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in

Sulas L. (ed.).
Legumes for Mediterranean forage crops, pastures and alternative uses

Zaragoza : CIHEAM
Cahiers Options Méditerranéennes; n. 45

2000
pages 79-83

Cytogenetic study of forage grasses and legumes

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Summary - A cytotaxonomic and cytogenetic characterization was conducted on forage legumes and grasses, such as *Lotus corniculatus, Lotus alpinus, Trifolium alpinum, Phleum alpinum, Phleum pratense, Festuca rubra* and *Festuca pratensis* collected at different altitudes in the Italian Alps, with the purpose of analysing the germplasm structure and identifying those species most suitable for establishing new improved varieties. Ploidy level, chromosome morphology, somatic karyotype and chromosome behaviour at meiosis were determined in different accessions of each species. For a better characterization of the *Lotus* spp. FISH was also used. Cytological analysis detected atypical chromosome numbers in some species, as well as variable chromosome numbers among individuals of the same accession in other species. Furthermore some of the species displayed variable numbers of supernumerary B-chromosomes. The origin and significance of those peculiarities in the chromosome sets are discussed and evaluated with a view to utilising the species analysed in breeding programmes.

Key words: forage species, cytotaxonomy, B-chromosomes, FISH

Résumé *- La caractérisation cytotaxonomique et cytogénétique d'un certain nombre de légumineuses et graminées fourragères a été entreprise dans le but d'en analyser la structure du germoplasme et d'identifier les espèces les mieux indiquées pour la constitution de variétés améliorées. Les espèces ayant fait l'objet de l'étude, récoltées à différentes altitudes dans les Alpes italiennes, comprenaient*: Lotus corniculatus, Lotus alpinus, Trifolium alpinum, Phleum alpinum, Phleum pratense, Festuca rubra, *et* Festuca pratensis. *Le degré de ploïdie, la morphologie des chromosomes, le caryotype somatique et le comportement des chromosomes en méiose ont été relevés dans plusieurs accessions pour chacune des espèces. L'analyse par FISH a également été utilisée pour une meilleure caractérisation des* Lotus *spp. L'analyse cytologique a révélé des nombres chromosomiques atypiques dans certaines espèces , ou bien variables suivant les individus d'une même accession dans d'autres espèces. Quelques espèces présentaient également des chromosomes B surnuméraires. L'origine et la signification de telles particularités des ensembles chromosomiques sont discutées et évaluées en vue d'une utilisation des espèces étudiées dans des programmes d'amélioration génétique.*

Mots clés : *espèces fourragères, cytotaxonomie, chromosomes B, FISH*

Introduction

In the northern part of the Mediterranean area, the Alps represent a unique environment for their genetic potential in the breeding of forage species, firstly, because of the considerable variety of species and ecotypes still to be found there, in contrast with the decreasing variety of cultivated forage species in the lowlands, and secondly, because forages from mountain pastures are of great interest for animal feeding (Mariani *et al.*, in press). However, only slow progress has been made to date in the breeding of those species, both because of peculiarities of their reproductive biology and on account of factors associated with their overall economic value. Based on these considerations, a cytotaxonomic and cytogenetic characterization of a number of forage legumes and grasses from various sites in the Italian Alps, was undertaken. The objectives of the study were (*i*) to gain a better understanding of the germplasm structure, (*ii*) to determine the role and potential of those species and (*iii*) to assess their genetic variability for the purpose of transferring useful

characters through appropriate breeding methods, but primarily for constituting improved varieties adapted to the mountain environment.

Materials and methods

The species studied *Lotus corniculatus, Lotus alpinus, Trifolium alpinum, Phleum pratense, Phleum alpinum, Festuca pratensis* and *Festuca rubra* were collected from various sites in the Italian Alps, at altitudes varying between 1000 m and 2000 m (collection was made by the Istituto Sperimentale per le Colture Foraggere-MiPA, Lodi, Italy). For *L. corniculatus* and *F. rubra*, commercial varieties also were used as control. From 2 to 3 accessions per species were used, and 20 to 30 individuals (seeds and/or plants) per accession were analysed. Determination of the somatic chromosome number and chromosome morphology was made on root tips pretreated in ice (24 h) and alfa-bromonaphtalene (3 h), fixed in ethanol:acetic acid (3:1), then stained according to the Feulgen method. 30 to 40 well-spread metaphases were analysed for each accession. For microsporogenesis, analysis conducted in order to determine the degree of homology and pairing behaviour of the meiotic chromosomes, inflorescences were fixed in ethanol:acetic acid (3:1) and then Feulgen stained after 30 sec hydrolysis in 1N HCL at 60 °C. A cytological and molecular analysis of *L. corniculatus, L. alpinus* and *L. tenuis* - the latter being used as control - was performed using fluorescent *in situ* hybridization (FISH) and $AgNO_3$ staining according to the method described by Basili et al. (in press).

Results and discussion

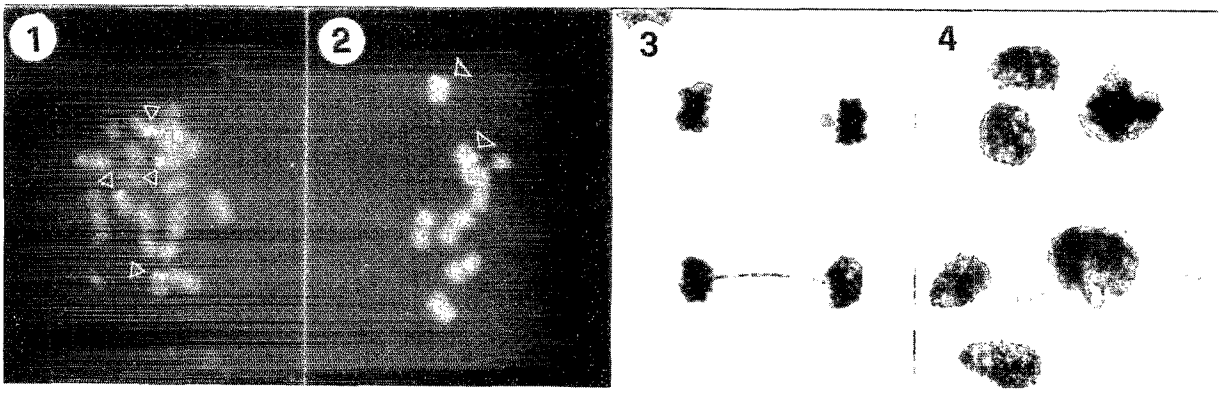
In some of the species, karyological analysis detected regular chromosome numbers, as in *T. alpinum* and *P. pratense*, whose all accessions exhibited 2n=16 and 2n=42 , respectively. Differently, other species were found to have atypical chromosome numbers. In particular, one accession of *F. pratensis* and two of *P. alpinum* had 2n=42 with six satellites instead of the typical chromosome number of 2n=14 with two satellites. For all of the species studied, the number of satellites was confirmed by the number of nucleoli in interphase cells. In fact, the number of nucleoli is known to be coincident with the number of nucleolar organizers present on the satellited chromosomes. Two of the eleven accessions of tetraploid *L. corniculatus* (2n=24) were scored as diploids with 2n=12. Since differences between those accessions with 2n=12 and *L. corniculatus* were also detected by morphological analysis, the diploids were thought to belong to *L. alpinus*, considered by previous authors to be, respectively, a subspecies of *L. corniculatus* (Fiori, 1969), its ancestor, or a differentiated type adapted to higher altitudes (Pignatti, 1982).

Noteworthy, however, is the fact that *L. corniculatus* (2n=24) has been reported showing considerable polymorphism, with wide variability particularly in some of those morphological characters which are expected to distinguish the species (Beuselinck *et al.*, 1996). In microsporogenesis, both the diploid and tetraploid *Lotus* accessions displayed an equal frequency of irregular meiotic stages at first and second divisions. Based on these observations, two accessions each of *L. corniculatus*, *L. alpinus* and of *L. tenuis*, itself a diploid with 2n=12, used as control, were examined in order to characterize the diploid and tetraploid types of *Lotus* on a chromosomal basis and thus determine their interrelationships. A cytological and molecular approach (FISH) was taken because conventional cytological methods alone were considered unsuitable to answer the purpose. FISH analysis, therefore, was used to study the localization and expression of the ribosomal rDNA genes 18S-5.8S-25S present at the nucleolus organizing regions (NORs), because the number of NOR sites

coincident with the number of satellites, and their location are used for characterizing species within a genus and establishing their phylogenetic relationships (Cuéllar *et al.*, 1999). In addition to FISH, silver staining of metaphase chromosomes was performed to assess transcriptional activity of the NOR sites. The number of nucleoli per interphase nucleus was also determined, because the maximum number of nucleoli generally is coincident with the number of AgNORs (silver stained nucleolus organizing regions) (Moscone *et al.*,1995; Lima-Brito *et al.*, 1998). The two accessions of *L. corniculatus* 2n=24 showed four hybridization sites (Fig. 1) both in metaphase chromosomes and interphase nuclei. Furthermore, $AgNO_3$ staining of metaphase chromosomes and maximum number of nucleoli showed all four hybridization sites to be transcriptionally active. One of the two accessions of *L. alpinus* 2n=12 displayed, at both metaphase and interphase, only two hybridization signals (Fig. 2), that positive $AgNO_3$ staining showed to be both transcriptionally active. Also, the maximum number of interphase nucleoli confirmed all rDNA regions as being able to organize nucleoli. The other accession of *L. alpinus* generally exhibited two more intense signals and two of lower intensity probably due to a lower gene copy number (Basili *et al.*, in press). Silver staining and the number of nucleoli observed seem, however, to indicate that only two of the sites as detected by FISH are transcriptionally active. The two *L. tenuis* accessions mostly displayed metaphases with two hybridization sites, while other two sites of lower intensity were observed in few cells only. Further studies will be required for *L. tenuis*, not only to verify the results from FISH analysis but also in relation to the evidence provided by silver staining and by the number of nucleoli observed, since in both accessions there would seem to be two active sites in some metaphases and four in others, two of which very weak. There were generally 1 or 2 nucleoli per cell, but as many as four could occasionally be seen.

With respect to *F. rubra*, as this species had shown variability of the chromosome number among individuals of the same accession and exhibited supernumerary B-chromosomes (Mariani *et al.*, in press), a study was undertaken on plants of one variety collected at three different altitudes, i. e. plain (Lodi), 1000 m and 2000 m, to verify possible environment effects on ploidy level and chromosome behaviour at meiosis in relation to genome plasticity. Modifications of the genome could, in fact, act as growth regulators and factors of environmental adaptation. Cytological analysis could only be carried out on materials collected in the plain and at 1000 m, to the exclusion of plants collected at 2000 m which had a very poor bloom, probably due to poor weather conditions, and therefore produced no seed. Seeds from the "Lodi" plants and from a commercial variety used as control germinated normally, while seeds from plants collected at 1000 m had a very low germination rate (30%). Chromosome numbers varying from 2n=58 to 2n=68 were recorded for the "Lodi" plants, as well as three to four B-chromosomes in a few individuals. In plants collected at 1000 m variability was less marked (2n=54-58) and again a few individuals were found which had 2 to 3 B-chromosomes. The commercial variety was stable with 2n=64. The presence of B-chromosomes in *F. rubra* and in some accessions of *T. alpinum* deserves particular attention. Although they may vary among individuals of one species, or between cells, tissues or organs of the same individual, these chromosomes, which are very frequent in plants, are always negatively correlated with plant growth and fertility (Jones and Rees, 1982). Thus, in Alpine species, such chromosomes will be worth further investigations with respect to their number and behaviour. In relation to plants of *F. rubra* collected at the three altitudes, microsporogenesis analysis was initiated to study chromosome behaviour at meiosis, so as to gather information on fertility in those materials. Preliminary data have provided evidence that meiotic stages are in general more regular in the "Lodi" plants than in plants growing at 1000 m, in which a high frequency of meiotic cells with a number of anomalies was found at both I and II divisions (Fig. 3).

Cells with various kinds of anomalies such as desynapsis, chromatin bridges, laggards and syncytia, were also observed at I and II divisions in the very few inflorescences produced by plants growing at 2000 m (Fig. 4), suggesting that a similar meiotic behaviour may be associated with low pollen viability.



Figs. 1-2 Fluorescent *in situ* hybridization (FISH) on chromosomes of (1) *Lotus corniculatus* (2n=24): four hybridization sites; (2) *L. alpinus* (2n=12): two hybridization sites. Magnification 1600x.
Figs. 3-4 Microsporogenesis of (3) *Festuca rubra* collected at 1000 m: ana-telophase II with a chromatin bridge and a laggard and (4) *F. rubra* collected at 2000 m: syncytia with six nuclei. Magnification 1000x.

Conclusions

The results achieved by cytotaxonomic and cytological/molecular analyses suggest that chromosome-based characterization of the species studied is possible and reveals genetic variability. A similar approach may be used to investigate phylogenetic and evolutive relationships between species of one genus and select the most suitable types for the constitution of improved varieties.

Knowledge about the germplasm structure and fertility and stability of the species will provide a valuable basis for the study of possible associations between environmental factors (altitude, etc.) and modifications of genome size and organization.

Ackowledgements

This research was financially supported by MiPA as part of the proiect "Gestione delle risorse prato pascolive alpine"

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