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A genetic linkage map of *Picea abies* Karst., based on RAPD markers, as a tool in population genetics

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Abstract Norway spruce (*Picea abies* Karst.) is a most important species among European forest trees for both economical and ecological reasons. However, this species has suffered from a lack of information on the genetic side due to the scarcity of linkage data. In this study we have used a population of 72 megagametophytes from a single tree in a natural Italian stand to produce a genetic linkage map by means of RAPD markers. Ninety-six random decamers used as primers yielded 185 polymorphic loci showing Mendelian inheritance. Analysis of the segregation by multipoint analysis allowed us to define 17 major linkage groups covering a total distance of 3 584 cM, with an average spacing between markers of 22 cM. Possible uses of a genetic linkage map with respect to population ecology and genetics are discussed.

Key words *Picea abies* · RAPD · Population genetics · Linkage map

Introduction

An unparalleled explosion in the production of genetic linkage maps in plants has taken place in recent years due to the availability of molecular genetic markers. Maps have been produced either as the final goal of the research or as a side result in studies concerning the detection of QTLs.

This rising tide has only marginally touched the shores of forest trees genetics, mainly because most of the initial effort has been devoted to crops. Relatively few maps are available for forest trees (Neale and Sederoff 1991; Tulsieram et al. 1992; Gerber et al. 1993). Because of the lack of suitable pedigrees, genetic maps in conifers have been based on the analysis of a population of haploid megagametophytes from a single individual. Moreover, the very large DNA content per haploid genome ($30–40 \times 10^9$ bp, Govindaraju and Cullis 1991) and the presence of extended regions of repeated DNA (Neale and Williams 1991) are the main factors that have hampered the use of RFLPs in mapping conifer genomes. Thus, to achieve a significant number of markers, all studies have so-far relied on PCR-based techniques, such as RAPDs, even though this has the drawback of using dominant markers.

Norway spruce (*Picea abies* Karst.) is one of the most important species of European trees, both from an economic and an ecological point of view. Studies performed so far on the population genetics of this species have been based mainly on isozymes and/or morphological traits (Tigerstedt 1973; Lundkvist and Rudin 1977; Borghetti et al. 1988; Lagercrantz and Ryman 1990). Although some paper have been published on linkage analysis between isozyme markers in forest trees (Strauss and Conkle 1986; Geburek and von Wuehlisch 1989) few have dealt with the importance of linkage analysis in the population genetics of forest tree species (Mitton et al. 1980; Epperson and Allard 1987). Therefore, both population genetics and evolutionary studies would greatly benefit from the availability of a genetic linkage map.

In this paper we present the first linkage map of Norway spruce. It has been obtained by means of RAPD markers and is intended to represent a framework for subsequent improvements by using other kinds of molecular markers and to serve as a starting point to answer questions concerning the ecology and population genetics of this species.

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Materials and methods

Plant material

Cones were collected from trees growing in the Forest of Campolino, near Pistoia, Italy (W 10° 15′, N 44° 07′). This forest is believed to be a relic population from the Quaternary ice-age (Chiarugi 1936; Giannini et al. 1991).

After maintaining the cones at room temperature for several days to induce their opening, seeds were collected and stored at 4 °C. The megagametophytes have been isolated by removing the inner and outer coats of the seed and the embryo working under a stereomicroscope. They were then frozen and stored at −80 °C until DNA extraction.

DNA extraction

DNA from 72 megagametophytes of a single tree was extracted according to a modification of the protocol by Lee and Taylor (1990): tissue was homogenized in 200 µl of 50 mM Tris-HCl pH 7.2, 50 mM EDTA pH 8.0, 3% SDS and 1% β-mercaptoethanol, incubated at 65 °C for 1 h and extracted with Phenol:chloroform:isoamylalcohol (25:24:1). After centrifugation, the supernatant was extracted with chloroform:1-octanol (24:1). Following precipitation with sodium acetate and 2.5 v/v 100% ethanol, DNA was resuspended in TE and incubated with 5 µl of RNAseA (stock 10 mg/ml) at 37 °C for 2 h. After re-precipitation, DNA was suspended in 15 µl of TE. The yield was highly satisfactory, with a maximum of 8 µg of DNA/megagametophyte, and an average of about 4.0 µg.

DNA amplification

The original protocol for RAPD reactions was followed (Williams et al. 1990) with minor modifications: the total reaction volume was 24 µl, and the reaction was 3.0 ng of template DNA, 5 pmoles of primer (random decamers), 3 nmoles of each dNTP, 2.4 µl of *Taq* buffer (10 × Boehringer Mannheim) and 0.75 units of *Taq* DNA polymerase (Boehringer Mannheim), overlaid with 50 µl of mineral oil.

All reaction mixtures were set up by using a Hamilton Microlab ATplus robot in 96-well microtitre plates. After being covered with mylar film, the plates were run in MJ Research PTC-100 programmable temperature cyclers.

The amplification profile was 5 s at 94 °C, 1 min 55 s at 92 °C for starting and 45 cycles of 5 s at 94 °C, 55 s at 92 °C, 1 min at 35 °C and 2 min at 72 °C. A final extension of 7 min at 72 °C followed, before incubating at 4 °C until the products were loaded.

The reactions were run in 2% agarose gel in 1 × TAE at 3 V/cm, stained with ethidium bromide, visualized under UV light, and photographed using a Polaroid camera.

Scoring the RAPDs

In the first phase, DNA from eight megagametophytes was tested with an array of 328 primers, 10-base oligonucleotides with random sequence, either obtained from Operson Technologies (Alameda, Calif.) or from J. E. Carlson (University of British Columbia). Based on the number of polymorphic bands revealed, and on their sharpness, 96 primers were chosen for the mapping effort. All of the 96 were used to amplify the population of 72 megagametophytes comprising the eight already tested and used as a reproducibility assay.

The amplification products showing hints of non-repeatibility were not considered further. All polymorphic bands were scored as 1 for present and 0 for absent and tested for the expected 1:1 segregation by a chi-square test at $P < 0.05$. Mendelian markers obtained as described were then divided into two classes: class A was made up by those markers showing a very bright amplification product for the "1" allele and a blank "0" allele and class B by those markers that, although unambiguously scored, showed not so bright amplification products for the "1" allele and/or some background for the "0" allele.

Linkage analysis

MAPMAKER/EXP version 3.0 (Lander et al. 1987; Lincoln et al. 1992) was used for the analysis of the segregation using an F_2 backcross data file. First, a skeleton map was produced by taking into account only the class A markers at a LOD score of 4.0 and $\Theta = 0.4$. Having thus established a reliable framework, the whole data set was analyzed. Unlinked markers were then assigned to the linkage groups obtained by using full multipoint analysis at more relaxed thresholds of 3.0 and 2.0 for log-likelihood. The orders of the markers for each linkage group were tested by comparing their likelihood toward the likelihood of the allowed permutations.

Results

The total number of amplification reactions performed in this study was 9 536. The analysis of the amplification products obtained by running the 72 megagametophyte samples against 96 random decamer primers revealed the presence of 185 polymorphic bands whose segregation ratio was not statistically different from 1:1 (chi-square test, $P < 0.05$). Therefore, we considered these bands as suitable Mendelian genetic markers. A typical result for this mapping effort is shown in Fig. 1, where the products resulting from the amplification of the entire 72 samples by using two different primers are visible. Out of 96 primers chosen for the mapping effort, 20 yielded no Mendelian markers, 22 yielded one, 21 yielded two, 20 yielded three, six yielded four, five yielded five and two primers yielded six Mendelian markers (1.93 markers per primer on average). One-hundred-and-twenty-one polymorphisms were grouped in class A (see Materials and methods) and used for the first ordering of the markers.

The matrix of data was analyzed for cosegregation of the markers by multipoint analysis with MAPMAKER/EXP: 152 markers out of 185 fell into linkage groups with at least another marker at a minimum LOD score of 3.0 and a maximum distance of 40.0 cM. Thirteen other markers were assigned to the linkage groups using 2.0 as a LOD threshold.

In this way, we obtained 17 groups each with more than three associated markers, two triples, and seven pairs of markers. Twenty markers remained unlinked. The seventeen larger groups are shown in Fig. 2 and will henceforth be considered as a partial genetic map of *Picea abies*. The large interval between markers C6670421 and C1231080 was maintained, although exceeding 40% of recombination, due to the strong likelihood supporting the linkage between those two markers.

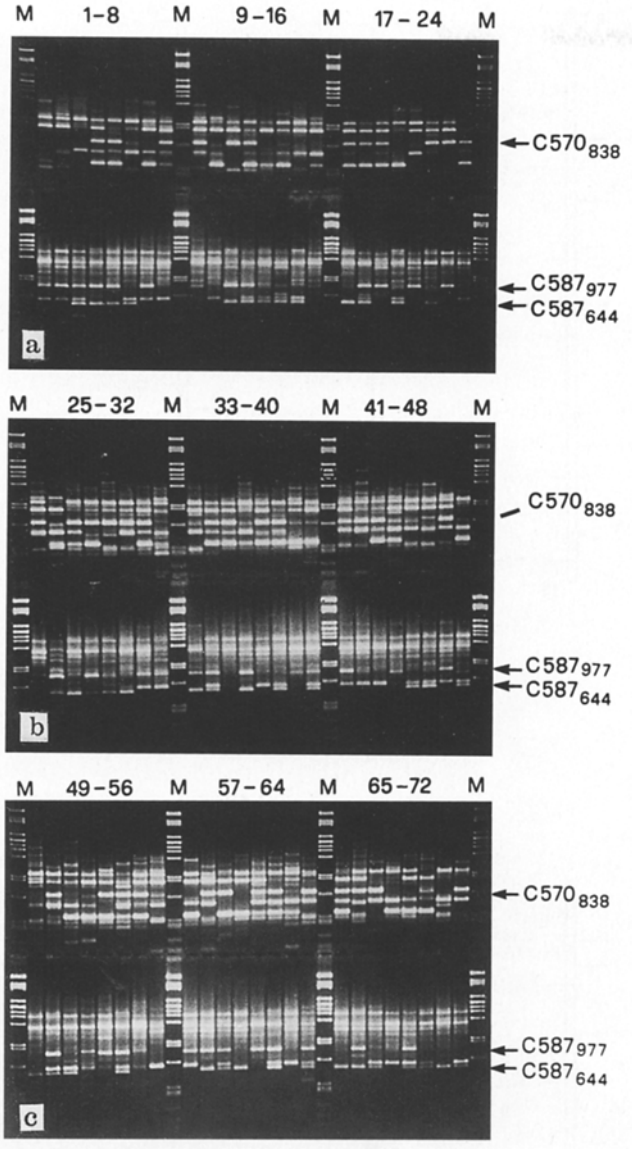

Fig. 1a–c RAPD amplification of 72 megagametophytes by two different 10-mer primers **a**, upper half, megagametophytes from #1 to #24 amplified with primer C570; lower half, megagametophytes #1 to #24 amplified with primer C587. **b and c** The same for megagametophytes #25–#48 and #49–#72, respectively. *Arrows* indicate 1:1 segregating polymorphic bands, which are designated by the primer name and their molecular size. *M* = size markers, lambda DNA digested with *Pst*I

The total distance covered by our linkage groups is 3 584 cM (triplets and pairs included), with an average distance between markers of 22.0 cM. It has to be pointed out, however, that map distances estimates are very sensitive to sampling errors: although sufficient for the purpose of this study, our 72 megagametophytes probably do not represent a good sample. Moreover, for some of the groups, the order of the markers at the ends of the chromosomes is not established with absolute certainty. Although linkage with other markers was assured by LOD scores higher than 2.0, the differences in likelihood between the first three or four more probable orders were less than 1.0. In these cases, a larger population size would probably be more efficient to discriminate between different orders.

Discussion

The genome of *Picea abies* has been ordered, in this study, into 17 major linkage groups covering 3 584 cM, with an average distance between markers of 22 cM. One-hundred-and-eighty-five Mendelian markers (RAPDs) have been scored, but only 165 fell into a linkage group with at least another marker in it: the 20 markers we were not able to locate with respect to the others would probably extend the dimensions of our map. These 20 unlinked markers probably reflect the very large genome of this species, considering also that the mapped RAPDs are quite regularly spaced and do not cluster, thus indicating random sampling. The length of the genome of *Picea abies* falls in the range of total genetic distances obtained in conifer mapping: 1 687 cM in loblolly pine (Neale and Sederoff 1991), 836.8 cM in white spruce (Tulsieram et al. 1992), and 3 360 cM in slash pine (Nelson et al. 1993).

Taking into account that the amount of DNA in the genome of *P. abies* is in the range of 4×10^{10} bp, and that maize, for example, lags one order of magnitude behind, this estimate of genome size is probably not much exaggerated, although total genetic length depends mainly on the length of the units of recombination (Binelli et al. 1992).

The number of chromosomes per haploid genome in *Picea* is 12. In this study, we found 17 major linkage groups, plus two triples and six pairs of markers. Large gaps not filled by any marker between some of the minor linkage groups identified may be due to an inadequate sampling of the very large genome of Norway spruce. Further analyses increasing the number of primers and/or megagametophytes will be needed in order to fill in gaps.

The accuracy (and usefulness) of a genetic linkage map is directly related to the coverage of the genome achieved and the reliability of the genetic markers employed. The first issue is partially covered by the map presented in this paper. As for the second, in a previous study on the feasibility of the RAPD approach in Norway spruce (Bucci and Menozzi 1993), 17 primers (random decamers) were used to screen 34 megagametophytes rom a single tree. Fifty-four markers were found to show a 1:1 segregation and six out of these were checked again for Mendelian behaviour on six megagametophytes from each of five other trees. Five markers showed 1:1 segregation for all the five trees, the last one showing distorted segregation for one tree only. In a recent study on black spruce (*Picea mariana*), Isabel et al. (1993) have shown that RAPDs whose Mendelian segregation had been tested at the megagametophyte level are useful to assess the genetic stability of embryogenesis-derived trees. Additionally, Heun and

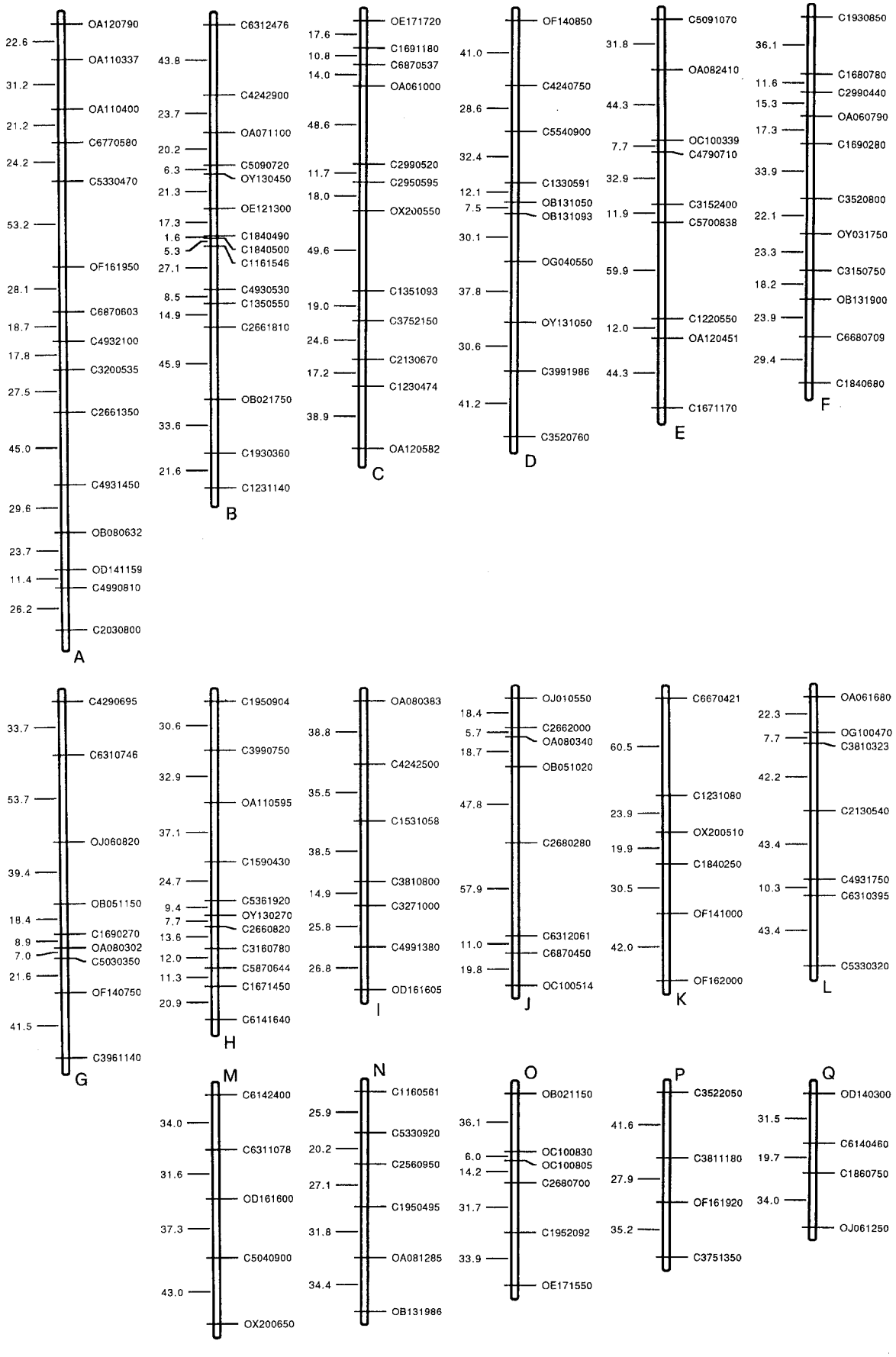
A
OA120790
22.6
OA110337
31.2
OA110400
21.2
C6770580
24.2
C5330470
53.2
OF161950
28.1
C6870603
18.7
C4932100
17.8
C3200535
27.5
C2661350
45.0
C4931450
29.6
OB080632
23.7
OD141159
11.4
C4990810
26.2
C2030800
B
C6312476
43.8
C4242900
23.7
OA071100
20.2
C5090720
6.3
OY130450
21.3
OE121300
17.3
C1840490
1.6
C1840500
5.3
C1161546
27.1
C4930530
8.5
C1350550
14.9
C2661810
45.9
OB021750
33.6
C1930360
21.6
C1231140
C
OE171720
17.6
C1691180
10.8
C6870537
14.0
OA061000
48.6
C2990520
11.7
C2950595
18.0
OX200550
49.6
C1351093
19.0
C3752150
24.6
C2130670
17.2
C1230474
38.9
OA120582
D
OF140850
41.0
C4240750
28.6
C5540900
32.4
C1330591
12.1
OB131050
7.5
OB131093
30.1
OG040550
37.8
OY131050
30.6
C3991986
41.2
C3520760
E
C5091070
31.8
OA082410
44.3
OC100339
7.7
C4790710
32.9
C3152400
11.9
C5700838
59.9
C1220550
12.0
OA120451
44.3
C1671170
F
C1930850
36.1
C1680780
11.6
C2990440
15.3
OA060790
17.3
C1690280
33.9
C3520800
22.1
OY031750
23.3
C3150750
18.2
OB131900
23.9
C6680709
29.4
C1840680
G
C4290695
33.7
C6310746
53.7
OJ060820
39.4
OB051150
18.4
C1690270
8.9
OA080302
7.0
C5030350
21.6
OF140750
41.5
C3961140
H
C1950904
30.6
C3990750
32.9
OA110595
37.1
C1590430
24.7
C5361920
9.4
OY130270
7.7
C2660820
13.6
C3160780
12.0
C5870644
11.3
C1671450
20.9
C6141640
I
OA080383
38.8
C4242500
35.5
C1531058
38.5
C3810800
14.9
C3271000
25.8
C4991380
26.8
OD161605
J
OJ010550
18.4
C2662000
5.7
OA080340
18.7
OB051020
47.8
C2680280
57.9
C6312061
11.0
C6870450
19.8
OC100514
K
C6670421
60.5
C1231080
23.9
OX200510
19.9
C1840250
30.5
OF141000
42.0
OF162000
L
OA061680
22.3
OG100470
7.7
C3810323
42.2
C2130540
43.4
C4931750
10.3
C6310395
43.4
C5330320
M
C6142400
34.0
C6311078
31.6
OD161600
37.3
C5040900
43.0
OX200650
N
C1160561
25.9
C5330920
20.2
C2560950
27.1
C1950495
31.8
OA081285
34.4
OB131986
O
OB021150
36.1
OC100830
6.0
OC100805
14.2
C2680700
31.7
C1952092
33.9
OE171550
P
C3522050
41.6
C3811180
27.9
OF161920
35.2
C3751350
Q
OD140300
31.5
C6140460
19.7
C1860750
34.0
OJ061250

Fig. 2 Genetic linkage map of *Picea abies*. The 17 linkage groups found are identified by the letters *A to Q*. For each locus, the first four characters indicate the primer used for amplification, while the last four digits indicate the approximate size in nucleotides. Genetic distances are in cM (Kosambi's function)

Helentjaris (1993) have tested the behaviour of RAPDs in the different genetic environment provided by 16 F_1 hybrids derived from five different maize inbreds, showing that 95% of the unambiguously scored RAPDs segregated according to a completely dominant fashion, thus indicating that RAPDs are well suited for linkage analysis once their Mendelian inheritance has been tested. Therefore, we are confident that our map represents a good starting point for further studies.

The availability of a genetic linkage map will be useful for both population biology studies – by allowing the choice of unlinked markers – and evolutionary studies – by facilitating the identification of genomic region(s) involved in selective processes. Previously reported studies have demonstrated that several isozymic markers turned out to be linked in different conifer species (Lundkvist 1979; Cheliak et al. 1984; Muona and Szmidt 1985; Geburek and von Wuehlisch 1989). Lowered recombination rates between markers can introduce severe bias in the estimates of the genetic parameters of the population(s) studied (Nei 1987). Moreover, linkage disequilibrium between markers is considered an important indicator of epistatic selection, inbreeding or migration in natural populations (Hastings 1990) and can strongly influence evolutionary processes (Clegg et al. 1972; Allard 1975).

Applications in the fields of population genetics and ecology of the information obtained by the map presented could be greatly enhanced if the markers were unambiguously characterized and localized. To this end we plan to apply the SCAR (Sequence Characterized Amplified Regions) technique (Paran and Michelmore 1993) to a selected set of suitably spaced RAPDs. With this approach the amplification would be specific for a given locus, maintaining both the randomness and the RAPDity of the approach but limiting the chance of error due, for example, to the comigration of bands produced by different loci. Finally, as described by these same authors, the probability exists that some of the RAPD markers will exhibit a codominant behaviour once transformed into SCARs.

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