Searching for DNA Markers Linked to Leaf Colour in Copper Beech, *Fagus sylvatica* L. var. atropunicea[1])

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Summary

Purple leaf colour in *Fagus sylvatica* is a rare spontaneous mutation. The trait is presumably inherited by a completely dominant gene. Accordingly, open-pollinated offspring of a heterozygous copper beech tree should segregate for leaf colour. We have used such offspring to screen for DNA markers that are possibly linked to the locus controlling this trait. Green and purple seedlings were used in preparing bulk DNA samples for the 2 segregating types. First, these samples were screened for differences in patterns of randomly amplified DNA, employing 640 different 2-primer combinations of 10mers and 'repetitive' primers. Of these combinations, 299 resulted in patterns of well-separated bands. While there were minor differences between the bulks in a number of instances, 10 primer combinations showed up differences in banding patterns which were investigated further. For this second step, 24 green and 24 purple seedlings were analysed individually. Five markers produced were useful for mapping. One marker band was unequally distributed among green and purple seedlings, supporting weak linkage to the 'green' allele at 35 cM. As 3 more markers formed a separate linkage group, this example shows that single-tree genetic linkage maps can be constructed from open-pollinated offspring by using markers absent from the pollen pool.

Key words: Fagus sylvatica var. *atropunicea,* bulked segregant analysis, genetic markers, leaf colour, random DNA amplification, repeat primers.

FDC: 165.3; 164.5; 165.51; 176.1 *Fagus sylvatica* var. *atropunicea.*

Introduction

Copper beech (*Fagus sylvatica* L. var. *atropunicea*) is a purple leaf mutant of European beech. It is planted as an ornamental tree in many parks and gardens in the temperate climate zones. The colour pigment that accumulates in the leaves has been identified as an anthocyanin (ROBINSON and SMITH, 1955). Copper beech trees have been rarely, but repeatedly discovered in European beech forests (KRÜSSMANN, 1965, *loc. cit.* p. 130). Offspring of dark coloured trees is not necessarily true-to-type (KRÜSSMANN, 1949, *loc. cit.* p. 198; and 1965). Studies with controlled crosses and open-pollinated families support the hypothesis that single dominant genes control presence/absence of pigmentation in individual trees. JAHN (1934) found a segregation ratio close to 1:1 for green and purple seedlings from an open-pollinated copper beech tree. However, more than 10% of the copper seedlings were faintly coloured or variegated. BLINKENBERG *et al.* (1958) included copper beech trees in their controlled crosses. Progeny from seed and pollen parents in crosses, as well as open-pollinated offspring segregated at an approximate 1:1 ratio (39% to 57% purple seedlings in single-tree progeny; pooled ratio 959 green : 970 purple). Similar purple leaf colour has been found in other genera (HATTEMER, 1991); among them, birch (*Betula pendula* ROTH.), filbert (*Corylus avellana* L.), and peach (*Prunus persica* [L.] BATSCH) have been studied genetically. While in birch a single dominant gene was suggested (HATTEMER *et al.,* 1990, *loc. cit.* p. 45), THOMPSON (1985) concluded that more than one gene influences this trait in filbert. CHAPARRO *et al.* (1994) were able to map a gene for red leaf colour in peach, which exhibited epistatic interaction with a gene for white flower (CHAPARRO *et al.,* 1995).

The objective of this study was to identify DNA Markers linked to the locus controlling leaf pigmentation in open-pollinated offspring of a copper beech specimen by bulked segregant analysis (MICHELMORE *et al.,* 1991). This technique uses phenotypic pools of segregating progeny, usually from controlled crosses (e.g., OH *et al.,* 1994; MAISONNEUVE *et al.,* 1994; KESSELI *et al.,* 1993) or near-isogenic lines (e.g., BARUA *et al.,* 1993). In tree species, this approach was successfully taken up by HORMAZA *et al.* (1994) for a sex-determinating gene in *Pistacia vera* L., by CHAPARRO *et al.* (1994) in *Prunus persica* for leaf pigmentation, and by DEVEY *et al.* (1995) for blister rust resistance in sugar pine (*Pinus lambertiana* DOUGL.).

Materials and Methods

Plant material

The arboretum of the Research Station 'Mariabrunn' has 2 copper beech specimens at a distance of approximately 50 m from each other. There are 2 other green-leaved beech trees further away in the arboretum; more beech trees are located in gardens and beech forests in the surroundings. Beech nuts were collected under the larger copper beech tree in autumn and sown; 1009 seedlings were available by summer 1995.

Leaf colour in copper beech varies with exposure to sunlight, with a gradual fading of purple colour in shaded leaves. However, the veins of the leaf blades allow of a clear assessment. Colour intensity is also dependent on leaf age and changes during summer. We visually assessed the seedlings in June, when colour expression was easily scored.

Sampling strategy

Our strategy of searching for DNA markers linked to the leaf colour trait consisted of preparing two pools of DNA (MICHELMORE *et al.,* 1991), 1 from 50 green seedlings, and 1 from 50 purple seedlings. Pooled DNA was amplified in polymerase chain reactions (PCR) using combinations of random 10mer primers (WELSH and MCCLELLAND, 1990; CAETANO-ANOLLÉS *et al.,* 1991; LIVAK *et al.,* 1991) with simple sequence repeat (SSR) primers (ZIETKIEWICZ *et al.,* 1994 GUPTA *et al.,* 1994) to produce genetic markers that differ between the 2 pools. In a second step, individual seedlings were analysed with primer combinations that showed up differences in screening of pooled DNA. Here, 24 seedlings of each leaf colour, different from those in the DNA pools, were used.

[1]) Dedicated to Prof. Dr. H. MELCHIOR on occasion of his 70th birthday.

DNA preparation

Standard molecular biology techniques were employed (SAMBROOK *et al.,* 1989). The pools of 'green' and 'purple' DNA were prepared from young leaves (0.10 g/plant to 0.15 g/plant). Fifty green and 50 purple seedlings were sampled in June. The leaves were pooled (green and purple separately), homogenized in liquid nitrogen with a porcelain pestle and mortar, and extracted with 20 ml extraction buffer and 15 ml chloroform for 20 minutes (min.). Extraction buffer (modified from SAMBROOK *et al.,* 1989; and HEINZE, 1994) contained: 100 mM Tris pH 8.0, 500 mM NaCl, 20 mM EDTA, 10 mg/ml SDS, 10 mg/ml PEG 6000, 20 µg/ml beta-mercaptoethanol, 4 mg/ml DEDTCA, and 250 µl/ml Proteinase K (Boehringer, Mannheim, Germany). The aqueous phase was removed for further DNA purification; the remaining cell homogenisate was extracted with fresh buffer and chloroform as above. The pooled aqueous phase was treated with an equal volume of phenol, then chloroform. From this solution, DNA was precipitated by adding an equal volume of 20% (w/v) PEG 6000 – 1.4 M NaCl and incubating for 1 hour (h) at 4 °C, redissolved in PCR-TE (10 mM Tris pH 8.0, 0.1 mM EDTA), treated with RNAase A (2 µl of 10 mg/ml RNAase A [Boehringer, Mannheim, Germany] per 100 µl of PCR-TE) for 45 min. at 37 °C or overnight at 4 °C, extracted with phenol:chloroform (1:1), precipitated in 0.6 volumes isopropanol – 0.3 M sodium acetate, and finally dissolved in PCR-TE.

DNA from individual seedlings was prepared from 3 tiny 'leaf discs' (DERAGON and LANDRY, 1992) per seedling, cut out from freshly harvested young, soft leaves with the cap of 1.5 ml microfuge tubes. This material was homogenised in microfuge tubes after adding 100 µl of extraction buffer (see above) and little glass powder. A plastic pestle mounted onto an electric stirrer was used for homogenising. More extraction buffer (400 µl) was added after a few min. at room temperature (RT), together with 500 µl chloroform. These components were mixed gently by hand. After 20 min. at RT, and centrifugation to separate phases, the aqueous phase was mixed with 400 µl phenol and 400 µl chloroform. After centrifugation, the new aqueous phase was mixed with 500 µl of 20% PEG – 1.4 M NaCl, incubated at –20 °C for 15 min., then at 4 °C for 45 min. DNA was pelleted (10 min. at full speed in microfuge), washed in 50% ethanol and 70% ethanol, dried, and dissolved in 100 µl of PCR-TE containing 2 µl of RNAase A solution (see above), incubating for either 45 min. at 37 °C or overnight at 4 °C.

DNA concentration was measured by Hoechst 33258 fluorometry (SAMBROOK *et al.,* 1989) and adjusted to 1 ng/µl to 12 ng/µl with PCR-TE.

Amplification of random genetic markers

Primers were obtained from Clontech, Palo Alto, CA, USA (random 10mers 'CT 1–11') and NAPS unit, University of British Columbia, Vancouver, BC, Canada (set # 9, consisting of mainly 16mers and 17mers with simple sequence repeat motifs such as 2-base, 3-base and 4-base repeats interrupted at one end, see ZIETKIEWICZ *et al.,* 1994; GUPTA *et al.,* 1994; primer sequence lists are available from Dr. JOHN HOBBS, NAPS unit, UBC; e-mail address: hobbs@unixg.ubc.ca, and from the authors). Amplification reactions contained 200 µm of each dNTP, 2 primers (1 random 10mer and 1 repeat primer) at 200 nM each, dNTPs at 200 µm each, RNAase A at 0.1 µg/µl (HEINZE, 1994), 0.5 units of DynaZyme DNA Polymerase (Finnzymes Oy, Espoo, Finland) and 1x supplier's buffer, in a reaction volume of 11 µl. For bulk DNA samples, 5 ng to 10 ng DNA was used per reaction, and for individual seedlings, 0.5 ng to 2 ng DNA, respectively. The cycling program in an MJ Research (Watertown, MA, USA) PTC-100 thermocycler (with heated lid installed) consisted of: 1 min. at 94 °C, 45 cycles of 94 °C – 30 seconds (sec.), 36 °C – 30 sec., and 72 °C – 1 min. 30 sec., a final 72 °C – 5 min., and 4 °C until recovery. DNA fragments were separated in 1.5% or 2% agarose gels. Photographs were taken over UV light, and negatives were scored for band presence/absence.

Data analysis

For the primer screening using bulk DNA, banding patterns were compared visually. Most reactions were carried out in duplicate in this phase. Two separate amplification reactions were done for each DNA bulk/ primer set combination. These were compared on the same gel side-by-side. In the analysis of individual seedlings, presence/absence of polymorphic bands was recorded, and their frequencies were compared between 24 green and 24 purple seedlings. All uncertain scorings were treated as missing data.

Tests for linkage comprised χ^2 and log likelihood (LANDER *et al.,* 1987; LINCOLN *et al.,* 1992; MAPMAKER v. 3.0b) analyses. Map distances were calculated using KOSAMBI's (1944) function.

Results

Leaf colour assessment in seedlings

We scored 509 seedlings as green and 500 as purple, which fits a 1:1 ratio of segregation. Among seedlings scored as purple, there was a seemingly continuous variation in colour intensity, ranging from deep purple (nearly black) to greenish with purple veins in shaded leaves. This variation was more striking later in summer, when purple colouration was fading in a significant number of seedlings, and at the same time newly emerging leaves showed yellowish or orange colours. A few seedlings showed chimeric colour expression, with sectors of one or more leave blades reverting from purple to green (these seedlings were scored as purple for the segregation analysis and not used for DNA preparation).

Screening of primer combinations

We screened the 2 DNA pools with 640 primer combinations, of which 299 produced at least one discernible band. The total number of bands produced by all these combinations approximated 1000. Some primers, when used in different combinations, gave characteristic patterns largely independent of the second primer employed. For instance, primer '807' produced only 1 band of identical molecular weight in most combinations; in the reaction products of nearly every primer combination including 'CT 1', a characteristic band of > 3000 basepairs (bp) was present. Other primers (e.g., 830, 860, or 869) very often produced 'smears' of unresolved bands regardless of the second primer present in the reaction. We therefore pre-selected primers on the basis of their performance in the first few combinations tested.

Minor differences in the banding patterns of the green and purple pools were detected with several primer combinations. We chose to further investigate 10 of those combinations by analyzing individual seedlings.

Differences in marker frequencies among individual green and purple seedlings

All segregating bands produced in the individual seedling DNAs were recorded. This included bands that had not shown differences between the bulks. Among all those bands, 5 were useful for linkage analysis (present in copper beech parent tree, segregating 1:1). Among them, a band produced by primers 'CT 6' (sequence, 5'-CTCAGCCCAG-3') and '882' (5'-

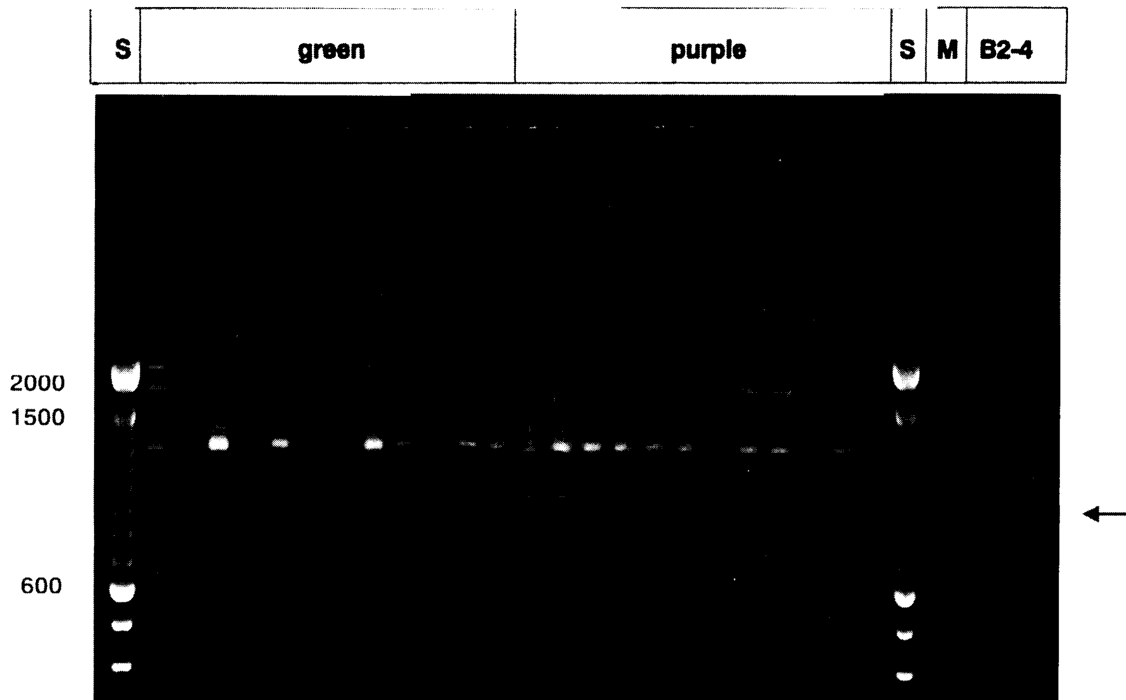

Figure 1. – Segregation of a genetic marker (arrow on the right) produced by primers 'CT 6' and '822' in green and purple seedlings. M, mother tree; B2–4, beech trees in the arboretum; S, size standards (100 bp ladder, fragment sizes given in bp on the left). Missing lanes are failed amplification reactions.

[A/C/G][C/G/T][A/C/G]ATATATATATATAT-3') was weekly linked to the 'green' allele at the leaf colour locus. The approximate size of the band was 900 bp (*Figure 1*). Numbers of green and red seedlings showing band presence or absence, respectively, are given in *table 1*. The linkage of the marker was supported by χ^2 (significant at 1% level) and log likelihood (LOD score 1.50, distance 35 cm) tests.

Other markers that were polymorphic between the pools turned out to be either weak and not clearly scorable in most seedlings, absent from the mother tree, or deviating from the 1:1 segregation requirement.

Linkage was supported between three more markers among the five, forming a linkage group: one pair was linked at LOD score 7.42 cM and 9.2 cM, the other pair was linked at LOD score 2.50, 27.5 cM. The group covers 44.8 cM as determined by 3-point linkage analysis ('map' command in MAPMAKER).

Table 1. – Co-segregation analysis of purple leaf trait and genetic marker produced by primers 'CT 6' and '882'. χ^2 values: marker locus, 2.814; leaf colour locus, 0.023; linkage, 6.721 ($\chi^2_{1,\,0.01} = 6.63$).

	marker	
	present	absent
green	18	4
purple	9	12

Discussion

Segregation of leaf colour trait

Colour assessment in our open-pollinated copper beech offspring suggests a segregation ratio of 1:1. Similar observations were obtained by Blinkenberg *et al.* (1958) and Jahn (1934). In our experiment the number of progeny from a single tree was very high. The data strongly suggests the involvement of a single gene with a dominant mode of inheritance in our material. A second copper beech tree is present in the arboretum which may have sired an unknown proportion of the seedlings. However, we assume that at least these two copper beech specimens are closely related genetically. Comparing banding patterns of randomly amplified DNA from these two trees, the level of polymorphism was approximately the same as among the half-sib seedlings. In the case of a close genetic relationship, significant (consanguineous) mating between the two copper beeches is unlikely because European beech usually exhibits a very low self-fertility (Nielsen and Schaffalitzky de Muckadell, 1954).

It is remarkable that just as the trees investigated by Blinkenberg *et al.* (1958; 5 trees) and Jahn (1934; 1 tree), our copper beech specimen is heterozygous for a locus controlling the trait, too. It would be interesting to know whether the same genes are involved in purple leaf pigmentation in different copper beech trees, or if different genes involved in the anthocyanin pathway are affected in different trees. Two purple birch trees investigated by Hattemer *et al.* (1990) were found to possess a 'purple gene' in the heterozygous condition. In contrast, Chaparro *et al.* (1994) observed homozygotes for 'red leaf' in peach.

Interaction with the environment, or with other genes, may influence colour intensity in our seedlings. Changeable colouration types in copper beech offspring are also reported by Krüssmann (1949). Chaparro *et al.* (1995) observed epistatic inter-

action between 2 genes controlling pigmentation in leaves and flowers, respectively, in peach.

A few of the seedlings exhibited leaf variegation or reversion of sectors, just as reported by KRÜSSMANN (1965) and BLINKENBERG *et al.* (1958). This may be caused by an activated transposon. Transposons have been identified in a wide number of plant genera by classical genetic (NEVERS *et al.*, 1986) and molecular (HIROCHIKA and HIROCHIKA, 1993; VOYTAS *et al.*, 1992) methods. Such a tansposon would have first influenced a gene involved in leaf pigmentation/anthocyanin production, resulting in the original green to purple mutation in copper beech. In some copper beech progeny (those that revert to green leaf colour), it would transpose again to restore the normal gene functions in the anthocyanin pathway. Other possible explanations for leaf variegation include somatic rearrangements or somatic co-suppression.

Screening set-up

We chose to prepare DNA in bulks for the initial screening because this greatly reduced the workload (MAISONNEUVE *et al.*, 1994). However, contributions of individual seedlings to the bulk may not have been constant. This may be tolerable with the high number of seedlings used. The more seedlings represented in the pools, the more recombinants between trait and putative markers will be present, too. Therefore, it may be expected that only markers closely linked to the trait show up in the screening. However, random primer DNA amplification is a complex competition reaction with poorly understood kinetics (CAETANO-ANOLLÉS *et al.*, 1992). Markers present at a low 'allelic concentration' (< 5%) in the pools may go undetected (MICHELMORE *et al.*, 1991).

A number of laboratories have used primers in pairs for random amplification (e.g. WELSH and MCCLELLAND, 1991; CAETANO-ANOLLÉS, *et al.*, 1991; KAEMMER *et al.*, 1992; MICHELI *et al.*, 1993). This saves on primer costs, as many combinations produce new bands not found among the reaction products from single primers. However, by far not all the 1000 bands recorded in our screening experiments were unique to only 1 primer combination. We did not score the proportion of unique bands, but it is certainly considerably smaller. Among these, only markers heterozygous in the mother and absent from the pollen pool are useful in the setting we used. CHAPARRO *et al.* (1994) have screened 522 primers to construct a genetic linkage map in peach from controlled crosses, covering 396 cM. The genome of *Fagus sylvatica*, an outcrossing forest tree, may be much larger than that. More primers will have to be screened to cover the genome of our copper beech tree at a sufficient density to detect markers more closely linked to the purple trait. Estimates for the number of markers needed to saturate a genetic linkage map of a typical eukaryote range in the order of several hundred (NEALE and WILLIAMS, 1991). Our screening experiments, however, have been conducted in a very short time, which is a great advantage of this approach.

The repeat primers we used are expected to direct amplification preferably from sequences in the genome that are adjacent to microsatellites (GUPTA *et al.*, 1994; ZIETKIEWICZ *et al.*, 1994), where the level of sequence polymorphism may be higher than elsewhere in the genome. Therefore, the primers are not entirely 'random'. Several repeat primers produced mainly 'smears', which may in part be due to their sequence. For instance, primers consisting of GT-repeats were prone to resulting in smears under our conditions. On the other hand, '875' (sequence, $[CTAG]_4$) which is perfectly self annealing usually produced well-defined bands in combinations with 10mers.

Linkage of trait to molecular marker

In our set-up, it is equally probable to find markers linked to either the 'green' or the 'red' allele of the mother tree. As only a subset of markers polymorphic in the seedlings is useful for linkage analysis (requirements are presence in mother and 1:1 segregation in progeny), more effort is needed to find markers closer to the trait locus. Just how much more markers are needed cannot be estimated from the data.

Linkage of the marker to the 'green' allele was only weekly supported by log likelihood estimates – usual LOD scores are 2.5 to 3.0, whereas our marker scored only 1.5. However, χ^2 analysis resulted in a comfortable significance level (< 1%). GERBER and RUDOLPHE (1994) compared the 2 mapping methods and found that the log likelihood estimate, which does not allow for the selection of a significance level, is in general more conservative than χ^2.

The 3 more markers that formed a separate linkage group confirm that the open-pollinated progeny approach can be used to establish genetic linkage maps of single trees, albeit with considerable effort.

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Variation in Temperature Sum Requirement for Flushing of Beech Provenances

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Summary

The time of bud-burst of provenances of beech (*Fagus sylvatica* L.) is important especially on sites with frequently occurring late frosts. On a field trial in the nursery of the Institute of Forest Genetics at Grosshansdorf, Germany, the time of bud-burst was scored in 2 different ways on 158 3 year old provenances representing most part of the natural distribution of beech. Also the air temperatures were logged to calculate temperature sums at the time of bud-burst. A cline became evident with provenances from the east and south-eastern part of the range of distribution to flush early and provenances of the western part of the range to flush late. Also provenances from higher elevations tend to burst bud earlier than those from lower elevations. At a base temperature of 5 °C starting from January 1st early flushing provenances require 8,500 degree hours for bud-burst whereas late flushing provenances require 11,000 degree hours. Averagely 9,750 degree hours were required for bud-burst. The first single trees to flush required 7,600, the last 14,750 degree hours before bursting bud. According to the local risk of late frost occurrence during the period when the freshly flushing beech leaves are prone to frost it can be predicted which provenances could be potentially planted without late frost risk at a certain site and for which the late frost risk is too high.

Key words: Fagus sylvatica L., bud-burst, flushing, temperature sum, provenance variation, phenology.

FDC: 165.53; 181.22; 181.8; 232.12; 176.1 *Fagus sylvatica.*

Introduction

Many studies of temperate tree species have shown that the character "bud-burst" is highly heritable and the time of flushing of populations relative to another between years is highly correlated (e.g. WORRALL, 1983, 1993; VON WUEHLISCH et al., 1995). This results from the adaptation to the local climate allowing the trees to flush early enough to profit from the favourable spring and early summer growing conditions but flushing late enough not to be at too high a risk of late frost damage. Also beech (*Fagus sylvatica* L.), a species covering large areas in Central, Western, and Southern Europe is known to flush differently in the different regions (BORGHETTI and GIANNINI, 1982; MUHS, 1985; RECK, 1972; ŠINDELÁŘ, 1985; SITTLER, 1982; TEISSIER DU CROS et al., 1988; THOMASIUS and GÄRTNER, 1985). Generally, provenances from higher elevations and more eastern origin flush earlier than populations from lower elevations or atlantically influenced areas (MUHS, 1985; MADSEN, 1995; VON WUEHLISCH *et al.,* 1993, 1995). This adap-