Biológia, Bratislava, **48**/1: 95 – 100, 1993

Karyological, anatomical and restriction fragment length polymorphism characteristics of the interspecific hybrid *Pinus banksiana* x *Pinus contorta*

ANDREJ KORMUŤÁK, RADOSLAVA MATÚŠOVÁ[1], ALFRED SZMIDT, DAG LINDGREN[2]

[1] *Institute of Plant genetics Slovak Academy of Sciences, Akademická 2, P.O.B. 39A, 950 07 Nitra, Slovakia*
[2] *Swedish University of Agricultural Sciences, Institute of Forest Genetics and Plant Physiology, S–901 83, Umeå, Sweden*

Kormuťák, A., Matúšová, R., Szmidt, A., Lindgren, D., Karyological, anatomical and restriction fragment length polymorphism chracteristics of the interspecific hybrid *Pinus banksiana* x *Pinus contorta*. Biológia (Bratislava) 48, 95 – 100, 1993.

Comparative study of the North American species *Pinus banksiana* Lamb. and *Pinus contorta* Dougl. and their hybrid combination *P. banksiana* x *P. contorta* has involved karyological characteristics, needle anatomy and restriction fragment polymorphism data of cpDNA. At the anatomical level the hybrid individuals are distinct by their needles containing the resin ducts which have not been revealed in any of the parental species. Karyologically all the three taxa under investigation are indistinguishable. The restriction profiles of the chloroplast DNAs are uniform in both the parents and hybrid individuals consisting of 2–3 fragments by which they deviate strikingly from the remaining five species and two interspecific hybrids of pines compared so far.

Key words: *Pinus,* interspecific hybrids, chromosomes, needles, cpDNA.

The lodgepole pine (*Pinus contorta* Dougl.) and the jack pine (*Pinus banksiana* Lamb.) are typical representatives of the genus Pinus in North America whose areas extend as far as from the Yukon Territory of Canada to the Baja California in the USA in the former species and from Nova Scotia to the Northwest Territories of Canada in the latter one (Mirov, 1967). The region around Alberta together with the area of the Mackenzie River in the northwestern part of Canada are the only places where the extensive ranges of both species overlap and where they intercross spontaneously (Moss, 1949; Scotter, 1974). The high hybridological affinity of these species has also been confirmed artificially by Righter, Duffield (1951) and Liddicoet, Righter (1961).

The progeny obtained so far was shown to possess the distinct symptoms of heterosis not only during embryogeny (Buchholz, 1945) but also with respect to the height growth of juvenile trees (Johnson, Critchfield, 1978). The biochemical characteristics of the hybrid have also become available including the isozyme markers (Dancik, Yeh, 1983) and restriction frangment length polymorphisms of both the chloroplast nad nuclear DNAs (Wagner et al., 1987; Govindaraju et al., 1989).

The objective of presented study was to enrich these data with the karyological chracteristics and needle anatomy of the interspecific hybrid *P. banksiana* x *P. contorta*. Further extending of the data on restriction fragment length polymorphisms of its cpDNA was made with the purpose of comparing these traits of the hybrid with the restriction profiles of chloroplast DNAs in five additional species and two interspecific hybrids of pines.

Material and methods

Seeds and seedlings

The hybrid seeds of *Pinus banksiana* x *Pinus contorta* originating from a controlled crossing of the species in 1983 (Kormuťák, , Lanáková, 1988) were sown in a nursery together with the control offspring of *P. banksiana* resulting from an intraspecific crossing of three individuals. The height

parameters of seedlings produced so far were measured at the age of four years.

Karyological analyses

The hybrid seeds as well as those obtained from intraspecific pollinations of both *P. banksiana* and *P. contorta* were utilized in karyological studies in which the root tip meristems were used. The pretreatment procedure has involved the exposure of germinating seeds to 0.1 per cent of colchicine aqueous solution for a period of 12 hours. Following fixation and hydrochloric acid hydrolysis, the excised root tips were stained in *Feulgen solution* and squashed in 45 per cent acetic acid.

Histological analysis

The needle anatomy was analysed on cross–sections prepared from the middle part of 1–year old needles which were collected from the seedlings of all the three taxa mentioned above. The needle segments were processed according to the modified paraffin method by Tang–Shui Liu (1971) using formalin–acetic acid fixative, glycerin and butyl alcohol as dehydrating agents and paraffin for embedding.

Restriction analysis of chloroplast DNA

Except of the species *P. banksiana* Lamb. and *P. contorta* Dougl. of the section Banksia and their hybrid *P. banksiana* x *P. contorta*, the restriction analysis included also the species *P. sylvestris* L., *P. nigra* Arn. and *P. thunbergii* Parl. from the section Eupitys and their hybrid combination *P. nigra* x *P. thunbergii* and *P. nigra* x *P.sylvestris*. The sections *Cembra* Spach., *Strobus* Sweet ex Spach and Paracembra Koehne were represented by one species of each, e. g. by *P. cembra* L., *P. strobus* L. and *P. aristata* Engelm.

The chloroplast DNA was extracted according to the method described by Sandbrink et al. (1989) using the mixture of 10 g of needles from ten seedlings and 50 mM TRIS–HCl buffer, pH 8.0 with 1,25 M NaCl, 10 mM 2–mercapto–ethanol, 7mM EDTA, 1 per cent (w/v) of bovine serum albumin, and 5 per cent (w/v) of polyvinylpyrrolidone, added respectively.

Isolated cpDNA was digested with the endonucleases Taq I, Hind III, Sau 3A and Dra I, followed by the electrophoretic separation of the fragments in 0.8 per cent agarose using TBE buffer, pH 7.8 (10 mM TRIS, 1 mM EDTA.Na_2 salt). The restriction fragments of cpDNA transferred from a gel to the Hybond N matrix by Southern blotting were hybridized with the nik-translated probe of psbA isolated from *Spinacia oleracea* L.

Results and discussion

The interspecific hybrid *P.banksiana* x *P. contorta* belongs according to the growth characteristics to the heterotic combinations of pines only slightly differing from the parental species by the anatomy of its needles but karyologically indistinguishable from them. Biochemically the parental species as well as their hybrid are characterized by the uniform restriction fragment profiles of their cpDNA by which they differ markedly from the remaining species and interspecific hybrids of pines studied so far.

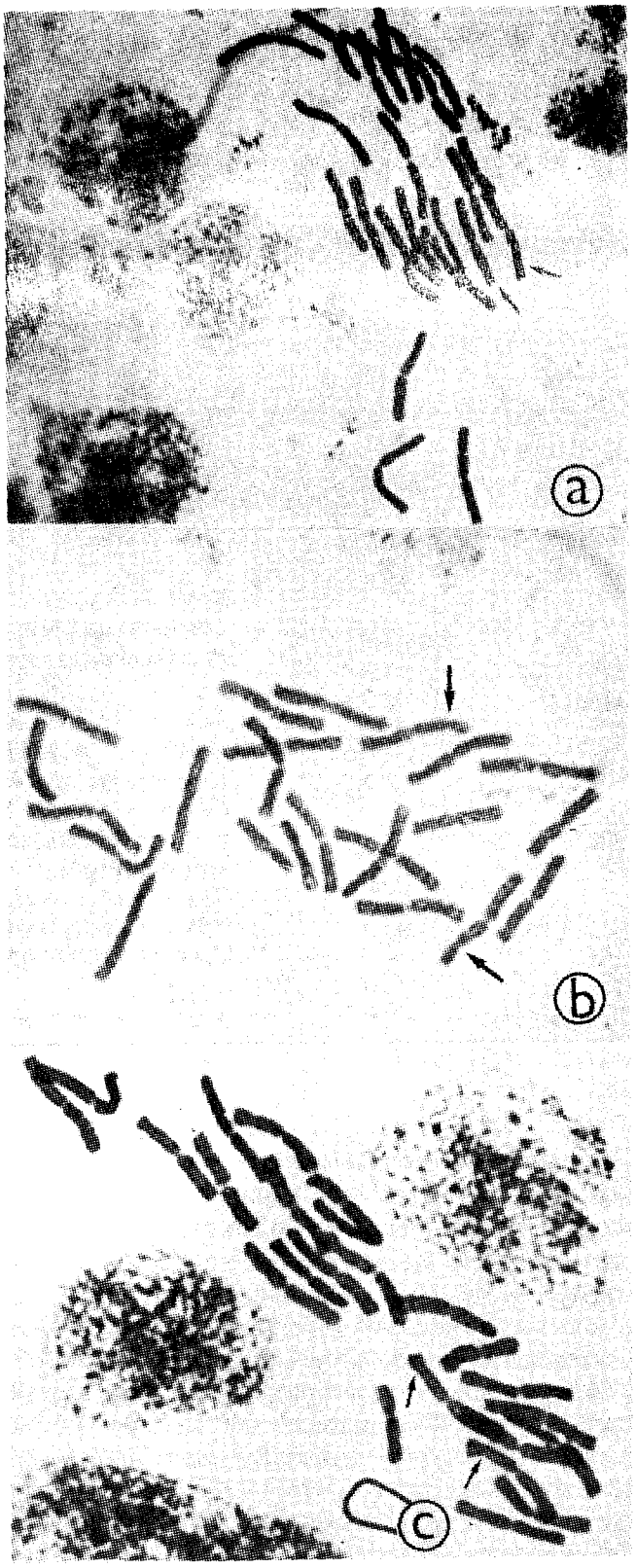
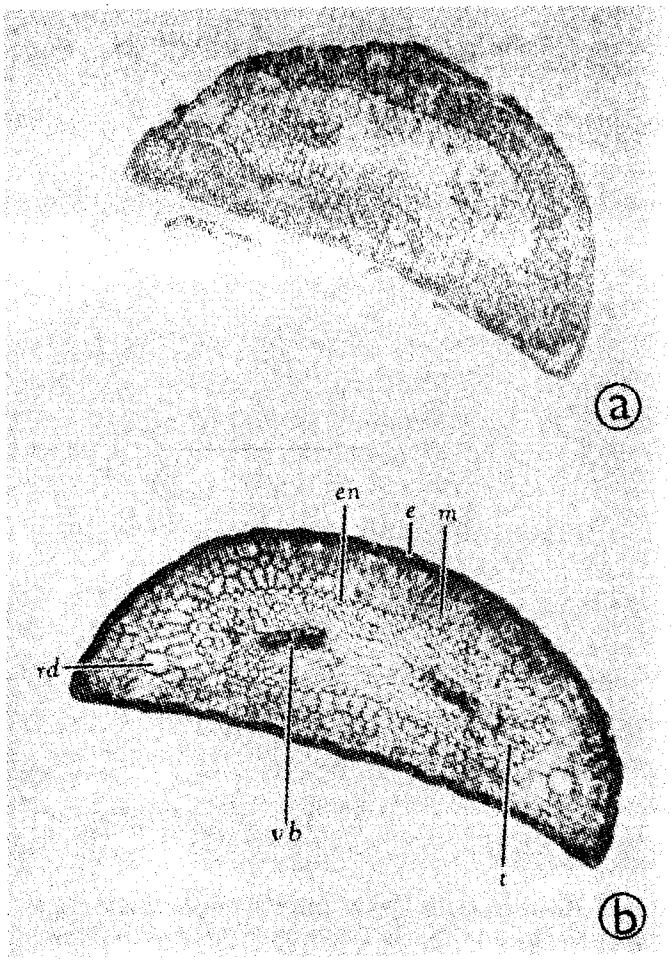


Fig. 1. Diploid chromosome sets of *Pinus banksiana*,*P. banksiana* x *P. contorta* and *P. contorta*.

The intrasectional hybrid of jack pine and lodgepole pine of the section Banksia is the only verified hybrid with *P. banksiana* involved as the parental species (Rudolph, Yeatman, 1982). Despite the references that it suffers from *Cronartium comptoniae* at some places of Canada (Yeatman, Holst, 1972), there are no indications of susceptibility of the interspecific hybrid to this infectious fungus in Slovakia. An excelent performace of the hybrid under our climatic condition has been illustrated by its heterotic growth during the first four years of its development. The evaluated sample of 91 hybrid individuals has reachen 13.37 cm of height on an average as compared with the mean value of 7.07 cm characterizing the growth of a control offspring from intraspecific crossing of *P. banksiana*. Statistically highly significant difference manifests unequivocally the growth potential of this hybrid.

Karyologically the parental species as well as the hybrid combination *P. banksiana* x *P. contorta* are very similar. As fig. 1 illustrates, there can be distinguished 18 chromosomes of a long size with the median position of their centromeres and 6 shorter chromosomes within the diploid chromosome set of each of them. One pair of the longer chromosomes contains secondary constrictions, while two of the shorter chromosomes are of heretobrachial type. All these features conform to the scheme of karyological structure of pines derive by Sax and Sax (1933). Some controversy arises only in relation to the data published recently by Kozubov and Muratova (1986) who postulate the existence of two chromosome pairs with secondary constrictions in *P. contorta* and even as many as six chromosome pairs of the kind in *P. banksiana*. The variable degree of spiralization and contraction of chromosomes during pretreatment procedure is usually offered as a possible explantation of this variation (Matern, Simak, 1968).

Of the internal traits of needles, it is the presence of resin ducts by which the hybrid form deviates conspicuously from the parental species both of which lack this structural feature in their needles (fig.2). One pair of ducts revealed in hybrid needles had as a rule been constant in all the samples processed so far. It is of interest to mention that the absence of resin ducts in needles of *P. contorta* is postulated also by Mirov (1967). Keng nad Little (1961) have the opinion that this character of needle anatomy is exceedingly variable in pine hybrids. In the combination *P. contorta* x *P. banksiana* they had observed no resin ducts in some individuals as compared with 1–3 resin ducts which were detected in the remaining hybrid individuals. The vascular region consisting of the double vascular bundle (vb), transfusion tissue (t) and endodermis (en) follows the elliptical shape of needles in a cross-



.Fig. 2. Cross–sections of needles as revealed in *P. banksiana* (a), *P. banksiana x P. contorta* (b), (e – epidermis, m – mesophyll tissue, en – endodermis, vb – vascular bundles, t – transfusion tissue, rd – resin ducts).

-section and is similar in this respect in all of the three compared samplex (fig.2).

In contrast to the cytological evidence supporting a close genetic relationship of jack and lodgepole pines, the results of restriction analysis of their cpDNAs indicate also the taxonomic uniqueness of these representatives of the section Banksia. The uniform restriction profiles obtainded after enzymatic digestion of cpDNAs of both species and their hybrid had as a rule differed markedly from the corresponding digests of the species from the section *Eupitys* represented by *P. nigra, P. sylvestris, P. thunbergii* and their hybrids *P. nigra* x *P. sylvestris* and *P. nigra* x *P. thunbergii*, respectively, as well as from the representatives of the subgenus *Haploxylon P. cembra* and *P. strobus* belonging systematically to the equally named sections of *Cembra* and *Strobus* (Pilger, 1926).

As shown in fig. 3, the parental variants of *P. banksiana* (h) and *P. contorta* (j) with the two frag-

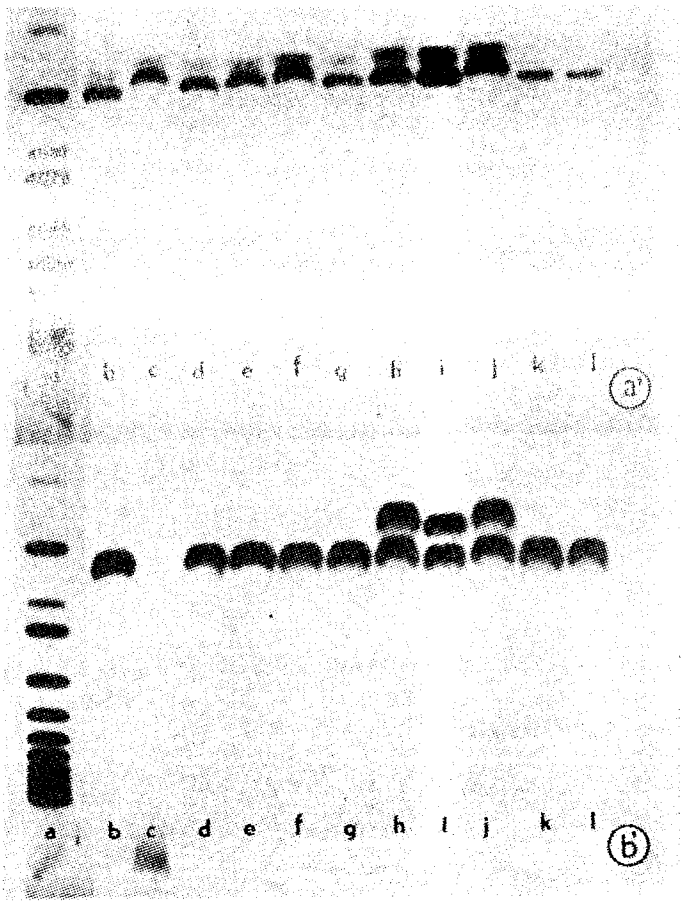


Fig. 3. Restriction profiles of cpDNA obtained after digestion with Sau 3A (a') and Taq I (b') in pines given below.

a– 1 kb ladder (BRL)
b– *P. nigra*
c– *P. nigra* x *P. sylvestris*
d– *P.sylvestris*
e– *P.nigra*
f– *P.nigra* x *P. thunbergii*
g– *P.thunbergii*
h– *P.banksiana*
i– *P.banksiana* x *P. contorta*
j – *P.contorta*
k– *P. cembra*
l– *P. strobus*

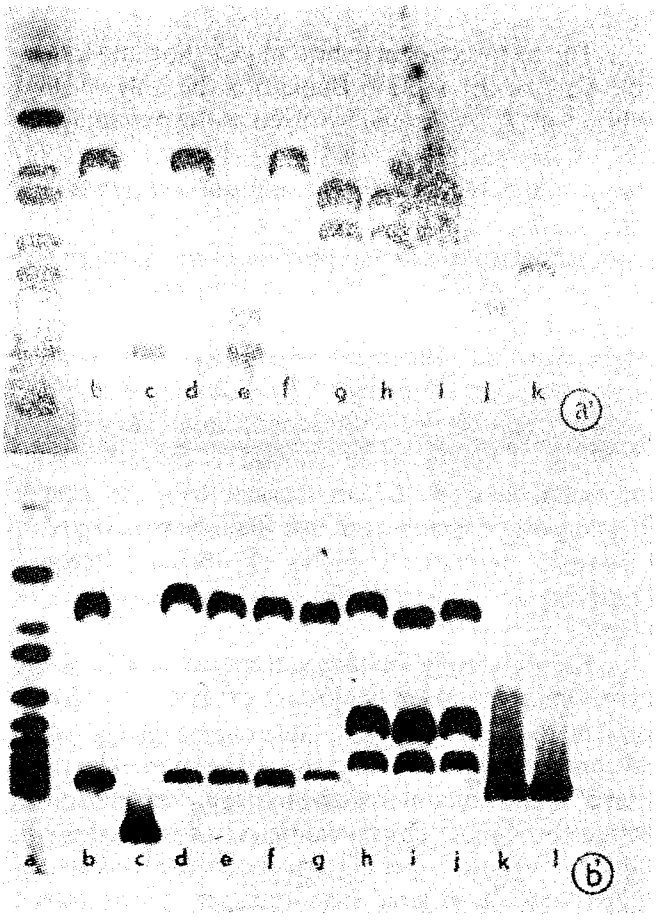


Fig. 4. Restriction fragment polymorphism of cpDNA obtained after digestion with Dra I (a') and Hind III (b') in pines given below.

a': a– 1 kb ladder (BRL)
b– *P.sylvestrix*
c– *P.nigra* x *P. sylvestris*
d– *P.nigra*
e– *P.nigra* x *P.thunbergii*
f– *P.thunbergii*
g– *P.banksiana*
h– *P.banksiana x P.contorta*
i– *P.contorta*
j– *P.cembra*
k– *P.strobus*

b': a– 1 kb ladder (BRL)
b– *P.nigra*
c– *P.nigra* x *P.sylvestris*
d– *P.sylvestris*
e– *P.nigra*
f– *P.nigra* x *P.thunbergii*
g– *P.thunbergii*
h– *P.banksiana*
i– *P.banksiana x P.contorta*
j– *P.contorta*
k– *P.cembra*
l– *P.strobus*

ments of cpDNA which have also been revealed in their hybrid (i) have been obtained after digestions with the endonucleases Sau 3A and Taq I. The fragments of Sau 3A corresponding to approximately 2 kb sizes have occurred uniformly in all the species and hybrids compared (fig. 3a'), whereas the two fragment restriction patterns of Taq I were typical only for the above mentioned representatives of the section *Banksia* and contrasted with only one fragment of cpDNA revealed in the remaining samples (fig. 3b'). The interspecific hybrid *P. nigra* x *P. sylvestris* (c) failed in this respect completely as its cpDNA had not been cleaved by the enzyme Taq (fig. 3b').

The two distinct fragments of a longer size (approx. 5 kb) have also been obtained after digestion of cpDNA of *P. banksiana, P. contorta* and their hybrid with Dra I (fig. 4a' g–i). On the other hand, the species *P. sylvestric* (b), *P. nigra* (d), *P. thunbergii* (f), *P. cembra* (j), and *P. strobus* (k), respectively, contained only one fragment of a conspicuous difference in its size in the last two species. However, it has not been possible to confirm the hybrid nature od the interspecific combinations *P. nigra* x *P. sylvestris* (c) and *P. nigra* x *P. thunbergii* (e) using the molecular markers of Dra I.

The treatment of cpDNAs of *P. banksiana, P. contorta* and their hybrid with Hind III have resulted in the highest number of fragments produced so far. It can be seen from fig.4b' that this pair of pines and the interspecific hybrid *P. banksiana* x *P. contorta* (h–j) deviated by their three fragments strikingly from the

species *P. sylvestris* (d), *P. nigra* (e) and *P. thunbergii* (g) as well as from the hybrid *P. nigra* x *P. thunbergii* (f). The attempts to digest cpDNA of the hybrid combination *P. nigra* x *P. sylvestris* (e) and that of the species *P. cembra* (k) and *P. strobus* (l) have on the other hand finished with a failure.

Owing to the fact that chloroplast DNA was extracted from the mixtures of needles which were collected from several seedlings of individual species, it was not possible to follow the mode of inheritance of cpDNA in the interspecific crosses. However, with respect to the postulated paternal inheritance of cpDNA in conifers (Neale et al., 1989,1991) it seems that Sau 3A phenotype of *P. nigra* x *P. sylvestris* (fig. 3a') and Hind III restriction fragments of *P. banksiana* x *P. contorta* (fig. 4b') reflect to some degree the patroclinial mode of cpDNA transfer in both of the above mentioned crosses.

It is evident that in comparison with the results of karyological and anatomical studies the restriction analysis revealed the existence of variation which lies beyond the resolving power of both the applied cytological approaches. Neither the karyological peculiarities nor the anatomical structure of needles are of help in distiguishing the North American species *P. banksiana* and *P. contorta* as well as their hybrid from the remaining pines studied so far. It fully justifies the opinion of Williams et al. (1990) that restriction lenght polymorphisms data provide a large number of new genetical markers which will be valuable for studies on conifer population genetics and evolution. This statement is of particular importance in relation to the genus *Pinus* the homoploid nature of which makes it difficult to differentiate karyologically between individual species. Also, the vascular bundles have been known since the last century to have the diagnostic value only at the subgenera level (Koehne, 1983). The restriction profiles of cpDNA along with the isoenzymes represent therefore the most promising approaches to the genetical discrimination between pine species. With respect to the jack pine and lodgepole pine, Wheeler and Guries (1987) were able to confirm the discrete taxonomic status of these species using morphological and electrophoretic data. Equally, Dancik and Yeh (1983) have provided evidence for a profound differentiation of allele frequence in seven loci of the lodgepole and jack pines. Govindaraju et al. (1989) had on the other hand identified a large number of cpDNA variants in two populations of *P. banksiana* and *P. contorta* sympatric region near Carson Creek and Windfall in Alberta, among them also those of species specific. The authors suggest that interspecific hybridization could lead to novel polymorphism in the chloroplast genome. The restriction endonucleases applied by us produced the uniform restriction profiles in *P. banksiana, P.contorta* and *P. banksiana* x *P. contorta*. Their Sau 3A restriction phenotypes were similar to those of the remaining species and hybrids under comparison but on the background od this uniformity it was possible to distinguish the above representatives of the section Banksia on the basis of their Taq I, Dra I and Hind III signals consisting of two instead of one (Taq I, Dra I) and of three instead of two fragments of cpDNA (Hind III), respectively. It is obvious therefore that none of the applied enzymes has the capacity to differentiate between the two parental species and that the only way how to proceed further in this respect is to choose a proper restriction nuclease. In relation to the jack pine it is the enzyme Sst I which according to Wagner et al. (1987) efficiently detects polymorphism of its cpDNA. The established status of *P. banksiana, P. contorta* and their hybrid deserves a special attention. According to Szmidt et al. (1988) *P. contorta* and *P. banksiana* differ from other pines with respect to unusual duplication of the psbA gene which codes for D1–protein engaged in the photosystem II. So far, this duplication appears to be unique not only to other pines but to all higher plants.

References

BUCHHOLZ, J. T., 1945: Embryological aspects of hybrid vigour in pines. Science, 102: 135–142.

DANCIK, B.P., YEH, F. C., 1983: Allozyme variability and evolution of lodgepole pine (*Pinus contorta* var. *latifolia*) and jack pine (*P. banksiana*) in Alberta. Can. J. Genet. Cytol., 25: 57–64.

GOVINDARAJU, D. R., DANCIK, B. P., WAGNER, D. B., 1989: Novel chloroplast DNA polymorphism in a sympatric region of two pines. J. Evol. Biol.: 2, 49–59.

JOHNSON, L., C., CRITCHFIELD, W. B., 1978: The production of functional pollen and ovules by pine seedlings less than 1 year old. For. Sci., 24: 467–468.

KENG, H., LITTLE, E.L., 1961: Needle characteristics of hybrid pines. Silvae genetica, 10: 131–146.

KOEHNE, E., 1983. Deutsche Dendrologie. F. Enke, Stuttgart, 157 pp.

KORMUŤÁK, A., LANÁKOVÁ, M., 1988: Biochemistry of reproductive organs and hybridological relationships of selected pine species (*Pinus sp.*) Acta Dendrobiologica. Veda–Bratislava, 119 pp.

KOZUBOV, G. M., MURATOVA, E. N., 1986: Sovremennyje golosemennyje. Nauka, Leningrad, 126 pp.

LIDDICOET, A. R., RICHTER, F. I., 1961: Trees of Eddy Arboretum. Inst. For. Genet., Plecerville, Calif., (Rev. ed.), US For. Serv., Pacific Southwest Forest and Range Exp. Sta., Misc. Paper No. 43: 13–18.

MATERN, B., SIMAK, M., 1968: Statistical problems in karyotype analysis. Hereditas, 59: 280–288.

MIROV, T. N., 1967: The genus *Pinus*. The Ronald Press Company, New York, 602 pp.

Moss, E. H., 1949: Natural pine hybrids in Alberta. Can. Jour. Res., Sect. C., Bot. Sci., 27: 218–229.

Neale, D.B., Marshall, K.A., Sederoff, R. R., 1989: Chloroplast nad mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl., Proc. Natl. Acad. Sci. USA,86: 9347–9349.

Neale, D. B., Marshall, K. A., Harry, D. E., 1991: Inheritance of chloroplast and mitochondrial DNA in incense–cedar (*Calocedrus decurrens*). Can. J. For. Res., 21: 717–720.

Pilger, R., 1926: Genus Pinus. In: A. Engler und K. Prantl, Die natürlichen Pflanzenfamilien. Vol. XIII. Gymnospermae. Wilhem Engelmann, Leipzig, p. 331–342.

Richter, F. I., Duffield, J. W., 1951: Interspecific hybrids in pines. Jour. Hered., 52: 75–80.

Rudolph, T. G., Yeatman, C. W., 1982: Genetics of jack pine. Res. Paper No. Wo–38, Washington, DC: Forest Service, US Department of Agriculture: 64 pp.

Sandbrink, J.M., Vellekoop, P., Van Ham, R., Van Brederode, J., 1989: A method for evolutionary studies on RFLP of chloroplast DNA; applicable to a range of plant species. Biochem. System. Evol., 17: 45–49.

Sax, K., Sax, H.J., 1933: Chromosome number and morphology in the conifers. Jour. Arnold Arboretum, 14: 357–375.

Scotter, G. W., 1974: Distribution of pines in the south Nahani and Flat Rivers region, northwest territories. Can. J. For. Res., 4: 555–557.

Szmidt, A.E., Sigurgeirsson, A., Wang, X. R., Hälgren, J. E., Lindgren, D., 1988: Genetic relationships among *Pinus species* based on chloroplast DNA polymorphism. Proc. of the Frans Kempe symposium „Molecular genetics of forest trees" in Umeå, June 14–16, 1988. Swedish University of Agricultural Sciences, report 8: 33–47.

Tang Shui Liu, 1971: A monograph of the genus *Abies*. Taiwan National University, Taiwan, 608 pp.

Wagner, D. B., Furnier, G. R., Saghai–Maroof, M. A., Williams, S. M.,Dancik, B. P., Allard, R. W., 1987: Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc. Natl. Acad. Sci., USA, 84: 2097–2100.

Wagner, D. B., Govidaraju, D. R., Yeatman, C.W., Pitel, J. A., 1989: Paternal chloroplast DNA inheritance in a diallel cross of jack pine (*Pinus banksiana* Lamb.). Jour. Hered., 80: 483–485.

Wheeler, N.C., Guries, R. P., 1987. A quantitative measure of introgression between lodgepole and jack pines. Can. J. Bot., 65: 1876–1885.

Williams, G. C., Neale, B. D., Greenwood, S.M., 1990: Use of genome mapping in conifer breeding programs. Proc. IUFRO XIX World Congr., Montreal, Canada, 5–11/8/1990, Divis.2: 150.

Yeatman, C.W., Holst, M. J., 1972: Hardiness of jack pine hybrids and provenances in the boreal forest. Forest Chronicle, 48: 7–9.

Received November 18, 1991