COMPARATIVE CHROMOSOME AND MOLECULAR STUDIES OF SOME SPECIES OF GENUS *ARUM* FROM EASTERN SLAVONIA AND BARANYA REGION IN CROATIA

ANITA LENDEL[1], MARIJA BEDALOV[2], MIRJANA SABO[3], TOMISLAV BAČIĆ[4], LJILJANA KRISTIN[4], TIHANA MARČEK[3]

[1] Institut für Systematische Botanik
Zollikerstrasse 107, CH – 8008 Zürich, Switzerland

[2] Universite de Neuchâtel, Laboratorie de Botanique Evolutive
Rue Emile-Argand 11, 2007 Neuchâtel, Switzerland

[3] Faculty of Food Technology, J.J. Strossmayer University
Kuhačeva 13, 31000 Osijek, Croatia
e-mail: mirjana.sabo@ptfos.hr

[4] Faculty of Philosophy, J.J. Strossmayer University
L. Jägera 9, 31000 Osijek, Croatia

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ABSTRACT

Karyological and molecular studies were done in this paper on three species of genus *Arum*; *Arum italicum* Mill. and *Arum maculatum* L., with two varieties, and *Arum alpinum* Schott & Kotschy, also with two varieties. The main goal of this paper was to establish whether they were regularly determined exclusively on the principle of morphological parameters. Karyological studies showed that the number of chromosomes for *Arum italicum* Mill. amounted to 2n=84, for *Arum maculatum* L. 2n=56 and for *Arum alpinum* Schott & Kotschy 2n=28. This confirmed that these species are not only clean and separated, but also support the regularity of the morphological determination. Molecular studies, e.g. RAPD method showed that two genetically separated species groups correspond to the three mentioned species. *Arum italicum* Mill. is the least homogenous species closely related and the variability between populations is high. *Arum maculatum* L. is more homogenous within the species, two varieties could be differentiated whereas they are closely genetically related and the variability between the populations is too high. *Arum alpinum* is strongly homogenous and within these species two varieties could also be differentiated. That means that they are closely related and the variability between the populations is very high. These observations mostly coincided with previous morphological investigations.

KEY WORDS: *Arum* species, chromosomes, molecular studies.

INTRODUCTION

This work is a continuation of our research presented in the previous paper (Lendel et al. 2004). As it has already been said, the three species, the two with two varieties, were determined in general as regular ones on the five localities exclusively on the basis of morphological parameters. The species are: *Arum italicum* Mill., *Arum maculatum* L. var. *maculatum* L., *Arum maculatum* L. var. *immaculatum* Reichb., *Arum alpinum* Schott & Kotschy var. *pannonicum* Terpó and *Arum alpinum* Schott. & Kotschy var. *intermedium* Terpó. The species *Arum maculatum* L. var. *immaculatum* Reichb. and *Arum alpinum* Schott. & Kotschy, both with two varieties, are not mentioned in Flora Europaea (1964-1980) or in Flora of Croatia (Domac 1994). The habitats are: Zablaće, Katunište and Normanci in Eastern Slavonia and Bilje and Branjin Vrh in Baranya. The regular identification of the species of genus *Arum* has presented a great problem from the beginning (Boys 1993). Studies of the species of genus *Arum* go back into the past (Reinchenbach 1830; Schott and Kotschy 1851; Schott 1860; Prime 1961; Terpó 1971, 1973, 1992; Bedalov 1975-1977, 1981, 1983; Bedalov and Fischer 1994; Bedalov et al. 1998), but the identifications of the separate species have not been sufficient until today. The purpose of this study is to establish, through investigations of chromosomes and molecular studies, whether the previous morphological studies were exactly true.

MATERIAL AND METHODS

Habitats, species and samples

One of the leaves of each species of genus *Arum* was chosen for the studies in each of the following localities in Eastern Slavonia and Baranya region in Croatia. The localities in Eastern Slavonia were: Zablaće, Katunište and Normanci in which *Arum italicum* Mill., *Arum maculatum* L. var. *maculatum* L. and *Arum maculatum* L. var. *immaculatum* Reichb are growing. The localities in Baranya were Bilje and Branjin Vrh. In these habitats two *Arum* species were: *Arum alpinum* Schott & Kotschy var. *pannonicum* Terpó and *Arum alpinum* Schott & Kotschy var. *intermedium* Terpó.

Karyological investigations

The observations and counting of the number of chromosomes of the species of genus *Arum* were performed in mitotic cells placed in apical meristem of rootlets apex. The rootlets were fixed during October when its growth is rapid. The pre-treatment consisted of rootlets treatment by saturated water solution of α-bromonaphtalen during 2.30 to 4.30 hours. After that followed the fixation in aceti alcohol (1:3) and dying with acetocarmine and ferrum acetate. After warming up, during two minutes, "squash" of cells was made. Chromosomes were counted by light microscope (Dialux 20 EB, Leitz, Germany) at the immersion magnification of 1000×.

Molecular measurements

Molecular measurements involved the following treatments: DNA extraction, quantification of extracted DNA, random amplification of polymorphic DNA (RAPD), and gel electrophoresis.

DNA extraction

DNA was extracted from leaf tissue dried in silica gel of each investigated species of the genus *Arum*. The extraction was done by Quiagen Dneasy™ Plant Mini Kit (Quiagen, Basel) and performed with liquid nitrogen (N_2) that destroyed plant tissues and cells. The destroyed cells were then lysed by adding a lysis buffer (400 µl API, Quiagen) and incubated at 65°C during ten minutes. In such a sample Rnaze (4 µl Rnase A, Quiagen) was added to destroy RNA. After polysaccharide and proteins precipitation at 40°C a centrifuge removed the other cell remains.

Determination of extracted DNA concentration

The concentration of extracted DNA was determined by the Lightwave UV/VIS Diode-Array Spectrophotometer (WPA Ldt, Cambridge). Aliquot of DNA stock solution was prepared to ~30 µg/µl by a sample concentration or dilution and stored at 4°C for further use.

Random amplification of polymerase DNA

This method is a treatment with molecular marks. The method is based on different segments of DNA, which can be isolated from the plants, e.g. polymorphic enzymes, and then mutually compared. In the analysis of genome of the species of genus *Arum* the RAPD method, or AFLP, RFLP; SSR and SNP, was chosen. The principle of this method is a multiple randomly selected DNA sequence between two monomial DNA if they are at a certain distance. The result of this reaction is visualised as electrophoregram with either one or more visible bands. The bands represent DNA fragments and serve for genotype identification, similarity comparison among analysed genotypes, or for the link analysis among some useful properties. The occurrence, number and arrangement of bands are stable for one genotype and as a rule do not depend on ecological factors. In case of amplification of the parts of genome of the species of genus *Arum* RAPD, the solution was modified according to Guadagnuolo et al. (2001) and consisted in the volume of 25 µl.

Polymerase chain reaction

The method of polymerase chain reaction enables the multiple DNA in vitro. For this treatment one molecule of DNA is sufficient and it needs to be multiple (so-called template molecule of DNA), one molecule of monomial DNA (so-called primer) which will set the boundaries of the template place, four molecules of deoxyribonucleotide phosphates, enzyme polymerase and a source of warmth. The number of new DNA, which needs to be synthesised, is increased exponentially to 2n during several hours (Fig. 1). One DNA molecule produced about 1 048 576 new DNA molecules during 20 cycles. The whole process was performed in about 40 cycles and the result of this multiple DNA is finally 10^9 molecules (Delić 1997). For multiple DNA fragments of the species of genus *Arum* Biometra T gradient and T3 Termocycler (Whatman Biometra, Göttingen) were used with the following treatment: denaturation during 5 minutes at 94°C, 40 cycles in a minute at 93°C, the link of DNA primer and the link of DNA template during one minute at 40°C to 44°C and the DNA chain length during one minute at 72°C.

Electrophoresis

This is the method for separating macromolecules either of DNA, of RNA or proteins of different electrical charge in an agar gel in electrical field through the opposite electrical charge from itself, as well as their isolation, cleanse and characterisation (Delić 1997). Nucleic acids are slightly negative and therefore they are attached to the positive electrode. The dimension of DNA segments in comparison with the known marks gives the electrophoresis. Bands of DNA fragments with this gel product could be seen by ad-

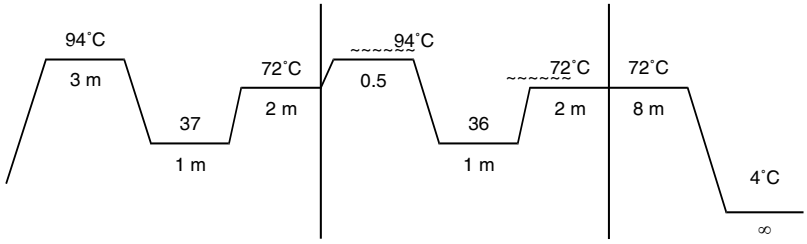

Fig. 1. Thermal Cycler condition for RAPD--PCR reaction.

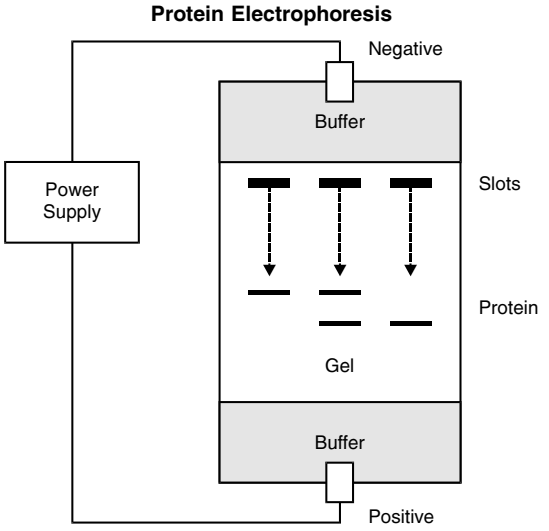

Fig. 2. Scheme of the gel electrophoresis.

ding an intercalary colour ethidium bromide, which in UV light is fluorescent. Multiple products of DNA fragments of the species of genus *Arum* were separated in 1.6% (w/v) agar gel in 0.5% TBE (tries borate) at 100 V, coloured by fluorescence ethidium bromide and photographed at UV light with the gel documentation system (BioRad Hercules, Ca, USA) (Fig. 2).

Statistical analysis of obtained data by RAPD method

The treatment of the obtained data by the RAPD method begins by record making of the electropherogram with molecular analysis software version 1.5 (BioRad, Hercules, CA, USA). For each version number 1 was recorded, when the band individuals existed, or 0 if the band did not exist. The band presence/absence was recorded in the Excel table. The matrix of genetic resemblance was accounted from the binary table according to Jaccard asymmetrical coefficient (Jaccard 1980): Sj=a/(a+b+c) (a – number of fragments shared by both individuals, b and c – number of fragments characteristic for certain individuals).

For statistical meaning of the species and population, as well as for the separation, the Mantel test was used (Mantel 1967) within R4 parcel (Phillipe Casgrain & Pierre Legendre, Universities de Montreal).

The similarity matrix was converted to the distance of matrix according to the formula: $D_1=1-S_i$ and compared with the matrix in which the two individuals of the same species had values 1, whereas the two individuals of different species or population had values 0. The obtained r – values were interpreted as a correlation coefficient. The distance matrix as principal coordinates analysis (PCoA) was used (Gower 1996).

RESULTS AND DISCUSSION

Karyological investigations

Studies of chromosomes pointed out that number of chromosomes for each of the individuals within the species *Arum italicum* Mill. accounted 2n=84, in *Arum maculatum* L. 2n=56, and in *Arum alpinum* Schott & Kotschy 2n=28. Between the two varieties of *Arum maculatum* L., var. *maculatum* L. and var. *immaculatum* Reichb., as well as between varieties of *Arum alpinum* Schott & Kotschy var. *pannonicum* Terpó and var. *intermedium* Terpó, no differences in the number of chromosomes were found. On the basis of these parameters no hybrids were found either. But such finding does not mean that hybrids exist at these localities. They are probably present in this region, but have not been detected so far. (Bedalov et al. 1994). Therefore future studies should be dedicated to this problem. It should be also stressed that these investigations of chromosomes did not only completely coincide with, but were also supported by the studies of Bedalov (1975-1977, 1981, 1983) and Bedalov et al. (1998).

Results obtained by RAPD method

By this method 46 individuals were studied and they belonged to three species and represented 11 populations from the 4 localities. For their studies 5 monomials were used as follows (Table 1). Under the assumption that every band with different molecular weight represents a unique locus, as well as fragments amplified with different primers (Schoenenberger 2001), these 5 monomials gave either 70 locus or in average of 14, according to the primers. However, this number varies from 8 to 17 fragments according to the primers (Fig. 3). Since the RAPD markers are dominant, it is supposed that each band represents the phenotype at the simple biallelic locus (Williams at al. 1990).

TABLE 1. Primers DNA used in RAPD method.

Primers	Sequence (5'-3')
OPB 15	GGAGGGTGTT
OPB 20	GGACCCTTAC
OPT 6	CAAGGGCAGA
OPT 7	GGCAGGCTGT
OPT 18	GATGCCAGAC

The Jaccard's coefficients (Jaccard 1980) of similarities were used to make the dendrogram with arithmetic mean algorithm (Sokal and Rohlf 1981). For this the program of Cluster Package Software (Brzustowski 1999) Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used. The dendrogram was visualised by the use of the program Treeveiw Package Softwere (Page 1998) (Fig. 4). In the dendrogram 46 individuals were separated; 10 individuals belonged to the species *Arum italicum* Mill. (Zablaće: Z_{11}-Z_{15} and Normanci: N_{11}-N_{15}); 10 individuals belonged to the species *Arum maculatum* L. var. *maculatum* L. (Normanci: N_6-N_{10} and Zablaće: Z_6-Z_{10}); 10 individuals belonged to the species *Arum maculatum* L. var. *immaculatum* Reicbh. (Normanci: N_1-N_5 and Zablaće: Z_1-Z_5) and 16 individuals belonged to the species *Arum alpinum* (Bilje: B_1--B_{10} and Branjin Vrh: BV_1-BV_5). Three species were particularly visibly separated. These were: *Arum italicum* (Z_{11}--N_{15}); *Arum maculatum* (N_5-N_6) and *Arum alpinum* (V--BV_3). Inside the group of *Arum maculatum* (N_5-N_6) it was possible to differ two groups: N_6-N_9 in which was *Arum maculatum* L. var. *maculatum* L. and N_2-N_5 in which was *Arum maculatum* L. var. *immaculatum* Reicbh.)

In analysing principal coordinates (PCoA) the matrix of distance was used (Gower 1966). This (PCoA) scatter plots

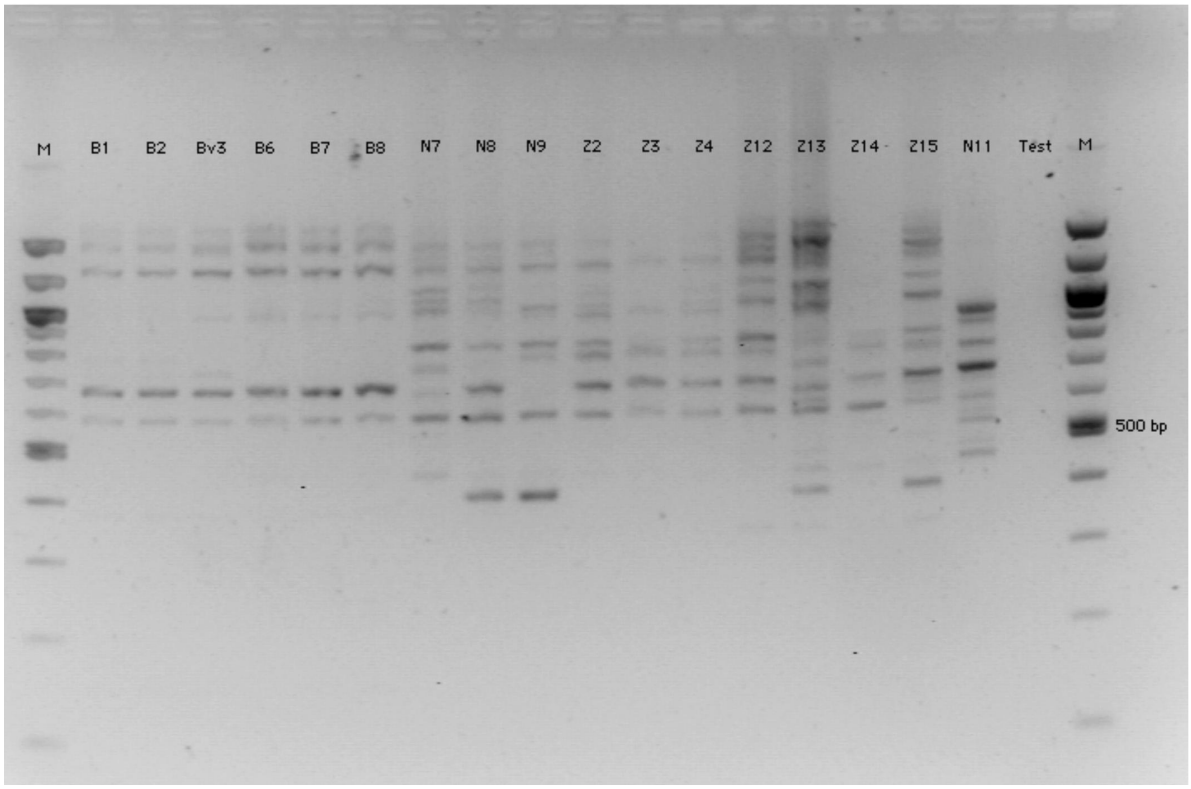

Fig. 3. Example of RAPD amplification: electropherogram obtained by using primer OPT 7.

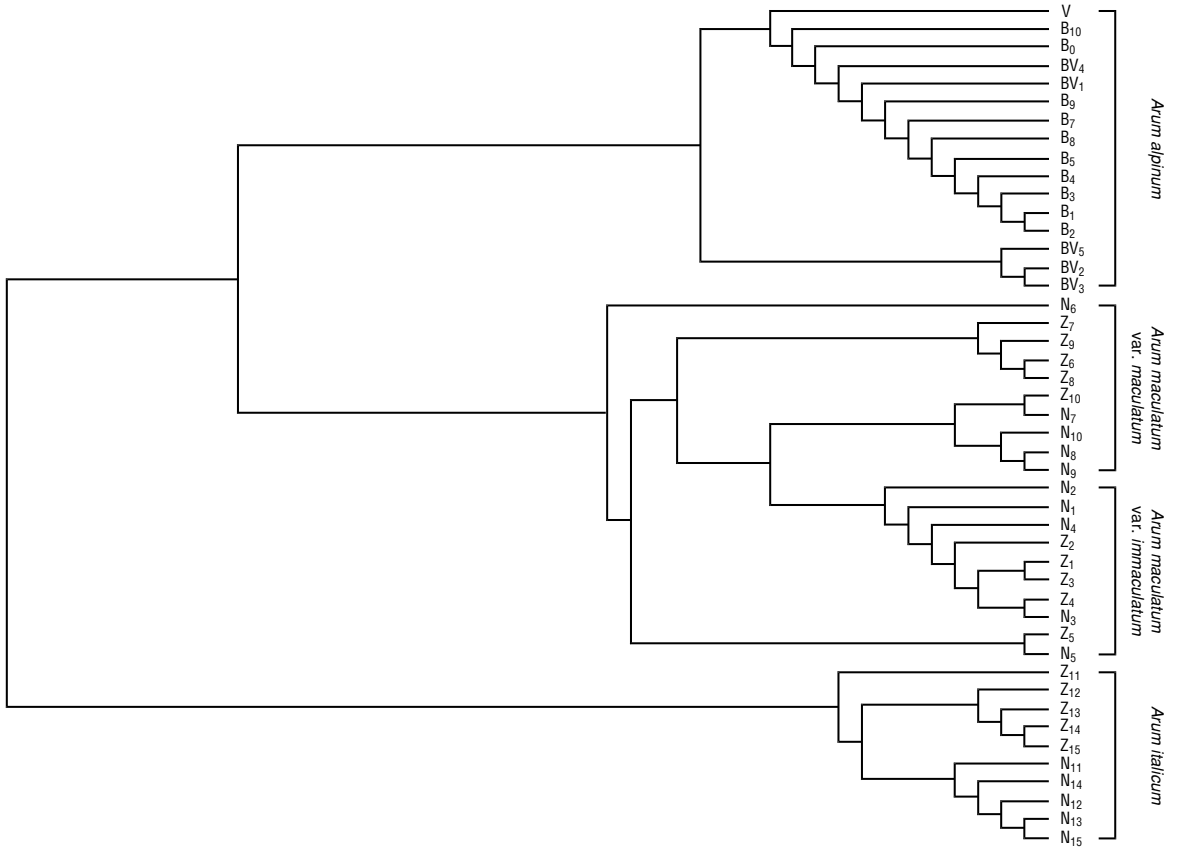

Fig. 4. UPGMA Dendrogram of species of genus *Arum* in Slavonia and Baranya region in Croatia obtained on the principle of amplification with five primers.

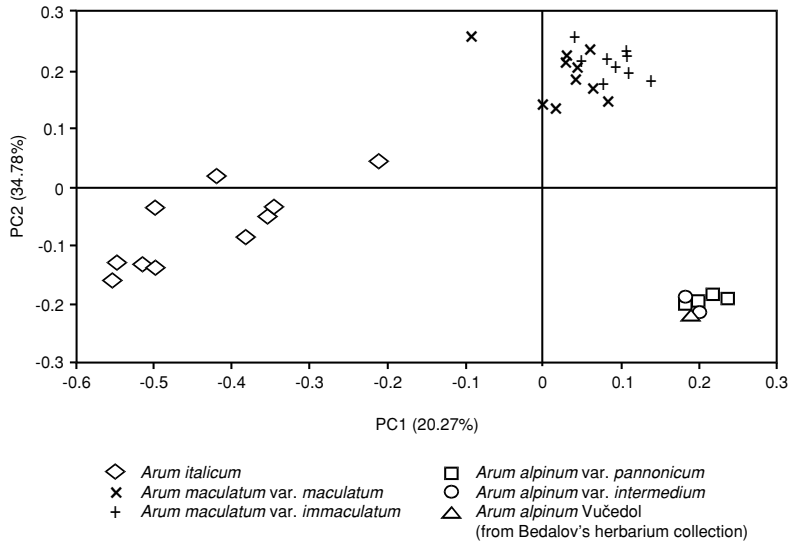

Fig. 5. PCoA of species of genus *Arum* found in Slavonia and Baranya region in Croatia.

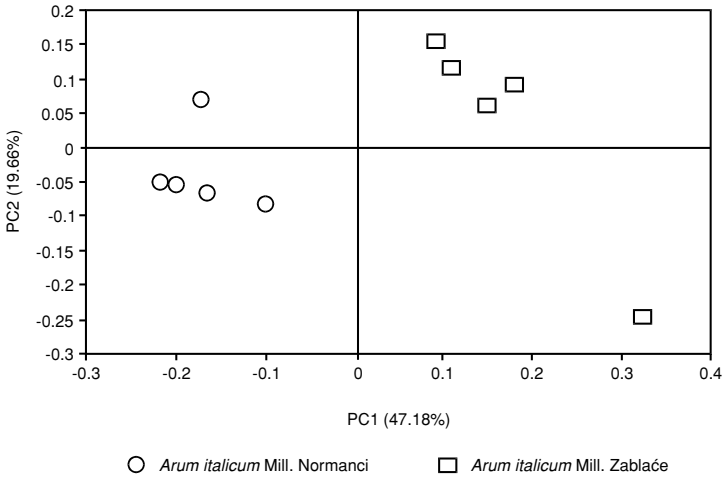

Fig. 6. PCoA. Variability between populations of species *Arum italicum* Mill.

gave the following pieces of information that are clearly evident.

The existence of separation of the species of genus *Arum* in Eastern Slavonia region in Croatia as the obtained results by RAPD methods showed (Fig. 5).

The detected individual variability inside populations and the variability between populations of the species *Arum italicum* Mill. in the habitats of Normanci and Zablaće in the Eastern Slavonia region were found (Fig. 6).

The detected individual variability inside populations and the variability between populations of the species *Arum maculatum* L. var. *maculatum* L. in the habitats of Normanci and Zablaće in Eastern Slavonia region were determined (Fig. 7).

The detected individual variability inside populations and between populations of the species *Arum maculatum* L. var. *immaculatum* Reicbh. in the habitats of Normanci and Zablaće in Eastern Slavonia region were shown (Fig. 8).

The detected individual variability inside populations and between populations of the species *Arum alpinum* Schott & Kotschy var. *pannonicum* in the habitats of Bilje and Branjin Vrh in Baranya region were found (Fig. 9).

The variability among varieties of the species *Arum* in the Baranya region in Croatia as the obtained results by RAPD method show:

The detected variability between varieties of the species *Arum alpinum* Schott & Kotschy var. *pannonicum* Terpó and *Arum alpinum* Schott & Kotschy var. *intermedium* Terpó in the habitats of Bilje (Fig. 9).

It could be noted that there was also the variability between varieties of the species *Arum italicum* Mill., *Arum maculatum* L. var. *maculatum* L., *Arum maculatum* L. var. *immaculatum* Reicbh. and *Arum alpinum* Schott & Kotschy var. *pannonicum* Terpó and var. *intermedium* Terpó in the habitats in Eastern Slavonia and Baranya region (Fig. 10). The species *Arum italicum* Mill. varied the most and the species *Arum alpinum* Schott & Kotschy varied the least. By condensing the results obtained it is possible to differ two genetically separated groups corresponding to the species *Arum italicum* Mill., *Arum maculatum* L and *Arum alpinum* Schott & Kotschy.

The group of individuals of the species *Arum italicum* Mill. is the least homogenous and its individuals are mutually related. However, they are very closely separated and

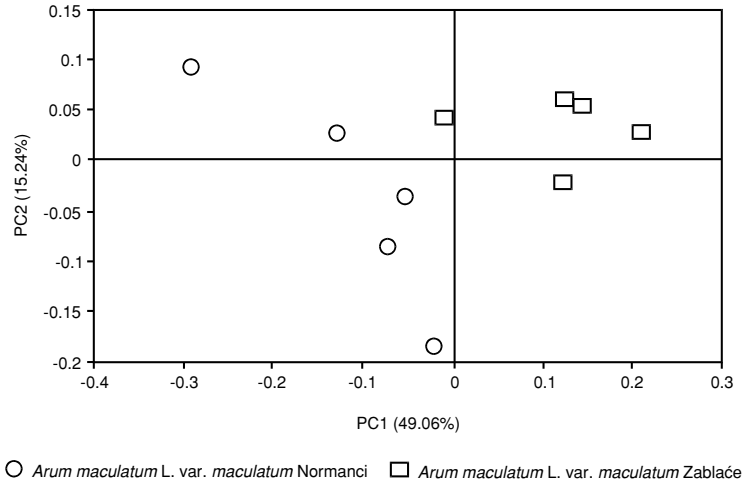

Fig. 7. PCoA. Variability between populations of species *Arum maculatum* L. var. *maculatum* L.

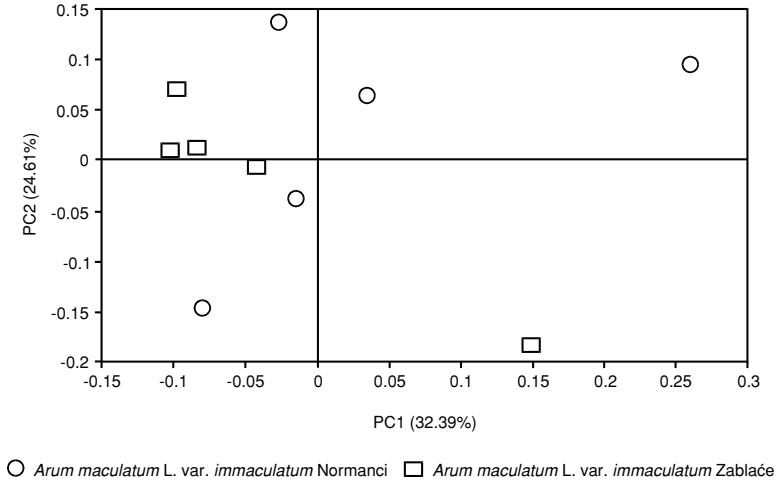

Fig. 8. PCoA. Variability between populations of species *Arum maculatum* L. var. *immaculatum* Reichb.

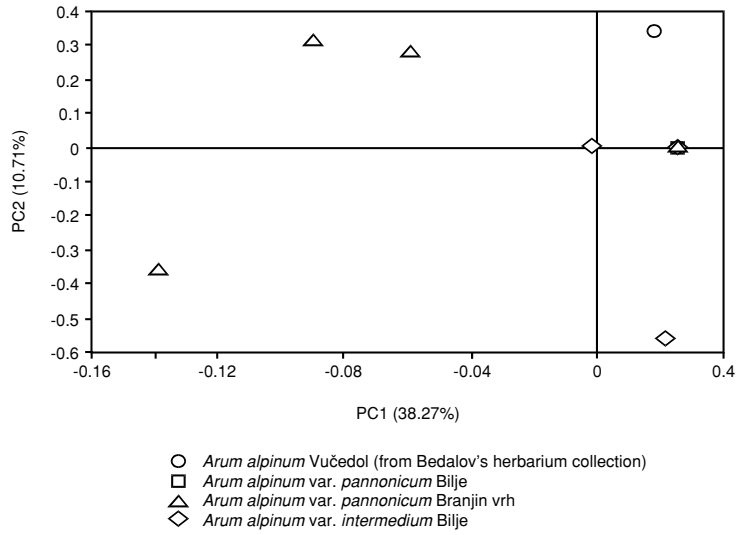

Fig. 9. PCoA. Variability of the species *Arum alpinum* Schott. & Kotschy var. *pannonicum* Terpó and. var. *intermedium* Terpó.

do not show relationships with the other species group of genus *Arum*. These species are more distinguished in relation to the other two groups. It should be also noticed that the variability of this species between populations is very high because they are mutually separated, but are still close to each other.

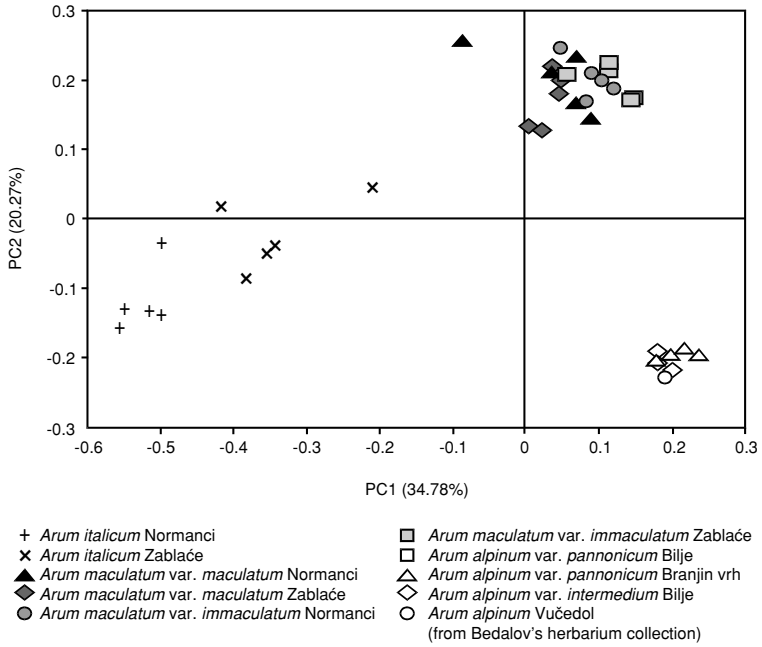

Fig. 10. PCoA. Variability of the species *Arum* in Eastern Slavonia and Baranya region in Croatia.

The group of individuals of the species *Arum maculatum* L. is more homogenous and within it could be differed the two more or less separated groups corresponding to the two varieties, var. *maculatum* L. and var. *immaculatum* Reicbh. Therefore, they are genetically related, but not maximally since it is possible to differ the two varieties. Moreover, they do not show any relationship with the other species of genus *Arum*. It should be also remarked, that the variability between populations is high, particularly between populations of var. *immaculatum*, as it is difficult to differ two mutually separated groups of populations.

The group of individuals of the species *Arum alpinum* Schott & Kotschy is very homogenous and it is extremely difficult to differ the two varieties of the species, var. *pannonicum* Terpó and var. *intermedium* Terpó, so it could be said that they are very closely related and as result of this they closely resemble. Also, they do not show any relationship with the other species of genus *Arum*. In addition the variability between populations of the species *Arum alpinum* is too high, but only in the habitat of Bilje, since the population var. *pannonicum* is very homogenous, the individuals of varieties *intermedium*, however are less homogeneous. Individuals of populations var. *pannonicum* in the habitat Branjin Vrh are either more or less scattered. It is necessary to note that it is also extremely difficult to differ the one from the other varieties.

Finally, it is visible from figure 10 that the species *Arum italicum* Mill., *Arum maculatum* L. and *Arum alpinum* Schott & Kotschy are separated and clean and they are mutually minimally related. Observations by RAPD method indicate that there were found no data for the differentiation between two varieties of *Arum alpinum* Schott & Kotschy, var. *pannonicum* Terpó and var. *intermedium* Terpó, although the morphological studies show clear differences between them. In contrast to two varieties of the species *Arum maculatum* L., var. *maculatum* L. and var. *immaculatum* Reicbh., between which data for their differentiations obviously exist, which is in concordance with the morphological parameters (Lendel et al. 2004). Moreover, it has also been noted in general that the variability of individuals existed either inside the same population or between different populations.

The new assumption (Lendel et. al. 2004) suggested that the species *Arum alpinum* Schott & Kotschy, with two varieties, var. *pannonicum* and var. *intermedium* Terpó, is actually *Arum cylindraceum*. The studies made by the RAPD method showed that *Arum cylindraceum* can clearly be distinguished from both *Arum italicum* Mill. and *Arum maculatum* L.

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