



Citation: Fernando Tapia-Pastrana (2023). Cytogenetics of *Diphysa americana* (Mill.) M. Sousa (Leguminosae-Papilionoideae-dalbergioid clade), a rare species from the coast of Oaxaca, Mexico. *Caryologia* 76(2): 51-57. doi: 10.36253/caryologia-2300

Received: September 6, 2023

Accepted: October 27, 2023

Published: December 31, 2023

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Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

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Cytogenetics of *Diphysa americana* (Mill.) M. Sousa (Leguminosae-Papilionoideae-dalbergioid clade), a rare species from the coast of Oaxaca, Mexico

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Abstract. *Diphysa* Jacq. is an essentially Mexican and Central American genus that includes 21 species and only one cytogenetic report. In this work, a surface spread and air-drying method was used to obtain the karyotype of *Diphysa americana* (Mill) M. Sousa, a rare native tree that grows in a coastal town in the State of Oaxaca, Mexico. Metaphase cells showed a $2n = 20$, consistent with the predominant diploid number in the dalbergioid clade. This number contrasts with a previously reported $2n = 16$. The karyotypic formula $5m + 5sm$, first proposed for a species of the genus, denotes a slightly asymmetric karyotype. The presence of secondary constrictions associated with satellites on the short arms of a pair of sm chromosomes and other cytogenetic parameters require studies in other species of the genus to verify their taxonomic utility. In addition, cells in prometaphase exhibited a circular fragment of unknown origin like that observed in a species of the genus *Aeschynomene*, also dalbergioid. This fragment could be related to extrachromosomal circular DNA (eccDNA) observed in other plants. *Diphysa* is a small, cytogenetically favorable genus, and further studies will exhibit the karyotypic diversity that underlies its diversification.

Keywords: basic number, dalbergioid clade, karyotype, SAT-chromosomes.

INTRODUCTION

Leguminosae is the third largest family within angiosperms and exhibits its enormous ecological, genomic, cytological, chemical, and morphological diversity (Doyle and Luckow 2003; Lewis et al. 2005). The Papilionoideae subfamily is the largest and most widespread of the three traditional Leguminosae subfamilies, with an estimated 478 genera and 13,860 species (Lewis et al. 2005; Cardoso et al. 2013). Phylogenetic reconstructions point to it as a monophyletic group with highly specialized papilionate flowers that have a clearly distinctive standard petal, wings, and keel as well as partially fused stamens that wrap around the ovary, although there are unusual lineages with

marked radial floral symmetry (Pennington et al. 2001; Wojciechowski et al. 2004; Lavin et al. 2005; McMahon and Sanderson 2006; Cardoso et al. 2012, 2013; LPWG 2013). By using molecular data Lavin et al. (2001) detected a pantropical monophyletic group of papilionate legumes called “dalbergioid” legumes, which is another example of a typical cryptic clade with an estimated age of 55.3 ± 0.5 million years (Lavin et al., 2005). All dalbergioids belong to one of three well-supported subclades, the Adesmia, Dalbergia, and Pterocarpus clades.

Circumscribed as up to now, the dalbergioids comprise 54 genera and more than 1300 species of perennial and annual trees, shrubs, and herbs (Lavin et al. 2001; Wojciechowski et al. 2004; Cardoso et al. 2012; Moraes et al. 2020). Economically important species are included as hardwood species (*Dalbergia* spp. and *Pterocarpus* spp.), forage legumes (*Stylosanthes* spp. and *Aeschynomene* spp.), but also weeds of rice crops (Martins et al. 2021), grown for consumption human (*Arachis hypogaea*, an allopolyploid) and some endangered taxa (*Centrolobium paraense*) and several *Dalbergia* species (Cervantes et al. 2019).

Diphysa Jacq. (subclade Dalbergia) is an essentially Mexican and Central American genus with extensions to the southwestern United States and northern South America (Lavin et al. 2000; Lewis et al. 2012; Rzedowski et al. 2016) and includes 21 usually unarmed tree or shrub species (WFO 2023). *Diphysa americana* (Mill.) M.Sousa is a 4-15 m tall tree that grows mainly in the seasonally dry tropical biome (Sousa 1990; WFO 2023). It is characterized by a fissured bark and leaves 8-14 cm long, imparipinnate. Its leaflets (5-21) are alternate or sub-opposite, generally 1.5-3.5 cm long and 0.5-1 cm broad, oval, or obovate with entire margin, dark green above, paler below, and glabrous rachis. The inflorescences with 6-7 yellow papilionate flowers (Fig. 1A), a standard with a macula with reddish edges (Fig. 1 B-C) and the turbinate calyx 6-9 mm (Fig. 1 D). Fruits 5.3-8.4 cm long and 1-1.8 cm wide, stipitate, reticulate, glabrous, veined margins and light brown seeds 6 x 3 mm (Fig. 1 E) (Martín et al. 2000; Rzedowski et al. 2016; Rojas-Rodríguez and Torres-Córdoba 2018).

In Mexico, *D. americana* is an uncommon native species of tropical deciduous, seasonally dry, and evergreen forests. Its distribution encompasses both coastlines as well as the center of the country at altitudes of 100 to 1000 m a.s.l. (Rzedowski et al. 2016; Villaseñor 2016). It is one of the first elements to colonize coastal dunes and is commonly found solitary in abandoned sites that were once used for agriculture (Acosta 1993; Ramírez-Pinero 2012). The trees have a high use value in agroforestry systems where the flowers are used as food

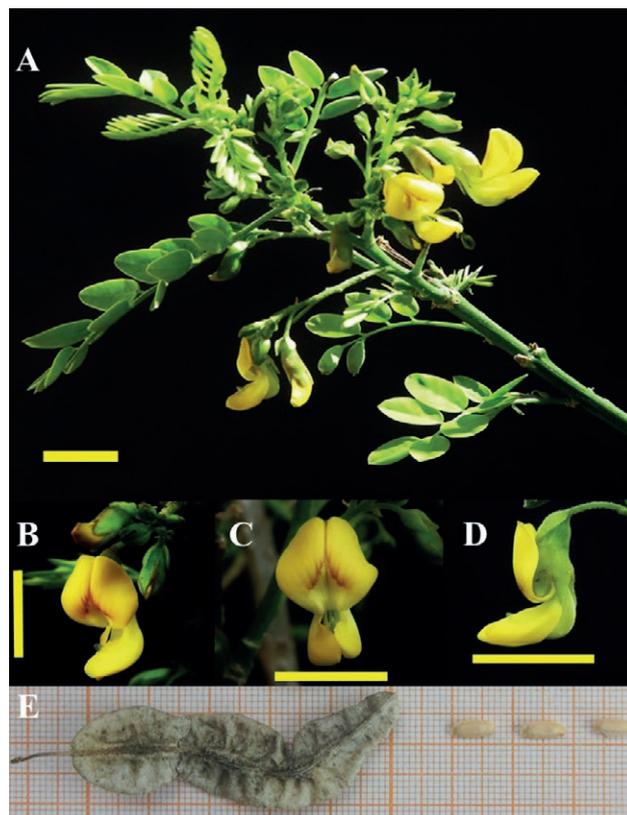


Figure 1. Morphological aspects in *Diphysa americana*. A. Inflorescences. B-D, Three-quarter, frontal, and lateral views of individual flowers. E, Fruit, and seeds. Scale bars = 1 cm.

cooked in salt or fried with egg (Manzanero-Medina et al. 2020), they can also be used as live fences to delimit cultivation areas and provide shade for coffee plantations. In addition, the stem is used as firewood or for the construction of houses (Pascual-Mendoza et al. 2020). It is also used in traditional medicine and is highly prized for the uses of its wood (Rzedowski et al. 2016; WFO 2023). Additionally, its influence on the structure and ecological dynamics of the dune vegetation describes it as a nucleating species (Ramírez-Pinero et al. 2018). It is known by the common names of Amarillo, Chilillo, Chipil, Chipilín, Flor de gallito, Cochipili, Cuachepil Guachipilín, Guachipilín, Macano, Palo amarillo, Quebracho, Quiebracha (CONABIO 2023; Rzedowski et al. 2016). It is not considered a frequent plant, however, the IUCN (2021) places it in the category of least concern.

The genus *Diphysa* records a $2n = 16$ chromosome count for *Diphysa robinoides* Benth., a synonymy of *D. americana* (Atchison 1951; Sousa 1990; WFO 2023). However, this number is far from the basic number $x = 10$ and the diploid number $2n = 20$ that predominate in genera belonging to the dalbergioid clade of Papilionoide-

ae (Goldblatt 1981; Lavin et al. 2001; Tapia-Pastrana et al. 2020) and therefore requires a cytogenetic reevaluation where, in addition to verifying its chromosome number, a detailed description of its karyotype is obtained.

The objective of this work is to carry out a detailed analysis of the chromosomal characteristics of a Mexican population of *D. americana*, establish its karyotype and compare it with previous cytogenetic descriptions carried out in the genus and in other species belonging to the dalbergioid clade of the Papilionoideae subfamily.

MATERIALS AND METHODS

During June 2016, ripe fruits of *Diphysa americana* were collected from two individuals separated by at least two kilometers in the vicinity of the municipality of Santiago Pinotepa Nacional in the coastal region of the state of Oaxaca, Mexico at 16°20'N, 98°03'W and 210 m a.s.l. The area is characterized by a warm sub-humid climate, with an average annual temperature of 26.2 °C and a rainfall of 1,237.5 mm. The vouchers of the studied specimens were deposited in the National Herbarium (MEXU) of the Instituto de Biología, UNAM.

The mitotic cells were obtained from meristems of seeds germinated in Petri dishes lined with cotton moistened in distilled water. Chromosome preparations were made by surface spreading and air-drying (Tapia-Pastrana and Mercado-Ruaro 2001). All meristems were collected from 3-5 mm long roots pretreated with 2 mM 8-hydroxyquinolin for 5 h at room temperature and fixed in the fixative (ethanol: acetic acid=3:1). They were then treated with a mixture of 20% pectinase (Sigma) and 2% cellulase (Sigma) in 75 mM KCl for 60 min at 37 °C. After centrifugation at 1500 rpm for 10 min, the cell pellet was transferred to 75 mM KCl solution for 13 min at 37 °C. After two successive rinses with the KCl solution, they were again fixed in the fixative and subsequently rinsed twice more. One or two drops of the suspension of pellet were placed on clean slides, air-dried, and stained in 10% Giemsa solution for 13 min for conventional karyotyping. At least ten metaphase and prometaphase plates with well-distributed chromosomes were photographed using a Carl Zeiss A1 axioscope. Five photographs of metaphases in which the chromosomes showed comparable degrees of contraction were used to determine: the diploid number ($2n$), the length difference between the longest chromosome and the shortest chromosome (range), total haploid chromosome length (THC), average chromosome size (AC) and the longest chromosome/shortest chromosome ratio (Ratio, L/S). The asymmetry index (TF %) was obtained following

Huziwara (1962). Chromosomes were classified as metacentric (m), submetacentric (sm) according to their morphology and arm proportions (Levan et al. 1964). Chromosome size was estimated using a Mitutoyo Digimatic Caliper CD-G'' BS digital caliper. Karyotypes were prepared from photomicrographs by cutting individual chromosomes, organizing them in descending order of length and matching according to their morphology.

RESULTS

A total of 184 cells in typical metaphase and well-distributed chromosomes were analyzed (Fig. 2 A-D), from which the *Diphysa americana* karyotype was prepared (Fig. 2 E). All of them exhibited a $2n = 20$ and complements constituted by chromosomes m and sm, with a size that oscillated between 1.22 μm and 2.33 μm (Table 1). In some complements, the presence of one or two sm chromosomes carrying secondary constrictions and microsatellites on short arms was clearly visible (Fig. 2B-D). The observation of prometaphase cells revealed, on the one hand, the association between secondary constrictions and a single nucleolus (Fig. 3A) and, on

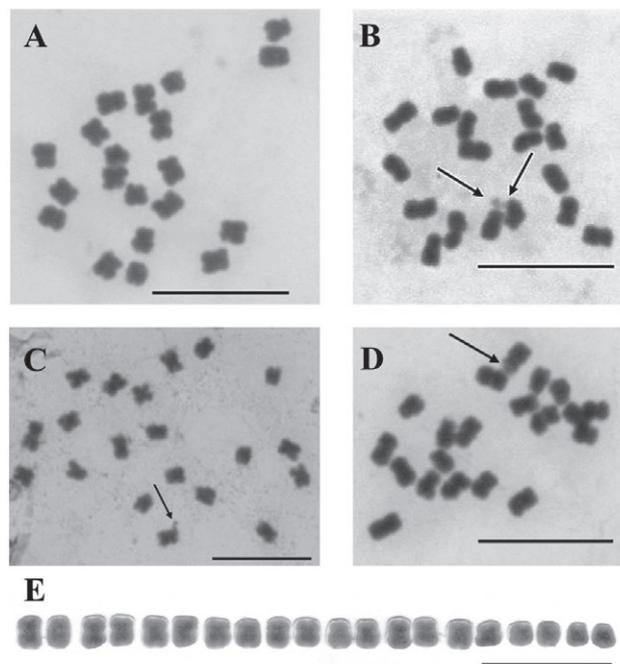


Figure 2. Mitotic cells in metaphase $2n = 20$ and karyotype of *Diphysa americana*. A-D, Metaphase chromosome plates with optimal distribution. Arrows point to secondary constrictions and satellites on short arms of submetacentric chromosomes. E, Karyotype 5m + 5sm. Chromosomes are aligned by the centromere and arranged in decreasing order. Scale bars = 10 μm .

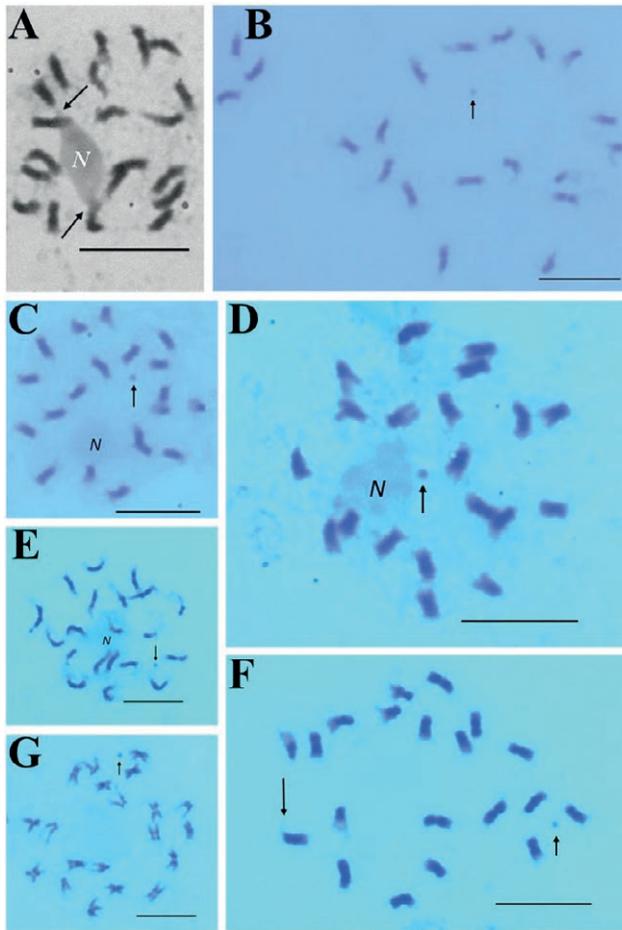


Figure 3. Prometaphase plates $2n = 20$ of *Diphysa americana*. **A.** Arrows indicate satellites of NOR chromosomes associated with the nucleolus. **B-G.** Presence of circular fragments (arrows) of unknown origin not associated with the nucleolus or aligned with the chromosomes. In **F** is shown for comparison, one of these fragments and a secondary constriction indicated by an arrow. **N** = nucleolus. Scale bars = 10 μm .

the other hand, the presence of a small circular-looking fragment that is not associated with the nucleolus or with any chromosome (Fig. 3B-G). This fragment cannot be confused with a secondary constriction or microsatellite, and it stains with the same color and intensity as the rest of the chromosomes (Fig. 3F). The proposed

Table 1. Mean chromosome measures in *D. americana*.

CP	TCL (μm)	LLA (μm)	LSA (μm)	r	S
01	2.61 \pm 0.35	1.43 \pm 0.15	1.17 \pm 0.20	1.22	m
02	2.38 \pm 0.33	1.31 \pm 0.16	1.06 \pm 0.18	1.23	m
03	2.32 \pm 0.33	1.32 \pm 0.18	0.98 \pm 0.16	1.34	m
04	2.23 \pm 0.32	1.47 \pm 0.20	0.74 \pm 0.12	1.98	sm
05	2.19 \pm 0.32	1.23 \pm 0.17	0.95 \pm 0.15	1.29	m
06	2.15 \pm 0.36	1.48 \pm 0.29	0.66 \pm 0.07	2.24	sm*
07	2.08 \pm 0.29	1.17 \pm 0.18	0.90 \pm 0.11	1.30	m
08	2.05 \pm 0.30	1.44 \pm 0.21	0.60 \pm 0.09	2.40	sm
09	1.98 \pm 0.29	1.37 \pm 0.21	0.60 \pm 0.09	2.28	sm
10	1.82 \pm 0.23	1.26 \pm 0.17	0.54 \pm 0.06	2.33	sm

CP=Chromosome pair; TCL=total chromosome length; LLA=length long arm; LSA=length short arm; \pm =SD; r=arms ratio; S=shape after Levan et al. (1964). *Chromosomes with secondary constrictions on short arm.

karyotypic formula for *D. americana*, the position of the SAT chromosomes, and other characteristics of its karyotype are summarized in Tables 1 and 2.

DISCUSSION

Diphysa americana is a species with a wide distribution in the New World and the results of its cytogenetic analysis could be of importance in the understanding of chromosome evolution in the dalbergioid clade of Papilionoideae. It represents the only species of its genus so far studied karyologically. This is the first report of both diploid number and karyotype of *Diphysa americana* obtained in a Mexican population. The $2n = 20$ confirms on the one hand the predominance of $x = 10$ as the basic chromosome number of the dalbergioids and on the other hand *Diphysa* as a cytogenetically favorable taxon. The number of metaphases analyzed (184) and the absence of nuclei with a different set of chromosomes allow us to state with certainty that the chromosome number recorded here for *D. americana* is correct and consistent with what is expected for a genus included in the dalbergioid clade. A $2n = 20$ ($x = 10$) recorded

Table 2. Karyotype analysis of the taxon under study.

Species	NA	$2n$	Karyotype formula	Sat	THC \pm S.E (μm)	AC \pm S.E (μm)	Range \pm S.E (μm)	L/S \pm S.E	TF%
<i>D. americana</i>	184	20	5m + 5sm	2	21.81 \pm 3.14	2.18 \pm 0.21	0.79 \pm 0.12	1.43 \pm 0.02	37.59

NA = Nuclei analyzed; Sat = Number of satellites; THC = Total haploid chromosome length; AC = Average chromosome size; TF% = Asymmetry index.

here differs from the $2n = 16$ ($x=8$) shown by Atchison (1951) for a Central American population. In this regard, it is worth mentioning that Lewke Bandara et al. (2013) highlight the existence of variations in the number of chromosomes and the level of ploidy in some species of the genera *Onobrychis*, *Hedysarum* and *Sulla* (Hedysareae: Papilionioideae) and even information available in the IPCN database (tropicos.org, Missouri Botanical Garden) shows basic numbers far from $x=10$ in *Acosmium*, *Dalea* and *Ormocarpum* (dalbergioid clade: Papilionioideae). Without overlooking the possible existence of cryptospecies with similar morphology and different chromosome numbers, the predominance of $x = 10$ recognized in dalbergioids allows us to assume that numbers as low as $2n = 16$ in a diploid species can hardly be explained by decreasing aneuploidy. Clarifying this disagreement requires broader population sampling.

The karyotypic formula proposed here ($5m + 5sm$) shows that in the dalbergioid chromosome complements obtained so far, chromosomes with a medium (m) or slightly displaced (sm) centromere predominate (Table 1), and therefore they are symmetric or slightly asymmetric karyotypes (Tapia-Pastrana et al. 2020). Likewise, the presence of secondary constrictions associated with microsatellites in short arms of sm chromosomes (SAT chromosomes) corroborates a trend in plant species where secondary constrictions are located preferentially in short arms (Lima de Faria 1976; Lim et al. 2001), particularly in legumes (Biondo et al. 2006; Tapia-Pastrana 2012; Tapia-Pastrana and Tapia-Aguirre 2018; Tapia-Pastrana et al. 2020). Likewise, their role in the organization of the nucleolus is evident since they were observed associated with it in prometaphase cells (Fig. 3A) and considered nucleolar organizing regions (NOR) or at least part of them. In addition, its shape and behavior resemble those exhibited by species belonging to the genus *Aeschynomene*, Serie Americanae (Tapia-Pastrana et al. 2020).

On the other hand, the values of THC, AC, Range, L/S and TF% (Table 2) are parameters obtained for the first time in the genus *Diphysa* and to demonstrate their taxonomic value, a greater number of species must be studied. However, with respect to THC ($21.81 \pm 3.14 \mu\text{m}$) and AC ($2.18 \pm 0.21 \mu\text{m}$), it can be stated that they differ little from the values obtained for species of the recently reestablished genus *Ctenodon* (Tapia-Pastrana et al. 2020; Cardoso et al. 2020) and *Dalbergia spinosa* (Jena et al. 2004) also in the dalbergioid clade. Regarding the presence of small circular fragments, for the moment there is no information on their origin and function. It is noteworthy that similar structures in shape and size were also recorded in *Aeschynomene americana* var. *glandulosa*, another dalbergioid (Tapia-Pastrana et al.

2020). It remains to mention that this material is like extrachromosomal circular DNA (eccDNA) detected by electron microscopy in plants, which mainly contains repeated sequences derived from chromosomal DNA involved in the evolution of B chromosomes and rDNA mobility (Cohen et al. 2008). They also resemble the satellite-like structures recorded in the chromosomes of Giemsa-stained prometaphase cells of *Nicotiana kawakamii* Y. Ohashi (Nakamura et al. 2001). Deciphering this enigma will require molecular cytogenetic techniques and the analysis of a greater number of species.

Circumscribed as so far, the dalbergioid clade is composed of more than 1300 species (Lavin et al. 2001; Wojciechowski et al. 2004; Cardoso et al. 2012; Moraes et al. 2020) and only about 300 ($\approx 28\%$) are known cytogenetically. As shown here, the genus *Diphysa*, which includes few species, is cytogenetically favorable and represents an opportunity to corroborate not only the constancy of the basic chromosome number ($x = 10$) exhibited in dalbergioid taxa, but also to verify the karyotypic diversity that surely underlies their evolutionary and speciation processes.

Characterizing the genome architecture of higher plants is an important scientific task. Its first approach is to visualize chromosomal domains by obtaining detailed karyotypes that reveal the physical organization of DNA in chromosomes. Comparative cytogenetic studies can be taxonomically relevant and complement phylogenies based on molecular data (Tapia-Pastrana et al. 2020; Cordeiro et al. 2020), so this task should continue.

ACKNOWLEDGEMENTS

The author is grateful for the support of the Division of Postgraduate Studies and Research, Faculty of Higher Studies Zaragoza.

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