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Karyotypic Diversity in European Black Pine (*Pinus nigra* ARN.) from Bulgarian Provenances

By

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With 5 Figures

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Summary

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Analysis of chromosome morphology in natural populations of *Pinus nigra* ARN. (European Black Pine) in Bulgaria showed a karyotype diversity of 30% among populations. Seventy-nine Black Pine trees from 9 natural provenances were examined. Based on average values for all 12 chromosomes pairs (short arm S, long arm L, total length T, arm ratio and index class) for each provenance, a total of 9 different karyotypes were discovered, depending on the average values of the lengths of short arm and long arm of the chromosomes. Inter-population variability of the karyotype was summarized with Principal Component Analysis/ Factor Analysis. These analyses showed that European Black Pine in Bulgaria can be divided into three basic groups: (1) "Western Black Pine Formation"; (2) "Marginal Black Pine Formation" and (3) "Central Rhodopean Mountain Black Pine Formation". This pattern of inter-population differentiation suggest that after the warming of the climate, migration of European Black Pine to higher altitudes and recolonization of sites also with other tree species would have occured at different speeds with varying success. This has

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probably led to disruption of the range of *Pinus nigra*, and creation of isolated, small groups of populations.

Zusammenfassung

NAYDENOV K., TREMBLAY F. & GANCHEV P. 2003. Karyotyp-Diversität der Schwarzföhre (*Pinus nigra* ARN.) bulgarischer Herkünfte. – Phyton (Horn, Austria) 43 (1): 9–28, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Analyse der Chromosomenmorphologie von *Pinus nigra* ARN. (Schwarzföhre) in Bulgarien zeigte eine Karyotyp-Diversität von 30% zwischen den Populationen. 79 Bäume von 9 natürlichen Herkünften wurden untersucht. Auf der Basis der Mittelwerte (für kurzer Arm S, langer Arm L, Gesamtlänge T, Arm-Verhältnis und Index-Klasse) für alle 12 Chromosomenpaare wurden insgesamt 9 verschiedenen Karyotypen gefunden. Die Karyotyp-Variabilität zwischen den Populationen wurde mit Hauptkomponenten-Analyse und Faktorenanalyse geprüft. Diese Analysen zeigen, daß Schwarzföhren-Populationen in Bulgarien in drei Gruppen gegliedert werden können: (1) "Western Black Pine Formation"; (2) "Marginal Black Pine Formation" and (3) "Central Rhodopean Mountain Black Pine Formation". Dieses Muster der Differenzierung zwischen den Populationen legt nahe, daß nach der Erwärmung des Klimas die Wanderung der Schwarzföhre in höhere Lagen und die Wiederbesiedlung von Standorten zusammen mit anderen Baumarten mit verschiedenen Geschwindigkeiten und unterschiedlichem Erfolg geschehen ist. Dies hat möglicherweise zum Zerreißen des Schwarzföhren-Areals und zum Entstehen isolierter, kleiner Gruppen von Populationen geführt.

1. Introduction

Karyotype analyses in coniferous forest trees were initially used for taxonomic and evolutionary studies (SAX & SAX 1933, SAX 1960, SAYLOR 1961, SIMAK 1964). The majority of these investigations were carried out on a relatively small number of individuals and populations. The discussion of the results were most often based on secondary constrictions, chromosome length and symmetry (SIMAK 1964, 1966, MOULALIS & ILLIES 1975, SCHLARBAUM & TSUCHIYA 1976, ONO 1977). In some cases however, the results were unexpected as the karyotypes of different individuals from one species differed (HAQUE 1984). For example, KORMUTAK 1975 and NATARAJAN & al. 1961 reported different idiograms for *Pinus sylvestris* L.

Chromosome length, metacentricity, and secondary constrictions vary both within and between populations of *Pinus koraiensis* SIEB. & ZUCC., *Pinus pumila* (PALLAS) REGEL in KUESTER & al., *Pinus sylvestris* L. and *Eucalyptus camaldulensis* DEHN. (MURATOVA 1979a, 1979b, ABATUROVA 1978, GRUNWALD & KARSCHON 1979). Some authors have focused their attention on investigations of the presence and number of B-chromosomes (accessory chromosomes) and nucleoli (KRUKLIS 1971, TERASMAA 1975, REES & al. 1977, PRAVDIN & al. 1978). Karyotypes within tree species vary widely in relation to both altitude and latitude (GRANT 1976, TEOH & REES 1977). Karyotypes may also be used to identify individual varieties and clones

and to a lesser extent, to determine individual forms and inter-species hybrids (BENTZER 1977, MOIR & FOX 1977, TODA 1976). Morphological characteristics of the karyotypes are generally described with the following variables: length of long arm, length of short arm, total chromosome length, arm ratio, centromere index, chromosome index, symmetry index, and size grading index (MATERN & SIMAK 1968, TEPPNER 1974, SAUER & LEEP 1979, BORZAN 1988, KOHLER & al. 1995). Many classifications and nomenclatures for chromosome metacentric determination have been proposed, the most popular being those by WILSON 1928, TJIO & LEVAN 1954, LEVAN & al. 1964, GULIAEV & MALCHENCO 1975 and SCHLARBAUM & TSUCHIYA 1984. The last two classifications are modified versions of the TJIO & LEVAN 1954 and LEVAN & al. 1964 proposals.

European Black Pine (*P. nigra* ARN.) is an important forest tree species in Europe with a distribution that covers nearly all mountains in the southern part of the old continent from Spain to the Balkan Peninsula, the Crimea and Asia Minor. It is also found on some islands in the Mediterranean as well as in certain isolated sites of the Northern African littoral. During the glacial recession, this species had no major migration, and with time it has formed relatively isolated populations (STEFANOV 1943, HUTTUNEN & al. 1992, WILLIS 1994). In Bulgaria, European Black Pine is found in the southern part of the country, mainly in the mountains of Rila, Pirin, Slavyanka, the Rhodopes, and Osogovo. It is found in regions from 100 to 1800 m in altitude (ALEXANDROV & al. 1988). During the last 10 to 15 years, efforts have been directed towards a better characterization of population variability at biochemical and cytological levels. Previous investigations have shown a variation of chromosome length and secondary constriction position in European Black Pine (CHINCHALADZE & TODUA 1971, KORMUTAK 1975, BORZAN 1977, 1981, GANCHEV & TSVETKOVA 1985, KAYA & al. 1985). This study was carried out to more clearly establish the morphology of chromosomes and the karyotypic diversity in some natural populations of European Black Pine in Bulgaria .

2. Materials and Methods

The progeny from 79 European Black Pine trees from 9 natural provenances in the Osogovo, Slavianka, Pirin, Rila and Rhodopes mountains were examined (Table 1, Fig. 1). Within each site, a sample area of 1 ha was established. The populations sampled were representative of the genetic resources of European Black Pine in Bulgaria, as populations over most of the range were sampled. Trees of good health and physical condition were chosen for cone collections. Cones were collected from the middle of the crown of 6 (pop-6/Djenda) to 11 (pop-1/G.Delchev) selected trees that were evenly distributed in each site. The average number of investigated trees in each population was 8.8, which is greater than in previous investigations (COULAUD & al. 1999, MCARTHUR & SANDERSON 1999). Seeds were removed and kept in darkness at 4° C. Ten seeds from each tree were chosen for karyological analysis after stratifica-

Table 1. Geographical location of the investigated native provenances of European Black Pine (*Pinus nigra* ARN.) from Bulgaria.

Trial	Provenances[1]								
	Pop-N1	Pop-N2	Pop-N3	Pop-N4	Pop-N5	Pop-N6	Pop-N7	Pop-N8	Pop-N9
Latitude (North)	41°26'	42°06'	41°54'	41°44'	42°06'	41°48'	41°39'	41°52'	41°53'
Longitude (Est)	23°42'	22°42'	24°40'	24°14'	24°08'	25°08'	23°21'	23°23'	23°33'
Altitude (m)	1100	1000	1300	1200	1000	1000	1000	1050	1050
No. of trees	11	10	10	10	10	6	8	8	6
Average No. of seedling cells observed	3.0	3.1	3.3	3.2	3.3	5.2	4.0	4.1	5.3
Ages of trees (years)	180–200	180–200	180–200	180–200	180–200	180–200	180–200	180–200	180–200

[1] Provenance's names - N1-G.Delchev, N2-Gabra, N3-Hvoina, N4-Borino, N5-Rakitovo, N6-Djenda, N7-Sandanskii, N8- Razlog-I and N9- Razlog-II

tion. They were placed in Petri dishes at 26° C under a light regime 14 h. Fifteen days after germination, one seedling from each tree was chosen at random for further analysis following a modified method of PRAVDIN & al. 1976. Briefly, root tips (10–15 mm) were treated with a 0.2% solution of colchicine for 8 h followed by fixa-

Fig. 1. Natural distribution area of European Black Pine (*Pinus nigra* ARN.) – geographic location of the nine investigated provenances in Bulgaria and their three groups as determined by karyological analysis: W – "Western Black pine Formation"; C – "Central Rhodopean Mountain Black Pine Formation"; and the remainder is the "Marginal Black Pine Formation".

tion in Klarck's solution. Maceration was carried out in 1 N HCl for 10 min at 60° C. The chromosomes were stained with a 1:1 mixture of Schiff's solution and aceto-orcein for 4 h (PRAVDIN & al. 1976). A minimum of 3 plates per individual were photographed. Between 31 to 33 plates per site were analysed.

Black-and-white photographs of the complete chromosome complement in somatic cells were taken with a CETOPAN microscope (800x). Photographs were scanned with a HP/Scan Jet 4C with separation capability 1200 dpi using Software-Desk Scan II, Version-2,5/Hewlett-Packard Co (Fig. 2). The images obtained were imported and processed by Software-Corel DRAW 8, Version-8,232/Corel Corporation Lengths of short arm, long arm and total length were transformed into relative values, where the longest chromosome No. 1 together with secondary constriction (if any) was assumed to be 100%. B-chromosomes and nucleoli were observed in some of the preparations, but were not taken into consideration. Due to a lack of consistency, the positions and frequencies of the secondary constrictions were not investigated in this work.

The chromosomes are arranged in pairs, longest to shortest, and corresponding by numbered 1–12. The arm measurements are averages of the arms in each pair of chromosome for each tree. Indexes from each chromosomes pair are similar to those described in KOHLER & al. 1995 and COULAUD & al. 1999. The karyotype of all provenances is represented for each pair of chromosomes with the following characteristics divided into two main groups:

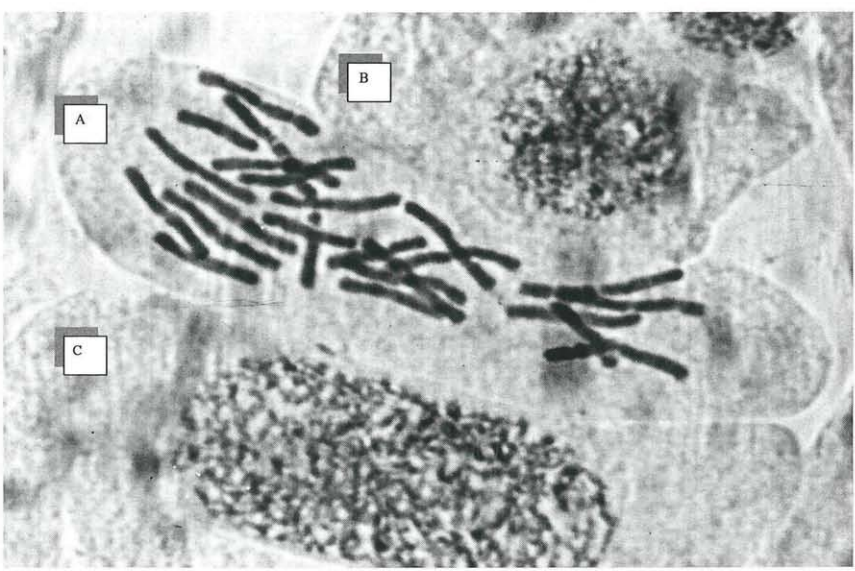


Fig. 2. Metaphase chromosomes (A) of European Black Pine (*Pinus nigra*) from pop-N3-Hvoina, tree N2 (photograph N3), interphase nulceus (B) and prophase nucleus (C).

Table 2. Averages of numerical karyotype of the 9 investigated native provenances of European Black Pine-*Pinus nigra* ARN. (SD in parentheses).

	Chromosomes pairs											
Trial	1	2	3	4	5	6	7	8	9	10	11	12
	Pop-N1-G.Delchev											
S[1]	47.16 (2.17)	44.95 (3.10)	42.08 (4.52)	41.54 (4.20)	40.53 (4.74)	41.57 (2.67)	40.20 (2.66)	40.20 (2.66)	36.93 (4.95)	34.99 (2.04)	29.68 (4.88)	25.52 (3.81)
L	52.84 (2.17)	48.66 (4.00)	48.65 (3.24)	47.33 (4.09)	46.42 (6.00)	41.99 (2.90)	42.50 (3.74)	40.63 (2.67)	39.79 (2.89)	38.32 (3.85)	37.69 (5.67)	32.63 (2.19)
T	100.00	93.61 (5.45)	90.72 (5.01)	88.86 (7.07)	86.94 (6.94)	83.56 (5.41)	82.70 (5.38)	80.84 (5.14)	76.73 (6.78)	73.31 (5.12)	67.36 (6.71)	58.15 (4.60)
S/L	0.89 (0.08)	0.93 (0.09)	0.87 (0.12)	0.88 (0.09)	0.89 (0.14)	0.99 (0.03)	0.95 (0.08)	0.99 (0.03)	0.93 (0.11)	0.92 (0.08)	0.81 (0.19)	0.78 (0.12)
Si	89.95 (3.34)											
	Pop-N2-Gabra											
S	49.21 (1.26)	46.42 (2.34)	45.31 (3.00)	44.22 (2.45)	42.65 (2.00)	41.55 (2.26)	40.99 (2.15)	39.91 (3.34)	37.69 (4.97)	32.73 (5.08)	28.83 (6.61)	26.17 (5.86)
L	50.79 (1.26)	49.21 (2.79)	47.60 (2.36)	45.89 (2.68)	44.74 (3.53)	43.94 (2.81)	42.60 (2.25)	42.60 (2.25)	41.55 (2.26)	39.91 (3.35)	36.29 (4.58)	32.20 (6.31)
T	100.00	95.62 (4.23)	92.90 (4.51)	90.10 (4.37)	87.40 (4.47)	85.49 (4.33)	83.59 (3.56)	82.51 (4.19)	79.24 (5.23)	72.65 (7.65)	65.12 (9.90)	58.37 (12.0)
S/L	0.97 (0.05)	0.94 (0.06)	0.95 (0.06)	0.96 (0.06)	0.96 (0.07)	0.95 (0.06)	0.96 (0.06)	0.94 (0.09)	0.91 (0.13)	0.82 (0.10)	0.79 (0.15)	0.81 (0.05)
Si	91.91 (2.11)											
	Pop-N3-Hvoina											
S	49.44 (1.17)	47.36 (2.41)	45.72 (2.68)	44.67 (2.78)	44.18 (2.09)	43.64 (1.85)	42.01 (3.52)	39.44 (2.64)	37.29 (4.19)	35.14 (3.64)	32.03 (5.20)	27.40 (2.39)
L	50.56 (1.17)	48.94 (1.80)	47.89 (2.40)	46.78 (2.45)	44.69 (2.80)	43.66 (1.86)	44.72 (2.31)	44.16 (1.39)	42.04 (2.62)	38.89 (4.18)	36.28 (4.01)	32.53 (3.04)
T	100.00	96.31 (3.46)	93.61 (4.27)	91.46 (4.49)	88.87 (4.69)	87.30 (3.72)	86.73 (3.88)	83.60 (3.06)	79.33 (5.41)	74.03 (6.45)	68.32 (8.29)	59.92 (3.64)
S/L	0.98 (0.04)	0.97 (0.05)	0.96 (0.06)	0.96 (0.06)	0.99 (0.03)	1.00 (0.00)	0.94 (0.10)	0.89 (0.06)	0.89 (0.10)	0.91 (0.10)	0.88 (0.11)	0.85 (0.12)
Si	93.70 (0.97)											
	Pop-N4-Borino											
S	49.20 (1.35)	45.30 (4.31)	45.30 (4.31)	44.16 (4.03)	41.36 (4.23)	39.32 (5.10)	38.69 (5.38)	38.36 (3.94)	36.68 (3.43)	32.09 (6.19)	26.23 (3.79)	23.49 (2.36)
L	50.80 (1.35)	47.52 (4.50)	45.30 (4.31)	45.30 (4.31)	45.77 (5.83)	46.14 (7.55)	41.92 (3.32)	41.10 (5.84)	39.62 (4.70)	37.51 (3.21)	33.37 (4.84)	29.90 (4.27)
T	100.00	92.82 (8.31)	90.60 (8.61)	89.46 (7.99)	87.13 (9.09)	85.46 (10.1)	80.61 (7.81)	79.45 (8.67)	76.30 (6.55)	69.60 (7.77)	59.60 (6.01)	53.39 (5.33)
S/L	0.97 (0.05)	0.95 (0.06)	1.00 (0.00)	0.98 (0.05)	0.91 (0.09)	0.86 (0.12)	0.92 (0.11)	0.94 (0.10)	0.93 (0.11)	0.86 (0.16)	0.80 (0.17)	0.80 (0.13)
Si	91.29 (2.38)											

Pop-N5-Rakitovo												
S	48.06 (2.11)	44.65 (4.48)	45.10 (3.72)	44.77 (4.34)	44.30 (3.57)	40.84 (2.49)	37.77 (3.13)	37.22 (3.39)	36.64 (1.78)	34.46 (2.84)	29.84 (2.76)	27.57 (4.54)
L	51.94 (2.11)	49.35 (3.14)	47.36 (3.72)	46.59 (2.94)	45.48 (3.10)	44.93 (2.67)	45.62 (3.38)	41.96 (4.06)	40.14 (4.48)	39.60 (5.00)	38.16 (3.21)	32.47 (4.99)
T	100.00	93.99 (5.44)	92.46 (5.37)	91.36 (5.86)	89.78 (6.20)	85.77 (3.68)	83.39 (4.53)	79.17 (3.85)	76.77 (3.89)	74.06 (5.25)	68.01 (4.39)	60.05 (7.79)
S/L	0.93 (0.08)	0.91 (0.11)	0.96 (0.10)	0.96 (0.09)	0.97 (0.05)	0.91 (0.08)	0.83 (0.09)	0.90 (0.13)	0.92 (0.11)	0.88 (0.13)	0.79 (0.10)	0.86 (0.15)
Si	90.01 (2.56)											
Pop-N6-Djenda												
S	47.90 (3.16)	45.08 (5.23)	43.49 (4.20)	44.18 (2.45)	43.64 (1.51)	42.60 (2.93)	40.57 (5.87)	40.28 (5.64)	38.79 (2.56)	33.08 (5.56)	25.56 (5.24)	23.86 (5.34)
L	52.10 (3.16)	49.68 (4.22)	47.98 (3.29)	47.24 (3.43)	44.18 (2.45)	43.64 (1.51)	44.62 (1.50)	44.81 (2.86)	42.08 (3.40)	40.87 (6.66)	36.69 (6.91)	32.99 (5.57)
T	100.00	94.76 (4.06)	91.47 (4.93)	91.42 (4.93)	87.82 (3.85)	86.23 (3.90)	85.19 (5.85)	85.09 (5.79)	80.86 (5.21)	73.96 (9.82)	62.25 (11.7)	56.85 (9.19)
S/L	0.92 (0.11)	0.92 (0.15)	0.91 (0.11)	0.94 (0.07)	0.99 (0.03)	0.98 (0.06)	0.91 (0.14)	0.90 (0.14)	0.92 (0.07)	0.82 (0.15)	0.70 (0.08)	0.73 (0.16)
Si	89.00 (2.05)											
Pop-N7-Sandanskii												
S	49.54 (1.16)	47.15 (3.26)	45.42 (2.27)	44.52 (1.48)	44.52 (1.48)	42.00 (4.62)	40.52 (2.98)	39.89 (2.37)	37.87 (4.99)	33.73 (7.47)	30.20 (4.88)	24.37 (4.00)
L	50.46 (1.16)	48.96 (2.13)	48.54 (3.58)	46.95 (2.87)	46.32 (2.65)	45.87 (2.11)	44.35 (2.93)	43.01 (3.63)	41.47 (3.44)	39.61 (3.06)	34.49 (6.18)	30.41 (6.17)
T	100.00	96.11 (4.37)	93.96 (4.90)	91.47 (3.03)	90.85 (2.70)	87.87 (5.78)	84.86 (3.95)	82.90 (5.09)	79.35 (6.92)	73.35 (7.56)	64.69 (10.1)	54.78 (8.52)
S/L	0.98 (0.04)	0.96 (0.07)	0.94 (0.07)	0.95 (0.07)	0.96 (0.07)	0.92 (0.09)	0.92 (0.09)	0.93 (0.07)	0.92 (0.12)	0.86 (0.20)	0.89 (0.13)	0.82 (0.16)
Si	92.14 (1.40)											
Pop-N8-Razlog-I												
S	47.61 (3.06)	47.12 (2.72)	44.82 (4.38)	44.76 (2.71)	44.14 (3.19)	41.56 (2.00)	40.99 (2.72)	38.34 (3.58)	36.54 (4.35)	33.36 (4.33)	30.61 (4.16)	26.67 (5.14)
L	52.39 (3.06)	48.65 (1.95)	49.24 (2.29)	45.36 (2.76)	45.39 (3.29)	45.95 (3.66)	44.75 (2.72)	42.58 (3.07)	39.48 (2.50)	38.94 (3.98)	37.47 (5.87)	33.19 (4.33)
T	100.00	95.77 (3.75)	94.06 (4.55)	90.12 (5.20)	89.53 (5.42)	87.51 (3.52)	85.75 (3.48)	80.92 (6.03)	76.02 (6.21)	72.30 (6.77)	68.09 (8.73)	59.86 (8.55)
S/L	0.91 (0.11)	0.97 (0.06)	0.91 (0.10)	0.99 (0.03)	0.97 (0.07)	0.91 (0.10)	0.92 (0.09)	0.90 (0.06)	0.92 (0.09)	0.86 (0.11)	0.83 (0.12)	0.80 (0.13)
Si	91.05 (1.34)											
Pop-N9-Razlog-II												
S	48.02 (2.50)	45.31 (3.84)	45.31 (3.84)	42.71 (3.03)	41.72 (2.14)	41.73 (2.13)	39.82 (2.78)	38.89 (1.60)	38.89 (1.60)	35.20 (1.73)	29.58 (3.14)	28.64 (4.60)
L	51.98 (2.50)	51.14 (3.80)	50.16 (3.99)	47.28 (3.38)	46.24 (3.33)	45.40 (2.78)	43.45 (2.97)	43.50 (3.81)	38.89 (1.60)	38.88 (3.87)	34.18 (4.03)	30.48 (3.63)
T	100.00	96.45 (5.48)	95.47 (5.23)	89.99 (5.08)	87.96 (4.01)	87.13 (4.04)	83.26 (3.74)	82.39 (4.07)	77.79 (3.21)	74.08 (3.92)	63.76 (4.86)	59.12 (6.94)
S/L	0.93 (0.09)	0.89 (0.10)	0.91 (0.11)	0.91 (0.08)	0.91 (0.08)	0.92 (0.06)	0.92 (0.09)	0.90 (0.09)	1.00 (0.00)	0.91 (0.10)	0.88 (0.14)	0.94 (0.14)
Si	91.23 (1.45)											

[1] S- short arm, L- long arm, T- total length, S/L- arm ratio and Si- symmetry index

(1) basic variables:
S – short arm;
L – long arm;
T = (S+L) – total length;
(2) composite variables:
S/L - arm ratio
Si = (total lengths of the short arms/total lengths of the long arms) × 100 – symmetry index;
r = L/ S – index class (by SCHLARBAUM & TSUCHIYA 1984).

In addition to descriptive statistics, multivariate statistics Factor Analysis were also used for provenance's differentiation. For inter-population investigation we took only the "basic" variable – S and L. To obtain the normal distribution of the variables implicated, we used the simple mathematical transformation-$\sqrt{\alpha}$ (tested with SHAPIRO-WILLK's 1968). The total error for each provenances (calculated by method of ZHIVITOVSKY 1984) is less then 0.05, which makes the statistical analysis representative. The total number of variables for Principal Component Analysis (PCA) is equal to the number of chromosomes pairs (n=12), multiplied by the number of "basic" variables (S and L). Thus, 24 variables were generated. Statistica 99th Edition, Kernel release 5.5 A/Stat Soft was used for the statistical processing of the data. Further, the first axis of the PCA for each karyotype characteristic variable (L, S, T, Si and r) as analyzed with a multivariate analysis of variance (MANOVA) using PROC GLM (SAS institute 1990) with population and tree as main effects.

3. Results and Discussion

The average values for all 12 chromosome pairs (short arm S, long arm L, total length T, arm ratio and index class) for each provenance are shown in Table 2. On the basis of these data, the idiograms for each provenance, were prepared (Fig. 3.). Nine different karyotypes were discovered, depending on the average values of the lengths of the short arm and the long arm of the chromosomes. Chromosome pairs 10, 11, and 12 showed the greatest variability and chromosome pairs 1, 2, 4, 5, and 6, showed the lowest variability.

3.1. Basic Variables

The average values of S, varied from 23.49 (pop-4/chromosome No. 12) to 49.54 (pop-7/chromosome No. 1), while the SD was within the limits of 1.16 (pop-7/chromosome No. 1) to 7.47 (pop-7/chromosome No. 10). Chromosome numbers – 1, 5 and 6 showed the lowest variability while the "short chromosomes", numbers 10, 11 and 12, showed the greatest. Average values for L varied from 29.90 (pop-4/chromosome No. 12) up to 52.84 (pop-1/chromosome No. 1). The lowest SD value was found for chromosome No. 1 from pop-7, (1.16), and the greatest in chromosome No. 6 from

pop-4 (7.55). The variability of this index was more often the lowest with chromosome numbers 1, 7, and 8 and the highest with numbers 10, 11, and 12 ("short chromosomes"). For all the provenances, chromosome No. 1 was the longest and used in the karyotype as a standard (100%) The lowest average value of total length occurred in pop-4 (chromosome No. 12). The highest value of SD was 12.0 (pop-2/chromosome No. 12), The lowest variability out of the remaining 11 chromosomes was seen in numbers 7 and 2, while "short chromosomes" numbers 10, 11 and 12 showed the highest variability.

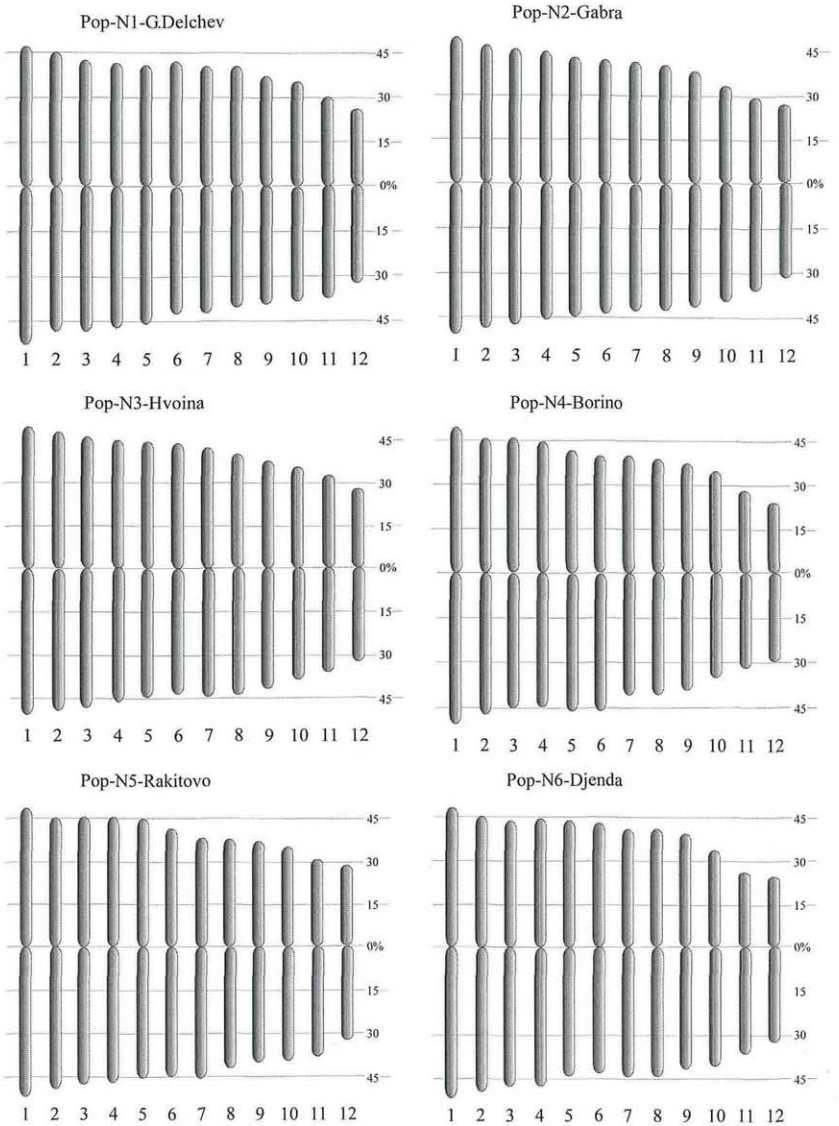
3.2. Composite Variables

Arm ratio varied from 0.70 in chromosome No. 11 (pop-6) to 1.00 in chromosomes No. 6 (pop-3), No. 3 (pop-4) and No. 9 (pop-9). This index showed the lowest standard deviation, reaching 0.20 in chromosome No. 10 (pop-7). The variability was the lowest in chromosome numbers 3, 4, and 6, and the highest in the "short chromosomes" 10, 11 and 12.

The remaining index of the group of "composite" variables Si characterized the idiotype as a whole and not the individual chromosomes (Table 2). The average Si values varied from 89.00 (pop-6) to 93.70 (pop-3). The lowest value of SD (0.97) was found in pop-3 and the highest value of SD (3.34) in pop-1.

Metacentricity is determined with great precision. The number of metacentric chromosomes ("median point") varied in the individual provenance from one (pop-9) to six (pop-3). They were encountered most often in chromosomes: 4, 1, 2, 4, and 5. The "median-submedian" chromosomes were the "short chromosomes" with numbers 11 and 12. There were no karyotypes in which "submedian", "subterminal", "terminal" and "terminal point" chromosomes could be found (Table 3).

The typical characteristics of phylogenetical age of the karyotype suggested by RIEGER & MICHAELIS 1958, such as greater numbers of metacentric chromosomes, support the hypotheses of a low rate of migration of this species on the Balkan Peninsula during the "glacial depression". Our results represented 25% of all investigated populations of European Black Pine to date. The obtained kayotype by centromere position for Bulgarian provenances is different then other investigated provenances (Table 3). The differences are clear at individual, population and group (formation) levels. Other karyological studies have reported up to 12 median region (m) and up to 9 median point (M) chromosome pairs (SAYLOR 1964, TARNAVSCHI & CIOBANU 1965, PEDERICK 1970, CHINCHALADZECH & TODUA 1971, MIHAILESCU & DALU 1971, 1972, KORMUTAK 1975, BORZAN & PAPE 1978, BORZAN 1981, KAYA & al. 1985) in *Pinus nigra* (Table 3).



3.3. Inter-Population Variability

Inter-population variability was investigated with the Principal Component Analysis/ Factor Analysis (Unirotated). Six canonical variables were determined with their Eigenvalues (Table 4). For Factor-1 and Factor-2, variables with the greatest loading (marked loading >0.7) were S

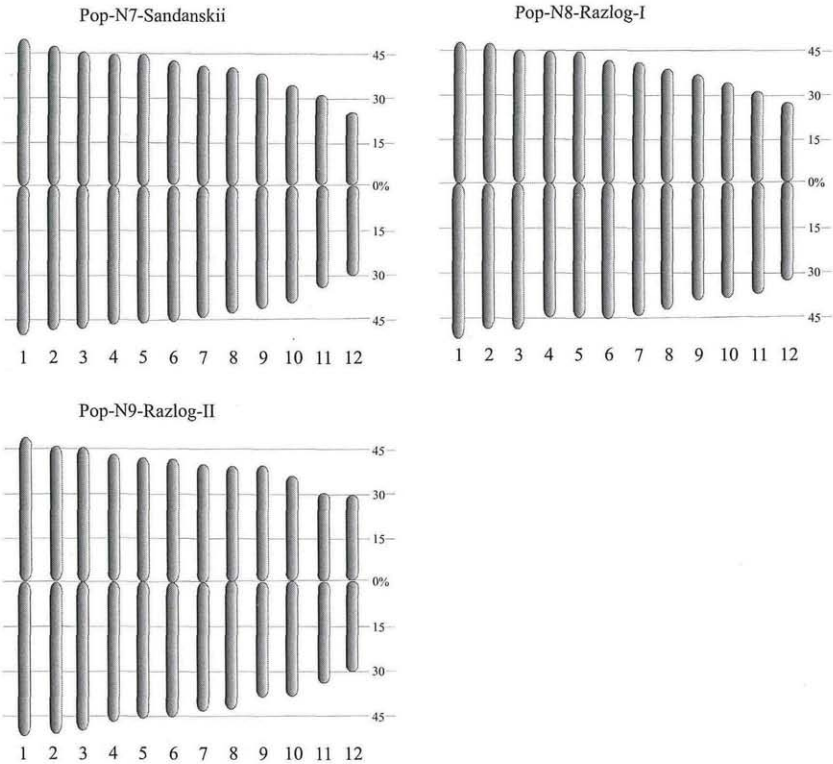


Fig. 3. The mean idiograms without NORs of the nine investigated native European Black Pine (*Pinus nigra*) provenances from Bulgaria.

(short arms) in chromosomes 1, 3, 4, and 7; and L (long arms) in chromosomes pairs 1, 4, 5, 6 and 9. The cumulative additional total variance for all 6 canonical variables was 71.51%, the highest for Factors 1, 2, and 3.

Two methods were used to determine the inter-population variability: (1) average and (2) median values of factor score coefficient for each tree examined. Figure 4 and 5 shows their distribution in the two-dimensional space and the differentiation of the investigated populations in three groups:

Group 1 – Includes the provenances pop-2, 7, 8, and 9 and can be referred to as "Western Black Pine Formation". This formation covers Rila, Pirin and Osogovo mountains, in the western par of country;

Group 2 – Includes the provenances pop-1, 5, and 6. These are above all the provenances of Black Pine characteristic for the borders of the mountain range, the Rhodopes and Slavyanka and can be referred to as the "Marginal Black Pine Formation";

Table 3. Chromosome types by index classes of European Black Pine (*Pinus nigra* ARN.) according to previous authors and to the present study.

	Chromosome pairs[1]											
Provenances[2]	1	2	3	4	5	6	7	8	9	10	11	12
unidentified[2a]	m	M	M	M	M	m	m	M	m	m	msm	msm
var.*banatica*[2b]	M	M	M	m	m	M	M	M	m	msm	msm	M
var.*austriaca*[2b]	M	M	M	m	M	M	M	M	M	M	msm	msm
Corsica[2c]	m	m	m	m	m	m	m	m	m	m	msm	msm
Krim (Ukraine)[2d,3]	m	m	m	m	m	m	m	msm	msm	msm	msm	msm
var.*calabrica*[2e]	m	m	m	m	m	m	m	m	m	m	m	m
var.*corsicana*[2e]	m	m	m	m	m	m	m	m	m	m	m	m
var.*dalmatica*[2e]	m	m	m	m	m	m	m	m	m	m	m	m
var.*austriaca*[2e]	m	m	m	m	m	m	m	m	m	m	m	m
var.*koekaelare*[2e]	m	m	m	m	m	m	m	m	m	m	m	m
Romania-N[2f]	m	m	m	M	m	m	m	m	m	m	msm	msm
Slovakia[2g]	m	m	m	m	m	m	m	m	m	m	m	m
Croitia[2h,4]	m	m	m	m	m	m	m	m	m	m	msm	msm
Austria[2i]	m	m	m	m	m	m	m	m	m	m	m	m
France[2i]	m	m	m	m	m	m	m	m	m	m	m	m
Greece[2i]	m	m	m	m	m	m	m	m	m	m	m	msm
Turkey[2i]	m	m	m	m	m	m	m	m	m	m	m	msm
Yugoslavia[2i]	m	m	m	m	m	m	m	m	m	m	m	msm
Pop-2/ western[2j]	M	m	M	M	M	m	M	m	m	m	m	m
Pop-7/ western[2j]	M	M	m	m	M	m	m	m	m	m	m	m
Pop-8/ western[2j]	m	M	m	M	M	m	m	m	m	m	m	m
Pop-9/ western[2j]	m	m	m	m	m	m	m	m	M	m	m	m
Pop-1/ marginal[2j,]	m	m	m	m	m	M	m	M	m	m	msm	m
Pop-5/ marginal[2j,]	m	m	m	M	M	m	m	m	m	m	m	m
Pop-6/ marginal[2j,]	m	m	m	m	M	M	m	m	m	m	msm	msm
Pop-3/ central[2j]	M	M	M	M	M	M	m	m	m	m	m	m
Pop-4/ central[2j]	M	M	M	M	m	m	m	m	m	m	m	m

[1] all chromosomes types are presented according to LEVAN & al. 1964, modified by SCHLARBAUM & TSUCHIYA 1984. Some publications do not give the description of the index classes and/or the names of the authors who classified the chromosomes according to centromer position. In these cases, the types of chromosomes are calculated with respect to the S (Short arms) and L (Long arms) values presented by the authors.

[2] names of provenances and varieties are presented according to the author's description: [2a] – SAYLOR 1964; [2b] – TARNAVSCHI & CIOBANU 1965; [2c]– PEDERICK 1970; [2d] – CHINCHALADZE & TODUA 1971; [2e & 2f] – MIHAILESCU & DALU 1971, 1972; [2g] – KORMUTAK 1975; [2h] – BORZAN & PAPE 1978; [2i] – KAYA & al. 1985; [2j] Bulgaria/ Present study.

[3] description according to additional information by personal communication from GANCHEV.

[4] similar publication BORZAN 1981.

Table 4. General results of the Principal Component Analysis/ Factor Analysis (Unirotated): Eigenvalues in % of the total variance for 9 native provenances of European Black Pine (*Pinus nigra*) in Bulgaria.

AXIS[1]	Eigenvalues	Variance total %	Cumul. Eigenvalues	Cumul. %
1	7.882	32.844	7.882	32.844
2	3.257	13.572	11.140	46.416
3	1.820	7.586	12.960	54.002
6 (max)	1.221	5.090	17.162	71.509

[1] Marked with more than 0.7 loadings for axis-1 and axis-2 are Short arms (S) for chromosome pairs N 1, 3, 4, 7 and Long arms (L) for chromosome pairs N 1, 4, 5, 6, 9.

Group 3 - Includes pop-3 and 4. This is a group of the typical "Central Rhodopean Mountain Black Pine Formation".

Both methods, the average and median values of factor score coefficient, gave similar results. A slight shift for the provenances G.Delchev (pop-1) and Djenda (pop-6) was observed however, they remained in Group-2.

Enzyme analysis studies for *P. nigra,* where the study area was five times greater in size than ours (covering the majority of the natural range of European Black Pine), revealed a variability of 6%–13.5% among populations (BONNET-MASIMBERT & BIKAY-BIKAY 1978, FINESCHI 1983, NICOLIC & TUCIC 1983, SCALTSOYIANNES & al. 1994a, b). Greater between population differentiation is found with analysis of secondary metabolites such as monoterpenes, flavonoids, and others (LEE 1968, ARBEZ & al. 1974, WHEELER & al. 1976, FINESCHI & GROSSONI 1981, NAYDENOV & al. 1993/1996; GERBER & al. 1995). Karyological analysis of *P. nigra* populations has already been published by KAYA & al. 1985, but it did not cover the whole area of distribution of European Black Pine and the Balkan Peninsula is not completely represented. Our data for centromere position are different than the average values reported by KAYA & al. 1985.

In comparison with enzyme and metabolite analyses, the karyological analyses have a higher capacity to reveal differences among provenances in Bulgaria. According to the 65 publications cited by LEDIG 2000, enzyme analysis, in general, expresses not more "10%, and often less than 5%", of inter- population variability in the genus *Pinus*. To our knowledge microsatelite (SSR), RFLP, AFLP or other DNA analysis have not yet been published for *P. nigra*.

In this study, the maximal distance among the provenances was 300–330 km. An analysis of variance (ANOVA) of chromosome karyotype indicated that the differences between provenances and trees are statistically significant and variability among provenance explained between 30–40%

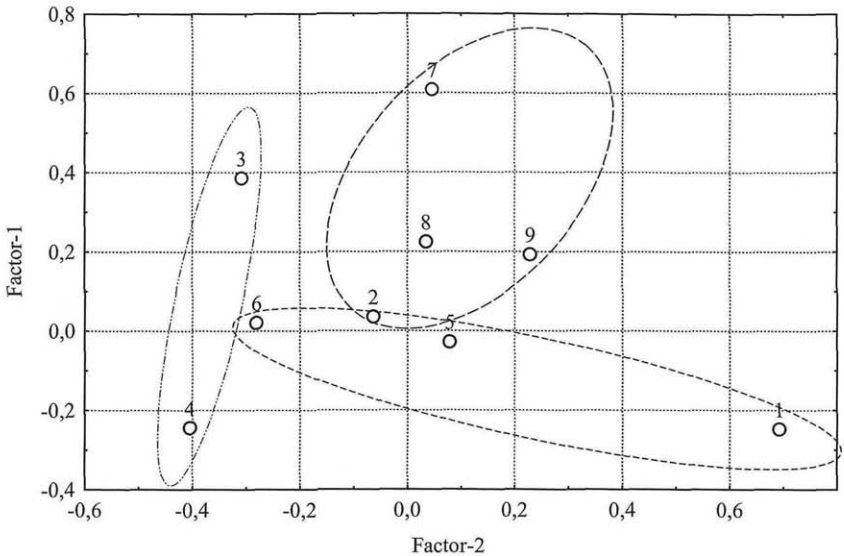


Fig. 4. Identification of the 9 natives provenances of European Black Pine (*Pinus nigra*) on the first two axis (Factor-1 and Factor-2) of the Principal Component Analysis/Factor Analysis (Unirotated): sample determined by median of factor score coefficients (pop-1/G. Delchev; pop-2/Gabra; pop-3/Hvoina; pop-4/Borino; pop-5/ Rakitovo; pop-6/Djenda; pop-7/Sandanskii; pop-8/Razlog-I and pop-9/Razlog-II.

of the total variance (Table. 5). This is more than the inter-population variability, calculated by enzyme analysis for the same provenances (NAYDENOV & al. 1993/1996). Perhaps the centromere position and length variation in chromosome arms are population determined (i.e. resulting from population structure and evolution). Different processes, such as pericentric inversions, unequal translocations, duplication and deletion, occurring during meiosis could cause variation in length and centromere position and contribute to population differentiation (MIKSHE 1967, 1968, PEDERICK 1969, SAYLOR 1969, STEBBINS 1971, 1976, KAYA & al. 1985).

Previous studies based on morphology of European Black Pine populations from Bulgaria have revealed the presence of significant differentiation of provenances; this is consistent with our results. Specific forms have been described that differ in growth characteristics such as: form of the apophysis, size of the cone, leaves and seeds; thickness and texture of the bark, angle of the branches as well as other morphological markers (DOBRINOV & IAGZIDIS 1968, IVANOV 1971, KOSTOV 1974, DOBRINOV & al. 1982, DOBRINOV 1983, MIHAILOV 1983, 1987, 1993, 1998, VELKOV & al. 1983). The extent of genetic control on these traits and the potential for selection however, still need to be determined as well as the exact relationship

Table 5. Results of the multivariate analysis of variance (MANOVA) first axis of the principal component analysis. Analysis of variance (ANOVA) on the basic and composite variables.

Sources	Hotelling-Lawley Trace	F	df	P
First axis (PCA)				
Pop	3.3737	2.37	48, 107	**0.0001**
Tree	1.0034	1.1385	30, 82.8	0.3235
Sources	**Mean square**	**F**	**df**	**P**
Short arm				
Pop	0.5665	0.41	8	0.9103
Tree	0.2524	0.18	5	0.9682
Long arm				
Pop	29.0186	3.6273	8	**0.0004**
Tree	8.5201	1.7040	5	0.0717
Total arm				
Pop	3.9648	0.4956	8	0.9353
Tree	1.6380	0.3276	5	0.9434
Arm ratio				
Pop	28.0226	3.6278	8	**0.0006**
Tree	9.4289	1.8857	5	0.5284
Symetry index				
Pop	24.2083	3.0260	8	**0.0003**
Tree	4.6862	0.9372	5	0.2057

among the different forms. It has been suggested that some of the specific forms of European Black Pine were a result of spontaneous among-species hybridisation between *Pinus nigra* and *Pinus sylvestris* and also between *Pinus nigra* and *Pinus heldreichii* Christ (DOBRINOV & al. 1982, DOBRINOV 1983).

A correlation between the present karyotypes and some phenotypic characteristics such as: edafotype, form of apophysys, bark texture and size of cones and seeds was not observed (data not shown).

Some authors have suggested that Bulgarian European Black Pine populations have not migrated substantially following the "glacial depression". Due to the height of the mountains in the Balkan Peninsula there has been only secondary "additional glacial depression" (STEFANOV 1943, HUTTUNEN & al. 1992, WILLIS 1994). After climate warming, migration of European Black Pine to higher altitudes and recolonization of sites also with other tree species would have occurred at different speeds with varying success. This has probably led to disruption of the area of *P. nigra*, and creation of isolated, small groups of populations, and the possible emergence of different forms. Our results show that this pattern of migration is

revealed by the two groups of Rodopean mountain "Marginal Black Pine Formation" and "Central Rhodopean Mountain Black Pine Formation".

In conclusion, European Black Pine in Bulgaria can be divided into three basic groups: (1) "Western Black Pine Formation"; (2) "Marginal Black Pine Formation" and (3) "Central Rhodopean Mountain Black Pine Formation". If these three formations of *P. nigra* are confirmed by other studies, seed collection of this species must take this into account. This may also lead to a differentiated approach to choosing provenances for reforestation. We established that variability is distributed between a great number of canonical variables – 6, due to the extremely large number of non-canonical variables – 24. When their number is high, the ordinary variables restrict the use of other methods of multivariable statistics (Cluster Analysis, etc.). In species with a large number of chromosomes, analysis of the karyotypes in this manner will be even harder to carry out. Similar difficulties may also arise with the Giemsa banding method of chromosome staining. With differentiated staining, the various bands can be used as variable values. Therefore, the investigator is faced with the possibility of compromise where the variables would not undergo a secondary differentiation depending on the chromosome number. This would make it possible for the canonical variables to be fewer in number but with sufficiently high variability. We will attempt to verify this possibility in our next investigation.

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