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Original article

Chromosomal differentiation between *Pinus heldreichii* and *Pinus nigra*

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Abstract – Two tertiary relict pines, *Pinus heldreichii* and *P. nigra,* have been considered as taxonomically very close species. Both species have very discontinuous geographical distributions which overlap in some localities in Balkan Peninsula. The pattern of heterochromatin regions distribution (AT-, GC-rich and DAPI positive heterochromatin) and activity of nucleolar organizing regions (NORs) were analyzed for these two pines by fluorochrome banding and silver staining respectively. Morphometric data for karyotype of *P. heldreichii* were presented here for the first time. Chromomycin (CMA) banding pattern was particularly species specific, *P. heldreichii* possessed 12 and *P. nigra* 24 bands. In comparison with other species from subsection *Pinus, P. heldreichii* displayed particular CMA-banding pattern. Hoechst banding was less specific and similar to other pines. DAPI staining was applied after DNA denaturation/renaturation and revealed important differences in number and position of bands between the two species. Number of secondary constrictions (SCs), of NORs and of nucleoli also differed between *P. heldreichii* (10) and *P. nigra* (12). Our results proved a clear interspecific differentiation at the chromosomal level between the two species and add some data to discuss about the possible new subsectional placement of *P. heldreichii.*

***Pinus* / heterochromatin / fluorochrome banding / G-C and A-T rich DNA / interspecific differentiation**

Résumé – Différentiation chromosomique entre *Pinus heldreichii* et *Pinus nigra.* Deux reliques tertiaires, *Pinus heldreichii* et *P. nigra,* sont considérées comme espèces taxonomiquement très proches. Leur distribution géographique est très discontinue, se chevauchant dans quelques localités des Balkans. La répartition des régions hétérochromatiques (AT-, GC- riche et hétérochromatine constitutive nonspécifique) et l'activité des organisateurs nucléolaires (NORs) sont analysées par fluorochrome banding et coloration au nitrate d'argent respectivement. Les données morphometriques du caryotype de *P. heldreichii* sont présentées ici pour la première fois. La distribution des bandes GC-riches est particulièrement spécifique, *P. heldreichii* possède 12 et *P. nigra* 24 bandes. En comparaison avec d'autres espèces de la subsection *Pinus*, *P. heldreichii* présente un chromomycin banding particulier. Les bandes AT-riches sont moins spécifiques et leur localisation est similaire aux autres pins. La coloration au DAPI, effectuée après la dénaturation/renaturation de l'ADN, révèle des différences importantes en nombre et position des bandes entre deux espèces. Le nombre des constrictions secondaires (SCs), des NORs et des nucléoles diffère également entre *P. heldreichii* (10) et *P. nigra* (12). Nos résultats montrent une différentiation nette au niveau chromosomique entre ces deux espèces et ouvrent la discussion sur un changement éventuel de subsection pour *P. heldreichii.*

***Pinus* / hétérochromatine / fluorochrome banding / ADN riche en G-C et A-T / différentiation interspécifique**

1. INTRODUCTION

Pinus heldreichii Christ. (Bosnian pine) and *P. nigra* Arnold (European black pine) have been considered as taxonomically very close Tertiary relict pine species [49]. Both pines were classified into subsection *Pinus* but additional confirmation of subsectional placement of *P. heldreichii* is suggested [33, 42]. Certain authors, investigating morphoanatomical features of Bosnian pine, proposed inclusion of *P. heldreichii* into group of species close to *P. halepensis* Mill. [10, 14]. Recent chemical and molecular studies provided additional evidences for exclusion of *P. heldreichii* from subsection *Pinus* and included it into group of "Mediterranean pines" [35, 45, 51]. Geographical distribution of *Pinus heldreichii* is limited to Balkan high mountains and few localities in South Italy while *P. nigra* occurs mostly in mountains of Mediterranean region [1]. Black pine is highly variable Mediterranean species including five subspecies with many varieties [15, 49]. On the contrary, only two

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Table I. Origin of investigated material.

Species	Locality
P. heldreichii	Mt. Hranisava, Bosnia and Herzegovina, 1780 m
	Mt. Rujiste, Bosnia and Herzegovina, 1050 m
	Blidinje (Mt. Cvrsnica), Bosnia and Herzegovina, 950 m
	Mt. Sar-planina, Serbia and Monte Negro, 1600 m
P. nigra subsp. *nigra**	Pale, Bosnia and Herzegovina, 900 m
P. nigra subsp. *nigra*	Konjic, Bosnia and Herzegovina, 780 m

* Individuals from Arboretum of Faculty of Forestry, University of Sarajevo.

varieties of *P. heldreichii* (var. *heldreichii* Christ and var. *leucodermis* Ant.) can be recognized [15, 49]. Both species have very discontinuous geographical distributions which overlap in some localities of the Balkan Peninsula [12]. Their putative spontaneous hybrids (*P.* × *nigraedermis* Fuk. et Vid.) were reported from Mt. Rujište (Bosnia and Herzegovina) [13].

Conventional cytogenetics revealed same chromosome number and prominent karyotype uniformity in both species, which is a characteristic of genus *Pinus*, and even of entire family Pinaceae [21]. Recent investigations employing modern molecular-cytogenetic techniques are focused to physical genome mapping, molecular chromosome structure and genome size. Fluorochrome banding was firstly reported for *Pinus nigra* var. *maritima* (Aiton) Melville [23], and then for other pines [24, 25, 27] detecting specific heterochromatin pattern in karyotype. Recently employed techniques of fluorescent in situ hybridization (FISH) revealed organization of ribosomal genes in *Pinus* species [9, 26, 28, 34]. Last decade was particularly marked by genome size investigations of *Pinus* species [2, 20, 29, 39, 40, 48, 50]. All these methods are very useful for consideration of phylogenetic and systematic relationships among pine species.

There are many chromosome reports for *P. nigra* and those were obtained by conventional [3, 21, 36, 43] and molecular-cytogenetical methods [23, 28]. Only few data exist concerning *P. heldreichii* karyotype [7, 43]. So, chromosomal organisation of this species is scarcely known and there are still not morphometric data for its karyotype.

Therefore, the aim of the present study is to provide and complete the data on karyotype features and chromosome organization for *P. heldreichii* and *P. nigra*. We also discuss some systematic aspects in the light of new cytogenetic data for these two pines.

2. MATERIALS AND METHODS

2.1. Material

Plant material originated from natural populations of *P. heldreichii* and *P. nigra* except of a population of Black pine from the arboretum (Tab. I). Seeds from adult trees were collected and used for cytological analysis. Seeds were soaked in 5% sodium hypochlorite to sterilize surface for 20 min, and rinsed twice in distilled water [21].

Identification and nomenclature of investigated species followed usual classification system [15, 33, 42]. Vouchers corresponding to studied pine individuals were deposited in Herbarium of Faculty of Forestry, University of Sarajevo.

2.2. Methods

Seeds were germinated on moist filter paper in Petri dishes at 23 °C temperature in incubator. Root tips were pretreated during 23–24 h in 0.05% aqueous solution of colchicine. Pretreated material was fixed in acetic-alcohol (3:1, absolute ethanol:glacial acetic acid, v/v) and placed in refrigerator (+4 °C) for 24 h [11]. After fixation plant material was washed in distilled water and finally stored in 70% ethanol at –20 °C until use.

Chromosome spreads were obtained using protoplast technique [16] with slight modifications. Hydrolysis was made by enzymatic treatment during 50 min in moist chamber at 37 °C. A universal enzymatic mix was used to hydrolyze plant material and to release chromosomes from cytoplasm: 3% cellulase R10 (Yakult Honsha Co.), 1% pectolyase Y-23 (Seishin corporation, Tokyo, Japan), 4% hemicellulase (Sigma Chemical Co.) in citrate buffer (pH = 4.2).

After hydrolysis, meristems were gently squashed in a drop of 45% acetic acid to gain protoplast suspension. The quality of chromosome spreads was controlled under phase-contrast microscope. After removal of the coverslips with liquid CO_2 [8], the slides were dehydrated in absolute ethanol and air-dried for at least 12 h at room temperature.

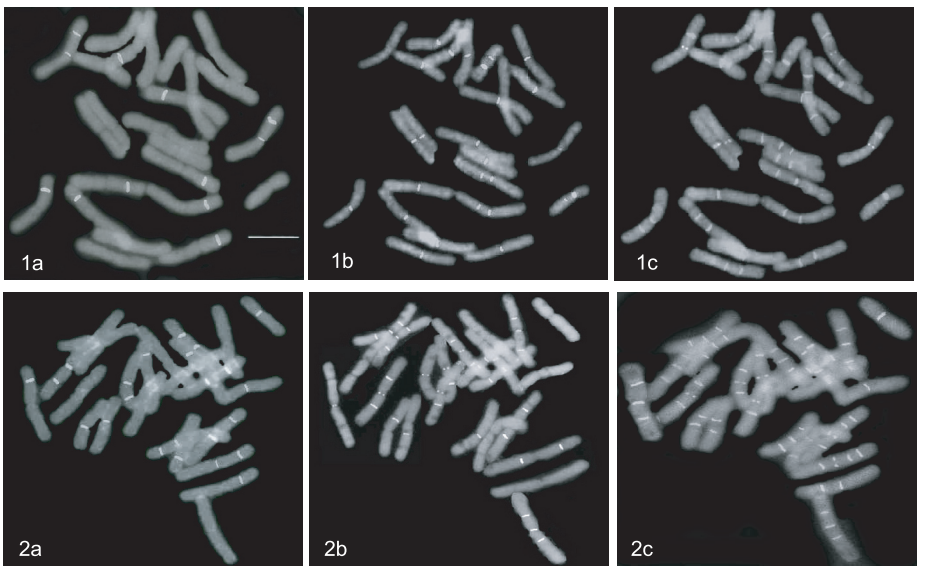
To observe GC- and AT-rich regions, chromosomes were stained with chromomycin A_3 (CMA) (Sigma) [31, 46] and Hoechst 33258 (Ho) (Sigma) [37], with minor modifications: CMA_3 concentration was 0.2 mg/mL [47]; 5 mM $MgSO_4$ was added in McIlvain buffer [31] instead of 10 mM $MgCl_4$ [46]; dystamicin A_3 staining was avoided because it did not improve CMA staining.

After staining with CMA and Hoechst same plates were also destained and prepared for DAPI (4,6 diamino-2-phenylindol) staining. The chromosomes were denatured using 70% formamide in 2×SSC, 2 min at 75 °C, then 5 min at 55 °C and incubated at 37 °C in mixture sond containing 2×SSC overnight following protocols for FISH experiment [47]. Staining was performed in 2 μg/mL of DAPI in McIlvain buffer (pH = 7) for 15 min.

For silver staining of nucleoli germinated root tips were fixed in standard fixative solution (3:1 absolute ethanol/glacial acetic acid) and stored 24 h in refrigerator. Meristem tissues were squashed in a drop of 45% acetic acid. Coverslips were removed by freezing with liquid CO_2 and dehydrated with absolute ethanol, then air-dried at room temperature for at least 12 h. Silver nitrate solution (50% in distilled water) was dropped on slides and incubated at moist chamber for 15 h at 60 °C [19]. Nucleoli number was determined on 100 interphase nuclei per individual. At least three individuals from both species were analyzed.

The chromosome observations were performed using an epifluorescent Zeiss Axiophot microscope with filter set 01 (excitation 365, emission 480 nm long pass) for Ho and DAPI staining and filter set 07 (excitation 457, emission 530 for long pass) for CMA. Images were captured with a Princeton Micromax CCD Camera, using Metavue analyser.

Numerical karyotype analysis was done for four populations of *P. heldreichii* and two populations of *P. nigra* (five individuals per population). Chromosomes were identified and ordered according to their total length, arm ratio and fluorochrome banding patterns. Following karyological features were analyzed: */TL* (total length of each chromosome) = *l* (long arm) + *s* (short arm)*/*, the ratio between long and short arm (*r*), the relative length of each chromosome ($RL = 100 \times TL/\Sigma TL$), the global asymmetric index ($AsI = \Sigma l \times 100/\Sigma TL$), and the ratio between the longest and shortest chromosome pairs (*R*). The chromosome types were characterized according recommended classification [32, 44]. Ideograms were drawn from mean values of long and short arms for each chromosome pair.



Figures 1. *Pinus heldreichii* (1) and *P. nigra* (2): CMA (a), Hoechst (b) and DAPI (c) banding patterns.

Table II. Morphometric data for karyotypes of *Pinus heldreichii* and *P. nigra.*

	P. heldreichii				*P. nigra*			
ChP	*l* ± SD (μm)	*s* ± SD (μm)	*TL* ± SD (μm)	*r/t*	*l* ± SD (μm)	*s* ± SD (μm)	*TL* ± SD (μm)	*r/t*
I	7.78 ± 0.35	7.15 ± 0.45	14.93 ± 0.74	1.09/m	*6.63 ± 0.18	5.89 ± 0.29	12.53 ± 0.20	1.12/m
II	7.44 ± 0.45	*6.98 ± 0.43	14.33 ± 0.85	1.06/m	6.45 ± 0.19	5.59 ± 0.31	12.04 ± 0.35	1.15/m
III	7.33 ± 0.46	6.71 ± 0.44	14.05 ± 0.81	1.09/m	6.39 ± 0.21	*5.62 ± 0.61	12.02 ± 0.51	1.15/m
IV	7.16 ± 0.52	6.54 ± 0.43	13.70 ± 0.86	1.09/m	6.16 ± 0.20	*5.65 ± 0.28	11.81 ± 0.33	1.09/m
V	6.94 ± 0.46	*6.47 ± 0.47	13.41 ± 0.91	1.07/m	*6.16 ± 0.17	5.43 ± 0.18	11.60 ± 0.32	1.13/m
VI	6.83 ± 0.42	*6.37 ± 0.45	13.21 ± 0.82	1.07/m	5.89 ± 0.30	5.48 ± 0.16	11.37 ± 0.40	1.07/m
VII	6.67 ± 0.37	*6.30 ± 0.41	12.97 ± 0.73	1.06/m	*5.68 ± 0.26	5.18 ± 0.32	10.87 ± 0.57	1.09/m
VIII	6.60 ± 0.38	6.14 ± 0.42	12.75 ± 0.75	1.07/m	5.56 ± 0.22	5.13 ± 0.22	10.69 ± 0.62	1.08/m
IX	6.43 ± 0.40	*5.94 ± 0.40	12.38 ± 0.75	1.08/m	5.46 ± 0.34	*4.95 ± 0.25	10.42 ± 0.56	1.10/m
X	6.08 ± 0.40	5.51 ± 0.27	11.60 ± 0.60	1.10m/	5.04 ± 0.16	4.61 ± 0.36	9.65 ± 0.51	1.09/m
XI	5.97 ± 0.30	4.34 ± 0.32	10.32 ± 0.54	1.38/m-sm	5.14 ± 0.14	3.87 ± 0.04	9.01 ± 0.17	1.32/m-sm
XII	5.72 ± 0.43	3.22 ± 0.31	8.94 ± 0.60	1.78/sm	4.70 ± 0.29	2.85 ± 0.29	7.55 ± 0.52	1.65/m-sm
THL (μm)	152.74				129.60			
AsI (%)	53.04				53.48			
R	1.66				1.66			

ChP: chromosome pair; *l*: long arm; *s*: short arm; *TL*: total length of chromosome; *r*: ratio long/short arm; *t*: morphological type of chromosome; *THL*: total length of haploid chromosome set; *: position of secondary constrictions; *R*: ratio of the longest and shortest chromosome pairs; SD: standard deviation.

3. RESULTS

3.1. Karyotype analysis

Karyotypes of both species present the same diploid number 2n = 24 (Fig. 1). Morphometric data of chromosome lengths for *P. heldreichii* ranged from 8.94 to 14.93 μm (Tab. II). Decreasing in chromosome lengths was in generally continual and gradual in each metaphase plate (Fig. 2A, Tab. II). Two smallest chromosome pairs decreased sharply and possessed submedial centromeres, particularly pair 12. Chromosome pair 11 belonged to meta-submetacentric (*m-sm*) type. Hence, chromosome complement was symmetric and homogenous (*AsI*% = 53.04). Mean value of total haploid karyotype length was 152.74 μm. Value of *r* ranged from 1.06 to 1.78. The first ten chromosomes had *r* values ranging from 1.02 to 1.1, while this value varied from 1.30 to 1.78 for chromosome pairs 11 and 12 (Tab. II). Significant differences were

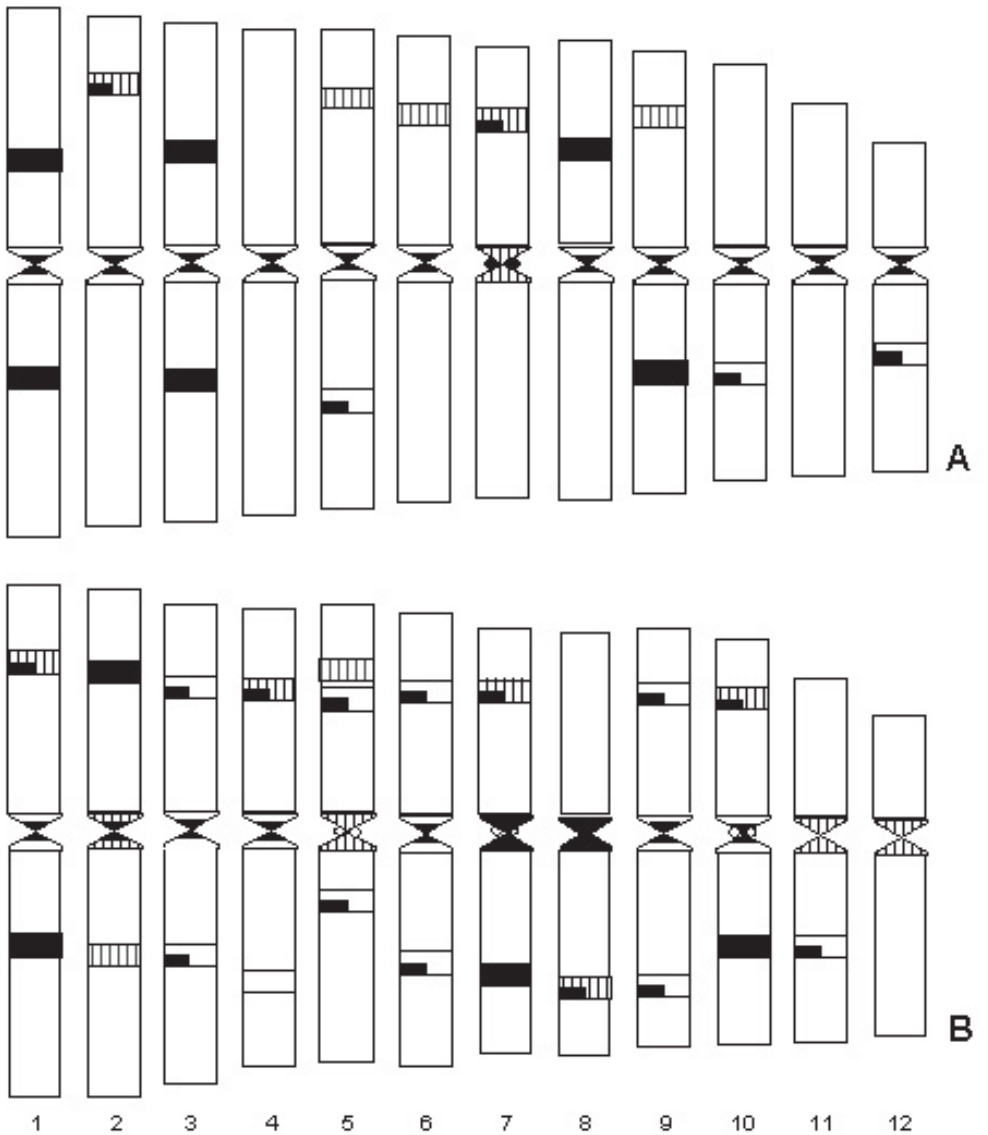


Figure 2. Idiograms of *P. heldreichii* (A) and *P. nigra* (B) showing CMA (lines), Hoechst (white), DAPI (black) banding patterns.

Table III. Comparison of some morphometric karyotype data among studied populations of *P. heldreichii.*

	H	B	R	S
THL (μm)	152.41	153.63	151.30	153.60
Σ*TLl* (μm)	81.3	80.93	80.19	81.55
AsI%	53.34	52.67	53.00	53.09
R	1.68	1.66	1.60	1.73

THL: Total length of haploid chromosome set; Σ*TLl*: total length of long chromosome arms; *AsI%*: Asymmetric index; *R*: ratio of the longest and shortest chromosomes lengths; H: Hranisava; B: Blidinje; R: Rujiste; S: Sar-planina.

not detected by ANOVA test among populations for mean chromosome lengths. Secondary constrictions (SCs) were located at five chromosome pairs: 2, 5, 6, 7 and 9 (Tab. II). The highest values of total lengths of haploid chromosome sets were observed for Sar-planina (153.60 μm) and Blidinje (153.63 μm) samples (Tab. III).

Similar karyotype pattern is observed for *P. nigra* (Figs. 1 (2a) and 2B), but mean values of chromosome lengths were lower than in *P. heldreichii*. Therefore, mean value of total length of haploid chromosome set (*THL*) was only 129.60 μm (Tab. II). Chromosome length values decreased continually and gradually, from 12.53 to 7.55, and sharply decreased from 9th to 12th chromosome pair (Tab. II). Strong decreasing was

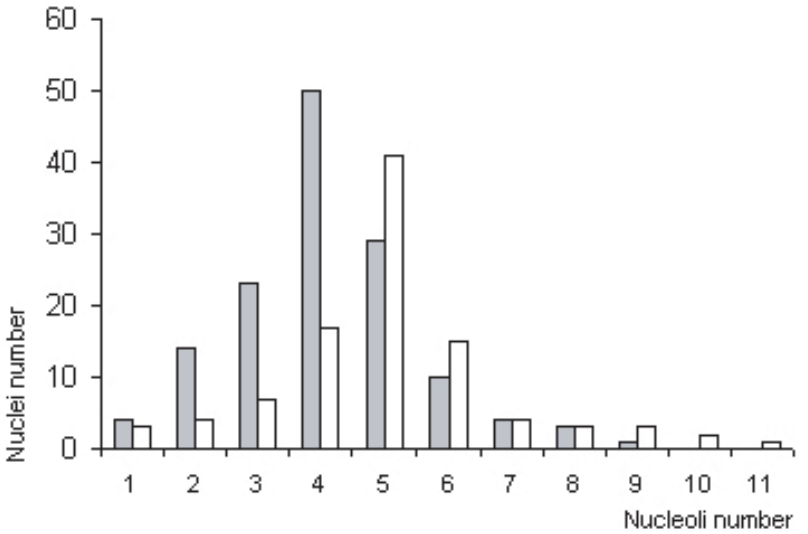


Figure 3. The frequency of observed nucleoli in *P. heldreichii* (gray) and *P. nigra* (white) nuclei.

recorded between chromosome pair 11 and 12. Karyotype was composed from ten metacentric and two smallest meta-submetacentric pairs. Karyotype was also very symmetric and homogenous (*AsI%* = 53.48) like in *P. heldreichii*. The *r* values of first ten chromosome pairs ranged from 1.07 to 1.15, while chromosome pairs 11 and 12 had *r* values 1.32 and 1.65, respectively. Chromosome complement of black pine possessed 12 secondary constrictions which were located on chromosome pairs: 1, 3, 4, 5, 7 and 9 (Tab. II).

3.2. Fluorochrome banding and silver staining

In *P. heldreichii* CMA staining displayed 12 GC - rich bands. Ten intercalary bands were located on short arms, while chromosome pair 7 possessed also the centromeric band. Three types of chromosomes could be distinguished in *P. heldreichii* karyotype by CMA staining: chromosome pairs without any CMA bands (1, 3, 4, 8, 10, 11 and 12), chromosome pairs with intercalary bands (2, 5, 6 and 9) and one chromosome pair (7) with both intercalary and centromeric bands (Figs. 1 (1a) and 2A). Sporadically, some individuals were characterized with 13th intercalary band on the short arm of chromosome pair 11.

Karyotype of *P. nigra* was characterized by more of CMA bands (24 bands). In contrast to *P. heldreichii*, black pine possessed only three chromosome pairs that lacked bands (3, 6 and 9), 4 chromosome pairs with intercalary CMA bands (pairs 1, 4, 7 and 8), 2 with only centromeric bands (pairs 11 and 12) and 3 pairs (2, 5 and 10) having both intercalary and centromeric bands (Figs. 1 (2a) and 2B).

In karyotype of *P. heldreichii* 26 Hoechst (Ho) bands were registered mostly in centromere regions (Fig. 1 (1b)). Only pairs 5 and 10 had intercalary Ho bands on their long arms (Fig. 2A). CMA bands appeared Ho negative.

As for CMA staining, *P. nigra* possessed more of Ho bands (34) (Fig. 1 (2b)). Only three pairs (2, 8 and 12) lacked Ho signals. Most of signals were located in centromeric regions and some were intercalary on one or both arms. Weak spot signals were observed at centromeres of chromosome pair 10 (Fig. 2B). All CMA bands appeared negative after Ho staining.

DAPI staining after DNA denaturation/renaturation revealed many bands at the centromeric region in most chromosomes of both species, and also many intercalary bands (Fig. 1 (1c, 2c)). This staining produced more prominent banding pattern for both pines. Numerous DAPI bands coincided with CMA and Hoechst signals, but also additional DAPI signals were detected (Figs. 2A and 2B). According to the position of DAPI bands (44 bands) four types of chromosomes can be recognized for *P. heldreichii*: chromosome pairs with only centromeric band (pairs 4, 6, 10 and 11), chromosome pairs with centromeric and intercalar bands on short arm (pairs 2, 7, and 8), chromosome pairs with centromeric and intercalar bands on long arm (pairs 5, 9 and 12) and chromosome pairs with centromeric band and intercalar bands on both arms (pairs 1 and 3). DAPI signals were observed at CMA positions of chromosome pairs 2 and 7. Also, DAPI coincided with all Ho centromeric signals and intercalary signals of chromosome pairs 5 and 12. DAPI banding displayed the highest number of signals (58) in karyotype of *P. nigra.* The number and position of mentioned specific bands are presented on ideograms (Figs. 2A and 2B).

The nucleoli number of analyzed nuclei was different and range from 2 (minimum number) to 10 (maximum number), with five nucleoli per nuclei as the most frequent case in *P. heldreichii* (Fig. 3). Maximum number of nucleoli for *P. nigra* was 12, minimum number was 2, and the most frequent nucleoli number was 6 (Fig. 3).

4. DISCUSSION

Basic karyological features of *P. heldreichii* have already been known [7, 43]. Authors reported the chromosome number,

Table IV. Comparison of some cytogenetic characters of *P. heldreichii* and *P. nigra*.

	T	THL (μm)	SC	Nl	CMA	Ho	DAPI	2C DNA (pg)
P. heldreichii	10 m, 1 m-sm, 1 sm	152.74	10	10	12	26	44	50.01
P. nigra	10 m, 2 m-sm	129.60	12	12	24	34	58	45.50

T: morphological type of chromosome; THL: total length of haploid chromosome set; SC: number of secondary constrictions; Nl: number of nucleoli; CMA: number of CMA bands; Ho: number of Hoechst bands; DAPI: number of DAPI bands; 2C DNA: mean value of genome size (from [2]).

details on chromosome morphology and the number of secondary constrictions (SCs): one chromosome pair bearing SCs was observed, but the origin of plant material is unknown [43], while karyotype from Calabria population showed three pairs with SCs [7], The first morphometric data on *P. heldreichii* karyotype is presented here. Chromosomes of *P. heldreichii* are long, ranging from 8.93 to 14.93 μm, these values being the highest within subsection *Pinus* [21].

Chromosomal reports on *P. nigra* are numerous [4, 5, 21, 30, 36, 41, 43]. Chromosome lengths (12.79–7.55 μm), much lower than those observed in *P. heldreichii* (14.93–8.94 μm), reflect also obvious differences in genome size which was 45.5 pg and 50.01 pg respectively (Tab. IV) [2]. Values of *r* and *AsI* (%) for both species are in concordance with the results obtained by other authors [4, 21, 43] which emphasizes karyotype uniformity found by conventional cytogenetical techniques. Discordance in number and position of SCs is particularly obvious, because of arm lengths and total chromosome lengths that are nearly identical. Thus, inversion in the position of individual chromosomes in karyotype may easily occur.

Despite the earlier mentioned facts certain relations can be discussed only when same methods are applied. Hence, the two species differ in mean values of chromosome lengths, number and position of SCs as well as morphological types of chromosomes. Two smallest chromosome pairs of *P. nigra* and the last chromosome pair of *P. heldreichii* belong to *m-sm* type (Tab. IV).

In this study fluorescent-banding methods were used to identify individual chromosomes and analyze interspecific relationships. Thus, banding pattern displayed marked differences between the two species (Tab. IV), but also common features with other *Pinus* species [23–25, 27] were evident. Karyotype of black pine showed much more of CMA, Hoechst and DAPI signals than *P. heldreichii* (Tab. IV). In spite of great differences in the number of CMA bands (12 bands for *P. heldreichii* and 24 for *P. nigra*) these two species possess some common features: chromosomes having interstitial signals and those having both interstitial and centromeric signals. Further, chromosomes with both interstitial and centromeric signals are found for species from subgenus *Pinus*, while it was not yet recorded in subgenus *Strobus* [27], but the authors had analysed only one species from soft pines group. Centromeric bands were not observed using C-banding technique for *Strobus* investigated pines either [36].

Both species have four chromosome pairs with only interstitial CMA signals, but only one pair with both interstitial and centromeric signals in *P. heldreichii* and three in *P. nigra* were observed. Three pairs with interstitial signals have the same positions in the complements (2, 5, 7). The second, fifth and tenth pair of *P. nigra* possesses centromeric signals as well, while *P. heldreichii* has CMA centromeric bands in pair 7. Two smallest chromosome pairs of *P. nigra* have centromeric bands. Present CMA banding pattern is very similar to previous results for *P. nigra* var. *maritima* [23]. We found same number of CMA bands (24), but some differences exist in band positions and chromosome type. While six chromosome pairs with interstitial signals were described in var. *maritima* [23], present study showed one more chromosome pair with both interstitial and proximal band in *P. nigra* subsp. *nigra*. However, intraspecific variation in number and size of heterochromatic bands is not rare case in plant karyotypes [18].

Recent study on pines pointed out the presence of centromeric CMA bands in two smallest chromosome pairs which sequences have been used as PCSR (*Proximal CMA-band specific repeats*) probes in FISH experiment [28]. Proximal CMA signals of pairs 11 and 12 are characteristic for most investigated pines, especially for all species belonging to subsection *Pinus*: *P. densiflora* Sieb. et Zucc. (both pairs), *P. thunberghii* Franco (pair 12), *P. luchuensis* Mayr (pair 12), *P. yunannensis* Franch (both pairs), *P. tabuleformis* Carr. (both pairs) [27]. The correlation between PCSR and CMA signals was found at proximal regions of chromosomes [28], but our results showed one chromosome pair with both proximal and interstitial signal more than in Black pine from Slovakia.

The source of these slight differences may be linked to different investigated taxa of black pine, thus supposing possible karyotypic differentiation between geographical races within species. Interindividual variation of CMA bands has already been observed in *P. nigra* var. *maritima* [23] and *P. densiflora* and *P. thunberghii* [25].

However, *P. heldreichii* is very interesting and unique because it does not possess any proximal CMA signal at chromosome pairs 11 and 12. In comparison to *P. nigra*, *P. heldreichii* has half as much CMA signals (Tab. IV). An interesting fact is also the number of chromosome pairs with both interstitial and proximal signals. Evidently, Asian pines display in general more CMA signals, higher number of chromosomes having both interstitial and proximal signals and higher number of long chromosomes with proximal signal [27]. *Pinus heldreichii* and European black pine have more chromosomes with interstitial signals.

The results of hybridization in situ of PCSR, telomere repeats and rDNA in *P. densiflora, P. sylvestris, P. thunberghii* and *P. nigra* showed that *P. nigra* is not so close to the other three species [28]. To our knowledge *P. heldreichii* is pine with the lowest number of CMA bands except of *P. bungeana* [27] that belongs to subgenus *Strobus*. In *P. heldreichii* the number of CMA signals (12) corresponded to numbers of SCs and

nucleoli. This confirms that all NORs could be active. Black pine has the 6 chromosome pairs with a secondary constrictions carried a NOR which corresponded to the 6 nucleoli in the most observed nuclei and that was already reported [36], while 14 intercalary CMA signals were observed. Consequently, two signals do not correspond to NORs.

Distribution of AT-specific regions in chromosomes showed generally similar pattern for both species. Ho signals were mostly observed at centromeric positions of *P. heldreichii* karyotype except in chromosome pair 7 which possess CMA centromeric signal, but DAPI confirmed weak spot signals also. Centromeric position of Ho bands is confirmed for other conifers, such as *Cedrus* spp. [6]. Authors confirmed positive CMA signals that appeared Ho negative, and this pattern was also found for *Picea* species [47]. These results are typical for plant chromosomes after use of DAPI fluorochrome [46], and particularly for *Pinus* chromosomes [23, 25]. In our case DAPI was applied after denaturation of DNA and in this way it detects constitutive heterochromatin. All centromeric DAPI signals coincided to Ho signals, but also signals corresponding to a new DAPI positive heterochromatin in *P. heldreichii* karyotype were detected. DAPI coincided with CMA signals at two chromosome pairs (2 and 7). CMA-bands localized at the SCs usually contain 18S-5.8S-26S rRNA [22, 26]. CMA signals appeared Ho negative except in chromosome pair 10 in *P. nigra*, but in this case DAPI confirmed both CMA and Ho signals and also displayed new regions of DAPI positive heterochromatin.

Intercalary DAPI signals, AT-rich, are specific for chromosomes of *Pinus* spp., too. So, numerous intercalar DAPI signals corresponding to telomere repeat sequences were detected by hybridization in situ [9, 28]. Unspecific DAPI heterochromatic signals may be explained by possible alternation of AT- and GC- rich repetitive DNA sequences, with a number of base repetitions not sufficient to allow binding of specific fluorochrome (5 AT and 3 GC are minimum motifs for Ho and CMA fluorescence respectively, [17]). Hence, these signals are expressed as DAPI positive heterochromatin. Always intensive intercalary DAPI band of chromosome pair 11 were located in long arm for *P. sylvestris*, *P. densiflora*, *P. thunberghii* and *P. nigra* [28], but *P. heldreichii* had it on a long arm of pair 12 (Fig. 2A).

Recent investigations of phylogenetic relationships of *Dyploxylon* pines (subgenus *Pinus*), based on plastid sequence data, include *P. heldreichii* into group of Mediterranean pines (subsections *Halepenses*, *Canarienses* and *Pineae*) [35, 51]. Actually, it forms sister clade with Mediterranean pines. According to the classical taxonomic schemes *P. nigra* is regarded as the closest relative of *P. heldreichii* but incongruence between taxonomic and phylogenetic systems is not rare. Different classification schemes of species emerge as the result of paucity of discrete characters, homoplasy of morphological characters and their plesiomorphic nature in the genus *Pinus* [35]. However, it has been showed that *P. heldreichii* had different terpen composition than *P. nigra* [38]. Seed protein analysis also supported *P. heldreichii* being more closely related to Mediterranean pines than other members from subsection *Sylvestres* [45]. Probably, *P. heldreichii* and *P. nigra* shared common evolutionary history, but diverged separately in circummediterranean area. Our results provide additional corroboration indicating high genomic differentiation between these two species (Tab. IV). Concerning all these facts, subsectional replacement of *P. heldreichii* into group of "Mediterranean pines" seem to be natural and future investigations will be focused on interspecific relationships with species from that group.

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