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Genetic variability of *Hedysarum coronarium* L. using molecular markers

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Summary - The study of genetic diversity in *Hedysarum coronarium* L. has been performed by the help of RFLP analysis in 75 individuals representing five local populations from various geographical origins. Taking advantages of the large variation shown by the ribosomal RNA genes, we were interested in the use of nuclear ribosomal genes as RFLP markers to analyse the genetic variation among and within several spontaneous populations of *H. coronarium*. A homologous rDNA probe was used for southern hybridization to generate patterns from five spontaneous populations. The distribution of the generated polymorphic bands was studied using a multivariate analysis. It has been assumed that a high degree of rDNA polymorphism was observed. On the other hand, the geographical origin was not a determinant criterion for population clustering. In addition, a considerable genetic diversity has been detected in the case of populations characterized by a maximum of phenotypic variability (opposite geotropism). Our data are in good agreement with results based on isozyme markers.

Key-words: *Hedysarum coronarium*, RFLP markers, genetic diversity

Résumé *- Analyse de la variabilité génétique chez* Hedysarum coronarium *L. par des marqueurs moléculaires. L'étude de la diversité génétique de* Hedysarum coronarium *L. a été entreprise par la technique du polymorphisme de la longueur des fragments de restriction (RFLP) chez 75 individus représentant 5 populations spontanées locales d'origines géographiques différentes. Tenant compte de la grande diversité offerte par les gènes rDNA, nous avons utilisé une sonde homologue rDNA. Les résultats qui émanent de l'analyse multivariée montrent une importante variabilité génétique indépendamment de l'origine géographique. De plus, une diversité génétique considérable a été mise en évidence pour des populations caractérisées par des différences morphologiques (géotropisme opposé).*

Mots-clés: Hedysarum coronarium*, marqueurs RFLP, diversité génétique*

Introduction

In Tunisia, local phytogenetic resources have been currently damaged by a severe genetic erosion due to overgrazing and reduction of range land. Thus, pastoral areas are currently relegated to the dry lands. Among these crops, *Hedysarum* species are in a great interest and would provide an alternative approach usable to valorise and enhance phytogenetic resources. It's worth noting that among Mediterranean group species, which are nutritious and highly palatable to sheep, only *Hedysarum coronarium* L. (also called Sulla or Spanish sainfoin) is grown for fodder in several countries, specially in Spain, Italy and North Africa.This species appears to be distributed in all western Mediterranean countries except France (Boussaid *et al.*, 1995). In Tunisia, the semi temperate ecological domain of *H. coronarium*, is the north of the Tunisian dorsal. Vegetative shoot system in this species is characterized by an orthotropic main stem bearing some lateral plagiotropic shoots. In order to elaborate a conserving and valorising strategy of these spontaneous phytogenetic resources, we are interested in the analysis of the genetic diversity in this crop. In this scope, previous studies based on morphological and allozymic variations have been conducted within a set of spontaneous

populations and cultivars of *H. coronarium*. Hence, both of these forms exhibit a high allozymic diversity in spite of their distinctiveness by their opposite geotropism (Trifi-Farah *et al.*, 1989).

As part of our work aiming at the search of molecular markers to precise the genetic diversity, we became interested in the use of nuclear ribosomal genes as RFLP markers. The rDNA-RFLP analyses constitute an efficient method widely used to determine genetic polymorphisms in higher plants and to differentiate the species (Kabbaj *et al.*, 1995; Amarger and Mercier, 1996; Parani *et al.*, 1997; Lebrun *et al.*, 1998; Anamthawat-Jonson *et al.*, 1999).

The purpose of this study was to take advantage of the large variation shown by ribosomal DNA (r-DNA) within and among plant populations to quantify the genetic diversity in *Hedysarum coronarium* L. Here we report the use of RFLP markers to determine the overall degree of polymorphism and to detect similarities among genotypes.

Materials and methods

Five accessions from *H. coronarium* were collected from natural populations prospected throughout Tunisia. These are: Bizerte (Bi), Mateur (Ma), Tunis (Tu), Jebel Zit (Zi) and El-Haouaria (Eh). Randomly collected seeds from each accession are germinated. The resultant seedlings are transferred in Petri dishes and incubated in a greenhouse until emergence of roots and cotyledons.

Total cellular DNA was isolated from the resultant seedlings according to Dellaporta *et al.*, (1983) procedure. For inter and intra population RFLP analysis, genomic DNA from each of the 75 selected plants was digested with Hpa II, Alu I, Sma I and Bgl I enzymes. The restricted DNA was electrophoresed in 0.8% agarose gel and transferred to nylon membrane (Hybond N+, Amersham) by Southern blotting (1975). The membranes were prehybridized and hybridized in 5xSSC, 5x Denhardt, 0.5% SDS, 45% Formamide at 42°C overnight with random prime labelled probes. The membranes were washed and exposed overnight on x-ray film at –80°C (Sambrook *et al.*, 1989).

A fragment of 1.6 kb corresponding to the intergenic spacer was used as homologous DNA probe. It designs a PCR amplified DNA using appropriate oligonucleotides flanking the conserved sequences in the main repeated rDNA unit.

RFLP bands were scored for presence-absence. Values registered were used to perform a principal component analysis (PCA) and a Factorial Analysis of correspondence (FAC).

Results and discussion

Five *H. coronarium* accessions were studied to determine the overall degree of polymorphism and to detect similarities among genotypes. Using all the probe × enzymes combinations, a total of 39 polymorphic RFLP bands are generated. These represent a high degree of genetic variation at the DNA level. Among these bands, 8 are generated from Sma I, 9 from Hpa II, 10 from Bgl I and 12 from Alu I. For each enzyme, bands were labelled starting from 1 for the slowly one.

Data were then computed to study the genetic diversity using PCA and FAC procedures. The resultant PCA groupings of *H. coronarium* genotypes were based mainly on the first three PC that account for 29,20% of the variability observed, i.e. for 12,27%, 9,61% and 7,32% respectively (Table 1). The most important variables integrated by PC1 were markers of the four used enzymes. However, PC2 and PC3 were correlated mainly to Hpa II markers. The clustering analysis using PC1 and PC2 (Fig.1), has greatly supported differentiation between genotypes characterised by their opposite geotropism form. This is well examplified by Ma and Zi accessions which are characterised by their orthotropic form and are easily

distinguishable from the other ones (i.e. Eh, Tu and Bi). We may assume that this morphological trait seems to be strongly correlated with the polymorphisms generated by Hpa II enzyme. The main contribution of markers generated by this enzyme in the definition of PC2 axis is favourable in this assumption.

Table 1: Eigenvalues, proportion of variation and eigenvectors associated with the first three axes of the PCA in *H. coronarium* populations.

Principal components (axes)	1	2	3
Cumulated proportion of variation	12.27	21.88	29.20
Markers		Eingenvectors	
HpaII1	-0.09	0.42	0.12
HpaII2	0.01	-0.40	-0.24
HpaII4	0.34	-0.11	-0.16
HpaII5	0.16	-0.37	-0.14
HpaII9	-0.03	-0.22	0.17
AluI5	-0.23	0.12	-0.02
SmaI5	0.26	0.13	-0.03
BglI5	-0.26	0.03	-0.03
BglI8	0.22	0.08	0.00

In addition, data analysis using FAC procedure has generated similar associations (data not shown). Hence, the close agreement of both analyses may indicate that RFLP banding patterns are genetically informative of the tested accessions. These patterns help to examine the genetic diversity at the DNA level and to provide evidence of RFLP markers correlated with agronomic traits.

Conclusions

The present study was conducted with only one RFLP probe that generated a high degree of polymorphism. Opportunely, our data constitute an inspection of the use of RFLPs to analyse the genetic diversity in *H. coronarium* genotypes and to shed the light on the domestication process in *H. coronarium*. It has been established that such polymorphism is greatly correlated with agronomic trait particularly the geotropic form. The geographical origin was not a determinant criterion for population clustering.

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Fig. 1 Principal component analysis of five randomly selected accessions of *H. coronarium* based on RFLP markers. (Zi): Oued Zit; (Ma): Mateur; (Tu): Tunis; (Bi): Bizerte and (Eh): El Haouaria.