Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *75* (10) 1337–1346 (2010)
JSCS–4056

UDC 547.21:665.944.55:633.94
Original scientific paper

n-Alkanes in the needle waxes of *Pinus heldreichii* var. *pančići*

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(Received 22 March, revised 14 June 2010)

Abstract: This is the first report of *n*-alkanes in needle epicuticular waxes of the variety Bosnian pine, *Pinus heldreichii* var. *pančići*. *n*-Hexane extracts of needle samples, originating from seven isolated localities in Serbia, were analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). The results evidenced *n*-alkanes ranging from C_{18} to C_{33} in epicuticular waxes. The most abundant alkanes were C_{27}, C_{23}, C_{25} and C_{29} (12.53 %, 12.46 %, 12.00 % and 10.38 % on average, respectively). The carbon preference index (CPI_{total}) of *Pinus heldreichii* var. *pančići* ranged from 1.1 to 2.1 (1.6 on average), while the average chain length (ACL_{total}) ranged from 25.0 to 25.8 (25.3 on average). A high level of individual quantitative variation in all of these hydrocarbon parameters was also found. The obtained results were compared with the bibliographic references for *Pinus heldreichii* var. *leucodermis* and other species of the *Pinus* genus.

Keywords: Bosnian pine; Pinaceae; needles; *n*-alkanes.

INTRODUCTION

Bosnian pine, *Pinus heldreichii* Christ. (Pinaceae family), is a relict and subendemic tree species naturally distributed in fragmented areas in Italy, Bosnia and Herzegovina, Serbia, Montenegro, Albania, Macedonia, Bulgaria and Greece. The Bosnian pines, which grow in southwestern Serbia and northern Montenegro, are regarded as *Pinus heldreichii* var. *pančići* Fukarek.[1–3] These trees grow individually or in smaller groups, in relict and geographically isolated popula-

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doi: 10.2298/JSC100322089N

tions (mainly up to 10 trees). Concerning the site and morphology of the branches, needles and/or cones, var. *pančići* is similar to *Pinus nigra* Arn. and partially to two natural hybrids: *Pinus x mugodermis* Fukarek[4] and *Pinus x nigradermis* Fukarek and Vidaković,[5] and one intermedial form, *Pinus nigra* f. *leucodermoides* Fukarek and Nikolić,[6] but differs clearly from other *Pinus heldreichii* varieties: *Pinus heldreichii* var. *typica* Markgraf, *Pinus heldreichii* var. *leucodermis* (Ant.) Markgraf and *Pinus heldreichii* var. *longiseminis* Papaioannou.[1] Differences between populations of Bosnian Pine from Serbia and Montenegro (regarded as *Pinus heldreichii* var. *pančići* and *Pinus heldreichii* var. *leucodermis*,[2] respectively) in the compositions of the essential oil have also been noticed.[7]

n-Alkanes are among the most common hydrocarbons in cuticular waxes of numerous higher plants. In leaf waxes, they show abundances up to 73 %.[8] In waxes of conifers, polar lipids are dominant (above 90 %), whereas there is often a small percentage of non-polar lipids, particularly leaf *n*-alkanes (up to 4.5 %),[9] with the exception of *Wollemia nobilis* Jones, Hill and Allen (up to 22 %).[10]

Hitherto, the research of *n*-alkanes has most frequently been used in chemotaxonomic studies of trees[11–16] and herbaceous plants.[17–22] *n*-Alkanes in plants, in combination with other chemical markers, are also valuable objects for analyses in other fields: phylogenetic studies,[23] hybrid detection,[24] air pollution studies,[25,26] studies of nutrition,[27,28] *etc*.

The parameters which are often used for the description of the *n*-alkane distribution patterns are: abundance of long-chain *n*-alkanes (LNAs), carbon preference index (*CPI*)[29] and average chain length (*ACL*).[30] They can be used as chemotaxonomic markers at the genus level,[31] in environmental studies,[30] in palaeoenvironmental reconstructions,[32] *etc*.

The *n*-alkanes in conifer trees have been most extensively researched in *Picea*[11,33] and *Pinus*[11] genera. The *n*-alkanes in pines are present as mixtures with chain lengths ranging from 18 to 34 carbon atoms, whereby odd-numbered alkanes are dominant.[11]

The composition of the epicuticular wax of needles of *Pinus heldreichii* var. *leucodermis* has already been studied[11] but, to the best of our knowledge, this is the first report of the composition and variability of *n*-alkanes in the needles of Bosnian pine from natural populations. In addition, this is the first report of the composition of pine needle epicuticual wax from the variety *pančići*.

EXPERIMENTAL

Plant material

The population density of Bosnian pine in Serbia is low, *ca*. 200 trees, which are very old (up to 400 years) and tall (10–20 m). These trees grow individually or in small groups,[2] mainly on difficult to access terrains. In order to cover the altitudinal range of Bosnian pine in Serbia, seven trees were selected from seven natural sites, *i.e.*, from Mt. Zlatibor to Mt. Pešter, at the following localities: 1) Negbina (village, Mt. Zlatibor), 2) Sjeništa (village, Mt. Zlatibor), 3) Kamena gora (Mt. near Prijepolje), 4) Lastva (Milakovići village near Brodarevo, Mt. Ozren), 5) Trijebine (Prijevorac village, located between Mt. Ozren and Mt. Giljeva), 6) Bare (Krajinovići village, Mt. Giljeva), and 7) Lokva (Krajinovići Village, Mt. Giljeva) (Table I).

TABLE I. Geographic and geologic characteristics of the study area of *Pinus heldreichii* var. *pančići*

Localities	Negbina	Sjeništa	Kamena gora	Lastva	Trijebine	Bare	Lokva
	1	2	3	4	5	6	7
Latitude (N)	43°32’	43°32’	43°20’	43°16’	43°16’	43°10’	43°10’
Longitude (E)	19°47’	19°47’	19°33’	19°42’	19°55’	19°52’	19°52’
Altitude, m	1206	982	1273	1430	1313	1360	1335
Exposition	SE	NE	E	SW	E	SW	S
Geological substratum	Lime-stone	Serpen-tinite	Limestone	Serpen-tinite	Neogene sediments	Limestone horno-stone tuff	Serpen-tinite

Two-year-old needles were collected at the end of the photosynthetic active season, around the lower third of the unshaded tree crown, to ensure comparability of the sample collections. The second, more practical reason for this kind of plant collection, is the fact that climbing and collecting of needles from the higher parts of crown could be very difficult and unsafe, since many of the investigated trees from natural populations were located on difficult to access terrains. The needles were kept in polyethylene bags (with the labels of the sample plot, date of collection and age of the needles) in a hand fridge and transported to a freezer (–20 °C).

Extraction and isolation of needle wax

The total wax of each sample was extracted by immersing 3 g of needles in 10 ml of *n*-hexane for 45 s. After extraction, the solvent was removed under vacuum at 60 °C. The concentrated extracts were chromatographed on a small-scale column using a Pasteur pipette filled with silica gel 60, previously activated at 120 °C.[34] The wax was obtained by elution with 5 ml of *n*-hexane. The wax samples were stored at –20 °C until further analysis.

Chemicals and reagents

n-Hexane (HPLC grade) and silica gel 60 (0.2–0.5 mm) were purchased from Merck (Darmstadt, Germany).

GC and GC–MS analysis

Gas chromatography (GC) and gas chromatography–mass spectrometric (GC–MS) analyses were performed using an Agilent 7890A GC equipped with an inert 5975C XL EI/CI mass spectrometer detector (MSD) and flame ionisation detector (FID) connected by capillary flow technology 2-way splitter with make-up. A HP-5MS capillary column (30 m×0.25 mm×0.25 μm) was used. The GC oven temperature was programmed from 60 to 300 °C at a rate of 3 °C min^{-1} and held for 10 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). An auto-injection system (Agilent 7683B Series Injector) was employed to inject 1 μL of sample. The sample was analysed in the splitless mode. The injector temperature was 250 °C and the detector temperature 300 °C. MS data was acquired in the EI mode with scan range 30–550 *m*/*z*, source temperature 230 °C, and quadrupole temperature 150 °C; the solvent delay was 3 min.

The components were identified based on their retention index and comparison with reference spectra (Wiley and NIST databases) as well as by the retention time locking (RTL) method and the RTL Adams database. The retention indices were experimentally determined using the standard method of Van Den Dool and Kratz[35] involving retention times of *n*-alkanes, injected after the sample under the same chromatographic conditions. The relative abundance of the *n*-alkanes (Table II) was calculated from the signal intensities of the homologues in the GC-FID traces.

Calculations of the CPI *and* ACL *values*

Carbon preference index of total odd-numbered and even-numbered LNAs (CPI_{total}) was calculated by formula of Mazurek and Simoneit[36] (Table III). The average chain length of the total odd-numbered and even-numbered LNAs (ACL_{total}) was calculated by the Poynter and Eglington method.[37] In order to compare the obtained results with those from literature sources,[9,38,39] CPI_{25-33}, CPI_{20-36}, CPI_{15-21} and CPI_{25-31} were also calculated using the formula of Bray and Evans,[40] as well as ACL_{23-35} value using the formula of Poynter and Eglington.[37] The relative proportions of short, mid and long chain n-alkanes[39] (n-C_{18-20}, n-C_{21-24} and n-C_{25-33}, respectively) were also calculated.

RESULTS AND DISCUSSION

In the epicuticular waxes of two-year old needles of *Pinus heldreichii* var. *pančići*, the *n*-alkanes ranged from C_{18} to C_{33} (Fig. 1, Tables II and III). It can be noticed that the trees at the northernmost sites, Negbina (No. 1) and Sjeništa (No. 2), had a narrower range of *n*-alkanes (C_{18} to C_{31}), as well as *Pinus heldreichii* var. *leucodermis*.[11] Some pines from the section Sylvester (*Pinus sylvestris* L., *Pinus sylvestris* var. *iberica* Svoboda, *Pinus mugo* var. *pumilio* (Haenke) Zenari, *Pinus mugo* Haenke, *Pinus thunbergii* Parl., *Pinus engelmannii* Carr. and *Pinus montezumae* Lamb.) and the section Strobus (*Pinus cembra* L. and *Pinus wallichiana* A. B. Jacks.).[9,11,38] also had the same or a narrower range of needle *n*-alkanes than *Pinus heldreichii* var. *pančići*.

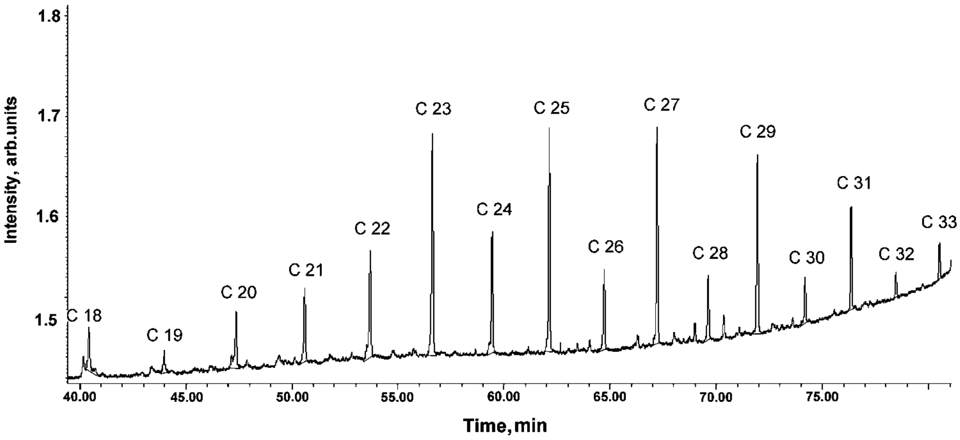

Fig. 1. *n*-Alkanes in needle wax of *Pinus heldreichii* var. *pančići*, Lastva locality (No. 4).

TABLE II. Chemical composition of needle *n*-alkanes of *Pinus heldreichii* var. *pančići*

n-Alkane	Localities							Statistical parameters	
	1	2	3	4	5	6	7	X^a	SD^b
C_{18}	2.41	4.21	4.36	3.19	6.58	3.94	4.84	4.22	1.32
C_{19}	0.00	0.00	1.31	0.89	1.24	1.23	0.88	0.79	0.57
C_{20}	0.00	7.10	3.90	4.03	5.28	3.83	3.63	3.97	2.14
C_{21}	4.48	4.77	4.91	5.21	6.22	5.90	4.51	5.14	0.68
C_{22}	9.11	10.40	7.20	7.80	8.94	10.57	8.49	8.93	1.25
C_{23}	13.43	11.50	9.51	14.44	10.90	14.23	13.21	12.46	1.85
C_{24}	8.58	9.74	8.85	8.17	6.73	7.15	7.57	8.11	1.04
C_{25}	13.12	10.90	10.45	14.03	9.13	12.76	13.64	12.00	1.85
C_{26}	4.89	5.41	7.08	5.10	4.55	4.57	4.37	5.14	0.93
C_{27}	15.16	10.93	11.39	12.49	11.40	12.61	13.76	12.53	1.50
C_{28}	3.76	4.56	5.77	3.51	3.97	3.53	3.83	4.13	0.80
C_{29}	12.50	8.10	10.11	9.77	12.13	9.59	10.47	10.38	1.52
C_{30}	2.49	3.21	4.14	2.31	2.86	2.19	2.51	2.82	0.68
C_{31}	5.94	4.90	5.59	5.47	5.54	4.90	5.41	5.39	0.38
C_{32}	1.50	1.99	2.66	1.53	1.64	1.46	1.16	1.71	0.49
C_{33}	2.63	2.28	2.77	2.06	2.89	1.54	1.72	2.27	0.52

[a]Mean value; [b]standard deviation

In *Pinus heldreichii* var. *pančići* needles, the most abundant alkanes were the four odd-numbered *n*-alkanes: C_{27}, C_{23}, C_{25} and C_{29} (12.53, 12.46, 12.00 and 10.38 % on average, respectively, Table II). Most trees had a maximal abundance at C_{23} (four trees). *n*-Alkane C_{27} was first-ranked at the localities Kamena gora and Lokva (No. 3 and No. 7, respectively). *n*-Alkane C_{29} was first-ranked only in the tree which grows in the southernmost part of the research altitudinal range (Trijebine, No. 5). Two even-numbered *n*-alkanes, C_{22} and C_{24}, were also abundant (8.93 and 8.11 % on average, respectively). Variation of each *n*-alkane is listed as the value of the standard deviation (*SD*, Table II). The most variable were C_{20}, C_{23}, $C_{25,}$ C_{29} and C_{27} (*SD* values: 2.14, 1.85, 1.85, 1.52 and 1.50, respectively). According to literature sources, other pine species are often rich only in one *n*-alkane, C_{31},[11] rarely C_{33}.[9] However, in *Pinus heldreichii* var. *pančići*, no correlation between the C_{max} values of needle *n*-alkanes and the distances of the trees from villages or adjacent roads was found.

For the calculation of the *CPI* and *ACL* values of *n*-alkanes of *Pinus heldreichii* var. *pančići* (Table III), the relative values from Table II were used. CPI_{total} of *Pinus heldreichii* var. *pančići* (from 1.1 to 2.1; 1.6 on average) was a little higher than in wood smoke particular matter of *Pinus elliottii* Engelm.[41] The maximum *CPI* values of long chain *n*-alkanes of *Pinus heldreichii* var. *pančići* (Table III) were 2.2 ($CPI_{20–36}$), 3.0 ($CPI_{25–31}$) and 3.1 ($CPI_{25–33}$), while the short chain *n*-alkanes ($CPI_{15–21}$) ranged from 0.3 to 1.1 (0.6 on average) and exhibited an even/odd predominance (*EOP*) (because $CPI < 1$ indicates *EOP*, $CPI > 1$ denotes *OEP*[39]). The ACL_{total} values of *Pinus heldreichii* var. *pančići*

TABLE III. Analytical data for *n*-alkanes in needles of *Pinus heldreichii* var. *pančići*

No. of tree	C_{range}	C_{max}	CPI_{total}[c]	CPI_{25-33}[d]	CPI_{20-36}[e]	CPI_{15-21}[f]	CPI_{25-31}[g]	ACL_{total}[h]	ACL_{23-35}[i]	In % of total *n*-alkanes (C_{18-33})		
										n-C_{18-20}	*n*-C_{21-24}	*n*-C_{25-33}
1	[illegible]	23	2.1	3.1	2.2	1.1	3.0	25.8	26.8	2.4	35.6	62.0
2	18, 20 – 33	23	1.1	2.0	1.4	0.3	1.9	25.0	26.6	11.3	36.4	52.3
3	18 – 33	27	1.3	1.7	1.5	0.6	1.7	25.6	27.0	9.6	30.5	60.0
4	18 – 33	23	1.8	2.8	2.1	0.6	2.8	25.2	26.5	8.1	35.6	56.3
5	18 – 33	29	1.5	2.6	1.9	0.5	2.5	25.1	27.0	13.1	32.8	54.1
6	18 – 33	23	1.7	2.9	2.0	0.7	2.8	25.0	26.4	9.0	37.9	53.2
7	18 – 33	27	1.7	3.1	2.1	0.5	3.0	25.2	26.5	9.4	33.8	56.9
Range	18 – 33	23 – 29	1.1 – 2.1	1.7 – 3.1	1.4 – 2.2	0.3 – 1.1	1.7 – 3.0	25.0 – 25.8	26.4 – 27.0	2.4 – 13.1	32.8 – 37.9	52.3 – 62.0
Average	18 – 33	27	1.6	2.6	1.9	0.6	2.5	25.3	26.7	9.0	34.6	56.4

[c] $CPI_{total} = \Sigma odd\ C_n / \Sigma even\ C_n$ (ref. 36); C_n is the concentration of *n*-alkane containing *n* carbon atoms;

[d] $CPI_{25-33} = [\Sigma(C_{25}–C_{33})odd/\Sigma C_{24}–C_{32})even+\Sigma(C_{25}–C_{33})odd/\Sigma C_{26}–C_{34})even]/2$ (ref. 40);

[e] $CPI_{20-36} = [\Sigma(C_{20}–C_{36})odd/\Sigma(C_{19}–C_{35})even+\Sigma(C_{20}–C_{36})odd/\Sigma(C_{21}–C_{37})even]/2$ (ref. 40);

[f] $CPI_{15-21} = [\Sigma(C_{15}–C_{21})odd/\Sigma(C_{14}–C_{20})even+\Sigma(C_{15}–C_{21})odd/\Sigma(C_{16}–C_{22})even]/2$ (ref. 40);

[g] $CPI_{25-31} = [\Sigma(C_{25}–C_{31})odd/\Sigma(C_{24}–C_{30})even+\Sigma(C_{25}–C_{31})odd/\Sigma(C_{26}–C_{32})even]/2$ (ref. 40);

[h] $ACL_{total} = n\Sigma C_n/\Sigma C_n$ (ref. 37);

[i] $ACL_{23-35} = (23C_{23}+25C_{25}+27C_{27}+29C_{29}+31C_{31}+33C_{33}+35C_{35})/(C_{23}+C_{25}+C_{27}+C_{29}+C_{31}+C_{33}+C_{35})$ (ref. 37)

ranged from 25.0 to 25.8 and the $ACL_{23–35}$ values ranged from 26.4 to 27.9 (Table III).

The relative proportion of short chain *n*-alkanes of *Pinus heldreichii* var. *pančići* (n-$C_{18–20}$ = 9.0 %) is significant (Table III) and similar to that in the leaves of *Acacia* sp. ($C_{14–20}$ = 10.1 %).[40] There is no great dominance of long chain (n-$C_{25–33}$ = 56.4 %) over mid chain *n*-alkanes (n-$C_{21–24}$ = 34.6 %), which could explain lower *CPI* and *ACL* values of *Pinus heldreichii* var. *pančići* (Table III) compared to those of some other species of the section *Pinus*.[9,38]

The differences in the composition, abundance, C_{max} values and other characteristics of *n*-alkanes between *Pinus heldreichii* var. *pančići* and other pines from the literature can be the consequence of the time of the needle sampling. In this research, the needles were sampled in the autumn, whereas some authors sampled the needles in the spring.[9,11,38] The crown exposition and illumination can also be a cause of the differences between *Pinus heldreichii* var. *pančići* and other pines. A large number of factors including light intensity, humidity, use of recycled CO_2, osmotic stress, CO_2 concentration, temperature, altitude and plant age influence the carbon isotopic composition of *n*-alkanes,[32,42,43] which is often regarded together with *ACL* values.[43] Trends to longer *n*-alkane chains and less negative carbon isotopic values are evident from rain forest over C_3 savanna to C_4 vegetation.[43] Lipids from inner shade leaves of *Quercus robur* L. and *Fagus sylvatica* L. were consistently more depleted in ^{13}C than those from the corresponding sun-lit leaves.[32] In contrast, in the case of *Quercus castaneifolia* C. A. Mey., the concentrations of C_{27} and C_{31} *n*-alkanes remained at constant levels throughout the whole growing season.[44] As the amount of seasonal variation is unknown for *Pinus* needles, the influence of different sampling season on sample comparison is unknown.

It is important to emphasize that in many other studies, chloroform[9] and not *n*-hexane was used as the dissolving agent.

CONCLUSIONS

According to the dominant *n*-alkanes, *Pinus heldreichii* var. *pančići* differs clearly from *Pinus heldreichii* var. *leucodermis*. The fact that *n*-alkanes can be successfully used in the determination of the variety of conifers has already been proved for *Juniperus communis* L.[16] and *Picea omorika* (Pančić) Purkyne.[45]

According to several alkane patterns, it was found that *Pinus heldreichii* var. *pančići* is more similar to some pines from subsections *Ponderosae* (section Pinus), *Cembrae* and *Strobi* (section Strobus) than to some of closely related pines (subsection *Sylvestres*, section Pinus).[9,11,38,39,41] Low values of odd/even predominance of *Pinus heldreichii* var. *pančići* leaf *n*-alkanes as well as low *CPI* and *ACL* values are the consequence of large amounts of short and mid chain *n*-alkanes, which are not typical for most pines and other C_3 gymnosperm and angyosperm plants, where long-chain *n*-alkanes strongly dominate.[46]

Populations adapted to warmer and more arid conditions have higher proportions of alkanes with longer chains in their waxes than those adapted to cooler and more humid conditions.[47] It is presumed that a strong genetic influence on the adaptation of hydrocarbons to environmental conditions exists (in the Cupressaceae family).[48] Similarly to the composition and variability of essential oils,[7] the specific profile of *n*-alkanes of *Pinus heldreichii* var. *pančići* could also be explained by the relict character of this species, the variability and genetic adaptation of which was expressed during great tectonic changes and glaciations.

Although in this paper the composition of alkanes in *Pinus heldreichii* on the different natural sites were analysed for the first time, the study of a small number of samples cannot give the complete picture on the variability at the species level, but only a picture of its variety *pančići*. Due to this fact, more detailed population research is recommended to be conducted in the future outside the borders of Serbia.

Acknowledgment. This research was supported by a grant from the Ministry of Science and Technological Development of the Republic of Serbia.

ИЗВОД

n-АЛКАНИ У ВОСКОВИМА ИГЛИЦА *Pinus heldreichii* var. *pančići*

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Ово је прво саопштење о *n*-алканима у восковима иглица варијетета мунике, *Pinus heldreichii* var. *pančići*. *n*-Хексански екстракти узорака иглица који потичу са седам изолованих локалитета у Србији анализирани су гасном хроматографијом (GC) и гасном хроматографијом–масеном спектрометријом (GC–MS). Резултати су показали да се *n*-алкани налазе у опсегу од C_{18} до C_{33}. Међу њима су најобилнији C_{27}, C_{23}, C_{25} и C_{29} (12,53, 12,46, 12,00 и 10,38 % у просеку, редом). Угљенични преференцијални индекс (CPI_{total}) *Pinus heldreichii* var. *pančići* је био у опсегу од 1,1 до 2,1 (просечно 1,6), а дужина низа угљеникових атома (ACL_{total}) у опсегу од 25,0 до 25,8 (просечно 25,3). Такође је утврђен висок ниво индивидуалне квантитативне варијабилности у свим анализираним параметрима ових угљоводоника. Добијени резултати су упоређени са литературним подацима који се односе на *Pinus heldreichii* var. *leucodermis* и друге врсте рода *Pinus*.

(Примљено 22. марта, ревидирано 14. јуна 2010)

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