NOTE

Randomly amplified polymorphic DNA linkage relationships in different Norway spruce populations

M. Troggio, T.L. Kubisiak, G. Bucci, and P. Menozzi

Abstract: We tested the constancy of linkage relationships of randomly amplified polymorphic DNA (RAPD) marker loci used to construct a population-based consensus map in material from an Italian stand of ***Picea abies*** (L.) Karst. in 29 individuals from three Norwegian populations. Thirteen marker loci linked in the Italian stand did show a consistent locus ordering in the Norwegian population. The remaining 16 unlinked marker loci were spread over different linkage groups and (or) too far apart both in the population map and in this study. The limited validity of RAPD markers as genomic "hallmarks" resilient across populations is discussed. We also investigated the reliability of RAPD markers; only 58% of the RAPD markers previously used to construct the consensus map in the Italian population were repeatable in the same material. Of the repeatable ones 76.3% were amplified and found polymorphic in 29 megagametophyte sibships from three Norwegian populations.

Résumé : Les auteurs ont voulu verifier, chez 29 individus provenant de trois populations norvegiennes de *Picea* ***abies*** (L.) Karst., la stabilité des relations de liaison entre les loci de marqueurs d'amplification au hasard des polymorphismes de I'ADN (RAPD) utilisés pour construire une carte consensus de population à partir de materiel qui origine d'un peuplement italien de la même espèce. Un groupe de 13 loci de marqueurs du peuplement italien a démontré un ordre similaire des loci chez les populations norvegiennes. Les autres I6 loci de marqueurs non liés Ctaient répartis entre différents groupes de liaison et (ou) Ctaient trop éloignés Ies uns des autres, à la fois sur la carte de la population italienne et dans la présente etude. Les auteurs discutent de la validité limitée des marqueurs RAPD comme marqueurs génomiques universels stables d'une population à l'autre. Les auteurs ont également étudié la fiabilité des marqueurs RAPD. Seulement 58% des marqueurs RAPD utilisés précédemment pour construire la carte consensus de la population italienne Ctaient reproductibles chez Ie même materiel. Parmi les marqueurs reproductibles, 76,3% ont pu être amplifies et ont démontré du polymorphisme chez les 29 familles de mégagamétophytes des trois populations norvegiennes.

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Introduction

In the last decade, randomly amplified polymorphic DNA (RAPD) markers have been used extensively in forest tree species' mapping projects for their useful characteristics: relative simplicity, large multiplex ratio, suitability for automation, etc. (Williams et al. 1990; Hadrys et al. 1992).

When multiple genomes from different provenances are studied, transferability of RAPD markers (as any other type of marker) and consistency of locus ordering should be verified by testing the robustness of their linkage relationships (Hurme and Savolainen 1999; Verhaegen and Plomion 1996). Comparing three single-tree linkage maps (72 megagametophytes/tree), the orthologous origin of 20 RAPD markers was demonstrated in *Pinus* ***sylvestris*** L. (Hurme and Savolainen 1999). Consistency of RAPD markers loci across similar genomes has also been reported by comparing linkage maps from the same individual genotype using different tissues (megagametophytes and needles) or different crosses (selfed and open-pollinated seeds) sharing one of the parents (Plomion and O'Malley 1996; Plomion et al. 1995; Kubisiak et al. 1996). By pooling linkage information over sibships of megagametophytes from different individuals of the same population, a "consensus" map has been constructed for ***Picea abies*** (L.) Karst. (Bucci et al. 1997) and heterogeneity

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of recombination fraction across different individuals evaluated (Bucci et al. 1997).

RAPDs have been claimed to be scarcely reproducible using different experimental conditions (Perez et al. 1998; Jones et al. 1997; Haig et al. 1994). Sensitivity of RAPDs to slight changes in reaction conditions is well know (Hadrys et al. 1992; Jones et al. 1997). In snap bean (*Phaseolus vulgaris* L.), the percentage of scorable RAPD bands in a replicated sample set was 76% (Skroch and Nienhuis 1995). Perez et al. (1998) found a proportion of repeatable bands of 23% for deer, 36% for boar, and 26% for fruit fly in two replicate reactions per individual. Plomion et al. (1995) using a replicated design found 70% of the markers in two sets of megagametophytes from a self- and open-pollinated family sharing a common parent.

In this study we investigated the locus ordering and the linkage relationship obtained analyzing 29 individuals from three Norwegian populations of *Picea abies* in comparison with a "consensus" mapping analysis (48 individuals) from an Italian population of the same species (Binelli and Bucci 1994; Bucci et al. 1997).

Materials and methods

Plant material and DNA extraction

We used material from Norway (29 Norway spruce adult trees randomly chosen from three Norwegian populations) made available to us from diallel crosses whose genetics is under study in our laboratory (Skröppa and Tho 1990). Megagametophytes from 12 open-pollinated seeds per tree were extracted and analyzed. Seeds were germinated following standard methods, and megagametophytes were stored at -80°C after germination and just prior to the seed coat being cast. Genomic DNA was extracted from megagametophytes using the hexadecyltrimethyl ammonium bromide based method of Doyle and Doyle (1987) with slight modifications.

As a control sample set for reproducibility, we used the same DNA samples from a sibship of 12 megagametophytes previously used in a consensus map experiment (Bucci et al. 1997).

DNA amplification

Polymerase chain reactions (PCRs) were set up using a Hamilton Microlab At_{plus} robotic pipettor (Reno, Nev.) in 96-well microtitre plates and cycled in MJ Research PTC-100 thermal engines (Watertown, Mass.). Amplification conditions matched those previously reported by Binelli and Bucci (1994), except for the use of V-bottom plates (Type M, Costar Corporation, Cambridge, Mass.) instead of U-bottom plates as specified in the previous experiment.

RAPD marker screening

RAPD marker screening was carried out following a two-step procedure, involving a total of 10 080 PCR reactions.

The first step was aimed to assess the reproducibility of marker bands in the control set of megagametophytes. Sixty-six nuclear chromosomal loci were chosen among those previously used for the construction of a population consensus map (Bucci et al. 1997). Based on the consensus map, 38 of these marker loci were linked in eight different groups, while the remaining were either unlinked or spread too far apart on the other groups for linkage to be detected ($\theta > 0.4$). The total number of reactions in this step was 336 reactions (28 chosen primers x 12 megagametophytes of the control set).

The second step was aimed to assess the reliability of linkage relationships of the above marker loci in the 29 sibships of 12 megagametophytes from the Norwegian populations. Some 9744 reactions (28 primers x 12 megagametophytes x 29 trees) were carried out in this step to characterize the 29 parental Norwegian trees. RAPD bands showing segregation in the 29 sibships from the three Norwegian populations were tested for Mendelian inheritance. Segregation analysis for each marker locus was carried out pooling segregation ratios within each sibship of megagametophytes over all heterozygous parents showing at least 11 unambiguous scores (Bucci et al. 1997). The observed segregation ratios within sibships over the whole population were tit to their binomial expectation using a Kolmogorov-Smirnov (K-S) test ($a = 0.05$).

Linkage analysis

Linkage relationships among marker loci were estimated using the maximum likelihood approach implemented in the MAPMAKER version 2.0 software package (CEPH data type format; Lander and Green 1987). Linkage analysis was restricted to double heterozygous trees showing at least seven unambiguous dilocus, haploid scores. Marker loci were assigned to linkage groups using a likelihood of odds (LOD) of 2.0 and recombination fraction of 0.3. Map distances in centiMorgans (cM) were calculated using the Kosambi mapping function.

Homogeneity of two-point recombination estimates between marker loci across the 29 sibships of megagametophytes was veritied using a χ^2 test using the JMHET module of the JOINMAP version 2.0 software package (Stam 1993).

Results

RAPD marker reproducibility in the control set

The 28 primers used in the previous consensus map experiment were expected to yield 66 nuclear chromosomal loci in a control set of megagametophytes (Table 1). Thirty-eight (57.6%) matched those obtained in the previous study and 10 (15.2%) were too faint to score. Sixteen (24.2%) were not amplified in the present analysis and two (OJ100550, 0X200510) (3.0%) exhibited a different pattern of segregation (Table 1).

Marker screening in the Norwegian sibsbips

Of the 38 marker loci reproducible in the control sample set, 29 (76.3%) were amplifiable and polymorphic, showing the expected size in the 29 Norwegian sibships. Eight (21%) gave faint bands that could not be unambiguously scored, and only one did not amplify at all (OY 130270). Only two (C3160780, C5030350) of 29 marker loci amplifiable in the 29 Norwegian sibships showed significant deviations from the expected Mendelian ratios in the population. Pooling segregation ratios over all the 29 polymorphic marker loci and heterozygous trees, no significant deviations from the binomial expectation were found (K-S, $d = 0.101$, $p > 0.05$).

Linkage analysis and comparison of linkage relationships

The 29 polymorphic marker loci successfully amplified in the Norwegian sibships were tested for their independent segregation. Of 406 possible pairwise combinations of marker loci (29 x 28/2), 103 were noninformative (i.e., no double heterozygous trees for these marker loci where available in the population analyzed). A total of 9076 pairwise

Table 1. Results of RAPD marker reproducibility and screening in the Norwegian sibships.

Marker	Primer sequence	Control sample set[a]	Norwegian sibships[b]	Segregation ratio test[c]	Italian consensus map[d]
C 1230646	GTCTTTCAGG	R	P	ns	winF
Cl231080	GTCTTTCAGG	R	P	ns	winB
Cl231 140	GTCTTTCAGG	R	P	ns	winA
C1350550	AAGCTGCGAG	R	P	ns	winB
Cl351093	AAGCTGCGAG	F	—	—	U
Cl671170	CCAATTCACG	F	—	—	U
C1671450	CCAATTCACG	R	P	ns	U
C 1680780	CTAGATGTGC	R	P	ns	winF
C 1690270	ACGACGTAGG	R	P	ns	winGE
Cl690280	ACGACGTAGG	R	P	ns	winF
Cl691 180	ACGACGTAGG	N			U
C 1930360	TGCTGGCTTT	R	P	ns	winA
C1931000	TGCTGGCTTT	F	—	—	winD
C2660820	CCACTCACCG	R	P	ns	winA
C2661205	CCACTCACCG	F	—	—	U
C2661350	CCACTCACCG	F	—	—	U
C2661810	CCACTCACCG	F	—	—	winB
C2662000	CCACTCACCG	N	—	—	winGE
C2680280	AGGCCGCTTA	R	P	ns	U
C2950595	CGCGTTCCTG	R	F	—	winD
C2990440	TGTCAGCGGT	R	F	—	winF
C2990520	TGTCAGCGGT	R	P	ns	winD
C2990850	TGTCAGCGGT	R	F	—	winGD
C3 160780	CCTCACCTGT	R	P	<0.05	winA
c399 1900	TTGCTGGGCG	N	—	—	winGD
C3991986	TTGCTGGGCG	N	—	—	winGD
C4930400	CCGAATCACT	R	P	ns	U
C4930530	CCGAATCACT	R	P	ns	winB
c493 1450	CCGAATCACT	R	F	—	U
c493 1750	CCGAATCACT	N	—	—	U
C4932 100	CCGAATCACT	N	—	—	U
C4990340	GGCCGATGAT	R	P	ns	winF
C5030350	ATCGTCCAAC	R	P	<0.05	winGE
cso9 1040	ACAGAGACTG	N	—	—	winE
C5091070	ACAGAGACTG	R	F	—	winE
C5870644	GCTACTAACC	R	P	ns	winA
C5870944	GCTACTAACC	R	P	ns	U
C6140460	GTAGTCTCGC	N			U
C6141640	GTAGTCTCGC	F	—	—	winA
C6 142400	GTAGTCTCGC	N	—	—	U
C6870450	ATACAAGGGG	F	—	—	U
C6870537	ATACAAGGGG	R	P	ns	U
C6870603	ATACAAGGGG	R	P	ns	U
014070680	GAAACGGGTG	R	F	—	U
OA07 1100	GAAACGGGTG	R	F		U
OA080302	GTGACGTAGG	R	P	ns	winGE
OA080340	GTGACGTAGG	R	P	ns	winGE
OA080383	GTGACGTAGG	R	P	ns	U
OA082410	GTGACGTAGG	N	—	—	winE
OB05 1020	TGCGCCCTTC	R	P	ns	winGE
OB0511 SO	TGCGCCCTTC	R	P	ns	winGE
OB05 1300	TGCGCCCTTC	R	F	—	U
OE171000	CTACTGCCGT	N	—	—	U
OE171650	CTACTGCCGT	N	—	—	winD
OE171720	CTACTGCCGT	N	—	—	winC

Table 1 (concluded).

Marker	Primer sequence	Control sample set[a]	Norwegian sibships[b]	Segregation ratio test[c]	Italian consensus map[d]
OF1 40750	TGCTGCAGGT	R	P	ns	U
OF1 40850	TGCTGCAGGT	R	P	ns	U
OF141000	TGCTGCAGGT	F	—	—	U
OG 100470	AGGGCCGTCT	F	—	—	U
OJ010550	CCCGGCATAA	NR	—	—	winGE
0x2005 IO	CCCAGCTAGA	NR	—	—	winB
OX200550	CCCAGCTAGA	N	—	—	winD
OX200650	CCCAGCTAGA	N	—	—	U
OY 130270	GGGTCTCGGT	R	N	—	winA
OY **130450**	GGGTCTCGGT	N	—	—	U
OY131050	GGGTCTCGGT	R	P	ns	winGD

"Reproducibility in the control sample set: R, perfectly reproducible (correct size and score); F, too faint to be scored accurately; N, no bands of the expected size amplified; NR, nonreproducible (different score in the control sample set).

"Amplifiable or polymorphic in the Norwegian sibships: P, perfectly scorable and polymorphic in the sibships; F, faint band (discarded); N, no bands of the expected size amplified.

'Goodness-of-tit test to the expected (Mendelian), within-family segregation ratios over the population (see Materials and methods). ns, not significant.

"Map localization on the Italian population, consensus map: labels refer to windows of recombination (win) within linkage groups previously identified (Bucci et al. 1997); see also Fig. 1; U, either unlinked or falling out the windows of recombination, at a distance too large for linkage to be detected (for more details, see Bucci et al. 1997).

meioses were coinformative. The average number of coinformative meioses per marker loci pair was 29.95, while the mean number of heterozygous loci per individual was 7.45 (ranging from 2 to 13).

Of the 29 marker loci above, 10 were grouped at LOD > 2 and $\theta < 0.3$, while three additional marker loci were grouped at LOD > 1.70 and $\theta < 0.3$. The remaining 16 marker loci showed no significant linkage at LOD 1.7 ($\alpha = 0.02$). Overall, three triplets and two doublets were obtained (Fig. 1): C2660820, C1930360, and C3160780 (mapped in the recombination window WinA in the consensus map experiment); C 1230646, C4990340, and C 1690280 (WinF); C 1690270, OA080302, and OA080340 (WinGE); C5870644 and C1231140 (WinA); and OB051020 and OB051150 (WinGE). The most likely order for each triplet (Fig. 1) was obtained by the three-point analysis implemented in the MAPMAKER version 2.0 package. The average distance between marker loci was 14.2 cM.

Of the 16 marker loci showing no significant linkage at LOD 1.7 in the Norwegian populations, nine (C5870944, C4930400, 014080383, C 1671450, OF1 40750, C2680280, OF140850, C687537, and C6870603) were unlinked in the Italian consensus map and turned **out** to be unlinked in this study also. Two marker loci (C2990520, OY 13 1050) were spread over different linkage groups and (or) too far apart in the consensus map to show significant linkage in this study and, therefore, were considered as unlinked. As for the remaining five previously mapped marker loci (C1231080, C1350550, C4930530 in WinB; C1680780 in WinF; C5030350 in WinGE, the latter showing uncertain linkage relationships and possible alternative arrangements in the consensus map experiment), no linkage was detected, although in one case (locus C1671450) independent segregation was rejected using a χ^2 test (a = 0.05).

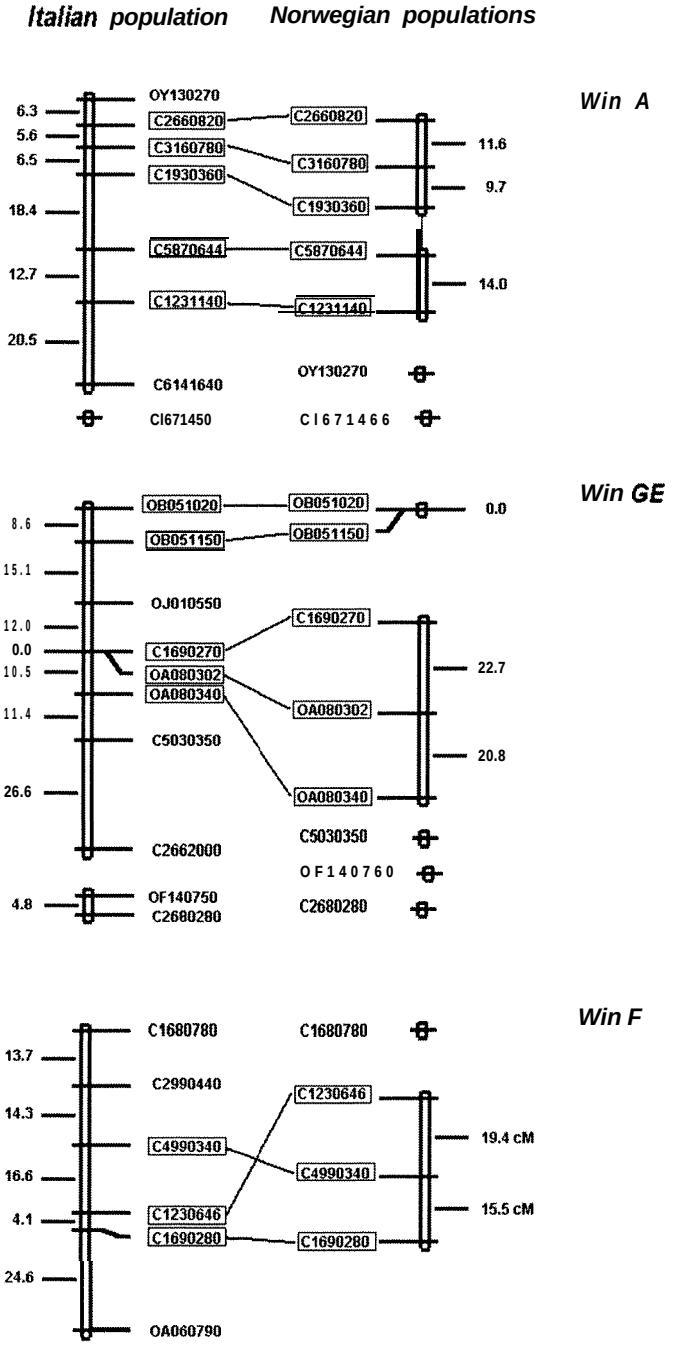
Homogeneity of the pairwise recombination fraction over the Norwegian sibships was verified for all pairs of mapping marker loci. Of 303 pairwise combinations of marker loci, only two (C3160780, C5030350) were heterogeneous across the population at a = 0.05, a proportion lower than what expected by chance.

Discussion

In this investigation, linkage relationships of RAPD marker loci previously identified and mapped in an Italian Norway spruce population (at the southern margin of its range) were investigated in individuals from three Norwegian populations (northern margin of the species' range). Marker loci reproducible in the control sample set were found reproducible in fairly high proportion (76.3%) in the Norwegian sibships. All such marker loci were polymorphic in at least one of the individuals analyzed. A subset of these marker loci (13 of 29) showed linkage relationships consistent with those previously obtained in the consensus map experiment (Bucci et al. 1997) (Fig. 1). Of the remaining 16 marker loci five belonged to "windows of recombination" in the Italian map experiment but showed independent segregation in this study. This could be due to several causes: (i) miscoring of the markers in the Norwegian sibships, due to comigration of unrelated bands, can introduce some noise in the data set; (ii) the low number of coinformative meioses detected for most of these marker loci may reduce the pairwise LOD score with other mapped marker loci; (iii) genomic divergence between Italian and Norwegian populations may have produced genomic rearrangements, puzzling linkage relationships among marker loci.

For forest tree species, there are very few reports on linkage map comparison across different individuals of the same

Fig. 1. Comparison between the linkage structure found in a Norwegian population of *Picea abies* (29 individuals) and the map that was constructed with a common set of markers for an Italian population (Binelli and Bucci 1994; Bucci et al. 1997). Vertical solid lines connecting two rods represents interval supports <1.7. Common loci are shown in box and interconnected with solid lines. Distances are shown in centiMorgans. No amplification product was observed for marker OY 130270. Markers C1680780, C5030350, OF140750, and C2680280 turned out to be unlinked at a LOD threshold of 1.7. Independent segregation for marker Cl671450 was rejected using a χ^2 test (a = 0.05).



species using RAPD markers. For three individuals of Scots pine, linkage relationships among 22 RAPD loci segregating in at least two trees were compared (Hurme and Savolainen 1999). Of the 22 loci, 20 (91%) were linked and in the same order between or among trees, the remaining two loci being unlinked because nonhomologous. Sewell et al. (1999) constructed a "consensus" map for loblolly pine (using RFLPs, RAPDs, isozymes) integrating the linkage data from two unrelated three-generation outbred pedigrees, but very few RAPD marker loci were used to align homologues linkage groups. Krutovskii et al. (1998), comparing two hybrid individuals of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) for 28 RAPD loci, found three pairs of loci closely located in both maps, three closely linked loci in one hybrid but unlinked in the other, while the others were too far apart to detect linkage. Verhaegen and Plomion (1996) investigated the consistency of the linkage relationships comparing two single-tree RAPD maps constructed for ***Eucalyptus*** *urophylla* S.T. Blake and ***Eucalyptus*** *grandis* Hill ex Maid. (based on segregation of RAPDs in a F_1 interspecific progeny) with those constructed by Grattapaglia and Sederoff (1994) on a progeny from a different interspecific cross. Similar recombination fractions and locus ordering between the two maps were found for eight RAPD loci in ***E. urophylla*** and for seven RAPD loci in *E. grandis*. Kubisiak et al. (1995) constructed linkage maps for the parents of a longleaf pine (*Pinus* ***palustris*** Mill.) x slash pine (*Pinus* ***elliottii*** Engelm.) F_1 family. By taking advantage of the information provided by loci heterozygous in both parents (and segregating 3:1), they found four of the longleaf-parent linkage groups potentially homologous counterpart to five different slash-parent linkage groups.

The low reproducibility of RAPD markers that we found is not surprising given the changes in the amplification conditions in comparison with those of the previous experiment (the main difference was the V-bottom polycarbonate versus U-bottom polyethylene microtiter plates, dramatically influencing heat transfer from thermal block to PCR mixture). Since our main goal was to identify RAPD markers resilient to divergent genomes, ad hoc altering of the experimental conditions for improving reproducibility (see Skov 1998) was not pursued.

Our results are a further confirmation of the caution with which the outcomes of RAPD analysis should be considered. This has been pointed out previously (Isabel et al. 1999). We are aware of the need of integrating investigations carried out with RAPDs with different more reliable markers such as microsatellites (Paglia et al. 1998) and expressed sequence tag polymorphisms (Perry et al. 1999).

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