Cytogenetic Studies in the Rare South American *Lathyrus hasslerianus* Burk. (Leguminosae)

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Summary A detailed analysis of the karyotype and the meiotic behavior of the South American endemic *Lathyrus hasslerianus* is provided in this study. The karyotype obtained is the most asymmetrical of section Notolathyrus, which together with the particular morphological characters and ecological requirements supports a derived state of this species. Meiotic analysis showed the occurrence of univalents and out of plate bivalents in metaphase I, which ultimately leads to the formation of micromicrospores or micronuclei at the sporad stage. Mechanisms of aberrant sporad formation are discussed. Even though, pollen viability was reduced, it does not compromise the species fertility as shown by the analysis of the seed set.

Key words *Lathyrus*, Karyotype, Meiosis, Seed set.

The genus *Lathyrus* comprises around 150 species of annual or perennial herbs. It is distributed mainly throughout the temperate regions of the northern hemisphere, but has 2 minor centers of diversity in tropical East Africa and South America (Kupicha 1983). The nearly 23 endemic South American species (Burkart 1935, 1942), together with *L. pusillus* of North America, have been included by Kupicha (1983) within the section Notolathyrus. This section constitutes an interesting group, both in itself and in the context of the whole genus, because its species have a reticulate pattern of variation of the vegetative and reproductive traits (Kupicha 1983). South American species mainly occur in temperate to cold regions, from mesic hills and from flooded lowlands to the Patagonian forest (Burkart 1935, 1942, Seijo personal observations). However, very few entities were recorded for subtropical regions. One of them, is the perennial *L. hasslerianus*, a very poorly collected species that lives in some more or less lightly patches of the subtropical Paranaense rainforest, usually close to water streams (Seijo personal observations). Besides its exclusively ecological requirements, this species has some particular morphological features like, long climbing stems (up to 3 m high), latifoliate leaves, yellow flowers, and big black seeds, all of which are very rare or unique among the Notolathyrus species.

From a cytological point of view, the karyotypes of nearly half of the Notolathyrus species have been analyzed (Batistín and Fernández 1994, Klämt and Schiffino-Wittman 2000, Seijo and Fernández 2003), and very few studies concerning the meiotic division were so far reported in this group (Seijo 1994, Schiffino-Wittmann *et al.* 1994). In this context, *Lathyrus hasslerianus* remains almost unknown except for the karyotype of one population which was included in a recent general chromosome analysis of the section (Seijo and Fernández 2003). Based on the phenetic analysis of karyotype features it was suggested that this species would be the most differentiated within the section. However, more information about this rare taxon would be desirable for the biosystematic and phylogenetic comprehension of the Notolathyrus members. New collection of *Lathyrus*

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germplasm in South America have included a new population of *L. hasslerianus*, and additionally, we have obtained more seeds and some floral buds of the population studied before (Seijo and Fernández 2003) from plants that have been growing in the greenhouse. To our knowledge these are the only 2 populations collected described by Burkart (1935) based on an incomplete specimen. With this material available and in order to provide new insights in the chromosome analysis, both of *L. hasslerianus* and of the section as a whole, in this paper we perform a detailed karyotype characterization, a complete analysis of the meiotic behavior as well as an estimation of pollen viability and the seed set of this species.

Material and methods

Voucher specimens of the studied material are deposited at the Herbarium of the Instituto de Botánica del Nordeste (CTES), Corrientes, Argentina. The populations studied are: Seijo 2000 (S 2000), Argentina, Misiones Province, Dept. San Pedro, A° Tambero; and Keller & Cocolmet 1181 (K&C 1181), Argentina, Misiones Province, Dept. Iguazú, Paraje Aguaray.

Somatic chromosome number and karyotype analysis were carried out on meristematic cells of root tips obtained from germinated seeds of population (K&C 1181). Roots were pre-treated at 0°C for 24 h, then fixed in ethanol:lactic acid 5:1 (Fernández 1973) and kept in 70% ethanol. Staining was performed following the Feulgen's technique and the meristems were macerated in a drop of 3% acetic orcein before squash. Permanent slides were prepared using Euparal.

Chromosome measurements were done from drawings using a Wild camera lucida attached on a Zeiss microscope. The karyotypes were described according to the chromosome morphology determined by the centromeric index ($ci = \text{short arm} \times 100 / \text{total chromosome length}$) as suggested by Levan *et al.* (1964). Chromosomes were classified in two categories: metacentrics (m) with $ci = 50\text{--}37.5$, and submetacentrics (sm) with $ci = 37.5\text{--}25$. For the idiogram construction, chromosomes were firstly arranged by morphology and then by size. The criteria for satellite classification followed Seijo and Fernández (2003). The relative length was calculated for each pair as follows, $lr = (\text{chromosome length} / \text{haplotype karyotype length}) \times 100$. Karyotype asymmetry has been determined according to the Romero Zarco (1986) intrachromosomal (A_1) and interchromosomal (A_2) indices, as well as by the classification proposed by Stebbins (1971).

Suitable floral buds for meiotic studies were only obtained from population S 2000. They were fixed in ethanol:lactic acid (5:1) for 24 h, transferred to 70% ethanol and kept in refrigerator until use. Pollen mother cells (PMCs) were stained with acetic orcein and slides were made permanent as described above for mitotic analysis. Chiasmata distribution was established by dividing the chromosome arm in thirds from the centromere to the telomere, and chiasmata were considered as proximal, interstitial and distal depending on which third they were located. The recombination index was calculated according to Darlington (1965) by the addition of the haploid number to the mean chiasmata number per PMC. Mean chiasmata frequency per bivalent was calculated dividing the chiasmata frequency per PMC by the number of bivalents. Meiotic index was determined by the ratio between abnormal sporads/total sporads analyzed $\times 100$.

Stainability with carmine glycerine or fluorescein diacetate-phenosaphranine (Widholm 1972) was used as an estimation of pollen viability. Seed set was determined by the number of viable seed/number of ovules per pod $\times 100$ in 50 pods.

Results

Karyotype characterization

All the plants analyzed have $2n = 14$ chromosomes (Fig. 1A) in their somatic cells. Both populations showed the same karyotype formula, $2m + 12sm$. Chromosome morphology and measure-

Fig. 1. Meiotic behavior of *L. hasslerianus*. A) Mitotic metaphase stained with Feulgen's technique. B) Normal prophase, early diplotene. C) Metaphase I with one out of plate bivalent. D) Metaphase I with two monovalents. E) Interphase with two micronuclei of one chromatid. F) Interphase with one micronucleus of two chromatids. G) Metaphase II with one out of plate chromosome. H) Telophase II with two micronuclei of one chromatid. I) Telophase II with two micronuclei of two chromatids. Bar = 10 μ m.

Table 1. Mean chromosome measurements by pairs and chromosome types according to the classification of Levan *et al.* (1964) in *L. hasslerianus*. s: short arm, l: long arm, tl: total length, ci: centromeric index, and lr: relative length. Measurements are provided in μ m.

Pair	Chromosome length (μ m)			ci	lr %	Type
	s	l	tl			
1	2.79 $\pm$ 0.04	4.32 $\pm$ 0.12	7.11 $\pm$ 0.15	39.30 $\pm$ 0.57	15.59	m
2	2.50 $\pm$ 0.08	4.40 $\pm$ 0.15	6.90 $\pm$ 0.23	36.22 $\pm$ 0.33	15.14	sm
3	2.26 $\pm$ 0.05	4.37 $\pm$ 0.17	6.63 $\pm$ 0.20	34.31 $\pm$ 0.66	14.55	sm
4	2.16 $\pm$ 0.08	4.42 $\pm$ 0.13	6.58 $\pm$ 0.19	32.78 $\pm$ 0.60	14.44	sm
5	1.91 $\pm$ 0.08	4.35 $\pm$ 0.16	6.26 $\pm$ 0.24	30.50 $\pm$ 0.56	13.74	sm
6	1.79 $\pm$ 0.08	4.46 $\pm$ 0.15	6.25 $\pm$ 0.23	28.56 $\pm$ 0.50	13.71	sm
7	1.57 $\pm$ 0.05	4.28 $\pm$ 0.09	5.85 $\pm$ 0.13	26.78 $\pm$ 0.54	12.82	sm-SAT

Table 2. Karyotype formulae, total chromosome length (TCL), mean chromosome length (CL), chromosome length range (Range), mean centromeric index (CI), intra (A_1) and interchromosomal (A_2) asymmetry indices, and the Stebbins' category of asymmetry (S) in *L. hasslerianus*. Measurements are provided in μm .

Formulae	TCL	CL	Range	CI	A_1	A_2	S
2 m+12 sm	45.57 $\pm$ 1.25	6.51 $\pm$ 0.14	5.84–7.11	32.66 $\pm$ 1.03	0.51	0.07	3A

Table 3. Frequency of bivalent configurations in metaphases I of *L. hasslerianus*.

Configuration	7 Ring	6 Ring+1 Rod	5 Ring+2 Rod	4 Ring+3 Rod	3 Ring+4 Rod	Total
No of cells	27	17	2	2	1	49
Frequency	0.55	0.35	0.04	0.04	0.02	1

Table 4. Meiotic analysis of *L. hasslerianus*. Frequencies of normal and abnormal configurations are given for each meiotic stage.

Configurations	Zygotene	Diakinesis	Metaphase I	Interphase	Metaphase II	Anaphase II	Telophase II
Normal	100	100	91.7	91.4	88.9	94.1	87.4
2 monovalents			1.2				
1 out of plate bivalent			7.1		11.1		
1 out of nuclei monovalent				5.4			
1 out of nuclei bivalent				3.2			
1 univalent with extra spindle						5.9	
1 micronucleus with 1 chromatid							5.3
1 micronucleus with 2 chromatids							3.2
2 micronuclei with 1 chromatid							1.1
4 micronuclei with 1 chromatid							1.5
2 micronuclei with 1 chromatid+							1.1
1 with 2 chromatids							
2 micronuclei with 2 chromatids							0.4
Total number of analysed cells	50	50	84	58	18	17	277

ments for each pair are given in Table 1. The karyotype formulae, length for the haploid karyotype, mean chromosome length, mean range of chromosome length centromeric index, and asymmetry indices are detailed in Table 2. All the chromosomes had similar size, being the m pair the longest and the sm pair which borne a macrosatellite in the long arm the shortest of the complement. The satellite comprises the 30% of the arm length and in prometaphases and early metaphases appeared far from the proximal segment, resembling small acentric chromosomes.

The karyotype was moderately symmetrical according to the A_1 and A_2 indices, and it laid in the 3A category of Stebbins, which includes the higher number of sm chromosomes among the symmetrical karyotypes (A categories).

Meiotic analysis

Prophases were normal (Fig. 1B) and 7 bivalents were formed in metaphase I, most of which were of the ring type. Table 3 shows the meiotic configurations observed in metaphase I. The mean chiasma frequency was 1.95 per bivalent and 13.67 per PMC. Chiasmata were mainly terminal, some interstitial, while proximal ones were very rarely observed. The recombination index was 20.7.

Table 5. Analysis in *L. hasslerianus*. Frequencies of each type are given based on $n=310$.

Sporad constitution	Scheme	Frequency	Sporad constitution	Scheme	Frequency
Normal tetrad		89.7	Tetrad, 1 microspore with 1 micronucleus and 1 collapsed microspore		0.3
Tetrad+1 micromicrospore with 2 chromatids		1.3	Tetrad, 1 microspore with 2 micronuclei		2.0
Tetrad+2 micromicrospores with 1 chromatid		0.7	Tetrad, 2 microspores with 2 micronuclei		0.7
Tetrad+4 micromicrospores with 1 chromatid		0.3	Tetrad, 2 microspores with 1 micronucleus and 1 microspore with 2 micronuclei		0.3
Tetrad+2 micromicrospores with 1 chromatid+1 with 2 chromatids		0.3	Tetrad, 1 collapsed microspore		3.3
Tetrad, 2 microspores with 1 micronucleus		0.3	Tetrad, 2 collapsed microspores		0.3
Tetrad, 2 microspores with 1 micronucleus and 1 microspore with 2 micronuclei		0.3			

Two abnormal phenomena were detected in metaphase I (Table 4), in which 1 “out of plate” bivalent in a 7.1% of the PMCs (Fig. 1C), and 2 monovalents (1.2%, Fig. 1D) were observed. Both phenomena altered the regular chromosome segregation, since in most cases these chromosomes showed spindle misintegration, with the consequent difficulty to be incorporated into the telophase I nuclei. If out of plate bivalents segregate, the chromosomes remained almost always as laggards and did not reach the poles, since their segregation occurred independently of the main spindle. On the other hand, the monovalents behavior was variable, being possible to remain in the cytoplasm or be randomly incorporated into the telophase nuclei.

As it is shown in Table 4, the frequency of the out of nuclei chromosomes observed in the 8.6% interphasic PMCs (Fig. 1E, F) was similar to the joint frequency of out plate chromosomes and monovalents in metaphase I. At the same time, this frequency agreed with the 11.1% of PMCs having out of plate chromosomes in metaphase II (Fig. 1G). In anaphase II, the most striking phenomenon observed was the presence of chromosomes segregating independently of the main spindles. These chromosomes corresponded to the out of plate chromosomes which in metaphase II were still composed by 2 chromatids, and which underwent equational segregation in an additional spindle. However, this phenomenon not always happened and some chromosomes retained their bichromatidic constitution. These chromosomes, either composed by 1 or 2 chromatids, usually remained out of the telophase II nuclei, leading to the origin of independent micronuclei.

A great diversity of micronuclei, both in number and in chromosome constitution, was observed in telophase II (Table 4). PMCs having 1 to 4 monochromatidic micronuclei (Fig. 1H) or 1 to 2 bichromatidic micronuclei were recorded (Fig. 1I). The percentages of micronuclei observed in this stage agreed with those expected to arise from the out of plate chromosomes observed in metaphase II.

The analysis of the meiotic products showed a great variety of sporads (Table 5) with a meiotic index of 89.7. Besides the 4 expected normal sized microspores (Fig. 2A), 1 to 4 micromicrospores containing 1 monochromatidic or bichromatidic chromosome each were observed in 2.6% sporads (Fig. 2F). Additionally, normal sized microspores bearing 1 or 2 micronuclei were observed (Fig. 2B–D). In most cases, when 2 micronuclei were present in a microspore, they were closely attached (Fig. 2C, D). The number of micronuclei presented by the microspores of any sporad were almost always paired, *i.e.* 2 microspores having 1 micronucleus each (Fig. 2B), 1 microspore with 2 mi-

Fig. 2. Sporads of *L. hasslerianus*. A) Normal tetrad. B) Tetrad with two microspores containing one micronucleus. C) Tetrad with one microspore having two paired micronuclei. D) Tetrad with two microspores with two paired micronuclei. E) Tetrads with one (right) and with two (left) collapsed microspores. F) Tetrad with one collapsed microspore and one micromicrospore. Bar = 10 μ m.

cronulei (Fig. 2C), or 2 microspores with 2 micronuclei each (Fig. 2D, Table 5). For each chromosome that became a micromicrospore or a micronucleus in any sporad, it is expected 1 aneuploid microspore with $n-1$ chromosomes.

Degeneration of the microspores protoplast was also observed in some sporads (Fig. 2E, F). This phenomenon affected different proportions of microspores within each sporad as it is shown in Table 5—1 collapsed: 3 normal (3.6%), and 2 collapsed: 2 normal (0.3%)—. No relationship could be established between the presence of micronuclei and the collapsed microspores, since only 10% of the sporads with collapsed microspores also showed micronuclei or micromicrospores. On the other side, most of the sporads with micronuclei in the microspores did not show evidences of protoplast degeneration.

The estimation of pollen viability by carmine glycerine and flourescein diacetate were comparable with a mean of 94.3% stained grains. Among the grains considered as no viable we recorded non-stained grains (4%), micrograins (1.5%), and macrograins (0.2%). Seed set analysis showed 85% of well developed seeds.

Discussion

Karyotype characterization

The karyotype formulae obtained for the material analysed here agree with those informed previously for *L. hasslerianus* (Seijo and Fernández 2003). Even though the karyotype was symmetrical, either considering the Romero Zarco (1986) and Stebbins (1971) classifications, the prevalence of sm chromosomes is restricted to this taxon, which has the highest degree of asymmetry when compared with the data published for other species of section *Notolathyrus* (Seijo and Fernández 2003). Considering the primary tendency toward asymmetry within a related group of

species postulated by Stebbins (1971), the karyotype of *L. hasslerianus* would be derived from the most symmetrical ones borne by the other perennial species of *Notolathyrus*.

Considering the geographical distribution of the species and their cpDNA sequences, it has been suggested that the dispersion of *Lathyrus* into South America may have occurred from North America through the Andean mountains (Kupicha 1983, Asmussen and Liston 1998). Furthermore, it was also proposed that these mountains, in which species with most ancestral characters are still found, probably constitute the primary South American centre of diversification of the section *Notolathyrus*; and that from this centre, specialized taxa may have derived (Seijo 2002). In this context, the particular features of the *L. hasslerianus*, in karyotype, morphological characters and ecological requirements, supports the hypothesis that this subtropical species constitutes a derived entity within *Notolathyrus*, that may have arisen during a secondary radiation process of the genus in South America.

Meiotic analysis

Chromosome behavior was normal in general terms. Even though this species formed mostly ring bivalents in metaphase I, it showed a higher percentage of rod bivalents and a chiasmata distribution slightly different from that reported for other few perennial taxa of *Lathyrus* (Narayan and McIntire 1989, Seijo 2002). However, the recombination index was similar to that expected for species of similar life span.

The normal meiotic behavior was reduced by the presence of monovalents and out of plate bivalents. The first abnormality may have arisen as a consequence of precocious segregation or late desynapsis, as was reported for some other species of the tribe *Lathyrus* (Seijo 2002). The second one, however, was rarely cited in plants (Guillin *et al.* 1995, Seijo 2002), and the causes that lead to the misintegration of only 1 bivalent per PMC to the metaphasic plate still remain very obscure.

Our analysis of the whole meiotic division showed that the out of plate chromosomes and univalents would lead to the formation of additional micromicrospores. The diversity, both in size and number, of the micromicrospores formed per sporad is close related to the chromosomes with abnormal behavior. In this sense, the size of the micromicrospores is determined by the constitution of the chromosomes from which they have arisen, *i.e.* mono- or bichromatidic. On the other hand, the number of micromicrospores observed in each sporad would be determined by the differential pattern of segregation of the chromosomes with irregular behavior, either in the first or in the second division. However, this number will be also affected by the rate of fortuitous integration of these chromosomes to the main nuclei.

Even though the occurrence of micromicrospores is directly related to the presence of out of nuclei chromosomes in telophase II, it seems that such chromosomes not always lead to the formation of a micromicrospore. This statement is supported by our findings of micronuclei in some normal size microspores. The mechanism that determines whether an out of nuclei chromosome in telophase II will become a micromicrospore or a micronuclei is still unknown, but it would be related with the previous behavior of that chromosome. In this sense, the chromosomes that have had equational division in the first meiosis, and that were unable to induce the formation of an additional spindle in anaphase II, would probably remain as micronuclei in the sporads. On the other hand, those that retained the bichromatidic constitution and that formed a spindle in the second division would lead to the formation of a micromicrospore. Among these later chromosomes, those which segregate would form 2 monochromatidic micromicrospores, while those which fail to segregate would form a single bichromatidic micromicrospore.

Other rare phenomenon observed in *L. hasslerianus* is the protoplast degeneration in some microspores of a given sporad, even when they are still within the callose. A similar phenomenon was observed in some meiotic mutants of *Pisum* by Nirmala and Kaul (1994). These authors related the microspore abortion to a *msg 6* mutant gene, which induces a great amount of chromatin distur-

bances and reduces the callose to a very thin layer, that disappears at the early sporad stage. In *L. hasslerianus*, however, the abortion of sporads can not be directly explained by the action of the same gene because the callose layer remained thick during the whole meiotic division as in other *Lathyrus* species (Seijo 2002) and chromosome abnormalities were less frequent than those described for the msg 6 mutant gene. Therefore, other mechanism may be leading to protoplast breakdown of microspores in *L. hasslerianus*, but additional studies are needed to address this topic.

Pollen viability was higher than that expected from the meiotic behavior observed. This fact may indicate that aneuploid microspores (n-1) may reach anthesis without detectable alterations. On the other hand, microspores showing protoplast degeneration at the sporad stage would not form pollen grains, phenomenon that will reduce the total amount of pollen but will not reduce the viability estimations at anthesis.

Seed set is a trait that can be affected by a great number of factors. However, it is one of the most effective ways to evaluate the fertility of plants in both germ lines (Marshall and Schmidt 1954, Sapra and Heyne 1973). In this sense, the populations analyzed here showed similar seed set than other species of *Lathyrus* (Seijo 2002), which suggest that the meiotic disorders observed in the male strain do not severely affect the reproductive efficiency of *L. hasslerianus*.

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