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Use of genomic fingerprinting techniques for revealing DNA polymorphism in *Ginkgo biloba* L., a medicinal woody species

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Abstract:

The main goal of our study was to use two DNA fingerprinting techniques (RAPD and AFLP) for revealing the possible intraspecific genetic variation between Ginkgo biloba individuals grown in different sites. Ten of the twenty RAPD (Random Amplification of Polymorphic DNA) decamer primers yielded scorable amplification patterns and generated polymorphic bands among the genotypes studied.

Of the 165 AFLP (Amplified Fragment Length Polymorphism) primer combinations used, Ecs-AGG+MCG have revealed the largest number of polymorphic bands (96) among samples of Ginkgo biloba originating from Romania and Denmark. The least number of polymorfic bands (74) was detected using primer combination Ecs-AGG+MGA.

Individuals of Ginkgo, analyzed by the techniques mentioned above, clustered into two main groups and the values of genetic similarity coefficients between data shows some differences at DNA molecular level.

Keywords: Ginkgo biloba, DNA polymorphism, RAPD, AFLP, fingerprinting techniques

Introduction

In recent years, Ginkgo leaf extract has been extensively studied for its various medicinal qualities (B.J. DIAMOND & al.) [1], (S. LOGANI & al.) [2]. 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 Egb 761, (BODEA) [3].

The use of drugs containing standardized EGb761 extract as a memory enhancing supplement is widespread among the elderly; however, the efficacy of these supplements varies widely (KIDD) [4]. Such variation has been hypothetically attributed to the different methods of leaves extract preparation, the formulation of the supplements and the genetic diversity of the plant stocks used to prepare the extract (Z. PENG. & al.) [5]. Genetic diversity (R.KUDDUS & al.) [6], (G.J. YI & al.) [7] of individuals and populations, genetic relationship and sex markers (X.M. WANG & al.) [8, 9], were studied by isozyme, allozyme, RAPD, ISSR and other techniques. DNA fingerprinting ensures presence of the correct genotype but does not reveal the contents of the active principle or chemical constituents. Proper integration of molecular techniques and pharmacognostic techniques for chemoprofiling such as HPLC (High Performance Liquid Chromatography),

SFC (Supercritical Fluid Chromatography), NMR (Nuclear Magnetic Resonance Spectroscopy), NIR (Near Infrared Spectroscopy), GC (Gas Chromatography) etc. will lead to the development of a comprehensive system of botanical characterization that can be conveniently applied at the industry level for quality control of botanicals (A. SHARMA & al.) [10]. Using these methods together will determine the opportunity to identify elite plants (with high content in pharmaceutical useful substances). These plants can be cloned by *in vitro* propagation methods and thus may stimulate production of secondary metabolites for pharmaceutical manufacturing (BODEA) [3].

Materials and methods

DNA isolation

Biological material used for DNA isolation was the young leaves of *Ginkgo biloba* originated from mature trees harvested in spring 2008 from Botanical Garden Cluj-Napoca, Botanical Garden Craiova, Student Campus Hasdeu Cluj-Napoca – Romania, KVL Garden Copenhagen, Arboretum KVL , Botanical Garden Copenhagen- Denmark and Private Garden Roskilde-Denmark (provenance used in addition for AFLP analysis) . Prior to isolation, approximately 400mg of young leaves were grinded in liquid nitrogen into a fine powder. Three isolation protocols were tested: first described by DOYLE and DOYLE [11], the second - a protocol described by S.O. ROGER & al. [12] and third a modified version (R. POP & al.) [13] of the protocol published by M.A. LODHI et al. [14]. In our experiments extraction buffer was supplemented with 5 mM ascorbic acid, 4 mM diethyldithiocarbamic acid and the concentration of polyvinilpyrolidone was enhanced at 2 %. DNA concentration and the absorbance ratio at A_{250}: A_{280} was quantified with Nano Drop Nd-1000 Spectrophotometer (*Nanodrop Technologies*).

RAPD analysis

Reaction mixture for PCR in 25 µl volume consisted of 50 ng DNA, 200 µM of each dNTP (Promega), 0,2 µM primer, 2,5 mM $MgCl_2$, 2,5 mM 10 x Buffer, 1 U Taq DNA Polymerase (*Promega*), 2% PVP (*Sigma*) and bidistillated sterile water. Amplification was performed in a Eppendorf Mastercycler Gradient programmed for the following thermal profile: an initial denaturation step 3 min at 95^{o}C, followed by 45 cycles of 1 min. at 93^{0}C, 1 min. at 34^{0}C, 1 min. at 72^{0}C.

A final extension step at 72^{0}C was performed for 10 minutes. We used the following decamer primers : OPA 03, OPA 01, OPAB 11, OPAB 18, OPA 04, OPAL 20, OPE 14, OPC 02, OPC 04, OPD 16, OPD 19 and OPF 20 (*Microsynth Lab*.). The amplicons obtained were migrated in 1 % agarose gel (*Promega*) using 100 bp DNA Ladder Step (*Promega*) molecular weight and 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 analyzed individuals 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.

AFLP analysis

DNA samples were digested with two different endonucleases: MseI and EcoRI. The restriction was performed in a Thermocycler (*PTC 100 MJ Research*) overnight at 37^{0}C. EcoRI and MseI restriction sites of genomic DNA fragments were ligated to the doubled

stranded adaptors carrying EcoRI and MseI complementary ends, respectively, for three hour at 37^0C. Pre-selective amplification primers were reffered to as AEO and AM0 and the cycling conditions were 20 cycles of three steps as 94^0C for 20s, 56^0C for 30s, 72^0C for 2 min (P. VOS & al.) [15]. After final amplification the samples were mixed with a loading buffer consisting of deionized formamide and ROX-500 internal size standard, prior to loading on the ABI 377 sequencer. Table 1 shows the primers used of AFLP technique for revealing DNA polymorphism in *Ginkgo biloba* individuals.

Table 1. Primers used of AFLP technique for revealing DNA polymorphism in *Ginkgo biloba*

Name of primer	Sequence of primer	Label	Description	Number of bp
A-Ad- E1	CTCgTAgACTgCgTACC	-	Eco-RI- Adapter 1	17
A-Ad- E2	AATTggTACgCAgTC	-	Eco-RI- Adapter 2	15
A-Ad- M1	gACgATgAgTCCTgAg	-	Mse- Adapter 1	16
A-Ad- M2	TACTCAggACTCAT	-	Mse- Adapter 2	14
A- E0	gACTgCgTACCAATTC	-	EcoRI- Primer: 0	16
A-M0	gATgAgTCCTgAgTAA	-	Mse- Primer: 0	17
A-E-AAC	gACTgCgTACCAATTCAAC	NED	EcoRI- Primer-AAC	19
A-E-AAG	gACTgCgTACCAATTCAAg	JOE	EcoRI- Primer-AAG	19
A-E-ACA	gACTgCgTACCAATTCACA	5-FAM	EcoRI- Primer-ACA	19
A-E-ACC	gACTgCgTACCAATTCACC	NED	EcoRI- Primer-ACC	19
A-E- ACG	gACTgCgTACCAATTCACg	JOE	EcoRI- Primer-ACG	19
A-E-ACT	gACTgCgTACCAATTCACT	5-FAM	EcoRI- Primer-ACT	19
A-E-AGC	gACTgCgTACCAATTCAgC	NED	EcoRI- Primer-AGC	19
A-E-AGG	gACTgCgTACCAATTCAgg	JOE	EcoRI- Primer-AGG	19
A-E-AGT	gACTgCgTACCAATTCAgT	5-FAM	EcoRI- Primer-AGT	19
A-P-AC	gACTgCgTACATgCAgAC	NED	Pst- Primer- AC	18
A-P-CA	gACTgCgTACATgCAgCA	JOE	Pst- Primer- CA	18
A-P-CC	gACTgCgTACATgCAgCC	5-FAM	Pst- Primer-CC	18
A-P-ACA	gACTgCgTACATgCAgACA	NED	Pst- Primer- ACA	19
A-P- ACG	gACTgCgTACATgCAgACG	JOE	Pst- Primer- ACG	19
A-P- ACT	gACTgCgTACATgCAgACT	5-FAM	Pst- Primer- ACT	19
A-M-CAA	gATgAgTCCTgAgTAACAA	-	MseI- Primer- CAA	19
A-M-CAC	gATgAgTCCTgAgTAACAC	-	MseI- Primer- CAC	19
A-M-CAG	gATgAgTCCTgAgTAACAG	-	MseI- Primer- CAG	19
A-M-CAT	gATgAgTCCTgAgTAACAT	-	MseI- Primer- CAT	19
A-M-CCT	gATgAgTCCTgAgTAACCT	-	MseI- Primer- CCT	19
A-M-CGA	gATgAgTCCTgAgTAACGA	-	MseI- Primer- CGA	19
A-M-CGT	gATgAgTCCTgAgTAACGT	-	MseI- Primer- CGT	19
A-M-CTA	gATgAgTCCTgAgTAACTA	-	MseI- Primer- CTA	19
A-M-CTC	gATgAgTCCTgAgTAACTC	-	MseI- Primer- CTC	19
A-M-CTG	gATgAgTCCTgAgTAACTG	-	MseI- Primer- CTG	19
A-M-CTT	gATgAgTCCTgAgTAACTT	-	MseI- Primer- CTT	19

It is possible to multiplex primer combinations allowing three final amplification reactions involving different fluorescent labelled primers to be run in a single well.
We mixed 0,4 µL of each dye (yellow, green, blue) amplification product and 1,4µL of the loading buffer. The mixed samples for each dye were heated at 95^0C for 2 min. and cooled rapidly on ice and kept on ice until loading. The data were analyzed using GeneScan 3.1 software (*Applied Biosystems, Foster city, CA, USA*) following the instuctions on the ABI TM 377 DNA Sequencer: Gene Scan TM Chemistry Guide. PPB (percentage of polymorphic bands) were calculated on the proportion between the number of common bands and the total number of bands generated by primers. The dendrograms among individuals were constructed

based Jaccard's genetic distance. Cluster analysis was performed with NTSYS-pc version 2.10 t (*Applied Biostatistics, Inc., 2000*), a numerical taxonomy and multivariate analysis software package using an unweighted pair-group method, arithmetic average (UPGMA).

Results and discussions

Using DNA isolation protocol C, representing a modified version (RODICA POP & al.) [13] of the protocol published by Lodhi et al. (1994), the purity of ginkgo DNA samples had the values comprised between 1.87-2.01, higher than in protocol A (1.67-1.85 purity values) and B protocol (1.79- 1.90 purity of isolated DNA). The addition of PVP-40, DIECA and ascorbic acid in the extraction buffer in protocol C minimized the damage caused by contaminants like polyphenols and polysaccharides to the nucleic acids. Although *Ginkgo biloba* has been considered a *recalcitrant plant taxa* (M.Y. KHAN & al.)[16] our results indicate that by improving the extraction protocol one can obtain good quality DNA for RAPD and AFLP analysis. In our study, RAPD primers have generated a variable number of polymorphic bands: primer OPA 04 produced 7 polymorphic bands, primer OPD 16 generated 6 polymorphic bands, OPA 01 and OPC 04 primers gave 3 polymorphic bands and OPF 20 just one.

These results are in concordance with those reported by LIU & al., [17] which shows that there are differences at DNA molecular level between analysed Ginkgo individuals provided from China (PPB calculated values was 30,5%). In our study we found that the genomic similarity between individuals harvested from Romania was notable (calculated values for the percentage of polymorphic band were only 3,0-3,2%) compared with those coming from Denmark (6,02 % for individuals cultured in Arboretum KVL and 40,3% PPB value for Ginkgo trees grown in Copenhagen Botanical Garden. 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 origins (Japan and China).

The built dendrogram (Fig.1), generated after RAPD analysis, illustrates the genetic relationships among individuals grown in different sites in Romania and Denmark.

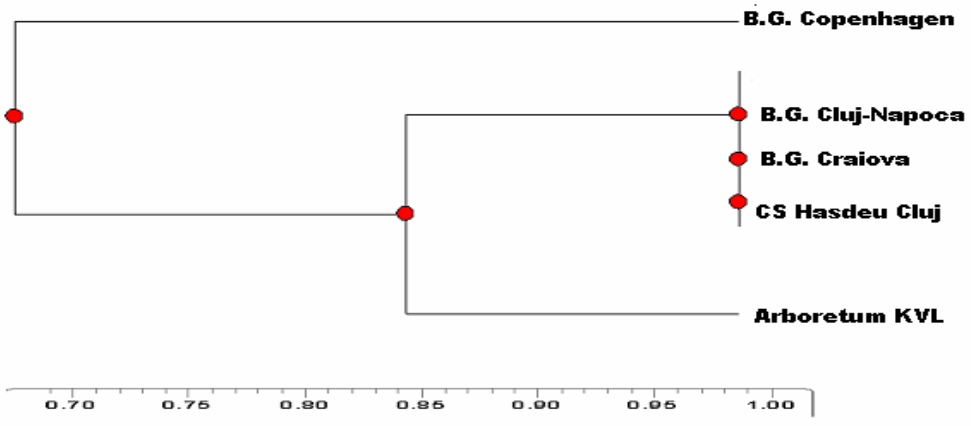

Fig. 1. Neighbor Joining dendrogram illustrating genetic relationships among *G. biloba* individuals grown in different Romanian and Danish sites (Botanical Garden Copenhagen- B.G. Copenhagen; Botanical Garden Cluj-Napoca – B.G. Cluj-Napoca; Botanical Garden Craiova– B.G. Craiova; Student Complex Campus Hasdeu- CS Hasdeu Cluj-Napoca, KVL – Arboretum).

Concerning AFLP analysis, our results shows that primer combinations Ecs-AGG+MCG, have revealed the largest number of polymorphic bands (96) among samples of

Ginkgo biloba originated in Romania and Denmark. The least number of polymorfic bands (74) was detected using primer combination Ecs-AGG+MGA. The percentage of polymorphic bands of each provenance for ginkgo individuals varied considerably, ranging from 8,9 % (B. G. Cluj-Napoca provenance) to 24,7% (KVL Arboretum). In *Ginkgo biloba* samples originated from Romania there have been registered few polymorphic bands (102, 123, 284), with band size comprised between 240-380 bp, while in those provided from Denmark the number of polymorphic bands has been much greater (more than 300), with band size comprised between 240-500 bp. Genetic relationships among individuals grown in different sites in Romania and Denmark (Botanical Garden Cluj-Napoca-Romania, Botanical Garden Craiova-Romania, Student Complex Hasdeu Cluj-Napoca, Botanical Garden Copenhagen-Denmark, KVL Garden-Denmark and Private Garden Roskilde-Denmark) can be seen in Fig 2.

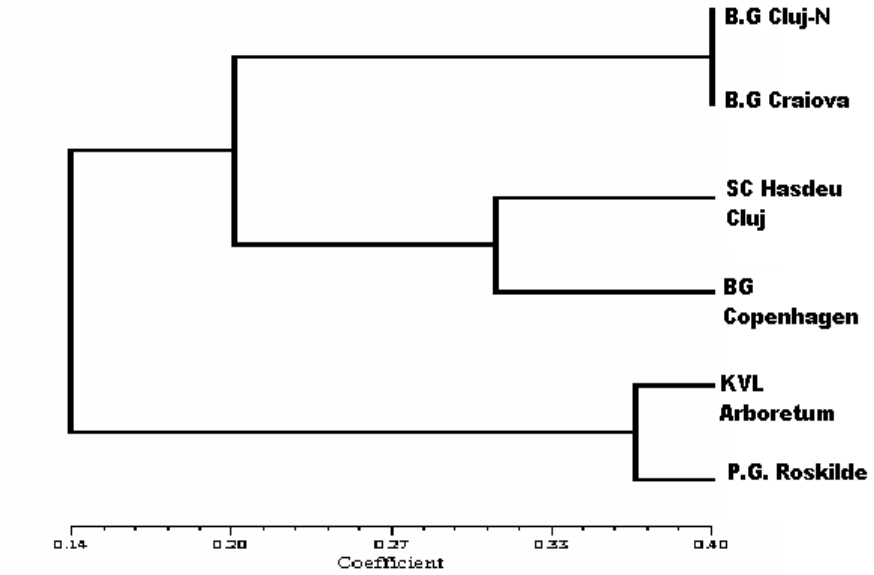

Fig.2. UPGMA dendrogram illustrating genetic relationships among *G. biloba* individuals grown in different Romanian and Danish sites

The above results indicated that the Ginkgo individuals from the same country provenance did not completely belong to one cluster. An explanation of these groups may be due to different origin of specimens from Denmark (China and Japan). It is possible to assert that high genetic similarity between individuals provided from Romania were due probably to a common unknown origin (China or Japan).

Conclusions

The addition in the DNA extraction buffer of PVP-40, DIECA and ascorbic acid in protocol C minimized the damage caused by contaminants like polyphenols and polysaccharides to the nucleic acids and have prevented the browning of the extract. Although *Ginkgo biloba* has been considered a *recalcitrant plant* (M.Y. KHAN & al.) [16] our results indicate that by improving the extraction protocol one can obtain good quality DNA for RAPD and AFLP analysis.

In our study we found that the genomic similarity between individuals provided from Romania was notable compared with those coming from Denmark.

AFLP analysis shows that in *Ginkgo biloba* samples originated from Romania there have been registered few polymorphic bands (102, 123, 284), with band size comprised between 240-

380 bp, while in those provided from Denmark the number of polymorphic bands has been much greater (more than 300), with band size comprised between 240-500 bp.

This preliminary study will be completed in the future by analyzing at DNA molecular level of other individuals cultivated in different parts of Romania especially since some research groups claim that the intraspecific genetic variations in *Ginkgo biloba* grown in different sites could be a possible explanation for the observed variations in the efficacy of medications with different *G.biloba* extracts (R. KUDDUS & al.) [6].

Based of these results it could be stated that AFLP and RAPD techniques, involving primers with the previously mentioned base pair sequences, can be successfully used to reveal molecular polymorphism among DNA samples of *G. biloba* originating from various sites.

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