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Quality of forest reproductive material in the field of the application of European Community rules

Qualité du matériel forestier de reproduction et application des directives communautaires

Edited by : Daniel Terrasson

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Preface

The proceedings of E.C. Scientific Workshop "Quality of forest reproductive material in the field of the application of E.C. rules" are reported in this publication. The workshop was held under the auspicies of the Commission of the European Community, Directorate General for Agriculture, in Paris on December 9-10, 1993.

The participants in the workshop came from Forest Research Institutes, Universities, Forest Agencies and the Commission of the European Communities. In total, 47 forest researchers and managers from 15 countries (European Union, Norway) attended the meeting.

Objective and context

Until a recent past foresters used to think that selection realised during the life of a stand exempted themselves from paying attention to the genetic quality of forest material. But the increament of afforestation practise, and some ill-considered genetic transfers led to occasional obvious failures. These situations have stressed the necessity to take into account the origin of forest reproductive material, and to have a minimum selection for seed-stands. Therefore European Community decided to rule the commerce of forest reproductive material, which was completed through directives 66/404 and following texts.

On the other hand, because of genetic improvement achievements, foresters are now offered a choice between a "wild" material croped on natural stands, and an "obtained" material produced by more and more complex techniques.

At a scientific level, the application of the European rules raise three main issues :

- how to **identify** forest material ?
- This identification is defined in legal term by the trilogy : Distinction, Homogeneity, Stability, in which the two last words are related to the conformity with a standard ;
- how to **evaluate** the material ?

- This covers scientific legal and operational aspects with a different view for "wild" or "obtained" materials ;

- how to **use** the material ?

This issue is raised, not only by tree-planters, but also by the social community as a whole, since everybody feels strongly concerned about the stability of forest stands in the long term (conservation of biodiversity, managment of genetic resources).

Publication

This publication contains the papers presented at the Scientific Workshop. The presentations where organized into four sessions :

1. Identification of forest reproductive material

2. Evaluation of forest reproductive material

3. How to use forest reproductive matérial ?

4. Conservation of genetic resources

At the end of the seminar the participants identified gaps in knowledge and European directives, and encircled promising topics for forest research and international actions. This is reported in the synthesis at the end of the proceedings.

The commission of the European Communities thanks Mr Daniel TERRASSON, Cemagref, for his help in organizing the seminar, and the participants for their interesting contributions to the lectures and the discussions.

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Identification of forest reproductive material

Chairman : Svern M.G. DE VRIES

Use of biochemical and molecular markers for identification of forest reproductive material

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Abstract

This contribution addresses the applicability of biochemical and molecular markers for identification of forest reproductive material, originating from actual forest stands. Material originating from seed orchards is not considered. Genetic prerequisites of markers for identification purposes are first discussed. The different markers are compared for the level of polymorphism and differentiation that they reveal. Current available results are reviewed for identification of (i) single trees, (ii) populations and (iii) groups of populations. Finally, a general methodology is proposed combining genetic information of nuclear and cytoplasmic markers. It consists in a two step procedure : delineation of regional units based on multilocus analysis of nuclear markers and stand identification based on maternally inherited cytoplasmic markers.

Key-words : *Terpenes, isozymes, DNA, seed certification, forest trees, polymorphism, differentiation.*

Introduction

In the past thirty years, various biochemical and molecular markers have been developed in forest trees. These have been widely used for investigating levels and distribution of polymorphism in natural populations. For example, 935 citations were reported in a bibliographic review on isozymes in forest trees (Paule, 1990). Despite their success in genetic inventories, their application towards identification purposes has only been scarcely addressed. Among the 935 citations, only 15 concerned seed certification. It is the objective of this contribution to examine and compare critically the applicability of different markers for identification purposes : (1)

prerequisites of markers will be outlined, (2) genetic properties will be reviewed, (3) current potential applications will be stressed and finally (4) details of a proposed methodology with current available techniques will be given.

There are three different kinds of markers routinely used for genetic inventories in forest trees : secondary metabolites (terpenes), isozymes and DNA fragments. Separation techniques will not be detailed in this contribution ; they are given elsewhere (Baradat and Marpeau ; 1991, for terpenes ; Bergmann, 1991, for isozymes ; Szmidt and Wang, for DNA fragments, 1991). Only their application for identification of single trees and natural populations (or groups of population) will be addressed. Genetic units of artificial origin (seed lots from various seed orchards) will not be considered.

1. Prerequisites of genetic markers to be used for identification purposes (Table 1)

1.1 Phenotypic expression

Phenotypic expression of markers should be free of environmental influence, e.g. heritability of theses traits should be 1. In a similar way, their expression should be independent from the developmental stage of the tree. Whereas tissue specific, or development specific terpenes or isozymes have been reported, DNA markers have the great advantage to be present in any organ or tissue.

Marker	Separation technique	Inheritance	Gene action	Locus specificity	Reproducibility
Secondary metabolites	Gaz chromatography	Mendelian	Dominance	?	Fair
Isozymes	SGE or PAGE	Mendelian	Codominance	Yes	Fair
DNA Fragments					
* Cytoplasmic	RSBA or Uniparental PCR		Yes	Good	
* Nuclear	RSBA	Mendelian	Codominance	Yes	Excellent
	Targeted PCR	Mendelian	Dominance	Yes	Excellent
	Random PCR	Mendelian	Dominance	?	Poor

SGE : Starch gel electrophoresis PAGE : Polyacrymalide gel electrophoresis
RSBA : Restriction Southern Blot Analysis (leading to RFLP : restriction fragment length polymorphism)
PCR : Polymerase chain reaction

Table 1 - **Prerequisites of biochemical and molecular markers**

1.2 Inheritance

Inheritance of the markers should be well established beforehand in segregation studies based on controlled crosses. Two modes of inheritance may be considered :

- biparental, for nuclear markers. In this case, mendelian segregations must be checked.
- uniparental, for cytoplasmic DNA markers. Complete uniparental inheritance for organelle DNA markers may not be always the case ; preferential but not exclusive uniparental transmission has been reported in some cases (Wagner *et al.*,1991)

1.3 Gene action

Codominant markers permit the identification of all classes of genotypes and should always be preferred to dominant markers. However if numerous dominant markers are available, their resolution for identification purposes is as powerful as a few codominant markers. In this respect the RAPD technique (Random Amplified Polymorphic DNA), which allows to assess numerous fragments should not be discarded, even if most of them are dominant.

1.4 Locus specificity

Markers with identical phenotypic expression (for example, same length for DNA fragments) should correspond to the same chromosome location. Locus specificity arises as a problem particularly for PCR (Polymerase Chain Reaction) derived markers. When genomic amplification is used to obtain markers in two genotypes A and B, electrophoretic profiles may exhibit fragments of similar sizes in both genotypes. Similar size does not necessarily refer to the same locus. The same difficulty may occur when electrophoretic profiles comprise numerous fragments and fragments of similar size can be confounded. In either case, locus specificity can be checked using molecular hybridization with radiolabelled probes.

1.5 Repeatability of separation techniques

Identification of reproductive material necessarily implies the application of separation techniques in different laboratories. Therefore these techniques should be standardized beforehand.

2. Desired properties of genetic markers to be used for identification purposes

Optimal properties of markers to be used for identification purposes are (1) that they are polymorphic, e.g. that there are present with different variants in a species and (2) that these variants are unevenly distributed between the genetic units that have to be identified, e.g. that these units can be differentiated. Experimental results and theoretical predictions show that the current markers differ largely in the level of polymorphism and differentiation that they reveal. These two properties are independent: highly polymorphic markers are not necessarily those that differentiate genetic units and *vice versa*.

2.1 Polymorphism (Table 2)

The level of polymorphism that a genetic marker can reveal can be expressed at different levels (alleles, genotype, gamete, zygote). The corresponding parameters used to estimate polymorphism all depend on the number of the existing variants at these levels and on their frequency distribution. It is out of the scope of this contribution to review the different parameters, however the different markers currently available differ largely in their ability to reveal polymorphism.

Terpenes are limited in providing polymorphism. Quantitative assessments of these molecules only permit to identify two variants for a given locus (richness or poorness of the concentration). The separation of these variants becomes even more difficult when dominance relationships exist. Furthermore only a small number of terpenes have been so far genetically characterized : 3 monoterpenes and 2 sesquiterpenes in the case of Maritime pine (Baradat, 1988).

Whereas numerous variants can be electrophoretically separated for isozymes, their frequency distributions show that there is usually one highly frequent allele and several alleles with low frequency. As a result the effective number of alleles, for polymorphic isozymes, varies between two and three. The number of isozymes that can be assayed is also limited. In most studies, the number of loci varies between 10 and 20.

Provided that resources are available, the number of loci that can be investigated with nuclear DNA markers is unlimited, whatever current technique is used (restriction-hybridization or DNA amplification). Chloroplast DNA molecules should be considered as one single locus, if one assumes that no recombination occurs. The nuclear genome is extremely heterogeneous as regards the polymorphism. Mutation rates vary by a factor 100 from coding regions to hypervariable minisatellites and microsatellites.

These latter genomic zones are constituted by short DNA sequences that are repeated in tandem throughout the genome, or located in specific zones. Satellites vary in the number of repeats, and the polymorphism is so high that they can be used to fingerprint various genotypes.

Marker	Mean Number of loci	Number of variants per locus		Total number of variants		
		Alleles	Genotypes	Alleles	Genotypes	
Secondary metabolites	5	2	3	32	243	a
Isozymes	15	2	3	32768	$14*10^6$	b
DNA Fragments						
Cytoplasmic	1	10	10	10	10	c
Nuclear	?	?	?	?	?	

a) - Baradat and Marpeau, 1991
b) - Hamrick et al., 1992
c) - Demesure and Petit, personal communication

Table 2 - **Comparative levels of polymorphism revealed by each marker**

2.2 Differentiation among populations (Table 3)

Differentiation among populations is of main concern here for identification purposes of populations or groups of populations. Actual levels of differentiation among forest tree populations result from a balance between selection and genetic drift on one hand and migration among populations on the other hand. Various parameters are used to assess levels of differentiation (coefficient of gene differentiation of Nei (1973), Gst ; average differentiation of Gregorius (1987)). Levels of differentiation may vary markedly between neutral and non neutral markers.

Neutral markers

For neutral markers, levels of differentiation can be predicted from theoretical developments taking into account population sizes, and gene flow (Petit *et al.* 1993b). Forest trees are mostly allogamous and have large populations sizes. As a result, among population variation of variant frequencies of the nuclear genome is therefore expected to be low, as confirmed by experimental results reviewed by Hamrick and coworkers (1992). As shown by their review, Gst values obtained from isozyme studies in most forest trees are less than 10%. To illustrate this result, differentiation between two hypothetic populations, in the case of a diallelic locus, amounts to 9% when one allele exists with frequency 0.3 in one population and 0.5 in the other population. There have been extensive results obtained with isozymes on more than 160 species from the temperate to the boreal zones. Exceptions to

the low differentiation are found for species with scattered geographic distribution, where populations may have been isolated. These expectations need however to be confirmed by experimental data. Results obtained from population surveys with neutral nuclear DNA markers are rare, however the expectations are that the level of differentiation should not be higher than with isozymes, even if the markers used exhibit higher levels of polymorphism. The reasons are that their actual levels of differentiation depend on the same evolutionary factors and that at equilibrium between these factors, an identical level of differentiation is expected, regardless of the level of polymorphism.

Marker	Pattern of population differantiation	Reference
Secondary Metabolites	Monolocus level : fixation or loss of a given allele in particular populations. Multilocus level : regional differentiation	a, b, c
Isozymes	Monolocus level : extremely low differantiation (Gst<5%) Multilocus level : regional differantiation	d
DNA fragments		
Cytoplasmic	Extremely high differantiation for maternally inherited organelles (Chloroplasts and mitochondria in broadleaves) Low differantiation for paternally inherited organelles (chloroplasts in conifers)	e, f
Nuclear	No data available on range wide studies. Expected levels of differantiation for neutral markers are similar to those obtained with isozymes. Higher levels are expected for markers linked to adaptive traits.	

a) Baradat and Marpeau, 1991 - b) Müller-Starck et al., 1992 - c) Hanover, 1992
d) Hamrick et al., 1992 - e) Petit et al., 1993 - f) Petit et al., 1994

Table 3 - **Comparative levels of differanciation revealed by each marker**

A clear difference should be made here between uniparental and biparental inherited markers. Since cytoplasmic markers are only transmitted by one parent, populations sizes corresponding to their genes, and migration rates should be lower than for nuclear genes. As a result, the former should be more affected by genetic drift than the latter and higher levels of differentiation should expected for cytoplasmic than for nuclear genes (Petit *et al.*, 1993b). The discrepancy of the level of differentiation between cytoplasmic and nuclear genes should be proportional to the difference in migration between uniparentally and biparentally inherited genes. Differentiation should be much higher for markers with maternal inheritance than paternal inheritance, since seed migrate generally at lower distances than pollen. These theoretical predictions were confirmed by experimental results obtained in oaks and pines. In oaks, chloroplasts are maternally inherited : migration of organelle genes are therefore limited by seed dispersal. On a range wide scale in *Q. petraea*, the coefficient of differentiation was 2 % for isozymes (nuclear markers) and 86% for