin relationship; $r > 0.25$) or null hypothesis (unrelated; $r < 0.25$). Secondly, we tested the hypothesis of parent–offspring relationship ($r = 0.50$) vs. half-sib relationship ($r = 0.25$). In this way, we assigned individual trees to categories of relationship to the Lovran Marron as parent–offspring, second-order relative or unrelated.

Results

We revealed that the 26 marron trees from Lovran are, in fact, a single clone with no variations between individuals. Therefore, in the population genetic analysis, we included only wild sweet chestnut populations. Analysing 10 microsatellite loci in 301 sweet chestnut trees from 15 wild populations, a total of 125 alleles were detected. The smallest number of alleles was found on locus EMCs15 (5 alleles) and the greatest on locus CsCAT3 (32 alleles). The values of *PIC* ranged from 0.381 (locus OAL) to 0.875 (locus CsCAT2), with the mean value of 0.710 (Table S2). The majority of loci had high *PIC* values, above 0.7, which confirm their efficiency in assessing the genetic diversity and structure of populations.

Genetic diversity parameters for each population based on allelic frequencies are summarized in Table 1. The average number of alleles per population ranged from 4.8 (P09 Cres, HR) to 6.7 (P13 Prealps, IT). The number of alleles independent of the number of samples in the analysed population (allelic richness, N_{ar}) ranged from 4.039 (P15 Nagymaros, HU) to 6.059 (P13 Prealps, IT), with a mean value of 4.901. The number of private alleles per population ranged from one to four, and 30 unique alleles were found in total. The greatest number of private alleles was found in the Macedonian population P01 Rečane and the Romanian population P14 Baia Mare. The lowest observed heterozygosity (H_O) was found in

Table 1 Population, country, sample size and genetic diversity estimates based on data from ten microsatellite loci in 15 wild *Castanea sativa* populations

No.	Population	Country	*n*	N_a	N_{ar}	N_{pr}	H_O	H_E	F_{IS}	$P_{Bottleneck}$
P01	Rečane	MK	20	4.900	4.503	4	0.450	0.386	−0.167 ns	0.539
P02	Skudrinje	MK	20	5.200	4.752	2	0.350	0.416	0.158 ns	0.246
P03	Kosovo	XK	20	4.900	4.616	2	0.600	0.505	−0.188 ns	0.188
P04	Cazin	BA	20	5.400	5.005	2	0.350	0.303	−0.157 ns	0.161
P05	Petrova gora	HR	20	5.300	5.003	1	0.300	0.271	−0.107 ns	0.313
P06	Moslavačka gora	HR	20	4.900	4.607	2	0.150	0.190	0.208 ns	*0.042*
P07	Samoborsko gorje	HR	20	5.300	4.883	1	0.300	0.261	−0.152 ns	0.246
P08	Medvednica	HR	20	6.100	5.564	3	0.500	0.501	0.003 ns	0.216
P09	Cres	HR	20	4.800	4.613	1	0.500	0.465	−0.077 ns	*0.009*
P10	Lovranska Draga	HR	26	5.800	4.967	2	0.654	0.519	−0.261 ns	0.385
P11	Učka	HR	15	4.900	4.846	1	0.500	0.385	−0.300 ns	0.097
P12	Buje	HR	20	5.800	5.305	2	0.350	0.396	0.116 ns	0.615
P13	Prealps	IT	20	6.700	6.059	2	0.200	0.317	0.369 ns	0.722
P14	Baia Mare	RO	20	5.100	4.759	4	0.250	0.224	−0.118 ns	0.161
P15	Nagymaros	HU	20	4.200	4.039	1	0.100	0.097	−0.027 ns	0.116

P values lower than 0.05 are indicated in italics

MK Macedonia, XK Kosovo, BA Bosnia and Herzegovina, HR Croatia, IT Italy, RO Romania, HU Hungary, *n* number of individuals per population, N_a average number of alleles per locus, N_{ar} allelic richness, N_{pr} number of private alleles, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient, ns non-significant values, $P_{Bottleneck}$ probability results of Wilcoxon signed-rank tests used to assess population bottleneck

the Hungarian population P15 Nagymaros (0.100) and the highest in the Croatian population P10 Lovranska Draga (0.654). The values of expected heterozygosity (H_E) in the majority of populations were somewhat lower and ranged from 0.097 (P15 Nagymaros, HU) to 0.519 (P10 Lovranska Draga, HR).

There was no evidence of allele dropout in the data according to the Micro-Checker programme. Null alleles were found in five of 150 alleles × population combinations. Their frequency was estimated using FreeNA and ranged from 0.109 for locus CsCat1 in the Hungarian population P15 Nagymaros to 0.176 for locus CsCat16 in the Croatian population P07 Samoborsko gorje.

No significant departures from the HW equilibrium were observed at any loci in any population. A significant surplus of heterozygosity in relation to population heterozygosity which is in mutation–drift balance ($H_E > H_{EQ}$), assuming a two-phase model, was found in two populations, P09 Cres and P06 Moslavačka gora (Table 1).

The average F_{ST} value across all populations was 0.133. According to the obtained F_{ST} values (Table S3), it was found that genetic differentiation was most pronounced between the population P01 (Rečane, MK) and all other studied populations. The highest F_{ST} value was found between populations P01 (Rečane, MK) and P15 (Baia Mare, RO), $F_{ST} = 0.302$, and the lowest between populations P10 (Lovranska Draga, HR) and P11 (Učka, HR), $F_{ST} = 0.001$. Genetic differentiation values were statistically significant for all population pairs, except between populations P10 (Lovranska Draga, HR) and P11 (Učka, HR). The AMOVA analysis showed that the largest proportion of molecular variance was attributable to the differences between individuals within populations (86.43%).

The unrooted Fitch–Margoliash tree clearly demonstrated that the populations were grouped by their geographical proximity into three major clusters (Fig. 1). Populations from continental Croatia, Bosnia and Herzegovina, Italy and Romania exhibit a clear genetic differentiation from populations from the Mediterranean region of Croatia (P09 Cres, P10 Lovranska Draga, P11 Učka), the Hungarian population P15 Nagymaros and populations from the southern area of study (P01 Rečane, P02 Skudrinje, P03 Kosovo). This result was supported by the bootstrap value of 54%. The Hungarian population Nagymaros and the Mediterranean populations from Croatia also grouped together, supported by the bootstrap value of 62%. Within that group, the separation of Croatian Mediterranean populations from the Hungarian population Nagymaros was supported by the bootstrap value of 99%.

The genetic structure of the 15 chestnut populations was inferred using the STRUCTURE software. The most probable division with the highest ΔK value was detected at $K = 3$ (Supplementary material, Fig. S1). The estimated population structure inferred for $K = 3$ is shown in Fig. 1. If the proportion of a certain population was equal to or higher than 0.75, it was assumed that the population belonged to one cluster, and if it was lower than 0.75, it was assumed that the population had a mixed origin. The populations from Macedonia were grouped together into genetic cluster A. The Kosovo population was of mixed ancestry, with the dominant proportion of its genetic makeup coming from genetic cluster A. The populations from continental Croatia, Bosnia and Herzegovina, Italy and Romania grouped together into genetic cluster B. The populations from the Mediterranean region of Croatia and the Hungarian population Nagymaros belonged to genetic cluster C. The results obtained with the STRUCTURE software were congruent with the clustering obtained with the unrooted Fitch–Margoliash tree.

The AMOVA analysis (Table 2) demonstrated that the majority of genetic diversity stems from the differences between individuals within populations (84.1%). However, a small, but highly significant, percentage of variation was explained by differences among populations within genetic clusters (8.92%) and by differences between clusters (6.67%).

The Konovalov and Heg's maximum-likelihood estimates of relatedness (r) between the Lovran Marron and the individual trees sampled from 15 wild sweet chestnut populations ranged from −0.149 to 0.796 (Supplementary material, Table S4). Average relatedness per population ranged from −0.085 (P06 Moslavačka gora, HR) to 0.414 (P10 Lovranska Draga, HR). Positive average relatedness was observed in four out of 15 populations (P09 Cres, HR; P10 Lovranska Draga, HR; P11 Učka, HR; P15 Nagymaros, HU), all belonging to genetic cluster C. Box plot of relatedness coefficients per population is shown in Fig. 2. Forty-six individuals that had a

Table 2 Analysis of molecular variance (AMOVA)

Source of variation	*df*	Variance components	% Total variation	ϕ statistics	$P(\phi)$
Among populations	14	0.495	13.57	0.136	<0.0001
Within populations	587	3.156	86.43		
Among groups	2	0.249	6.67	0.067	<0.0001
Among populations within groups	12	0.334	8.92	0.096	<0.0001
Within populations	587	3.156	84.41	0.156	<0.0001

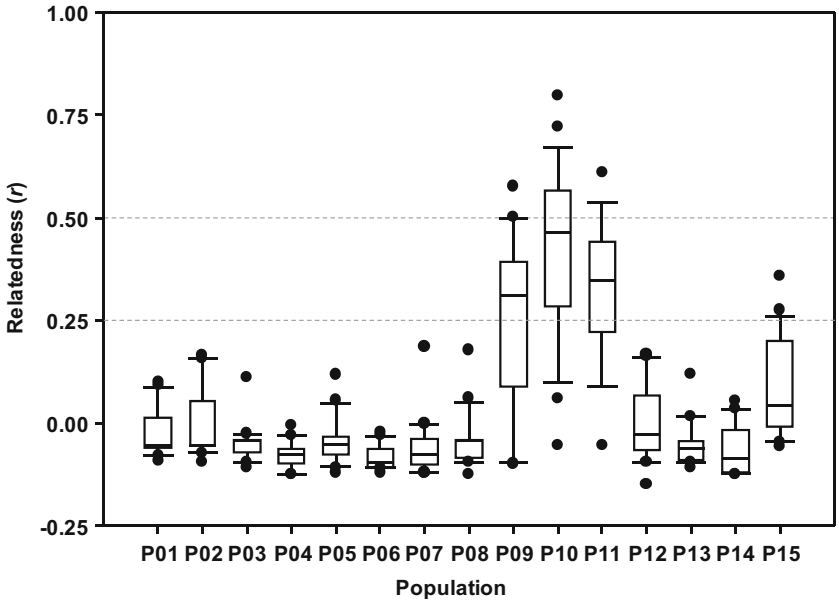

Fig. 2 Box plot of Konovalov and Heg's maximum-likelihood estimator of relatedness (*r*) between 'Lovran Marron' and individual trees sampled from 15 *Castanea sativa* populations. Expected critical relatedness coefficients ($r = 0.50$ between first-order relatives; $r = 0.25$ between second-order relatives) are indicated

relatedness coefficient higher than 0.25 were found exclusively in four abovementioned populations, and in all cases, *P* values were lower than 0.05, as obtained by the resampling procedure. Out of 46 individuals, 13 had a relatedness coefficient higher than 0.50 (expected in the case of first-order relatives) and were found exclusively in three populations close to Lovran (2 in P09; 10 in P10 and 1 in P11; Fig. 3). The likelihood ratio test gave similar results. Significant likelihood ratio tests between the primary hypothesis of kin relationship ($r > 0.25$) vs. the null hypothesis of unrelatedness ($r = 0.00$) were observed for all but one individual having a relatedness coefficient higher than 0.25. Similarly, by testing the hypothesis of parent–offspring relationship ($r = 0.50$) vs. half-sib relationship ($r = 0.25$), significant tests were obtained in the case of 11 out of 13 individuals having a relatedness coefficient higher than 0.50 (Fig. 3). As expected, in the population P10 from Lovranska Draga, the maximum number of both first- and second-order relatives was detected. In addition, it was found that allele 311 of locus OAL in the marron is the only allele which was not present in any of the analysed populations, except in populations P09, P10 and P11, and could therefore be used as a diagnostic marker.

Discussion

Genetic diversity and structure of sweet chestnut populations

This study represents the first report on the genetic diversity and structure of sweet chestnut populations in Central Europe

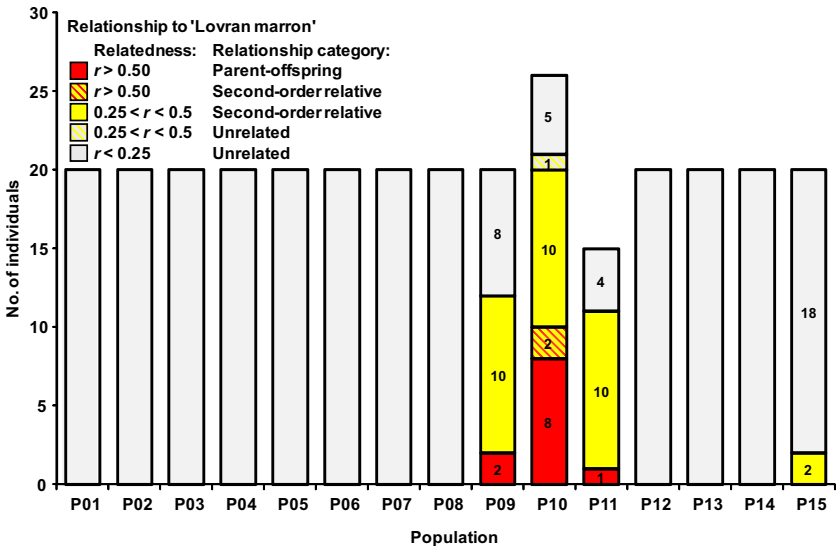

Fig. 3 Number of individuals in each of the 15 *Castanea sativa* populations classified according to their relatedness to 'Lovran marron' based on (a) the value of relatedness coefficient and (b) the category of relationship as assigned by likelihood-ratio tests

and the western part of the Balkan Peninsula. The study revealed the existence of three genetically and, to a large extent, geographically distinct and well-defined groups of sweet chestnut populations. Two not entirely separated groups of populations were detected in the northern part of the studied area and one in the southern. Genetic differentiation of populations was most pronounced between the southern populations from Macedonia (P01 and P02) and Kosovo (P03) and all other studied populations from Croatia, Bosnia and Herzegovina, Hungary, Romania and Italy. Increased divergence of southern and northern populations could be the result of colonization processes after the last ice age. Although the history of the sweet chestnut after the last ice age is still not entirely clear, it is very likely that, just as the majority of European tree species, it survived in glacial refugia on Mediterranean peninsulas (Krebs et al. 2004). The association between the chestnut populations' genetic data and the available data on chestnut refugia was established by Mattioni et al. (2013). The results of that study suggest a clear genetic divergence between eastern and western European populations. Similar results were also obtained in this study. The clear genetic divergence of north-western and south-eastern chestnut populations indicates that populations from the southern area of this study originate from the refugia in the southern part of the Balkan Peninsula, or even Asia Minor, while the populations from the northern area of this study originate from the refugia in the Apennine Peninsula. Long-term persistence of the sweet chestnut in these refugia caused distinct evolutionary processes and led to the differentiation of new genetic lineages (Hewitt 1996, 1999, 2001; Gassert et al. 2013).

In addition to the high level of genetic differentiation between the northern and the southern populations, a slightly lower level of genetic differentiation between southern populations was also observed. It could be explained by their origin from different refugia in the southern part of the Balkan Peninsula. On the other hand, the detected divergence between southern populations could indicate their relict character. In fact, the western and southern areas of the Balkan Peninsula were highlighted on several occasions as important refugia of the European flora (Huntley and Birks 1983; Petit et al. 2002; Heuertz et al. 2004a, b, 2006; Magri et al. 2006; Liepelt et al. 2009; Cornille et al. 2013; Temunović et al. 2012, 2013; Havrdová et al. 2015). According to the refugium probability index, Macedonia is located in the area for which a lower probability of the existence of a shelter zone was indicated (Krebs et al. 2004). However, in the same study, Krebs et al. (2004) note that no definite conclusion can be drawn about the origin of the sweet chestnut in the territory of former Yugoslavia, because a small number of paleontological data are available. In the study of Quaternary refugia of north European tree species, Bennett et al. (1991) claim that trees occupied the mid-altitude areas in the mountains of southern Europe, especially of the western Balkans. The same authors claim that it is very likely that many species survived the ice age in those refugia, but in such small population densities that they could not be detected in pollen records. Therefore, it is possible that the southern populations of the sweet chestnut in this study represent relict populations that survived in situ from the last ice age to the present day. Such relict populations are usually small, with a lower genetic diversity due to long-term isolation, and are usually very old, genetically more divergent and better adapted to the local, frequently suboptimal environmental conditions (Petit et al. 2003; Hampe and Petit 2005). This hypothesis is also supported by the conclusions of Lusini et al. (2014), who, on the basis of microsatellite data, postulated the existence of relict Bulgarian sweet chestnut forests in the adjacent region.

The 'mixed origin' of the Kosovo population (P03) is of particular interest, because our data indicate a certain gene flow between this population and the populations in the northern area of our study. However, this can be entirely ruled out, because the sweet chestnut is completely absent from central Bosnia and Herzegovina, while the high mountain massifs of the Balkans create a natural barrier to the gene flow between northern and southern populations. There is evidence of anthropogenic origin of chestnut populations in this region since Roman times (Sučić 1953; Brande 1973). Therefore, the admixed pattern of the Kosovo population is probably the result of human-mediated transplanting of plant material and/or gene flow between the natural populations and those introduced by the Romans.

Among the two groups detected in the northern part of the studied area, the populations of continental Croatia, Bosnia and Herzegovina, Italy and Romania grouped together within genetic cluster B. The presence of the Romanian population Baia Mare within this cluster can be explained by its anthropogenic origin (Conedera et al. 2004; Botu 2009). If the Romanian population Baia Mare is excluded from the Fitch–Margoliash tree, it is clearly visible that the populations from genetic cluster B are grouped according to their geographic distances. Although the sweet chestnut's distribution and genetic diversity were greatly influenced by humans, it is hard to believe that such a pattern of genetic structure could be created by human impact alone. In the study conducted in Spain by Martín et al. (2012), it was found that populations which were only 16 km apart belonged to different genetic clusters. Such a form of irregular geographic distribution of genetic diversity is associated with intentional expansion exclusively under human impact. The genetic structure of the populations in this study was in line with their geographic distribution, which suggests their natural origin.

Sweet chestnut forests in the continental part of Croatia and in north-western Bosnia and Herzegovina form a continuous part of the distribution of this species from the Prealps in north-eastern Italy, highlighted as one of the main refugia during the last ice age by Krebs et al. (2004). Therefore, it is

possible that the north-eastern part of Italy could have served as the point of origin for postglacial expansion of the sweet chestnut in Croatia and Bosnia and Herzegovina. The genetic diversity of peripheral chestnut populations in continental Croatia and Bosnia and Herzegovina is reduced, forming a west–east gradient of reduction, supporting this theory. Moreover, in one of the marginal populations, P06 Moslavačka gora, significant excess of heterozygosity was detected, suggesting a possible bottleneck event in the past. These results can be attributed to successive founding events during the postglacial recolonization (Comps et al. 2001). Hewitt (2000), Comps et al. (2001), Magri et al. (2006) and Liepelt et al. (2009) report that higher levels of genetic diversity can be expected in the refugia compared to areas colonized after the last ice age. Furthermore, our results are very similar to those obtained for *Fagus sylvatica* (Comps et al. 2001) and *C. sativa* (Mattioni et al. 2013). Comps et al. (2001) revealed a decrease in allelic richness (N_{ar}) and a simultaneous increase in gene diversity (H_E) and genetic differentiation among populations (F_{ST}), from the refugia towards newly colonized areas. These findings were attributed to long-distance founding events during the colonization, selection during population establishment and increased gene flow in lower population densities.

As opposed to the hypothesis of successive natural colonization events, the origin of the Croatian coastal populations could be explained by the strong human impact on this species. Results of pollen analysis (Beug 1977; Jahns and van den Bogaard 1998; Schmidt et al. 2000) and analyses of plant macrofossils (Šoštarić and Küster 2001) showed that the sweet chestnut was brought to the Mediterranean part of Croatia by the Greeks and Romans. Non-native origin of the population Cres can additionally be confirmed by the evidence of a human-mediated bottleneck event. Furthermore, Croatian coastal populations along with the Hungarian population Nagymaros exhibit a clear genetic differentiation from other northern populations and are grouped together in genetic cluster C. These results are consistent with morphometric studies of wild chestnut populations in Croatia (Idžojtić et al. 2009; Poljak 2014). The morphological traits that significantly differentiated populations Učka and Cres from other populations in Croatia were fruit weight (Idžojtić et al. 2009) and the shape of the leaf lamina (Poljak 2014), i.e. they had the highest average nut weight and narrower leaves. The presence of the Hungarian population Nagymaros in genetic cluster C could be also explained by anthropogenic impact, since there are no certain records of chestnut pollen in this area. The lowest values of genetic diversity, observed in this study, confirm opinions that the chestnut might have been introduced into this area from Italy in the fourteenth century (Zeller 2013) or earlier, by the Romans (Huntley and Birks 1983).

Cultivated-to-wild gene flow

In the Mediterranean area, in addition to the naturally growing sweet chestnut trees in forest stands, there are also cultivated plantations primarily grown for the production of large, high quality fruit (Fernández-López and Pereira 1993; Conedera et al. 1994; Hennion 2009; Bounous 2009; Bouffier and Maurer 2009; Gomes-Laranjo et al. 2009; Pereira-Lorenzo et al. 2001a, b, 2009; Martín et al. 2007; Martín et al. 2010b, c, Soylu et al. 2009). When regarding the nut quality, the best and most widely known cultivars are those of the marron type. Marrons are only those varieties with the best quality tasty and large fruits. In the Mediterranean part of Croatia, the only known traditional Croatian variety Lovran Marron is mostly grown within natural populations P09 Cres, P10 Lovranska Draga and P11 Učka (Idžojtić et al. 2012; Ježić et al. 2014). In these areas, cultivated-to-wild gene flow has been confirmed in our study. Therefore, this is the first report of hybridization between wild and cultivated sweet chestnuts. In fact, 30% of the individual trees from the population P10 Lovranska Draga are in the parent–offspring relationship to the Lovran Marron. Since the Lovran Marron is astaminate (Idžojtić et al. 2012), cultivated-to-wild gene flow could only be derived from marrons seeds. Actually, in these forest/orchard mixed populations, the so-called Marušnjak trees (local name) can be found, denoting sweet chestnut trees with fruits similar to the marrons. According to Poljak et al. (2016), Marušnjak trees are grown from the Lovran Marron seeds, and since they are not grafted, they can be easily distinguished from locally cultivated marron trees. Furthermore, Poljak et al. (2016) revealed that Marušnjak trees mostly have intermediate morphological and chemical characteristics of fruits compared to the Lovran Marron and trees from the natural population. Our results confirm that the Marušnjak trees are hybrids of marrons and of wild sweet chestnut trees. In addition, it should be noted that the Marušnjak trees are not astaminate, i.e. they produce male fertile catkins. In that case, gene flow between hybrids and wild sweet chestnut trees can be mediated not only by seeds but also by pollen. If we assume that these cultivated–wild crosses can yield viable and fertile progeny, backcrossing between hybrids and wild progenitors can occur and therefore maintain cultivated genes in the wild trees' gene pool. As there are no records on when exactly the growing of the Lovran Marron began, it is difficult to determine the exact time when the introgression commenced. The first records of the Lovran Marron date back to the early seventeenth century. According to Medak et al. (2009), the fruits of this cultivar were exported as early as the seventeenth century and in addition to olives, grapevine and sweet cherries, they were one of the crops that had been providing livelihood to the population of the region for centuries. Therefore, we assume that the introgression of the Lovran Marron genotype into the surrounding wild populations probably started a few centuries

ago. Unlike the population P10 Lovranska Draga, neighbouring populations P09 Cres and P11 Učka from the same region had a significantly smaller number of direct relatives with the Lovran Marron. This can be explained by the fact that the latter two populations are considerably more distant from cultivated populations than is the case with the population P10 Lovranska Draga.

Introgression from cultivated-to-wild populations has been observed in other woody species: *Prunus* spp. (Delplancke et al. 2011), *Theobroma cacao* (Chumacero de Schawe et al. 2013), *Macadamia* spp. (O'Connor et al. 2015) and *Malus* spp. (Coart et al. 2003; Cornille et al. 2015). It is reported that gene flow from grafted trees can cause a reduction in the genetic diversity of wild populations (Ellstrand et al. 1999; Wolf et al. 2001). Nevertheless, Croatian Mediterranean populations have maintained relatively high values of genetic diversity. It was also observed that genetic differentiation among these populations is decreased, which is obviously a consequence of anthropogenic activities, i.e. a long history of plant material transplanting and hybridization with cultivars. On the other hand, there is no evidence of cultivated-to-wild gene flow in populations from clusters A and B. Thus, our results indicate that cultivated-to-wild introgression in the sweet chestnut is dependent on the presence of orchards within the landscape. As is the case with the Lovran County and the island of Cres, Nagymaros has a long tradition of chestnut cultivation, dating back several centuries. In addition to forest stands, indigenous and Italian marrons are also grown in this area (Zeller and Bürgés 2013). Nevertheless, no genotypes related to marrons have been detected in the Hungarian Nagymaros population. These findings could be explained by the fact that marrons from the Nagymaros area were not included in our research. However, the relatedness coefficient of this population was positive and somewhere in between the relatedness coefficients observed in populations P09 Cres, P10 Lovranska Draga and P11 Učka and all other analysed populations. In this case, the possibility of gene flow via seed dispersal from grafted varieties into local areas cannot be ruled out either. The genetic makeup of the populations from genetic cluster C, and their clear differentiation from other populations from the northern area of study, is very likely a consequence of their anthropogenic origin, long history of cultivation and hybridization with local varieties.

Conclusions

Our results indicate that the genetic structure of sweet chestnut populations in Central Europe and the western part of the Balkan Peninsula is the result of both natural colonization events and significant and lengthy human impact. Furthermore, it has been proven that gene flow between the cultivated/grafted trees' and wild chestnut stands can influence their genetic structure and that the Marušnjak trees are hybrids of marrons and of wild sweet chestnut trees. These insights could play an important role in breeding programmes. We also believe that our results could be valuable baseline data for the development of more efficient conservation and management plans for this noble hardwood species.

Acknowledgements This study was supported by the Croatian Ministry of Science Education and Sport (project 068-0242108-2773), Swiss National Science Foundation (SCOPES project No. IZ73Z0_152525/1) and Croatian Science Foundation (Project No. 5381).

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Data archiving statement

Microsatellites data will be published on the DRYAD repository.