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CROSS-SPECIES AMPLIFICATION OF NUCLEAR EST-MICROSATELLITES DEVELOPED FOR OTHER *Pinus* SPECIES IN *Pinus nigra*

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Šarac Z., J. Aleksić, T. Dodoš, N. Rajčević, S. Bojović, and Petar Marin (2015*): Cross-species amplification of nuclear EST-microsatellites developed for other Pinus species in Pinus nigra.*- Genetika, Vol 47, No. 1, 205-217.

Due to the current lack of nuclear microsatellites (simple sequence repeats – SSRs) specifically developed for *Pinus nigra*, an important European coniferous species, we cross-species amplified 12 EST-SSRs (expressed sequence tagged SSRs) developed for other *Pinus* species in *P. nigra* in order to delineate loci which can be used for assessing levels of genetic diversity and genetic structuring in this species. We amplified these loci in individuals from seven populations from the central Balkans representing four recognized infraspecific taxa of *P. nigra* (ssp. *nigra*, var. *gocensis*, ssp. *pallasiana*, and var. *banatica*). Contrary to expectations on high transferability of EST-SSRs into related species, only three out of 12 tested loci were successfully amplified in *P. nigra*, but they displayed lack/low levels of polymorphism or generated multilocus amplification products. Thus, our estimates on levels of genetic diversity (H_E = 0.183) and genetic differentiation (F_{ST} = 0.007) were based on variability of a single locus harboring four alleles only and they should be taken with cautions. Our study highlights the need for the development of high-resolution molecular markers, such as co-dominant genic or genomic SSRs or predominantly biallelic SNPs, or utilization of anonymous dominant markers, such as AFLPs, for genotyping in *P. nigra.*

Key words: cross-species amplification, EST-SSRs, genetic diversity, *Pinus nigra,* the Balkans

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INTRODUCTION

Pinus nigra J.F. Arnold (Austrian or black pine) is a coniferous species of the tertiary origin. It is one of the economically and ecologically most important pine species in Europe which represents a typical component of Mediterranean and submediterranean coniferous forests. Its natural range, which extends from the North Africa through the northern Mediterranean and eastwards to the Black Sea (TUTIN *et al*., 1993), is highly fragmentary. Due to the high morphological polymorphism throughout its range, several infraspecific taxa have been described to date (e.g. VIDAKOVIĆ, 1991; TUTIN *et al*., 1993; STRID and TAN, 1997; RICHARDSON, 1998, QUÉZEL and MÉDAIL, 2003, etc.). Within the central Balkans, the Flora of Serbia (SARIĆ, 1992) recognizes two subspecies of *P. nigra* (ssp. *nigra* and ssp. *pallasiana* (D. Don) Holmboe) and several varieties of these taxa. Varieties *nigra* and *gocensis* Đorđ. (ssp. *nigra*), distributed from Austria to central Italy and the Balkans, are widespread throughout western and central Serbia, while varieties *pallasiana* and *banatica* Georg. et Ion. (ssp. *pallasiana*) are found only in eastern Serbia at few sites because their main natural ranges extends over Asia Minor, the Crimea, south-eastern part of the Balkan Peninsula, and the Southern Carpathians (TUTIN *et al*., 1993; BOŞCAIU and BOŞCAIU, 1999). Interestingly, recent biochemical studies revealed three distinct chemotypes (*nigra*, *pallasiana*, and *banatica*) within the central Balkans (BOJOVIĆ *et al*., 2012; ŠARAC *et al*., 2013).

The knowledge on levels of genetic diversity in plant populations and their genetic structuring is required not only for developing suitable conservation strategies but also for future breeding efforts (FRANKHAM *et al*., 2002). Studies accumulated over the past few decades reveal that at the nuclear (nuDNA) level, outcrossing conifers generally display high levels of genetic diversity in their large populations characterized by large effective population sizes, and low genetic differentiation of populations due to the effective gene flow through pollen and seed, and high connectivity of populations (HAMRICK *et al*., 1979; SORANZO *et al*., 1998; AL-RABAB'AH and WILLIAMS, 2002; NAYDENOV *et al*., 2014). However, the genetic profiling of *P. nigra* populations is not that advanced as compared to other conifers, and, at the nuclear DNA level, populations of this species have been studied to date only with allozymes (SCALTSOYIANNES *et al*., 1994; 2009; AGUINAGALDE *et al*., 1997; TSAKTSIRA *et al*., 1998), RAPD (LIBER *et al*., 2003; LUČIĆ *et al*., 2010; 2014) and ISSR markers (RUBIO-MORAGA *et al*., 2012).

To the best of the authors' knowledge, nuclear microsatellites (simple sequence repeats – SSRs) have not yet been developed for *P. nigra*. However, nuclear SSRs are versatile molecular markers which are preferred molecular tools for genotyping in plant and animal species due to their hypervariability, multiallelic and co-dominant nature, high reproducibility and amenability to automation and high throughput genotyping (ELLEGREN, 2004; VARSHNEY *et al*., 2005; SELKOE and TOONEN, 2006; HAYDEN *et al*., 2008). Genomic SSRs, found usually within non-coding and anonymous regions of the nuclear genome (MORGANTE *et al*., 2002; ELLEGREN, 2004), have traditionally been developed in conifers from genomic libraries (e.g. ELSIK and WILLIAMS, 2001). This approach, however, has been only partially successful because of the large and repetitive genomes of conifers which may results in complex multilocus amplification products at genomic SSR loci (ELSIK and WILLIAMS, 2001; BÉRUBÉ *et al*., 2007). To overcome this obstacle, low-copy libraries (e.g. ELSIK and WILLIAMS, 2001) and other approaches (reviwed in SHEPARD *et al*., 2002) have been used for developing single-copy genomic SSRs. However, these procedures are usually costly and time consuming and thus, cross-species amplification of orthologous genomic SSRs among pines has been suggested as a valued methodology and applied by several authors

(reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004). The success of this approach was variable and dependent on numerous issues including the divergence time between species for which genomic SSRs were developed and species in which SSRs were tested (reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004).

On the other hand, the availability of genomic resources and EST (expressed sequence tags) databases in conifers enabled development of EST-SSRs found within transcribed but untranslated portion of the genome (e.g. RUNGIS *et al*., 2004; BÉRUBÉ *et al*., 2007). Although EST-SSRs are belived to be less variable than genomic SSRs, they have less null alleles and, as a major advantage, high level of transferability to related species (ELLEGREN, 2004; VARSHNEY *et al*., 2005; HAYDEN *et al*., 2008). To date, EST-SSRs have been developed for *P. taeda* (LIEWLAKSANEEYANAWIN *et al*., 2004; BÉRUBÉ *et al*., 2007; ECHT *et al*., 2011), *P. taeda* and *P. pinaster* (CHAGNE *et al*., 2004), and *P. contorta* (LESSER *et al.*, 2012).

The aim of the present study is to cross-species amplify EST-SSRs developed for other pine species (i.e. *Pinus taeda*, subgenus *Pinus*, section *Trifoliae*, subsection *Australes*, CHAGNE *et al*., 2004, LIEWLAKSANEEYANAWIN *et al*., 2004, ECHT *et al*., 2011; and *P. contorta*, subgenus *Pinus*, section *Trifoliae*, subsection *Contortae*, LESSER *et al*., 2012) in *Pinus nigra* (subgenus *Pinus*, section *Pinus*, subsection *Pinus*), and to employ transferable loci to assess levels of genetic diversity and genetic differentiation among seven native *P. nigra* populations from the central Balkans representing all recognized infraspecific taxa of this species found within this region.

MATERIALS AND METHODS

Plant Material. Plant material from seven typical native populations of known *Pinus nigra* taxa from the central Balkans was used - *P. nigra* ssp. *nigra* was represented by three populations: I - Banjska stena, II - Omar, III - Zmajevački potok; *P. nigra* var. *gocensis* by two populations: IV - Priboj–Crni vrh, and V - Goč-Gvozdac; *P. nigra* ssp. *pallasiana* by one population: VI – Jarešnik; and *P. nigra* var. *banatica* by one population: VII - Lazareva reka. Young twigs with needles from the lowest third of the tree crown were collected in the late summer to the early fall 2009 from 15 individuals per population with one exception only – this was population VII represented by 14 trees. Sampled trees were randomly selected and they were distant at least 30 m. Plant material deposited in labeled polyethylene bags (sample plot, date of collection, and age of the needles) was immediately transferred to a freezer and stored at –20 °C prior to DNA extraction. Details about locations and ecological conditions of the populations used in the present study have been reported previously (BOJOVIĆ *et al.*, 2012; ŠARAC *et al*., 2013).

Selection of EST-SSRs. EST-SSRs used for cross-species PCR amplification in *P. nigra* were selected among 53 EST-SSRs reported by CHAGNE *et al*. (2004), 14 EST-SSRs of LIEWLAKSANEEYANAWIN *et al*. (2004), 21 EST-SSRs of ECHT *et al*. (2011) and 184 EST-SSRs of LESSER *et al.* (2012). The selection criteria included: 1) repetition motif - only EST-SSRs having a dinucleotide AT repetition motif were selected because such microsatellites are the most common in plants (e.g. MORGANTE and OLIVIERI, 1993, MORGANTE *et al*., 1996; RUNGIS *et al*. 2004); 2) the number of repeats - only loci displaying more than 10 repetitions of a dinucleotide AT motif were selected; 3) localization of a microsatellite within EST sequence - only microsatellites found within 5'- untranslated region (UTR) or 3'-UTR were selected, and 4) successful PCR amplification in all species used for validation. Thus, we used three EST-SSRs of CHAGNE *et al*. (2004) developed for *P. taeda* and validated in *P. pinaster*, *P. radiata*, *P. sylvestris*, *P. halepensis*,

P. pinea, *P. canariensis* (SsrPt_ctg7444, SsrPt_ctg7731, and SsrPt_ctg865), two EST-SSRs of LIEWLAKSANEEYANAWIN *et al.* (2004) developed for *P. taeda* and validated in *P. contorta* ssp. *latifolia*, *P. ponderosa*, *P. sylvestris* (Lop2 and Lop11), two EST-SSRs of ECHT *et al.* (2011) developed for *P. taeda* (PtSIFG_6015 and PtSIFG_6044), and five EST-SSRs of LESSER *et al.* (2012) developed for *P. contorta* and validated in *P. banksiana*, *P. ponderosa*, *P. palustris*, *P. elliotii*, *P. halepensis*, *P. longaeva*, *P. edulis*, *P. flexilis* (Pico_19, Pico_46, Pico_60, Pico_81, and Pico_182), yielding a total sample of 12 EST-SSRs used for cross-species amplification in *P. nigra*.

Isolation of DNA. Frozen plant tissue of each individual was homogenized with TissueLyser II (Qiagen, Valencia, CA, USA) and c. 30 mg of homogenized plant tissue per individual was used for DNA extraction following procedure of ALEKSIĆ *et al.* (2012). Genomic DNA was quantified and assessed for purity utilizing NanoVue (GE Healthcare Europe, Freiburg, Germany), and DNA solutions were diluted to a working concentration of 50 ng/µl.

Cross-species PCR amplification of EST-SSRs developed for other Pinus species in P. nigra. Twelve EST-SSRs selected for cross-species amplification in *P. nigra* were initially validated in a panel of seven *P. nigra* individuals representing each out of seven studied populations. PCR conditions and PCR amplification profiles are provided elsewhere (CHAGNE *et al.*, 2004 for PCR amplification of loci SsrPt_ctg7444, SsrPt_ctg7731, and SsrPt_ctg865; LIEWLAKSANEEYANAWIN *et al.*, 2004 for amplification of loci Lop2 and Lop11; ECHT *et al.*, 2011 for amplification of loci PtSIFG_6015 and PtSIFG_6044; and LESSER *et al.*, 2012 for amplification of loci Pico_19, Pico_46, Pico_60, Pico_81, and Pico_182). We used 2.5 µl of each PCR product for separation by electrophoresis using 2% agarose gels. The length of PCR products was assessed utilizing GeneRuler 50 bp DNA ladder (Fermentas UAB, Vilnius, Lithuania). For visualization of PCR products, we used Midori Green DNA Stain (NIPPON Genetics EUROPE GmbH, Dueren, Germany) added to agarose gel (2.0 µl for 100 ml of the agarose gel), and Vilber Lourmat ECX-F20.M transilluminator (Cedex 1, France).

Upon validation of 12 EST-SSRs in seven *P. nigra* individuals, three loci (SsrPt_ctg7444, SsrPt_ctg7731 and SsrPt_ctg865) were further amplified in the remaining individuals of *P. nigra*. For these PCR assays, we used forward (F) primers from each primer pair labelled with one out of four different fluorescent dyes from the Dye set G5 (DS-33, Applied Biosystems, USA) to enable fragment sizing on an automated DNA fragment analyzer: F primer for PCR amplification of SsrPt_ctg7444 was labeled with 6-FAM (blue), SsrPt_ctg7731 with NED (yellow), and SsrPt_ctg865 with PET (red). PCRs were performed in a total volume of 25 µl containing 50 ng template DNA, 1 × *Taq* buffer with $(NH_4)_2SO_4$ (Fermentas UAB, Vilnius, Lithuania), 2 mM $MgCl_2$ (Fermentas UAB, Vilnius, Lithuania), 0.2 mM dNTPs, 0.1 µM of each forward and reverse primers of a single EST-SSR locus, and 0.5 U *Taq* DNA polymerase (Fermentas UAB, Vilnius, Lithuania). PCRs were carried out in a peqStar 96 Universal thermal cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) with the following protocol: 5 min initial denaturation at 94 °C, 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C for EST-SSR SsrPt_ctg7444, 51°C for SsrPt_ctg7731, and 45°C for SsrPt_ctg865, 1 min extension at 72 °C, and 10 min final extension at 72 °C.

To downscale consumables and resources required for automated fragment sizing, we used a multipooling approach (HENEGARIU *et al.*, 1997), i.e. pooling of products of PCR amplification of individual EST-SSR loci in each out of 104 *P. nigra* individuals from seven populations. All PCR products were separated comercially via capillary electrophoresis by

Macrogen Europe, Amsterdam, the Netherlands (http://dna.macrogen.com/eng/) using 96-capillary 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Inc. USA). Sizing of fragments was performed manually with GeneMapper ver. 4.0 (Applied Biosystems, Foster City, USA).

Parameters of genetic diversity and genetic differentiation. The following parameters of genetic diversity were assessed for each microsatellite locus, population and at the species level: the number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E, unbiased estimator of NEI, 1978), and inbreeding coefficients (F_{IS}, WEIR and COCKERHAM, 1984) using ARLEQUIN 3.0 (EXCOFFIER *et al*., 2005) and GENEPOP 4.0 (ROUSSET, 2008). The F_{ST} parameter of genetic differentiation was calculated using ARLEQUIN 3.5.1.2, employed also for the analysis of molecular variance (AMOVA, EXCOFFIER *et al*., 1992).

RESULTS

Cross-species PCR amplification of EST-SSRs developed for other Pinus species in P. nigra. The validation of 12 EST-SSRs revealed that only loci SsrPt_ctg7731 and SsrPt_ctg865 were successfully amplified in majority of seven tested *Pinus nigra* individuals, while amplification of both SsrPt_ctg7444 and Pico 46 was successful in three individuals each (Fig. 1). However, the amount of PCR products (inferred from the intensity of bands on agarose gels) was higher upon amplification of SsrPt_ctg7444 than Pico 46, and thus, the former locus was selected for amplification in the remaining individuals of *P. nigra* along with SsrPt_ctg7731 and SsrPt_ctg865.

The cross-species amplification of three EST-SSRs developed for *P. taeda* in 104 individuals of *P. nigra* was partially successful. At loci SsrPt_ctg7444 and SsrPt_ctg7731, amplicons of expected length were detected in 52 and 77 individuals, respectively, while at locus SsrPt_ctg865, multiple amplification products were observed in all individuals in which PCR amplification of this locus was successful (67 individuals).

Parameters of genetic diversity and genetic differentiation. At locus SsrPt_ctg7444, only one allele, 275 bp long, was detected in 52 individuals, revealing a complete lack of variability at this locus in *P. nigra*. Four alleles, 208, 210, 218 and 220 bp long, were detected in 77 individuals at locus SsrPt_ctg7731. Due to the low polymorphism of this locus, invariability of SsrPt_ctg7444, and occurrence of multiple amplification products at SsrPt_ctg865, additional PCRs in individuals for which PCR products were not generated, were not performed for any of these loci. These PCR assays would be required for distinguishing between null alleles at loci in question from a simple failure of a PCR reaction. Nonetheless, the parameters of genetic diversity were calculated for locus SsrPt_ctg7731 (Table 1). The observed heterozygosity (H_O) ranged from 0.000 in population Omar to 0.250 in population Goč and averaged 0.176. The expected heterozygosity (H_E) ranged from 0.000 in Omar to 0.289 in Zmajevački potok, and averaged 0.183. The inbreeding coefficient (F_{IS}) was low in overall sample (0.007), negative in populations Banjska stena, Goč, Jarešnik, and Lazareva reka, equal to zero in population Priboj, and relatively high and positive in population Zmajevački potok.

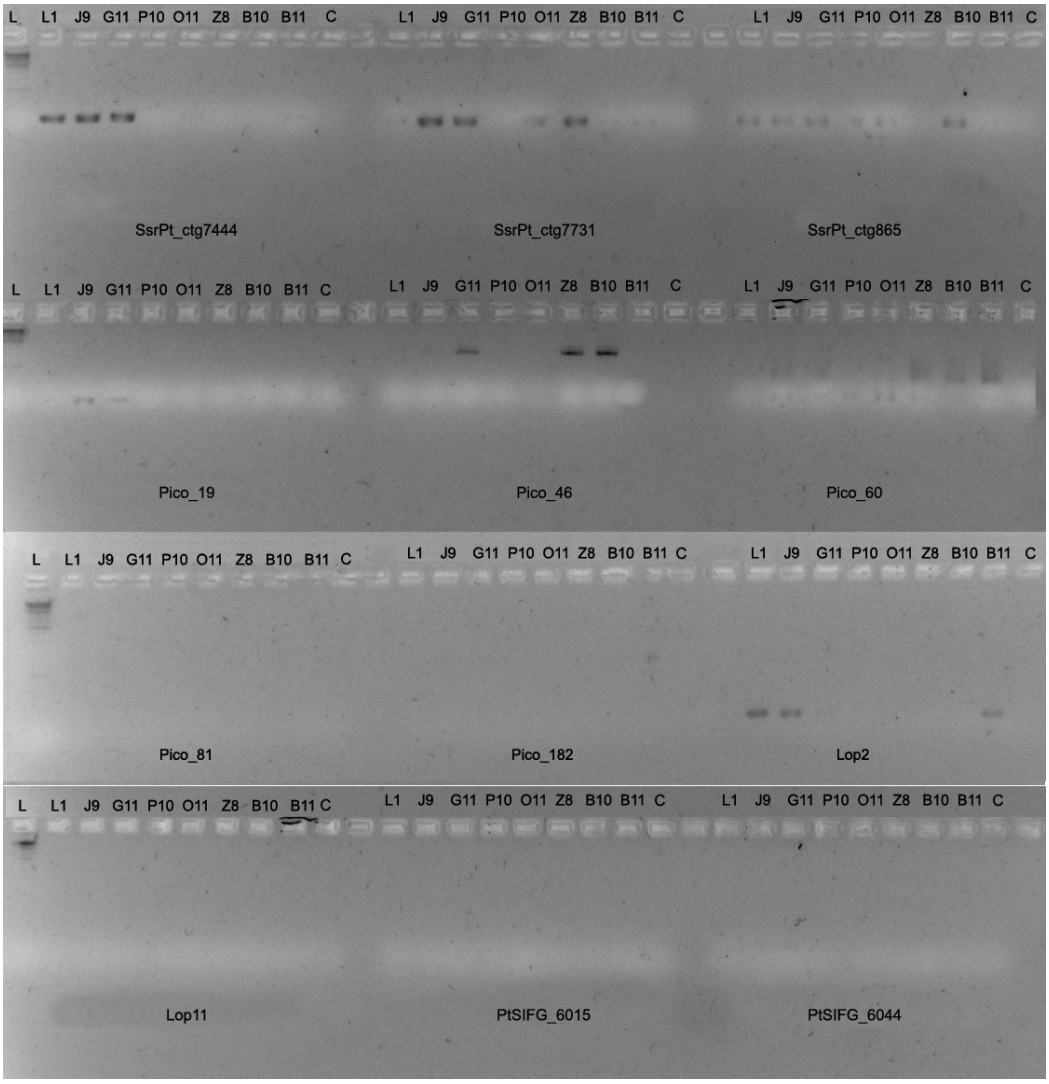

Fig. 1. The outcomes of PCR amplification of 12 EST-SSRs developed for other *Pinus* species in *P. nigra*
L – DNA ladder; L1, J9, G11, P10, O11, Z8, B10 and B11 – *P. nigra* individuals from seven populations; C – control (PCR amplification without DNA); SsrPt_ctg7444, SsrPt_ctg7731, and SsrPt_ctg865 (CHAGNE *et al*., 2004); Lop2 and Lop11 (LIEWLAKSANEEYANAWIN *et al*., 2004); PtSIFG_6015 and PtSIFG_6044 (ECHT *et al*., 2011); Pico_19, Pico_46, Pico_60, Pico_81, and Pico_182 (LESSER *et al*., 2012).

Table 1. Parameters of genetic diversity in P. nigra from the central Balkans based on variability of a single EST-SSR locus (SsrPt_ctg7731)

Population[a)]	*N*	*A*	H_O	H_E	F_{IS}
I - Banjska stena	15	2	0.200	0.189	-0.059
II - Omar	15	1	0.000	0.000	-
III - Zmajevački potok	15	4	0.231	0.289	0.200
IV – Priboj (Crni vrh)	15	2	0.125	0.125	0.000
V - Goč	15	3	0.250	0.235	-0.065
VI - Jarešnik	15	2	0.231	0.212	-0.091
VII – Lazareva reka	14	2	0.200	0.189	-0.059
I-VII	**104**	**4**	**0.176**	**0.183**	**0.007**

N – sample size; *A* - the number of alleles; H_O - observed heterozygosity; H_E - expected heterozygosity; F_{IS} - inbreeding coefficient.

Genetic differentiation and analysis of molecular variance. The only polymorphic locus in *P. nigra*, SsrPt_ctg7731, was used also for calculations of genetic differentiation and AMOVA. The value of the F_{ST} parameter was low, negative and insignificant (-0.020, $p = 0.822$, Table 2), suggesting that certain individuals from different populations may be genetically more similar among themselves than to individuals from their own populations. AMOVA revealed also the lack of genetic structuring due to the low and negative percentage of molecular variance assigned to among population variation, and high and positive percentage of molecular variance assigned to within population variation (Table 2).

Table 2. Analysis of molecular variance (AMOVA) and F_{ST} in P. nigra from the central Balkans based on variability of a single EST-SSR locus (SsrPt_ctg7731)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	6	0.309	-0.00183	**-2.04**
Within populations	147	13.477	0.09168	**102.04**
Total	153	13.786	0.08984	

F_{ST} = -0.020, p = 0.822

DISCUSSION

P. nigra represents one of the economically and ecologically most important pine species in Europe. The knowledge on levels of genetic diversity and genetic structuring in this species is required for both conservation of its genetic diversity and tree-improvement programs (FRANKHAM *et al*., 2002). However, to date, large-scale studies on genetic patterns in *P. nigra* were mainly based on variability at the chloroplast (cpDNA) (e.g. NAYDENOV *et al*., 2006; AFZAL-RAFII and DODD, 2007; JARAMILLO-CORREA *et al*., 2010; SOTO *et al*., 2010) and mitochondrial (mtDNA) level (e.g. JARAMILLO-CORREA *et al*., 2010). Nuclear diversity in this species has been studied using rather diverse molecular markers (listed below), but not with nuclear microsatellites, which are the markers of choice for population genetics and other studies in plant and animal species

(ELLEGREN, 2004; VARSHNEY *et al.*, 2005; SELKOE and TOONEN, 2006; HAYDEN *et al.*, 2008). To the best of the authors' knowledge, these molecular markers have not yet been developed for *P. nigra*.

Nonetheless, EST-SSRs, characterized by a high transferability into the related taxa (ELLEGREN, 2004; VARSHNEY *et al.*, 2005; SELKOE and TOONEN, 2006; HAYDEN *et al.*, 2008), have been developed for *P. taeda* (LIEWLAKSANEEYANAWIN *et al*., 2004; BÉRUBÉ *et al*., 2007; ECHT *et al*., 2011), *P. taeda* and *P. pinaster* (CHAGNE *et al*., 2004), and *P. contorta* (LESSER *et al.*, 2012). In order to infer genetic patterns at the nuclear genome in seven populations of *P. nigra* from the central Balkans representing all recognized infraspecific taxa found within this region (SARIĆ, 1992), we cross-species amplified EST-SSRs developed for other *Pinus* species in *P. nigra* to delineate loci which can be used in this species. However, contrary to expectations on high transferability of EST-SSRs into related taxa, we found that only three out of 12 EST-SSR loci (25%) tested in a panel of seven *P. nigra* individuals were successfully PCR amplified in this species, revealing a rather poor transferability of these markers into *P. nigra* (Fig. 1). Such a limited cross-species transferability was in the range or even lower than transferability of genomic SSRs among pine species (reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004).

Moreover, we found that even the loci which were successfully transferred and amplified in *P. nigra* may be invariable (SsrPt_ctg7444) or may generate multilocus amplification products (SsrPt_ctg865) and thus, must be discarded from future work. The only locus which was successfully amplified and was polymorphic in *P. nigra* was SsrPt_ctg7731. The number of alleles at this locus, however, was low (four). On the other hand, GONZÁLEZ-MARTÍNEZ *et al.* (2004) found that the number of polymorphic trans-specific genomic SSRs in tested *Pinus* species was variable, and that those which were polymorphic *P. nigra* displayed up to 8 alleles per locus upon testing in c. 15 individuals. Thus, a rather low number of alleles observed at a single EST-SSR locus in the present study in comparison to the findings of GONZÁLEZ-MARTÍNEZ *et al.* (2004) is actually in agreement with the expected higher polymorphism of genomic SSRs than EST-SSRs (ELLEGREN, 2004; VARSHNEY *et al.*, 2005; SELKOE and TOONEN, 2006; HAYDEN *et al.*, 2008).

However, due to the low number of alleles detected at locus SsrPt_ctg7731 in *P. nigra*, the estimate of genetic diversity in this species was low (H_E = 0.183). This was rather unexpected due to the life history traits of all conifers, which usually translates into high levels of genetic diversity in their populations and species *per se* (HAMRICK *et al*., 1979). For instance, rather high levels of genetic diversity have been reported in several *Pinus* species based on genomic SSR variability (e.g. H_E of 0.850 in one population of *P. sylvestris*, SORANZO *et al*., 1998; 0.679 in 5 populations of *P. taeda*, AL-RABAB'AH and WILLIAMS, 2002; 0.508 in 17 populations of *P. recinosa*, BOYS *et al*., 2005; 0.403 in 27 populations of *P. pinaster*, NAYDENOV *et al*., 2014). Nonetheless, a low level of nuDNA diversity has been reported in stone pine, *P. pinea*, a species which experienced a prolonged bottleneck during the Quaternary (VENDRAMIN *et al.*, 2008) that has led to the severe decrease in levels of diversity not only at the nuclear level (H_E of 0.015 based on allozyme variability, FALLOUR *et al*., 1997; H_E of 0.11 based on nuSSR variability, PINZAUTI *et al*., 2012), but also at the cpDNA level (H_E = 0.019, VENDRAMIN *et al.*, 2008). On the other hand, RUBIO-MORAGA *et al*. (2012) studied ISSR diversity in Spanish and Moroccan *P. nigra* populations and found variable and relatively low levels of genetic diversity among eight populations (H_E ranging from 0.123 to 0.242) explained by past forest management which has led to the decrease of levels of genetic diversity in studied populations.

Although a low level of genetic diversity observed in *P. nigra* from the central Balkans at a single EST-SSRs locus may imply that this species may have been submitted to such past population dynamics that has led to its current low genetic diversity at the nuclear DNA level, that assumption may be in disagreement with observations on rather high levels of genetic diversity observed in *P. nigra* at cpSSRs (NAYDENOV *et al*., 2006; AFZAL-RAFII and DODD, 2007; JARAMILLO-CORREA *et al*. 2010; SOTO *et al*. 2010). This is because cpSSRs are characterized by lower mutation rates as compared to the nuSSRs (ANGIOI *et al*., 2009; ISMAIL, 2010). Nonetheless, it is worth mentioning that low levels of genetic diversity at the nuclear genome along with the high levels of genetic diversity at the chloroplast genome have been observed in plant species, and such pattern has been explained by processes such as genetic drift followed by isolation and local adaptation, association of microsatellite loci to loci that are under the purifying selection and other processes.

Unfortunately, LUČIĆ *et al*. (2010; 2014) and LIBER *et al*. (2003) studied RAPD diversity in *P. nigra* but without reports on levels of genetic diversity. SCALTSOYIANNES *et al*. (1994; 2009), AGUINAGALDE *et al*. (1997), and TSAKTSIRA *et al*. (1998), however, reported rather high levels of allozyme diversity in *P. nigra* (H_E ranging from 0.180 to 0.438). These findings would imply that our estimates of levels of genetic diversity are most likely biased because the used EST-SSR locus may not accurately reflect levels of genetic diversity in *P. nigra*. This is because EST-SSRs, which may be informative in species for which they have been developed, may be less variable or monomorphic in related species in which they have been cross-species amplified. Such a pattern has been observed at some genomic SSRs (reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004) and may hold not only for locus SsrPt_ctg7731 but also for locus SsrPt_ctg7444, the latter being invariable in *P. nigra*. Thus, all EST-SSR loci used in the present study should be omitted from future studies in this species. This would imply also that the observed low genetic differentiation of *P. nigra* populations from the central Balkans, which was assessed based on F_{ST} values (0.007) and AMOVA analysis and which prevented further taxonomic and/or other inferences in this species that were possible based on biochemical data (BOJOVIĆ *et al*., 2012; ŠARAC *et al*., 2013), should be taken with cautions. Nonetheless, low genetic differentiation among populations of coniferous species may be expected due to the effective pollen and seed flow in these species and high connectivity of their populations (HAMRICK *et al*., 1979).

CONCLUSION

Contrary to expectations on high transferability of EST-SSRs into related species (ELLEGREN, 2004; VARSHNEY *et al.*, 2005; SELKOE and TOONEN, 2006; HAYDEN *et al.*, 2008), cross-species amplification of EST-SSRs developed for other *Pinus* species was only partially successful in *P. nigra*. Namely, transferability success was 25%, and was in the range or even lower than transferability of genomic SSRs among pine species (reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004). Moreover, even loci that were successfully amplified in *P. nigra* individuals from seven populations from the central Balkans, representing all recognized infraspecific taxa of this species found within this region, displayed lack/low levels of polymorphism or generated multilocus amplification products, a pattern observed also in some trans-specific genomic SSRs (see GONZÁLEZ-MARTÍNEZ *et al.*, 2004 and references therein). Thus, in contrast to previous reports on relatively successful cross-species amplification of genomic SSRs among pine species (reviewed in GONZÁLEZ-MARTÍNEZ *et al.*, 2004), we cannot support this approach as suitable for tested EST-SSRs in studied species, and our estimates on levels of genetic diversity and genetic

differentiation in *P. nigra* based on variability of a single locus harboring four alleles only should be taken with cautions. However, it is worth mentioning that we used a rather low number of specifically selected EST-SSRs. Based on outcomes of our study, we highlight the need for the development of high-resolution nuclear markers, such as co-dominant genic or genomic SSRs or predominantly bi-allelic SNP, for genotyping in *P. nigra*, or utilization of anonymous dominant markers, such as AFLP, because their application does not require the knowledge on the primary nucleotide sequences of primer binding sites.

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INTER-SPECIJSKA AMPLIFIKACIJA NUKELARNIH EST-MIKROSATELITA RAZVIJENIH ZA DRUGE VRSTE BOROVA KOD *Pinus nigra*

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Izvod

U nedostatku nuklearnih mikrosatelita (simple sequence repeats – SSRs) specijalno razvijenih za crni bor, *Pinus nigra*, važne evropske četinarske vrste, urađena je inter-specijska amplifikacija 12 EST-SSRS razvijenih za druge vrste borova kod crnog bora da bi se detektovali lokusi koji se mogu koristiti za utvrđivanje nivoa genetičkog diverziteta i genetičke diferencijacije kod ove vrste. Odabrani lokusi su amplifikovani kod individua iz sedam populacija crnog bora iz centralnog Balkana koje pripadaju različitim infraspecijskim taksonima (ssp. *nigra*, var. *gocensis*, ssp. *pallasiana* i var. *banatica*). Suprotno očekivanoj visokoj transferabilnosti EST-SSR u srodnim vrstama, samo tri od 12 testiranih lokusa su uspešno amplifikovani kod crnog bora, ali su oni pokazali odsustvo/nizak nivo polimorfizma ili generisali multilokus amplifikacione produkte. Prema tome, utvrđene vrednosti nivoa genetičkog diveziteta (H_E = 0.183) i genetičke diferencijacije (F_{ST} = 0.007) su dobijene na osnovu varijabilnosti jednog lokusa sa samo četiri alela i moraju biti uzete sa rezervom. Ova studija ukazuje na potrebu razvijanja markera visoke rezolucije na nivou jedarnog genoma, kao što su ko-dominantni genski ili genomski SSRs ili pretežno bi-alelni SNP-ovi, ili korišćenje anonimnih dominantnih AFLP markera za genotipizaciju crnog bora.

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