Arch. Biol. Sci., Belgrade, 64 (1), 191-199, 2012 DOI:10.2298/ABS1201191S

THE REPRODUCIBILITY OF RAPD PROFILES: EFFECTS OF PCR COMPONENTS ON RAPD ANALYSIS OF FOUR *CENTAURIUM* SPECIES

MARIJANA SKORIĆ[1], B. ŠILER[1], TIJANA BANJANAC[1], JASMINA N. ŽIVKOVIĆ[1], SLAVICA DMITROVIĆ[1], DANIJELA MIŠIĆ[1] and D. GRUBIŠIĆ[1]

[1] *Institute for Biological Research "Siniša Stanković", University of Belgrade,* 11000 Belgrade, Serbia

Abstract - Random amplified polymorphic DNA (RAPD) analysis is a simple and reliable method used to detect DNA polymorphism. Several factors can affect the amplification profiles, thereby causing false bands and non-reproducibility of the assay. In this study, we analyzed the effects of different concentrations of primer, magnesium chloride, template DNA and Taq DNA polymerase to develop and standardize a RAPD protocol for *Centaurium* species. The optimized PCR reaction mixture included: 50 ng of DNA extracted using a CTAB based protocol, 2.5 mM $MgCl_2$, 7.5 pmol primer and 2 U of Taq polymerase in a final volume of 25 μl. Each of the five primers used in experiments (OPB11, OPB15, OPB18, OPF05 and OPH02) generated reproducible and distinguishable fingerprinting patterns of four *Centaurium* species. The obtained optimized RAPD protocol and the selected primers are useful for our further work in the genetic diversity studies of *Centaurium* species.

Key words: Centaurium, RAPD, optimization, DNA, reproducibility

Abbreviations: CTAB - Hexadecyltrimethylammonium bromide; TBE - Tris borate-EDTA; PCR - Polymerase chain reaction; RAPD - random amplified polymorphic DNA

INTRODUCTION

The medicinal properties of Centauries (genus *Centaurium,* fam. Gentianaceae) have been well known for centuries. There are only a few plants possessing so many different and successful uses in traditional medicine as Centauries (Grieve, 1971). The majority of *Centaurium* species, are a rich source of bitter secoiridoid glycosides, xanthones and β-monoglycosides (van der Sluis, 1985) which are reported to have a wide range of bioactivity, with antifungal and antibacterial activity being the most investigated ones (van der Sluis and Labadie, 1981; Tan et al., 1998; Kumarasamy et al., 2003; Šiler et al., 2010).

The similar phenotypic characteristics of different *Centaurium* species perplexed botanists and herbalists during the last century (Pringle, 1987). Some species of this genus are often found alongside each other in the same habitat where interspecies hybrids arise readily, causing morphological variability and resulting in taxonomic divergences (European Medicines Agency, 2009; Mansion, 2004; Mansion et al., 2005). For instance, in areas of the British Isles and northern Europe, hybridization between *Centaurium erythraea* Rafn subsp. *erythraea* and *C. littorale* (D. Turner) Gilmour subsp. *littorale* has resulted in the production of a new allohexaploid species *Centaurium intermedium* (Wheldon) Druce (Ubsdell, 1979).

Various types of morphological, chromosomal, biochemical and molecular markers are widely used for the analysis of genetic diversity and the relationships among and between plant populations, species, and varieties. Until recently, methods for the identification of Centauries were primarily based on the analysis of phenotype markers (different morphological traits, chemical constituents, etc.). However, these markers are generally unreliable indicators of plant genotype, and are influenced by both genetic and environmental factors. Thus, discrimination among closely and related *Centaurium* species was often extremely difficult and questionable. In practice, analysis of molecular markers (e.g. analysis of macromolecules: DNA, RNA and proteins) is often used for the analysis of genetic diversity. The advantages of DNA markers are their stability and detectability in most of the plant tissues and organs, independent of the growth phase. Furthermore, they are not influenced by environmental factors or by pleiotropic and epistatic interactions. Random Amplified Polymorphic DNA (RAPD) is one of the most efficient molecular methods in terms of ability to produce abundant polymorphic markers within a short span of time and limited budget. Since its introduction only two decades ago (Williams et al., 1990), RAPD has proved to be a valuable tool in various areas of plant research, for instance, in studying inter- and intra-species genetic variations, patterns of gene expression, and identification of specific genes using nearly isogenic variants (Caetano-Anollés et al., 1991; Barker et al., 1999; Kuddus et al., 2002; Bauvet et al., 2004). However, the use of RAPD as an approach to molecular typing can be greatly limited by the low repeatability of the amplification patterns. Thus, careful optimization of each step of the amplification reaction is needed to achieve the satisfactory reproducibility of the RAPD data (Micheli et al., 1994). The PCR conditions for RAPD analysis can be optimized by varying the concentrations of the reaction mixture components, e.g. type and concentration of thermostable polymerase, deoxynucleotide triphosphates, Mg^{2+} ions, primer and DNA template concentration, and other reaction factors such as primer annealing, primer extension, denaturation time and temperature (Doulis et al., 2000; Padmalatha and Prasad, 2006).

The RAPD method can be a very valuable tool in studies involving the molecular identification of species, particularly in cases such as the *Centaurium* species where natural hybridization has been frequently reported and may be one of the major causes for morphological variability and resulting taxonomic divergences reported (Ubsdell, 1979; Pringle, 1987; Mansion, 2004; Mansion et al., 2005). The aim of this work was to optimize several components of the PCR reactions used for generating RAPD profiles from genomic DNA of *Centaurium* species, to develop a reliable fingerprinting method for species identification and further extensive genetic studies.

MATERIALS AND METHODS

Plant material

Seeds of *Centaurium littorale* (Turner) Gilmour were purchased from the Botanical Gardens in Nantes in 2004. *C. maritimum* (L.) Fritch seeds were collected in 2002 in the area of Podgorica (Montenegro), whereas seeds of *C. erythraea* Rafn and *C. pulchellum* (Swartz) Druce were collected in 2006 in Serbia (Bele Vode and Koštunići, respectively). The seeds were stored at -20°C until use. Voucher specimens are stored at the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

The collected seeds were surface-sterilized with 20% NaOCl, thoroughly washed 5 times with sterilized distilled water and aseptically plated on medium containing ½ MS salts (Murashige and Skoog, 1962), 20 g l^{-1} sucrose and 7 g l^{-1} agar. Two week-old seedlings were transferred to 760 ml glass jars covered with transparent polycarbonate caps and cultured on the same culture medium under light with a photon flux density 50 μmol m^{-2} s^{-1} (16 h light/8 h dark cycle), at 24 ± 2°C. The plants were grown for an additional 8 weeks until harvesting.

DNA isolation

Freshly harvested *in vitro* grown plants (0.15 – 0.2 g) were ground in liquid nitrogen and DNA was extracted using a CTAB-based procedure described earlier by Zhou et al. (1994). Isolation of DNA was performed in triplicate for each species, using separate individuals. The remaining RNA was eliminated with RNaseA (Fermentas International Inc., Canada) and the suspension was incubated for one hour at 37°C. After another extraction with an equal volume of chloroform-isoamyl alcohol (24:1), the aqueous phase was conserved at -20°C. The DNA concentration was estimated by reading absorbance at 260 nm using a UV-visible spectroscopy system (Agilent 8453, Agilent Technologies, Germany). The purity of the DNA samples was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm and 230 nm. The integrity of the DNA samples was examined by electrophoresis in 1% agarose gel in TBE buffer (TBE 1x) containing ethidium bromide (0.5 μg ml^{-1}) followed by visualization using a UV transilluminator.

RAPD optimization

The isolated DNA of *C. littorale,* as well as OPB15, OPB18 and OPF05 decamer primers (Operon Technologies, Alameda, California USA), were used to optimize the RAPD protocol for *Centaurium* species. The sequences of used primers are presented in Table 1. The initial 25 μl PCR reaction mixture consisted of 50 ng of template DNA, 2.5 mM $MgCl_2$, 2U Taq DNA polymerase (Fermentas International Inc., Canada), 200 μM dNTPs (of each dATP, dCTP, dGTP and dTTP) and 5 pmol primer in 1x $(NH_4)_2SO_4$ reaction buffer supplied with the polymerases (Fermentas International Inc., Canada). The reactions were assembled on ice and overlaid with approximately 40 μl of mineral oil to prevent evaporation. PCR amplifications were performed on a Genius thermocycler (Techne, Cambridge, UK) following amplification profile: an initial denaturation step at 95°C for 5 min, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. The final extension step was 10 min at 72°C and a 4°C hold temperature at the end. The optimization experiments were performed with different concentrations of primer (1, 2.5, 5, 7.5 and 10 pmol), $MgCl_2$ (1.5, 2, 2.5, 3, 3.5, 4 mM), template DNA (5, 10, 25, 50, 75 and 100 ng) and Taq DNA polymerase (0.5, 1, 1.5, 2, 2.5 and 3 U). A master mix of all components was prepared and only the varying components were adjusted individually so that any experimental error would be evenly distributed throughout all the treatments. All the reactions were carried out in triplicate to estimate experimental error and to verify the RAPD reproducibility. Reactions without DNA were used as negative controls.

The amplification products were separated by electrophoresis on 1% agarose gels in 1x TBE buffer at 90 V for 1.5 h. The gels were stained with ethidium bromide (0.5 μg ml^{-1}) and visualized by a UV transilluminator. The 1kb DNA ladder (Fermentas International Inc., Canada) was used as fragment size markers.

The band intensity of RAPD-PCR products, separated on agarose gels, was analyzed by densitometry, using ImageJ 1.32j software (National Institutes of Health, USA). Obtained values were normalized to the highest value, and the results are presented as the relative intensity of DNA fragments.

RAPD profiles reproducibility

The concentration of components that generated the highest number and intensity of bands was selected as the most optimal. Thus, for further analysis of RAPD reproducibility we used the 25 μl PCR reaction mixture containing 50 ng of template DNA, 2.5 mM $MgCl_2$, 2 U Taq DNA polymerase, 200 μM dNTPs (of each dATP, dCTP, dGTP and dTTP) with 7.5 pmol primer in 1x reaction buffer. The reproducibility of the banding patterns obtained using the optimized RAPD method was tested by comparing the fingerprints generated from three replicates of each of the DNA samples extracted from four *Centaurium* species (*Centaurium erythraea*, *C. littorale, C. maritimum* and *C. pulchellum*). In these experiments, five random 10-mer primers were used: OPB11, OPB15, OPB18, OPF05 and OPH02 (Table 1).

Table 1. Primers with arbitrary sequences tested in the RAPD analysis of four *Centaurium* species.

Primer name	Sequence
OPB11	5'-GTAGACCCGT-3'
OPB15	5'-GGAGGGTGTT-3'
OPB18	5'-CCACAGCAGT-3'
OPF05	5'-CCGAATTCCC-3'
OPH02	5'-TCGGACGTGA-3'

RESULTS

DNA isolation

The isolated DNA was of high quality, as demonstrated by parameters ranging between 1.6 and 1.7 after calculating the ratio of absorbance 260/280 nm. The obtained DNA yield ranged from 0.5 to 1 μg μl^{-1}.

Optimization of RAPD protocol

In order to optimize the RAPD technique for *Centaurium* species, several concentrations of primers, $MgCl_2$, template DNA and Taq DNA polymerase were individually tested, while keeping constant concentrations of the remaining PCR reaction components with the same amplification profile in the thermocycler. Data presented in Figs. 1-4 show the variations in the amplification product patterns in *C. littorale*, obtained using OPF05 primer.

The relative importance of primer concentration on the obtained results is demonstrated in Fig. 1. The application of low (1 and 2.5 pmol) and high (10 pmol) primer concentrations resulted in low or no visible amplification of DNA fragments. The best fingerprinting pattern was obtained using 7.5 pmol primer.

The relationship between the concentration of $MgCl_2$ and the obtained RAPD profiles is represented in Fig. 2. The number of detectable products increased at 2.5 mM $MgCl_2$. Similar profiles were obtained using 3 and 3.5 mM $MgCl_2$. In the presence of 4 mM $MgCl_2$, the amplification of the larger fragments was reduced. In terms of the number and intensity of PCR products, 2.5 mM $MgCl_2$ was selected as the optimal concentration.

Identical profiles were obtained using a range of concentrations of template DNA from 10 ng to 100 ng per reaction, although the relative intensity of bands at 75 ng was affected (Fig. 3). The application of a low DNA concentration (5 ng) resulted in hardly detectable amplification.

With regard to the Taq polymerase concentration in the PCR reaction mixture (Fig. 4), there was a general increase in both the number and intensity of the detectable bands by increasing the concentrations of Taq polymerase. Two U was the minimal concentration of Taq polymerase, for which the RAPD profiles were identical (Fig. 4).

The same results were obtained with the other two primers (data not shown). We did not observe amplification bands in the negative controls (reactions without template DNA), indicating that there was no contamination in the assays.

The reproducibility of RAPD profiles

The optimal concentrations of PCR mixture parameters for further experiments were selected according to their relative effects on RAPD amplifications as regards the highest number and intensity of bands. The particular concentrations are given in Materials and Methods. Obtained RAPD profiles of *C. erythraea* and *C. littorale* using two primers (OPB11 and OPB15) are represented in Fig. 5, whereas Fig. 6 demonstrates the RAPD patterns of *C. maritimum* and *C. pulchellum* using OPF05 and OPB15 primers. The identical RAPD profiles for each of the three specimens of the particular species indicated high reproducibility of the standardized method. Clear distinguishing of species and reproducible polymorphic patterns were achieved using all of the five primers (data not shown). The amplified fragment size ranged from 300 bp to 2000 bp.

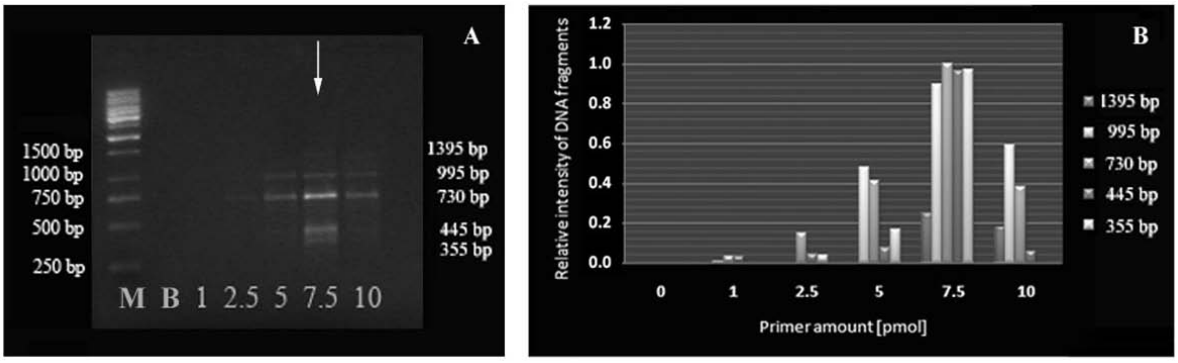

Fig. 1. Effect of primer (OPF05) concentrations on RAPD patterns of *C. littorale*. Increasing concentrations of primer (1, 2.5, 5, 7.5 and 10 pmol) were tested in the initial 25 µl PCR reaction mixture consisted of 50 ng of template DNA, 2.5 mM $MgCl_2$, 2 U Taq DNA polymerase. Lane B – negative control with all the reaction components except template genomic DNA. Lane M – l kb ladder size marker (Fermentas International Inc., Canada). Arrow represents the optimal concentration. Relative band intensity of DNA fragments analyzed by densitometry. Obtained values were normalized to the highest value, and the results are presented.

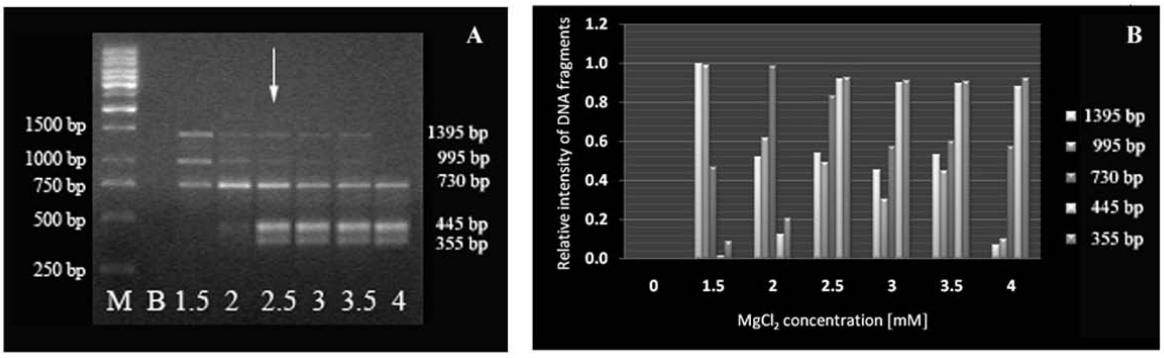

Fig. 2. Effect of template DNA concentrations on RAPD patterns of *C. littorale* using OPF05. Increasing amounts of *C. littorale* DNA (5, 10, 25, 50, 75 and 100 ng) were amplified in the initial 25 µl PCR reaction mixture consisted of 2.5 mM $MgCl_2$, 2 U Taq DNA polymerase and 5pmol primer. Lane B – negative control with all the reaction components except template genomic DNA. Lane M – l kb ladder size marker (Fermentas International Inc., Canada). Arrow represents the optimal concentration. Relative band intensity of DNA fragments analyzed by densitometry. Obtained values were normalized to the highest value, and the results are presented.

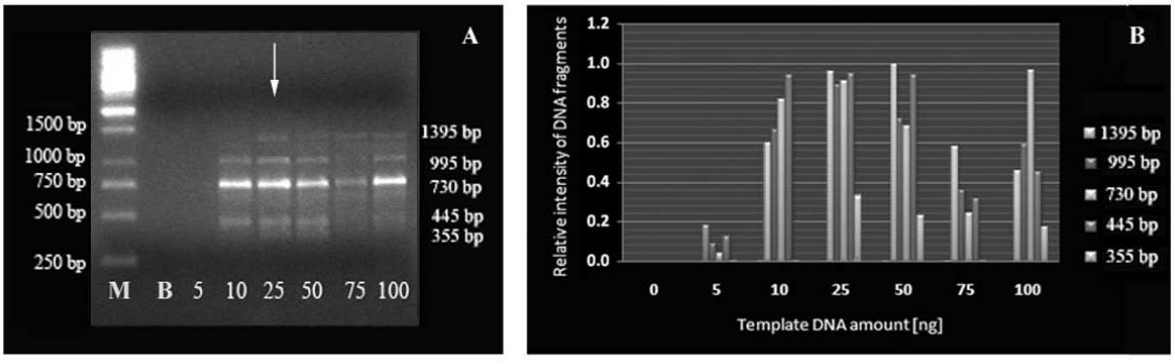

Fig. 3. Effect of magnesium chloride concentrations on RAPD patterns of *C. littorale* using OPF05 primer. Varying concentrations of $MgCl_2$ (1.5, 2, 2.5, 3, 3.5, 4 mM) were used in the initial 25 µl PCR reaction mixture consisted of 50 ng of template DNA, 2 U Taq DNA polymerase and 5pmol primer. Lane B – negative control with all the reaction components except template genomic DNA. Lane M – l kb ladder size marker (Fermentas International Inc., Canada). Arrow represents the optimal concentration. Relative band intensity of DNA fragments analyzed by densitometry. Obtained values were normalized to the highest value, and the results are presented.

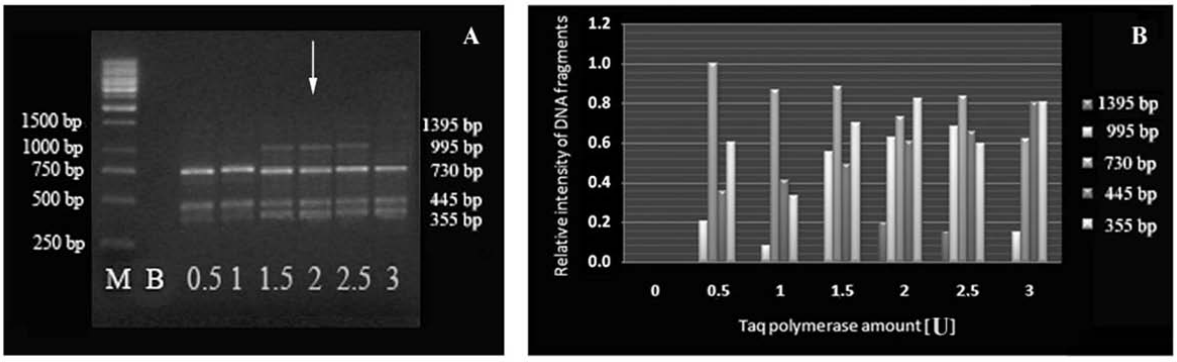

Fig. 4. Effect of Taq DNA polymerase concentrations on RAPD patterns of *C. littorale* using OPF05 primer. Varying concentrations of Taq DNA polymerase (0.5, 1, 1.5, 2, 2.5 and 3U) were used in the initial 25 µl PCR reaction mixture consisted of 50 ng of template DNA, 2.5 mM $MgCl_2$U and 5pmol primer. Lane B – negative control with all the reaction components except template genomic DNA. Lane M – l kb ladder size marker (Fermentas International Inc., Canada). Arrow represents the optimal concentration. Relative band intensity of DNA fragments analyzed by densitometry. Obtained values were normalized to the highest value, and the results are presented.

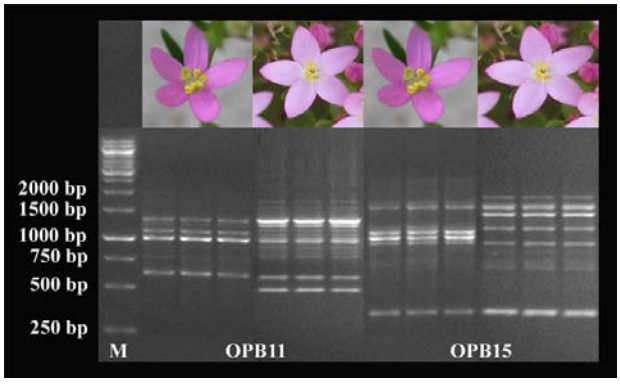

Fig. 5 - RAPD banding patterns obtained from three specimens of the *C. littorale* and *C. erythraea* using the optimal conditions. Lane M – 1 kb DNA ladder (Fermentas International Inc., Canada); Lanes 1-3 and 7-9 – the RAPD- PCR products amplified from genomic DNA of *C. littorale* using the OPB11 and OPB15 primer. Lanes 4-6 and 9-12 – the RAPD-PCR products amplified from genomic DNA of *C. erythraea* using the OPB11 and OPB15 primers, respectively.

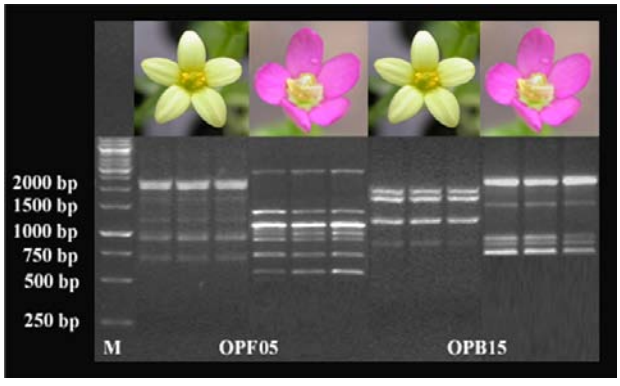

Fig. 6 - RAPD banding patterns obtained from three specimens of the *C. maritimum* and *C. pulchellum* using the optimal conditions. Lane M - 1kb DNA ladder (Fermentas International Inc., Canada); Lanes 1-3 and 7-9 – the RAPD- PCR products amplified from genomic DNA of *C. maritimum* using the OPF05 and OPB15 primers. Lanes 4-6 and 9-12 – the RAPD-PCR products amplified from genomic DNA of *C. pulchellum* using the OPF05 and OPB15 primer, respectively.

DISCUSSION

Performing RAPD analysis using the plant material obtained under *in vitro* conditions has a few advantages: growth under aseptic conditions, a year-round availability of plant material, and the high multiplication level. Using techniques such as micropropagation, the appearance of somaclonal variations is limited. The obtained *in vitro* plant material is free of viruses and other pathogens, hence the contaminations of the isolated plant DNA with foreign DNA is excluded. This is especially important when isolated DNA is further used as a template for RAPD analysis, where small unspecific primers are used. Thus, the obtained results are more reliable.

The isolation and purification of DNA, especially from medicinal and aromatic plants, can result in the degradation of DNA caused by endonucleases, co-isolation of highly viscous polysaccharides, inhibitor

compounds such as polyphenols, and other secondary metabolites that directly or indirectly interfere with the enzymatic reactions. Certain polysaccharides are known to inhibit RAPD reactions. They can distort the results in many analytical applications and thereby lead to incorrect interpretations (Kotchoni et al., 2003). Polysaccharide co-precipitations could be avoided by adding a selective precipitant of nucleic acids, i.e., cetyltrimethylammonium bromide (CTAB) (Dellaporta et al., 1983). Therefore, in the present work, the CTAB DNA extraction protocol was applied (Zhou et al., 1994).

Many DNA isolation procedures yield large amounts of RNA, particularly 18 S and 25 S rRNA (Doyle and Doyle, 1987). The reduced yield of a PCR reaction can be caused by large amounts of RNA in the sample. Because of this, in our work we used RNaseA treatment to degrade the RNA into small ribbon nucleosides that do not contaminate DNA preparation and yielded RNA-free pure DNA.

The alterations in the different parameters tested had varying degrees of influence on the RAPD patterns and their reproducibility. An efficient protocol for RAPD analysis should be reasonably resistant to variations in template DNA concentrations. For the majority of plant species, qualitative results have been achieved using 50 to 100 ng in 50 µl reaction mixtures (Caetano-Annollés et al., 1991*)*. In our experiments, identical profiles were obtained using a range of concentrations of template DNA from 10 ng to 100 ng in 25 µl reaction, showing that the limits of template DNA were not narrow and the patterns obtained were relatively constant, even with a 10-fold increase in template concentration (Fig. 2). Similar observations have been reported for chrysanthemum DNA where amplification was relatively constant between 1 and 500 ng of the template DNA (Wolff et al., 1993).

The primer concentration is a factor that rather affects RAPD reproducibility. Higher concentrations lead to primer-dimer formation (Padmalatha and Prasad, 2006; Harini et al., 2008). In the present study, the final primer concentration of 7.5 pmol (Fig. 1) was optimal to ensure a maximal visibility of bands in the RAPD profiles.

Since Mg^{2+} is a co-factor of the Taq DNA polymerase enzyme, it influences the DNA amplification process and affects the quality of RAPD profiles obtained (Munthali et al., 1992). Generally, increasing amounts of Mg^{2+} will result in the accumulation of non-specific amplification products, although insufficient Mg^{2+} will reduce the yield (Williams et al., 1993). The use of $MgCl_2$ concentrations higher than 1 mM has been reported to be essential for sufficient quantities of DNA amplification when using bacterial and plant DNAs (Bassam et al., 1992). Usually, $MgCl_2$ concentrations in the range from 1 to 8 mM have been reported as optimal in most RAPD assays. In the present study, $MgCl_2$ concentration was important to the RAPD pattern obtained, having only a minor influence on the yield of amplified DNA. After this study, 2.5 mM $MgCl_2$ is considered to be the standard for RAPD analyses of *Centaurium* (Fig. 2).

Another factor that affects the reproducibility of a RAPD pattern is the quantity of Taq polymerase. Although only small increasing numbers of bands were observed with increasing enzyme concentration, these bands were more distinct at concentrations above 2 U in the final 25 µl reaction mixture volume. Still, it must be pointed out that the efficiency of the same polymerase can vary considerably depending on the nature of the target sequence, the primer sequences, and the reaction conditions (Eckert and Kunkel, 1991). Therefore, the effectiveness of Taq polymerase reported here might not essentially reflect the efficiency of a different PCR that would be carried out under different conditions.

Since the RAPD technique has achieved an extensive application in the studies of genetic diversity in the natural populations of some species (Demeke and Adams, 1994; Hensena et al., 2005; Vyas et al., 2009), the distinction of true polymorphism from artifactual variation is a critical point. Therefore, analysis of RAPD reproducibility is required in studies involving molecular identification, particularly in species such are those belonging to the genus *Centau-*

rium, which hybridize readily and their ranges extensively overlap. The screening of primers showed that OPB11, OPB15, OPB18, OPF05 and OPH02 gave a reproducible polymorphism, thereby recommending these primers as suitable for use in genetic studies of *Centaurium* species. The usefulness of primers was evaluated according to two criteria: (1) whether they revealed polymorphisms between different species; and (2) whether the polymorphisms were reproducible among samples of the same species. As such, they have the potential to be used for RAPD analysis in screening for inter- and intra-species genetic diversity within the *Centaurium* genus.

In conclusion, all of the tested parameters for the successful RAPD-PCR protocol establishment, including DNA template, primer, $MgCl_2$ and Taq polymerase concentrations, were optimized. A standardized protocol enabled the generation of clear, scorable, amplified products suitable for RAPD analyses of *Centaurium* species. The reproducibility of RAPD patterns of the four tested *Centaurium* species was of satisfactory quality. The optimized reaction described in the present work provides a rapid molecular tool for different applications related to the genetic diversity studies of *Centaurium* species.

Acknowledgement - We dedicate this work to our dear colleague Professor Dr. Dragoljub Grubišić, whom we remember with affection. This work was supported by the Ministry of Education and Science of the Republic of Serbia, Contract No. 173024.

REFERENCES

Barker, J. H. A., Matthes, M., Arnold, G. M., Edwards, K. J., Åhman, I., Larsson, S., and *A. Karp* (1999). Characterization of genetic diversity in potential biomass willows (*Salix* spp.) by RAPD and AFLP analysis. *Genome* **42**, 173-183.

Bassam, B. J., Caetano-Anollés, G., and *P. M. Gresshoff* (1992). Amplification fingerprinting of bacteria. *Appl. Microbiol. Biot.* **38**, 70-76.

Bauvet, J. M., Fontaine, C., Sanou, H., and *C. Cardi* (2004). An analysis of the pattern of genetic variation in *Vitellaria paradoxa* using RAPD marker. *Agroforest. Syst.* **60**, 61-69.

Caetano-Anollés, G., Bassam, B. J., and *P. M. Gresshoff* (1991). DNA amplification fingerprinting: A strategy for genome analysis. *Plant Mol. Biol. Rep.* **9**, 294-307.

Dellaporta S .L., Wood J., and *J. B. Hicks* (1983). A plant DNA mini-preparation: version II. *Plant Mol. Biol. Rep*. **1,** 19-21.

Demeke, T. and *R. T. Adams* (1994). The use of PCR-RAPD analysis in plant taxonomy and evolution, In: *PCR Technology: Current Innovations* (Eds. H.G. Griffin and A.M. Griffin), 179-189. CRC Press, Boca Raton, Florida.

Doulis A. G., Harfouche A. L., and *F. A. Aravanopoulos* (2000). Rapid, High Quality DNA Isolation from Cypress (*Cupressus sempervirens* L.) Needles and Optimization of the RAPD Marker Technique. *Plant Mol. Biol. Rep.* **17**, 1–14.

Doyle J. J. and *J. L. Doyle* (1987). A rapid DNA isolation procedures for small quantities of fresh leaf tissues. *Phytochem. Bull.* **19**, 11-15.

Eckert, K. A. and *T. A. Kunkel* (1991). DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl.* **1**, 17-24.

European Medicines Agency, (2009). Evaluation of Medicines for Human Use. Available on-line at: http://www.emea.europa.eu/pdfs/human/hmpc/centaurii_herba/10553508en.pdf

Grieve, M. (1971). A Modern Herbal: The medicinal, culinary, cosmetic and economic properties, cultivation and folklore of herbs, grasses, fungi, shrubs, & trees with all their modern scientific uses. Dover Publications. Available online at: http://www.botanical.com/products/learn/c/centaury.html

Harini, S. S., Leelambika, M., Shiva Karmeswari, M. N., and *N. Sathyanarayana* (2008). Optimization of DNA isolation and PCR-RAPD methods for molecular analysis of *Urginea indica* (Kunth). *Int. J. Integr. Biol.* **2**, 138-144.

Hensena, I., Oberprielerb, C., and *K. Weschea* (2005). Genetic structure, population size, and seed production of *Pulsatilla vulgaris* Mill. (Ranunculaceae) in Central Germany. *Flora* **200,** 3-14.

Kotchoni, S. O., Gachomo, E. W., Betiku, E., and *O. O. Shonukan* (2003). A homemade kit for plasmid DNA mini-preparation. *Afr. J. Biotechnol.* **2**, 88- 90.

Kuddus, R. H., Kuddus, N. N., and *I. Dvorchik* (2002). DNA polymorphism in the living fossil *Ginkgo biloba* from the Eastern United States. *Genome* **45**, 8–12.

Kumarasamy, Y., Nahar, L., and *S. D. Sarker* (2003). Bioactivity of gentiopicroside from the aerial parts of *Centaurium erythraea. Fitoterapia* **74**, 151-154.

Mansion, G. (2004). A new classification of the polyphyletic genus *Centaurium* Hill (*Chironiinae - Gentianaceae*): description of the New-World endemic *Zeltnera*, and reinstatement of *Gyrandra* Griseb., and *Schenkia* Griseb. *Taxon* **53**, 719-740.

Mansion, G., Zeltner, L., and *F. Bretagnolle* (2005). Phylogenetic patterns and polyploid evolution within the Mediterranean genus *Centaurium* (Gentianaceae - Chironieae). *Taxon* **54**, 931–950.

Micheli, M., Bova, R., Pascale, E., and *E. D'Ambrosio* (1994). Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Res.* **22**, 1921-1922.

Munthali, M., Ford-Lloyd, R. V., and *H. J. Newbury,* (1992). The random amplification of polymorphic DNA for fingerprinting plants. *PCR Methods Appl.* **1**, 274-276.

Murashige, T. and *F. Skoog,* (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plantarum* **15**, 473-497.

Padmalatha, K. and *M. N. V. Prasad* (2006). Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from Peninsular India. *Afr. J. Biotechnol*. **5,** 230-234.

Pringle, J. S. (1987). Proposal to reject the name *Centaurium minus* Moench (*Gentianaceae*). *Taxon* **36**, 482–483.

Tan, R. X., Kong, L. D., and *H. X. Wei* (1998). Secoiridoid glycosides and an antifungal anthranilate derivate from *Gentiana tibetica. Phytochemistry* **47**, 1223-1226.

Šiler, B., Mišić, D., Nestorović, J., Banjanac, T., Glamočlija, J., Soković, M., and *A. Ćirić* (2010). Antibacterial and antifungal screening of *Centaurium pulchellum* crude extracts and main secoiridoid compounds. *Nat. Prod. Commun.* **5**, 1525 – 1530.

Ubsdell, R. A. E. (1979). Studies on variation and evolution in *Centaurium erythraea* Rafn and *C. littorale* (D. Turner) Gilmour in the British Isles, 3. Breeding systems, floral biology and general discussion. *Watsonia* **12**, 225-232.

van der Sluis, W. G. and *R. P. Labadie* (1981). Fungitoxic activity of the secoiridoid glucoside gentiopicrin (gentiopicroside). *Planta Med.* **42**, 139-140.

van der Sluis, W. G. (1985). Chemotaxonomical investigations of the genera *Blackstonia* and *Centaurium* (*Gentianaceae*). *Plant Syst. Evol.* **149**, 253-286.

Vyas, G. K., Sharma, R., Kumar, V., Sharma, T. B., and V. Khandelwal (2009). Diversity analysis of *Capparis decidua* (Forssk.) Edgew. using biochemical and molecular parameters. *Genet. Resour. Crop Ev.*, **56**, 905-911.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and *S. V. Tingey* (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.

Williams, J. G. K., Hanafey, M. K. , Rafalski, J. A., and *S. V. Tingey* (1993). Genetic analysis using random amplified polymorphic DNA markers. *Method. Enzymol.* **218**, 704-741.

Wolff, K., Schoen, E. D., and J. Peters-Van Rijn (1993). Optimizing the generation of random amplified polymorphic DNAs in chrysanthemum. *Theor. Appl. Genet*. **86**, 1033-1037.

Zhou, X., Guangcheng, C., Rufa, L., Yongru, S., and L. Wenbin (1994). A rapid and efficient DNA extraction method of genus *Fagopyrum* for RAPD analysis, In: *Proceedings of the International Colloquium on Impact of Plant Biotechnology on Agriculture* (Eds. B. Javornik, B. Bohanec, and I. Kreft), 171-175. University of Ljubljana, Ljubljana.