

Karyotype analysis of some plants of Boraginaceae

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Abstract: The family Boraginaceae Juss. (borage or forget-me-not) is characterized by alternately arranged linear or lanceolate leaves, bisexual flowers and coiled shaped inflorescence. The flower has usually five-lobed calyx; bell-shaped to tubular with five lobes, five stamens and one style with one or two stigmas. The family includes a number of garden ornamentals, such as *Heliotropium* (heliotrope), *Martensia virginia* (bluebell), *Phacelia* (Scorpionweed), *Pulmonaria* (Lungwort), and *Myosotis* (forget-me-not) etc.

In the present investigation systematic relationships among three closely related species of Boraginaceae, viz., *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum* on the basis of resemblances and differences compared to the modern findings of biosystematic relationship has been established on the basis of their karyotypes. The results revealed that all specimens of *Heliotropium indicum* collected from fifteen different localities of Gaya showed a complement with $2n = 4x = 44$. The specimens of *Trichodesma indicum* collected from fifteen different localities of Gaya showed a complement with $2n = 4x = 6x = 66$. Similarly, all specimens of *Trichodesma zeylanicum* collected from fifteen different localities of Gaya showed a chromosome complement with $2n = 4x = 48$.

The karyological observations on fifteen varieties of each of the three species of Boraginaceae viz., *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum*, collected from different localities of Gaya were carried out with special reference to chromosome number, chromosome morphology, and chromosome behavior at meiosis to give a contribution to the cytotaxonomy of the family. It is clear that the tetraploids are quite distinct from the known diploids in their habitat preferences and general physiological vigor. The diploids are fairly strong competitors and generally prefer a relatively mesic habitat while the tetraploids and hexaploids ($6x=66$ in some plants of *T. indicum*) are rather poor competitors and are generally found growing in arid habitats, often under great water stress, occurring from April to July in Gaya. This does not indicate, of course, whether the tetraploids arose as autopolyploid or allopolyploid types, as both mechanisms have been demonstrated to result in these kinds of changes.

The present study is the first to investigate phylogenetic relationships of the Boraginaceae using cytological data of fifteen different morphovars of three species viz. *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum*.

Key Words: Karyotypes, Biosystematics, Chromosome formula, Phylogeny, Boraginaceae

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I. Introduction

Boraginaceae Juss. (borage or forget-me-not), the family of dicotyledonous angiosperm includes about 148 genera and more than 2700 species. Plants of this family are frequently herbaceous and hairy and can be annuals or perennials. Some are vines or trees, and a few are obligate parasites. Plants have alternately arranged leaves, or a combination of alternate and opposite leaves. The leaf blades usually have a narrow shape; many are linear or lance-shaped. They are smooth-edged or toothed, and some have petioles. Most species have bisexual flowers, but some taxa are dioecious. Pollination mostly occurs by hymenopterans and bees. Most species have coiled shaped inflorescence. The flower has usually five-lobed calyx. The corolla varies in shape from rotate to bell-shaped to tubular, but it generally has five lobes. It can be green, white, yellow, orange, pink, purple, or blue. There are five stamens and one style with one or two stigmas. The fruit is a drupe. The family includes a number of garden ornamentals, such as *Heliotropium* (*heliotrope*), *Martensia virginia* (bluebell), *Phacelia* (Scorpionweed), *Pulmonaria* (Lungwort), and *Myosotis* (forget-me-not) etc.

Biosystematists have become deeply involved in cytological studies, particularly of the chromosomes. These studies provide one good example of clear cut species differences at the cellular level. Chromosome number is studied in preparations of dividing cells, usually from root tips or from pollen mother cells.

Chromosome numbers are not always constant with a species, although they are usually reliable. Sometimes, chromosome morphology is valuable in biosystematic studies. Most commonly, chromosome length are examined, and relative distances between position of the centromeres and ends of chromosomes are tabulated (Oyewole and Mustapha, 1990) [1]. The number and type of chromosome aberrations present at meiosis may also be important. Again cytological studies have provided insights into evolutionary mechanism (Mustapha, 1987) [2].

Family *Boraginaceae* Juss. has one of its main centres of diversity in the Mediterranean basin and central-western Asia (Hilger *et al.*, 2005; 2004) [3, 4]. The value of chromosome characters in the systematics of the family is known since the early studies of Strey (1931) [5], Smith (1932) [6] and Britton (1951) [7], which brought to the light a considerable variation in terms of ploidy level, chromosome number, size and morphology. Later cytotaxonomic investigations have contributed significantly to the systematics and phylogeny of taxonomically difficult groups, such as *Myosotis* (Grau 1965) [8], *Omphalodes* (Grau 1967) [9], *Onosma* (Teppner 1971) [10], and *Pulmonaria* (Sauer 1975) [11]. This has been further demonstrated by later studies on other genera (Luque 1983, 1984, 1989, 1990, 1992, 1995; Selvi and Bigazzi 2002; Bigazzi and Selvi 2001, 2003) [12, 13, 14, 15, 16, 17, 18, 19, 20]. In spite of this, the family is karyologically still poorly known. It has been estimated that only about 35% of the species of *Boraginaceae* are known for their chromosome number (Coppi *et al.*, 2006; Al-Shehbaz 1991) [21, 22]. Coppi, *et al.*, (2006) [21] have described the results of karyological observations on 32 taxa in the Mediterranean and Near-East, to give a contribution to the cytotaxonomy of the family *Boraginaceae*.

Mekki *et al.*, (1987) [23] have studied the Giemsa C-banded karyotype of diploid *Symphytum officinale* (*Boraginaceae*) and observed twelve pairs of chromosomes of the diploid cytotype of *Symphytum officinale* L. ($2n = 24$) which can be distinguished individually with the use of their Feulgen-Giemsa banding pattern in combination with the relative length of the chromosomes and the position of the centromeres. The karyotypes of plants from France, Hungary and the Netherlands do not differ significantly, except for the number of satellites. Cytotaxonomic studies in the genus *Symphytum* of *Boraginaceae* have largely been reviewed by Gadella and E. Kliphus (1967; 1971) [24, 25].

The aim of this study is to evaluate the systematic relationships among three closely related species of *Boraginaceae*, viz., *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum* on the basis of resemblances and differences compared to the modern findings of biosystematic relationship.

II. Materials and Methods

Twenty five plants of each of the three species of *Boraginaceae*, viz., *Heliotropium indicum*, *Trichodesma indicum* and *T. zeylanicum*, which differed from each other slightly in their morphological features were collected from fifteen localities of District Gaya (Bihar). The localities from where the plants of each of the three species collected were as follows:

Delha (DLH) MU campus, Bodh Gaya (BG), Bramhyoni Pahari (BP), Gaya College Campus, Rampur, Gaya (GC), MahaBodhi College Campus, Bela Ganj, Gaya (MB), Manpur (MP), Fatehpur (FP), Mohanpur (MOP), Paraiya (PRA), Tekari (TK), Chekand (CK), Bara (BR), Tankappa (TKP), Panchanpur (PNP), Ramshila (RMS).

The cytological investigations were carried out on a population of twenty five plants of each of the three species of *Boraginaceae* collected from fifteen different localities of Gaya District for assessing the genetic variations among population of the same species. The meiotic behaviour was made from anther squash preparation. Similarly the mitotic behavior was made from the root tips of freshly grown plants of each of the three species. Karyotype of all the three species was studied following Huziwara's method (1962) [26]. All slides for cytological investigation were made permanent following Celarier's method (1956) [27].

For study of meiosis the pollen-mother cells (PMC's) from young floral buds of each of the three species were collected in the field and immediately placed in a killing and fixing solution. The cytological experiments were carried out in replicates of ten for all the three species. For this purpose four standard solutions were used (Sass, 1958) [28]: Nawaschin formula I, Farmers, Carnoys, and that of Newcomer (1953) [29]. The results obtained from all four fixatives were essentially the same, so after the initial study Farmers fluid was used almost exclusively. After a minimum of 24 hours the floral buds were rinsed and transferred to a 70% ethyl alcohol solution. For staining the floral buds were placed in the alcoholic hydrochloric acid-carminic stain of Snow (1963) [30] for 24 hours at 60⁰ C. After rinsing in 70% ethyl alcohol the anthers were then removed from the buds and placed in a drop of 45% acetic acid on a glass slide. As originally proposed by Beeks (1955) [31] permanent preparations were produced by adding a drop of Hoyer's gum Arabic-chloral hydrate mixture just prior to application of the cover slip. The cover slip was then tapped gently to separate the cells and pressure applied as outlined by Benson (1962, p. 430) [32]. Snow's stain, besides providing excellent staining of chromosomes without excessive darkening of the cytoplasm, has an added advantage, that of

hydrolysis of the pectic compounds of the middle lamella and spore wall. This provides for easy separation of all cells of the anther and maximum flattening.

Mitotic divisions for chromosome counts were generally obtained from the root-tips of germinating seeds, although some were obtained from potted plants or newly dug transplant material, in preparation for seed germination, nut lets were soaked for 24 hours in water. The carpel material and integument layer was then cut away from the cotyledon end of the nut let and the embryo removed and placed on moistened filter paper in a Petri dish. The Petri plates were then placed in a controlled environment chamber programmed for a 12 hour day-night regime with 7°C night and 24°C day temperatures. This relatively low temperature program allowed for maximum germination of embryos with minimal disease or damage.

Regardless of their source, root tips of each of the three species of Boraginaceae viz., *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum* were placed in a saturated aqueous para-dichlorobenzene solution for 3 hours at 12°C (Sharma and Sharma, 1965) [33]. The tips were quickly washed and placed in Farmers fluid for killing and fixing. At this point three different methods were employed in preparing and staining the root tips. The first was that of Warmke (1935) [34], but the results were less than satisfactory. The second procedure used was that described previously for the handling of PMC material. This was quite satisfactory but did not provide intensely stained chromosomes. The third procedure was a modification of the schedule established by Lowry (1963) [35]. The fixed root-tips were hydrolyzed for 10-15 minutes at 60°C in a mordant containing: 2% aqueous iodic acid, 2% aluminum alum, and 2% chrome alum in 1 N hydrochloric acid. The root-tips were then washed three times in distilled water to remove excess mordant and hydrochloric acid and placed in vials containing the aceto-iron-hematoxylin stain. The stain contains 4% hematoxylin and 1% iron alum in 45% acetic acid and was prepared according to the method prescribed by Wittman (1962) [36]. The root-tips were removed after two hours, washed briefly in distilled water, and mounted according to Beek's method previously described. This method resulted in intensely stained chromatin material with very little cytoplasmic staining. The results obtained have been presented in Table- 1, 2 and 3; Photoplate-1-25.

Table 1: Showing Somatic chromosome numbers (2n), base numbers (X), ploidy levels, karyotype formulas and mean chromosome length (L) of the fifteen natural variants of *Heliotropium indicum* collected from different localities of Gaya.

Localities	2n	X	Ploidy level	Formula	Length in μm
DLH	22	11	2X	12m + 2mSAT + 8sm	3.4
BG	22	11	2X	16m + 2sm + 4st	1.5
BP	22	11	2X	8m + 10sm + 4st	1.6
GC	22	11	2X	8m + 12sm + 2st	2.5
MB	22	11	2X	10m + 6sm + 4st + 2st ^{SAT}	2.5
MP	44	11	4X	16m + 16sm + 12st	2.2
FP	44	11	4X	16m + 12sm + 16st	1.6
MOP	44	11	4X	16m + 18sm + 10st	1.7
PRA	22	11	2X	12m + 4m ^{SAT} + 6sm	3.4
TK	22	11	2X	8m + 10sm + 4st	1.4
CK	22	11	2X	8m + 12sm + 2st	2.5
BR	44	11	4X	16m + 16sm + 14st	2.0
TKP	22	11	2X	8m + 10sm + 4st	1.2
PNP	44	11	4X	16m ^{SAT} + 12sm + 16st	2.4
RMS	44	11	4X	16m + 12sm + 16st	2.7

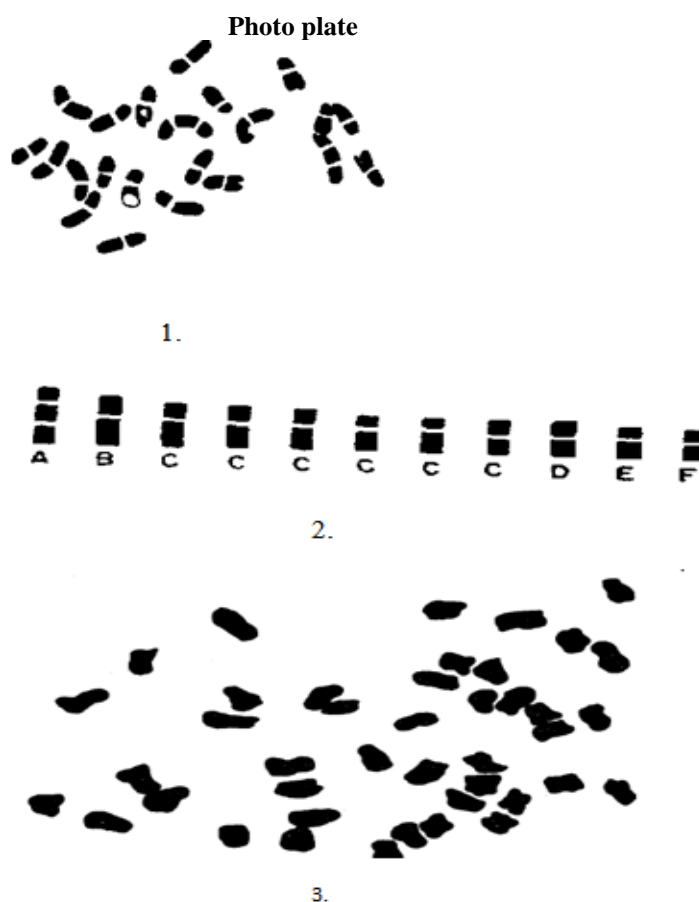
Table 2: Showing Somatic chromosome numbers (2n), base numbers (X), ploidy levels, karyotype formulas and mean chromosome length (L) of the fifteen natural variants of *Trichodesma indicum* collected from different localities of Gaya.

Localities	2n	X	Ploidy level	Formula	Length in μm
DLH	22	11	2X	12sm + 4sm ^{SAT} + 6st	5.6
BG	22	11	2X	12sm + 4sm ^{SAT} + 6st	3.4
BP	22	11	2X	10sm + 6smSAT + 6st	2.5
GC	44	11	4X	16m + 18sm + 10st	1.7
MB	44	11	4X	18m + 12sm + 12st + 2st ^{SAT}	6.0
MP	44	11	4X	16m + 16sm + 2smSAT + 8st	4.9

FP	66	11	6X	22m+24sm+20st	0.5
MOP	44	11	4X	16m+2m ^{SAT} +12sm+14st	1.5
PRA	22	11	2X	10sm+6sm ^{SAT} +6st	2.7
TK	44	11	4X	16m+2m ^{SAT} +10sm+16st	1.5
CK	44	11	4X	16m+12sm+4st ^{SAT} +12st	4.5
BR	66	11	6X	24m+22sm+20st	1.7
TKP	66	11	6X	20m+26sm+20st	2.0
PNP	66	11	6X	26m+20sm+20st	2.3
RMS	44	11	4X	16m+18sm+8st+2sm ^{SAT}	2.5

Table 3: Showing Somatic chromosome numbers (2n), base numbers (X), ploidy levels, karyotype formulas and mean chromosome length (L) of the fifteen natural variants of *Trichodesma zeylanicum* collected from different localities of Gaya.

Localities	2n	X	Ploidy level	Formula	Length in μm
DLH	24	12	2X	8m + 10sm + 6st	1.2.
BG	24	12	2X	8m+8sm+6st+2sm ^{SAT}	3.5
BP	24	12	2X	6m+10sm+8st	2.7
GC	48	12	4X	16m + 16sm + 16st	2.0
MB	48	12	4X	16m+18sm+16st	2.6
MP	48	12	4X	18m+2m ^{SAT} +12sm+14st	2.4
FP	48	12	4X	16m+2m ^{SAT} +16sm+10st	1.7
MOP	48	12	4X	16m+2m ^{SAT} +16sm+10st	1.9
PRA	48	12	4X	20m+20sm+8st	2.0
TK	48	12	4X	16m+16sm+16st	1.5
CK	24	12	2X	8m + 12sm + 4st	2.3
BR	24	12	2X	8m + 12sm + 4st	2.4
TKP	48	12	4X	20m+2m ^{SAT} +12sm+14st	1.8
PNP	48	12	4X	18m+2m ^{SAT} +14sm+14st	1.7
RMS	24	12	2X	10m + 6sm + 6st + 2st ^{SAT}	5.3





4.



5.



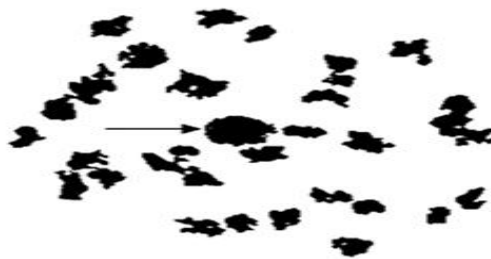
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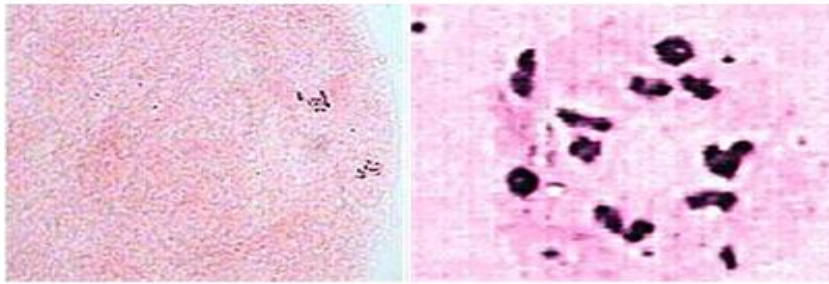
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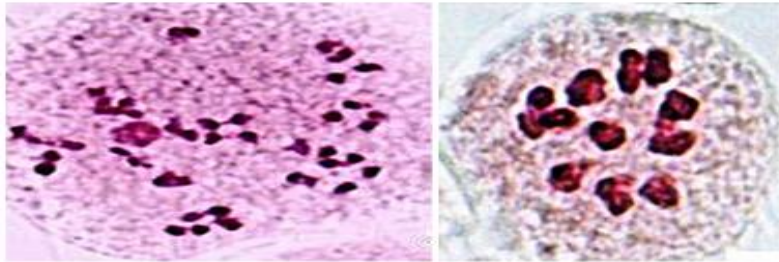


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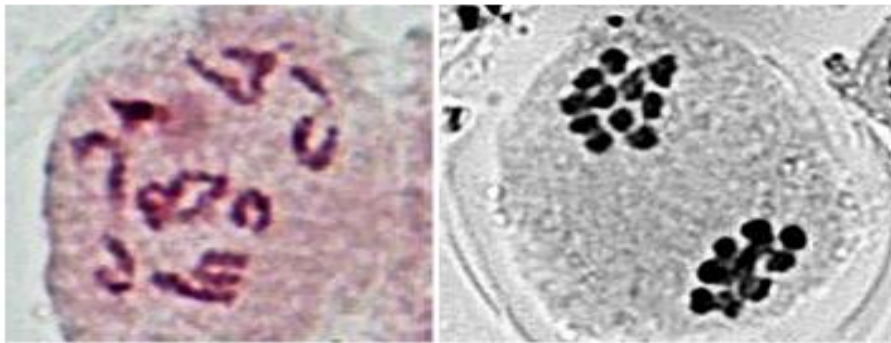
10: 5 μ m

11: 10 μ m



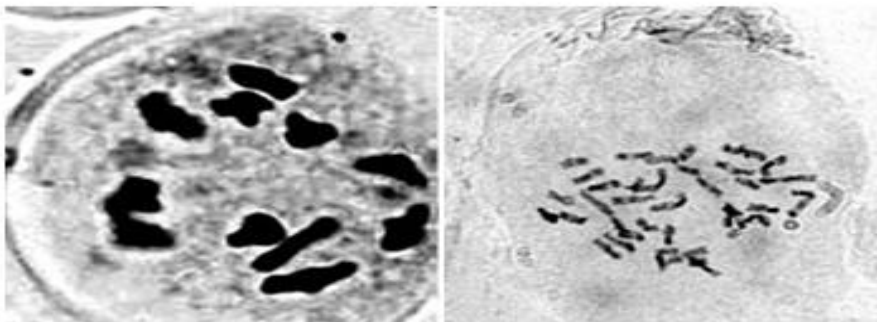
12: 5 μ m

13: 10 μ m



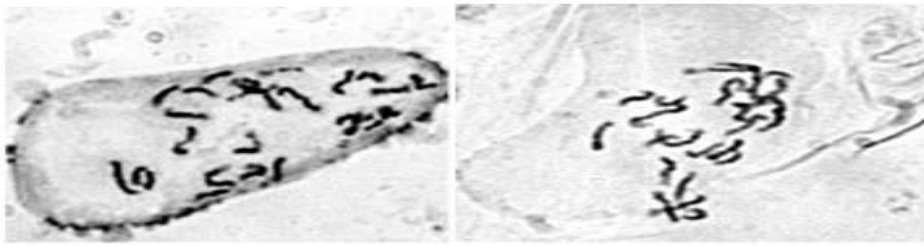
14: 10 μ m

15: 10 μ m



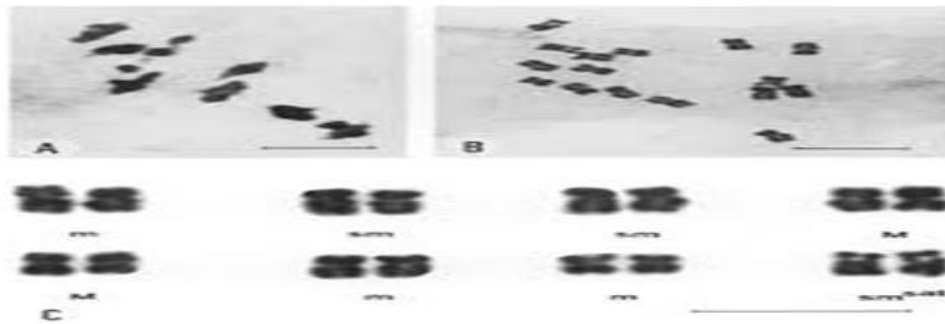
16: 10 μ m

17: 5 μ m

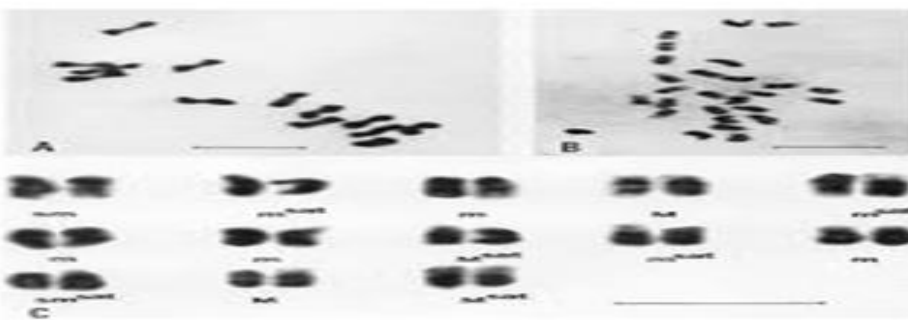


18: 10 μm

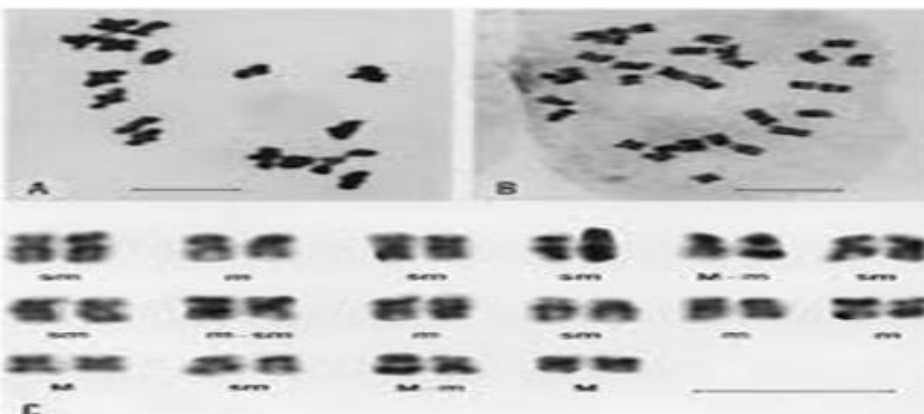
19: 5 μm



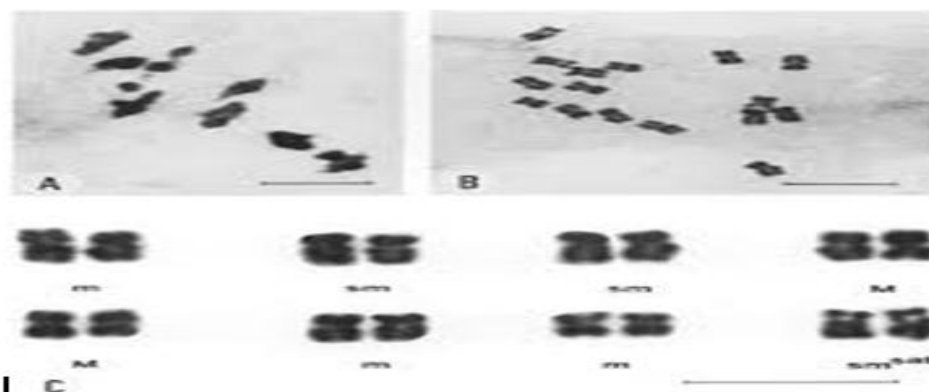
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21.



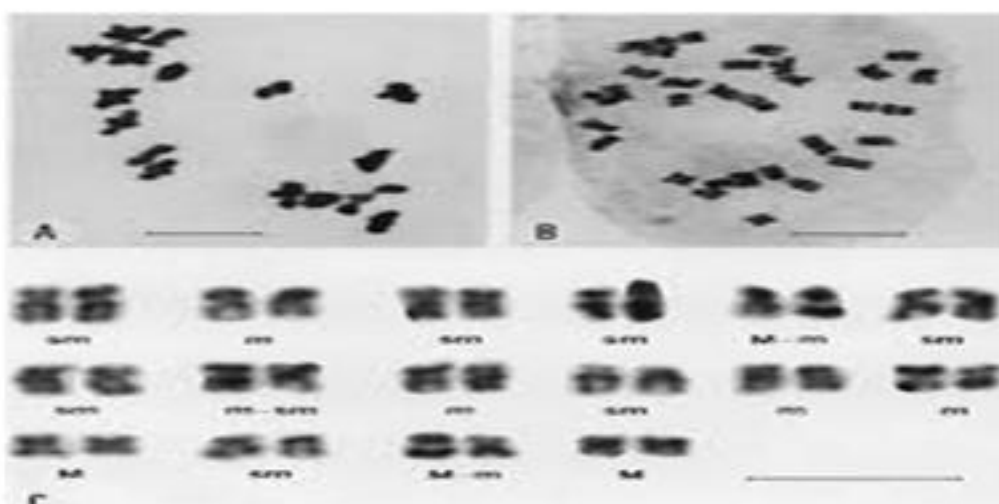
22.



23



24



25

EXPLANATION OF PHOTOPLATE (KARYOTYPES)

Photoplate-1: Camera lucida diagram of somatic metaphase plate of *Heliotropium indicum* showing 22 chromosomes. X2450

Photoplate-2: Camera lucida diagram of Diploid karyotype of *Heliotropium indicum*, Idiogram. X2450

Photoplate-3: Camera Lucida diagram of Tetraploid karyotype (Mitotic chromosomes) of *Heliotropium indicum*. X2450.

Photoplate-4: Camera lucida diagram of Diakinesis of *Heliotropium indicum*. X2450

Photoplate-5: Camera lucida diagram of Bivalents in Metaphase I of *Heliotropium indicum*. X2450

Photoplate-6: Camera lucida diagram Showing regular distribution of the chromosomes at Metaphase II of *Heliotropium indicum*. X24500

Photoplate-7: Camera lucida diagram of Diakinesis of *Heliotropium indicum*. X2450

Photoplate-8& 9: Camera lucida diagram showing meiotic study in *Heliotropium indicum*. X2450

Arrow indicates nucleolus

Photoplate-10: Meiotic anaphase-I of *Heliotropium indicum*

Photoplate-11: Meiotic metaphase-I of *Trichodesma zeylanicum* (n= 12)

Photoplate-12: Meiotic Diakinesis of *Trichodesma indicum* (n=22)

Photoplate-13: Meiotic metaphase of *Trichodesma indicum* (n=11)

Photoplate-14: Diakinesis of *Trichodesma zeylanicum* (n=12)

Photoplate-15: PMC at anaphase-I of *Heliotropium indicum* (n=11)

Photoplate-16: PMC at anaphase-I of *Trichodesma indicum* (n=11)

Photoplate-17, 18 & 19: Mitotic metaphase of *Trichodesma zeylanicum* (2n=24)

Photoplate-20 & 23: A, B & C Karyotype & Idiogram of *Heliotropium indicum*.

Photoplate-21 & 24: A, B & C Karyotype & Idiogram of *Trichodesma indicum*

Photoplate-22 & 25: A, B & C Karyotype & Idiogram of *Trichodesma zeylanicum*

III. Results

The results of the Chromosome numbers, ploidy levels, karyotype formulas and mean chromosome lengths of the investigated taxa of three species of Boraginaceae have been presented in Table- 1, 2 and 3 and photo plate- 1 to 25.

***Heliotropium indicum:* (Photoplate- 1 to 10, Plate- 1& 2 mitotic diploid karyotype, plate- 3 tetraploid karyotype; plate- 4- 10 Meiotic karyotype)**

All specimens of *Heliotropium indicum* collected from fifteen different localities of Gaya showed a complement with $2n = 4x = 44$. The plants of *H. indicum* collected from DLH, BG, BP, GC, MB, PRA, TK, CK and TKP showed normal diploid karyotype. The specimens collected from MP, FP, MOP, BR, PNP and RMS, on the other hand exhibited tetraploid karyotype. The diploid karyotype of specimen collected from DLH consisted of eleven pairs of chromosomes of which twelve were metacentric, eight submetacentric and two satellite metacentric. Chromosomes were large sized and relatively uniform. The specimens exhibiting diploid karyotypes showed slightly different chromosome formulae. The specimens from BG, BP, GC and MB exhibited diploid karyotype of $16m+2sm+4st$, $8m+10sm+4st$, $8m+12sm+2st$ and $10m+6sm+4st+2st^{SAT}$ respectively. Similarly the specimens from PRA, TK, CK and TKP exhibited chromosome karyotype formula of $12m+4m^{SAT}+6m$, $8m+10m+4st$, $8m+12m+2st$ and $8m+10m+4st$ respectively. Out of fifteen specimens only six specimens viz. MP, FP, MOP, BR, PNP and RMS of *H. indicum* showed tetraploid karyotype with chromosome formula of $16m+16sm+12st$, $16m+12sm+16st$, $16m+18sm+10st$, $16m+16sm+14st$, $16m+12sm+16st$ and $16m+12sm+16st$ respectively (Table-1; Plate- 1 to 10).

***Trichodesma indicum:* (Fig- 21 and 24 A, B and C mitotic karyotype; plate- 12, 13 and 16 meiotic karyotype)**

All specimens of *Trichodesma indicum* collected from fifteen different localities of Gaya showed a complement with $2n = 4x = 6x=66$. The plants of *Trochodesma indicum* collected from DLH, BG, BP and PRA showed diploid karyotype with chromosome formula of $12sm+4sm^{SAT}+6st$, $12sm+4sm^{SAT}+6st$, $10sm+6sm^{SAT}+6st$, $10sm+6sm^{SAT}+4st$ respectively. The plants of *T. indicum* collected from GC, MB, MP, MOP, TK, CK, BR and RMS exhibited tetraploid karyotype ($2n=4x=44$) with chromosome formula of $16m+18m+10st$, $18m+12sm+12st+2stSAT$, $16m+16sm+2smSAT+8st$, $16m+2mSAT+12sm+14st$, $16m+2mSAT+10sm+16st$, $16m+12sm+4stSAT+12st$ and $16m+18sm+8st+2smSAT$ respectively. Similarly the specimens collected from FP, BR, TKP and PNP showed hexaploid karyotype with chromosome formula of $22m+24sm+20st$, $24m+22sm+20st+20m+26sm+20st$ and $26m+20sm+20st$ respectively (Table-2; Plate-21 and 24 A, B and C mitotic karyotype; plate- 12, 13 and 16 meiotic karyotype).

***Trichodesma zeylanicum:* (Plate- 17, 18, 19, 22 and 25 A, B and C mitotic karyotype; plate- 11, 14 meiotic karyotype)**

All specimens of *Trichodesma zeylanicum* collected from fifteen different localities of Gaya showed a chromosome complement with $2n = 4x = 48$. The specimens of *T. zeylanicum* from DLH, BG, BP, CK, BR and RMS showed diploid karyotype ($2n=24$) with chromosome formula of $8m+10sm+6st$, $8m+8sm+6st+2sm^{SAT}$, $6m+10sm+8st$, $8m+12sm+4st$, $8m+12sm+4st$ and $10m+6sm+6st+2st^{SAT}$ respectively. Similarly the specimens of *T. zeylanicum* from GC, MB, MP, FP, MOP, PRA, TK, TKP and PNP exhibited tetraploid karyotype with chromosome formula of $16m+16sm+15st$, $16m+18sm+16st$, $18m+2m^{SAT}+12sm+14st$, $16m+2m^{SAT}+16sm+10st$, $16m+2m^{SAT}+16sm+10st$, $20m+20sm+8st$, $16m+16sm+16st$, $20m+2m^{SAT}+12sm+14st$ and $18m+2m^{SAT}+14sm+14st$ respectively (Table- 3; Plate- 17, 18, 19, 22 and 25 A, B and C mitotic karyotype; plate- 11, 14 meiotic karyotype).

IV. Discussion

For the three species of Boraginaceae viz., *Heliotropium indicum*, *Trichodesma indicum* and *T. zeylanicum* the chromosome numbers $2n = 22=44$ (*H. indicum*); $2n=24=44=66$ (*T. indicum*) and $2n=24=48$ (*T. zeylanicum*) have been reported. The present findings are in agreement with Mekki *et al.*, (1986) [23]; Gadella and Kliphuis 1967, 1978 [37, 38] who observed $2n=40$ and $2n=48$ karyotype in *Symphytum officinale* L. of Boraginaceae. In addition the entire chromosome numbers between $2n=22$, $2n=44$, $2n = 48$, $2n=66$ occur occasionally in nature due to hybridization of the two tetraploid cytotypes (Gadella 1972, Gadella and Kliphuis, 1971) [37, 38]. The diploid cytotype of *H. indicum* is morphologically indistinguishable from the tetraploid plants with the chromosome number $2n =22$ and $2n= 44$, except for the colour of the flowers which is always white in the diploid plants whereas it is white or purple in the tetraploid plants. The diploid cytotypes are fairly common and widely distributed throughout Gaya, but the tetraploid plants have only been found in a small number of scattered populations in MP, FP, MOP, PNP and RMS.

The karyotypes of the fifteen plants of *H. indicum* from different localities of Gaya differed slightly in their karyotype. The idiogram of the haploid karyotype consists of three long chromosomes (2-2.4 micron), two very short chromosomes (1.1-1.3 micron) and seven chromosomes of intermediate length (1.5-2 micron). The chromosomes have a heterochromatic terminal band in the short arm, except for chromosome three and the smallest chromosome in both of which the short arm is Giemsa negative. The long arms of all the chromosomes have a distinct heterochromatic band in the proximal half near the centromere and a narrow, usually faint terminal band, except for the two smallest chromosomes in which the long arms are completely heterochromatic. The distinct band in the proximal half of the long arms of the third chromosome and the smallest chromosome are very close to the centromere. A third, intercalary band was observed only in the long arms of the longest chromosome and chromosome three. Some of the chromosomes have a satellite attached to the short arm. However, the position of the satellites is apparently not fixed and their numbers also vary. In the plants from Gaya localities on average three satellites were observed in each plate. *T. indicum* showed $2n= 22= 44= 66$ karyotype i.e. diploid, tetraploid and hexaploid, whereas *T. zeylanicum* showed $2n=24=48$ i.e. diploid and tetraploid karyotype.

Out of the 45 taxa (fifteen from each of the species *H. indicum*, *T. indicum* and *T. zeylanicum*) investigated, 12q were still karyologically unknown allowing a few points to be commented. At the tribe level, the present results suggest that *Boragineae* have the broadest variation in base numbers, $x = 11$ (*H. indicum*), and $x= 12$ (*T. indicum* and *T. zeylanicum*). A. Coppi *et al.*, (2006) [21] also reported the broadest variation in base numbers, with $x = 7$ (*Paraskevia*), 8 (*Anchusa*, *Phyllocara*, *Hormuzakia*), 9 (*Cynoglottis*), 10 (*Elizaldia*, *Symphytum*, *Nonea*) and 15 (*Elizaldia*, *Nonea*). Adding also $x = 6$ of *Brunnera* (Bigazzi and Selvi 2001) [19] and $x = 11$ of *Pentaglottis* (Luque, 1989) [14] we have a broad series of base numbers that is likely to reflect a complex history of chromosomal evolution. This is matched by wide variations in ploidy levels (e.g. *Trachystemon orientalis*) and chromosome structure, as in the case of the large heterochromatic segments and secondary constrictions in *Elizaldia* and *Nonea vesicaria*. In *Lithospermeae* we found $x = 7$ (*Onosma*, *Alkanna*), 8 (*Cerithe*, *Echium*, *Arnebia*) and 9 (*Buglossoides*); the base number of *Alkanna hirsutissima* remains uncertain. Tribal karyological variation is certainly considerable, though probably not as wide as in *Boragineae*.

Members of *Boraginaceae* investigated showed the lowest variation, with only $x=11$ and $x=12$ as haploid number in *H. indicum* and *T. indicum* and *T. zeylanicum* as also observed by A. Coppi *et al.*, (2006) [21] in *Cynoglosseae* $x = 12$ as haploid number in *Cynoglossum*, *Omphalodes*, *Paracaryum*, *Pardoglossum* and *Solananthus*. This matches the findings of Luque & Valdes (1986) [39] on Spanish species of *Cynoglossum*, all with $2n = 24$. Although *Omphalodes* is known to include species with different numbers (Grau, 1967) [9], radiation and evolution of new forms in this tribe seem to have involved minor chromosomal rearrangements with respect to *Boragineae* and *Lithospermeae*, also in terms of changes in ploidy levels. Hence, cytotaxonomy provides little help in the systematics of *Cynoglosseae*, first of all for the definition of the generic limits in the critical *Cynoglossum/Solananthus/Pardoglossum/Paracaryum* group. The relatively high base number $x = 12$ is possibly derived from lower ones, such as $x = 6$ and this may support the traditional view that *Cynoglosseae* represent “the most highly specialized tribe in the family” (Johnston 1924; Britton, 1951) [40, 41]. In tribe *Eritrichieae*, the base number $x = 10$ is confirmed for *Rochelia* (*R. cardiosepala* and *R. disperma*), where it coexists with $x = 11$ (*R. disperma*, Luque, 1992) [15]. In the genus *Lappula* our finding of $2n = 48$ in *L. sessiliflora* matches the report for *L. squarrosa* from Spain (Luque 1992; 1989; 1983; 1984; 1990; 1986 [15, 14, 12, 13, 15, 42] and Luque and Valder, (1984) [39], confirming that $x = 12$ is one of the two base numbers in the genus together with $x = 11$ (*L. microcarpa* Ledeb. (Vasudevan, 1975) [43]. The base $x = 12$ and small chromosome size are therefore karyological features shared with most members of tribe *Cynoglosseae*. Johnston (1924) [40] viewed the two tribes as derived from the “more primitive” groups of *Lithospermeae* and *Boragineae* in view of synapomorphic characters such as the columnar/pyramidal gynobase and appendaged mericarpid. Some of the new reports deserve some more comments to highlight further systematic implications. In tribe *Boragineae*, the two taxa of *Elizaldia* endemic to Morocco, *E. calycina* subsp. *embergeri* and *E.*

heterostemon, have two different numbers, $2n = 20$ and $2n = 30$, respectively. While the latter was already known in *Elizaldia calycina* subsp. *Multicolor* from Morocco (Grau, 1971) [44], the former is here reported for the first time in genus *Elizaldia*. Accordingly, chromosome characters support the elevation of *E. calycina* subsp. *embergeri* at the species rank, in line with its morphological and auto-ecological peculiarities (Dobignard, 1997) [45]. On the other hand, it is worth of note that $2n = 20$ is characteristic of several *Nonea* species, either annual (*N. obtusifolia* (Willd.) DC.) or large-sized perennial such as the Anatolian endemics *N. intermedia* Ledeb., *N. pulmonarioides* Boiss. & Balansa and *N. monticola* (Rech. fil.) Bigazzi & Selvi (Selvi and Bigazzi 2002; Bigazzi and Selvi 2003) [18, 20]. Hence, the present finding supports the close relationship between *Elizaldia* and *Nonea* which has recently emerged from also morphological and molecular studies (Selvi *et al.*, 2002; Hilger *et al.*, 2004; 2005) [18, 46, 47]. Such link is further corroborated by the strong karyotypic similarity found between *E. heterostemon* and *N. vesicaria* in terms of number and chromosome structure. The derived base number $x = 15$, the presence of secondary constrictions and of large heterochromatic segments represent synapomorphic characters which are not found in any other species of *Nonea*. This suggests to exclude mere parallelism as a cause for such similarity but instead a common history of chromosome evolution that may have involved an event of amphidiploidy between annual *Nonea* species with $x = 7$ and $x = 8$ (Fernandes and Leitao, 1972; Luque 1995; Selvi and Bigazzi, 2002) [48, 17, 18]. Alternatively, the number $2n = 30$ may have resulted from the union of reduced and unreduced gametes of a taxon with $2n = 20$, a common phenomenon in several angiosperm groups. Ongoing phylogenetic studies on the *Nonea/Elizaldia* group using molecular tools will provide further insights on this subject.

For the three species of Boraginaceae viz., *Heliotropium indicum*, *Trichodesma indicum* and *T. zeylanicum* the chromosome numbers $2n = 22=44$ (*H. indicum*); $2n=24=44=66$ (*T. indicum*) and $2n=24=48$ (*T. zeylanicum*) have been reported. In addition all the chromosome numbers between $2n=22$, $2n=44$, $2n = 48$, $2n=66$ occur occasionally in nature due to hybridization of the two tetraploid cytotypes. The diploid cytotype of *H. indicum* is morphologically indistinguishable from the tetraploid plants with the chromosome number $2n = 22$ and $2n = 44$, except for the colour of the flowers which is always white in the diploid plants whereas it is white or purple in the tetraploid plants. The diploid cytotypes are fairly common and widely distributed throughout Gaya, but the tetraploid plants have only been found in a small number of scattered populations in MP, FP, MOP, PNP and RMS.

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In the present investigation the karyological observations on fifteen varieties of each of the the three species of Boraginaceae viz., *Heliotropium indicum*, *Trichodesma indicum* and *Trichodesma zeylanicum*, collected from different localities of Gaya were carried out with special reference to chromosome number, chromosome morphology, and chromosome behavior at meiosis to give a contribution to the cytotaxonomy of the family.

It is clear that the tetraploids are quite distinct from the known diploids in their habitat preferences and general physiological vigor. The diploids are fairly strong competitors and generally prefer a relatively mesic habitat while the tetraploids and hexaploids ($6x=66$ in some plants of *T. indicum*) are rather poor competitors and are generally found growing in arid habitats, often under great water stress, occurring from April to July in Gaya. This does not indicate, of course, whether the tetraploids arose as autopolyploid or allopolyploid types, as both mechanisms have been demonstrated to result in these kinds of changes.

Of the fifteen varieties of each of the three species of Boraginaceae presented in this paper nine varieties of *H. indicum*, four varieties of *T. indicum* and six varieties of *T. zeylanicum* are diploids ($2n=22$, $2n=22$ and $2n=24$ respectively). Two of these, *H. indicum* and *T. indicum* closely related and are doubtless of common origin. These two species have similar habitat preferences and differ in only minor morphological characters. Both are found in meadows, forest clearings, or along stream banks and roadsides. Besides the obvious corolla color difference they differ slightly in the size of the corolla features, in the shape of the fornix

protuberance, in the surface texture of the corolla appendage, in the length of the fruiting pedicel, and in the size of the nutlet. Unfortunately these taxa have been very poorly collected and are known from only a few localities. However, the morphological features separating these two taxa are constant in the known populations and therefore these two entities should remain as distinct species at this time. The third diploid *Trichoderma zeylanica*, is unlike any of the other known diploids and must stand alone in any discussion of phylogeny.

Of the tetraploids, *H. indicum* (4x=44), *T. indicum* (4x=44) and *T. zeylanicum* (4x=48) are distinct enough morphologically to make their alignment with the other tetraploids difficult. There is evidence of natural hybridization of *H. indicum* and *T. indicum* with *T. zeylanicum*. If these two taxa, which are the most clearly defined of all the tetraploids, will hybridize with *H. indicum* where they are neighboring sympatric, one must conclude that the tetraploids are all fairly closely related. Nevertheless *H. indicum*, *T. indicum*, and *T. zeylanicum* are unique and therefore of unknown phylogenetic relationship.

Chromosomal study is useful to understand the morphological diversity and genomic variation within a species (Stace, 2000) [49]. Generally the researchers identify the plant species based on their morphological characteristics. The cytological analysis especially the study of chromosome number can further authenticate the identity of the plant species.

It might be suggested that the tetraploid varieties of *H. indicum*, *T. indicum* and *T. zeylanicum* arose from a diploid varieties of *H. indicum* species which was separate and distinct from the diploid involved in the evolution of the other tetraploid species. The remaining tetraploids specimens of *H. indicum* and *T. indicum* are so closely similar and sometimes overlapping in morphological characters, that one must suspect they have a recent common origin. Of these, the specimens of *H. indicum* collected from MP, FP, MOP, BR, PNP and RMS whose habitat preferences and morphological characters remain fairly constant throughout their respective ranges. If these were not for this constancy, one would have difficulty justifying their species status, since on strictly morphological grounds they might best be placed as varieties in the *H. indicum* complex. In the present study six tetraploid taxa of *H. indicum* treated as varieties of *H. indicum*; six tetraploid and four hexaploid taxa of *T. indicum* and nine tetraploid taxa of *T. zeylanicum* treated as varieties of *T. indicum* and *T. zeylanicum* respectively.

V. Conclusions

The present study is the first to investigate phylogenetic relationships of the Boraginaceae using cytological data. From these phylogenies, it is evident that additional species-level phylogenetic studies should be undertaken on specific clades in which large, widespread genera, such as *Myosotis*, *Cynoglossum*, *Eritrichium*, and *Anchusa*, are resolved as non-monophyletic. Further analyses of these genera and their relatives will help to determine the most appropriate manners in which to circumscribe genera. In future family-level studies of Boraginaceae, it will be important to include more Indian representatives of the family.

Conflict of interest: Authors declare no conflict of interest directly or indirectly

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