MATZIRIS, D. (1993): Variation in Cone Production in a Clonal Seed Orchard of Black Pine, Silvae Genetica **42**: 136–141.

SAS INST. INC. (1988): SAS/STAT User's Guide, Release 6.03 Edition, Cary, NC.

SHELBOURNE, C. J. A. (1969): Tree Breeding Methods. New Zealand Forest Research Institute, Technical Paper No: 55, ODC: 165. 3/7, New Zealand.

SHELBOURNE, C. J. A. (1992): Genetic Gains from Different Kinds of Breeding Population and Seed or Plant Production Population, Paper Presented at the IUFRO Symposium "Intensive Forestry: The Rule of Eucalyptus", Held in Durban, South Africa, in September 1991: 49–65.

TOLUN, A. A., E. VELIOĞLU, B. (NAZLIER) ÇENGEL and Z. KAYA (2000): Genetic Structure of Black Pine (*Pinus nigra* ARNOLD subspecies *pallasiana*) Populations Sampled from the Bolkar Mountains, Silvae Genetica **49**: 113–119.

VELIOĞLU, E., B. ÇENGEL and Z. KAYA (1999): Genetic Variation in Natural Black Pine (*Pinus nigra* Arnold. susp. *pallasiana* (Lamb.) Holmboe.) Populations Sampled from Kazdağlari, Forest Tree Seeds and Tree Breeding Research Directorate, Technical Bulletin No: 1, Ankara.

YALTIRIK, F. and A. EFE (2000): Dendroloji Ders Kitabı, İÜ Yayın No 4265, OF Yayın No 465, İstanbul.

YEATMAN, C. W. (1966): Joint Proceedings of the Second Genetics Workshop of the Society of American Foresters and the Seventh Lake States Forest Tree Improvement Conference; Res. Pap. NC-6. St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station: 28–36.

Chromosomal Mapping of 18S-25S and 5S Ribosomal Genes on 15 Species of Fagaceae From Northern Thailand

By P. CHOKCHAICHAMNANKIT[1),2)], K. ANAMTHAWAT-JÓNSSON[2)] and W. CHULALAKSANANUKUL[1),*)]

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Abstract

Fifteen species of Fagaceae from Chiang Mai province, northern Thailand, were investigated: eight *Castanopsis*, four *Lithocarpus* and three *Quercus* species. The species were generally diploid with the chromosome number 2n = 24, and the basic number x = 12 was confirmed in some species with meiosis. One tree belonging to *Q. lenticellatus* had 2n = 14. Chromosomal mapping of the highly repetitive 18S-25S and 5S ribosomal genes by fluorescence *in situ* hybridisation (FISH) was performed. Most species (from all three genera) showed four 18S-25S rDNA sites (two pairs: one subterminal major and one paracentromeric/intercalary minor loci) and two 5S rDNA sites (one pair: paracentromeric locus). *Quercus kerrii* also had two pairs of 18S-25S rDNA sites, but both were subterminal major loci. Two species, *C. argentea* and *Q. brandisianus*, only had one pair of 18S-25S rDNA sites. Two species, *C. calathiformis* and *L. vestitus*, showed an odd number of (unpaired) sites, and this indicated hybrid origin and/or polyploidy. Polyploid cells were detected in these species. The ribosomal gene maps based on both sequences together were genus-specific. In *Castanopsis*, the 18S-25S and the 5S genes were localized on three different chromosome pairs, and comprised species-specific maps. On the other hand, the ribosomal genes in *Lithocarpus* and *Quercus* were found only on two chromosome pairs, because one of the two 18S-25S rDNA loci was localized on the same chromosome as the 5S rDNA locus. The FISH markers may be used to clarify discrepancies arising from morphological assessments.

Key words: Fagaceae, *Castanopsis*, *Lithocarpus*, *Quercus*, fluorescence *in situ* hybridisation (FISH), ribosomal gene mapping, 18S-25S and 5S rRNA genes.

Introduction

Fagaceae (beech family) includes 7–12 genera and 600–1000 species distributed worldwide, apart from tropical and southern Africa (SOEPADMO, 1972; SCOGGAN, 1978; CHENGJIU et al., 1999). Fagaceae dominates forests in the temperate, seasonally dry regions of the Northern Hemisphere, with a centre of diversity found in tropical South-east Asia (SOEPADMO, 1972; MANOS et al., 2001). In Thailand (FORMAN, 1964; GARDNER et al., 2000; PHENGKLAI et al., 2005), this family comprises four genera: *Castanopsis* (D. Don) Spach. (chestnut, mostly evergreen, 33 species); *Lithocarpus* Blume (stone oak, mostly evergreen, 56 species); *Quercus* L. (oak, mostly deciduous, 29 species); and *Trigonobalanus* Forman (evergreen, one species). SOEPADMO (1972) emphasized that South-east Asia, Indo-China in particular, maintained the greatest assemblage and most primitive forms of *Castanopsis* and *Lithocarpus* as well as *Quercus* (subgenus *Cyclobalanopsis*), compared to other regions of the world. Many of these species, especially in the genus *Castanopsis*, form part of the montane forest distribution east of Himalaya, including eastern Nepal,

[1)] Department of Botany, Faculty of Sciences, Chulalongkorn University, Phayathai Road, Bangkok 10330, Thailand.

[2)] Biology Institute, University of Iceland, Askja – Sturlugata 7, Reykjavik IS-101, Iceland.

[*)] Corresponding author: Department of Botany, Faculty of Sciences, Chulalongkorn University, Phayathai Road, Bangkok 10330, Thailand. Tel: +66 2218 5482; Fax: +66 2218 5482; Email: warawut.c@chula.ac.th.

north-eastern India, Myanmar, Thailand, southern China, western Indo-China and southward to the Malay Peninsula. The species diversity of this family in South-east Asia is high and the taxonomy is known to be difficult.

Based on studies of Fagaceae from the temperate regions, the genetic diversity in this family is substantial, due to morphological variation, ecological adaptation, clinal differentiation, hybridisation, gene flow and introgression. These phenomena have been well documented, especially in economically important tree genera like oaks (*Quercus*), beeches (*Fagus*) and chestnuts (*Castania*) from Europe, North America and East Asia (e.g. MÜLLER-STARCK et al., 1992; DOW and ASHLEY, 1996; DUMOLIN-LAPEGUE et al., 1997; STREIFF et al., 1998; WANG, 2003; KANNO et al., 2004; BELLAROSA et al., 2005; CHUNG et al., 2005; LANG et al., 2006; MAGRI et al., 2006). According to floras (e.g. SCHWARZ, 1964; SCOGGAN, 1978; NIXON, 1997), species within each genus of Fagaceae, wind-pollinated species in particular, are often interfertile and hybrids are therefore common in regions where related species grow together. Such hybridisation, together with gene flow and introgression, makes taxonomic delimitation difficult.

Molecular cytogenetics has proven to be a useful tool in plant taxonomy: for identification of variation due to hybridisation and polyploidy; for studying genome and species relationships; and for identifying ancestral origin of natural hybrids and allopolyploids (ANAMTHAWAT-JÓNSSON et al., 1990; ANAMTHAWAT-JÓNSSON, 2001; BENNETT, 2004; KATO et al., 2005). However, advances in plant cytogenetics have been made essentially from studying large monocot chromosomes. For tree species, especially those with small chromosomes, cytogenetic data is not as extensive. The oak species examined so far have diploid chromosome number 2n = 24, and very similar karyotypes, regardless of the substantial genetic diversity within the genus *Quercus* (e.g. MEHRA et. al., 1972; OHRI and AHUJA, 1990; D'EMERICO et. al., 1995). In general, the family Fagaceae shows a very stable chromosome number with all genera and species having 2n = 24 except *Nothofagus* with 2n = 26 (CARR and MCPHERSON, 1986; OHRI and AHUJA, 1990 and 1991). Striking similarities in terms of genome size among different *Quercus* species, and genome organization based on mapping of the ribosomal repeats (18S-25S and 5S rRNA genes), have also been reported (ZOLDOS et al., 1998; ZOLDOS et al., 1999). Differentiation among some temperate oak species at the chromosome and whole genome level appears to be small, particularly between the two European white oak species, *Q. robur* and *Q. petraea*. The species status has been questioned by some oak specialists, who proposed that the two oaks should be ecophysiological types rather than good species. They are interfertile, as many pairs of oak species are, and the evidence based on biochemical and molecular markers supports the hypothesis that any differences, allelic or genomic, are too small for differentiation at species level (GOMORY et al., 2001; MUIR et al., 2001; ZOLDOS et al., 2001).

The knowledge about genetic differentiation among Fagaceae species in tropical South-east Asia is limited. The species have been identified taxonomically based on morphological characteristics (e.g. PHENGKLAI, 2004), but genetic or evolutionary relationships among taxonomically related species have not been systematically investigated. Most studies on tropical Asiatic species of Fagaceae involve analysis of genetic diversity and phylogeography based on molecular markers (CANNON and MANOS, 2003; BLAKESLEY et al., 2004; CHENG et al., 2005). The objective of the study reported here was therefore to characterize genetic relationships among selected Fagaceae species from northern Thailand, using molecular cytogenetic markers from 18S-25S and 5S ribosomal gene families. The ribosomal gene maps were expected to reveal species differentiation within this region, which could be used to clarify discrepancies arising from morphological assessments.

Materials and Methods

Plant materials

Plant samples were from *Khun Mae Kuong* Forest in Doi Saket district, Chiang Mai province, at approximately 18.87N/99.14E, northern Thailand. All samples were collected in the field during the period 2002 to 2005 by authors of this paper. Six locations, representing three types of habitats, were selected: hill-evergreen forest (code names **PA** & **NK**) and hill-evergreen forest with pine (**BA** & **RD**), both at relatively high elevation (1000–1800 m), and dry-deciduous forest at altitudes below 800 m (**PS** & **HH**). At each location, twenty trees or more, 5–30 m tall and 30–100 m apart, were randomly selected, marked with identification number, and local names recorded together with their position/map within the forest. Leaf buds, and flower buds if available, from individual trees were collected for chromosome isolation. In a few cases it was also possible to obtain root tips from just below the soil surface.

Twigs with fully grown leaves, fruits (acorns) and flowers were collected for taxonomic identification and kept as voucher specimens. Taxonomic identification followed Flora Malesiana (SOEPADMO, 1972) and the report on Fagaceae that was prepared for the Flora of Thailand (PHENGKLAI et al., 2005). The plants were identified to genus based on flowers, acorns, leaves and other vegetative characteristics, but species identification was based primarily on acorns. Acorns of *Castanopsis* are usually covered by spiny cupules, whereas acorns of *Lithocarpus* and *Quercus* are mostly or partly covered by non-spiny cupules (*Fig. 1*). Thirty different Fagaceae species were identified from this collection (unpublished results), but only fifteen species were included in this chromosome study: eight *Castanopsis*, four *Lithocarpus* and three *Quercus* species (*Table 1*).

Chromosome preparation

In the field, samples of leaf buds, flower buds or root tips were placed in iced water (4 °C) for 23–27 h, to arrest metaphases. After this, the samples were fixed in a 3:1 mixture of absolute ethanol and glacial acetic acid, and the samples kept at –20 °C in this fixative until use. Chromosomes from leaf buds were isolated according to ANAMTHAWAT-JÓNSSON (2003), with slight modification in the enzyme digestion step and the hypotonic treatment. Each sample was digested for at least 3–4 h at room

temperature in 100 µl of the enzyme mixture. Ten ml of this enzyme mixture contained 500 units of Cellulase Onozuka R10 (no. 102321, Merck, Germany), 280 units of Pectinase (P4716, Sigma, USA) in a buffer containing 75 mM KCl and 7.5 mM EDTA, pH 4. After digestion, the filtered protoplast suspension was treated with hypotonic solution (1.5 ml of cold 75 mM KCl) for 15 min at room temperature. The protoplasts were cleaned with fresh fixative 3–4 times, before being dropped onto microscopic slides. After staining with the fluorochrome 4, 6-diamidino-2-phenylindole (DAPI), chromosome number was determined under 1000x magnification in an epifluorescence microscope Nikon Eclipse 800. The images were captured with a Nikon DXM 1200F digital camera. The DAPI stained preparations were then used for fluorescence *in situ* hybridisation (FISH) experiments. Chromosomes from flower buds and root tips were isolated using the squash method as in ANAMTHAWAT-JÓNSSON (2001).

Fluorescence in situ hybridisation (FISH)

FISH was performed using a method modified from SCHWARZACHER and HESLOP-HARRISON (2000) and ANAMTHAWAT-JÓNSSON (2001). Two ribosomal DNA probes were used for double-target FISH: (1) Clone pTa71, a 9-kb fragment from wheat, which contained a part of 18S and the entire 5.8S and 25S coding region, together with non-transcribed spacers (GERLACH and BEDBROOK, 1979), was used as an 18S-25S rDNA probe. (2) Clone pTa794, which contained a complete 410-bp *Bam*HI fragment of the 5S rRNA gene and spacer regions from wheat (GERLACH and DYER, 1980), was used as a 5S rDNA probe. The rDNA probes were labelled by standard nick translation, using linearized cloned fragments as templates. Fluorescent labels used in this study were the followings: (1) Red labels from Rhodamine-4-dUTP (Amersham, no longer available), SpectrumRed-dUTP (Vysis, USA), ChromaTide Alexa Fluor 568-5-dUTP (Molecular Probes, USA), and Cy3-dUTP (Amersham, GE Healthcare, Sweden). (2) Green labels from Fluorescein-11-dUTP (Amersham, no longer available), Fluorescein-12-dUTP (Roche Applied Science, Germany), and ChromaTide Alexa Fluor 488-5-dUTP (Molecular Probes). The labelled probes were purified through ProbeQuant G-50 Micro Column (Amersham – GE Healthcare) following the manufacturer's protocol.

Before performing FISH experiments, the chromosome preparations were first treated with fresh fixative for 10 min at room temperature, washed twice with 96% ethanol, and air dried. The preparations were then treated with RNase-A (5 µg/ml) for 1 h at 37°C, proteinase-K (4–10 µg/ml) for 20 min at 37°C, and paraformaldehyde (4%, w/v) for 20 min at room temperature. In FISH experiments, 50 ng each of the 5S and the 18S-25S rDNA probes, which were labelled with different fluorescent colours, was applied to a chromosome preparation, together with 50% formamide, 20% dextran sulphate, 2xSSC and 0.5% SDS. The probe and the slide were denatured together at 89°C for 20 min, in a PTC-100 thermocycler with slide chambers (MJ Research, MA, USA), after which hybridisation was allowed to take place overnight at 37°C. The post-hybridisation washing steps included a stringent wash in 0.1xSSC at 60°C for 15 min. The chromosomes were stained again with DAPI and examined in an epifluorescence microscope using appropriate filters.

Results

The Fagaceae species under study had the expected 2n chromosome number of 24 (*Table 1*, an example shown in *Fig. 2a*). Each species was represented by at

Table 1. – Fagaceae species and individual trees examined. They were from different forest types: hill-evergreen forest (NK & PA), hill-evergreen forest with pine (BA & RD) and dry-deciduous forest (HH & PS). Chromosome number was determined from metaphases from leaf buds, and in some samples also from root-tips (*). FISH mapping of the 18S-25S and 5S ribosomal genes revealed the number of hybridisation sites per cell. Note: a) One pair major and one pair minor 18S-25S rDNA sites.

Species	Tree number	2n chromosome number	Number of 18S-25S rDNA sites	Number of 5S rDNA sites	Number of cells analysed for FISH	Note
Castanopsis acuminatissima (Blume) A.DC.	BA22, NK15	24, 24*(NK15)	4	2	3	a
Castanopsis argentea (Blume) A.DC.	PA14, RD24	24	2	2	3	
Castanopsis armata (Roxb.) Spach.	NK16, PA17	24	4	2	11	a
Castanopsis calathiformis (Skan.) Rehder & Wilson	NK5, PA25	24, 36*(NK5)	3	2	13	
Castanopsis cerabrina (Hickel & A. Camus) Barnett	NK23, PA26	24	4	2	12	a
Castanopsis diversifolia (Kurz) King & Hook.f.	NK1, PA12	24	4	2	8	a
Castanopsis indica (Roxb.) A.DC.	PA4, PA18	24	4	2	6 (+2 meiotic)	a
Castanopsis tribuloides (Sm.) A.DC.	RD4, RD14	24	4	NA	6	a
Lithocarpus ceriferus (Hickel & A. Camus) A. Camus	BA19, RD1	24	4	NA	3	a
Lithocarpus elegans (Blume) Harus ex Soepodmo	BA20, NK24	24	4	2	3	a
Lithocarpus polystachyus (A.DC.) Rehder	BA9, RD7	24	4	2	7	a
Lithocarpus vestitus (Hickel & A. Camus) A. Camus	NK10, PA11	24, 48*(NK10)	6	3	5	
Quercus brandisianus Kurz	RD3, PS9	24	2	2	1	
Quercus kerrii Craib	HH9, PS10	24	4	2	12	
Quercus lenticellatus Barnett	HH18, RD17	24, 14(RD17)	4	2	3	

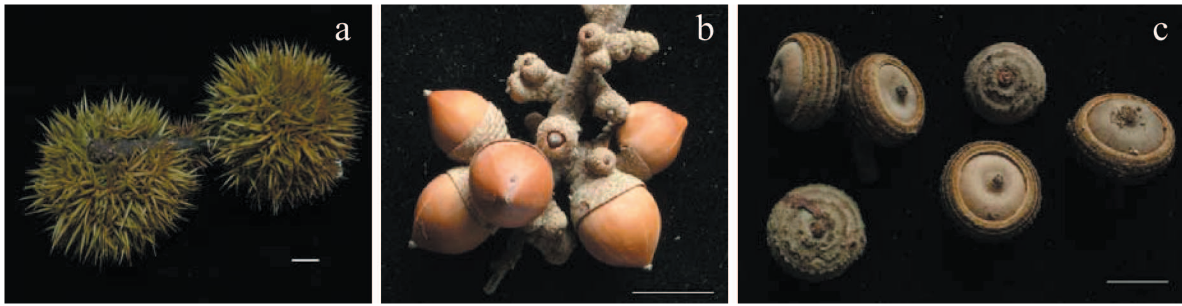

Figure 1. – Typical acorns of Fagaceae species in this study: (a) *Castanopsis argentea*, tree RD24; (b) *Lithocarpus polystachyus*, tree BA9; and (c) *Quercus kerrii*, tree HH9.

least two trees from different locations. The 2n chromosome number for each tree was determined from 10–30 well-spread metaphases isolated from several leaf buds, which were in some cases collected in the field at different times of the year. No variation in chromosome number within sample (tree) was detected using this type of tissue, i.e. leaf buds. Samples of root tips were collected from a few trees in the NK forest and two of these trees had polyploid roots. The tree NK5 (*Castanopsis calathiformis*) had triploid root cells with 36 chromosomes (*Fig. 2p*), while the tree NK10 (*Lithocarpus vestitus*) had tetraploid root cells with 48 chromosomes (*Fig. 2t*). Metaphases from root tips of other trees (for example *Fig. 2d*) showed the normal 2n = 24 as in leaf cells.

One sample belonging to *Quercus lenticellatus* (tree RD17) had the 2n chromosome number of 14 (*Fig. 2w*), whereas another tree from this species (HH18) had the normal 2n = 24. All other Fagaceae species and samples had 24 chromosomes in their diploid cells and therefore the basic number was x = 12. This was supported by a meiotic analysis of two species of *Castanopsis*, i.e. *C. indica* and *C. tribuloides*, whereby 12 bivalents were observed (*Fig. 2g*).

Fluorescence *in situ* hybridisation (FISH) mapping of the 18S-25S and 5S ribosomal genes on chromosomes of 15 Fagaceae species was performed and the results were brought together in *Table 1*. Examples of FISH results are shown in *Fig. 2* and ideograms showing ribosomal gene maps on selected species are presented in *Fig. 3*. Most species had four sites (two pairs) of 18S-25S rRNA genes and two sites (one pair) of 5S rRNA genes (*Fig. 2*). Based on FISH signal intensity, the four 18S-25S rDNA sites in *Castanopsis* and *Lithocarpus* consisted of one pair major and one pair minor sites. The major locus was subterminal (subtelomeric) and the minor locus paracentromeric or intercalary (*Fig. 3*). The major locus coincided with nucleolar organizing region (NOR) at the secondary constriction of satellite (SAT)-chromosomes (results not shown). *Quercus kerrii* also had two pairs of the 18S-25S rDNA sites, but both pairs were subterminal (*Fig. 2v* and *Fig. 3g*). Both were probably major loci, but this required confirmation with silver staining. Two species, *C. argentea* and *Q. brandisianus*, only had one pair of 18S-25S rDNA sites (*Fig. 2i*). The sample with 2n = 14 (*Q. lenticellatus*) showed two pairs of 18S-25S rDNA sites (*Fig. 2x*) and one pair of 5S rDNA sites, as did most of the Fagaceae species under study.

Odd numbers of ribosomal sites, or unpaired sites, were detected in two species, *C. calathiformis* and *L. vestitus* (*Table 1*). This observation was consistent in all cells examined, and in both trees of each species. *Castanopsis calathiformis* had three (unpaired) 18S-25S rDNA sites (*Fig. 2n* and *Fig. 2o*), whereas the 5S rRNA genes seemed to be in one pair of hybridisation sites, like in other species. Root tip chromosome preparations of one tree in this species (tree NK5) revealed a triploid chromosome complement (*Fig. 2p*); this triploid number (36) was seen in all metaphases examined and from several roots. However, leaf chromosome preparations produced variable results. The tree NK5 had a diploid chromosome number of 24 in the leaf cells (*Fig. 2m*), but the other tree belonging to this species (PA25) appeared to have two chromosome numbers, 24 and 28. The aneuploidy could cause reduced fertility of that particular tree. Neither of these trees produced flowers or acorns during the four-year period of field work. These two trees were from different locations, several kilometres apart. *Lithocarpus vestitus* was another species that showed multiple and unpaired ribosomal sites: six 18S-25S rDNA sites with variable FISH signal intensity and three sites of the 5S rRNA genes (*Table 1*, *Fig. 2s*). Chromosome preparations from root tips of the tree NK10 showed a tetraploid number of 2n = 48 (*Fig. 2t*), although the leaf chromosome number was 2n = 24. FISH results of *C. calathiformis* and *L. vestitus* indicated polyploidy in Fagaceae, but more trees need to be examined before a definite conclusion can be made.

Among the Fagaceae species under study, the 18S-25S ribosomal gene maps were variable both in terms of chromosomal location and the chromosomes bearing these genes, while the 5S rDNA maps were more conserved (*Fig. 3*). The 5S rDNA locus was always paracentromeric (see for example in *Fig. 2h* and *Fig. 2v*), and was localized on one pair of large submetacentric chromosomes. In *Castanopsis* (represented by five species in

Figure 2. – Fluorescence *in situ* hybridisation (FISH) of the 18S-25S and 5S rRNA genes to chromosomes of *Castanopsis* (a–p), *Lithocarpus* (q–t) and *Quercus* (u–x). The scale bar represents 5–8 µm. ***Castanopsis acuminatissima* (a–d)**, the most common *Castanopsis* species in this region, has two pairs of (green) 18S-25S rDNA sites and one pair of (red) 5S rDNA sites. The 18S-25S rDNA loci consist of one pair of major and one pair of minor sites (b & c). The major locus is subterminal, as seen in DAPI-stained metaphase (a), and in this cell the major sites lie very close to each other (b). This species, like most species in this study, is diploid with 2n = 24 in both leaf buds (a) and root tips (d). ***Castanopsis indica* (e–h)** also has two (major and minor) pairs of (green) 18S-25S rDNA sites and one pair of (red) 5S rDNA sites. The major 18S-25S locus is also subterminal (e & f), which is the case in all Fagaceae species. The 18S-25S and 5S rDNA genes in *Castanopsis* are on different chromosome pairs, and this is confirmed in meiosis (g & h) whereby all three ribosomal loci are on different bivalents. ***Castanopsis argentea* (i)** only has one pair of (green) 18S-25S rDNA sites, the major locus, and one pair of (red) 5S rDNA sites. ***Castanopsis cerabrina* (j & k)** and ***C. diversifolia* (l)** have two (major and minor) pairs of 18S-25S rDNA sites (red in j & l; green in k) and one pair of 5S rDNA sites (red in k; green in l). ***Castanopsis calathiformis* (m–p)** is mainly diploid in its leaf buds (m) but triploid in root tip cells (p). In the leaf metaphases, three sites of (red) 18S-25S rRNA genes can be seen (n & o), whereas the 5S rDNA locus (green) is on one chromosome pair (o). ***Lithocarpus polystachyus* (q)**, like *Castanopsis*, has two (major and minor) pairs of 18S-25S rDNA sites. ***Lithocarpus vestitus* (r–t)**, unlike all other species, has multiple ribosomal sites, i.e. six (green) 18S-25S rDNA sites with varying FISH signal intensity and three (red) 5S rDNA sites in its diploid leaf bud cells (r & s). Root tip cells are tetraploid (t). ***Quercus kerrii* (u & v)**, the most common *Quercus* species in this region, is diploid with 2n = 24 (u) and has two pairs of the 18S-25S rDNA sites (red, both are subterminal major loci) together with one pair of (green) paracentromeric 5S rDNA sites (v). The 5S rDNA locus is on the same chromosome arm as one of the major 18S-25S loci. ***Quercus lenticellatus* (w & x)**, tree RD17, has 14 chromosomes in all leaf metaphases examined (w). This tree has two pairs of 18S-25S rDNA sites (red) together with one pair of (green) 5S rDNA sites (w). The 5S locus is adjacent to one of the 18S-25S loci (indicated with an arrow).

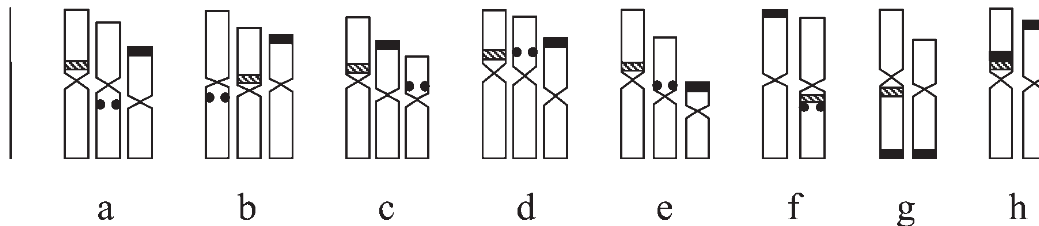

Figure 3. – Idiograms showing localization of 5S (hatched) and 18S-25S (solid black) ribosomal genes on chromosomes of *Castanopsis acuminatissima* (a), *C. armata* (b), *C. cerabrina* (c), *C. diversifolia* (d), *C. indica* (e), *Lithocarpus polystachyus* (f), *Quercus kerrii* (g), and *Q. lenticellatus* (h). Each chromosome represents a homologous pair, and the vertical scale bar represents 5 µm. Chromosome pairs were identified and arranged on the basis of chromosome length and arm ratio (LEVAN et al., 1964). The measurements were made on digital images of chromosomes which were captured with the maximum resolution of 12 megapixels.

Fig. 3), this locus was localized on the short arm, but it was on either the short arm or the long arm in other species. The 18S-25S ribosomal genes were, on the other hand, localized on one to two homologous pairs of metacentric and submetacentric chromosomes of varying sizes (*Fig. 3*). In samples with clear chromosome morphology, the major sites were always subterminal but the minor sites variable depending on species. In *Castanopsis*, the major 18S-25S rDNA locus was localized on medium-sized or small submetacentric chromosome pairs while the paracentromeric minor locus was mainly on large or medium-sized chromosomes. In other genera, the second 18S-25S rDNA locus was intercalary (minor sites in *L. polystachyus* and *Q. lenticellatus*) or subterminal (another major locus in *Q. kerrii*).

When both gene families were examined together, the ribosomal gene maps were also genus-specific (*Fig. 3*). In *Castanopsis*, the 18S-25S and the 5S rRNA genes were localized on different chromosome pairs. In species of *Lithocarpus* and *Quercus* from which accurate ribosomal gene maps were obtained, one locus of the 18S-25S rRNA genes was positioned on the same chromosome as the 5S rDNA locus. The 18S-25S and 5S rDNA loci were localized next to each other on the same chromosome arm near the centromere in *L. polystachyus*. The ribosomal gene maps were more variable in *Quercus*: both gene families either lie close to each other (*Q. lenticellatus*) like in *Lithocarpus*, or far apart (*Q. kerrii*), i.e. near the centromere (the paracentromeric 5S rDNA locus) and subterminally (the 18S-25S rDNA locus). In *Q. brandisianus*, the 5S and the 18S-25S genes were on different chromosomes (FISH not shown), but only one pair of these 18S-25S rDNA sites was detected, the subterminal major locus.

Discussion

Species of Fagaceae in this study (in the genera *Castanopsis*, *Lithocarpus* and *Quercus*) were found to be diploid with the chromosome number 2n = 24 and the basic number x = 12. The exception is that one species, *Q. lenticellatus*, showed the normal 2n chromosome number of 24 in one tree and 14 in another tree. FISH mapping of the ribosomal genes supported the diploid status of these samples, as the chromosomal loci were in homologous pairs. The basic number (x) for this tree could therefore be seven, but this required further investigation. As the tree with 14 chromosomes did not produce any flowers, there was no material for meiotic analysis. Trees with 24 chromosomes had normal meiosis with 12 bivalents. Other studies have shown that species of *Quercus* in Europe and Asia are also diploid with chromosome number 2n = 24 and the basic chromosome number x = 12 (e.g. WANG, 1986; OHRI and AHUJA, 1990; D'EMERICO et al., 1995; ZOLDOS et al., 1999). The same chromosome number was found in two *Castanopsis* species (HUANG et al., 1989), but there are no records on chromosome number of *Lithocarpus*. Cytogenetic data on tropical and subtropical species of Fagaceae has so far been limited, probably due to difficulty in obtaining plant materials for the conventional root-tip chromosome preparation together with difficulty in counting small chromosomes accurately. In the present study, a newly developed protocol for chromosome preparation from leaf buds (ANAMTHAWAT-JÓNSSON, 2003) was used. This type of plant material is always available in the field. In addition, the protoplast dropping technique can produce well-spread metaphases suitable for counting and karyotyping, as well as for molecular cytogenetic experiments.

The basic chromosome number x = 12 found in this study falls within the general range of the basic number mode of most woody plant genera. In the floras of the temperate zone, trees and shrubs have, on average, lower frequencies of polyploidy within a genus than perennial herbs, but they have higher basic numbers (STEBBINS, 1971). Woody plants of tropical regions resemble those of the temperate zone in the rarity of polyploid series within a genus, and their basic numbers are similar, with a mode at x = 11, 12, 13 and 14, which is significantly higher than the mode for temperate herbs, which has basic numbers x = 7, 8 and 9 (DARLINGTON and WYLIE, 1955). STEBBINS (1971) explained that basic numbers of modern woody genera were derived by ancient polyploidy, and that the original basic numbers of angiosperms, both woody and herbaceous, were x = 6 and x = 7. Although tropical floras are still much more poorly known cytogenetically than temperate ones, it is becoming evident that most woody families of angiosperms include basic numbers of x = 7–9, and that the great majority of these genera are tropical, in agreement with the hypothesis that temperate woody groups

have, in general, been derived from tropical ancestors (BRIGGS and WALTERS, 1997). Molecular data has begun to reveal the polyploid nature of many present-day diploid plant species (reviewed in SOLTIS and SOLTIS, 1999; SOLTIS et al., 2003; BENNETT, 2004). Our discovery that one *Quercus* tree has the somatic (leaf) chromosome number of 14 may indicate the existence of the basic chromosome number $x = 7$ among tropical Fagaceae trees. More trees from this region will be examined.

Two species, *C. calathiformis* and *L. vestitus*, showed unpaired rDNA sites in diploid cells. Some cells in these samples were polyploid or aneuploid. Unexpectedly, the polyploid cells were obtained from root-tip meristems, not from leaf tissues. On the other hand, the aneuploidy occurred in leaf buds. Such variation within a plant is difficult to explain. A genetic distance analysis using molecular markers (unpublished results) has indicated that *C. calathiformis* could be an interspecific hybrid involving *Lithocarpus*. This species has non-spiny acorns, which is an unusual character for *Castanopsis* (see typical acorns in *Fig. 1*). *Castanopsis* and *Lithocarpus* co-exist in hill-evergreen forests of this region and both are pollinated by generalist insects, whereas the wind-pollinated *Quercus* is dominant in deciduous forests. Further study will be carried out, both to verify the hybridisation or polyploidy in these samples and to explore the extent of such variation among Fagaceae trees in these forests.

Ribosomal gene maps of selected Fagaceae species from northern Thailand were found to be highly variable, both in terms of location of the 5S and 18S-25S rRNA genes and the chromosomes bearing these genes. A typical *Castanopsis* map consisted of three pairs of chromosomes bearing, separately, one paracentromeric 5S rDNA locus and two 18S-25S rDNA loci, one of which was a subterminal major locus and the other a paracentromeric or intercalary minor locus. In the few species of *Lithocarpus* and *Quercus* studied, the location of these genes varied considerably, and was completely different from the *Castanopsis* maps in that all of the loci were localized on only two pairs of chromosomes. The 5S rDNA locus was on the same chromosome arm as the minor or the second major 18S-25S rDNA locus. The ribosomal gene map of several European *Quercus* species was shown to be highly conserved (ZOLDOS et al., 1999), but the only similarity to our Fagaceae maps seems to be in the 5S rDNA gene. Genetic diversity within the family Fagaceae is undoubtedly large, especially within *Quercus*, and this is often reflected in the complex taxonomic classification. For example, *Quercus* spp from Europe belong to the subgenus *Quercus* while the subgenus *Cyclobalanopsis* comprises species from South-east Asia (MANOS et al., 2001). Present distribution of this genus is also extensive, covering both temperate and tropical regions, whereas *Castanopsis* and *Lithocarpus* are mainly found in the tropical and subtropical areas (SOEPADMO, 1972).

Ribosomal gene maps, constructed using both 18S-25S and 5S rRNA genes, could be used for tracing species origin, analysing species relationships and resolving taxonomic discrepancies. Ribosomal gene mapping has been shown to be useful, for example in differentiating between closely related tree species such as those found within *Picea* and *Pinus* genera (BROWN and CARLSON, 1997; SILJAK-YAKOVLEV et al., 2002; LIU et al., 2003). These species have large chromosomes, which are convenient for FISH karyotyping. Although broadleaf tree species tend to have small and compact chromosomes, the FISH application is becoming more visible, as shown in the present study and in others (e.g. ANAMTHAWAT-JÓNSSON and HESLOP-HARRISON, 1995; ZOLDOS et al., 1999; CORREDOR et al., 2004). A number of taxonomic questions arising from the present study will be examined further. For example, *Castanopsis fissa* appears to have a ribosomal gene map similar to that of *Lithocarpus*. Other evidence, molecular and morphological, also indicates that *C. fissa* is more closely related to *Lithocarpus* than to other species of *Castanopsis* (MANOS et al., 2001). Molecular cytogenetic markers offer distinct advantages as they can screen cryptic, cytotypic and/or ploidy variation that influence genetic architecture and reproductive potential of a population (LAVANIA, 2002). Some cytotypic variation has been detected in Fagaceae for the first time in the present study, and it is interesting to carry out further research on the extent and impact of this variation. It is important to know the structure and behaviour of chromosomes and genomes in order to elucidate the evolutionary potential of a population, which can help complement conservation plans.

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References

ANAMTHAWAT-JÓNSSON, K. (2001): Molecular cytogenetics of introgressive hybridization in plants. Methods in Cell Science **23**: 139–148.

ANAMTHAWAT-JÓNSSON, K. (2003): Preparation of chromosomes from plant leaf meristems for karyotype analysis and *in situ* hybridisation. Methods in Cell Science **25**: 91–95.

ANAMTHAWAT-JÓNSSON and J. S. HESLOP-HARRISON (1995): Molecular cytogenetics of Icelandic birch species: physical mapping by in situ hybridisation and rDNA polymorphism. Canadian Journal of Forest Research **25**: 101–108.

ANAMTHAWAT-JÓNSSON, K., T. SCHWARZACHER, A. R. LEITCH, M. D. BENNETT and J. S. HESLOP-HARRISON (1990): Discrimination between closely related *Triticeae* species using genomic DNA as a probe. Theoretical and Applied Genetics **79**: 721–728.

BELLAROSA, R., M. C. SIMEONE, A. PAPINI and B. SCHIRONE (2005): Utility of ITS sequence data for phylogenetic reconstruction of Italian *Quercus* spp. Molecular Phylogenetics and Evolution **34**: 355–370.

BENNETT, M. D. (2004): Perspectives on polyploidy in plants – ancient and neo. Biological Journal of the Linnean Society **82**: 411–423.

BLAKESLEY, D., G. PAKKAD, C. JAMES, F. TORRE and S. ELLIOTT (2004): Genetic diversity of *Castanopsis acuminatissima* (Bl.) A.DC. in northern Thailand and the selection of seed trees for forest restoration. New Forests **27**: 89–100.

BRIGGS, D. and S. M. WALTERS (1997): Plant Variation and Evolution, 3rd edition. Cambridge University Press, Cambridge.

BROWN, G. R. and J. E. CARLSON (1997): Molecular cytogenetics of the genes encoding 18S-5.8S-26S rRNA and 5S rRNA in two species of spruce (*Picea*). Theoretical and Applied Genetics **95**: 1–9.

CANON, C. H. and P. S. MANOS (2003): Phylogeography of the Southeast Asian stone oaks (*Lithocarpus*). Journal of Biogeography **30**: 211–226.

CARR, G. D. and C. MCPHERSON (1986): Chromosome number of New Caledonian plants. Annals of the Missouri Botanical Garden **73**: 486–489.

CHENG, Y.-P., S.-Y. HWANG and T.-P. LIN (2005): Potential refugia in Taiwan revealed by the phylogeographical study of *Castanopsis carlesii* Hayata (Fagaceae). Molecular Ecology **14**: 2075–2085.

CHENGJIU, H., Z. YONGTIAN and B. BARTHOLOMEW (1999): Fagaceae. Flora of China **4**: 314–400.

CHUNG, M. Y., K.-J. KIM, J.-H. PAK, C.-W. PARK, B.-Y. SUN, E. R. MYERS and M. G. CHUNG (2005): Inferring establishment histories in populations of *Quercus dentate* (Fagaceae) from the analysis of spatial genetic structure. Plant Systematics and Evolution **250**: 231–242.

CORREDOR, E., M. ROMAN, E. GARCIA, E. PERERA, P. ARUS and T. NARANJO (2004): Physical mapping of rDNA genes establishes the karyotype of almond. Annals of Applied Biology **144**: 219–222.

D'EMERICO, S., P. BIANCO, P. MEDAGLI and B. SCHIRONE (1995): Karyotype analysis in *Quercus* spp. (Fagaceae). Silvae Genetica **44**: 66–70.

DARLINGTON, C. D. and A. P. WYLIE (1955): Chromosome Atlas of Flowering Plants. George Allen and Unwin, London.

DOW, B. D. and M. V. ASHLEY (1996): Microsatellite analysis of seed dispersal and parentage of saplings in bur oak, *Quercus macrocarpa*. Molecular Ecology **5**: 615–627.

DUMOLIN-LAPEGUE, S., B. DEMESURE, S. FINESCHI, V. LE CORRE and R. J. PETIT (1997): Phylogeographic structure of white oaks throughout the European continent. Genetics **146**: 1475–1487.

FORMAN, L. L. (1964): *Trigonobalanus*, a new genus of Fagaceae. Kew Bulletins **17**: 381–396.

GARDNER, S., P. SIDISUMTHORN and V. ANUSARNSUNTHORN (2000): A Field Guide to Forest Trees of Northern Thailand. Kobfai Publishing Project, Bangkok.

GERLACH, W. L. and J. R. BEDBROOK (1979): Cloning and characterization of ribosomal genes from wheat and barley. Nucleic Acids Research **7**: 1869–1885.

GERLACH, W. L. and T. A. DYER TA (1980): Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Research **8**: 4851–4865.

GOMORY, D., I. YAKOVLEV, P. ZHELEV, J. JEDINAKOVA and L. PAULE (2001): Genetic differentiation of oak populations within the *Quercus robur*/*Quercus petraea* complex in Central and Eastern Europe. Heredity **86**: 557–563.

HUANG, S.-F., Z.-F. ZHAO, Z.-Y. CHEN, S.-J. CHEN and X.-X. HUANG (1989): Chromosome counts on one hundred species and intraspecific taxa. Acta Botanica Austro Sinica **5**: 161–176.

KANNO, M., J. YOKOYAMA, Y. SUYAMA, M. OHYAMA, T. ITOH and M. SUZUKI (2004): Geographical distribution of two haplotypes of chloroplast DNA in four oak species (*Quercus*) in Japan. Journal of Plant Research **117**: 311–317.

KATO, A., J. M. VEGA, F. HAN, J. C. LAMB and J. A. BIRCHLER (2005): Advances in plant chromosome identification and cytogenetic techniques. Current Opinion in Plant Biology **8**: 148–154.

LANG, P., F. DANE and T. L. KUBISIAK (2006): Phylogeny of *Castanea* (Fagaceae) based on chloroplast *trn*T-L-F sequence data. Tree Genetics and Genomes **2**: 132–139.

LAVANIA, U. C. (2002): Chromosome diversity in population: Defining conservation units and their micro-identification through genomic in situ painting. Current Science **83**: 124–127.

LEVAN, A., K. FREDGA and A. A. SANDBERG (1964): Nomencluture for centromeric position of chromosomes. Hereditas **52**: 201–220.

LIU, Z.-L., D. ZHANG, D.-Y. HONG and X.-R. WANG (2003): Chromosomal localization of 5S and 18S-5.8S-25S ribosomal DNA sites in five Asian pines using fluorescence in situ hybridization. Theoretical and Applied Genetics **106**: 198–204.

MAGRI, D., G. G. VENDRAMIN, B. COMPS, I. DUPANLOUP, T. GEBUREK, D. GÖMÖRY, M. LATAŁOWA, T. LITT, L. PAULE, J. M. ROURE, I. TANTAU, W. O. VAN DER KNAAP, R. J. PETIT and J.-L. DE BEAULIEU (2006): A new scenario for the Quaternary history of European beech populations: palaeobotanical evidence and genetic consequences. New Phytologist **171**: 199–221.

MANOS, P. S., Z. ZHOU and C. H. CANNON (2001): Systematics of Fagaceae: phylogenetic tests of reproductive trait evolution. Journal of Plant Science **162**: 1361–1379.

MEHRA, P. N., A. S. HANS and T. S. SAREEN (1972): Cytomorphology of Himalayan Fagaceae. Silvae Genetica **21**: 102–109.

MUIR, G., C. C. FLEMING and C. SCHLÖTTERER (2001): Three divergent rDNA clusters predate the species divergence in *Quercus petraea* (Matt.) Liebl. and *Quercus robur* L. Molecular Biology and Ecology **18**: 112–119.

MÜLLER-STARCK, G., PH. BARADAT and F. BERGMANN (1992): Genetic variation within European tree species. New Forests **6**: 23–47.

NIXON, K. C. (1997): Fagaceae. pp 436–506 in Flora of North America North of Mexico. Vol 3. Magnoliophyta: Magnoliidae and Hamamelidae, edited by Flora of North America Editorial Committee, Oxford University Press, New York.

OHRI, D. and M. R. AHUJA (1990): Giemsa C-banded karyotype in *Quercus* L. (oak). Silvae Genetica **39**: 5–6.

OHRI, D. and M. R. AHUJA (1991): Giemsa C-banding in *Fagus sylvatica* L., *Betula pendula* Roth and *Populus tremula* L. Silvae Genetica **40**: 72–75.

PHENGKLAI, C. (2004): Three new species and a new variety of Fagaceae from Thailand. Thai Forest Bulletin (Botany) **32**: 115–122.

PHENGKLAI, C., T. BOONTHAVIKOON, T. WONGPRASERT, P. PHONSENA and T. JONGANURAK (2005): The complete final report on Fagaceae in Thailand under the auspices of The Biodiversity Research and Training Program (BRT). Project number BRT R-145010.

SCHWARZ, O. (1964): *Quercus* L., pp. 61–64 in Flora Europaea, vol. 1, edited by T. G. TUTIN, V. H. HEYWOOD, N. A. BURGES, D. H. VALENTINE, S. M. WALTERS and D. A. WEBB, Cambridge University Press, Cambridge.

SCHWARZACHER, T. and P. HESLOP-HARRISON (2000): Practical *in situ* Hybridization. Bios Scientific Publishers, Oxford.

SCOGGAN, S. J. (1978): The Flora of Canada, Part 3, Fagaceae. National Museum of Natural Sciences, Canada.

SILJAK-YAKOVLEV, S., M. CERBAH, J. COULAUD, V. STOIAN, S. C. BROWN, V. ZOLDOS, S. JELENIC and D. PAPES (2002): Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*. Theoretical and Applied Genetics **104**: 505–512.

SOEPADMO, E. (1972): Fagaceae, pp. 265–388 in Flora Malesiana, series I, volume 7, edited by C. G. G. J. VAN STEENIS, Noordhoff International Publishing, Leyden.

SOLTIS, D. E. and P. S. SOLTIS (1999): Polyploidy: recurrent formation and genome evolution. Tree **14**: 348–352.

SOLTIS, D. E., P. S. SOLTIS and J. A. TATE (2003): Advances in the study of polyploidy since Plant speciation. New Phytologist **161**: 173–191.

STEBBINS, G. L. (1971): Chromosome Evolution in Higher Plants. Addison-Wesley Publishing Company, Massachusetts.

STREIFF, R., T. LABBE, R. BACILIERI, H. STEINKELLNER, J. GLOSSL and A. KREMER (1998): Within-population genetic structure in *Quercus robur* L. and *Quercus petraea* (Matt.) Leibl. assessed with isozymes and microsatellites. Molecular Ecology **7**: 317–328.

WANG, K.-S. (2003): Genetic diversity and temporal genetic structure in European beech (*Fagus sylvatica* L.). Silvae Genetica **52**: 100–106.

WANG, L.-M. (1986): A taxonomic study of the deciduous oaks in China by means of cluster and karyotype analysis. Bulletin of Botanical Research **6**: 55–69.

ZOLDOS, V., D. PAPES, S. C. BROWN, O. PANAUD and S. SILJAK-YAKOVLEV (1998): Genome size and base composition of seven *Quercus* species: inter- and intra-population variation. Genome **41**: 162–168.

ZOLDOS, V., D. PAPES, M. CERBAH, O. PANAUD, V. BESENDORFER and S. SILJAK-YAKOVLEV (1999): Molecular-cytogenetic studies of ribosomal genes and heterochromatin reveal conserved genome organization among 11 *Quercus* species. Theoretical and Applied Genetics **99**: 969–977.

ZOLDOS, V., S. SILJAK-YAKOVLEV, D. PAPES, A. SARR and O. PANAUD (2001): Representational difference analysis reveals genomic differences between *Q. robur* and *Q. suber*: implications for the study of genome evolution in the genus *Quercus*. Molecular Genetics and Genomics **265**: 234–241.

Evaluation of Early Rooting Traits of Eastern Cottonwood That Are Important For Selection Tests

By B. KOVACEVIC[1),*)], V. GUZINA[2)], M. KRALJEVIC-BALALIC[3)], M. IVANOVIC[4)] and E. NIKOLIĆ-ĐORIĆ[5)]

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Abstract

Vegetative propagation of superior genotypes via stem cuttings depends on their development of strong vigorous root system. Fourteen characters of cutting rooting were examined in multiannual tests with 12 genotypes of eastern cottonwood (*Populus deltoides* BARTR. EX MARSH) in course of evaluation of their utilization in selection tests. Variability and relationship among examined characters, and cutting survival rate were analyzed according to contribution of expected variances to the total variance and results of principal component analysis, stepwise regression analysis and path analysis. Along with total number and length of first-order roots, the characters that are regularly used in the assessment of rooting potential, our results signify dynamic shoot growth and uniform arrangement of roots on cutting at the beginning of growing period. The best results were obtained for shoot height at the second half of May. A rapid and non-destructive way of shoot characters' measurement allows testing of larger material and prevents losses in propagation material of interesting genotypes. Alone or together with total root number and length these alternative characters could be used for the improvement of selection tests and procedures for cultivar technology design.

Key words: Populus deltoides, cutting rooting characters, cutting survival rate, multivariate analysis.

1) PhD, Institute for Lowland Forestry and Environment, Antona Cehova 13, 21000 Novi Sad, Serbia.

2) PhD (retired), Institute for Lowland Forestry and Environment, Antona Cehova 13, 21000 Novi Sad, Serbia.

3) PhD, Faculty of Agriculture, Dositeja Obradovica 8, 21000 Novi Sad, Serbia.

4) PhD, Institute for Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia.

5) MSc, Faculty of Agriculture, Dositeja Obradovica 8, 21000 Novi Sad, Serbia.

*) Communicating author: BRANISLAV KOVACEVIC, Tel./Fax +381-21-540385. E-mail: branek@uns.ns.ac.yu.

Introduction

The hardwood cuttings of black poplars (section *Aigeiros* DUBY) are characterized by good rooting