Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

OPEN ACCESS

**Citation:** F. Tapia-Pastrana, A. Delgado-Salinas (2020) First cytogenetic register of an allopolyploid lineage of the genus *Aeschynomene* (Leguminosae, Papilionoideae) native to Mexico. *Caryologia* 73(4): 17-26. doi: 10.13128/caryologia-949

**Received:** May 24, 2020

**Accepted:** November 10, 2020

**Published:** May 19, 2021



**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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# First cytogenetic register of an allopolyploid lineage of the genus *Aeschynomene* (Leguminosae, Papilionoideae) native to Mexico

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**Abstract.** A conventional cytogenetics analysis revealed for first time an allopolyploid lineage of the genus *Aeschynomene* in Mexico. The hybrid condition is confirmed after all the prometaphase and metaphase nuclei of the hybrids exhibited only one pair of SAT-chromosomes, confirming the existence of nucleolar dominance and amphiplasty. The karyotype formula for this lineage was $2n = 4x = 40 = 34$ m + 6 sm with a total diploid chromosome length (TDCL) = 28μm and an average chromosome size (AC) = 1.40 μm. Comparison of the karyotype and other chromosomal parameters with recent cytogenetics records for other species of the subgenus *Aeschynomene* included in the Nod-independent clade allows propose to *Aeschynomene evenia* and *A. scabra* as possible progenitors. Furthermore, other comparison of seedlings focused at the number of leaflets of the first four eophylls of the proposed parents and of the hybrid individuals allowed to observe coincidences that support the proposal made from the cytogenetic analysis. Evidence of "gigas" effects on flowers and fruits of hybrids is also shown.

**Keywords:** cryptic taxa, cytotype, karyotype, nucleolar dominance, SAT-chromosomes, secondary constrictions, seedlings.

## I. INTRODUCTION

*Aeschynomene* Linnaeus (Leguminosae, Tribe *Dalbergieae* s. l.) is a diverse genus of subfamily Papilionoideae (Papilionoid legumes) distributed in the tropics and subtropics of the world (Lavin *et al.* 2001, Klitgaard and Lavin 2005). It comprises herbaceous and woody species, annual, repetitive and perennial with different ecological requirements. Several species contribute to supplement nitrogen to the soil through the production of nodular roots and stems in symbiosis with nitrogen fixing bacteria, so they are economically important as green manure (Alazar and Becker 1987; Fernandes 1996; Souza *et al.* 2012) and recently, *Aeschynomene evenia* C. Wright has been proposed as a model species in genetics to develop new agronomic

strategies in the engineering of nitrogen fixing nodules that enhance rice production (Arrighi *et al*. 2012, 2013). This taxon belongs to the group of 11 semi-aquatic species of *Aeschynomene* that have the property of being nodulated by photosynthetic *Bradyrhizobium* that lack the nodABC genes necessary for the synthesis of Nod factors and are grouped into the so-called Nod-independent clade (Chaintreuil *et al*. 2013; Brottier *et al*. 2018) and that correspond to the morphological series Indicae and Sensitivae (Rudd 1955).

The genus *Aeschynomene* traditionally included in the Aeschynomeneae tribe (Polhill *et al*.1981) and currently circumscribed in the Dalbergioid clade (Lavin *et al*. 2001; Wojciechowski *et al*. 2004) has evolved in different ecological niches and includes herbaceous forms, annual and perennial shrubs and trees up to 8 meters, with compound pinnate leaves and papilionoid flowers that are generally self-pollinated, although there is cross-pollination by bees (Rudd 1955; Fernandes 1996; Arrighi *et al*. 2014, Carleial *et al*. 2015). Other studies indicate that the genus *Aeschynomene* is not monophyletic and taxa with basifixed stipules and a campanulate calyx (subgenus *Ochopodium* Vogel) are more related to the genera *Machaerium* Persoon and *Dalbergia* Linnaeus f. than to taxa with medfixed stipules and a bilabiate calyx (subgenus *Aeschynomene* Léonard) (Ribeiro *et al*. 2007; Cardoso *et al*. 2012).

Currently *Aeschynomene* genus contains 170 (http://www.theplantlist.org) to 180 species (Klitgaard and Lavin 2005) 231taxa and cytotypes at four ploidy levels: diploid (2*x*), tetraploid (4*x*), hexaploid (6*x*) and octoploid (8*x*) (Index to Plant Chromosome Numbers; Kawakami 1930; Bielig 1997; Arrighi *et al*. 2012, 2014; Chaintreuil *et al*. 2016, 2018; Brottier *et al*. 2018). America, where most of the taxa are $2n = 20$ diploids, has been proposed as the center of origin of the genus, with a secondary distribution in Africa and Asia where polyploid species and some cases of aneuploidy predominate (Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020).

Although it is clear that in the Dalbergioid clade, diploid $2n = 20$ genera predominate, with some polyploid and aneuploid species, in *Aeschynomene* there is currently a renewed interest in knowing to what extent polyploidy has contributed to the diversification and radiation of the group. In this respect Arrighi *et al*. (2014) revealed multiple hybridization/polyploidization events, highlighting the prominent role of allopolyploidy in the diversification of Nod-independent clade. In addition Chaintreuil *et al*. (2016) studied African *Aeschynomene* species and their data support the idea that the whole African group is fundamentally tetraploid and revealed the allopolyploid origin of *A. afraspera* J. Léonard ($2n = 8x = 76$) and *A. schimperi* Hochst. ex A.Rich. ($2n = 8x = 56$), where variations in the number of chromosomes also indicated possible dysploidy/aneuploidy events. In Mexico, *Aeschynomene* is represented by 31 species and infraspecific taxa including several endemisms. An investigation about the patterns of chromosomal evolution in Mexican species, including six taxa of the Nod-independent clade, showed the predominant of a basic $2n = 20$ diploid structure and evolutionary patterns related to the corresponding morphological series (Tapia-Pastrana *et al*. 2020).

In the present research, a conventional cytogenetic study was carried out to obtain the karyotype and analyze the level of ploidy in a Mexican population initially described as *Aeschynomene scabra* G. Don, where the size of the flowers, fruits and seeds generated suspicions about a possible hybrid origin. In addition as the sampled individuals exhibited floral morphotypes similar to those of *A. evenia* C. Wright and *A. scabra*, whose collection records in Mexico would support their participation in the hybridization process, the growth pattern of the first four eophylls was also compared in putative hybrids and their parental assumptions.

## 2. MATERIAL AND METHODS

### *2.1 Collection sites*

Seeds of the putative hybrids were collected in the Municipio de la Huerta, Estado de Jalisco, Mexico, 19°29′N; 105°01′W (Carleial s/n, MEXU). The climate is semi-dry and warm. Mean temperature in the area is 25.2 °C, and there is a well-defined rainy season (average annual precipitation: 1107 mm) occurring from June to October (García-Oliva *et al*. 2002).

The seeds of *Aeschynomene evenia* and *A. scabra* were collected in the municipalities of Coyuca de Catalán (18° 19′ N; 100° 42′ W, JC Soto 15333 (MEXU)) and Arcelia (18°18′54′′N; 100°17′02′′W, JC Soto 15393 (MEXU)) respectively, in the State of Guerrero, Mexico. Both municipalities are part of the Tierra Caliente region. The predominant climate is warm subhumid with rains from June to September (average annual precipitation: 1100 to 1200 mm). The studied taxa are assigned to the infrageneric classification of Neotropical *Aeschynomene* sensu Rudd (1955) series Indicae of subgenus *Aeschynomene* and are part of the Nod-independent monophyletic clade (Chaintreuil *et al*. 2013), whose taxa are nodulated on roots and stems by photosynthetic *Bradyrhizobium* strains lacking the nod ABC genes necessary for the synthesis of Nod factors (Giraud *et al*. 2007).

*2.2 Chromosome and karyotype procedures in putative hybrids*

Seeds were collected in summer 2014 and from at least six plants. Batches of 40 seeds from each plant were used. The seeds were scarified and germinated in Petri dishes lined with a moist filter paper at room temperature and under natural light. Chromosomes at metaphase and prophase were obtained following the splash method (Tapia-Pastrana and Mercado-Ruaro 2001). All meristems were collected from 2-4 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 for 13 min. Preparations were made permanent using a synthetic resin.

At least ten metaphase plates of intact cells with well-spread chromosomes, no chromosome overlapping, and same contraction and ten prophase plates were photographed from each collection, using a microscope (Axioscope, Carl Zeiss) and analyzed for chromosome number determinations. Five photographs of metaphases with chromosomes having similar comparable degrees of contraction and centromeres clearly located were utilized to obtain the Total diploid chromosome length (TDCL), Total chromosome length (TCL), Average chromosome length (AC), the difference in length between the longest chromosome and the shortest chromosome (Range) and the longest/shortest chromosome ratio (L/S). The shapes of chromosomes were classified according to Levan *et al.* (1964) and the TF was obtained following Huziwara (1962). Furthermore, prometaphase cells were analyzed to verify both the number of nucleoli, and the behavior of the SAT chromosomes. The information thus obtained was compared with that recently recorded for *Aeschynomene evenia* and *A. scabra* in another cytogenetic study where the same method was used for karyotype analysis in *Aeschynomene* species and varieties (Tapia-Pastrana *et al.* 2020).

*2.3 Seedlings and Eophylls*

In order to compare seedling morphology in individuals of the supposedly hybrid population with those of *Aeschynomene evenia* and *A. scabra*, the development of 20 individuals grown in pots under greenhouse conditions was evaluated. Interest was particularly focused on the number of leaflets and the presence of hairs on their edges until the complete development of the fourth leaf. Eophylls at the first, second, third and fourth eophyllar nodes were referred to as E1, E2, E3 and E4, respectively following Schütz *et al.* (2019). Photographs of seedlings were taken with a Canon SX700 HS camera.

## 3. RESULTS

*3.1 Karyotype analysis*

A total of 410 cells were analyzed in metaphase and 16 in prometaphase and all exhibited a $2n = 4x = 40$ (Fig. 1 A-C). TDCL was 28 µm and AC 1.40 µm. The chromosomal range was 0.56 µm, the ratio 1.48 and a TF = 42.46. The karyotype formula was $2n = 4x =$ 34m + 6sm (Table 1). Consistently, in all prometaphase and metaphase nuclei, only one pair of submetacentric chromosomes was observed having lax secondary constrictions and macrosatellites in short arms (SAT-chromosomes) (Fig. 1 A-C). The karyotype exhibits small chromosomes (1.72-1.16 µm) clearly discernible, with predominance of metacentric chromosomes (m) and lacking subtelocentric chromosomes (st). This arrangement is consistent with a TF that describes a slightly asymmetric karyotype (Fig. 1D and Table 1). Occasionally the SAT-chromosomes were observed immersed in a single nucleolus.

*3.2 Seedlings and Eophylls*

The seedlings of the three taxa are illustrated in Fig. 2 A-C. Eophylls are stipulated, alternate, petiolate, pinnate, with alternate leaflets, have elliptic to oblong leaflets, a rounded apex, an entire margins, and one central primary vein in the three taxa under study. The leaflets did not present trichomes; both adaxial and abaxial surfaces are glabrous. The number of leaflets in the first four eophylls in seedlings of individuals of *Aeschynomene evenia*, *A. scabra* and putative hybrids are shown in Tables 2-4 respectively.

## 4. DISCUSSION

It is clear that the entire Dalbergioid clade (Adesmia, Dalbergia and Pterocarpus subclades) is dominated by $2n = 2x = 20$ species, with scattered polyploids and aneuploids (Lavin *et al.* 2001). In addition an ances-

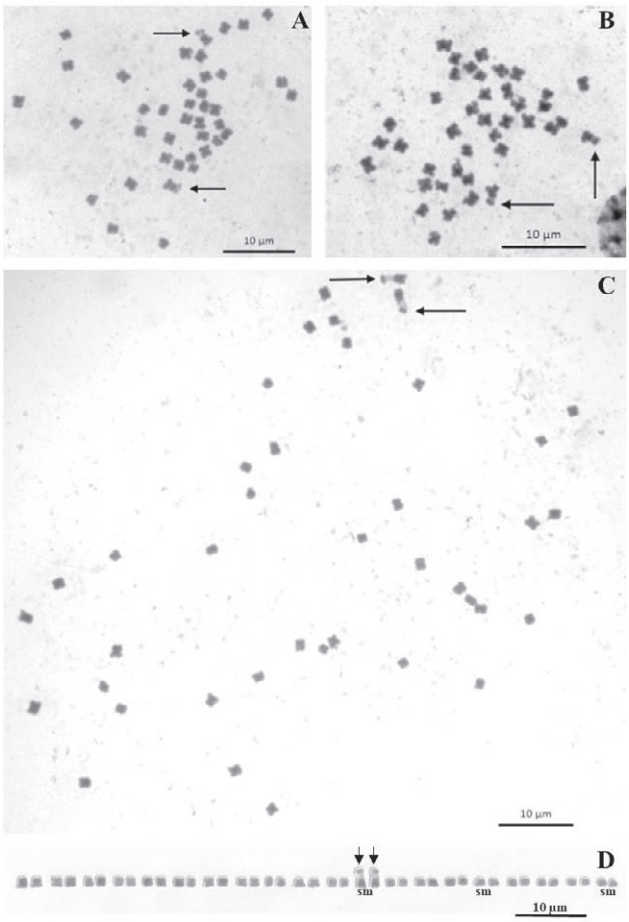


**Figure 1.** Mitotic metaphase cells of hybrid *Aeschynomene* 2*n* = 4*x* = 40. **A-C**, Metaphase chromosome plates in optimal spread; **D,** Karyotype 34m + 6sm. The chromosomes are aligned in decreasing order. Arrows point to secondary constrictions and satellites on short arms of submetacentric chromosomes.

**Table 1.** Average chromosome measurements obtained from five nuclei in metaphase of the hybrid population (2*n* = 4*x* = 40 = 34m + 6sm) under study.

| CP | TCL (µm) | LLA ( µm) | LSA (µm) | r | CT |
|---|---|---|---|---|---|
| 01 | 1.72 | 0.96 | 0.77 | 1.24 | m |
| 02 | 1.63 | 0.89 | 0.73 | 1.21 | m |
| 03 | 1.59 | 0.89 | 0.69 | 1.28 | m |
| 04 | 1.55 | 0.81 | 0.72 | 1.12 | m |
| 05 | 1.53 | 0.81 | 0.70 | 1.15 | m |
| 06 | 1.50 | 0.82 | 0.66 | 1.24 | m |
| 07 | 1.48 | 0.83 | 0.63 | 1.31 | m |
| 08 | 1.46 | 0.79 | 0.66 | 1.19 | m |
| 09 | 1.44 | 0.79 | 0.63 | 1.25 | m |
| 10 | 1.42 | 0.79 | 0.61 | 1.29 | m |
| 11 | 1.38 | 0.74 | 0.64 | 1.17 | m |
| 12 | 1.36 | 0.89 | 0.46 | 1.93 | sm* |
| 13 | 1.34 | 0.78 | 0.55 | 1.41 | m |
| 14 | 1.31 | 0.70 | 0.59 | 1.18 | m |
| 15 | 1.28 | 0.72 | 0.55 | 1.30 | m |
| 16 | 1.24 | 0.83 | 0.40 | 2.07 | sm |
| 17 | 1.23 | 0.69 | 0.52 | 1.32 | m |
| 18 | 1.19 | 0.67 | 0.54 | 1.24 | m |
| 19 | 1.19 | 0.66 | 0.49 | 1.34 | m |
| 20 | 1.16 | 0.78 | 0.36 | 2.16 | sm |
| TDCL | 28.00 | | | | |
| AC | 1.40 | | | | |

Abbreviations: CP- chromosome pair; TCL- total chromosome length; LLA- length long arm; LSA- length short arm; r- arm ratio; CT-chromosome type; TDCL- Total diploid chromosome length; AC- Average chromosome length; m- metacentric; sm- submetacentric; *- satellite. Abbreviations: CP- chromosome pair; TCL- total chromosome length; LLA- length long arm; LSA- length short arm; r- arm ratio; CT-chromosome type; TDCL- Total diploid chromosome length; AC- Average chromosome length; m- metacentric; sm- submetacentric; *- satellite.

tral state reconstruction performed in a phylogeny based on *ITS* + *matK* of the *Aeschynomene* genus and related genera indicated that diploidy is the ancestral condition in the entire group reviewed (Brottier *et al*. 2018). However, the role of allopolyploid speciation events in the origin of new taxa is now recognized (Arrighi *et al*. 2014).

As far as we know, the first assumption about of hybridization in *Aeschynomene* is attributed to Rudd (1955) who pointed out that the species with the widest distribution within the Indicae series (Nod-independent clade) tend to be more variable and intergrade with their neighbors. Later, Verdcourt (1971) suggested that specimens of *Aeschynomene rudis* Bentham (also into Nod-independent clade) with large flowers could be of polyploid origin, without pointing out the possible duplication mechanism involved, auto or allopolyploidy. To date, several studies have shown that the clade of *A. evenia* is mainly diploid (2*n* = 2*x* = 20), however some species such as *A. indica* Linnaeus (2*n* = 4*x* = 40, 2*n* = 6*x* = 60) seem to be of recent allopolyploid origin (Arrighi *et al*. 2014; Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020). Furthermore, it has been found that all species of the group *A. afraspera* are polyploid (2*n* = 4*x* = 28, 38, 40; 2*n* = 8*x* = 56, 76) and have a common AB genomic structure (Chaintreuil *et al*. 2016). In facts phylogenetic relationships between diploids and polyploids elucidated from *ITS* sequences show that in the Nod-independent clade, species such as *A. evenia*, *A. scabra* and *A. rudis* participate in the hybridization/polyploidization events and formation of polyploid complexes that have contributed to the radiation of this group (Arrighi *et al*. 2014).


**Figure 2.** Seedling morphology of *Aeschynomene* under study until the complete development of the fourth eophyll **A**, *Aeschynomene evenia*; **B**, *A. scabra*; **C,** hybrid of *Aeschynomene*.

**Table 2.** Number of leaflets up to the fourth eophyll in *Aeschynomene evenia*.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E1 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 9 | 10 | 9 | 8 | 10 | 9 | 10 | 10 | 8 |
| E2 | 12 | 12 | 12 | 12 | 11 | 12 | 14 | 12 | 10 | 14 | 12 | 12 | 14 | 12 | 10 | 13 | 12 | 11 | 10 | 11 |
| E3 | 15 | 16 | 15 | 14 | 14 | 16 | 17 | 15 | 14 | 16 | 16 | 15 | 17 | 12 | 12 | 16 | 16 | 12 | 14 | 12 |
| E4 | 16 | 18 | 16 | 16 | 16 | 16 | 18 | 18 | 14 | 16 | 18 | 16 | 18 | 16 | 15 | 16 | 16 | 14 | 16 | 15 |

**Table 3.** Number of leaflets up to fourth eophyll in *Aeschynomene scabra*.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E1 | 10 | 8 | 10 | 10 | 10 | 10 | 10 | 8 | 10 | 9 | 10 | 10 | 10 | 10 | 10 | 8 | 8 | 8 | 8 | 10 |
| E2 | 12 | 15 | 14 | 14 | 14 | 14 | 14 | 12 | 16 | 13 | 14 | 13 | 14 | 14 | 14 | 12 | 12 | 13 | 15 | 14 |
| E3 | 20 | 20 | 22 | 18 | 18 | 18 | 19 | 19 | 20 | 17 | 17 | 18 | 18 | 16 | 18 | 19 | 19 | 19 | 20 | 16 |
| E4 | 24 | 25 | 27 | 23 | 22 | 22 | 22 | 22 | 24 | 22 | 22 | 22 | 22 | 21 | 22 | 22 | 22 | 20 | 25 | 21 |

**Table 4.** Number of leaflets up to fourth eophyll in hybrids.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E1 | 10 | 10 | 8 | 10 | 10 | 9 | 10 | 8 | 8 | 10 | 8 | 10 | 8 | 10 | 10 | 8 | 10 | 10 | 8 | 10 |
| E2 | 14 | 14 | 12 | 14 | 13 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 16 | 14 | 14 | 14 | 14 | 14 | 10 |
| E3 | 18 | 20 | 18 | 20 | 20 | 19 | 20 | 20 | 20 | 19 | 18 | 20 | 18 | 20 | 20 | 20 | 20 | 15 | 18 | 14 |
| E4 | 21 | 25 | 22 | 23 | 23 | 26 | 23 | 24 | 23 | 24 | 23 | 26 | 22 | 27 | 22 | 22 | 23 | 22 | 23 | 14 |

In the present investigation, the chromosomal number obtained in all the nuclei analyzed from the individuals under study was $2n = 4x = 40$, which undoubtedly shows that they are polyploid cells and that the individuals from which they come integrate a polyploid lineage not previously detected in Mexico (Rudd 1955; Tapia-Pastrana *et al*. 2020). The origin of the polyploidy (auto or allopolyploidy) were established easily from the number of SAT chromosomes unambiguously identified both in nuclei in prometaphase and metaphase and by their position in relation to the nucleolus.

Indeed, polyploidy, the process of genome doubling that gives rise to organism with multiple sets of chromosomes, is recognized as an important process in plant evolution, a major mechanism of adaptation and is often invoked as a driver of diversification (Ramsey and

Schemske 1998; Soltis *et al*. 2009) and it is likely to be one of the most predominant mechanisms of sympatric speciation in plants (Otto and Whitton 2000). It can act alone, resulting in autopolyploidy, or in concert with hybridization, producing allopolyploids, and both modes lead to plant speciation. It should be mentioned that in the process of polyploidization by total gene duplication (autopolyploidy) the number of satellites present in a diploid species is also doubled, since this does not involve loss or suppression of the nucleolar function, the NOR regions associated with secondary constrictions in SAT-chromosomes are they show lax and therefore satellites are clearly appreciated. It is known that NORs contain tandemly arranged highly reiterated ribosomal rRNA genes coding for 18S-5.8S-26S rRNA whose expression is under epigenetic control (Pikaard 2000). For example, *Medicago sativa* Linnaeus, a recognized autotetraploid exhibits four macrosatélites in metaphase cells (Falistocco 1987). In contrast, plants of allopolyploid origin as cotton (*Gossypium hirsutum* Linnaeus $2n = 4x = 52$ AADD, Endrizzi *et al*. 1985), wheat (*Triticum aestivum* Linnaeus $2n = 6x = 42$, AABBDD, Lacadena and Cermeño 1985; Friebre *et al*. 1995) and canola (*Brassica napus* Linnaeus $2n = 4x = 38$ AACC; Xiong and Pires 2011) undergo inactivation of the regions of the nucleolar organizer (NOR) of one of the parental genomes, silenced by the effect of nucleolar dominance (Navashin 1934) and consequently a smaller number of satellites is recorded (Doyle *et al*. 2008; Ge *et al*. 2013). It is, rDNA loci may be additive in number, but then exhibit differences in gene expression. Interspecific hybrids often have rRNA genes of one parent functionally dominant over the rRNA of the other parent, and there are many examples of such regulation of rRNA gene activity in allopolyploids (Pikaard 2000; Pires *et al*. 2004). Comparative analyses of nucleolar organizer regions (NORs) of somatic metaphase chromosomes made by phase contrast, C-banding and silver staining have demonstrated that the activity of the NORs of certain chromosomes can be suppressed or partially inhibited by the presence of other SAT-chromosomes.

The NOR competition is cytologically expressed as amphiplasty: a term proposed to denote morphological changes which occur in chromosomes following interspecific hybridization (Rieger *et al*. 1976). The secondary constriction of the SAT-chromosome of one of the parental species is missing in the hybrid and the satellite is retracted onto the chromosome arm as a consequence (Lacadena and Cermeño 1985). Thus, in the *Hordeum murinum* Linnaeus complex (Poaceae, Triticeae), tetraploid and hexaploid cytotypes arising from hybridization exhibit only a pair of chromosomes with secondary and satellite constrictions (Cuadrado *et al*. 2013). In fact, the inactivation or epigenetic silencing of ribosomal genes is one of the most common phenomena in hybrid and polyploid members of Triticeae Linnaeus (Cermeño and Lacadena 1985; Carmona *et al*. 2016) and one of the first examples of differential gene expression discovered in plant hybrids nearly a century ago (Navashin 1934; Matyášěk *et al*. 2007). In the present work, the repressive effects on NORs from allopolyploid population are cytologically expressed (amphiplasty) as the suppression of a secondary constriction clearly observed in all their complements (Fig. 1 A-D).

The karyotype exhibited in hybrid individuals (34m + 6sm) (Fig. 1D and Table 1) coincides in several respects with that expected at a cross between *A. evenia* ($2n = 2x = 7$m + 3sm) and *A. scabra* ($2n = 2x = 10$ m) (Fig. 2 in Tapia-Pastrana *et al*. 2020). For example, the number of sm chromosomes in *A. evenia* agrees with the 6sm in hybrid individuals. In addition to submetacentric chromosomes, these individuals exhibit metacentric chromosomes whose predominance is consistent with the karyotype formulas described in their putative relatives, whose complements lack subtelocentric chromosomes (Tapia-Pastrana *et al*. 2020). There is a coincidence between THC and AC and even the morphology of the SAT-chromosomes (submetacentrics with macrosatellites in short arms) and their position in the karyotype is very similar to that recently described in *A. scabra* (Tapia-Pastrana *et al*. 2020). Therefore we propose to *A. evenia* and *A. scabra* as progenitors of the allopolyploid population ($2n = 4x = 40 = 34$m + 6sm) registered in this work. The reasoning is simple: if a diploid species is involved in the origin of a tetraploid cytotype, its chromosomes must be present in it. The same is true if tetraploid forms are involved in the origin of hexaploid forms (Cuadrado *et al*. 2013). In Mexico, recent collection data shows that populations of both species occupy overlapping ranges in some central areas of the country where *A. evenia* is considered an introduced species (Arrighi *et al*. 2013; Chaintreuil *et al*. 2018; Tapia-Pastrana *et al*. 2020).

This new proposal is not surprising, since previously the Indicae series species grouped within Nod-independent clade, including *A. evenia* and *A. scabra*, have been identified as progenitors in allopolyploids and in the formation of polyploid complexes, although attempts at hybridization have failed to form fertile individuals (Arrighi *et al*. 2014). Regarding the identity of the allopolyploid taxon recorded here, it can be argued that a detailed review of its complete morphological characters (data not shown) suggests that it shares characteristics described for *Aeschynomene rudis* particularly in the shape and size of flowers, fruits (hispidulous, verru-

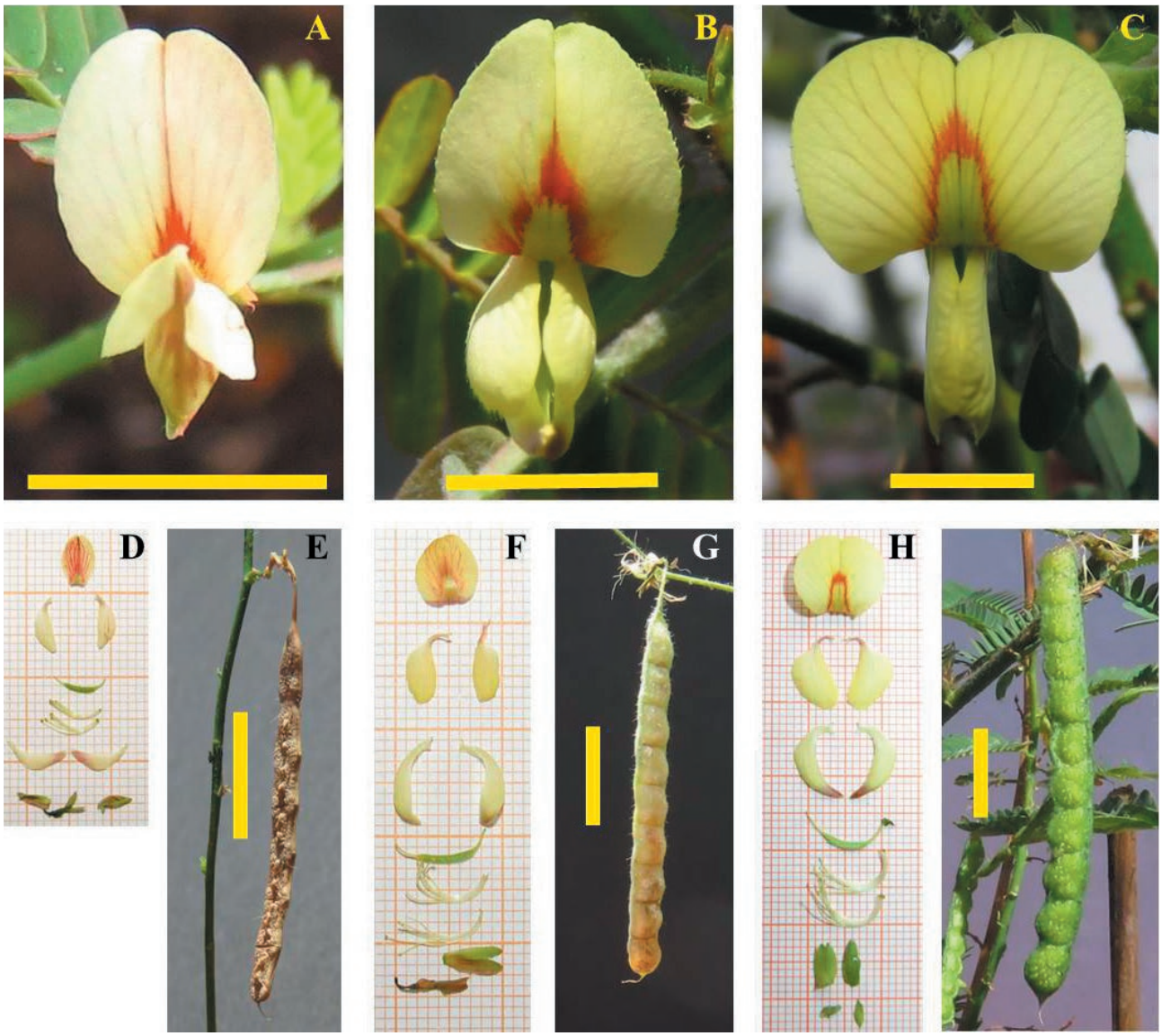


**Figure 3.** Floral morphotypes (above), dissected flowers and fruits (below) of the taxa under study. **A**, **D** and **E**, *Aeschynomene evenia*; **B**, **F** and **G**, *A. scabra*; **C**, **H** and **I**, hybrid of *Aeschynomene*. All three taxa exhibit typical pea or papilionoid flowers. These zygomorphic flowers comprise a standard (vexillum or banner) petal (adaxially placed), two lateral petals (wings) and two (usually partially fused and abaxially placed) keel petals, which conceal the androecium and gynoecium. The fruits have similar characteristics and are mainly differentiated by their size. Above scale bar = 0.5 cm, below = 1.0 cm.

coses, or muricate at the center) and seeds (Rudd 1955). However, it also recalls the robust version of *A. scabra* described by Rudd (1955). The existence of cryptic taxa in *Aeschynomene* as well as the need for broader sampling to detect new cytotypes has already been pointed out (Brottier *et al.* 2018, Chaintreuil *et al.* 2018) and the results of this study confirm this.

Regarding the results obtained from the seedling comparison, these seem to support a close relationship between the individuals of the three populations studied (Fig. 2, Tables 2-4). In principle, the observed intervals in the number of leaflets per eophyll (E1-E4) show some uniformity, particularly E1, whose interval (8-10 leaflets) was repeated in the three populations. In intermediate eophylls (E2-E4) a close concordance is observed between *A. scabra* and the hybrid population, while in *A. evenia* the number of leaflets was lower in correspondence with the taxonomic description of this species (Rudd 1955). Furthermore, the morphology of the eophylls was similar and in all populations the leaflets

exhibited entire margins, without trichomes and with a central primary vein.

Polyploids are known to often have novel phenotypes that are not present in their diploid progenitors or that exceed the range of parent species ("gigas" effects) (Ramsey and Schemske 2002; Ramsey and Ramsey 2014). In this sense, Fig. 3 shows floral morphotypes, dissected flowers and fruits of the populations studied here, where similarities are observed, but the differences in size of such characters are highlighted. The results obtained in this study confirm that in the Nod-independent lineage within the genus *Aeschynomene*, hybridization and polyploidization play a relevant role in the formation of species and those taxa such as the polymorphics *A. evenia* and *A. scabra* actively participate in it.

## ACKNOWLEDGEMENTS

This study is part of the doctoral thesis of the first author, F T-P, carried out at the Posgrado en Ciencias Biológicas of the Universidad Nacional Autónoma de México (UNAM). The authors thank to Dr. Samuel Carleial for seeds originally identified as *A. scabra* and to the Division of Postgraduate Studies and Research of the Faculty of Higher Studies, Zaragoza, UNAM for the support provided during the development of this research.

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