



Chromosomal analysis and distinctive morphological characteristics of *Aeschynomene denticulata* (Leguminosae: Papilionoideae: Dalbergieae), a predominantly hydrophytic species from Brazil

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Summary. A conventional chromosome analysis was performed on root meristems of the papilionoid legume *Aeschynomene denticulata* Rudd to obtain the karyotype, number of SAT chromosomes, their possible relationship with the NOR regions and the description of other karyological parameters. Furthermore, the distinctive morphological characteristics of this species are illustrated and described from healthy plants grown in a greenhouse. The diploid chromosome number $2n = 20$ recorded in *A. denticulata* confirms the basic number $x = 10$ common in the Dalbergioid clade. Likewise, the karyotypic formula obtained and the position and behaviour of the SAT chromosomes corroborate that *Aeschynomene* species have unique karyotypes and that the secondary constrictions carry the NOR regions or part of them. The morphological analysis indicated that the shape and size of the stipules, the denticulate-ciliate margins of vegetative and floral structures as well as the shape and indumentum of the fruits are reliable taxonomic characters in the identification of *A. denticulata*, which accompanied by a detailed chromosomal analysis will avoid confusing it with its phylogenetic sister species *A. ciliata*. Furthermore, the amplitude of the minimum generation time recorded here is explained in terms of nucleotypic theory.

Key Words. Dalbergia clade, karyotype, karyotypic formula, NOR regions, SAT chromosomes.

Introduction

Aeschynomene L. is a genus of flowering plants in the family Leguminosae, subfamily Papilionoideae, and recently assigned to the informal Dalbergia clade of Dalbergieae. It includes around 130 species of pantropical distribution commonly called jointvetch (Lavin *et al.* 2001; Wojciechowski *et al.* 2004; Cardoso *et al.* 2012, 2020; WFO 2024). Several species contribute to supplementing soil nitrogen through the production of nodular roots and stems in symbiosis with nitrogen-fixing bacteria, which is why they are economically important as green manure (Alazard & Becker 1987; Fernandes 1996; de Souza *et al.* 2012) and recently *Aeschynomene evenia* C.Wright (series Indicae) was proposed as a model plant to study the molecular genetics of the Nod-independent rhizobia-legume symbiosis (Arrighi *et al.* 2012, 2013), which has increased interest in genomics, genetics and cytogenetics of cogeneric species (Arrighi *et al.* 2014; Chaintreuil *et al.* 2016; Tapia-Pastrana *et al.* 2020; Tapia-Pastrana & Delgado-Salinas 2020).

There is agreement on the basic number of chromosomes $x = 10$ in the Dalbergieae clade (Lavin *et al.* 2001) and the cytogenetic studies carried out in *Aeschynomene* s.l. confirm this (Bir & Kumari 1977; Coleman & Demenezes 1980; Bairiganjan & Patnaik

1989), as well as $2n = 20$ for most species of the genus (Kawakami 1930; Fernández 1977; Renard *et al.* 1983; Vanni 1983; Kumari & Bir 1990; Seijo & Vanni 1999; Tapia-Pastrana *et al.* 2020). The karyomorphological information in *Aeschynomene* s.str. is scarce and to date karyotypic formulas have been described in thirteen species, infraspecific taxa and putative varieties, a considerably low number in a genus that according to its current circumscription comprises at least a hundred taxa, which limits access to information of taxonomic and evolutionary interest (Tapia-Pastrana *et al.* 2020; Cardoso *et al.* 2020). On the other hand, the Americas, where most taxa are diploid, has been proposed as the centre of origin of the genus, while Africa and Asia, with polyploid and aneuploid records, are considered secondary distribution centres (Chaintreuil *et al.* 2018; Tapia-Pastrana *et al.* 2020).

The morphologically-defined series *Indicae* and *Sensitivae* of *Aeschynomene* s. str. (Rudd 1955) include mainly hydrophytic species growing in swamps, rice fields, flooded meadows, along streams and riverbanks, exhibiting various levels of ploidy ($2x$, $4x$, $6x$) because of hybridisation. For example, in *A. indica* and *A. virginica* (*Indicae*) the existence of a polyploid complex $2n = 20$, 40 and 60 in the first and $2n = 40$ in the second has been recorded (Arrighi *et al.* 2014). It is also suspected that the

North American populations of *A. rudis* (*Indicae*) could be of hybrid origin (Tapia-Pastrana & Delgado-Salinas 2020). Following this line of thought, it has been proposed that *A. pratensis* (*Sensitivae*) is a $2n = 40$ allotetraploid (Arrighi *et al.* 2014). On the other hand, although there are cytogenetic records indicating that *A. denticulata* Rudd (*Indicae*) is a diploid species $2n = 20$ (Fernández 1977; Seijo & Vanni 1999); and it is suspected that it could be the third diploid progenitor involved in the formation of *A. indica* 6x (Arrighi *et al.* 2014), there are no descriptions of its karyotype or other karyological parameters useful in comparative cytogenetics.

Aeschynomene denticulata includes erect, branching subshrubs up to 1 m high. Stem and branches covered with glandular trichomes, yellow, tuberculated at the base. These are perennial and autogamous plants whose leaflets, bracts, bracteoles, and peltate stipules have denticulate or denticulate-ciliate margins and imparipinnate leaves between 3.5 and 8.2 cm long. Inflorescences in axillary racemes of up to 5 flowers with a pubescent rachis. The calyx is bibracteolate, c. 7 mm long, bilabiate, the upper lip bilobed, lower lip trilobed, and papilionaceous yellow, yellow-orange, or orange flowers measuring 9–12 mm; the banner petal 1.0×0.7 cm, circular glabrous, margin serrate-ciliate and macula orange traces; wings 0.8×0.3 cm, obovate, glabrous, with ciliated margin, sculpted on supra-basal-medial distal portion. Keel, the same dimensions, falciform, fused in the apex, glabrous and entire margin. Stamens are monadelphous, heterodyne, and glabrous and the gynophore is 8–11 mm long. Pods, lomenta $3.8\text{--}6.6 \times 0.3\text{--}0.6$ cm, narrow-oblong, straight or elliptical, hispidulous, light brown; articles $3\text{--}5 \times 3\text{--}4$ mm, quadrangular, or subquadrangular, setose, and bulging in the seminal cavity; seeds 4–13, dark brown kidney-shaped (Rudd 1955; Lima *et al.* 2006; de Souza *et al.* 2012; Vanni 2016; Chaintreuil *et al.* 2018; Sinani *et al.* 2019). Thrives in Bolivia, Brazil, Paraguay, and Argentina (Rudd 1955; Vanni 2016).

Molecular phylogenies reconstructed with the *trnL* intron of chloroplast DNA and the ITS/5.8S region of nuclear ribosomal DNA (Chaintreuil *et al.* 2013) and with concatenated ITS + *matK* sequences (Brottier *et al.* 2018) showed that *Aeschynomene denticulata* and *A. ciliata* Vogel are sister species. The close morphological similarity between both species and a certain resemblance to other species of the ser. *Indicae*, can give rise to important taxonomic errors since they tend to be misidentified in the field and/or in herbarium collections, which is why it has been suggested that *A. denticulata*, among other species of the same series, requires a taxonomic revision (Lima *et al.* 2006).

The general objective of this research is to provide a detailed and illustrated description of the chromosomal and morphological features of *Aeschynomene denticulata*, necessary to ensure its correct delimitation with respect to other closely related species.

Specifically, this work is aimed at (1) corroborating the diploid chromosome number of *Aeschynomene denticulata*; (2) describing its karyotype and other cytogenetic parameters; (3) determining the number of secondary constrictions and their possible participation as NOR regions; (4) characterising SAT chromosomes; and (5) verifying morphological characteristics in plants grown under greenhouse conditions.

Materials and Methods

Morphological characterisation

The seeds used to obtain root meristems come from a collection that accompanies an herbarium specimen of Brazilian origin labelled *Aeschynomene indica*, State of Mato Grosso do Sul, which is deposited in the National Herbarium, Department of Botany, Institute of Biology, UNAM (MEXU, 375752). However, reviews in the specialised literature and the morphological analysis of the plants grown from these seeds showed that they are actually *A. denticulata*. The morphological characteristics evaluated in the plants cultivated under greenhouse conditions were habit, height and indumentum of the plants, size of leaves, number and shape of leaflets, size and shape of the stipules, size, shape and colour of the flowers, presence of cilia on the margins of the petals, size and indumentum of the lomenta and dimensions of the seeds. All of these were used and recorded photographically for the easy and quick identification of *A. denticulata*. The vouchers of the studied specimens were deposited in the National Herbarium (MEXU) of the Institute of Biology, UNAM (IBUNAM), where there are 12 more specimens of this species, all native and cultivated in South America.

Chromosome and karyotype procedures

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 & Mercado-Ruaro 2001) and stained with a 10% Giemsa solution for conventional karyotyping. At least ten typical metaphase and ten prometaphase plates were photographed using a Carl Zeiss A1 Axioscope and analysed for chromosome number and behaviour of satellite-carrying chromosomes (SAT chromosomes). Five photographs of metaphases with chromosomes having comparable degrees of contraction were used to determine diploid number ($2n$), the difference in length between the longest chromosome and the shortest chromosome (range), the total haploid chromosome length (THC), average chromosome size (AC) and the proportion of the longest chromosome L/shortest chromosome S (Ratio L/S). The asymmetry index (TF %) was obtained following Huziwara (1962). The chromosomes were classified as

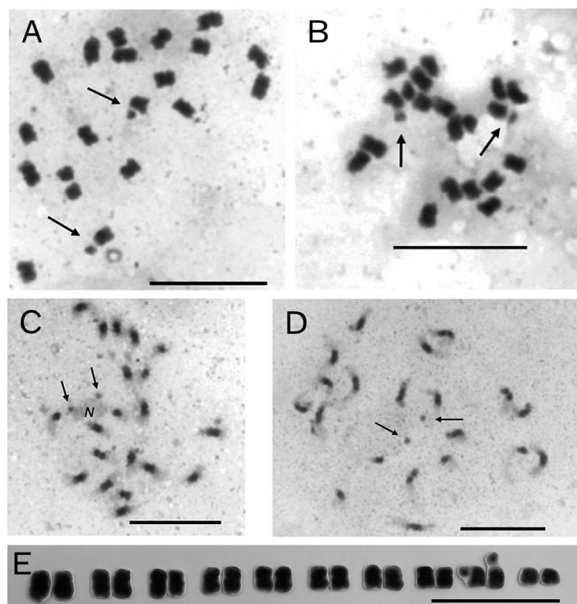


Fig. 1. *Aeschynomene denticulata*. **A – B** typical metaphase plates, complements show only m chromosomes and a pair of SAT chromosomes (arrows); **C – D** nuclei in prometaphase: **C** satellites (arrows) attached to remnants of the nucleolus (N) and **D** apparently isolated from it; **E** karyotype. The chromosomes are aligned by the centromere and in decreasing order.

metacentric (m) and submetacentric (sm) according to their morphology and arm ratios (Levan *et al.* 1964). Chromosome size was estimated using a Mitutoyo Digi-matic Caliper CD-G[®] BS digital caliper. The karyotype was prepared from photomicrographs by cutting out individual chromosomes, arranging them in descending order of length, and matching based on morphology.

Results

A total of 84 nuclei were analysed, 74 in typical metaphase and ten in prometaphase, and in all of them a diploid chromosome number $2n = 20$ was confirmed (Fig. 1A – D). In metaphase the predominance of metacentric chromosomes was evident as well as the presence of a single pair of SAT chromosomes with secondary constriction and macrosatellites in short arms (Fig. 1A, B). In prometaphase, satellites were observed attached to the nucleolus or free in the nuclear space (Fig. 1C, D). SAT chromosomes occupied the penultimate place in the descending order of the karyotype (Fig. 1E, Table 1). The symmetrical nature of the complements and other cytogenetic parameters are summarised in Table 2.

The minimum generation time, that is, the length of the period from germination to the first production of mature seed, observed in *Aeschynomene denticulata* was 18 weeks. Fig. 2A – G illustrates the morphology of plants

Table 1. Mean chromosome measures in *Aeschynomene denticulata*.

CP	TCL (μm)	LLA (μm)	LSA (μm)	R	S
01	1.99 ± 0.09	1.04 ± 0.04	0.93 ± 0.06	1.11	m
02	1.91 ± 0.10	1.00 ± 0.05	0.90 ± 0.05	1.11	m
03	1.84 ± 0.10	0.98 ± 0.03	0.86 ± 0.07	1.13	m
04	1.76 ± 0.08	0.92 ± 0.04	0.83 ± 0.04	1.10	m
05	1.68 ± 0.07	0.90 ± 0.07	0.77 ± 0.04	1.16	m
06	1.61 ± 0.07	0.89 ± 0.05	0.71 ± 0.04	1.25	m
07	1.53 ± 0.12	0.82 ± 0.06	0.69 ± 0.08	1.18	m
08	1.46 ± 0.11	0.79 ± 0.06	0.65 ± 0.05	1.21	m
09	1.42 ± 0.12	0.77 ± 0.06	0.64 ± 0.08	1.20	m*
10	1.26 ± 0.09	0.67 ± 0.05	0.58 ± 0.04	1.15	m

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

with healthy growth that exhibited the characteristics of *A. denticulata* described in the taxonomic keys, in particular the size, shape and denticulate-ciliate margins of the stipules and leaflets, as well as the straight shape and the hirsute/whitish indumentum of the fruits, characters that delimit this species and differentiate it from others closely related such as *A. ciliata* and *A. indica* (Table 3).

Discussion

Chromosomes have played an important role in plant systematics, because their changes in number and structure have always been linked to plant evolution and speciation (Rieseberg 2001; Raskina *et al.* 2008; Faria & Navarro 2010; Vimala *et al.* 2021). Furthermore, from a phylogenetic point of view, detailed cytological data are an important aid in deciphering differences and affinities, particularly in closely related taxa (Astuti *et al.* 2017; Vimala *et al.* 2021). In *Aeschynomene* s. str., the usefulness of this type of study was revealed when Tapia-Pastrana *et al.* (2020) superimposed karyotypes of species and varieties on a phylogenetic tree based on molecular data and observed a correspondence between the morphology, phylogeny, and cytogenetic characteristics of the included taxa. Furthermore, they noted that, although polyploidy has played an important role in the evolution of this genus, its speciation is also accompanied by chromosomal remodelling and changes in the number and position of secondary constrictions and associated satellites.

The results obtained here corroborate a diploid chromosome number $2n = 20$ recorded for *Aeschynomene denticulata* and confirm the intrageneric karyotypic diversity shown so far in the genus *Aeschynomene*, particularly in the series *Indicae* and *Sensitivae*. The karyotypic formula (10 m), the asymmetry index (TF % ≈ 46) and

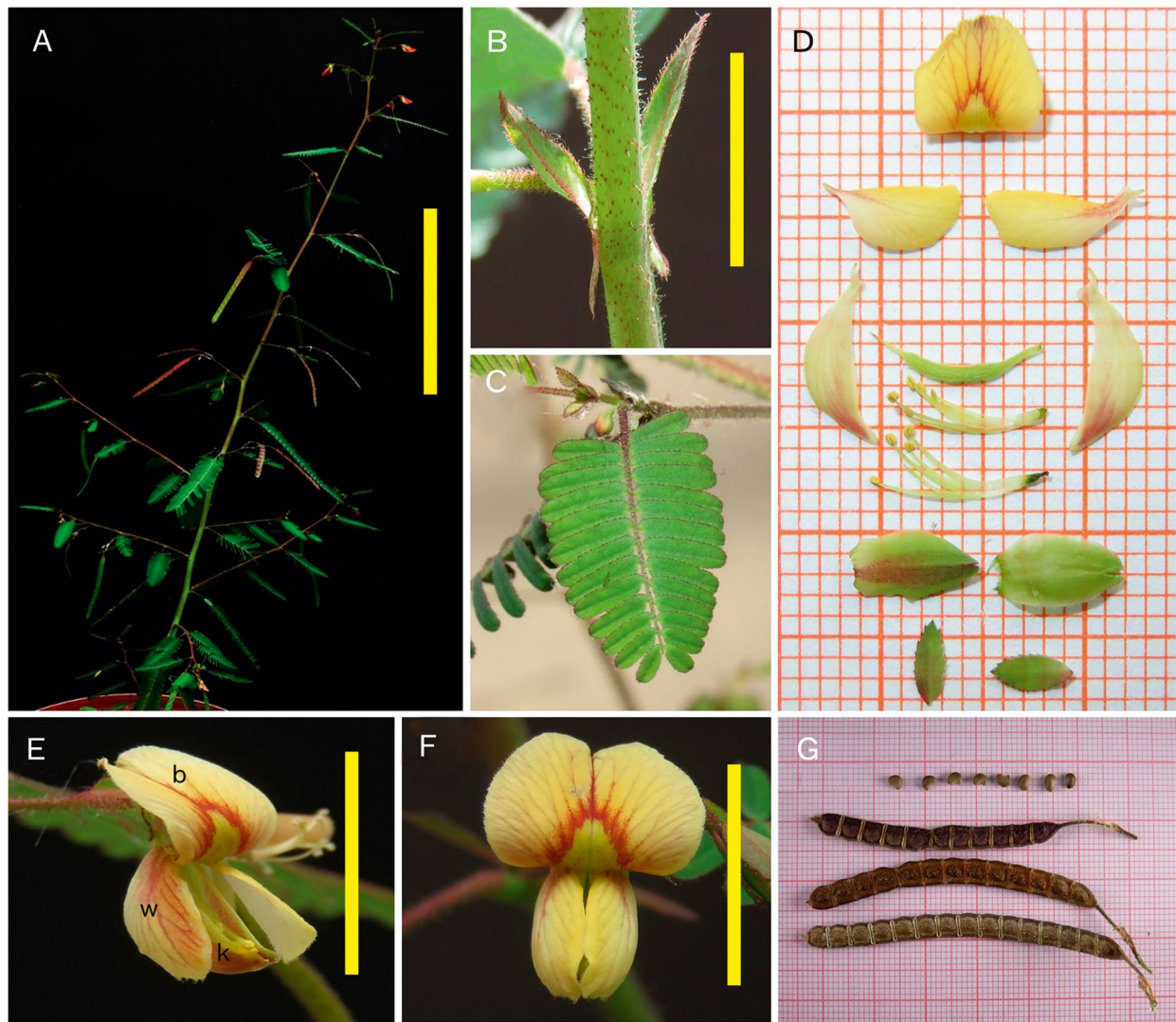


Fig. 2. *Aeschynomene denticulata*. **A** mature plant three months old in flowering and fruiting, erect and branched habit; **B** stipules, apical portion acuminate, slightly more than twice as long as basal auricle, acute to obtuse, margins narrowly denticulate-ciliate, stem and branches with abundant glandular hairs; **C** leaf with conspicuously denticulate leaflets; **D** dissected flower showing banner, wings and keel, gynoecium, androecium, calyx, and bracteoles; **E** flower with conspicuous orange traces, standard and wings with serrate-ciliate margins, entire keel; **F** flower, front view; **G** loment with quadrangular or subquadrangular articles and kidney shaped seeds. Abbreviations: **b** banner; **w** wings; **k** keel. Scale bars **A** 20 cm, **B**, **E**, **F** 1 cm.

other cytogenetic parameters recorded here for this species are very similar to those described for *A. scabra*, a species also belonging to ser. *Indicae* (Tapia-Pastrana *et al.* 2020). However, *A. denticulata* exhibits $THC = 16.47$

$\pm 0.95 \mu\text{m}$ and $AC = 1.64 \pm 0.09 \mu\text{m}$ (Table 2), which represent the highest values described so far for species of the ser. *Indicae* (Tapia-Pastrana *et al.* 2020). Furthermore, the position occupied by the SAT chromosomes

Table 2. Karyotype analysis of *Aeschynomene denticulata*.

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>A. denticulata</i>	84	20	10 m	2	16.47 ± 0.95	1.64 ± 0.09	0.73 ± 0.06	1.58 ± 0.08	45.92

NA = nuclei analysed; Sat = number of satellites; THC = total haploid chromosome length; AC = Average chromosome size; TF% = Asymmetry index.

Table 3. Comparison of *Aeschynomene denticulata* with closely allied species. Data compiled from Rudd (1955), Fernandes (1996), Lima *et al.* (2006), Antunes & Silva (2017), and WFO (2023), as well as author pers. obs.

Characters	<i>A. denticulata</i>	<i>A. indica</i>	<i>A. ciliata</i>
Leaflets	denticulate/ciliate	entire	serrulate/ciliate to subentire
Branches	abundant glandular hairs	glabrous	abundant glandular hairs
Stem	abundant glandular hairs	glabrous	abundant glandular hairs
Stipules	peltate, 10–15 × 2–3 mm, lanceolate, base rounded, apex acuminate, margin dentate-ciliate	peltate 10–15 × 2–3 mm, margin entire	peltate, 12–18 × 3–6 mm, ovate, rounded base, acute apex, ciliated margin
Flower colour	yellow-orangish	yellow-whitish	yellow-orangish
Petal margins	banner and wings serrate/ciliate; entire keel	entire, or sparsely ciliate	banner and wings conspicuously ciliate; entire keel
Loment	margins subentire, hispidulous, velvety, slightly verrucose or muricate at the centre	upper margin almost straight, the lower margin sinuous, glabrous, or scarcely hispid, sometimes muricate	margins entire or nearly so, hispidulous, rough
Development	perennial	annual	perennial

in the karyotype points out a notable characteristic that *A. denticulata* does not share, so far, with any other species from this same series (Tapia-Pastrana *et al.* 2020). Again, the number, size, and position of secondary constrictions and macrosatellites reveal their value as chromosomal markers of taxonomic utility, particularly in those taxa that are difficult to delimit (Palomino & Vázquez 1991; Solís Neffa & Fernández 2002; Tapia-Pastrana & Tapia-Aguirre 2018; Tapia-Pastrana *et al.* 2020). Besides, the close relationship between satellites and nucleoli observed in prometaphase cells allows us to propose that in *A. denticulata*, SAT chromosomes carry the NOR regions or part of them (Hasterok *et al.* 2001; Taketa *et al.* 2001; Kulak *et al.* 2002; Marasek *et al.* 2004; Hwang *et al.* 2011; Roa & Guerra 2012; Tapia-Pastrana *et al.* 2020).

On the other hand, although phylogenetic studies place *Aeschynomene denticulata* and *A. ciliata* as sister species (Chaintreuil *et al.* 2013; Brottier *et al.* 2018), their karyotypic formulas differ due to the presence in the latter of three pairs of submetacentric chromosomes (7m + 3sm), of which the two smallest pairs carry secondary constrictions and macrosatellites (Tapia-Pastrana *et al.* 2020). Furthermore, they present discrepancies in their chromosome sizes, since in *A. denticulata* a $\text{THC} = 16.47 \pm 0.95 \mu\text{m}$ and $\text{AC} = 1.64 \pm 0.09 \mu\text{m}$ were recorded (Table 3) while *A. ciliata* exhibited a $\text{THC} = 15.71 \mu\text{m}$ and $\text{AC} = 1.56 \mu\text{m}$ (Tapia-Pastrana *et al.* 2020) estimates that correspond well with their DNA contents (Arrighi *et al.* 2013). These differences do not interfere with the close morphological affinity exhibited between both species, which seems greater than that indicated with respect to other species of the same series (Rudd 1955; Vanni 2016; Sinani *et al.* 2019). In this regard, Table 3 describes the morphological and

developmental characteristics useful to differentiate them and includes *A. indica*, the type species of the ser. *Indicae*. Of particular importance were the shape and size of the stipules (Lima *et al.* 2006: fig. 1e), the margins of the banner and wings (Sinani *et al.* 2019: fig. 2k), and the shape and structure of the fruits (Rudd 1955; Lima *et al.* 2006).

The minimum generation time of about 18 weeks exhibited by *Aeschynomene denticulata* under greenhouse conditions was longer than the 12 weeks recorded by Arrighi *et al.* (2012) for *A. evenia*, also grown in a greenhouse. A possible explanation can be found in the different DNA contents estimated in both species. In fact, in *A. denticulata* a 2C DNA content of $1.29 \pm 0.03 \text{ pg}$ has been recorded, while in *A. evenia* it was $0.95 \pm 0.03 \text{ pg}$ (Arrighi *et al.* 2013). Under the nucleotypic theory (Bennett 1971), the minimum generation time is related to the nuclear DNA content so the time intervals will expand as the latter increases. Thus, the observed differences would be determined by the massiveness of the DNA (not by the information content) which does not modify the development pattern, but its extent and the rate (Bennett 1972). These observations support the hypothesis that nuclear DNA content and minimum generation time are correlated in herbaceous plants.

In *Aeschynomene*, a wide karyotypic diversity has been described even at the infraspecific level (Tapia-Pastrana *et al.* 2020). This diversity includes differences in chromosome size and in the number, position and size of constrictions and satellites (SAT chromosomes). In this sense, the karyotype described for *A. denticulata* confirms that the species of the genus have unique karyotypes and that the use of conventional cytogenetic techniques allows us to obtain valuable information on chromosomal changes involved in the evolution and speciation of plants.

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Declarations

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest The author declares that they have no conflict of interest.

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