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Computer-Aided Search for Codominant Markers in Complex Haploid DNA Banding Patterns – A Case Study in *Abies alba* MILL.

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Dedicated to GEORG HEINRICH MELCHIOR on the occasion of his 70th birthday

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Abstract

A genetic trait defines a gene marker in a collection of diplophase individuals only if each phenotype is produced by only a single genotype. This requires codominance as the mode of gene action at each of the involved loci (as opposed to dominance) and the absence of epistasis. Two computer programs are presented that aid in inheritance analysis of complex DNA banding patterns of single diploid individuals by searching the banding patterns of their haploid gametophytes for pairs of segregating bands representing codominant alleles of a locus as well as for single bands showing dominance over a "null allele" defined as absence of the band. The program MATRIX[3]) encodes densitometrical data obtained from DNA banding patterns as a 1/0 matrix. The program CoDo[3]) searches this matrix for pairs of bands showing 1:1 segregation and for single bands showing 1:1 segregation with a null allele. This system is demonstrated on both a fictitious data set and on banding patterns obtained by PCR using a primer pair designed from an M13 bacteriophage sequence in an individual of *Abies alba* (MILL.) and the haploid primary endosperm of a sample of its seeds. For the latter, no band pair showed codominance of gene action, but several bands were suggested for dominance. Reasons for the small number of bands showing 1:1 segregation are discussed.

Key words: DNA marker, haploid primary endosperm, inheritance analysis, gene marker, codominance, computer program, *Abies alba*, M13 fingerprint, population genetics.

FDC: 165.3; 165.42; 174.7 *Abies alba*.

Introduction

In many population genetic investigations, differences between the genetic structures of different populations of a species or of subpopulations of a population are analysed. The genetic structure of a collection of individuals is given as frequency distributions of alleles and genotypes at 1 or more loci. Often, the purpose of such investigations is to find associations between genetic structures and population genetic processes (e.g., to separate adaptive subpopulation differen-

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[3]) Microsoft Windows version of the program package CoDo and MATRIX is currently being developed and will soon be available.

tiation from stochastic differentiation by genetic drift). An application of particular current interest is the prediction of the adaptive potential of a population as an important criterion in the declaration of genetic resources. In some cases, studies of genetic structures and their functional associations require additional information obtainable by reconstruction of descent (e.g., identification of clones, species, provenances, or inference of parental genotypes or genealogical relationships between individuals).

Both quantification of genetic differences between collections of conspecific individuals and reconstruction of descent are performed with the help of genetic markers. Extensive population genetic studies of tree populations have been carried out during the past 25 years using isozyme gene loci, most of which classify as gene markers (see below) due to their codominance of gene action. In the past decade, genetic inventories have also used DNA markers, which results from a combination of electrophoretic separation of DNA fragments by length (i.e., number of base pairs) and some method of making certain fragments visible. Many DNA traits have been treated as gene markers, despite warnings against misinterpretation of the DNA banding patterns (LYNCH, 1988; WILLIAMS et al., 1990; GILLET, 1991 and 1993; WELSH et al., 1991; KENNARD et al., 1994).

Gene markers are defined on the basis of the following hierarchy of marker properties: DNA banding patterns are initially simply phenotypes, i.e., states of a trait that is defined by the specific methods of a particular DNA analysis. A set of characters of members of a collection, e.g., of conspecific individuals, defines a *trait* in the collection, if each member possesses exactly one of these characters as its *phenotype*. Precise description of the characters (including nuclear phase, ontogenetic stage, tissue type of the individual) and the environment in which they were scored (including exact laboratory methods for molecular markers) is necessary to ensure uniqueness of each individual's character and comparability of characters between individuals. As a rule, DNA traits are *genetic traits* over the set of all environments in which individuals of the species are viable, because the banding pattern of an individual does not change when the individual (or its genetically identical copy) is placed into any of the other environments. A genetic trait can be designated a *genetic marker* only after successful performance of an *inheritance analysis* to determine the *mode of inheritance* of the phenotypes. The latter consists of the *mode of transmission* (number of controlling loci, identification of the alleles at each locus) and the *mode of gene action* (type of interaction in phenotype production between alleles of each locus, e.g., dominance and codominance, and between genes of different loci, e.g., epistasis). Inheritance analysis yields the assignment of each phenotype to the set of (single or multi-locus) genotypes that produce it. Finally, a genetic marker defines a *gene marker*, if each phenotype is produced by only 1 genotype, since only in this case does each phenotype unequivocally identify all of the genes (alleles) at the involved loci. A genetic trait showing codominance of gene action at each of the involved loci and absence of epistasis qualifies as a gene marker.

DNA traits are expressed as patterns of bands (electropherograms) formed by DNA fragments that have been marked using some process involving DNA hybridization and separated electrophoretically according to length (i.e., number of base pairs). The length of a fragment visible as a band can be calculated from the position of the band relative to a standardized scale of bands of known fragment lengths. Each DNA trait is specified not only by properties of the individual expressing the trait (ploidy level, tissue type, ontogenetic stage, etc.) but also by the laboratory protocol by which it was produced; this protocol must be exactly followed for all individuals belonging to the collection under study.

In DNA analysis, a locus shows codominance of gene action, if in every individual all of the (transmission homologous) DNA fragments defining the locus are visible as fragments of the same length (homozygosity) or different lengths (heterozygosity). Hence, codominance is simply the absence of invisible DNA fragments at the considered locus. Epistasis is absent if fragments from different, recombining loci always have distinguishably different lengths.

However, many of the DNA traits which have been shown to be genetic markers controlled by one or more nuclear gene loci failed to be gene markers (BRADSHAW et al., 1994; KENNARD et al., 1994). Instead of each of the involved loci showing codominance as segregation of pairs of visible fragments, many of the inferred loci were expressed only as the phenotypes "presence" or "absence" of a visible fragment at a certain position in the banding pattern, the latter meaning that the transmission homologous fragment is invisible. An invisible fragment is often termed a "null allele". Invisibility results from differences in the DNA sequence at the same locus on a homologous chromosome that lead either to fragments of lengths outside of the range of visibility or to failure of the marking DNA to hybridize with the altered sequence (DEVLIN and RISCH, 1992). If a null allele is known to exist at a locus, then in diplophase individuals the visibility of a fragment belonging to the locus does not indicate whether the individual is homozygous for the fragment or heterozygous for the fragment and a null allele. Thus the genotype of the individual at this locus is not inferrable from its phenotype. If at least one of the loci expressing a trait shows such gene action, termed dominance, then the trait is not a gene marker. Epistasis is also frequently encountered in DNA fingerprints used for forensic purposes, as is documented by the the "fixed bin" approach discussed by DEVLIN and RISCH (1992) and WEIR (1992).

Markers with dominant or epistatic gene action are of little use for investigating allelic and genotypic frequency distributions in populations of diplophase individuals of unknown genealogical relationships, if only the phenotypes of the individuals are known. Estimation of allelic frequencies in populations according to expectations dependent on model assumptions, such as HARDY-WEINBERG-proportions under the assumption of random mating with respect to the marker, is not permissible as long as fulfillment of the assumptions has not been demonstrated. Inference of an individual's genotype by analysis of progeny phenotypes is expensive and time-consuming and thus impracticable for larger genetic inventories.

Uncertain results are also often obtained in analyses that attempt to infer genetic or genealogical relationships from DNA phenotypes that probably do not fulfill the properties of a gene marker. In the few cases among a wide range of organisms in which it has been performed, progeny analysis has shown that the frequency of loci showing heterozygosity for a visible fragment and a null allele in individuals is high in DNA traits that show the high degree of phenotypic variability desirable for reconstruction of descent, especially RAPDs (WILLIAMS et al., 1990) and RFLP-fingerprints (JEFFREYS et al., 1985a).

The ideal situation for inheritance analysis of genetic traits of diplophase individuals is that in which the involved loci are also expressed in haplophase gametophytes. Since the genetic information possessed by an individual's gametophytes is the product of meiotic segregation of the individual's DNA, the

alleles of a locus are recognizable by segregation of the gametophytes' phenotypes. Inheritance analysis of isoenzyme banding patterns (zymograms) in diploid tissue of conifers (needles, buds) has long been performed using the zymograms of the maternally transmitted haploid primary endosperm (megagametophyte) of seeds collected from single individuals, since it can eliminate the need to study offspring of controlled crosses (BERGMANN, 1973, and innumerable others since). Inheritance analysis of diploid DNA banding patterns has also been performed using the haploid patterns of megagametophytes (JERMSTAD et al., 1994). In angiosperms, methods of andro- or gynogenesis or single pollen analysis could provide analogous material.

Fundamental differences exist between DNA and isoenzyme banding patterns, however, which affect their genetic interpretation. The number of bands visible in complex DNA banding patterns, especially in individual-specific DNA fingerprints, is often a multiple of the number resulting from isozyme analysis of an enzyme system. Whereas zymograms usually can be divided into various "zones", which inheritance analysis shows to be expressed by the different enzyme loci, such prestructuring is not characteristic of DNA banding patterns. Fragments representing different alleles of the same locus can be of arbitrary length and appear as bands anywhere in the DNA patterns (or nowhere, in the case of a null allele).

The program CoDo was developed to aid in inheritance analysis of DNA banding patterns of diplophase individuals by searching the banding patterns of their gametophytes for segregating bands that represent the alleles of a locus with a codominant mode of gene action. Each band that is found to lack a transmission homologous counterpart in other gametophytes is also tested for the properties of a locus showing dominance of gene action. A second program, MATRIX, serves as an interface between densitometrical measurements of the DNA banding patterns and CoDo. Operation of the programs is demonstrated on a fictious set of banding patterns as well as on actual patterns observed in an individual of silver fir (*Abies alba* MILL.) and a sample of its megagametophytes. The latter were obtained by PCR using a primer pair designed from an M13 bacteriophage sequence.

Description of the Program CoDo

Characteristics of input data

The input data to the program CoDo are in the form of a matrix of 0's and 1's. The first column of the matrix represents the banding pattern of a diploid plant and each of the other columns the banding pattern of one of the plant's (haploid) gametophytes.

The number of rows of the input matrix is determined by the total number of different positions in which a band appears in the pattern of the plant or at least one of the gametophytes. In each column, a "1" in the row corresponding to a band position indicates the appearance of a band in the pattern of the corresponding gametophyte resp. plant, and a "0" the absence. A hypothetical example is shown in *table 1*.

Table 1. – A hypothetical banding pattern and the corresponding 1/0 matrix.

band position	*banding pattern*			*1/0 matrix*		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
1	—			1	0	0
2		—		0	1	0
3	—	—	—	1	1	1
4		—		0	1	0
5	—			1	0	0
6		—	—	0	1	1

The banding patterns of our experimental gels were scanned from Polaroid negatives (type 55). Scanning was performed by a personal densitometer (Personal Densitometer™ PC version) with integrated ImageQuaNT™ software (Molecular Dynamics). Fragment lengths, measured as numbers of base pairs, were assessed by FragmeNT Analysis software version 1.1 (Molecular Dynamics).

We developed a program MATRIX which determines bounds for the fragment length of each band according to gel specific migration characteristics and user-defined level of coarseness and which transforms the band positions into a 1/0 matrix (see above). MATRIX operates in 2 steps. In the first step the program sorts all fragments according to their lengths. In the second step, the user specifies the maximum number of base pairs (bp), termed the "coarseness" of the pattern interpretation, by which 2 fragments in different patterns are allowed to differ in length and still be assigned to the same band position. This accounts for the "noise" often observed in gels that causes identical fragments to appear in slightly different positions. For example, if the user chooses a coarseness of 20 bp, then the 2 fragments are assigned to the same band position if the difference in their length does not exceed 20 bp. If the length difference exceeds 20 bp, MATRIX considers the fragments to belong to 2 different band positions. If 2 fragments are of the "same" length with respect to the coarseness, then their lengths form the bounds of their common band position. Referring to the convention in DNA-analysis of picturing the path of electrophoretic migration from the top to the bottom of a banding pattern, thus locating smaller fragments below larger ones, the length of the smaller of 2 "different" fragments defines the upper bound of the lower band position, while the length of the larger fragment defines the lower bound of the upper band position. The lower the coarseness, the greater is the danger of assigning identical fragments to different positions due to the above-mentioned noise. Setting the coarseness too high can result in loss of information by causing fragments of different lengths to be assigned to the same position. The program MATRIX serves as interface between the densitometrical raw data and the inheritance analysis program CoDo.

Mode of Operation

Given the DNA banding pattern of a diploid plant and a sample of its gametophytes, a band position defined with the help of MATRIX corresponds to a range of fragment lengths within which the plant or at least one of its gametophytes exhibits a band. The width of this range is specified in MATRIX as the coarseness. In the *first* part of CoDo, all band positions are identified that fulfill the following 3 elementary prerequisites for correspondence of the position to a codominant or dominant allele at a locus at which the plant is heterozygous:

1. If a band appears at this position in at least one of the plant's gametophytes, then a band appears at the same position in the plant: Non-fulfillment can be caused by somatic mutation in the plant in a cell line different from those produced the gametophytes or in a gametophyte, or by meiotic recombination.

2. If a band appears at this position in the plant, then a band appears at the same position in at least one of the plant's gametophytes: Non-fulfillment may stem from an organelle that is not transmitted by the plant to the investigated gametophytes or may be the result of somatic mutation in the plant in

a cell line different from those that produced the gametophytes.

3. The band position shows variation, in that not all of the plant's gametophytes possess a band at this position: Non-fulfillment, i.e., "fixation" of the band, implies either that the plant is homozygous at the locus corresponding to the band or that the band stems from an organelle whose (extranuclear) DNA is fixed (i.e. non-variable) in the plant.

In the *second* part, CoDo searches for pairs of band positions among those identified in the first part that have the following additional properties that are characteristic of transmission homologous DNA fragments showing codominance of gene action:

4. The bands at these 2 positions segregate among the gametophytes, i.e., each gametophyte has a band in 1 but never both of the positions. (Part 1 guarantees that a band appears at either position in at least 1 gametophyte and that bands appear at both in the plant.) This is the prerequisite for transmission homology of the 2 corresponding DNA fragments.

5. The proportions of gametophytes in which either band appears correspond to regular (1:1) meiotic segregation, as tested using the log likelihood ratio (G) and the Chi^2 tests of goodness-of-fit.

If a pair of band positions fulfills 4. and 5., the 2 fragments appearing as the bands in these positions are strong candidates for *codominant* alleles of a locus. The probability that some pair of non-allelic bands would falsely obey 4. depends on how many of the possible gametophyte banding patterns have actually been observed, which in turn depends on the sample size and the actual number of variable gene loci contributing to the pattern; false fulfillment of 4. may be recognizable as a violation of step 5. On the other hand, violation of 4. unequivocally disqualifies a band pair from representing alleles of a locus, if epistasis due to the appearance of fragments of different loci at the same band position can be ruled out and assuming that the assignment of each band its position is correct.

The problem of correct assignment of band positions is particularly acute in the case of complex DNA fingerprints, in which a wide spectrum of many different fragment lengths is visible. Accordingly, distinction of neighboring band positions can be difficult. (Assignment of a band to the wrong position mimics epistasis.) Nevertheless, if pre-analysis is performed to identify those positions that have a good chance of representing codominant alleles of a locus, then a second electrophoretic screening could magnify the region around these positions and thus allow accurate assignment and analysis. CoDo can aid in such a *pre-analysis* by allowing prior specification of an error threshold for the requirement in step 4. For this purpose, define 2 values for each pair of band positions:

a. The observed relative frequency SP of those of the plant's gametophytes that show simultaneous presence of bands in both of the positions (co-presence).

b. The observed relative frequency SA of those of the plant's gametophytes that show simultaneous absence of bands in both of the positions (co-absence).

Given any pair of band positions fulfilling part 1 of CoDo, 2 alternatives exist. The one is that the fragments appearing at these positions are allelic with codominant gene action, in which case SP = SA = 0 (step 4.). The other is that the 2 band positions actually represent 2 different loci. Assuming random association of the alleles at the loci and regular segregation, SA and SP are both expected to equal 0.25. Yet even if segregation distortion or moderate genetic linkage are in effect, SA and SP should differ significantly from the value of 0 expected under the first hypothesis. If not, i.e., if SA or SP are close to but not equal to 0, then an error in band assignment is likely to have occurred. CoDo allows the user to define an *error* threshold "epsilon" less than 0.25 but greater than 0. If a band pair is found to have SA or SP greater than 0 but less than "epsilon", then the gametophytes showing simultaneous presence or absence are scored as being erroneous and are not considered in the test of 1:1 segregation of this band pair in step 5. If SA or SP is greater than "epsilon", the banding patterns of all gametophytes with respect to this pair of band positions are considered to be correct and the pair is ruled out as a candidate for codominant alleles.

In its *third* part, CoDo tests all band positions that fulfilled steps 1 to 3 for fulfillment of the property of *dominance* of gene action in the sense described in the introduction, such that the plant is heterozygous for "presence/absence" of a band at this position:

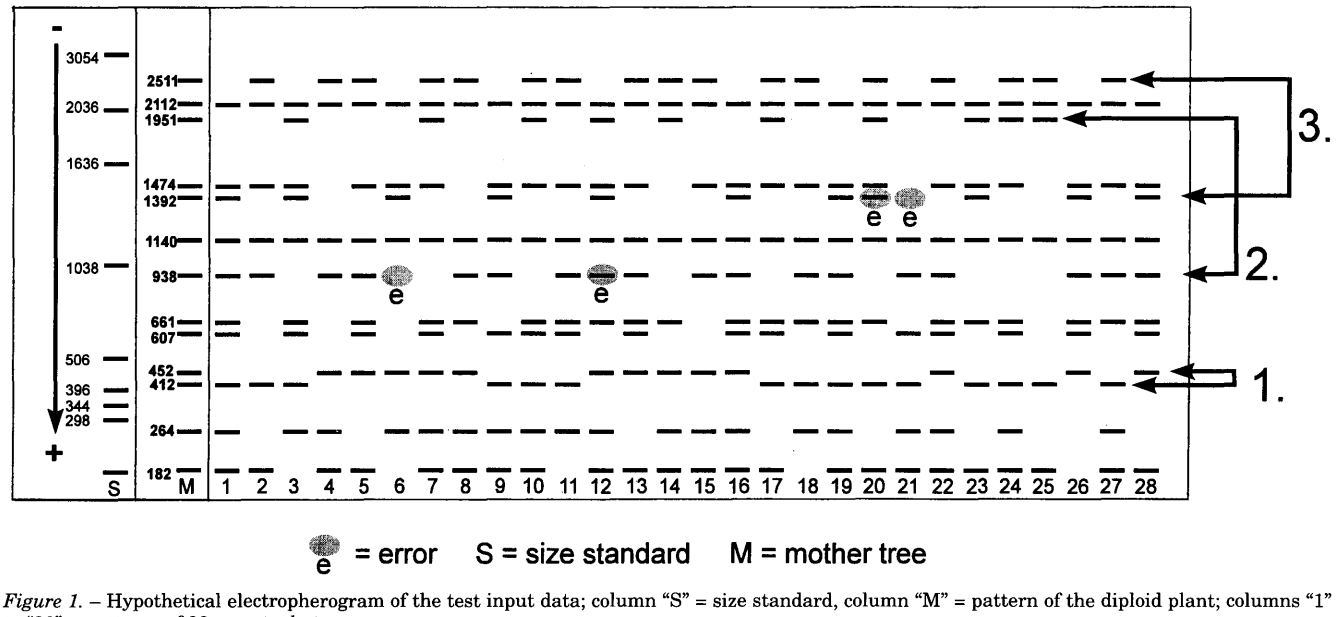

Figure 1. – Hypothetical electropherogram of the test input data; column "S" = size standard, column "M" = pattern of the diploid plant; columns "1" to "28" = patterns of 28 gametophytes.

6. The proportions of the plant's gametophytes that show "presence" as opposed to "absence" of a band at this position correspond to regular (1:1) meiotic segregation of these 2 types of gametophytes, as tested using the log likelihood ratio (G) and the Chi^2 tests of goodness-of-fit.

Note that both members of any pair of band positions that qualified as a candidate for codominance of gene action also fulfill 6.

Analysis of a Fictitious Data Set

Prior to analysis of real empirical data, we tested the power of CoDo on different fictitious data sets. Figure 1 shows 1 of the sets of fictitious but realistic DNA banding patterns. These patterns were subjected to the procedure described above (scanning, band identification, fragment length assessment, transformation of banding pattern to 1/0 matrix).

Every column represents the pattern of one individual for 13 band positions. Column "S" represents a size standard and column "M" (for "maternal") shows the pattern of the diploid plant. The other columns represent the pattern of 28 of the plant's gametophytes. The data set was designed to contain three candidates for loci with codominant alleles (see band regions marked by 3 pairs of arrows in *figure 1*). Pair No. 1 indicates 2 band positions, both of which are present in the diploid plant and are never simultaneously present or absent in any gametophyte. In contrast, the band pairs indicated by arrow pairs 2 and 3 have built in errors marked by an "e" in *figure 1*.

Table 2. – Absolute and relative frequencies for the remaining band positions after program step 3 for the gametophytes.

base pairs of the band	*absolute frequency*	*relative frequency*
264	19	.679
412	15	.536
452	13	.464
607	16	.571
661	21	.750
938	18	.643
1392	11	.393
1472	23	.821
1951	10	.357
2511	17	.607

After application of CoDo steps 1 to 3, 10 band positions remain to be considered in steps 4 to 6. *Table 2* shows the calculated sizes of the respective fragments in numbers of base pairs as well as the absolute and relative frequencies of the presence of these bands among the plant's gametophytes. Assuming regular segregation, each of these bands is expected to appear in one-half of the gametophytes. In *table 2* several bands approximate the corresponding value of 0.5.

The program also calculated SP and SA among the plant's gametophytes for each pair of the remaining ten bands in accordance with step 4. An error threshold of "epsilon" = 0.10 was chosen. All but the 3 pairs of bands shown in *table 3* were disqualified for codominance of gene action by values of SP or SA exceeding "epsilon". CoDo tested each of these three pairs for regular segregation (step 5), leaving supposedly erroneous gametophytes (showing simultaneous presence or absence of both bands of the respective pair) out of these calculations.

Table 3. – Pairs of bands, frequency of their co-absence and co-presence, and observed and expected segregation proportions; ns = no significant difference by G-test between expected and observed segregation properties on the 5% level.

combination of bands	*frequency of co-absence (SA)*	*frequency of co-presence (SP)*	*segregation observed / expected*	*G-test*
412/452	0%	0%	15:13/14:14	.14 ns
938/1951	3.6%	3.6%	17:9 /13:13	2.5 ns
1392/2511	3.6%	3.6%	10:16/13:13	1.4 ns

CoDo indicates that band positions 412bp and 452bp represent 2 codominant alleles of a locus (*Table 3*). No error was detected, and deviation from regular segregation is not significant. For the other 2 candidate pairs, the program suggested that 4 errors in assignment had been made and confirmed regular segregation after exclusion of erroneous gametophytes. This result suggests that reanalysis of these band positions may reveal errors in band position assignment, such that step 4. will be fulfilled by the correct data. If so, and only if the number of gametophytes is sufficient to rule out chance fulfillment of 4. and 5. by non-allelic bands, can a pair of bands qualify as codominant alleles of a locus.

In its third part, CoDo identified 2 additional bands (264bp, 607bp) as candidates for dominant gene action due to their fulfillment of steps 1 to 3 and 6.

Analysis of Highly Polymorphic DNA Patterns of *Abies alba* Mill.

In forest tree species, the detection of DNA markers showing codominance of gene action and allowing differentiation between individuals is expected to provide a powerful tool in population genetics for reconstruction of descent of individuals. DNA fingerprinting is a promising molecular technique that can produce the desired individual-specific patterns. This technique was originally based on hybridization of multiple tandem repetitive sequences or hypervariable minisatellites to unknown target DNA, and the generated patterns were termed hypervariable Restriction Fragment Length Polymorphisms = RFLPs (Jeffreys et al., 1985a and b; Vassart et al., 1987). More recently, fingerprints have been performed using the PCR techniques with random primers (RAPD) or primers from ubiquitous repetitive sequences (Williams et al., 1990; Caetano-Anollés et al., 1991; Lin et al., 1994; Chong et al., 1995). The AFLP technique (Amplification of Fragment Length Polymorphisms; patent of Zabeau and Vos, KeyGene) is also known to generate very complex and individual-specific DNA banding patterns (Peleman, 1995).

The fragments visible as bands in DNA fingerprints can represent either nuclear DNA alone or a mixture of nuclear and organelle DNA. Thus some fragments may follow biparental and others uniparental modes of inheritance. For purposes of identifying nuclear DNA loci with the desired codominance of gene action, the complex patterns must be subjected to inheritance analysis.

In various analyses reported on selected fingerprint fragments, both modes of gene action, dominance and codominance, could be found. For RAPD fingerprints, most of the fragments turned out to represent loci showing dominance of gene action (Williams et al., 1990; Carlson et al., 1991; Kazan et al., 1993; Bradshaw et al., 1994). The same was observed in AFLP fingerprints (Prabhu and Gresshoff, 1994). Nevertheless, some cases of codominant gene action have been reported for RAPD fingerprint fragments (Kawchuk et al., 1994; Kennard et al., 1994). DNA fingerprint patterns thus have to be regarded as a set of DNA phenotypes controlled by a mixture of loci of dominant and loci of codominant gene action. Thus a desirable aid to easy and rapid PCR fingerprinting techniques is the availability of computer-aided techniques, such as those described above, that can extract codominant loci out of highly

complex DNA banding patterns. This serves to automate and thus simplify the search in a species for codominant loci when a whole range of primer systems is used, each of which yields highly variable, presumably individual-specific DNA fingerprints.

Silver fir (*Abies alba*) MILL.) populations are mostly autochthonous throughout the wide range of the species. Moreover, most populations were founded by natural regeneration. Gene flow within and between populations is in the focus of interest of ecological geneticists.

For silver fir, a PCR fingerprint codominance test system is being developed. The basis for inheritance analysis is DNA from single tree megagametophytes (haploid primary endosperm). An initial primer pair has been run on this material, and the experimental gels serve to demonstrate how the developed computer programs MATRIX and CoDo work in practice.

This primer pair was designed from an M13 bacteriophage sequence. M13 bacteriophage sequences were found to cross-hybridizide with human DNA (VASSART et al., 1987) as well as with DNA of other animals and plants (RYSKOV et al., 1988; ZIMMERMAN et al., 1989). Individual-specific RFLPs generated by the M13 repeat probe have been reported for woody species, including both angiosperms and gymnosperms (ROGSTAD et al., 1988, 1991; NYBOM and ROGSTAD, 1990; NYBOM et al., 1990; NYBOM and HALL, 1991; VAHALA et al., 1991; KVARNHEDEN and ENGSTRÖM, 1992). Also, just recently, PCR fingerprinting was performed by use of an M13 universal primer with poplar and willow (LIN et al., 1994; CHONG et al., 1995) as well as with a fungus (STENLID et al., 1994).

We designed a primer pair from near the borders of an M13 bacteriophage DNA fragment that is bounded by *Bsm* I and *Cla* I restriction sites. This fragment has already been used as a probe in several RFLP fingerprintings (ROGSTAD et al., 1988; NYBOM and HALL, 1991). Our approach was guided by

- the necessity of obtaining DNA fingerprints from the small amounts of DNA that are extractable from single megagametophytes;
- the advantages of an easy and rapid PCR technique to analyze small amounts of DNA;
- the theoretically high probability of finding loci showing codominance of gene action by use of a primer pair that is likely to anneal to homologous chromosomal positions.

Material

PCR was performed on DNA isolated from megagametophytes contained in 50 seeds collected from one of the parents of a controlled crossing of *A. alba* (MILL.) as well as from needles of this parent. Crossed individuals are two individual mature trees, A and B, in a natural site in Nemcecy, Slowakia, growing a distance of about 90 m apart.

Methods

DNA-Extraction

Genomic DNA was extracted according to the minipreparation protocol of ZIEGENHAGEN et al. (1993). DNA concentration was measured in a fluorometer (HOEFER). Average yield of minipreparation was 1 µg DNA per megagametophyte and 2 µg per needle.

Amplification

Primers

Amplification of DNA was done by means of a primer pair from flanking regions of the *Bsm* I/*Cla* I restriction fragment of M13mp18 cloning vector (GenBank Accession: # X02513) The sequences are as follows: primer 1 (bp No. 1768 of M13mp18) 5' GTACTGGTGACGAAACTC 3' (1785 bp), primer 2 (bp No. 2531 of M13mp18) 5' ATCGATAGCAGCACCGTA 3' (2514 bp).

PCR-Conditions

Amplification was performed in 0.5 ml thinwalled reaction tubes (Perkin Elmer Corp.) within 25 µl volume of total reaction mixture. The PCR was run in a pre-heated DNA Thermal Cycler (Perkin Elmer Corp.) with cycle and temperature conditions as suggested by DEMESURE et al. (1995).

Visualization of PCR products

The PCR products were separated by electrophoresis in 0.7 % (w/v) agarose gel run in tris-borate buffer at 6V/cm for 45 min. followed by 8V/cm for 2 h 40 min. Within each run of PCR a contamination control (reaction mixture without template DNA) was simultaneously run. Visualization of patterns in the gel was done by UV fluorescence after staining with ethidium bromide (0.25 µg/ml). The patterns were scanned from Polaroid negatives (type 55). Scanning was performed by a personal densitometer (Molecular Dynamics).

Reproducibility of patterns

Reproducibility of the patterns was tested by 3 to 10 repeated PCR-runs for each megagametophyte. At least 1 repitition was performed using DNA template from needles taken from different positions of the maternal individual. Several reactions on the same template DNA were repeated in another Thermocycler (Type 9600, Perkin Elmer Corp.). All tests resulted in absolute reproducibility of the patterns, indicating that the patterns are very stable and that there is no intra-individual variation to be expected with respect to the investigated amplification products.

Specificity of amplification

Specificity of the amplification products was tested by increasing the stringency, either by raising the annealing temperature or lowering the salt concentration. Raising of annealing temperature up to 52 °C had no influence on the original patterns produced under annealing conditions of 49 °C. Also, patterns did not change when the salt concentration was lowered down to three-quarters of the original protocol.

Band determination

In order to standardize the starting position of the programme MATRIX, band determination in FragmeNT Analysis Software, version 1.1 (Molecular Dynamics) was fixed to 0.250 scale factor and 1.000 noise factor.

Results and discussion

Amplification of silver fir DNA by a primer pair from an M13 bacteriophage sequence generates hypervariable DNA fragment patterns. Of the 50 megametophytes subjected to PCR, the DNA of 32 of them was of sufficient quality to provide scorable fingerprints. *Figure 2* shows the banding patterns of the diploid mother tree A and the 32 megagametophytes.

The densitometrical data of the patterns transformed into fragment lengths were submitted to MATRIX. The assignment of bands to band positions was performed on 2 different user-defined levels of coarseness as described in Section 2.

For our experimental data we tried out 2 levels of coarseness that both guaranteed the correct assignment of obviously fixed bands to the same band positions with all megagametophytes.

Figure 3 shows the standardized banding patterns as output from MATRIX for our experimental lowermost level of coarseness = 20 bp. This pattern and the 1 for our experimen-

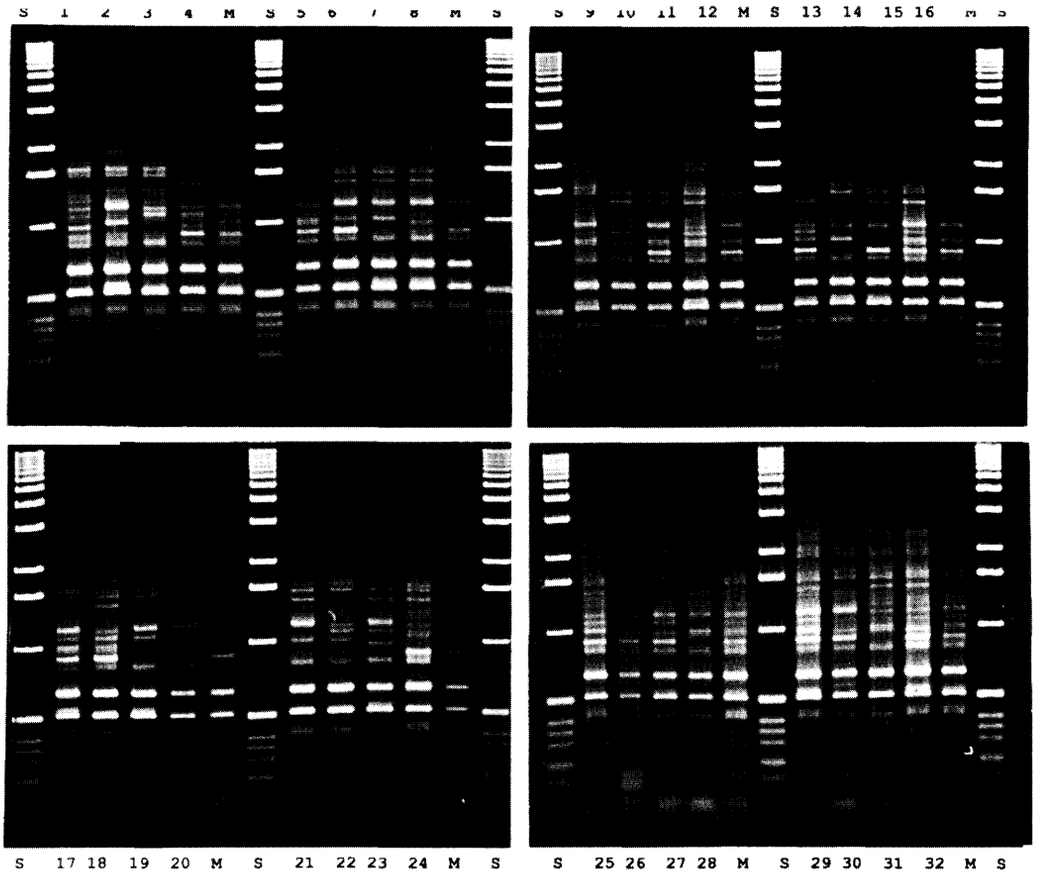

Figure 2. – Electropherogram of DNA from *A.alba* after amplification with a primer pair from an M13 bacteriophage sequence. M = banding pattern of diploid maternal tree; lanes 1-32: megagametophytes of maternal tree "M"; S = size standard (1KB Ladder, Gibco BRL).

tal uppermost coarseness = 15 bp (not shown) are transformed into a 1/0 matrix as input data set for CoDo.

Tables 4 and *5* give the results of the calculations in CoDo for matrices of both levels of coarseness (= 15 bp and = 20 bp).

Dependent on the 2 levels of coarseness, different numbers of band positions (94 resp. 74, see *Table 4*) are identified from the banding patterns. Of these, 71 bands (75 %) and 53 bands (71 %), respectively, were ruled out as candidates for co-dominant or dominant gene action by program steps 1 to 3 as follows:

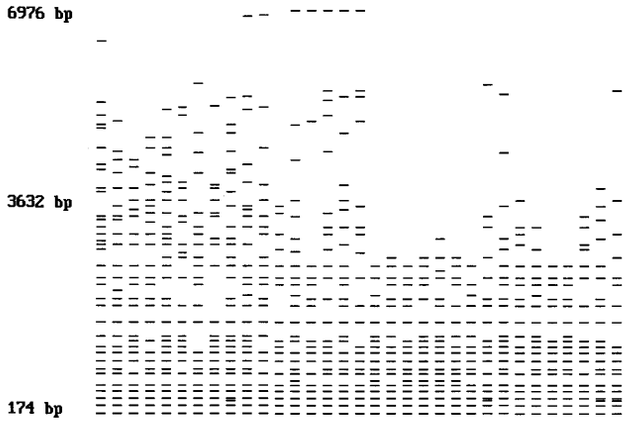

Figure 3. – Standardized banding pattern of our experimental banding pattern as output of MATRIX; first column represents fragment pattern of diploid mother tree, columns 2 to 33 represent fragment patterns of 32 megagametophytes; level of coarseness δ=20bp.

1. Bands were absent in most of the band positions in the mother tree (57 resp. 40). This might be due to the high frequency of meiotic recombination often discussed for mini-satellites whose core sequences are supposed to be putative recombination signals (JEFFREYS, 1986). Hypervariability could be caused especially by unequal crossing over (JEFFREYS, 1987).

2. Six (4) bands turned out to occur only in the mother plant. This could indicate that all gametophytes are recombinants for the corresponding fragments, although a recombination rate of 100 % would be surprising. Alternatively, these bands could stem from an organelle that is not transmitted to haploid endosperm (but see below).

3. Eight (9) of the band positions are fixed, i.e., bands are present there in the mother and all megagametophytes. If these represented nuclear DNA, they might stem from homozygous loci. An alternative explanation for the fixed bands is suggested by the detection in silver fir megagametophytes of haploid plastid DNA by a chloroplast DNA marker (ZIEGENHAGEN, unpublished data). This is noteworthy, since chloroplast DNA is paternally transmitted to the embryo in this species (ZIEGENHAGEN et al., in press). If contamination of the megagametophyte tissue by embryo DNA can be ruled out (see below), this suggests that plastids are present in the maternal gamete, but they are eliminated or "silenced" in the embryo.

Of the 23 (resp. 21) bands that fulfilled steps 1 to 3 of CoDo, none fulfilled the requirements of steps 4 and 5. No pair of bands was identified as candidates for codominant alleles, even if high error thresholds of "epsilon" = 0.2 for SP (simultaneous presence) and of "epsilon" = 0.5 for SA (simultaneous absence)

Table 4. – Summarized results of the calculations in CoDo for 2 levels of coarseness.

	level of coarseness δ	
	15bp	*20bp*
No. of identified bands	94	74
No. of bands absent in the mother tree	57	40
No. of bands absent in all gametophytes	6	4
No. of bands fixed in gametophytes	8	9
No. of remaining bands after step 1-3	23	21
No. of codominant band pairs	0	0
No. of dominant bands	5	6

Table 5. – Band position, frequency and observed and expected segregation proportions for candidates for bands with dominant gene action; ns = no significant difference by Chi²-test or G-test between expected and observed segregation properties on the 5% level.

level of coarseness δ *band position*	*frequency among gametophytes*	*segregation observed / expected*	*Chi²-test*	*G-test*
δ=15bp				
1478	0.625	(20:12/16:16)	2.0 ns	2.02 ns
2109	0.594	(19:13/16:16)	1.12 ns	1.13 ns
2360	0.625	(20:12/16:16)	2.0 ns	2.02 ns
3032	0.344	(11:21/16:16)	3.12 ns	3.18 ns
3209	0.344	(11:21/16:16)	3.12 ns	3.18 ns
δ=20bp				
1478	0.625	(20:12/16:16)	2.0 ns	2.02 ns
2109	0.594	(19:13/16:16)	1.12 ns	1.13 ns
2360	0.625	(20:12/16:16)	2.0 ns	2.02 ns
3024	0.375	(12:20/16:16)	2.0 ns	2.02 ns
3209	0.344	(11:21/16:16)	3.12 ns	3.18 ns
3330	0.375	(12:20/16:16)	2.0 ns	2.02 ns

were allowed. Simultaneous absence was a frequently observed phenomenon and might be due to faintness of the bands with large fragment lengths (*Figure 2*). Although endosperm was cautiously separated from the embryo, contamination of endosperm tissue by a minimal amount of tissue of the embryo cannot be ruled out. Especially in PCR fingerprints, this might cause the simultaneous presence of bands in the gametophytes.

In the third part of CoDo, 5 (6) candidates for dominant gene action were identified (*Table 5*). Comparison between the 2 levels of coarseness shows 4 of them to be "identical" which indicates the solid operation of MATRIX. Each candidate should be checked using an increased sample size to exclude systematic error.

In summary, our results are in accordance with those of other workers who have found codominance to be a rare event in DNA fingerprints (WILLIAMS et al., 1990; EPPLEN, personal communication). In this respect our PCR fingerprint codominance test system turned out to be well suited for a rapid and comprehensive screening with this and hopefully other primer systems. *In situ* hybridizations with minisatellite probes should principally contribute to answering the question: Is there any transmission homology of the minisatellite sequences which are used for DNA fingerprinting at all? Our test system did not find any such candidates. An explanation for the even rare event of dominance (in our case only 5 % resp. 8 % of all bands) could be the presence of transposable elements in repetitive sequences (MILGROOM et al., 1992). When care is taken to ensure reproducibility, DNA fingerprints are surely genetic markers. The banding patterns obtained in this investigation are, however, certainly not gene markers, since the maternal genotype cannot be inferred from its banding pattern alone (i.e., without the patterns of the gametophytes) nor does the maternal banding pattern contain any subpattern of fewer band positions that defines a gene marker, in that it is produced by one or more loci showing the required codominance of gene action. These results indicate that DNA fingerprints need to be handled very carefully in population genetics.

Outlook

The computer-based test system presented above can be applied to aid in the inheritance analysis of complex DNA banding patterns, in particular by enabling

1. efficient screening of molecular tools for fulfilment of the properties of genetic markers, and
2. differentiation between genetic markers and gene markers, as the highest step of the marker hierarchy presented in the introduction.

The results of such an analysis must be known before a decision can be made upon which elements of complex banding patterns can be utilized to study which population genetic questions.

The analysis provided by the computer program CoDo together with the data interface program MATRIX enables inheritance analysis of the DNA banding pattern of a single diploid individual using the patterns of its haploid gametophytes. The next step of development would be a comparative analysis of the modes of inheritance of DNA banding patterns of different individuals as the prerequisite for the identification of alleles of a single locus within an entire population. Only then can it make sense to apply diversity measures to characterize populations in terms of, for example, their suitability as gene resources.

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Characterization and Propagation of an Adult Triploid Pedunculate Oak (Quercus robur L.)

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Abstract

Investigations of "European oak decline" and genetic structure of more than 400 adult oaks showed that 1 tree was genetically variant because of unusual isozyme patterns. Further examination showed a variant leaf morphology and an increased stomatal length. Chromosome counts and isozyme analysis indicated that the tree is a triploid oak. The conservation of this remarkable genotype was achieved by rooted cuttings. The occurrence of flowering and fruit set of the adult tree offer opportunities for further research concerning problems of reproduction, genetics and stress resistance in oak with altered ploidy level.

Key words: isozyme markers, polyploidy, morphological markers, stomata, chromosome counts, rooted cuttings.

FDC: 165.3; 165.42; 165.44; 176.1 *Quercus robur*.

Zusammenfassung

Im Rahmen von Forschungsarbeiten zur Vitalität und genetischen Struktur an über 400 adulten Eichen fiel ein Baum aufgrund seiner ungewöhnlichen Isoenzym-Bandenmuster auf. Die Ergebnisse weiterer Untersuchungen zeigten eine abwei-

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