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Genetic patterns in *Pinus nigra* from the central Balkans inferred from plastid and mitochondrial data

Highlights
- Seven populations of *Pinus nigra* from the central Balkans, representing four infraspecific taxa, were analyzed with chloroplast microsatellites and a mitochondrial locus.
- Molecular data failed to support infraspecific circumscriptions.
- Levels of genetic diversities/differentiation at both genomes were in the range of those reported in western Mediterranean populations of *P. nigra*.
- Iberian/African and Balkans' populations share one mtDNA polymorphism and differ in three mutations.

Abstract
Pinus nigra J.F. Arnold, European black pine, is a typical component of Mediterranean and sub-Mediterranean coniferous forests with highly fragmentary distribution. Western Mediterranean populations of this species have been studied genetically to date, while eastern populations from the central Balkans, which are larger and more abundant, are still genetically understudied. We analyzed seven populations of *P. nigra* representing all infraspecific taxa recognized within the central Balkans (subspecies *nigra* with varieties *nigra* and *gocensis* Đorđević; and subspecies *pallasiana* (Lamb.) Holmboe with varieties *pallasiana* and *banatica* (Endl.) Georgescu et Ionescu), with three chloroplast microsatellites (cpDNA SSRs) and one mitochondrial (mtDNA) locus. Although our molecular data failed to support circumscription of studied infraspecific taxa, we found that genetic patterns at both genomes are in accordance with those found previously in westward populations of this species, that is – exceptionally high levels of genetic diversity ($H_T=0.949$) and low genetic differentiation ($G_{ST}=0.024$) at the cpDNA level, and moderate levels of genetic diversity ($H_T=0.357$) and genetic differentiation ($G_{ST}=0.358$) at the mtDNA level. Based on genealogical relations of mtDNA types currently present in Balkans' and Iberian/African populations, we inferred that the ancestral gene pool of *P. nigra* already harbored polymorphism at position 328 prior to the divergence to two lineages currently present in westward and eastward parts of the species range distribution. Subsequent occurrence of three mutations, which distinguish these two lineages, suggests their long-term isolation.

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1   Introduction

Pinus nigra J.F. Arnold, European black pine, is one of the economically and ecologically most important pine species in Europe. It is a typical component of Mediterranean and sub-Mediterranean coniferous forests, with highly fragmentary distribution extending from the North Africa, the northern Mediterranean Basin and eastwards to the Black Sea (Gaussen et al. 1993). Due to its Tertiary origin, relict nature and a prominent phenotypic plasticity throughout the range distribution, its taxonomic status and infraspecific classification are still somewhat unclear (e.g. Vidaković 1991; Gaussen et al. 1993; Christensen 1993; see also references in Naydenov et al. 2006 and Ganopoulos et al. 2013).

To date, fragmentary distributed natural populations of *P. nigra* from the western part of the species range have been studied genetically (e.g. Afzal-Rafii and Dodd 2007; Jaramillo-Correa et al. 2010; Soto et al. 2010). However, those from the Balkans and Turkey, which are larger and more abundant, have occasionally been studied from various aspects (e.g. Liber et al. 2003; Lučić et al. 2010; 2013; Gülsoy et al. 2014) but rarely from a population genetics aspect (e.g. Naydenov et al. 2006, 2015; Šarac et al. 2015). The genetic knowledge on these populations is required not only for shedding new light on species infraspecific circumscription and overall genetic and/or phylogeographic structure, but also for implementation of suitable conservation measures and future breeding efforts (Frankham et al. 2002). Studies of this kind, however, are not straight-forward because, on the one hand, *P. nigra* is one of the most commonly planted conifers in Europe and the origin of the planted material cannot be tracked in all cases, and, on the other hand, this predominantly outcrossing species is characterized by a rather high dispersal ability (pollination and seed dispersal by wind, Afzal-Rafii and Dodd 2007; Jaramillo-Correa et al. 2010; Soto et al. 2010) suggesting possible introgression of alien gene pools into native populations.

Within the central Balkans, two subspecies of *P. nigra* (ssp. *nigra* and ssp. *pallasiana* (Lamb.) Holmboe), and their varieties (var. *nigra* and *gocensis* Đorđević of the former subspecies, and var. *pallasiana* and *banatica* (Endl.) Georgescu et Ionescu of the latter subspecies), have been recognized (Jovanović 1992). It has been shown recently that these infraspecific taxa differ at the biochemical level (Bojović et al. 2012; Šarac et al. 2013). In the frame of the present study, we employ: 1) paternally inherited and potentially more variable chloroplast microsatellites (cpDNA SSRs) shown to be informative for assessing genetic patterns in *P. nigra* and other pines (Naydenov et al. 2006; Afzal-Rafii and Dodd 2007; Jaramillo-Correa et al. 2010; Soto et al. 2010), and 2) a more slowly evolving maternally inherited mitochondrial (mtDNA) locus *nad*7 intron 1, proven to be suitable for depicting phylogeographic structure in westward populations of *P. nigra* (Jaramillo-Correa et al. 2010), and test them in individuals of *P. nigra* from seven populations representing all recognized infraspecific taxa of this species from the central Balkans in order to: 1) assess whether cpDNA and/or mtDNA markers are suitable for distinguishing infraspecific taxa of *P. nigra* from the central Balkans; 2) estimate levels of genetic diversity and genetic structuring at cpDNA and mtDNA levels in these genetically understudied populations of *P. nigra*; and 3) use available variation at the studied mtDNA locus to make inferences on historical events that have led to the current genetic patterns in westward and eastward parts of the species range.

2 Material and methods

2.1 Plant material and DNA extraction

Samples (young twigs with needles) were collected in early fall 2009 from 14–15 trees per population from seven native populations of *P. nigra* from the central Balkans which represent all infraspecific taxa of this species (ssp. *nigra* represented by var. *nigra* and var. *gocensis*; and ssp. *pallasiana* represented by var. *pallasiana* and var. *banatica*) recognized by the Flora of Serbia (Jovanović 1992). We sampled 104 trees in total (Table 1). Trees sampled from each population were distant at least 30 m from each other, and sampled populations were not in the vicinity of known planted trees/populations of this species. Plant material, deposited in labeled polyethylene bags, was stored at –20 °C prior to DNA extraction.

Approximately 30 mg of the plant tissue of each individual, homogenized with TissueLyser II (Qiagen, Valencia, CA, USA), was used for DNA extraction following Aleksić et al. (2012).

2.2 Molecular markers

Nine chloroplast (cpDNA) microsatellites (Pt1254, Pt26081, Pt36480, Pt45002, Pt71936, Pt79951, Pt15169, Pt30204, and Pt87268, Vendramin et al. 1996) were initially tested in a panel of seven randomly selected individuals from each out of seven populations following PCR amplification procedure of Vendramin et al. (1996) and using a peqStar 96 Universal thermal cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Three loci, Pt15169, Pt30204, and Pt87268, showing a successful amplification in all individuals and a potential length polymorphism of PCR products upon separation by 2% agarose gel electrophoresis, were further used and PCR amplified using fluorescently labelled forward (F) primers (DS-33, Applied Biosystems, USA). 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).

Three mitochondrial (mtDNA) loci, the second intron of the NADH dehydrogenase subunit 1 gene - *nad*1 gene intron 2 (primers published by Demesure et al. 1995), *nad*5 intron 4 (Dumolin-Lapegue et al. 1997), and *nad*7 intron 1 (Jaramillo-Correa et al. 2004; Tian et al. 2010), were initially tested in the same panel of seven *P. nigra* individuals following PCR conditions provided in the corresponding publications. However, PCR products were obtained only at locus *nad*7 intron 1 using the F primer of Tian et al. (2010) and the R primer of Jaramillo-Correa et al. (2004), and the following PCR conditions: a total volume of 25 μl containing 25 ng template DNA, $1 \times$ *Taq* buffer with $(NH_4)_2SO_4$ (Fermentas UAB, Vilnius, Lithuania), 2.5 mM $MgCl_2$ (Fermentas UAB, Vilnius, Lithuania), 0.2 mM dNTPs, 0.1 μM of each forward and reverse primers, 0.80% BSA (Bovine Serum Albumin, Fermentas UAB, Vilnius, Lithuania), and 0.025 U *Taq* DNA polymerase (Fermentas UAB, Vilnius, Lithuania), and a touchdown amplification protocol: 4 min initial denaturation at 94 °C, 11 cycles of 1 min denaturation at 94 °C, 45 s annealing at 68 °C, 2 min extension at 72 °C with progressive decrease of the annealing temperature by 0.5 °C/cycle, followed by 26 cycles of 1 min denaturation at 94 °C, 45 s annealing at 56 °C, 2 min extension at 72 °C, and final extension of 10 min at 72 °C. PCR products were Sanger sequenced by Macrogen Europe using F or both (F and R) primers (see Results).

Table 1. The infraspecific taxa of *Pinus nigra* from the central Balkans used in the present study, characteristics of sampling localities, and estimates of chloroplast microsatellite (cpDNA SSR) and mitochondrial (mtDNA) diversity.

Taxon		Location	Pop. ID	Latitude (N°)	Longitude (E°)	Altitude (m a.s.l.)	Geologic substratum	N	cpDNA SSR						mtDNA		
									A	PA	ch	cph	H_S	H_T	mh	H_S	H_T
P. nigra ssp. *nigra*	var. *nigra*	Banjska stena	I	43°57′04″	19°24′04″	1040	Limestone	15	12	1	12	4	0.978		2	0.538	
	var. *nigra*	Omar	II	43°54′02″	19°23′18″	950	Limestone	15	11	2	8	2	0.876		2	0.533	
	var. *nigra*	Zmajevački potok	III	43°51′45″	19°25′05″	830	Serpentine	15	11	0	11	2	0.952		2	0.533	
	var. *gocensis*	Priboj-Crni vrh	IV	43°34′56″	19°35′06″	1100	Serpentine	15	14	1	10	4	0.857		1	0.000	
	var. *gocensis*	Goč-Gvozdac	V	43°33′32″	20°40′56″	790	Serpentine	15	12	1	9	2	0.933		1	0.000	
	var. *pallasiana*	Jarešnik	VI	42°22′32″	22°24′10″	1300	Crystalline slate	15	16	1	12	4	0.962		1	0.000	
P. nigra ssp. *pallasiana*	var. *banatica*	Lazareva reka	VII	44°01′02″	21°55′39″	750	Limestone	14	12	1	9	4	0.923		1	0.000	
Total or average								104	22	7	38	22	0.926	0.949	2	0.229	0.358

N – sample size; A – the total number of cpDNA SSR alleles; PA – the number of private cpDNA SSR alleles; ch – the total number of cpDNA haplotypes; cph – the number of private cpDNA haplotypes; H_S – haplotype diversity within populations; H_T – total haplotype diversity; mh – the total number of mtDNA haplotypes.

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2.3 Data analysis

At the cpDNA level, we assessed the following parameters of genetic diversity at the population level and in overall sample: the number of alleles (A), the number of private alleles (PA), the number of chloroplast haplotypes (ch), the number of private haplotypes (cph), unbiased within population gene diversity (H_S, equivalent to H_E for diploid data, Weir, 1996), and the total gene diversity (H_T) using CONTRIB 1.02 (Petit et al. 1998). Using the same software, we assessed also the number of mitochondrial haplotypes (mh), H_S and H_T at the mtDNA level in populations and at the species level. The standardized coefficients of genetic differentiation among populations, G_{ST}, at the cpDNA and mtDNA levels were also calculated in CONTRIB.

Pairwise population differentiation estimates, F_{ST}, based on cpDNA haplotype frequencies, and the analysis of molecular variance (AMOVA, Excoffier et al. 1992), based on the sum of squared number of repeat difference between cpDNA haplotypes (Slatkin 1995), were calculated using ARLEQUIN 3.11 (Excoffier et al. 2005). The significance of tests was examined by 15 000 permutations.

TESS 1.2 (François et al. 2006; Chen et al. 2007) was used to further study genetic differentiation of populations based on cpDNA data, by estimating the number of genetically similar clusters. TESS incorporates the spatial locations of all individuals into the analyses to assess genetic cluster membership. Spatial coordinates of individual trees within defined areas, corresponding to population sizes, were generated using population sample coordinates. Using no admixture model and interaction parameter of 0.6, 100 runs at each K ranging from 2 to 4 with 50 000 sweeps and a burn-in of 10 000 for each run were performed. At each K, 20 runs with the lowest deviance information criterion (DIC) were recorded and used for calculating average DICs. As recommended by Durand et al. (2009), the optimal number of clusters may be determined by plotting average DIC against K, and depicting K at the beginning of the plateau.

MUSCLE (Edgar 2004) implemented in MEGA 5.04 (Tamura et al. 2011) was used for the alignment of mtDNA sequences, which were checked and edited manually. Genealogical relationships among mtDNA haplotypes were assessed by constructing median-joining network with NETWORK 4.6.1.2 (Bandelt et al. 1999). For this analysis, we used also *nad*7 intron 1 sequences of *P. nigra* available in the GenBank (accessed on 01-12-2014): accessions HQ185286, HQ185287, HQ185288, and HQ185289 (Jaramillo-Correa et al. 2010), and sequences of *P. sylvestris* L. used as outgroup (DQ665913, DQ665914, and DQ665915, Naydenov et al. 2007). Sequence gaps were treated as a fifth character state after recoding insertions/deletions (indels) longer than 1 base-pair (bp) as single-bp indels.

3　Results

Three cpDNA SSRs were successfully amplified in 104 *P. nigra* individuals, yielding 5–9 alleles per locus, 22 alleles in total, and 38 haplotypes (Table 1). The number of PA and cph were high (7 and 22, respectively) as well as average H_S (0.926) and H_T (0.949). Genetic differentiation of populations was low (G_{ST}=0.024). MtDNA locus *nad*7 intron 1 was successfully amplified and sequenced with F primer in 99 *P. nigra* individuals while sequencing with R primer was successful in 73 individuals. The distribution of mtDNA haplotypes among populations was not random, and two detected haplotypes (T and G, see later) were found at more or less equal frequencies in populations of *P. nigra* ssp. *nigra* var. *nigra* (I, II and III), while populations IV to VII, representing the other studied taxa, were fixed for haplotype T. Thus, the average H_S and H_T were low to moderate (0.229 and 0.357, respectively), while G_{ST} was moderate (0.358).
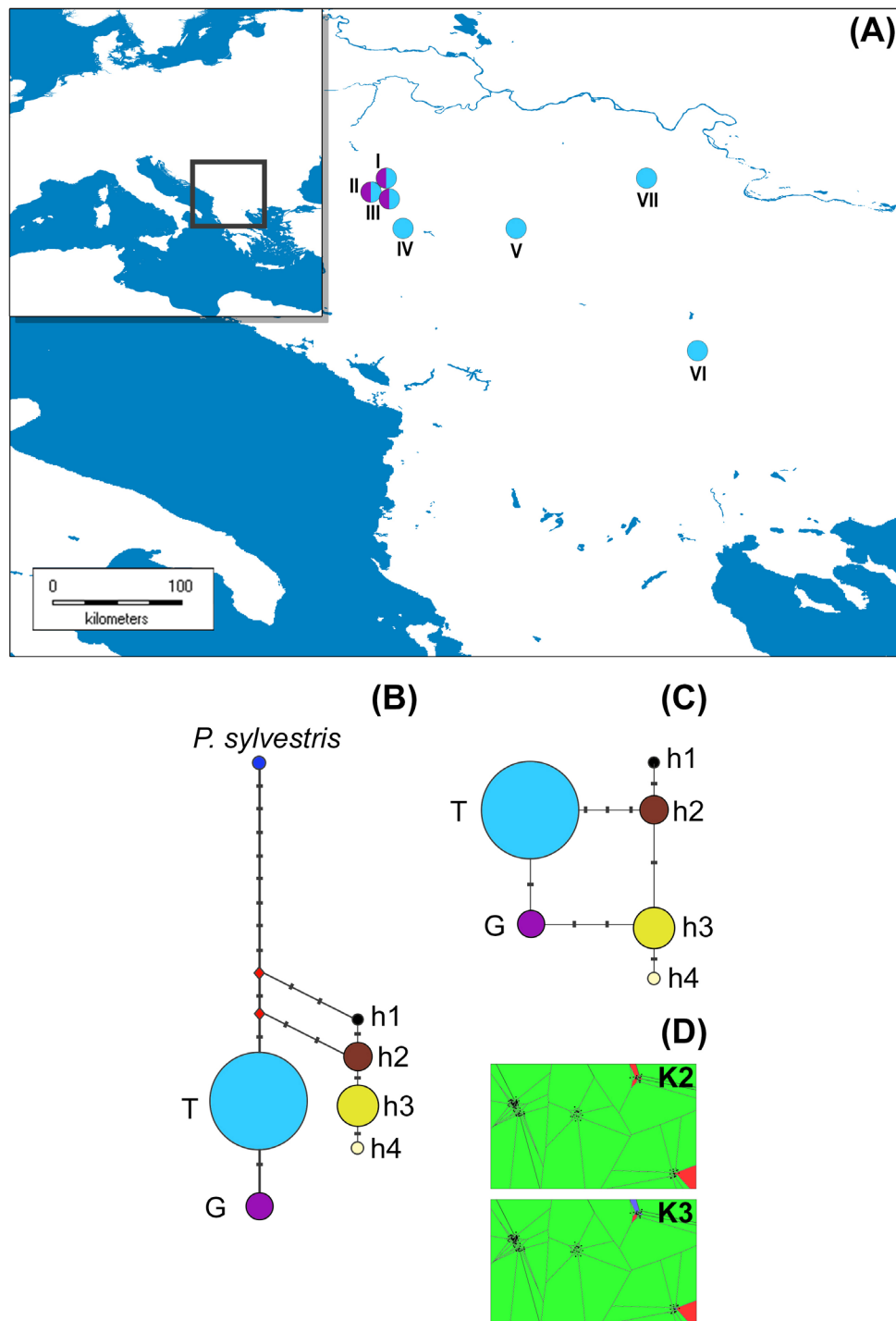
Fig. 1. The distribution of studied *P. nigra* populations within the central Balkans and the abundance of mtDNA haplotypes (A), haplotype networks based on mitochondrial data (B and C) and outcomes of TESS analyses based on chloroplast data (D).

Populations I, II and III represent *P. nigra* ssp. *nigra* var. *nigra*, populations IV and V represent *P. nigra* ssp. *nigra* var. *gocensis*, population VI represents *P. nigra* ssp. *pallasiana* var. *pallasiana*, and population VII represents *P. nigra* ssp. *pallasiana* var. *banatica* (see also Table 1); the size of circles in haplotype network rooted with *P. sylvestris* (B) and unrooted haplotype network (C) is proportional to the relative frequency of haplotypes T and G in eastward populations (see Table 1), and haplotypes h1 to h4 in westward populations, but the relative frequency of haplotypes in these regions is not comparable; mtDNA haplotype labels are given in the legend of Table 2; D – clustering of populations at K = 2 and K = 3 using TESS.

All population pairwise F_{ST} values were insignificant, and ranged from 0.066 (populations IV and V representing *P. nigra* ssp. *nigra* var. *gocensis*) to 0.000 or were negative (data not shown). Although AMOVA revealed that 1.72% and 98.28% of overall molecular variation were attributed to among and within population variation, respectively, these values were insignificant. When two subspecies of *P. nigra* were compared (populations I to V representing ssp. *nigra* vs. populations VI and VII of ssp. *pallasiana*), somewhat increased among population variation (2.06%), decreased within population variation (97.20%) and 0.73% of variation among populations within groups were observed. All values, however, were insignificant. The lack of genetic structuring of populations at the cpDNA level was revealed by TESS analysis as well, because at each tested *K*, all individuals were strongly assigned to a single gene pool, with a few individuals from populations VI and VII assigned to alternative gene pools (Fig. 1d). Therefore, calculations required for assessing the optimal number of clusters were not performed because individuals from all populations belong to a single gene pool.

The length of aligned matrices comprising *nad*7 intron 1 sequences of *P. nigra* generated in the present study, and those reported by Jaramillo-Correa et al. (2010), as well as sequences of *P. sylvestris* (Naydenov et al. 2007), were 985 bp, 985 bp and 991 bp, respectively. The positions of variable sites (point and length mutations) in the latter matrix are shown in Table 2. *P. nigra* mtDNA haplotypes from the Balkans harbored a single polymorphic site at position 328 (G/T transversion), yielding two mtDNA haplotypes: T (harboring T at position 328), and G (G at position 328), which were deposited in the GenBank (accessions KT343352 and KT343353). The Balkans' and Iberian/African mtDNA haplotypes of *P. nigra* shared variability at position 328, and differed in three mutations, at positions 435, 472 and 520 (Table 2). Nucleotide characters G and A at positions 435 and 520, respectively, are synapomorphic for individuals from Iberia/Africa, while a single-bp insertion (C) at position 472 is synapomorphic for individuals from the central Balkans. All *P. sylvestris* sequences harbored T at position 328, and differed from *P. nigra* sequences in 8 mutations, at positions 334, 337, 396, 445, 565, 775, 889, and 931 (see Table 2).

Haplotype networks constructed with and without outgroup, *P. sylvestris*, are shown in Fig. 1b and 1c, respectively.

4 Discussion

High levels of cpDNA SSR diversity (H_T=0.949) and low genetic differentiation of populations (G_{ST}=0.024) found in populations of *P. nigra* from the central Balkans are in the range of those detected in westward populations of this species (Afzal-Rafii and Dodd 2007; Jaramillo-Correa et al. 2010; Soto et al. 2010). These findings, along with upper Miocene fossil records of *P. nigra* in central and southern Serbia (Petković 1987), would suggest a long persistence of this species within the Balkans, supported also by findings of Naydenov et al. (2006). Interestingly, these authors found somewhat lower levels of cpDNA diversity and increased genetic differentiation of *P. nigra* populations in Bulgaria, with four groups of populations whose distinctiveness was explained by historical events, specific topography of the region and anthropogenic impact. The alternative clustering of these populations found at the nuclear genome, and discordance of cpDNA and nuclear data, was attributed to different migration patterns and effective radius of dispersal of *P. nigra* pollen and seed (Naydenov et al. 2015). On the other hand, we found moderately high levels of mtDNA diversity in eastward populations of *P. nigra* (H_T=0.357), and a non-random distribution of two mtDNA haplotypes, which has led to the moderate levels of genetic differentiation (G_{ST}=0.358). Since the observed levels of genetic differentiation at the mtDNA level are

Table 2. Positions of variable sites in 991 bp long aligned mtDNA *nad7* intron1 matrix comprised of six *Pinus nigra* haplotypes and three *Pinus sylvestris* haplotypes used as an outgroup.

Haplotype	317	318	328	334	337	342–373	392–395	396–427	428	Δ435	445–508	Δ472	Δ520	565–614	775–805	889	931–936
	A	1 bp insertion (T)	T	A	T	32 bp insertion	4 bp insertion	32 bp insertion	1 bp insertion (C)	C	64 bp insertion	1 b indel (C)	C	50 bp insertion	31 bp insertion	G	6 bp insertion
T	A	-	T	A	T	·	·	·	·	C	·	·	C	·	·	G	-
G	·	-	G	·	·	·	·	·	·	·	·	·	·	·	·	·	-
h1	C	-	·	·	·	·	·	·	·	G	·	-	A	·	·	·	-
h2	C	·	·	·	·	·	·	·	·	G	·	-	A	·	·	·	-
h3	·	·	G	·	·	·	·	·	·	G	·	-	A	·	·	·	-
h4	·	-	G	·	·	·	·	·	·	G	·	-	A	·	·	·	-
PS1	C	-	·	T	G	·	·	-	·	·	-	-	·	·	-	T	·
PS2	C	-	·	T	G	·	-	-	-	·	-	-	·	·	-	T	·
PS3	C	-	·	T	G	-	-	-	-	·	-	-	·	·	-	T	·

T and G – mtDNA haplotypes found in *P. nigra* individuals from the central Balkans; h1, h2, h3 and h4 – Iberian (h1–h3) and north African (h4) haplotypes of *P. nigra* reported by Jaramillo-Correa et al. (2010); PS1, PS2, and PS3 – *P. sylvestris* haplotypes available in GenBank (accessions DQ665913, DQ665914, and DQ665915, Naydenov et al. 2007); Δ – The positions of mutations which may be used for distinguishing westward and eastward lineages of *P. nigra* (nucleotide characters "G" and "A" at positions 435 and 520 are synapomorphic for *P. nigra* individuals from the Iberian Peninsula/northern Africa; one bp insertion (C) at position 472 is synapomorphic for individuals from the central Balkans)

usually expected at much larger studied areas than ours (e.g. Jaramillo-Correa et al. 2010; Tian et al. 2010; Aleksić and Geburek 2014 and references therein), we assumed that the Tara Mountain, with *P. nigra* populations characterized by increased levels of mtDNA diversity, may represent an interglacial refugium of this species, similarly as observed in another cold-adapted species, *Picea omorika* (Panč.) Purk. (Aleksić and Geburek 2014).

Although it has been shown recently that cpDNA variation in closely related spruces is more species-specific than mtDNA variation, and that the latter is often shared among related conifer species (Du et al. 2009), we were not able to use cpDNA nor mtDNA polymorphisms to support infraspecific circumscription of studied *P. nigra* taxa which has recently been implied at the biochemical level (Bojović et al. 2012; Šarac et al. 2013). The sole conclusions with regard to this aspect of our study are that at the cpDNA level, two studied populations of *P. nigra* ssp. *nigra* var. *gocensis* display the highest but insignificant genetic differentiation, and that populations VI and VII, representing varieties *pallasiana* and *banatica* of *P. nigra* ssp. *pallasiana*, respectively, may harbor genetically distinct individuals. It is worth mentioning that these populations are at the range-edge of *P. nigra* ssp. *pallasiana* distribution (Vidaković 1991) and thus, may be genetically different from populations of these taxa found within their main range (e.g. Bjedov et al. 2015 and references therein). At the mtDNA level, populations of *P. nigra* ssp. *nigra* var. *nigra* are genetically diverse while populations of the other studied infraspecific taxa are invariable. Nonetheless, mtDNA variation enabled new insights into historical events that have led to the current distribution of mtDNA diversity in westward and eastward parts of the *P. nigra* range.

Based on the haplotype network rooted with *P. sylvestris* (Fig. 1b), we may assume that *P. nigra* mtDNA haplotypes harboring G at position 328 (Table 2), which are currently less abundant in eastward but prevalent in westward populations (h3 and h4), are derived. This would imply that the ancestral gene pool of *P. nigra*, which initially spread throughout the Mediterranean Basin, harbored T at position 328, and that the same mutation (T → G at position 328) must have had occur twice, i.e. in the ancestral *P. nigra* gene pool within the Balkans, and in the ancestral gene pool of this species within the western Mediterranean Basin. Alternatively, due to the long persistence of *P. nigra* in the Mediterranean (Naydenov et al. 2006, 2015; Jaramillo-Correa et al. 2012) and its high dispersal ability, it is possible that the above mentioned mutation occurred at one site, and expanded later on to the other site. However, the more plausible and parsimonious scenario is that the polymorphism at position 328 was already present in the ancestral gene pool of *P. nigra* which inhabited the Mediterranean Basin, and that three mutations, which distinguish two lineages of this species confined to the Balkans and the Iberian Peninsula/northern Africa, occurred after their divergence. It is worth mentioning the nucleotide characters G and A at positions 435 and 520, respectively, and one bp insertion (C) at position 472 may be used as molecular diagnostic characters for distinguishing westward and eastward *P. nigra* lineages. These findings altogether further support long persistence of *P. nigra* in southern Europe, and long-term isolation of two lineages of this species detected in the present study.

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