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CYTOGENETIC ANALYSIS OF FIVE *CROTALARIA* SPECIES (PAPILIONACEAE)

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Abstract. Root tips and flower buds from plants of five *Crotalaria* species collected in Sucre and Monagas States, Venezuela, were squashed and stained in FLP orcein to analyze mitotic and meiotic chromosomes. *Crotalaria incana* (2n = 14), *C. retusa* (2n = 16), *C. purdiana* (2n = 16), *C. vittelina* (2n = 16), and *C. anagyroides* (2n = 16) exhibited small (1.7–4.5 μm), median point (M), median region (m) and submedian region (sm) chromosomes. Mean number of chiasmata per cell, in diplotene, diakinesis and metaphase I, ranged from 15.10 to 9.92. Dicentric bridges and acentric fragments in pollen mother cells, at anaphase I, showed that *C. purdiana* plants are heterozygous for a paracentric inversion, and may produce aneuploid organisms by non disjunction of a pair of homologous chromosomes. The 84.2% fertility in *C. purdiana* showed that a simple or double crossing over took place within reverse inversion loops between inverted and normal chromosome segments at pachytene, reducing fertility by producing genetically abnormal gametes. These species fit the genetic system, where bivalents have normal chromosome pairing at pachytene and a nonrandom chiasmata distribution, with one and two chiasmata in diplotene, diakinesis and metaphase I, and are essentially equal in crossover frequency. *Received: 25 June 2008, accepted: 12 February 2009.*

Key words. Chromosomes, *Crotalaria*, chiasma, bivalents, crossovers, meiosis, karyotype, Papilionaceae.

ANÁLISIS CITOGENÉTICO DE CINCO ESPECIES DE *CROTALARIA* (PAPILIONACEAE)

Resumen. Los ápices radicales y primordios florales de cinco especies de *Crotalaria* colectadas en los estados Sucre y Monagas, Venezuela, fueron aplastados y coloreados con orceina FLP para analizar los cromosomas mitóticos y meióticos. *Crotalaria incana* (2n = 14), *C. retusa* (2n = 16), *C. purdiana* (2n = 16), *C. vittelina* (2n = 16) y *C. anagyroides* (2n = 16)

mostraron cromosomas de punto medio (M), región media (m) y de región submedia (sm), con un tamaño que varió de 1,7 a 4,5 µm. El promedio de quiasmas por célula en diploteno, diacinesis y metafase I varió de 15,10 a 9,92. Puentes dicéntricos y fragmentos acéntricos en las células madres del polen en anafase I mostró que las plantas de *C. purdiana* fueron heterocigotas para una inversión paracéntrica y pueden originar plantas aneuploides por la no separación de un par de cromosomas homólogos. La fertilidad de 84.2% en *C. purdiana* indicó que la recombinación simple o doble tuvo lugar en paquiteno dentro del asa reversa de inversión entre un segmento normal e invertido de cromosomas que redujo la fertilidad por la producción de gametos genéticamente anormales. Estas especies se ajustan al sistema genético, donde los bivalentes se aparean normalmente en paquiteno con uno y dos quiasmas y los quiasmas tienen una distribución no aleatoria en diploteno, diacinesis y metafase I y son esencialmente iguales en frecuencias de apareamiento. *Recibido: 25 junio 2008, aceptado: 12 febrero 2009.*

Palabras clave. Cromosomas, *Crotalaria*, quiasmas, bivalentes, recombinación, meiosis, cariotipo, Papilionaceae.

INTRODUCTION

Members of the genus *Crotalaria* (Papilionaceae) are annual plants distributed in all central states in Venezuela (Matos 1978). Characteristics of these species include zygomorphic flowers, calyx of five sepals, papilionaceous corolla, diadelphous androecium (9-1), and a monocarpellary gynoecium (Lawrence 1951). Cytological studies of *Crotalaria* have indicated a basic chromosome number of n = 8 (Turner and Fearing 1959, Datta and Biswas 1962, Magoon *et al* 1963, Datta and Ghoshal 1969, Boulter *et al* 1970, Chennaveeraiah and Patil 1973, Patil and Chennaveeraiah 1975). Because these plants form nitrificant nodules in their roots, they are useful in improving nitrogen-poor soils, especially in places used for cattle forage.

In some pollen mother cells, different meiotic configurations such as dicentric bridges and acentric fragments are observed at meiosis I and II, if simple or double crossing-over takes place within reverse inversion loops between inverted and normal chromosome segments at pachytene. The configurations occur in paracentric inversions with diminished pollen fertility (McClintock 1938, Pickering 1991, Cequea *et al*. 2003, 2006).

Cytogenetic study of *Crotalaria* is important for species hybridization in order to obtain high-yield and disease resistant cultivars useful in biotechnology aplications (Moscone *et al*. 2003). This study: 1) reports the

chromosome number and types for five *Crotalaria* species: *C. incana*, *C. retusa*, *C. purdiana*, *C. vittelina*, and *C. anagyroides*, 2) quantifies the pairing of homologous chromosomes, and 3) determines if homologous chromosomes were behaving as normal diploids.

MATERIALS AND METHODS

We collected plants from wild populations in Sucre and Monagas states, Venezuela, during 2004. Seeds selected from five plants of *Crotalaria incana*, *C. retusa*, *C. purdiana*, *C. vittelina*, and *C. anagyroides* were germinated in 50% Hoagland´s solution. Seedlings ~7 cm long were planted in water expanded, Jiffy-7 peat pellets, until numerous roots developed. Seven week-old seedlings were transferred to 5 kg polyethylene sacs containing sand, peat and soil in a 1:1:1 ratio, and maintained in the greenhouse until plants reached maturity.

Root tips were excised between 9:00 and 9:30 h prior to peak mitotic activity in a dilute bromonaphthalene solution (1/100 ml in 10 ml of water) for 3 h to inhibit spindle fiber formation. After pretreatment, root tips were transferred to a 4:1 fixative (4 parts 95% ethanol: 1 part propionic acid) for 48–72 h at room temperature. Fixed root tips were hydrolyzed in 15% HCl, squashed and stained in orcein FLP 1.5%. Fifty well spread metaphase plates per plant were analyzed for chromosome number and morphology, and chromosome types were determined using the method of Levan *et al*. (1963).

Flower buds were harvested between 12:00 and 13:00 and fixed in 4:1 ethanol:propionic acid for 72 h. Anthers were removed from individual florets, squashed and stained in orcein FLP 1.5% (Cequea and Nirchio 1998). Meiotic configurations at anaphase I and II, telophase I and II, interkinesis, metaphase II and tetrad stage were scored for bridges, fragments, chromosome number at metaphase II and the number of microspores in pollen mother cells (PMCs). A minimum of 50 meiocytes per plant were scored for chromosome configurations and chiasmata number, at diplotene, diakinesis and metaphase I.

The following equations: oII = (Cx – n). No. of cells and cII = n – (Cx – n). No. of cells were applied to meiotic configurations observed at diplotene, diakinesis and metaphase I. These equations give the expected number of circle and chain bivalents in normal diploid individuals, with normal pairing and nonrandom (NR) distribution of chiasmata among bivalents (Jackson 1984). This was followed by a Chi-square test for observed chromosome configurations to those expected for each individual species.

Five hundred pollen grains per floret, in 10 plants, were stained with Buffalo Black NBR (1 g napthol Buffalo Black to 100 mL warm FLP solvent, cooled to room temperature and filtered). Pollen grains having uniform and darkly stained cytoplasm were considered normal, whereas those incompletely or lightly stained blue were considered abnormal.

The following symbols were used: oII = a bivalent with two chiasmata, cII = a bivalent with one chiasma, Cx = mean chiasmata number per cell, n = basic chromosome number, P = chiasma coefficient (derived by dividing the theoretically possible number of chiasmata per bivalent by the mean number of chiasmata observed), capital Q is 1 - P or lack of chiasmata, No. = number. Identification of the five *Crotalaria* species was collaborated by revising specimens deposited in the Universidad de Oriente IRBR Herbarium.

RESULTS

KARYOTYPE

Crotalaria incana L. chromosome complements were five median region chromosome pairs (d = 1, r = 1.67), and two submedian region chromosome pairs (d = 2.5, r = 1.8).

Crotalaria retusa L. chromosome complements were five median point chromosome pairs (d = 0, r = 1), and three median region chromosome pairs (d =1, r = 1.67).

Crotalaria purdiana chromosome complements were four median point chromosome pairs (1, 2, 6 and 7; d = 0, r = 1), and four submedian region chromosome pairs (3, 4, 5 and 8; d = 2.5, r = 1.8). Chromosome lengths ranged from 1.7 to 3.5 μm (Fig. 1).

Crotalaria vittelina chromosome complements were five median point chromosome pairs (1, 2, 3, 4 y 5; d = 0, r = 1), and three submedian region chromosome pairs (6, 7 and 8; d = 2.5, r = 1.80). Chromosome lengths ranged from 2.0 to 4.5 μm (Fig. 2).

Crotalaria anagyroides chromosome complements were five median region chromosome pairs (d = 0, r = 1), and three submedian region pairs (d = 2.5, r = 1.8).

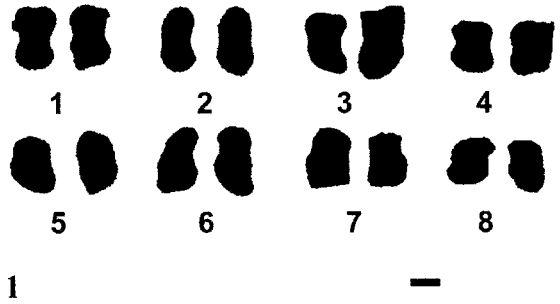

Figure 1. *Crotalaria purdiana* karyogram. Bar represents 1.75 µm.

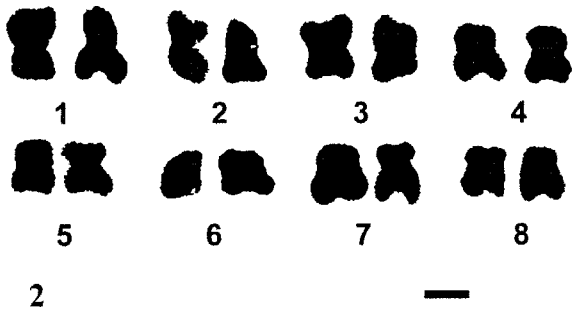

Figure 2. *Crotalaria vittelina* karyogram. Bar represents 2.81µm.

MEIOSIS

Crotalaria incana, *C. retusa*, *C. vittelina* and, *C. anagyroides* exibited normal meiosis in all stages and normal bivalents at diplotene (Fig. 3), diakinesis (Fig. 4), and metaphase I (Fig. 5), respectively. *Crotalaria purdiana* showed normal bivalents at diplotene, diakinesis and metaphase I, but one dicentric bridge and an acentric fragment in 20 meiocytes were observed in 65 cells analyzed at anaphase I (Fig. 6), one lagging chromosome in 5 meiocytes was observed in 55 cells analyzed at interkinesis (Fig. 7), and in 70 cells examined at metaphase II, 7 and 9 chromosomes in 10 meiocytes were observed (Fig. 8).

Figure 3. *Crotalaria retusa.* Diplotene with 8 circles.

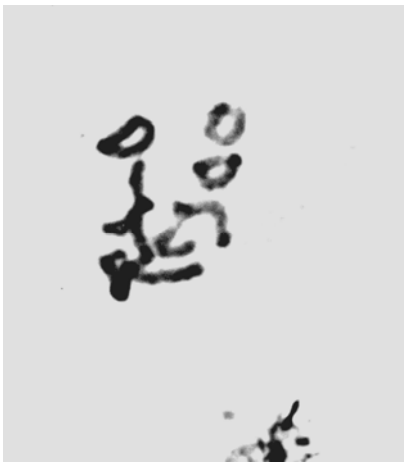

Figure 4. *Crotalaria retusa.* Diakinesis with 6 circles and 2 chain bivalents.

Figure 5. *Crotalaria purdiana*. Metaphase I with 3 circles and 4 chain bivalents. Bar represents 5.2 µm.

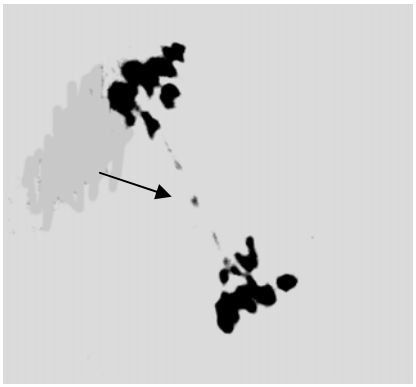

Figure 6. *Crotalaria purdiana*. Anaphase I with a dicentric bridge and acentric fragment (arrow).

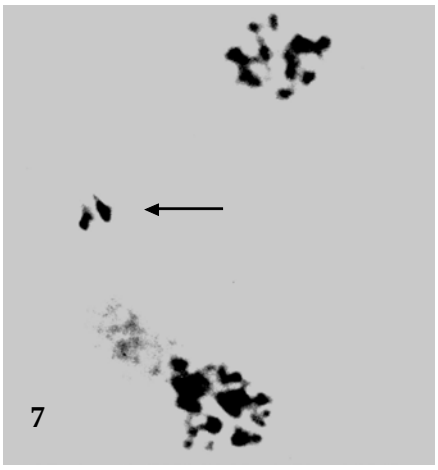

Figure 7. *Crotalaria purdiana*. Interkinesis with a lagging chromosome.

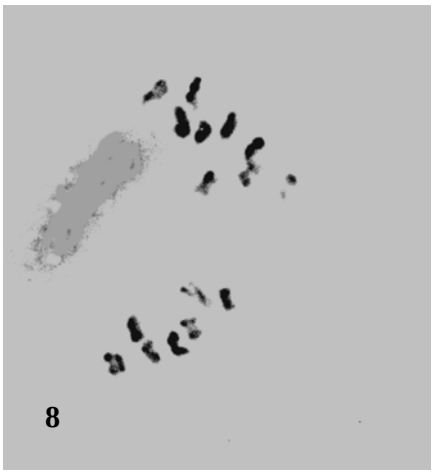

Figure 8. *Crotalaria purdiana.* Metaphase II with seven and nine chromosomes.

The results of chromosome pairing and crossing-over at pachytene indicated that the median point and median region chromosomes have a maximum of two chiasmata and the submedian region chromosomes have only one chiasma in these *Crotalaria* species. The meiotic configurations at diplotene, diakinesis, and metaphase I are shown in Table 1. The observed and expected of circle and chain bivalents (oII, cII), the mean number of chiasmata per cell (Cx), their fits to normal pairing and nonrandom chiasmata distribution, and the Chi-square values, are shown in Table 2. Mean pollen fertility was 95.0% in *C. incana*, 99.5% in *C. retusa*, 84.2% in *C. purdiana*, 96.8% in *C. vittelina*, and 97.5% in *C. anagyroides*.

DISCUSSION

KARYOTYPES

The chromosome number of 2n = 14 for *C. incana*, 2n = 16 for *C. retusa* and *C. anagyroides* agrees with the number previously reported for these species (Magoon *et al*. 1963, Boulter *et al*. 1970, Chennaveeraiah and Patil 1973, Raina and Verma 1979). The chromosome number for *C. purdiana* and *C. vittelina* is 2n = 16.

Table 1. Normal chromosome pairing at diplotene, diakinesis and metaphase I, in *Crotalaria incana*, *C. retusa*, *C. purdiana*, *C. vittelina*, and *C. anagyroides*.

Species	Meiotic Configurations	Meiotic State		
		Diplotene	Diakinensis	Metaphase I
C. incana	5 oII, 2 cII	15	5	5
	4 oII, 3 cII	205	140	40
	3 oII, 4 cII	30	75	135
	2 oII, 5 cII	0	25	0
	1 oII, 6 cII	0	5	0
C. retusa	8 oII	60	35	0
	7 oII, 1 cII	155	130	90
	6 oII, 2 cII	35	85	120
	5 oII, 3 cII	0	0	20
	4 oII, 4 cII	0	0	20
C. purdiana	4 oII, 4 cII	40	20	0
	3 oII, 5 cII	180	175	130
	2 oII, 6 cII	30	50	95
	1 oII, 7 cII	0	0	25
C. vittelina	5 oII, 3 cII	35	45	45
	4 oII, 4 cII	20	140	85
	3 oII, 5 cII	0	65	90
	2 oII, 6 cII	0	0	30
C. anagyroides	5 oII, 3 cII	5	0	5
	4 oII, 4 cII	165	65	60
	3 oII, 5 cII	80	180	175
	2 oII, 6 cII	0	5	10

Crotalaria incana, with a basic n = 7 chromosome number, may have originated from a karyotype similar to *C. purdiana* (2n = 16), by fission in the telomeric heterochromatic region of two submedian chromosomes. If later, a reciprocal translocation occurred, a 2n = 15 karyotype could be produced, with two small chromosome fragments lost during meiosis, because it may be impossible for them to segregate to the poles due to lack of centromeres. Normal, self-fertilized C*rotalaria* species may produce genetically balanced

Table 2. Normal chromosome associations observed (Obs), at diplotene, diakinesis and metaphase I, in *Crotalaria incana*, *C. retusa*, *C. purdiana*, *C. vittelina*, and *C. anagyroides*, tested by nonrandom (NR) model.

Meiotic State	Cx/Cell	oII	cII	I	X^2 Value
C. incana (250 Cells)					
Diplotene	14.94	Obs 197.00	153.00	0	
		NR 196.98	153.02	0	0
Diakinesis	10.46	Obs 173.00	177.00	0	
		NR 172.97	177.03	0	0
Metaphase I	9.92	Obs 146.00	204.00	0	
		NR 146.02	203.98	0	0
C. retusa (250 cells)					
Diplotene	15.10	Obs 355.00	45.00	0	
		NR 354.96	45.04	0	0
Diakinesis	14.80	Obs 340.00	60.00	0	
		NR 340.00	60.00	0	0
Metaphase I	14.12	Obs 306.00	94.00	0	
		NR 306.00	94.00	0	0
C. purdiana (250 cells)					
Diplotene	11.04	Obs 152.00	248.00	0	
		NR 152.00	248.00	0	0
Diakinesis	10.90	Obs 145.00	255.00	0	
		NR 145.04	254.96	0	0
Metaphase I	10.42	Obs 121.00	279.00	0	
		NR 121.04	278.96	0	0
C. vittelina (55 cells)					
Diplotene	12.64	Obs 51.00	37.00	0	
		NR 51.04	36.96	0	0
Diakinesis	11.92	Obs 196.00	204.00	0	
		NR 196.00	204.00	0	0
Metaphase I	11.58	Obs 179.00	221.00	0	
		NR 179.04	220.96	0	0
C. anagyroides (250 cells)					
Diplotene	11.70	Obs 185.00	215.00	0	
		NR 184.94	215.04	0	0
Diakinesis	11.24	Obs 162.00	238.00	0	
		NR 162.00	238.00	0	0
Metaphase I	11.24	Obs 162.00	238.00	0	
		NR 162.00	238.00	0	0

gametes of n = 8 and n = 7, and yield diploid individuals with 2n = 14 and 2n = 16. Karyotype alteration by central fusion or translocation has been observed in *Lycoris* (n = 6) and *Leucojum* (n = 7), and it also may produce an aneuploid reduction of n = 8 (Senn 1938).

High pollen fertility in these species (95.0%–99.5%) may be due to the controlled genetic system, which induces self-fertilization when pollen, produced by the anther, fertilizes the same flower. This kind of permanent self-fertilization allows segregation of lethal o sublethal genes which produce some weak, sterile or low fertility individuals. However, plants of these species must have gene associations, which increase adaptations to a wide distributional area. Thus, *C. retusa* and *C. incana* occupy extreme or marginal habitats, from 0 to 1,500 meters above sea level.

C*rotalaria purdiana* showed normal synapsis at pachytene, and only bivalents were observed at diplotene, diakinesis and metaphase I. Therefore, the dicentric bridge and an acentric fragment may be caused by small paracentric inversion or by a U-type interchange.

The 84.2% pollen fertility in *C. purdiana* may be caused by simple or double crossing-over taking place in the reverse inversion loops involving two and three chromatids between inverted and normal chromosome segments at pachytene, in paracentric inversions (Cequea *et al*. 2003, 2006) or by abnormal disjunction at anaphase I, where both homologous chromosomes were segregated to the same pole. The lagging chromosomes at interkinesis (Fig. 7) may reduce fertility, by producing abnormal gametes. However, 224 cells analyzed at tetrad stage showed 180 cells with 4 microspores and 20 with 5 microspores.

Predicted fertility from first anaphase, interkinesis and metaphase II classifications in *C. purdiana* is 87.5%, and corresponds well with observed values (84.2%). A chi-square test of observed and expected fertile and sterile pollen yields $X^2_{(1)} = 0.128$. We accept the hypothesis that sterility observed in this species is due to the inversion heterozygosity ($P > 50\%$), and in addition, crossover values in PMCs may not correspond exactly and may result in discrepancies between pollen grains and aberration proportions in PMCs (Sall *et al*. 1990).

The seven and nine chromosome groups, observed at metaphase II (Fig. 8), may be due to lack of disjunction of a pair of homologous chromosomes at anaphase I; also by failure of two homologous chromosomes to form

chiasmata,. Thus, the probability that both chromosomes will go to the same pole is equal to $2(1/2)^n$, where n represents the number of unpaired chromosomes. As a result, about 25 percent of the time, n + 1 gametes will be produced (Jackson 1971). In this way, *C. purdiana* may increase or lose a chromosome to form aneuploid organisms.

Analysis of 250 cells in diplotene, diakinesis, and metaphase I, showed absence of univalents (Table 1). Therefore, application of the nonrandom method for meiotic configurations gave Chi-square values of zero (Table 2). This indicates that *Crotalaria* species examined in this study fit the genetic system, that has normal pairing and normal, nonrandom chiasmata distribution. Chiamata terminalization was more evident in *C. incana* than in *C. retusa*, *C. purdiana*, *C. vittelina*, and *C. anagyroides*, judging by the mean chiasmata number per cell. The chiasmata distribution in these bivalents may be related to a genetic system, characteristic in each species, responsible for homologous normal pairing and location and number of chiasmata on specific bivalent sites.

In conclusion, *C. incana*, *C. retusa*, *C. vittelina* and *C. anagyroides* showed median point, median region and submedian region chromosomes. Sterility in *C. purdiana* may be caused primarily by single and three-stranded double crossovers in the inversion loop. These species fit the genetic system where bivalents have normal chromosome pairing at pachytene and a nonrandom chiasmata distribution, with one and two chiasmata in diplotene, diakinesis and metaphase I, and are essentially equal in crossover frequency.

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