Genetic Variability in *Nicotiana tabacum* and *Nicotiana* Species as Revealed by RAPD Markers:

1. Development of the RAPD Procedure*

by

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SUMMARY

At present there is no information about the level of genetic variability in *N. tabacum* and in the *Nicotiana* genus as revealed by random amplified polymorphic DNA (RAPD). Such knowledge could be useful for taxonomic and breeding purposes. The aim of this paper is to assess the potential application of the DNA polymorphisms generated by RAPD markers within this genus and in tobacco. As rigorously standardized reaction conditions are required to obtain a reproducible RAPD marker, four rapid DNA extraction methods were compared and several parameters of the reaction conditions for the random polymorphic DNA amplification were analysed and optimized. The DNA of six-week-old leaves of *N. tabacum* var. Samsun was obtained with the following methods differing in the strategy of purification: the cetyltrimethylammonium bromide (CTAB) method, that of Edwards, nucleon phytopure system and the method of Goring. Reproducible amplification profiles were obtained with all the methods except for Edwards'. As regards amplification conditions, the effects of primer-template annealing temperature, of a final extension step, of the number of cycles and of the length of extension time in each cycle were analysed. Moreover, the effects on amplification reaction of the DNA amount, of MgCl₂, primer and deoxynucleotide triphosphate (dNTP) concentration were evaluated. Then DNA of 12 *Nicotiana* species and *Nicotiana tabacum* was amplified with

primers OPA-01 and OPA-13 which revealed a considerable polymorphism. The same primers used to analyse 36 var. of *N. tabacum* belonging to different types, showed identical amplification profiles. Further amplification experiments were carried out with only 12 of the tobacco lines; three primers among the 12 assayed revealed one polymorphic fragment each. [Beitr. Tabakforsch. Int. 19 (2000) 1–15]

ZUSAMMENFASSUNG

Gegenwärtig ist über das Ausmaß der genetischen Variabilität in *N. tabacum* und in der Gattung *Nicotiana*, bestimmt durch das *random priming*-Verfahren RAPD (Random Amplified Polymorphic DNA), nichts bekannt. Diese Information könnte für taxonomische Zwecke und für die Tabakzüchtung nützlich sein. Ziel dieser Arbeit ist es, eine mögliche Anwendung der durch RAPD-Marker erzeugten DNA-Polymorphismen bei der Gattung *Nicotiana* und beim Tabak zu beurteilen. Da streng standardisierte Reaktionsbedingungen erforderlich sind, um reproduzierbare RAPD-Marker zu erhalten, wurden vier Methoden zur schnellen Extraktion der DNA miteinander verglichen und mehrere Parameter der Reaktionsbedingungen der *random* polymorphen DNA-Amplifikation analysiert und optimiert. Die DNA sechs Wochen alter Tabakblätter von *N. tabacum* der Varietät Samsun wurde mit Hilfe der nachfolgenden Methoden, die sich in der Art der Reinigung voneinander unterscheiden, gewonnen:

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der Cetyltrimethylammoniumbromid (CTAB)-Methode, der Methode nach Edwards, dem *Nucleon Phyt PURE* System und der Methode nach Goring. Reproduzierbare Amplifikationsprofile wurden mit allen Methoden, außer der von Edwards erhalten. Bei den Amplifikationsbedingungen wurden die Auswirkungen der Primerannealing-Temperatur, die Auswirkungen des letzten Extensionsschrittes, der Anzahl der Zyklen und der Dauer der Extensionszeit in jedem einzelnen Zyklus analysiert. Darüber hinaus wurden der Einfluß der DNA-Menge, der $MgCl_2$ -Konzentration sowie der Konzentration des verwendeten Primers und der Desoxynucleotridtriphosphate (dNTP) auf die Amplifikationsreaktion bestimmt. Dann wurde die DNA von 12 *Nicotiana*-Spezies und von *N. tabacum* mit den Primern OPA-01 und OPA-13 amplifiziert, was das Vorliegen einer beträchtlichen Anzahl von Polymorphismen zeigte. Dieselben Primer, mit denen 36 Varietäten von *N. tabacum*, die unterschiedlichen Sorten zugerechnet werden, untersucht wurden, ergaben identische Amplifikationsprofile. Weitere Amplifikationsexperimente wurden mit nur 12 Zuchtlinien durchgeführt; drei von 12 untersuchten Primern zeigten jeweils ein polymorphes Fragment. [Beitr. Tabakforsch. Int. 19 (2000) 1–15]

RESUME

On ne dispose pas actuellement d'informations sur le niveau de la variabilité génétique révélée par l'analyse des profils RAPD chez *N. tabacum* et le genre *Nicotiana*. De telles informations pourraient être utiles en taxonomie et en sélection. L'objectif de cette étude est d'évaluer une application potentielle des polymorphismes de l'ADN générés par marqueurs RAPD pour le genre *Nicotiana* et le tabac (*N. tabacum*). Les conditions de

réaction rigoureusement normalisées étant nécessaires pour obtenir un marqueur RAPD reproductible, quatre méthodes rapides d'extraction d'ADN ont été comparées, et plusieurs paramètres des conditions de la réaction d'amplification aléatoire polymorphe de l'ADN ont été analysés et optimisés. L'ADN de feuilles de *N. tabacum* var. Samsun âgées de six semaines, a été obtenu en appliquant des méthodes, qui diffèrent dans la stratégie de purification: méthode CTAB (cetyltrimethylammonium bromure), méthode d'Edwards, système *Nucleon Phyt PURE*, et méthode de Goring. Des profils d'amplification reproductibles ont été obtenus avec toutes les méthodes sauf celle d'Edwards. Pour les conditions d'amplification, on a analysé les effets de la température d'appariement, l'extension finale, le nombre de cycles de polymérisation et la durée du temps d'extension dans chaque cycle. En outre, on a évalué les effets sur la réaction d'amplification de la quantité d'ADN, de $MgCl_2$ et des concentrations en amorce et en dNTP. Ensuite l'ADN de 12 espèces de *Nicotiana* et de *N. tabacum* a été amplifié avec les amorces OPA-01 et OPA-13 qui ont révélé un polymorphisme important. Les mêmes amorces, utilisées pour analyser 36 variétés de *N. tabacum* de différents types, ont présenté des profils d'amplification identiques. D'autres essais d'amplification ont été réalisés avec seulement 12 lignées de tabac; trois amorces parmi les 12 testées ont révélé un fragment polymorphe. [Beitr. Tabakforsch. Int. 19 (2000) 1–15]

INTRODUCTION

Molecular genetic markers have become fundamental tools for plant scientists as they can provide a relatively unbiased method of quantifying genetic diversity in plants (1).

Table 1.
Tobacco lines examined in this study.

Burley Bu 49	Maryland Md 201	Erzegovina IST E 8A (*)
Burley IST G 14 (*)	Maryland Md 872	Erzegovina IST ER 21
Burley IST PS 186	Maryland Md 609 (*)	Erzegovina IST E 6B
Burley Bu 27	Kentucky IST T 371	Perustitza IST Pr 61 (*)
Burley IST 23 (*)	Kentucky W 39	Perustitza IST P 2B
Burley TN 86	Kentucky IST T 762 (*)	Perustitza IST P 1E
Bright IST G 19 (*)	Havanna IST T 720 (*)	Samsun Katerini 2/5 (*)
Bright IST G 9	Havanna II C	Samsun Katerini 227
Bright IST MS 82 (*)	Havanna IST 142	Samsun Katerini 228
Bright Coker 319	Xanthi IST X 5G	Samsun IST 97
Bright PY 10	Xanthi IST XR 21	Samsun T 16
Bright IST G 165	Xanthi IST X 2H (*)	Samsun T 1116 (*)

* DNA of all lines were amplified with primers OPA-01 and OPA-13. DNA of lines marked with symbol (*) were also amplified with other arbitrary primers.

Several methods have been developed such as restriction fragment length polymorphism (RFLP) technology and polymerase chain reaction (PCR) based techniques. RFLPs have been used extensively for genetic studies (2). However this approach is technically complex, costly, time consuming, and requires large amounts of DNA. Over the last few years, PCR technology has had a significant impact on almost all areas of molecular biology and modifications of the basic procedure have allowed the development of numerous assays for detecting variations at the nucleotide level (3). Among them, the technique named random amplified polymorphic DNA (4, 5, 6), based on PCR amplification of random DNA segments from single arbitrary primers, does not require prior DNA sequence information which is instead necessary for most of the other PCR based assays. Moreover, compared to that of RFLP, RAPD technique is rapid, easy to perform, non radioactive and requires only small amounts of sample DNA. The only disadvantage is that RAPD markers are dominant. This drawback may be overcome in some applications by using more than one closely linked marker (5).

RAPD assay has proved effective in fingerprinting genotypes (7, 8, 9), in phylogenetic studies (10, 11, 12), in the identification of markers linked to disease

resistance (13, 14, 15, 16), in the generation of linkage maps and in measuring genetic diversity-relatedness in several species (17, 18).

In tobacco the RAPD technique has been used mainly to identify markers linked to genes for resistance to pathogens (19, 20, 21, 22), while little information is available about the application of this technique to reveal genetic variability in *N. tabacum* and in the *Nicotiana* genus (23), that could be useful for taxonomic and breeding purposes.

The research described in this paper was conducted to optimize the parameters of RAPD assay for tobacco and to assess the potential application of the DNA polymorphisms revealed by RAPD technique within this genus and in tobacco.

MATERIALS AND METHODS

Plant materials

In this study 36 tobacco lines (Table 1) and 12 *Nicotiana* species and *Nicotiana tabacum* (Samsun line T 1116) (Table 2) were utilized. All species and lines came from the collection belonging to Istituto Sperimentale per il Tabacco in Scafati, Italy.

Table 2.
***Nicotiana* species examined in this study**

Subgenus	Section	Species	Somatic chromosome
<i>Rustica</i>			
	Paniculatae	<i>N. benavidesii</i>	24
	Rusticae	<i>N. rustica</i>	48
<i>Tabacum</i>			
	Tomentosae	<i>N. glutinosa</i>	24
	Genuinae	<i>N. tabacum</i> *	48
<i>Petunioides</i>			
	Alatae	<i>N. alata</i>	18
	Suaveolentes	<i>N. debneyi</i>	48
		<i>N. amplexicaulis</i>	36
		<i>N. velutina</i>	32
		<i>N. occidentalis</i>	42
		<i>N. simulans</i>	40
		<i>N. megalosiphon</i>	40
		<i>N. rotundifolia</i>	44
		<i>N. suaveolens</i>	32

* Samsun line T1116

DNA was prepared from leaves of six-week-old plants by means of the following methods:

A ▶ CTAB method (24, 25), partially modified: 100 mg of leaf tissue pulverized with liquid nitrogen were extracted in a polypropylene centrifuge tube containing 100 μ L of extraction buffer 2 $\times$ (Tris-HCl 100 mM pH 8.0, ethylenediaminetetraacetic acid (EDTA) 20 mM, NaCl 1.4 M, CTAB 2% and mercaptoethanol (added just before use) 2% (w/v)) at 65 °C. Homogenate obtained with a motor driven pestel, was treated with 200 μ L of extraction buffer 1 $\times$ (Tris-HCl 50 mM pH 8.0, EDTA 10 mM, NaCl 0.7 M, CTAB 1% and mercaptoethanol 1% (v/v)) and incubated at 65 °C for 30 minutes. 400 μ L of chloroform/isoamyl alcohol (24:1) were added at room temperature. After centrifugation for 10 minutes at 16 000 g, the supernatant was transferred into a clean tube and treated with 1.5 volumes of precipitation buffer 1 $\times$ (Tris-HCl 50 mM pH 8.0, EDTA 10 mM, CTAB 1%, mercaptoethanol 1% (v/v)). After centrifugation for 5 minutes at 4000 g, the supernatant was discarded and the pellet (CTAB-nucleic acid complex) was solubilized with 200 μ L of NaCl 1 M. DNA was precipitated with 2 volumes of cold absolute ethanol for 15 minutes at room temperature and centrifuged for 5 minutes at 4000 g. The pellet was washed with 70% cold ethanol, air-dried and resuspended in 25 μ L of TE buffer (tris-[hydroxymethyl]aminomethane hydrochloride (10 mM), ethylenediaminetetraacetic acid (1 mM), pH 8.0). The samples were stored at -20 °C.

B ▶ Edwards method (26)

C ▶ Nucleon phytopure system (Amersham Life Science)

DNA was extracted from 100 mg of leaf tissue pulverized with liquid nitrogen, according to the manufacturer's protocol including the presence of 10 mM mercaptoethanol in reagent A and RNase digestion (20 μ g/mL RNase) for 30 minutes at 37 °C after addition of reagent A. At the end of the preparation the pellet was resuspended in 25 μ L of TE buffer pH 8.0 and stored at -20 °C.

D ▶ Goring method (27), partially modified

100 mg of liquid nitrogen pulverized leaf tissue were extracted in a polypropylene centrifuge tube with 400 μ L of extraction buffer (Tris-HCl 50 mM pH 8.0, EDTA 100 mM, NaCl 100 mM, sodium dodecyl sulphate (SDS) 1% (w/v), mercaptoethanol 1% (v/v) (added just before use)). The suspension was added with proteinase K (0.5 mg/mL) and incubated at 60 °C for 30 minutes. Then the homogenate was treated with RNase (280 μ g/mL) for 30 minutes at 37 °C. After incubation, 400 μ L of phenol/isoamyl alcohol/chloroform (25:24:1) were added and the mixture was gently shaken for several minutes and centrifuged for 10 minutes at 16 000 g. The

supernatant was transferred into a fresh tube, treated with an equal volume of cold isopropanol and centrifuged for 5 minutes at 16 000 g. The pellet was washed with cold 70% ethanol, air-dried, resuspended in 25 μ L TE buffer pH 8.0 and stored at -20 °C.

The DNA amount was fluorimetrically quantified using bisBenzimide Hoechst no. 33258 (SIGMA) and DyNA Quant™ 200 Fluorometer (Hoefer). Calibration was performed using calf thymus DNA (SIGMA) as standard.

RAPD-PCR assay conditions for the analysis of Nicotiana species and tobacco lines

Primers were obtained from Operon Technologies Inc. 10 $\times$ buffer, MgCl₂, dNTPs and Taq DNA polymerase (AmpliTaq Gold™) were purchased from Perkin Elmer, gelatine from Boehringer.

The optimized reaction mixture consisted of 20 ng plant DNA and 10 pmol (0.4 μ M) of primer in 25 μ L of the following reaction solution: 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl₂, 100 μ M of each dNTP, 0.001% gelatine and 0.02 U/ μ L of Taq DNA polymerase. The reaction mixture was warmed in 0.2 mL polypropylene microtubes (Perkin Elmer) at 94 °C for 12 minutes then subjected to 45 cycles (94 °C for 0.5 min, 36 °C for 1 min, 72 °C for 1 min) followed by 10 minutes at 72 °C in a GeneAmp 2400 PCR-DNA thermal cycler (Perkin-Elmer).

Electrophoresis

Following PCR, the RAPD amplification products were separated by electrophoresis on 1.4% agarose gel (BIO-RAD) containing ethidium bromide (SIGMA) (1 μ g/mL) in TAE buffer (tris-[hydroxymethyl]aminomethane (40 mM), ethylenediaminetetraacetic acid (1 mM), pH 8.0), at constant voltage (5 V/cm), visualized with UV transilluminator and recorded by photography and by Gel Doc 2000 documentation system (BIO-RAD). 1 Kbp and 100 bp DNA ladder from GIBCO BRL; MM VI from Boehringer, were used as size markers.

Table 3.
Yields of DNA from different extraction procedures.

Method	Yield (μ g/mg of fresh tissue)
CTAB method	278 $\pm$ 40
Edwards method	219 $\pm$ 40
Nucleon phytopure system	196 $\pm$ 20
Goring method	197 $\pm$ 35

Data represent average $\pm$ standard deviation calculated from three experiments with 10 replications each

RESULTS

As rigorously standardized reaction conditions are required to obtain reproducible RAPD profiles, four rapid DNA extraction methods were compared and several parameters of the reaction conditions for the random polymorphic DNA amplification assay were analysed and optimized.

DNA isolation

Since it is not necessary for the extracted DNA to be of high molecular weight in order to produce moderately sized PCR products (28), several simple and rapid DNA extraction procedures are available, but not all are suitable for RAPD assay, according to the plant source investigated. It is necessary that DNA extracted is devoid of polysaccharides, tannins and other components that can inhibit DNA polymerase activity. Therefore in this study, the following DNA extraction methods were compared for *N. tabacum* utilizing leaves of line Samsun T 1116:

- A ▶ CTAB method, partially modified
- B ▶ Edwards method
- C ▶ Nucleon phytopure system
- D ▶ Goring method, partially modified

These methods differ in the strategy of purification: method A is based on the CTAB procedure, in which a precipitation step combined with chloroform extraction is remarkably effective for eliminating complex carbohydrates; method B requires only milligram quantities of fresh tissue and purifies DNA by isopropanol precipitation alone; method C is based on the use of SDS combined with a specially modified resin which binds polysaccharides; method D uses

SDS combined with proteinase K and phenol/chloroform/isoamyl alcohol (25:24:1, saturated with 10 mM Tris pH 8, 1 mM EDTA) purification step. In Table 3 the DNA yields obtained from the four procedures are reported; they slightly differed in DNA yield, which ranged from 200–280 ng/mg of fresh tissue. Three DNA preparations from each method were amplified with arbitrary primer OPA-01 according to the procedure reported in the Material and Methods section, to compare electrophoretic profiles. Reproducible RAPD patterns were obtained with the DNA from all the methods. Moreover, identical amplification profiles, consisting of five bands, ranging from 400–1000 bp, were revealed with DNA from the different extraction procedures except for that of Edwards, which showed only three of the five bands and all of low intensity (Figure 1).

RAPD-PCR parameters optimization

Because the concentration of template DNA, magnesium chloride, deoxynucleosides triphosphate and primer as well as thermal program conditions have an effect on the amplification profile, it is useful to optimize RAPD-PCR parameters when a new application is performed. The optimization was carried out by successive adjustments of standard RAPD conditions (5) and by taking into account the manufacturers' recommendation for each reagent and for the thermal cycler utilized.

Our experiments to optimize the reaction mixture employed four amounts of DNA template, five concentrations of $MgCl_2$ and six concentrations of deoxynucleosides triphosphate and primer. A summary of

Figure 1.

Amplification patterns obtained with primer OPA-01 of *N. tabacum* T 1116 line DNA extracted with four different methods; A = CTAB method, B = Edwards method, C = Nucleon phytopure system, D = Goring method modified. Three different DNA preparations were amplified for each method. Molecular weight (Kbp) markers are as indicated.

Table 4.
Evaluated and optimum concentrations for RAPD assay.

Variable	Concentration	
	Evaluated	Optimum
DNA template (ng/25 μ L)	20–40–60–80	20
MgCl ₂ (mM)	1.5–2.0–3.0–4.0–5.0	2
dNTP (μ M) (each)	50–100–150–200–250–300	100
Primer (μ M)	0.2–0.4–0.6–0.8–1.0–1.2	0.4

Figure 2.
Effect of different amount of DNA template on amplification pattern. The amplifications were performed with primer OPA-01 (a) and primer OPA-13 (b) in a reaction volume of 25 μ L. Molecular weight (Kbp) markers are as indicated.

evaluated concentrations is reported in Table 4. In the same table for each reagent the optimum value revealed by amplification profile analysis obtained with primers OPA-01 and OPA-13 is indicated.

Optimization of DNA concentration

The increase in DNA amount in the PCR mixture in the range 20–40 ng produced no differences in the patterns, while in the range 60–80 ng it resulted in the disappearance of some bands or in the decrease of their intensity (Figure 2); a further increase in DNA amount resulted in a smear of indistinct bands (data not shown).

Optimization of the magnesium chloride concentration

As regards Mg²⁺, concentrations less than 2.0 mM and greater than 3.0 mM caused a loss of bands with respect to patterns observed in the range 2.0–3.0 mM (Figure 3). With primer OPA-13, an increase of the amplification product (about 600 bp) was also observed (Figure 3b).

Optimization of the deoxynucleosides triphosphate concentration

A deoxynucleoside triphosphate concentration of 100–150 μ M for each of the four bases gave the best results in amplification profiles, as both at lower and at higher concentrations the intensity of bands became weaker (Figure 4).

Optimization of the primer concentration

As regards the effect of primer concentration, at 0.2 μ M using OPA-01 it was difficult to detect amplification products, while with OPA-13 fragments, in the range

Figure 3.

Effect of magnesium concentration on amplification pattern. The amplifications were performed with primer OPA-01 (a) and primer OPA-13 (b) in a reaction volume of 25 μ L. Molecular weight (Kbp) markers are as indicated.

1.6–1.0 Kbp, which disappeared at higher primer concentrations were noticed. In the range 0.4–1.2 μ M the increase in the intensity of small amplification products and the loss of some of the largest ones was observed for both primers (Figure 5). A primer concentration of 0.4 μ M was considered optimal.

Optimization of thermal profile

The annealing temperature (T_a) is one of most important parameters that affects the relative intensity of

amplified bands. Amplification with ten base primers is generally performed between 35–37 $^{\circ}$ C, and different results might be expected using different thermal cycling instruments. In order to value the effects of T_a variation in our conditions, four T_a 35; 36; 37; 38 $^{\circ}$ C were assayed.

For each temperature RAPD-PCR assay was performed with four different primers (OPA-01; OPA-013; OPB-12; OPD-03) and two DNA concentrations (20–40 ng in 25 μ L.). For all primers and both DNA concentrations, slight differences in amplification

Figure 4.

Effect of dNTP concentration on amplification pattern. The amplifications were performed with primer OPA-01 (a) and primer OPA-13 (b) in a reaction volume of 25 µL. Molecular weight (Kbp) markers are as indicated.

patterns were observed between 35 °C and 37 °C. The same patterns were obtained at 38 °C Ta but the intensity of some bands decreased.

The effects of the final extension step, the number of cycles and the length of extension time in each cycle were evaluated. The thermal profiles assayed are summarized in Table 5 and the adopted conditions are reported in Table 6.

As DNA polymerase utilized (AmpliTaq Gold™) is provided in an inactive state, it can be added to the

reaction mixture at room temperature without inducing the formation of specific PCR products, so an initial denaturation step 12 minutes long is performed to enable the recovery of enzyme activity. Moreover this step allows the initial complete denaturation of DNA template.

All experiments were conducted by performing RAPD-PCR assays with six primers (OPA-01, OPA-13, OPB-12, OPD-03, OPE-20 and OPL-17) and the results obtained are shown in Figure 6.

The tested PCR thermal profile programs revealed that

Figure 5.

Effect of primer concentration on amplification pattern. The amplifications were performed with primer OPA-01 (a) and primer OPA-13 (b) in a reaction volume of 25 μ L. Molecular weight (Kbp) markers are as indicated.

the increase of the primer extension step from 1 to 2 minutes produced remarkable differences in the banding patterns, thus inducing an increase in the intensity of the largest fragments or the appearance of even larger amplification products; a decrease in the intensity of the smallest fragments and a smearing in the banding patterns was often observed.

The variation of the number of cycles from 45 to 55 had no effect on the electrophoretic profile of amplification products, even if a slight increase in the intensity

of all fragments was observed. The presence of a final extension step always produced an increase in the intensity of amplified fragments, especially the largest ones.

RAPD-PCR analysis of tobacco and Nicotiana species

DNAs were isolated by means of nucleon phytopure system and amplified according to optimized assay

Table 5.
PCR thermal profiles assayed.

Number of cycles	Initial denaturation 94 °C	Denaturation 94 °C	Annealing 36 °C	Extension 72 °C	Final extension 72 °C
45	12 min	0.5 min	1 min	1 min	0 min
					10 min
				2 min	0 min
					10 min
55	12 min	0.5 min	1 min	1 min	0 min
					10 min
				2 min	0 min
					10 min

conditions reported in the Materials and Methods section.

Tobacco DNA (Samsun T 1116 line) was initially checked using 120 arbitrary primers. Twelve primers failed to amplify DNA, while 98 revealed 424 fragments, corresponding to an average number of bands per primer of 3.5 with a range from 1 to 11 different bands per primer. These amplification products had a size ranging from 0.2 to 2.6 Kbp. Primers that produced faint banding patterns or did not reproduce similar banding patterns when replicated at least three

times, were not used for further analysis. So a total of 59 primers were selected for the presence of clear and reproducible bands in the amplification profile.

An initial comparative RAPD analysis of genomic DNA extracted from 36 tobacco lines (Table 1) belonging to different types of tobacco was performed, using primers OPA-01 and OPA-13. The amplification profile obtained using these primers did not reveal the presence of polymorphic bands. Further analysis was carried out with only 12 of the tobacco lines (marked with * symbol in Table 1).

Table 6.
Adopted conditions of PCR thermal profile.

Initial denaturation	Cycle (no 45)			Final extension
	Denaturation	Annealing	Extension	
94 °C	94 °C	36 °C	72 °C	72 °C
12 min	0.5 min	1 min	1 min	10 min

Figure 6.

Amplification profiles obtained with six primers in different PCR thermal programs. Thermal program fixed conditions: Initial denaturation at 94 °C for 12 min, in the cycle denaturation at 94 °C for 0.5 min and annealing at 36 °C for 1 min. Thermal program varied conditions: 1.) extension time in cycle: 72 °C for 1 min (lanes A-B-C-D), 72 °C for 2 min (lanes E-F-G-H); 2.) number of cycles: 45 cycles (lanes A-B, E-F), 55 cycles (lanes C-D, G-H); 3.) final extension: 72 °C for 0 min (lanes A-C; E-G), 72 °C for 10 min (lanes B-D, F-H).

Examination of these lines was performed with 12 of the selected primers. Polymorphism was revealed only with primers OPE-20, OPL-07, OPL-17. Each of them evidenced only one polymorphic band. The amplification pattern obtained with OPE-20 showed an additional fragment of about 750 bp, present in Burley IST 23, Bright IST MS 82, Kentucky IST T 762, Xanthi IST X 2H, Perustitza IST Pr 61, Samsun Katerini 2/5 and Samsun T 1116 (Figure 7). In the amplification profiles obtained with OPL-07 a fragment of about 600 bp was absent in Bright IST MS 82 and in Perustitza IST Pr 61 (Figure 8). In the amplification pattern obtained with OPL-17 a band of about 800 bp was absent only in Burley IST G 14 and in Maryland Md 609 (Figure 9).

Comparative RAPD analysis of genomic DNA extracted from 12 *Nicotiana* species (Table 2) and *N. tabacum* (Samsun T 1116) line was performed using primers OPA-01 and OPA-13. Banding patterns of the amplification products obtained are shown respectively in Figures 10 and 11. The primers OPA-01 and OPA-13 generated, respectively, a total of 17 and 25

amplification products, having a size ranging from 300–1300 bp; for OPA-01 the number of amplified fragments ranged from 2 to 7 while for OPA-13 from 2 to 8. All 42 amplified products were polymorphic. All examined species showed a different amplification profile with OPA-13, while *N. Amplexicaulis*, *N. velutina* and *N. suaveolens* had the same pattern with OPA-01.

DISCUSSION

The first part of this study dealt with the determination of a RAPD assay protocol suitable for tobacco. Our experiments confirmed the importance of the step related to DNA extraction in a RAPD procedure (9). The Edwards method, although the quickest and the simplest of those compared, could not be used for tobacco, as it gave weak amplification profiles and also lacked some fragments. This result is in accordance with the findings of COUSSIRAT (23) who obtained no amplification bands using tobacco DNA extracted with the Edwards method. It is worth noting that the other

Figure 7.

RAPD profiles generated by primer OPE-20 with DNAs extracted from 12 tobacco lines. The following lines are included from lane 1 to 12: Burley IST G 14, Burley IST 23, Bright IST G 19, Bright IST MS 82, Maryland Md 609, Kentucky IST T 762, Havanna IST T 720, Xanthi IST X 2H, Erzegovina IST E 8A, Perustitza IST Pr 61, Samsun Katerini 2/5, Samsun T 1116. The arrow indicates a polymorphic product of about 0.75 Kbp.

Figure 8.

RAPD profiles generated by primer OPL-07 with DNAs extracted from 12 tobacco lines. The following lines are included from lane 1 to 12: Burley IST G 14, Burley IST 23, Bright IST G 19, Bright IST MS 82, Maryland Md 609, Kentucky IST T 762, Havanna IST T 720, Xanthi IST X 2H, Erzegovina IST E 8A, Perustitza IST Pr 61, Samsun Katerini 2/5, Samsun T 1116. The arrow indicates a polymorphic product of about 0.6 Kbp.

Figure 9.

RAPD profiles generated by primer OPL-17 with DNAs extracted from 12 tobacco lines. The following lines are included from lane 1 to 12: Burley IST G 14, Burley IST 23, Bright IST G 19, Bright IST MS 82, Maryland Md 609, Kentucky IST T 762, Havanna IST T 720, Xanthi IST X 2H, Erzegovina IST E 8A, Perustitza IST Pr 61, Samsun Katerini 2/5, Samsun T 1116. The arrow indicates a polymorphic product of about 0.8 Kbp.

Figure 10.

RAPD profiles generated by primer OPA-01 with DNAs extracted from 13 *Nicotiana* species. The following species are included from lane 1 to 13: *N. benevidesii*, *N. rustica*, *N. glutinosa*, *N. tabacum* (Samsun T 1116), *N. alata*, *N. debney*, *N. amplexicaulis*, *N. velutina*, *N. occidentalis*, *N. simulans*, *N. megalosiphon*, *N. rotundifolia*, *N. suaveolens*.

Figure 11.

RAPD profiles generated by primer OPA-13 with DNAs extracted from 13 *Nicotiana* species. The following species are included from lane 1 to 13: *N. benevidesii*, *N. rustica*, *N. glutinosa*, *N. tabacum* (Samsun T 1116), *N. alata*, *N. debney*, *N. amplexicaulis*, *N. velutina*, *N. occidentalis*, *N. simulans*, *N. megalosiphon*, *N. rotundifolia*, *N. suaveolens*.

three methods, though very different in the purification strategy, revealed the same reproducible amplification patterns. We decided to adopt the nucleon phytopure system of DNA extraction in our working protocol as it was quick and simple to use in research programs where large sample throughput is required. Optimum conditions of reagents in the amplification mixture, reported in Table 4, were selected for each component as the lowest concentration which produced reproducible profiles with the highest number of strong bands. Indeed, too much genomic DNA results in smears of bands in the gel (6); an excess of

Mg²⁺ ions may favour the amplification of aspecific products (29); high concentrations of dNTPs affect the fidelity of DNA polymerase (30); the precise ratio of primer to genomic DNA must be adjusted to avoid the lack of clear amplified bands or a high level of background smear (31).

The effects of DNA, magnesium and dNTP concentrations on amplification profiles, as evaluated in this study, agreed with the findings of WILLIAMS (6), while the effects of primer concentration were in variance. WILLIAMS reported that in the range 0.1–2.0 μM the primer concentration had no effect on the relative

intensities of amplification fragments. Our data revealed in the range 0.4–1.2 μ M an increase in the intensity of small fragments and the loss of some of the larger ones.

As regards the PCR program, since no differences were observed in banding patterns obtained with four primers in the range 35 °C – 37 °C, the annealing temperature step at 36 °C was considered suitable when other primers were screened. For all the primers utilized, no additional information was obtained on banding patterns when the number of cycles was increased from 45 to 55. Whereas, depending on the primer utilized, the increase of the extension time from 1 to 2 minutes could produce less informative banding patterns as for OPD-03 and OPE-20, So the initial conditions of thermal profile, arbitrarily defined by taking account of the standard RAPD procedure by WILLIAMS (5) and the recommendations of the thermal cycler manufacturer, were considered appropriate for carrying out RAPD assay.

Having thus established reproducible working conditions of the RAPD assay, amplification patterns of different tobacco lines and *Nicotiana* species were compared.

Concerning the intraspecific analysis performed in *N. tabacum*, the preliminary results obtained showed a low polymorphism, as the majority of the amplified fragments in the examined lines were monomorphic. Therefore, a low genetic variability was revealed in tobacco on the basis of random amplified polymorphic DNA and so this technique seemed to be inadequate for *N. tabacum* variety identification. Nevertheless a low genetic variability revealed by RAPD markers has also been reported for lentil, a predominant self pollinating species like tobacco. Lentil macrosperma and microsperma types had not been differentiated (32) in early studies, but in a subsequent report not only were the two types distinguished, but so were their accessions using additional informative primers (33).

Therefore we maintain that, though a low level of intraspecific polymorphism has been revealed by RAPD technique in this preliminary study, the screening of other carefully selected arbitrary primers is necessary to establish that this technique is not suitable for tobacco line identification and for breeding purposes.

Amplification profiles of the *Nicotiana* species examined, though obtained with only two primers, reveal a high degree of polymorphism, as all amplified products were polymorphic. On the basis of these preliminary results, it may be expected that RAPD technique is a useful tool to individuate molecular markers able to characterize all *Nicotiana* species using an appropriate number of primers. The availability of *Nicotiana* species fingerprints by means of the RAPD technique may contribute to a better characterization of this genus and may be a powerful monitoring tool in maintaining botanic collections.

REFERENCES

1. Clegg, M.T.: Molecular diversity in plant population; *in*: Plant population genetics, breeding, and genetic resource, edited by A.H.D. Brown et al., Sinauer Associates, Inc. Sunderland, M.A., 1990, pp. 98–115.
2. Paterson A.H., S.D. Tanksley and M.E. Sorrels: DNA markers in plant improvement; *Adv. Agron.* 46 (1991) 39–90.
3. Clarke, M.S.: Plant molecular biology. A laboratory manual; Springer Verlag, New York, N.Y., 1997, pp. 305–335.
4. Welsh J. and M. McClelland: Fingerprinting genomes using PCR with arbitrary primers; *Nucleic Acids Res.* 18 (1990) 7213–7218.
5. Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers; *Nucleic Acids Res.* 18 (1990) 6531–6535.
6. Williams J.G.K., M.K. Hanafey, J.A. Rafalski and S.V. Tingey: Genetical analysis using random amplified polymorphic DNA markers; *Methods Enzymol.* 51 (1993) 705–740.
7. Caetano-Anolles G., B.J. Bassam, and P.M. Gresshoff: DNA amplification fingerprint using very short oligonucleotide primers; *Bio-Technology* 9 (1991) 553–557.
8. Hu J. and C.F. Quiros: Identification of broccoli and cauliflower cultivars with RAPD markers; *Plant Cell Rep.* 10 (1991) 505–511.
9. Micheli M.R. and R. Bova: Fingerprinting methods based on arbitrarily primed PCR; Springer-Verlag Berlin Heidelberg, New York, 1997.
10. Halward T.M., H.T. Stalker, E.A. LaRue and G. Kochert: Genetic variation detectable with molecular markers among unadapted germoplasm resources of cultivated peanut and related wild species; *Genome* 34 (1991) 1013–1020.
11. Demeke T., R.P. Adams and R. Chibbar: Potential taxonomic use of random amplified polymorphic DNA (RAPD). A case study in Brassica; *Theor. Appl. Gen.* 84 (1992) 990–994.
12. Hoey B.K., K.R. Crowe and V.M. Jones: A phylogenetic analysis of *Pisum* based on morphological characters, and allozyme and RAPD markers; *Theor. Appl. Genet.* 92 (1996) 92–100.
13. Haley, S.D., P.N. Miklas, J.R. Stavelly, J. Byrum and J.D. Kelly: Identification of RAPD markers linked to a major rust resistance gene block in common bean; *Theor. Appl. Gen.* 86 (1993) 505–512.
14. Michelmore R.W., I. Paran and R.V. Kesseli: Identification of markers linked to disease resistant genes by bulked segregant analysis: A

- rapid method to detect markers in specific genomic regions using segregating population; Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 9828–9832.
15. Martin, G.B., J.G.K. Williams and S.D. Tanksley: Rapid identification of markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines; Proc. Natl. Acad. Sci. U.S.A., 88 (1991) 2336–2340.
 16. Lefebvre V. and A.M. Chèvre: Tools for marking plant disease and resistance genes a review; *Agro-nomia Plant Breeding Rev.* 15 (1995) 3–19.
 17. Tingey S.V. and J.P. del Tufo: Genetic analysis with random amplified polymorphic DNA markers; *Plant Physiol.* 101 (1993) 349–352.
 18. Virk P.S., B.V. Ford- Lloyd, M.T. Jackson and H.J. Newbury: Use of RAPD for the study of diversity within plant germplasm collections; *Heredity* 74 (1995) 170–179.
 19. Bai, D., R. Reedler and J.E. Brandle: Identification of two RAPD markers tightly linked with the *Nicotiana debneyi* gene for resistance to black root rot of tobacco; *Theor. Appl. Genet.* 91 (1995) 1184–1189.
 20. Noguchi S., T. Tajima, Y. Yamamoto and T. Kubo: Identification of RAPD markers linked to potato virus Y resistance gene in tobacco; *Bull. CORESTA*, Yokohama, 1996, Abstr. P9, p. 160.
 21. Johnson S.E.: Identification of RAPD markers to the black shank resistance gene in Coker 371-Gold; *Bull. CORESTA*, Yokohama, 1996, p.88–89.
 22. Rufty R., Y.H. Yi and E.A. Wernsman: RAPD markers linked to wildfire resistance in tobacco identified through bulked-segregant analysis; *Bull. CORESTA*, Montreux, 1997, Abstract AP 63, p.56.
 23. Coussirat J.C.: Genetic fingerprinting of tobacco by RAPD: Use of markers to screen somatic hybrids between *Nicotiana rustica* L; *Ann. du Tabac* 25, Section 2 (1993) 1–6.
 24. Murray H.G. and W.F. Thompson: Rapid isolation of higher molecular weight DNA; *Nucl. Acids Res.* 8 (1980) 4321.
 25. Doyle J.J. and E.E. Dickson: A rapid DNA isolation procedure for small quantities of fresh leaf tissue; *Phytochem. Bull.* 19 (1987) 11.
 26. Edwards K., C. Johnstone and C. Thompson: A simple and rapid method for the preparation of plant genomic DNA for PCR analysis; *Nucleic Acids Res* 19 (1991) 1349.
 27. Goring D.R., L. Thomson and S.J. Rothstein: Transformation of a partial nopaline Synthase gene into tobacco suppresses the expression of a resident wild-type gene; *Proc. Natl. Acad. Sci. USA* 88 (1991) 1770–1779.
 28. Kang F.Y., A. Van Deynze, and K.P. Pauls: Random amplified polymorphic DNA (RAPD) analysis; *in: Methods in plant molecular biology and biotechnology*, edited by B.R. Glick and J.E. Thompson, CRC Press, Inc., 1993, pp. 287–301.
 29. Vroh B.I., P. du Jardin, G. Mergeai and J.P. Baudoin: Optimization et application de la RAPD (Random Amplified Polymorphic DNA) dans un programme de selection recurrent chez le cotonnier (*Gossypium* spp.); *Biotechnol. Agron. Environ.* 1 (1997) 142–150.
 30. Eckert K.A. and T.A. Kunkel: The fidelity of DNA polymerases used in the polymerase chain reaction; *in: PCR a practical approach*, Vol. I, edited by M.J. Mc. Pherson, P. Quirke and G.R. Taylor, Oxford University Press, 1991, pp. 225–244.
 31. Michelmore R.W., R.V. Kesseli, D.M. Francis, M. G. Fortin, I. Paran, and C.H. Yang: Strategies for cloning plant disease resistance genes; *in: Molecular plant pathology, a practical approach*, Vol. II, edited by S.J. Gurr, M.J. Mc. Pherson, and D.J. Bowles, Oxford University Press, 1992, pp. 231–288.
 32. Abo-Elwafa A., K. Murai. and T. Shimada: Intra- and inter-specific variations in *Lens* revealed by RAPD markers; *Theor. Appl. Genet.* 90 (1995) 335–340.
 33. Alvarez M.T., P. Garcia. and M. Perez de la Vega: RAPD polymorphism in Spanish lentil landraces and cultivars; *J. Genet. Breed.* 51(1997) 91–96.

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