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MOLECULAR AND CYTOGENETIC DESCRIPTION OF *Vicia villosa* ROTH

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Vicia villosa Roth, known as hairy vetch, is an annual forage legume of economic importance. It is often cultivated in mixture mainly with oats for the production of green forage or silage. Given its potential in nitrogen fixation, it can be used as green manure. In this work the biological material consisted of a local registered variety and two accessions received from ICARDA. Our objective is to study the genetic diversity of these accessions using molecular markers (RAPD) and karyotype analysis. Molecular results revealed relatively high levels of genetic diversity within and between accessions of *Vicia villosa* Roth. Random Amplified Polymorphic DNA (RAPD) technique has proven effective and was able to distinguish the studied accessions. Among 15 primers tested, 4 primers showed variability within and between accessions. Therefore, in this study, we focused on optimistic application of RAPD molecular markers for the conservation of resources, genetic diversity and phylogenetic relationships. In conclusion, our results revealed a high level of genetic divergence between and within accessions among the genotypes studied of *Vicia villosa.* At karyological level, the existence of a single type of chromosomes in all accessions which consists of 7 pairs of submetacentric chromosomes (7sm) showed that their karyotype is symmetrical and consequently less evolved. Nevertheless, variation was observed among accessions for chromosomes size. This variation can be exploited in breeding programs and such characterization is considered as a priority if we want to safeguard and enhance the local forage and pasture plant genetic resources.
Keywords: Biodiversity, genetic resources, *Vicia*, RAPD markers, karyotype analysis, phylogenetic relationships

INTRODUCTION

The Mediterranean basin is the center of diversification of many plant species of forage and/or pastoral interest. Tunisian flora has a large specific and intra-specific diversity (over 2100 species) (Nabli, 1991; MEAT., 1998). The conservation and improvement of the best species is becoming an urgent need to maintain an adequate standard livestock number.

The genus *Vicia* comprises about 166 species located primarily in Europe, Asia and North America. It also extends to South America (temperate and tropical) and East Africa on high altitudes (Ildis, 1999; Meriç and Dane 1999). In Tunisia, the species of the genus *Vicia* of forage and pastoral vocation are well represented. They grow in different bioclimatic zones and on variable substrates and are very palatable by animals (Hassen, 2000; Al Faiz *et al.,* 2004).

Vetches are used in organic agriculture not only as green manure, but also for their strong root system for soil tillage. In addition to their biological role as plants fixing the atmospheric nitrogen, vetch can contribute to the development of arid and semi-arid areas, dominated by the barley monoculture offering a practical solution to the replacement of fallow and seeding the low fertile land.

In Tunisia, little work has been devoted to the study of vetch (Hassen, 2000; El Bok, 2005; El Bok *et al.,* 2014, 2015a,b). In the context of contributing to the improvement and management of this genetic resource, an appropriate characterization of different *Vicia villosa* accessions from different regions was conducted in this paper. The main objective of this work is to assess the diversity of *Vicia villosa* accessions by molecular and karyological approach to establish phylogenetic relationships among accessions.

MATERIALS AND METHODS

***Plant material*:** The plant material consists of two accessions *Vicia villosa* Roth acc. 2565 (P2) and acc. 3615 (P3) and one commercial variety of *Vicia villosa* Roth var. Sejenane (P1). They were stored as seeds at the National Institute of Agronomic Research of Tunisia. The variety Sejenane is a local variety obtained by agronomic assessment of natural populations from the region of Ras Rajel (wet bioclimatic zone with rainfall varying between 800 and 1000 mm and a minimum temperature of 6.3°C). The two accessions, acc. 2565 and acc. 3615, are from ICARDA. The characteristics of plant material are given in Table 1.

***Molecular analysis*:** Genomic DNA was extracted from young leaves of the three accessions using Wizard® Genomic DNA Purification Kit Promega. Four primers, which gave clear polymorphic bands with eleven test samples of each accession, were chosen from fifteen primers tested. The base sequences of polymorphic primer used in this study were as

follows (5' - 3'): (SBSA10) GTGATCGCAG; (SBSP17) TGACCCGCCT; (SBSQ18) AGGCTGGGTG; (SBSQ4) AGTGCGCTGA. Ten µl of reaction mixture was prepared as follows: 10X Buffer (1 µl), dNTPs (2.5 mM), 0.8 µl, $MgCl_2$ (50 mM) 0.3µl, primer (10 µM) 0.2 µl, taq polymerase (5U/µl) 0.2 µl, water 5.5 µl sample DNA 2 µl (50 ng/µl).The thermal cycle was: 3 min at 92°C; 40 cycles of 1 min at 92°C, 1 min at 40°C, 2 min at 72°C; followed by a final 10 min extension at 72°C then brought down to 4°C.

Table 1. Main characteristics of the studied *Vicia villosa* varieties and accessions.

Accession /variety	Code	Origin	Maturity	HSW (g)	Flower color
var. Sejenane	P1	Tunisia	Late	24.8	Violet
acc. 2565	P2	ICARDA	Early	36.9	Purple
acc. 3615	P3	ICARDA	Early	52.6	Purple

HSW: hundred seed weight.

The PCR products (10 µl) were mixed with 2µl gel loading buffer and loaded into an agarose gel (1.5% w/v) in 1X TBE (Tris-Borate- EDTA) buffer and was conducted to electrophoresis at 70 V for 150 min. A 100-bp DNA ladder and 1000-bp DNA ladder were used as standard molecular weight markers to get an estimated size of DNA fragments (Biomatik). Bands were detected by staining with ethidium bromide solution for 30 mn. Therefore, amplification was visualized using a Gel documentation system microDOC with UV-Transilluminator (Cleaver). The NTSYS-pc software ver. 2.02 was used to estimate genetic similarities with the Jaccard's coefficient. Principal coordinate analysis (PCoA) was conducted to visualize the dispersion of the genotypes in relation to the first two principal axes of variation. PCR products were scored as presence (1) and absence (0) of band for each of the three accessions analyzed. Only reproducible bands were scored. Then, cluster analysis was performed using the unweighted pair-group method with arithmetic averages (UPGMA) (Schluter and Harris, 2006).

***Karyotype analysis*:** Somatic chromosomes were studied in root meristems of germinating seeds which were pre-treated at room temperature for 2 h with 0.1% colchicine for the two accessions and with 1% olpha-bromo-naphtalene for the var. Sejenane. They were then fixed in ethanol acetic acid (3v:1v) during 24 h at 4°C and stored in 70% ethanol. Root tips were hydrolyzed with 1N HCL at 60°C during 25 min and stained according to the Feulgen technique. After that, root tips were washed briefly with distilled water. Meristematic regions of 1 mm length excised and squashed in a drop of 1% acetic orcein mixed with a drop of 45% acetic water (Jahier *et al*., 1992). The slides were examined under an optical Microscope type Hund (H 600) and photomicrographs were taken with the same microscope fitted with a BenQ camera using an oil immersion objective (100 X). Chromosomal nomenclature was carried out according to (Levan *et al*., 1964). For each chromosome cell, traits such a long arm (L), short arm (S) were measured and calculations were made to determine chromosome length, centromere position and arm ratio. For each accession, a karyogram was constructed by arranging the chromosomes in homologous pairs by order of their length. Karyotype data (total length of the chromosome, long arm (L), short arm (S), satellite length (SAT) long arm/short arm ratio (R)) were submitted to analysis of variance (ANOVA) with three classification factors (species, accession, chromosome pairs) to determine the importance of each factor on the variation of these parameters using the SAS program (SAS, 2000). The comparison of means between accessions to relevant parameters was performed by Duncan post-ANOVA test. All statistical tests were two-tailed, and a P-value of 0.05 or less was considered significant. Cluster analysis also was applied to karyological parameters in order to group accessions claimed in linkage averaging methodology based on their similarity.

RESULTS AND DISCUSSION

***Analysis of molecular variation*:** Fifteen random primers used for initial screening with three representative genotypes, only four primers amplified polymorphic patterns. These primers were used for RAPD analysis for studying genetic diversity among 33 genotypes. DNA samples were adjusted to a final concentration of 50 ng/µL and then stored at -20°C until use. The primers that gave clear and interpretable profile were detailed in Table 2.

Table 2. Polymorphism rates and calculation of the Polymorphism Information Content (PIC) for the 4 RAPD primers.

Primers	Number of polymorphic bands			Polymorphism rate	PIC
	P1	P2	P3		
SBS A10	8	9	9	54.16%	0.92
SBS P17	10	11	12	68.75%	0.90
SBS Q4	14	11	12	77.08%	0.93
SBS Q18	11	8	6	52.08%	0.92

P1: var. Sejenane; P2: acc.2565; P3: acc.3615; PIC: Polymorphism Information Content.

Figures 1, 2, 3 and 4 showed the profiles generated by the 4 primers visualized on an agarose gel (1.5% w/v). A total of 121 bands ranging from 300 bp to 3000 bp were identified in the studied accessions of *Vicia villosa* (Fig. 1, 2, 3 & 4). Revealed RAPDs have been converted into a binary matrix (0/1) where 0 indicates the absence of the band and 1 its presence. The number of bands obtained varied from 8 to 14 according to the primer used and the accession.

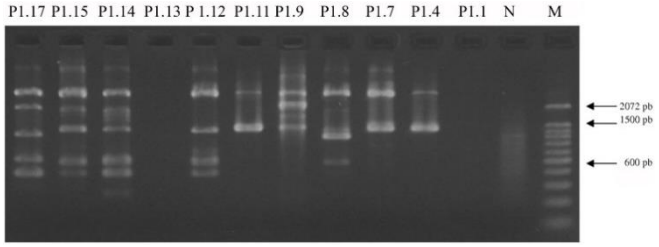


Figure 1. Randomly amplified polymorphic DNA profiles of var. Sejenane (P1) of *V. villosa* using SBSQ18 primer. N: negative control, M: molecular marker (100kb).

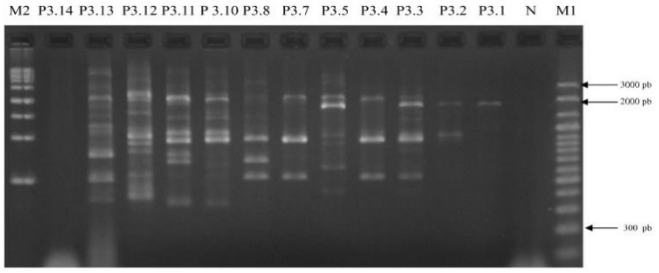


Figure 2. Randomly amplified polymorphic DNA profiles of acc. 2565 (P2) using SBS17 primer.
N = negative control, M1: molecular marker (100 kb), M2: molecular marker (1kb).

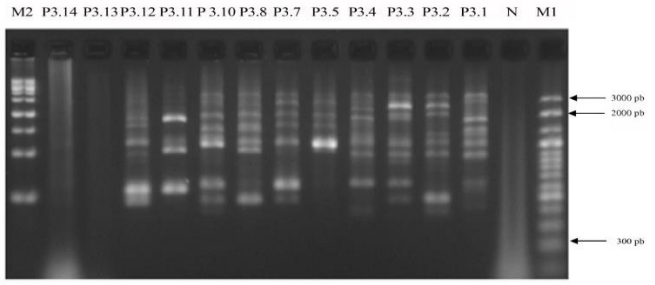


Figure 3. Randomly amplified polymorphic DNA profiles of acc. 3615 (P3) using SBSQ4 primer.
N = negative control, M1 = molecular marker (100 kb), M2 = molecular marker (1kb).

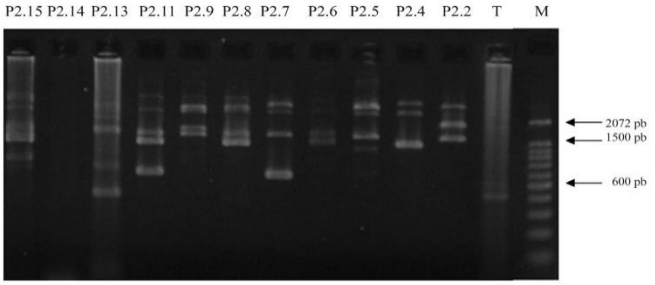


Figure 4. Randomly amplified polymorphic DNA profiles of acc. 2565 (P2) using SBSA10 primer.
N = negative control, M1 = molecular marker (100 kb), M2 = molecular marker (1kb).

Polymorphism rate or percentage of polymorphic fragments generated by each primer and the calculation of the PIC (Polymorphism Information Content) is summarized in Table 2**.** Polymorphism rate is estimated by the following formula:

*Polymorphism rate = (Number of polymorphic bands/Total number of bands) * 100.*

The total number of bands was estimated to 16 bands per primer in the 3 studied accessions.

The SBSQ4 primer has the highest percentage of polymorphism while the SBSQ18 has the lowest percentage. Regarding the calculation of the Polymorphism Information Content (PIC), the highest value was observed in the SBSQ4 which showed the important discriminating power of this primer. The lowest value of the PIC is represented by the primer SBSP17. The level of polymorphism found in our work is higher than that revealed by Yahia *et al.* (2014) analyzing the genetic diversity Tunisian faba bean (*Vicia faba* L.) accessions, by RAPD markers which revealed a polymorphism level of 60.63%. Same results were observed for *V. ramuliflora* a diploid population and new subspecies by Han *et al.* (2017). However, Han and Wang, (2010) showed a 100% polymorphism rate using the same primers of our work in a study of genetic diversity in two closely related *Vicia* species in northeastern China.

Principal coordinates analysis (PCoA): Principal coordinate's analysis (PCoA) is illustrated in Figure 5 and provided genetic similarities within and between the accessions. Indeed, it was possible to distinguish four groups genetically similar.

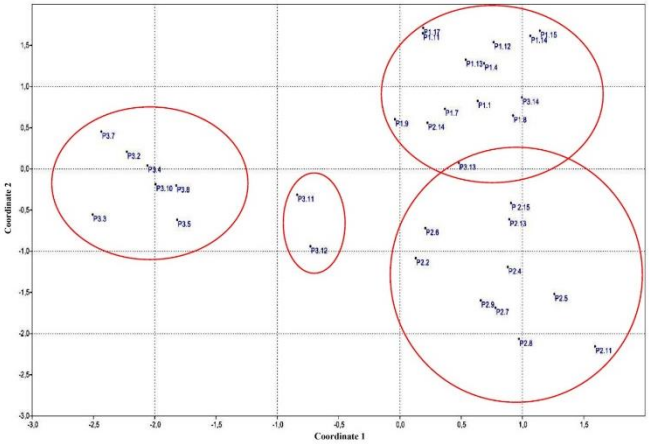


Figure 5. Principal Coordinates Analysis (PCoA) in 33 *Vicia villosa* genotypes using RAPD primers.

The first group included most of the P3 (acc. 3615) genotypes, the second group is formed by 2 genotypes of P3 (P3.11 and P3.12) distant from the other genotypes of P3 and enclosing a separate group. The third group is formed by all the genotypes of variety of Sejenane (P1) with one genotype of P2 (acc.

2565) and two genotypes of P3 (acc. 3615). The fourth group is represented by most of the genotypes of P2 (acc. 2565) but overlapping for a genotype of the third group. This result showed a gathering between the accession P2 and the commercial variety P2 while the accession P3 is relatively distant from the rest by using the PCo axis 1 (marked with dotted ellipse in Fig. 5).

Clusters analysis: The matrix of genetic distances between the 33 genotypes belonging to the three studied accessions allowed schematizing a phylogenetic tree. Figure 6 shows the dendrogram obtained by the UPGMA method. The analysis revealed two groups: Group 1 is represented by the majority of genotypes of the acc. 3615 (P3) accession. Group 2 is divided into 3 groups.

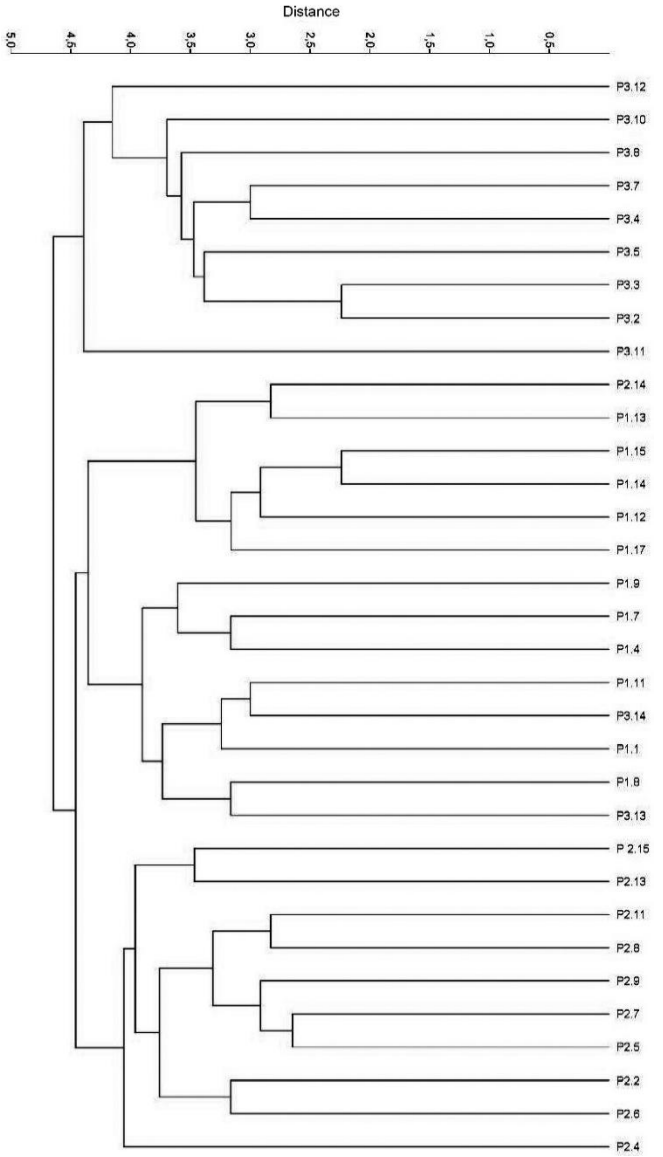


Figure 6. UPGMA dendrogram of the studied *Vicia villosa* Roth based on the genetic similarity matrix.

The first consists of the variety of Sejenane (P1) genotypes with one genotype of the acc. 2565 (P2.14) that is genetically close. The second consists of genotypes with the variety Sejenane (P1) and 2 genotypes of the accession acc. 3615 (P3.13 and P3.14). The third sub-group includes the majority of the genotypes of accession acc. 2565 (P2).

However, these results obtained by the UPGMA method showed that the local variety Sejenane (P1) and acc. 2565 (P2) are closer than the acc. 3615 (P3), which joins the results of the PCoA analysis. According to (Sakowick and Cieślikowski, 2006) phylogenetic analyzes of three sections of *Vicia* genus showed that RAPD markers could produce an information structure between species within the genus. Agar *et al.* (2006) found even though, that RAPD markers are particularly useful to resolve the phylogenetic relationships between closely related species. Overall, our results revealed a high level of genetic divergence between and within the studied accessions of *Vicia villosa* Roth.

***Karyotype characteristics*:** The observation and chromosome counts of metaphase plates revealed a chromosome number of 2n = 2x = 14 for all accessions (Fig. 7). This result is agreement with that of Lamine (2009), Yamamoto (1973), Tabur *et al.* (2001) and Gaffarzadeh-Namazi *et al.* (2008). However, significant differences between the accessions and the variety were observed for all measured parameters except the L/S ratio which is the same for all accessions (Table 3).

Table 3. Average of karyological parameters (in µm) of the variety Sejenane and the two accessions of *Vicia villosa* and their ranking according to the Duncan test at the 0.05 level.

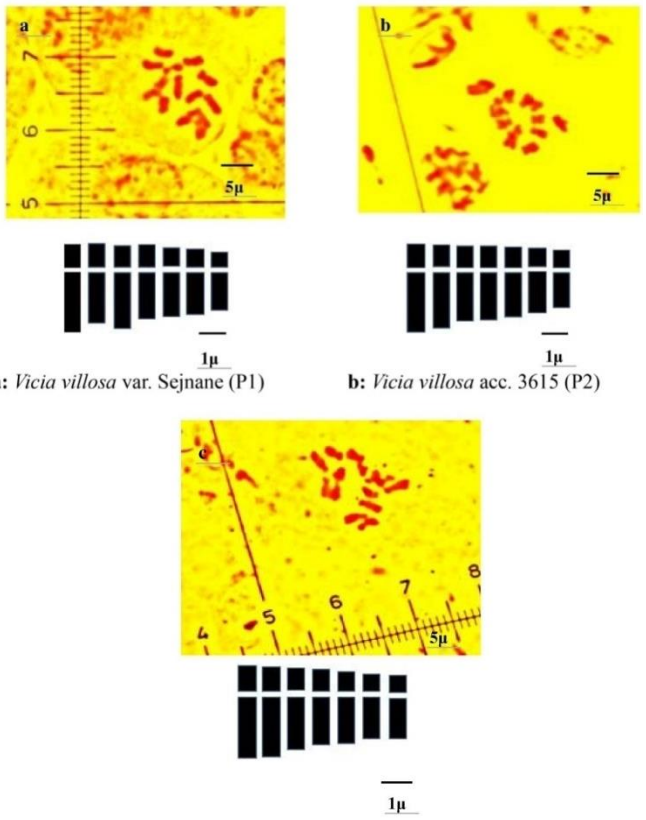
Accessions	Total length	Long arm	Short arm	L/S	Karyotype formula
var. Sejenane (P1)	2.69a	1.88a	0.81a	2.38a	14sm
acc 2565 (P2)	2.46b	1.73b	0.73b	2.40a	14sm
acc 3615 (P3)	2.80a	1.95a	0.84a	2.31a	14sm

P1: var. Sejenane; P2: acc.2565; P3: acc.3615.

According to Table 3, all accessions have the same karyotype formula namely 7 pairs of sub median chromosomes (7sm) while they have different chromosomes size. This result is consistent with that of Coulot and Raubate (2009) who found after analyzing plants belonging to *V. villosa* Roth subsp. *villosa*, *V. villosa* Roth subsp. *varia* and *Vicia villosa* Roth subsp. *eriocarpa*, diploid karyotype equivalent to 2n = 2x = 14 and having a similarity in the size and location of genes among tested plants. These authors also mentioned that tetraploidy (2n = 28) appears to exist in some accessions of *V. villosa* Roth but remained marginal. However, it is also noted that the three accessions have a homogeneous karyotype containing the same type of chromosomes, all of the submetacentric type which is considered a symmetric

karyotype according to the classification (Stebbins, 1971; Paszko, 2006).

Idiograms of the accessions and the variety of *Vicia villosa* are established on the basis of the centromeric index (Jahier *et al*., 1992) based on long arm (L) and short arm (S) ratios. Figure 7 illustrates the somatic chromosomes and idiograms of the two studied accessions, acc. 2565 (P2), the acc. 3615 (P3) and Sejenane variety (P1) of *V. villosa*. The two accessions as well as the variety Sejenane of *V. villosa* have a similar karyotype containing 7 pairs of sub median chromosomes. Cluster analysis applied to karyological data showed two groups of accessions (Fig. 7): one group including the variety Sejenane (P1) and the acc. 2565 (P2) and another group containing the acc. 3615 (P3). The latest one seemed to be very distant. This grouping is probably due to the geographic origin of accessions.



a: *Vicia villosa* var. Sejnane (P1) **b:** *Vicia villosa* acc. 3615 (P2)

c: *Vicia villosa* acc. 3615 (P3)

Figure 7. Somatic chromosomes and idiograms of the variety Sejenane and the two accessions of *Vicia villosa* (2n=2x=14); (a): var Sejenane (P1), (b): acc. 2565 (P2), (c): acc. 3615 (P3).

***Conclusion*:** Molecular results revealed relatively high levels of genetic diversity within and between the studied plant materials of *Vicia villosa* Roth. The RAPD technique has proved effective. In fact the 4 RAPD screened primers used generated 121 polymorphic fragments and a high number of phenotypes which varied considerably between primers, the two accessions and Sejenane variety. The analysis of the genetic diversity by the RAPD technique of the 3 accessions of *Vicia villosa* showed a higher level of polymorphism. The percentage of polymorphic fragments was 77%. In conclusion, variation of the genotypes of *Vicia villosa* based on RAPD has existed. Therefore, RAPD method can be used to assess variations among the *Vicia villosa* genotypes before starting a breeding program and can provide useful information in order to distinguish genotypes. However, the existence of a single type of chromosomes in the studied two accessions of *Vicia villosa* Roth acc. 2565 (P2) and acc. 3615 and also in the commercial variety (P1) of *Vicia villosa* Roth which include 7 pairs of submetacentric chromosomes showed that their karyotype is symmetrical and consequently less evolved. Nevertheless, variation was observed among the two accessions and among the variety for chromosomes size. The variation at karyological and molecular level can be explored in breeding programs. Also, this characterization is considered as a priority if we want to conserve and enhance the local forage and pasture plant genetic resources.

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