USE OF RAPD TECHNIQUE FOR REVEALING DNA POLYMORPHISM IN *GINKGO BILOBA* L. SPECIES

UTILIZAREA TEHNICII RAPD PENTRU EVIDENȚIEREA POLIMORFISMULUI ADN LA SPECIA *GINKGO BILOBA* L.

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***Abstract*:** *Random Amplification of Polymorphic DNA (RAPD) analysis is a valuable tool in studying genomic DNA polymorphism in plants. In this study, we used RAPD technique to reveal the possible intraspecific genetic variation in Ginkgo biloba grown in different sites in Romania and Denmark. Perhaps this variation could be a possible explanation for the observed variations in the efficacy of medications with different G. biloba extracts (KUDDDUS at all., 2002). Ten of the twenty decamer primers yielded scorable amplification patterns. These primers generated polymorphic bands among the genotypes studied. Some of the primers produced no amplification or unreadable gel smears. A dendrogram was constructed based on genetic distances using the program Tree View.The genotypes analyzed clustered into two main groups and the values of genetic distances between data shows that there are some DNA differences.*

Rezumat: *Tehnica RAPD este o metodă eficientă pentru evidențierea polimorfismului ADN–ului izolat de la plante. În acest studiu a fost utilizată tehnica RAPD pentru a pune în evidență prezumtiva variabilitate intraspecifică existentă între indivizi de Ginkgo biloba L. cultivați în zone din România şi Danemarca. Este posibil ca aceasta variabilitate să fie o explicație a eficacității diferite a unor medicamente fabricate din diferite extracte de ginkgo izolate de la indivizi de diferite proveniențe (KUDDDUS at all., 2002). Extracția ADN-ului total a fost realizată din embrioni zigotici maturi, utilizând metoda Lodhi et all. (1994) modificată de Pop R şi colab., 2003. Zece din cei 20 de primeri decamerici au generat fragmente de ADN amplificabile şi benzi polimorfice la genotipurile analizate. Unii primeri nu au generat produşi de amplificare PCR sau în geluri au apărut benzi intens colorate, nelizibile. Pe baza distanțelor genetice şi utilizând programul Tree View a fost întocmită o dendrogramă, existând anumite diferențe la nivelul genotipurilor analizate.*

Key words: DNA, RAPD, *Ginkgo biloba*, molecular polymorphism

INTRODUCTION

In recent years, ginkgo extract has been extensively studied for its various medicinal qualities (DIAMOND et al., 2000, LOGANI et al., 2000). Meanwhile hundreds of controlled scientific studies and research on the chemistry, pharmacology and clinical effects of the leaves have been conducted, mostly by European researchers over the last decades, using the German/ French extract EGb761, also called Kaveri, Tebonin, Tanakan, Rökan and Ginkgold.

Some studies (JACOBS B.P. and BROWNER W.S., 2000) shows that *Ginkgo biloba* has been considered a "recalcitrant plant taxa". There are a few reports regarding the possibility to use the molecular markers for detecting DNA polymorphism in *Ginkgo biloba* L. (KUDDUS at al., 2002, YONG-QI at al., 2003, FAN X. X. at al., 2004). The studies achieved in the last 10 years were referred to interest for an accurance botanic classification. For example, recent molecular analysis of the *G. biloba* genome, while far from complete, suggests a much closer relationship to the cycads than to the conifers (HASEBE, 1997).

We used RAPD analysis to reveal the intraspecific genetic variations in *Ginkgo biloba* grown in different sites in Romania and Denmark. Perhaps this variation could be a possible explanation for the observed variations in the efficacy of medications with different *G.biloba* extracts (KUDDUS at al., 2002).

MATERIAL AND METHODS

Biological material used for DNA isolation was represented by kernel of seeds collected in autumn 2006 from mature plants grows in Botanical Garden Cluj-Napoca, Botanical Garden Craiova- Romania, Student Residence Hasdeu Cluj-Napoca, Arboretum KVL and Botanical Garden Copenhagen- Denmark.

Prior to isolation, approximately 400 mg kernel seeds were grind in liquid nitrogen into a fine powder. Total DNA was extracted using the protocol developed by Lodhi *et al.* (1994) and modified by Pop *et al.* (2003). Extraction buffer has the following composition: 100 mM Tris-HCl, 20 mM EDTA, pH =8.0, 1.4 M Na Cl, 2% (w/v) CTAB and 2% PVP-40 and 0.2% β-mercaptoethanol. This buffer was also supplemented with 5 mM ascorbic acid and 4 mM DIECA. DNA concentration and the absorbance ratio at A_{260}:A_{280} was quantified in a BioPhotometer Eppendorf. Reaction mixture for PCR in 25 µl volume consisted of 50 ng DNA, 200 µM of each dNTP (Promega), 0,2 µM primer (Mycrosynth, see table no. 1), 2,5 mM $MgCl_2$, 2,5 mM 10 x Buffer, 1 U Taq DNA Polymerase (Promega), 2% PVP (Sigma), bidistillated sterile water.

Table 1

Primers used in DNA amplification of regenerants obtained

No	Primer	Sequence (5' – 3')	No. of polymorphic bands/primer
1	OPA 03	AGT CAG CCA C	2
2	OPA 01	CAG GCC CTT C	5
3	OPAB 11	GTG CGC AAT G	4
4	OPAB 18	CTG GCG TGT C	-
5	OPA 04	AAT CGG GCT G	7
6	OPAL 20	AGG AGT CGG A	3
7	OPE 14	TGC GGC TGA G	1
8	OPC 02	GTG AGG CGT C	-
9	OPC 04	CCG CAT CTA C	5
10	OPD 16	AGG GCG TAA G	6
11	OPD 19	CTG GGG ACT T	3
12	OPF-20	GGT CTA GAG G	1

Amplification was performed in a Eppendorf Mastercycler Gradient programmed for the following thermal profile: an initial denaturation step – 3 min at

95°C, followed by 45 cycles of 1 min. at 93°C, 1 min. at 34°C, 1 min. at 72°C. A final extension step at 72°C was performed for 10 minutes. The molecular marker used was 100bp DNA Step Ladder (Promega Corp., Madison, WI, USA). Gels were visualized on a UV light Biospectrum AC Imaging System (UVP BioImaging Systems, Upland, CA) after staining with 0,5 µg/µl Ethidium Bromide for 25 min.

Gel images were analyzed using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) and the bands resulted after RAPD amplification were scored as present (1) or absent (0), data entered into a binary matrix. The genetic distance between accessions was calculated using Nei and Li's coefficient of similarity. Cluster analysis was conducted with a Neighbor-Joining algorithm using FreeTree software and a dendrogram was constructed, using the TreeView software.

Table 1 shows the nucleotide sequences of RAPD primers used in DNA amplification of analyzed *Ginkgo* individuals. Sign (+) suggest the presence of specific, polymorphic DNA fragment generated in RAPD reaction with described primer and (-) the absence of specific, polymorphic DNA fragment generated in RAPD reaction with described primers.

RESULTS AND DISCUSSIONS

DNA fragments generated after PCR amplifications with RAPD primers had the length comprised between 200-1800 bp, respectively 300-1400 bp for the majority. The primer OPA 04 produced maximum 7 polymorphic bands; primer OPD 16 generated 6 polymorphic bands; OPA 01 and OPC 04 primers gave 5 polymorphic bands, OPF 20 1 polymorphic band. The agarose gels analysis reveal genetic differences among some of the individuals. The dendrogram illustrating the genetic relationships among individuals grown in different sites in Romania and Denmark (Botanical Garden Cluj-Napoca-Romania, Botanical Garden Craiova-Romania, KVL Arboretum Horsholm-Denmark, Botanical Garden Copenhagen-Denmark, KVL Garden-Denmark).

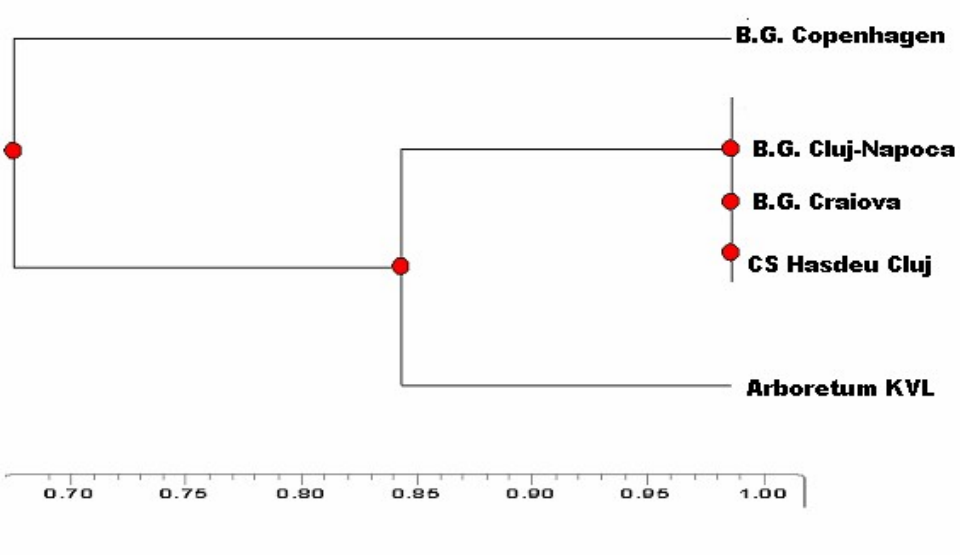

Fig. 1 Neighbor Joining dendrogram illustrating genetic relationships among *G. biloba* individuals grown in different Romanian and Danish sites.

In DNA samples originated in *Ginkgo biloba* provenances from Romania there have been registered few polymorphic bands, with band size comprised

between 400-680 bp, while in those originated in Danish *G. biloba* provenances the number of polymorphic bands has been much greater (more than 6), with band size comprised between 640-800 bp.

These results could be explained by the rather common origin of Romanian *Ginkgo biloba* samples while the Danish *G. biloba* provenances are known to have different origine (Japan and China).

CONCLUSIONS

1. Based of these results it could be staded that AFLP technique, involving primers with the previously mentioned base pair sequences, can be successfully used to reveal molecular polymorphism among DNA samples of *G. biloba* originated in various sites.

2. A through analysis of these polymorphic bands, including statistical ones, could render possible the identification of subspecies or clines within different provenances of *G. biloba*.

3. Such data are of real interest for medicinal use of various *G. biloba* geographic provenances.

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