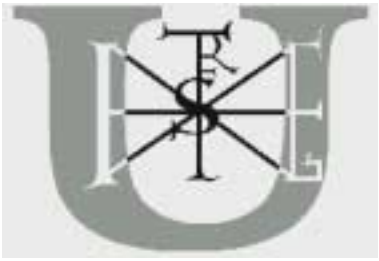

Molecular characterization on different genotypes of poplar, wheat, and hemp using RAPD, SSR, AFLP and SCAR

by

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A thesis

Submitted to the Institute of Ph.D. Studies of the Szent István University in Partial Fulfillment for the Degree of Doctor of Philosophy

Gödöllő, Hungary

July, 2001

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1. Priorities, objectives

For several decades genetic investigations focused at the phenotype increasing genetic variability of the cultivated crops have been based on the phenotype observations and analyses. The environmental effects, polygenic inheritance of quantitative traits, and complete or partial dominance greatly influence the selection effectiveness based on the examination of phenotype-genotype interaction. This is the main reason why the DNA based genetic markers have been getting more important in plant breeding at present and in future. The PCR-based methods supply the techniques suitable to detect the differences in nucleotide sequences i.e. allele-polymorphism.

In the present thesis I report on the molecular investigations of three different plant species: poplar, wheat, and hemp. The molecular techniques applied in these studies were the same, however, the specific objectives were different and subdivided into three chapters.

1.1. Molecular analysis of registered poplar clones

In Hungary the poplar plantations representing 9.5 percentage of the forest area supply 1/5 of the tree-felling. Cross fertilization in poplar breeding takes extremely long time, and breeding of a new hybrid would need several decades, too. Heterosis can be expected with the crossing of inbreds of non closely related genotypes. Therefore, the selection of the suitable parents is very important for this purpose molecular characterization of the clones of poplar species and hybrids can give background information.

In the present study to estimate the genetic diversity between the clones of the Hungarian registered poplars (*Populus sp.*), and to identify RAPD and AP-PCR markers was aimed in order to be able to differentiate these clones.

1.2. Molecular analysis of classically bred and doubled haploid (DH) wheat varieties

Genetic stability of doubled haploid lines of androgenic origin is the prerequisite of their breeding value. As a result of the multi-year studies in field trials, the DH lines and their varieties, and classically bred varieties seemed to be equivalent in efficiency of performance and adaptability. The cytological analysis did not manage to show significant differences between these varieties, either.

In the studies GK Góbé (classically bred variety), GK Délibáb (variety of androgenic origin), randomly selected individuals of their doubled haploid lines were analyzed by using PCR-based molecular techniques such as RAPD, SSR, STS, AFLP, in order to obtain information on the molecular background of homogeneity of the populations.

1.3. Identification and analyses of sex specific markers in hemp

In the absence of sexual dimorphism the gender of most dioecious plants can only be reliably determined at flowering. The dioecious hemp (*Cannabis sativa* L.) belongs to these plants in which the sex determination mechanism has not been elucidated yet.

In the present study we aimed at identification of sex associated markers in hemp. Since these marker types make sex determination possible at early phonological state an in addition to this background information can be obtained for clearing up sex determination mechanism, too.

2. Materials and methods

The polymorphisms originating from nucleotide sequential differences make molecular comparative analysis and detection of linked molecular markers possible. In order to detect polymorphism, PCR-based techniques such as RAPD, AP-PCR, SSR, STS, and AFLP were applied in poplar, wheat, and hemp.

In the genus of *Populus* the genetic distances of Hungarian registered clones were calculated using the Jaccard-index and Simple Matching similarity coefficient in case of dominant markers, and also Multidimensional Scaling analysis in the case of complete database.

On the basis of the male-linked marker sequences SCAR primers were constructed for sex determination in hemp.

3. Results

3.1. Molecular polymorphism in Hungarian registered poplar clones

40 scorable primers were used for the analysis of PCR-patterns of 19 Hungarian registered poplar clones of which 35 were suitable for detecting molecular polymorphism. PCR amplification bands were scored as present (1) or absent (0). On the basis of 162 DNA fragments of 18 RAPD primers cluster analysis was carried out, and Jaccard-index

and Simple Matching similarity coefficient were used for the estimation of genetic distances. The values of similarity coefficients varied from 0.350 to 0.832. As a result, the dendrogram clustered into four groups which species or hybrids the clones belong to. Subgroups were clustered within the *P. euramericana* group. Also, Multidimensional Scaling analysis was made in order to illustrate dimensionally and clearly the results.

Of 35 primers resulting in polymorphic bands 19 primers amplified species- or hybrid-specific PCR-patterns. In the case of *P. euramericana* clones one specific DNA-fragment was identified and amplified with the primer of OPX18. 10 RAPD primers resulted in such DNA fragments which were present only in the VIF (*P. alba*) clones. Additional 4 primers gave specific fragments for the UNA, RAS (*P. trichocarpa* × *P. deltoides*), and KOR (*P. pyramidialis* × *P. deltoides*) clones, respectively.

3.2. Molecular analysis of RAPD, SSR, STS, and AFLP in wheat varieties and their doubled haploid derivatives

RAPD analysis: As a first step we looked for primers being able to differentiate the varieties of GK Góbé and GK Délibáb. Out of 30 RAPD primers used the primers of OPAB09, NO11, OPH11, OPA16, OPQ14, OPX11 made it possible. All the samples were analyzed with the primers of OPAB09, OPX11, OPA16, and NO11. No individual-specific patterns were obtained within the groups of the varieties and their doubled haploid lines.

SSR and STS analysis: Out of 12 SSR and STS primer pairs there were 7 primer pairs resulted in different bands in the GK Góbé and GK Délibáb varieties, and their DH lines. Individual-specific patterns were found within the GK Délibáb group with the microsatellite primer of WMS186.

AFLP analysis: Out of 8 primer combinations 7 primers were able to detect molecular differences in the wheat samples studied resulting in a total of 81 polymorphic fragments. The number of polymorphic bands ranged from 4 to 20 per selective AFLP primer pair. A total of 47 markers were found which were present only in each individual of one of the GK Góbé and DK Délibáb varieties. The GK Góbé and GK Góbé doubled haploid individuals differed in 9 fragments from each other, and additionally 23 fragments were able to separate the individuals within the groups. Within the groups the numbers of polymorphic bands were as follows: GK Góbé (classically bred variety) - 4, GK Góbé

doubled haploid line - 11, GK Délibáb (doubled haploid variety) - 12, GK Délibáb doubled haploid line - 3.

3.3. Identification and analysis of sex specific markers in hemp

Out of 21 RAPD primers tested two male-linked RAPD markers were identified in the sex groups of three hemp varieties. PCR-analysis of individual samples proved these markers to be present in each male plant while they were absent from females and from monoecious individuals of hemp.

After cloning the male linked fragments Southern analysis was made. The RAPD marker identified by the OPD05 primer did not result in difference in the Southern patterns of both sexes. However the DNA fragment amplified with the UBC354 primer was able to differentiate the hybridization patterns of both sexes.

After sequencing SCAR primers were constructed for the 961 and 151 bp fragments. The segregation of the two SCAR markers and the male specific SCAR marker identified by Mandolino et al. (1999) were checked in F_2 generation.

Comparison of the sequences with the database sequences showed 50-55 % homology with several retrotransposon-like sequences of plant origin.

3.4. New scientific achievements

Molecular analysis of Hungarian poplar clones

1. On the basis of RAPD and AP-PCR analysis the 19 Hungarian registered poplar clones (*Populus* sp.) were subdivided into four groups and the molecular polymorphism and genetic distances among the clones were evaluated using cluster- and MDS analyses.
2. Primers were identified resulting in specific fragments for the *P. euramericana* and *P. alba*, and for the clones of *P. trichocarpa* × *P. deltoides*, *P. pyramidialis* × *P. berolinensis* hybrids. This makes the molecular identification of *Populus* species and hybrids possible in practice.

Molecular comparative analysis of classically bred and doubled haploid wheat varieties

3. According to RAPD, SSR, STS, and AFLP analysis it was proved that no significant genetic difference was found between the classically- and androgenetically bred varieties as for the polymorphism. This allows to conclude that the genetic homogeneity of classically bred and doubled haploid varieties is nearly equal.

4. Result of the AFLP and SSR analysis proved that molecular differences found between the homogenous varieties and individuals of doubled haploid progeny lines do not influence of the agronomic traits, yield, adaptability of these varieties. Cytological alterations could not be detected, either.
5. As it was mentioned in the sections 3 and 4, it was expected that there was no significant difference in the performance yield, and adaptability of classically bred and doubled haploid cultivars, the classically bred cultivars growing and maintaining under isolated conditions are as homogenous as the haploid or doubled haploid populations.
6. In addition to these results it was proved that AFLP is most while SSR is less suitable for the molecular comparative analysis of doubled haploid lines and their individuals.

Identification and analysis of sex specific markers in hemp

7. In different dioecious hemp varieties new male-specific DNA fragments of $OPD05_{961}$ and UBC_{354} were identified using by RAPD-PCR. It was proved also that these markers could not detected in the monoecious hemp variety of Fibrimon.
8. The new markers were cloned and sequenced. On the basis of the internationally known terminology the sequences were named MADC3 ($OPD05_{961}$) and MADC4 ($UBC354_{151}$). The sequences of the markers showed the 50-55 % homology with other retrotransposon-like sequences originated from different plant species.
9. The sequence-specific primers of $SCAR_{323}$ and $SCAR_{119}$ were constructed for the new markers. It was proved also that these sequences can effectively be applied for sex identification in hemp apart from the phenological stage of the plant.
10. On the basis of the molecular analysis of segregating F_2 population it was proved that the tester marker of $SCAR_{390}$, the newly isolated male-linked sequence-specific markers of $SCAR_{323}$ and $SCAR_{119}$ were closely related with each other and with sexes. According to our results it was expected that the MADC3 and MADC4 sequences can be found on the sex chromosome of Y in hemp.

4. Conclusions and recommendations

4.1. RAPD and AP-PCR analysis of poplar genotypes

DNA-fingerprinting methods make the evaluation of the genetic variability among and within populations possible. The calculation was carried out using similarity coefficients. For the evaluation of RAPD and AP-PCR patterns Jaccard-index is proposed to be applied because RAPD and AP-PCR methods result in dominant markers, and this index considers dominant markers to a larger extent than other similarity coefficients. Simple Matching coefficient differs from the Jaccard-index in such trait because during the comparison of pairs this takes into consideration DNA fragments which are absent from both genotypes or populations.

In the present investigation 19 Hungarian registered poplar genotypes were analyzed with 36 RAPD and 4 AP-PCR primers resulting in distinct patterns. There were 18 PCR patterns showing polymorphism. On the basis of RAPD and AP-PCR fragments amplified among the 19 poplar genotypes the VIF clone presented the least similarity with the value of 0.350-0.460 in comparison with the other clones, contrary the greatest similarity was evaluated between the clones of I15 and TRI due to their close relationship, especially the triploid TRI clone originated from the pollen donor clone of I15 and the another parent line of 438p. The latter line is bred as a result of the colchicine treatment of the I15 clone apical meristem. According to software analysis there were four main clusters found on the dendrogram as follows (i) VIF (*P. alba*), (ii) UNA (*P. trichocarpa* × *P. deltoides*), RAS (*P. trichocarpa* × *P. deltoides*), (iii) KOR (*P. pyramidialis* × *P. berolinensis*), (iv) others (*P. euramericana*). The group 4 was divided into subgroups. For example the ROB, SUD, PAN, I21, H32, BL, I45 and AGA clones, the I15, TRI and I27, as well as the KOP and KOL clones belong to the same subgroups. The BDA and PAR genotypes were sitting on separate cluster belonging to neither subgroups within the group 4. On the basis of binary data tables Multidimensional Scaling analysis was made in order to illustrate clearly the genetic distances between the clones. The genetic distances among the clones were evaluated and illustrated more correctly on the two dimensional MDS figure than on the dendrogram.

Our investigations proved that the molecular techniques of RAPD and AP-PCR can be successfully applied for the determination of genetic distances of *Populus* clones agreeing with international approaches.

Out of 35 primers resulting in polymorphic bands the 19 primers amplified species- or hybrid-specific PCR-patterns. In the case of *P. euramericana* clones one specific DNA-fragment was identified and amplified with the primer of OPX18. 10 RAPD primers resulted in such DNA fragment at about 550 bp in length which was present only in the VIF (*P. alba*) clone. Additional four primers gave specific fragments for the UNA, RAS (*P. trichocarpa* × *P. deltoides*), and KOR (*P. pyramidialis* × *P. deltoides*) clones, respectively.

4.2. Investigation on the homogeneity of wheat varieties and their dihaploid derivatives with molecular techniques

In our studies GK Góbé (classically bred variety), GK Délibáb (androgenic originated variety), and randomly selected individuals of their doubled haploid lines were analyzed using PCR-based molecular techniques. On the basis of our results RAPD, SSR, STS, and AFLP techniques proved to be suitable to detect molecular differences between the species. However, most of the polymorphic fragments were obtained with SSR and AFLP methods. These results agree with the international ones. Within the cultivar, and the populations of doubled haploid progeny lines there were no detectable differences using RAPD and STS methods. In average one polymorphic fragment per SSR primer tested, and 2.9 polymorphic fragments per AFLP primer combination were identified. Therefore, AFLP should be applied primarily, and SSR secondly for the detection of molecular differences among the individuals.

In AFLP analysis within the groups the numbers of polymorphic bands were as follows: GK Góbé (classically bred variety) - 4, GK Góbé doubled haploid line - 11, GK Délibáb (doubled haploid variety) - 12, GK Délibáb doubled haploid line - 3. As mentioned in the above the greatest number of polymorphic band (12 fragments) was found among the GK Délibáb individuals. The primer of WMS186 SSR resulting in polymorphism was only able to detect individual difference within this group. Of 10 wheat individuals there were about 130 bp fragment in four individuals, about 100 bp in one, nearly 130 and 100 bp fragments in five individuals amplified too. Also, there were molecular differences in large number (11 fragments) among the DH progeny individuals originating from the variety of Góbé. The largest degree of homogeneity was found within the DH lines of GK Délibáb where altogether the genetic variability was shown in

three DNA fragments. On the basis of molecular analysis the classically bred variety of GK Góbé is also homogenous.

As a result of the multi-year studies in field trials, the DH lines and varieties, and classically bred varieties seemed to be equivalent in performance and adaptability. The cytological analysis did not manage to exhibit significance differences among these varieties, either. Contrary there were molecular differences among the varieties and their DH lines, and also within the DH lines. But the classically bred variety or DH variety and their DH derivatives showed no significance difference.

4.3. Identification and analysis of sex linked RAPD markers in monoecious and dioecious hemp

The hemp (*Cannabis sativa L.*, 2n = 20) is a plant species with sex chromosomes. Recently a 47 Mbp size difference has been shown between the genomes of female and male hemp using by flow cytometry. This difference corresponds to 2.8 percentage of the entire genome. Considering and expecting that the RAPD primers are linked to genomic DNA randomly and uniformly, 2.8 % of the RAPD fragments would be linked to male plants. In our investigation 21 RAPD primers amplified 160 DNA fragments of which there were two fragments linked to male sex, that means 1.25 percentage. This is less than the theoretically expected 2.8 %, which can be interpreted that RAPD primers do not amplify fragments absolute randomly and uniformly within the genome, in additions to this the genomes of plant species contain repetitive sequences, and there may be homology in the male and female sequences, too.

The primer of OPD05 amplified a 961 bp DNA fragment while the primer of UBC354 a 151 bp fragment in the RAPD reaction of male individuals and the groups deriving from these individuals. In any case these fragments were absent from the females and monoecious hemp plants. The male-associated markers identified so far by other research groups were also absent from the PCR patterns of monoecious varieties.

The male-associated DNA sequences identified in hemp were named MADC1 and MADC2. Using this terminology the two new sequences detected and identified by us are named MADC3 ($OPD05_{961}$) and MADC4 ($UBC354_{151}$).

In comparative analyse of database sequences there was no significant homology with other nucleic acid sequences in any case. The retrotransposons deriving from different plant species showed the greatest homology (50-50 percentage) with it.

The sequence analysis was made by computer. According to six kinds of reading frames all the sequences may contain less or larger open reading frames from which sense protein synthesis is probably possible. Additionally according to one of the reading frames the MADC4 fragment has a stop codone with a short sequence. The MADC3 sequence may have many reading frames of 500-600 bp in length.

The genomic Southern analysis was made by the MADC3 and MADC4 sequences. The total DNAs of randomly selected male and female individuals were restricted by three kinds of restriction enzymes, and after gelelectrophoresis and hybridization followed by using radioactively labeled MADC3 and MADC4 sequences. The MADC3 sequence hybridized with the genomic DNA pattern in many sites (probably repetitive sequences in medium copies), and did not exhibit molecular difference between the sexes. Contrary the MADC4 sequence hybridized in fewer sites, and there were molecular variations in the Southern patterns of male and female individuals. So far the genomic differences were detectable only in the sexes of asparagus and papaya.

On the basis of the MADC3 and MADC4 sequences SCAR primers were constructed which make molecular detection possible. As a first step the DNA samples in the variety of KFF were tested with the primers of $SCAR_{323}$ and $SCAR_{119}$, and these were suitable for differentiating the individuals of both sexes as expected.

The 75 individuals of F2 generation were tested with the primers of $SCAR_{119}$ and $SCAR_{323}$ to be constructed by the new MADC3 and MADC4 sequences, and also with the known male-associated primer of $SCAR_{390}$ in which the sex ratio determining by phenotype was as follow: 41 females:32 males:2 monoecious. The sex ratios determined on the basis of phenotypes and SCAR markers differed from each other in only two cases. In no case recombination was detected within the three markers that allows to conclude that these sequences are closely located to each other in the genome, or contain chromosomal sections without recombination chance.

5. Publications relating to the topics of the thesis

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