

Genetic diversity of Christmas rose (*Helleborus niger* L.) natural local populations as revealed by AFLP markers

Andrej ŠUŠEK[1]*

[1]*University of Maribor, Faculty of Agriculture and Life Sciences, Pivola 10, 2311 Hoče, Slovenia*

ABSTRACT

Three naturally-growing local populations of Christmas rose (*Helleborusniger L.*) from two locations in Slovenia and one from neighbouring Croatia, were selected for molecular analysis based on AFLPs. 27 primer pairs were evaluated during the selective amplification step of the AFLP, and ten primer combinations were generated on the basis of the number of bands. The results from molecular analysis showed that the variation within populations was larger than that between populations. The molecular analysis showed that the genetic differences amongst populations were not high enough to state that the individuals of *H. niger* were grouped in clearly distinctive clusters. Small differences between the two Alpine populations (Bohinjska Bela and Peca) suggest that these are not isolated at the level of sexual reproduction. The migration of genes through pollen dissemination is probably always present, although pollen cannot migrate far.

Key words: Christmas rose, *Helleborus niger*, population diversity, molecular markers, AFLP

INTRODUCTION

The Christmas rose (*Helleborusniger L.*) that belongs to the family Ranunculaceae (Tamura 1993) is becoming more and more popular on the market as an ornamental plant (Armstrong 2002). However, it is a relatively new species regarding market-oriented production. It is important for its promotion to organise efficient and systematic genetic breeding, and to improve production technology. The efficiency of genetic breeding depends on several factors, such as available genetic resources, breeding methodology, breeders' experiences, and financial support.

Slovenia, one of the smaller European countries, appears to be very rich in terms of its biotic diversity (Martinčič, 1999). The main reasons for such diversity are its specific geographical position as well as its diverse climate, relief, and bedrock. *H.niger* grows within all phytogeographical regions (Wraber 1969) (Fig. 1) and Slovenia is considered as being at the centre of diversity regarding this species. The variation within the genus Helleborus appears to be extremely high and the majority of the more important traits are at least partly influenced by the environment (Šušek et al. 2005). Reliable data about existing diversity and variation within germplasm collections will be extremely important for breeders in the future.

The traditional evaluation of genetic variability, based on a description of morphological traits, cannot always ensure accurate estimates of the genetic variation. Many more reliable estimates of genetic variation can be obtained by using biochemical (e.g. isozymes) and molecular markers.

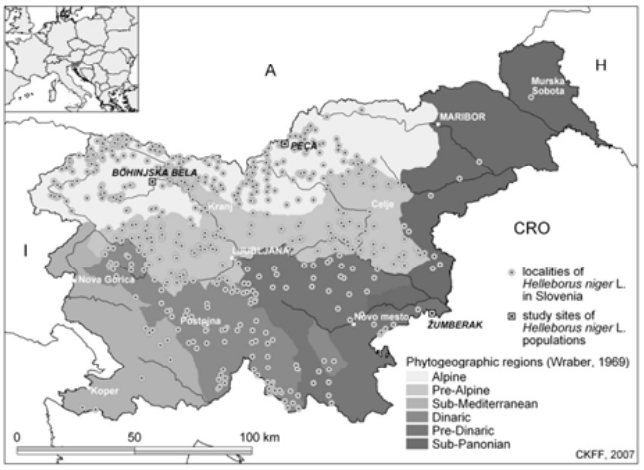

Figure 1: Localities of *Helleborusniger* in Slovenia (Jogan 2001). The map was adapted with the permission of the CKFF (the Slovenian Centre for the Cartography of Fauna and Flora)

The AFLP technique has been successfully used for evaluating genetic relationships within the genus *Helleborus*. Meiners et al. (2011) analysed the taxonomic subdivision within the genus *Helleborus*. The results supported the previously-suggested division of the genus and thereby approved AFLP data as being applicable for phenetic

*Correspondence to:
E-mail: Andrej Šušek@um.si

analyses. AFLP markers have found the widest application regarding the analyses of genetic diversity below the species level within plants, particularly during investigations into population structure and when differentiating between both cultivated and natural/rare populations (Gupta et al. 1999, Nissim et al. 2004). Such analyses are crucial from the applied breeding point of view and for genetic conservation, and the rapidity with which AFLP markers enable the delivering of crucial information within intense time-constraints.

Patzak (2001) compared its potential for assessing the genetic diversity of ten hop (*Humulus lupulus* L.) varieties by using RAPD, STS, ISSR, and the AFLP molecular approaches. All the molecular methods accurately distinguish between the tested varieties, with the exception of three clones, which can only be distinguished by AFLPs. The similarities between varieties were revealed by using cluster analysis, and similar results were found for all the molecular methods.

When AFLPs reveal low-levels of genotypical differentiation between sub-populations' and relatively high variations within populations, they cannot be used for determining the populations' geographical origins. The reason could be the migration (of seeds or pollen) amongst sub-populations. This migration is probably present frequently. If seed migrations were excluded, the most important factor could be the migration of pollen. Examples can be the studies of wild North American populations of the weedy-plant *Arabidopsis thaliana* (L.) Heynh, as conducted by Jorgensen and Mauricio (2004), and the analysis of population structure regarding *Arctophila fulva* var. *pendulina* (Laest) Holmb. (Kreivi et al. 2005). Similar results were also obtained by Nissim et al. (2004) when studying the genetic variations of wild populations and cultivated genotypes of *Anemone coronaria* (L.). Most of the genetic variations (about 80 %) were documented within populations and only about 20 % between populations.

Data about the level of genetic diversity within and between populations of *H. niger* are crucial for genetic breeders. They are also very important for the establishment and management of germplasm collections. Information about the genetic structuring of populations is crucial during evalution studies. However, at this moment in time, no such information is available.

AFLPs can be very helpful for studying the genetic relationships amongst populations, and for determining or identifying ecotypes, hybrid lines, and cultivars. The AFLP technique can also represent a useful tool during the breeding process of the Christmas rose, thus enabling early determinations of traits that can only normally be determined when the plants are fully mature, and traits that can be determined only within specific environments (e.g., in the presence of certain diseases or pests). We studied the genetic structures of three naturally-grown populations that were chosen on the basis of results from morphological analysis. The aim of this study was to estimate the usefulness of the AFLP molecular technique when examining the genetic diversities amongst and within populations.

MATERIALS AND METHODS

Plant material

The molecular analysis included randomly-chosen Christmas rose genotypes (individual plants) from two locations within Slovenia (Bohinjska Bela and Peca) and one in neighbouring Croatia (Žumberak). These locations were selected according to the results of morphological analysis that involved 8 populations (Šušek et al. 2005). From each selected population, we took (for the molecular analyses) 16 plants (a total of 48 plants). Each individual within the population was collected at a distance of 50-500 m from the others, in order to avoid clones.

DNA isolation and AFLP analysis

Genomic DNA was extracted from the leaves according to the established CTAB method using NucleoSpin Plant kits (Macherey-Nagel, GmbH & Co.KG, Düren, Germany) by following the manufacturer's protocol. The DNA quantities and qualities were estimated by measuring the absorbance at 260 nm and 280 nm, using an Eppendorf BioPhotometer (Eppendorf AG, Hamburg, Germany), and by gel electrophoresis by means of 0.8 % agarose gel electrophoresis.

AFLP analysis was performed according to Vos et al. (1995), with slight modifications. The AFLP core reagent and starter primer kits were purchased from ScienceTec (LI-COR Inc., Biotechnology division, Lincoln, Nebraska, USA). Approximately 100 ng of genomic DNA was double-digested using the restrictive enzymes mix *Eco*RI/*Mse*I. The genomic DNA was incubated within a 5 × reactive buffer (50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, and 250 mM K-acetate). Digestion was carried out within a final volume of 12.5 µl at 37 °C for 2 h, and then heated to 70 °C for 15 minutes in order to deactivate the enzymes.

*Mse*I and *Eco*RI adapters (Table 1) were subsequently ligated to the digested DNA fragments by adding 12 µl of adapter ligation solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate), and 0.5 µl of T4 DNA ligase (1 unit/µl in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50 % glycerol (v/v)). The ligation was incubated for 2 h at 20 °C. After the reaction, the digested-ligated DNA fragments were diluted (1:10) and used as templates for the first amplification reaction (the pre-amplification step prior to the selective amplification).

Pre-amplification reactions were performed within a 25.5 µl volume containing a 2.5 µl of 10 × PCR buffer for AFLP (100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl), 20 µl of pre-amplification primer mix (Table 2), 2.5 µl of diluted ligation and 0.5 µl of Taq DNA polymerase (Roche Molecular biochemicals, Penzberg, Germany). The PCR amplification was carried out in a PTC-100 Programmable Thermal Controller using the following cyclic profile:

Table 1: Adapter and +3 primer sequences (5'-3') used for AFLP analysis

Name	Enzyme	Type	Sequence (5'-3')
E-0	*Eco*RI	Adapter	5'-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5'
M-0	*Mse*I	Adapter	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-5'
M-CAG	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACA G
M-CTG	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACT G
M-CAC	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACA C
M-CTA	*Mse*I	Unlabelled primer +3	GAT GAG TCC TGA GTA ACT A
E-ACA	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAC A
E-ACC	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAC C
E-AAG	*Eco*RI	IRDye 700 labelled primer +3	GAC TGC GTA CCA ATT CAA G
E-ACT	*Eco*RI	IRDye 800 labelled primer +3	GAC TGC GTA CCA ATT CAC T
E-AGC	*Eco*RI	IRDye 800 labelled primer +3	GAC TGC GTA CCA ATT CAG C

Step	Temperature (°C)	Time	
1. denaturation	94	30 seconds	20 cycles total
2. annealing	56	60 seconds	
3. extension	72	60 seconds	
4.	4	hold	

After the reaction, pre-amplified DNA fragments were diluted (1:40) and used as templates for the selective amplification reaction. A volume of 11 µl PCR reaction combined with a 2 µl diluted (1:40) pre-amplification product, 0.5 µl of IRDye 700 labelled *Eco*RI primerA, 0.5 µl of IRDye 800 labelled *Eco*RI primerB, 2 µl of *Mse*I primer, and 6 µl of TaqMix. The TaqMix for 33 reactions contained 40 µl of 10×PCR amplification buffer (100 mM Tris-HCl, pH 9, 15 mM $MgCl_2$, 500 mM KCl), 158 µl of deionised water and 2 µl Taq DNA polymerase (5units/µl) (Roche Molecular Biochemicals, Penzberg, Germany). The second amplification was carried out in a PTC-100 Programmable Thermal Controller by programming a touch-down cyclical profile (Don et al., 1991). Touchdown PCR was performed by decreasing the annealing temperature from 66 °C to 56 °C by 0.7/30 s increments per cycle with each of the initial 12 cycles, followed by a touchdown annealing temperature for the remaining 25 cycles at 56 °C for 30 s with a final cycle of 72 °C for 1 min and a hold at 4 °C.

Following the amplification reaction, each product was mixed with 5.0 µl of blue-stop solution (98 % formamide,10 mM EDTA pH 8.0, and bromo-phenol-blue and xylene cyanol as tracking dyes). The resulting mixtures were heated at 94 °C for 3 min., and then quickly cooled by ice and covered to reduce exposure to light. The samples were stored at -20 °C for long-term usage

Gel electrophoresis was conducted using a Li-Cor Long ReadIR DNA sequencer (LI-COR Inc., Biotechnology division, Lincoln, Neb., USA).

Data analysis

Image analysis was performed using Saga 2 software (LI-COR Inc., Biotechnology division, Lincoln, Neb., USA). After the automated analysis, the placements of lanes, calibrations, desmile lines, and the scoring of bands was reviewed by the visual inspections of two independent readers. Only the bands' scores by both readers were accepted as reproducible. When considering of bands, the diffused or faint bands were not taken into account and neither were those bands that occurred at the extremes of the amplified size-range. Infrequent bands were eliminated. Only the bands with a frequency above 20 % were taken into account. Each AFLP marker was treated as a unit character and scored as a binary code (1/0).

Only polymorphic bands were used during the construction of a binary matrix, reflecting the presence or absence of those fragments obtained by AFLP, in different genotypes. The estimates of genetic similarities (GS) amongst all genotypes were calculated according to the Jaccard similarity coefficient (1908). The genetic distance was calculated as GD = 1 – J, using the data from the Jaccard's similarity coefficient matrix. The resulting distance matrices were subject to clustering methods by the Neighbour Joining Tree (NJTree) method.

The reliabilities and robustness of the phenograms were tested by bootstrap/jackknife analyses by drawing 1,000 samples.

Data analyses were done by Darwin, the non-commercial

software of CIRAD (Perrier et al. 2003). A bootstrap value was given to each edge and indicated the occurrence frequencies of this edge in the bootstrapped trees.

Principal component analysis (PCA) was used in an attempt to detect geographical patterns amongst the *H.niger* populations.

RESULTS

During the selective amplification step, the duplex of one *Mse*I with two IRDye labelled *Eco*RI primers was used for exploiting the capability of the LI-COR system when detecting two different dyes. The 27 primer pair combinations were evaluated with regard to their capacities for generating polymorphic bands using 4 genotypes, randomly chosen from the 3 populations being investigated (Table 2).

According to their capacities to generate polymorphic bands, it was decided to use a five duplex of one *Mse*I with two IRDye labelled *Eco*RI primers: (1) M-CAG, E-ACA (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (2) M-CTG, E-ACC (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (3) M-CAC, E-AAG (IRDye 700 labelled), E-ACT (IRDye 800 labelled); (4) M-CTA, E-ACC (IRDye 700 labelled), E-AGC (IRDye 800 labelled); (5) M-CTG, E-ACA (IRDye 700 labelled), and E-ACT (IRDye 800 labelled). Each selected primer combination generated a greater number of bands. The number of polymorphic fragments for each primer pair varied from 5 (for M-CTG/E-ACC) to 23 (for M-CAG/E-ACA), with an average of 8.9 per primer pair (Table 3).

The sizes of the AFLP fragments were determined by comparing an AFLP standard marker to the AFLP patterns. The AFLP fragments' sizes ranged from approximately 45 to 400 base pairs (bp). The polymorphic fragments were distributed across the entire size-range, with the major proportion being between 75 and 300 bp.

In order to assess the usefulness of AFLPs as phenetic markers, a similarity-matrix based on Jaccard's coefficient was constructed for estimating the level of relatedness amongst individuals from the three natural populations used during the study. Calculation of the Jaccard coefficient was based on the presence or absence of discrete characters (AFLP markers). The similarity matrix was then used to cluster the data using the NJTree method and to conduct PCA analysis. The 48 individuals appeared as being scattered along the resulting dendrogram constructed by the Jaccard coefficient, and by the NJTree clustering method (Fig. 2). Individuals from the Žumberak population were clustered into two sub-clusters of 5 and 10 individuals, and a sub-cluster of 6 individuals was identified for the Peca population. All the other individuals were clustered in groups of two to four individuals, irrespective of their geographic origins. The resulting dendrogram showed low bootstrap values for the main edges with a certain number of null values.

The similarity matrix was also used as input data for principal component analysis (PCA). The scatter-plot representation of the PCA showed a clear separation of 9 individuals from the Žumberak population in relation to the first two principal axes of variation (Fig. 3). When the plan was formed by axes 1 and 2 only, 18.11 % (9.76 % + 8.35 %) of the total variation was explained by the markers. The second

Table 3: Number of polymorphic fragments and their size-range

Primer combination	Scored polymorphic bands	Fragment size-range (bp)
M-CAG/E-ACA	23	50-370
M-CAG/E-AGC	10	50-370
M-CTG/E-ACC	5	40-340
M-CTG/E-AGC	6	50-360
M-CAC/E-AAG	13	45-340
M-CAC/E-ACT	5	50-400
M-CTA/E-ACC	6	50-370
M-CTA/E-AGC	8	45-370
M-CTG/E-ACA	8	50-255
M-CTG/E-ACT	5	50-370
Totals	89	-
Mean	8.9	-
Number of primer combination	10	-
Maximum	23	-
Minimum	5	-
Standard deviation	5.59	-

Table 2: Primer pair combinations tested for conducting selective amplification

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC		○		●	○		●	
E-AAG	○	●						
E-ACA		○	●			●		●
E-ACC				●	●	○	●	○
E-ACG	●	○		○				
E-ACT	○	●				○		○
E-AGC			●				●	
E-AGG		○	●		○			

plane (axes 3 and 4) accounted for 13.29 % (6.82 % + 6.47 %) of the total variation. This meant that the markers could not explain the highest portion of variation amongst the studied individuals. Similarly, in the case of the third plane (axes 4 and 5), only 12.17 % (6.47 % + 5.70 %) of the total variation could be explained.

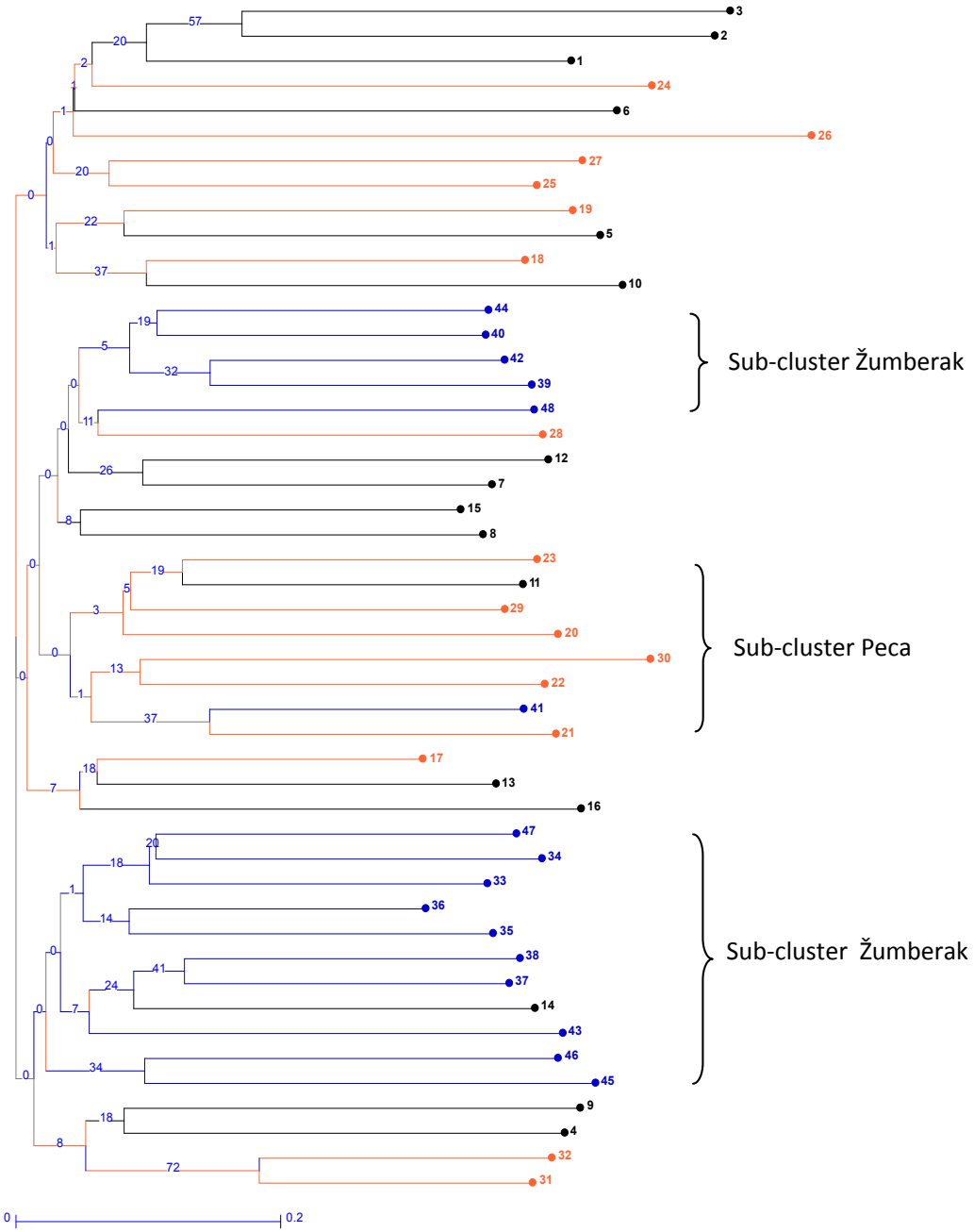

Figure 2: Unrooted dendrogram of individual plants from wild populations based on individual scoring for each AFLP band. The numbers represent individuals from various populations: Bohinjska bela (from 1 to 16); Peca (from 17-32); Žumberak (from 33 to 48). Neighbour-joining clustering was performed, using the genetic similarity coefficient of Jaccard calculated between every pair of the genotype. Bootstrap values (%) for each branch point are indicated

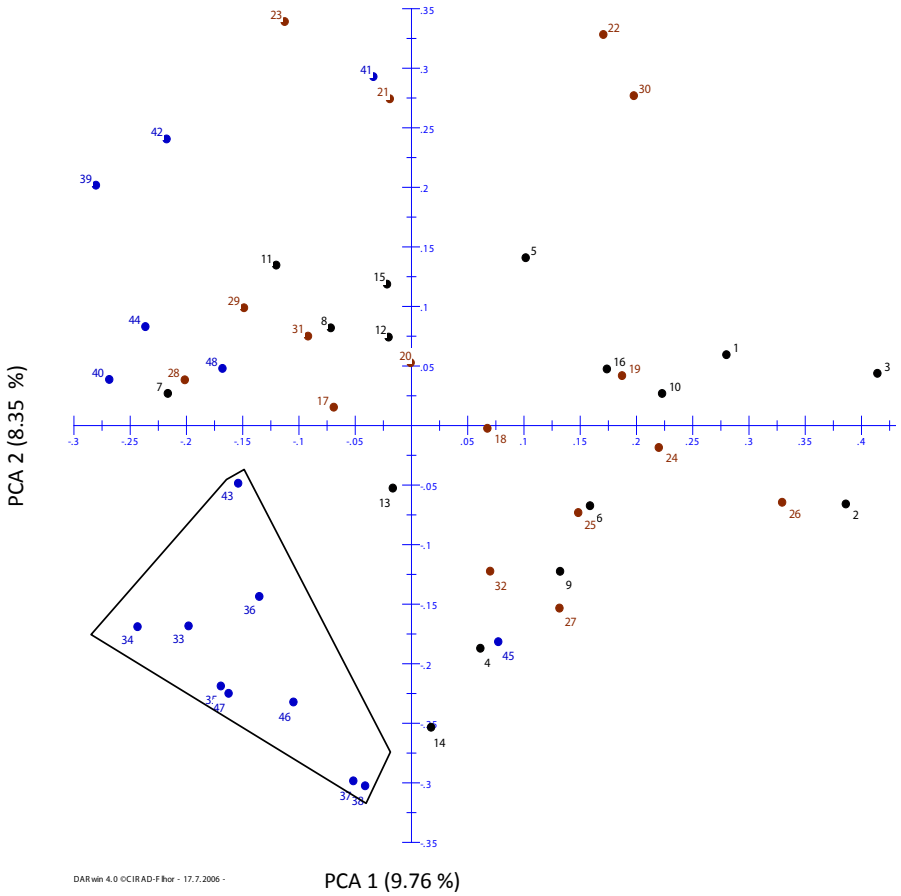

Figure 3: Principal component analysis (PCA) of AFLP data for *H. niger* naturally-growing populations. The numbers represent individuals from various populations: Bohinjska Bela (from 1 to 16); Peca (from 17-32); Žumberak (from 33 to 48). The percentage of total variance explained by the first two principal components is shown on the abscissa and ordinate axes, respectively.

DISCUSSION

The AFLP technique was used for evaluating the genetic diversities within and between three naturally-grown populations of Christmas rose. As this was the first work on the natural diversity of *H.niger*, sixteen individuals were selected from each population in order to gain some knowledge of their genetic diversities. For technical reasons, we decided to use different individuals as repetitions, being aware that this could not eliminate all the possible errors associated with the representativeness of the data.

For the AFLP assay, we used a relatively wide-range of primer combinations. From amongst the 27 AFLP primer combinations tested, 17 were discarded owing to the reduced number of obtained fragments, polymorphic or not. The genetic diversities were finally evaluated using 10 primer combinations that generated 89 polymorphic fragments and made the fingerprinting of each individual genotype possible.

Evaluation of the genetic diversity of the Christmas rose within naturally-occurring Slovenian populations did not show strong differentiations. Although several individuals from the same location appeared to be very tightly clustered, many of them were distributed all over the dendrogram (Fig. 2). The heterogeneity within clusters was higher than between clusters. PCA analysis showed that only a low percentage of the total variations amongst these individuals could be explained by the markers (Fig. 3). However, geographical distance tended to be a factor associated with genetic similarities. The Žumberak population, which was geographically the most distant when compared to the Bohinjska Bela and Peca populations, also tended to be genetically different to the two latter populations, to a certain extent (Figs. 2 and 3). A similar situation was observed by Šušek et al. (2005) on the basis of morphological traits where, on the basis of the first principal component, the Žumberak population was separated from the Peca and Bohinska Bela, which could not be distinguished one from the other.

One interpretation of the structure in the neighbour joining-tree and in the scatter plot from the PCA analysis could be that these Alpine populations were not genetically isolated. The only substance that enables genetic communication amongst populations is probably pollen, which is carried by various insects (e.g., bees).

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