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Cytological Studies in the Genus *Azolla*

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The heterosporous leptosporangiate fern *Azolla* survives only by seven species (Lumpkin and Plucknett 1982) in aquatic habitats at many parts of the world and is being traditionally used as biofertilizer for the rice crop in certain countries (Moore 1969, Singh 1979a, b, Lumpkin and Plucknett 1982). The cytological studies in *Azolla* are meagre, although there are several informations on its allied genera like *Marsilea* (Mehra and Loyal 1959, Abraham *et al.* 1962, Loyal and Kumar 1979) and *Salvinia* (Loyal 1958, Loyal and Grewal 1966). The chromosome numbers in *A. filiculoides* (Duncan 1940) and *A. pinnata* (Loyal 1958, 1972) are reported. Present work has been taken up to find out somatic chromosome number to analyse karyotype, to assign meiotic behaviour in microspore mother cell and to ascertain the possibility of existence of polyploidy among *Azolla* sps.

Materials and methods

Azolla species like *A. mexicana* (California, USA), *A. filiculoides* (Hawaii, USA), *A. pinnata* (Cuttack, India), *A. pinnata* (Hanoi, Vietnam, green isolate) and *A. pinnata* (Africa) were utilized in this study.

Sporophytes were treated with saturated solution of p-dichlorobenzene for 4 hours, then fixed in 3: 1 Carnoy's fluid for 6 hours. A maceration mixture was prepared from 1 part of 1 N HCl and 10 parts of 2% acetoorcein. Shoot-tips with apical meristem and young leaf primordia were boiled in maceration mixture taking in empty injection phials and kept in an incubator for 35 minutes at 60°C. When cooled, the shoot-tips were squashed with a drop of 45% acetic acid.

Young microsporocarps were dissected from field grown plants, and after their surface sterilised with 0.1% aqueous solution of $HgCl_2$ for 2 minutes, brushed, and washed in distilled water, they were soaked and then fixed in 3: 1 Carnoy's fluid for 6 hours. Microsporocarps were placed under dissecting microscope; the microsporangia were released by fine needles and squashed with 2% acetocarmine.

Measurements of important morphological parameters of three isolates of *A. pinnata* were also made.

Results

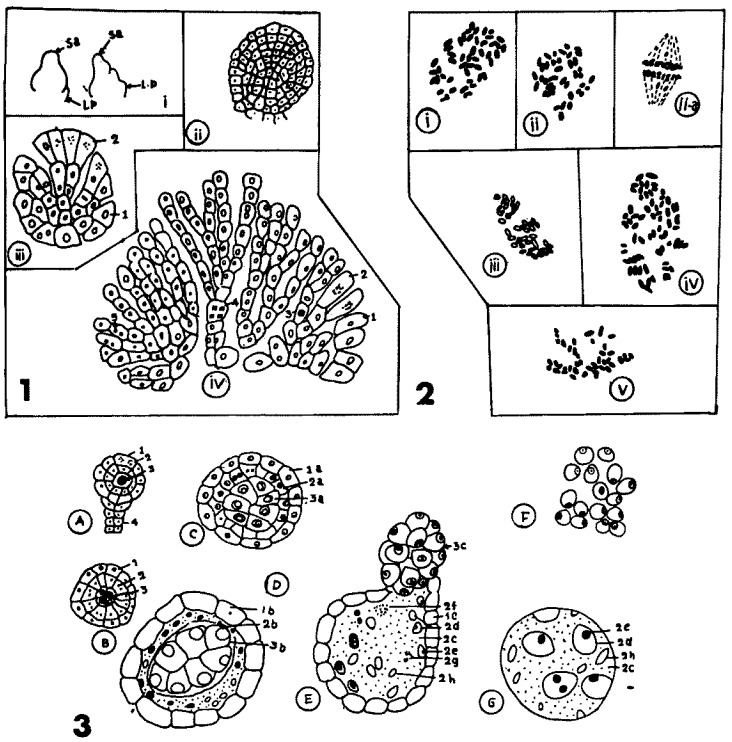
While dissecting shoot-tips, apical cell, its derivatives and cells of leaf primordia (Fig. 1) were frequently observed. The cytology of young leaf cells was studied (Fig. 1 ii, iii). The cells of young leaves were variously shaped (Fig. 1 iii, iv).

Mitotic events of leaf cells

During early phases, the nuclear membrane and nucleolus were not traceable in all species and isolates of *Azolla*. The chromosomes were thinly dispersed throughout the nucleus and found to be heterochromatic in nature. In early part of metaphase, the chromosomes accom-

plished their maximum state of condensation and the somatic chromosome number was counted to be 2n=48 in *A. mexicana*, USA (Fig. 2 i); 2n=40 in *A. filiculoides*, USA (Fig. 2 ii); 2n=44 in *A. pinnata*, India (Fig. 2 iii); 2n=66 in *A. pinnata*, Vietnam (Fig. 2 iv); 2n=44 in *A. pinnata*, Africa (Fig. 2 v).

The chromatid separation was simultaneous in all cases but in *A. filiculoides* it was irregular for certain cells, where at the onset of anaphase few chromosomes moved precociously tow-



Figs. 1–3. 1, leaf primordia. i, shoot-apex (s.a.), young leaf primordia (l.p.). ii, young upper lobe. iii, split rows of cells in young upper lobe. iv, at later stage. 2, somatic chromosomes at premetaphase (Camera-lucida impression×1000). i, *A. mexicana* 2n=48. ii, *A. filiculoides* 2n=40, ii-a. *A. filiculoides* anaphase showing precocious movement of one to two chromosomes. iii, *A. pinnata*, India, 2n=44. iv, *A. pinnata*, Vietnam, 2n=66. v, *A. pinnata*, Africa, 2n=44. 3, stages of microsporogenesis (hand drawings). A=young microsporangium, B and C=spore mother cell (SMC) inside microsporangium, D=later stages, E=oozed-SMC from microsporangium, F=quarters SMC, G=a portion of tapetum showing free nuclei, I=Jacket cells with nuclei, 2a=granulated cytoplasm and some with binucleate state in tapetum, 3=archesporial cells, 1b=nuclei diminished sized, 1c=hyaline jacket, 2c=granulated cytoplasm of exposed tapetum, 2d=tapetum with free nuclei, 2e=tapetum nucleoli, 2f=a tapetal free nucleus at premetaphase, 2h=vacuoles inside tapetum.

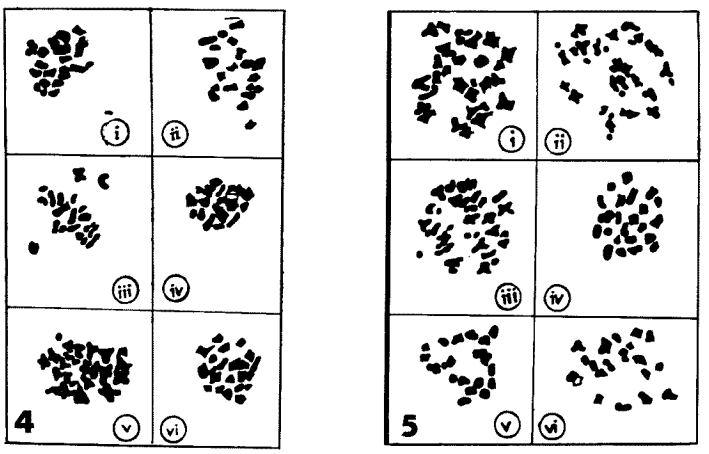
wards the poles (Fig. 2 ii-a). The nuclear membrance and nucleolus were not traceable even in daughter cells formed after establishment of cell plate (Fig. 3).

The karyotypic analysis of different species/isolates investigated in the present study was confined to premetaphase of somatic mitosis. As centromeres were not observed in any case the arm-ratio and types of chromosomes could not be determined. On the other hand, absolute length (AL), total chromatin length (TCL), average length and relative length (RL) were

calculated. In all cases, the chromosomes acquired simple form where even secondary constriction or satellite was not observed. Basing on AL the somatic chromosomes were grouped in different sets from A to N by virtue of their size in descending order.

i. *A. mexicana* (2n=48)

The total chromatin length was observed to be 61.38 μ in *A. mexicana*. Depending upon the absolute length, 48 somatic chromosomes at premetaphase were grouped into 7 sets (D, F, G, H, J. K and L) in descending order (Table 2). Biggest chromosomes of average 1.74 μ in length constituted the set D while smallest chromosomes of average 0.92 μ in length constituted Set L. Chromosomes of Set H contributed major portion (26.98%) of the genome (Table 2). In *A. mexicana* the karyotype was asymmetrical, The average chromosome length was found to be 1.28 μ. The karyotypic formula of *A. mexicana* constituted as 4D+4F+4G+12H+10I+8K+6L (Table 2).



Figs. 4–5. 4, meiotic stages (early metaphase 1) of microspore mother cell (Camera-lucida impressions). i, *A. mexicana* 24 IIs, ×1100. ii, *A. filiculoides* 20 IIs, ×1400. iii, *A. filiculoides* 20 IIs, meiotic appearance of some bivalents, 3 bivalents yet to reach the metaphase plate, ×1400. iv, *A. pinnata*, India 22 IIs, ×900. v, *A. pinnata*, Vietnam 17 IIIs+6 IIs+3 Is, ×900. vi, *A. pinnata*, Africa 22 IIs, ×900. 5, late prophase I of microspore mother cells (Camera lucida impression ×1100). i, *A. pinnata*, Vietnam: showing diplotene (10 IIIs+16 IIs+3 Is). ii, *A. pinnata* (Vietnam): showing Diakinesis (12 IIs+11 IIs+8 Is). iii, *A. pinnata* (Vietnam): diakenesis (7 IIIs+18 IIs+9 Is). iv, *A. mexicana* (24 IIs). v, *A. filicuoides* (19 IIs+2 Is). vi, *A. filiculiodes* (20 IIs).

ii. *A. filiculoides* (2n=40)

The chromosomes were narrow and rod shaped unlike those of *A. mexicana*. The total chromatin length in this species was comparatively smallest among all species utilized measuring 44.34 μ in average. Forty chromosomes were grouped into 7 sets (G, H, I, J, K, M and N) (Table 3). The longest chromosomes constituting set G were of 1.45 μ (Table 1). The chromosomes of Set H also shared major portion (37.08% genome). Extremely short chromosomes of 0.73 μ absolute length comprised in Set N. The average chromosome length was 1.11 μ. Karyotype was also asymmetrical in *A. filiculoides* and karyotypic formula became 4G+12H+2I+2J+6K+6M+8N (Table 2).

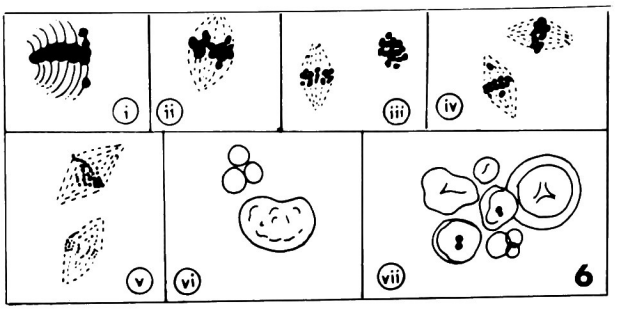
iii. *A. pinnata*, Cuttack, India (2n=44)

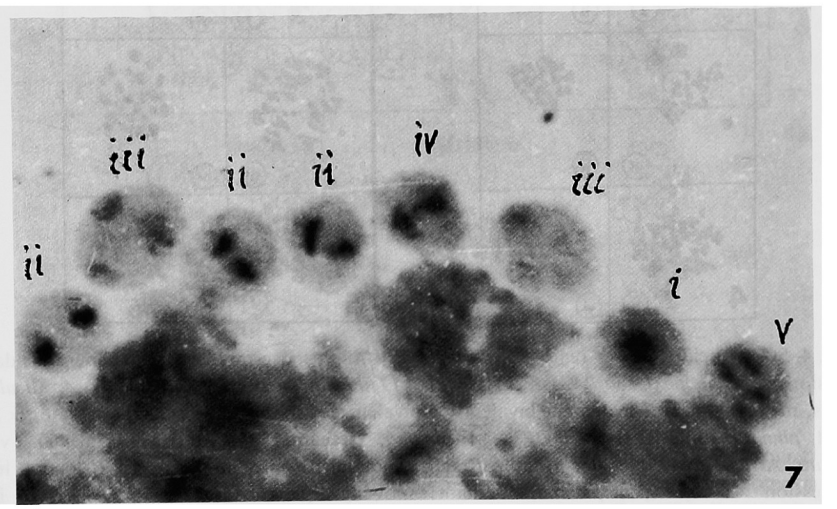
The total chromatin length was 69.38 μ. The 44 chromosomes were grouped into 8 sets of A, B, C, E, G, I, J and K. The longest chromosomes of average 2.31 μ were grouped in Set

A and shortest chromosomes of average 1.06 μ were grouped in Set K (Table 1). The average chromosome length was 1.58 μ. The karyotype was observed to be asymmetrical. The karyotypic formula would be 2A+4B+6C+8E+12G+6I+4J+2K (Table 2).

iv. *A. pinnata*, Vietnam, green isolate (2n=66)

Chromosomes of this isolate were almost similar to those of Cuttack isolate in shape and size, and usually bigger chromosomes were observed in periphery. The total chromatin length of 104.97 μ was observed to be longest among all species (Table 1). The average chromosome





Figs. 6–7. 6, meiotic stages of microspore mother cell (Camera lucida impressions). i, *A. filiculoides*: early anaphase I showing curved spindle and precocious movement of some chromosomes. ii, *A. filiculoides*: early anaphase showing normal spindle and precocious movement of some chromosomes. iii, *A. filiculoides*: late prophase II and metaphase II. iv, *A. filiculoides*: early anaphase II with precocious movement of some chromosomes and metaphase II. v, *A. pinnata* Vietnam: early anaphase II. vi, *A. filiculoides*: abnormal microspores. vii, *A. pinnata* Vietnam: abnormal and normal microspores. 7, microspore mother cells of *A. pinnata*, Vietnam at various meiotic stages. i, prophase I (leptotene). ii, metaphase II. iii, tetraphedral tetrad. iv, one at metaphase II, other at late prophase II. v, anaphase II.

length was 1.59 μ. The karyotype was highly asymmetrical and the formula became 3A+6B+9C+12E+18G+9I+6J+3K (Table 2).

v. *A. pinnata*, Aftrica (2n=44)

The total chromatin length was 52.54 μ (in average). The chromosomes were short in length than India and Vietnam isolates. The chromosomes were grouped into 8 sets (C, E, G, H, I, K, L and M). The absolute lengths of longest chromosome comprising the Set C were 1.82 μ and smallest chromosomes comprising the Set M were 0.85 μ (Table 1). The average

length of the chromosomes was 1.19 μ. The karyotype was asymmetrical and formula constituted as 2C+2E+4G+8H+6I+10K+8L+4M (Table 2).

Meiotic events of micro-spore mother cells

Meiotic stages of young micro sporangia (Figs. 3A to 3E) were almost same in all species. During squashing, 16 spore mother cells (SMC) were seen oozing out of microsporangium wall (Fig. 3E). The 16 spore mother cells took a spindle appearance in aggregation. During the interphase, the SMC appeared to be more conical than variously shaped due to coherance (Fig. 3F) where the cytoplasm was clear. In majority cases nuclei were eccentric in position and in few cases these were centrally placed (Fig. 3F). The nuclei were oval or elliptical in shape.

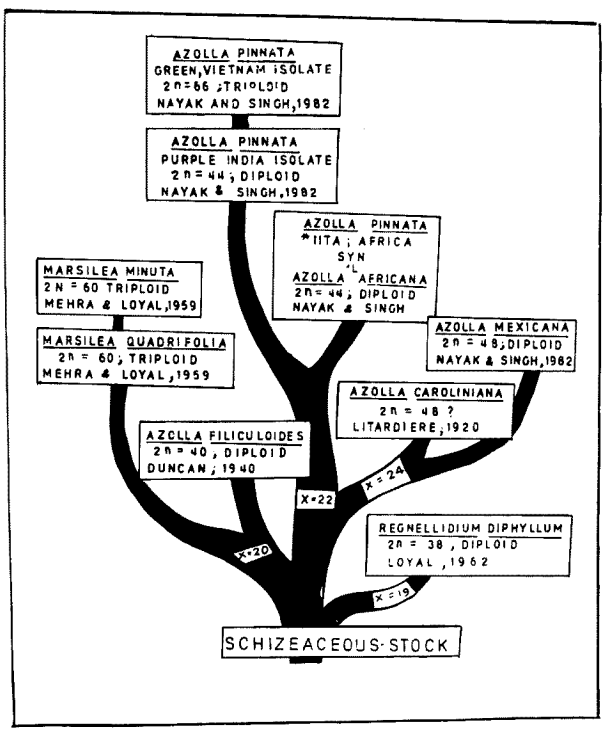


Fig. 8. The phylogram in *Azolla* reconstructed after Mehera (1961).

During leptotene, an association of 4 SMC or a group of 16 SMC was observed at a time (Fig. 3F) in all the cases. During substage zygotene, the SMC became more spherical, less vacuolar and more granular. In some of SMC, clumped chromatins were marked to one side of its nuclear region. During pachytene, chromatins were also clumped to one side. Heterochromatic bodies were predominant on their lengths.

At diakinesis, chromosomes were marked to be thicker beaded like and distributed throughout the cytoplasm. The 24 bivalents were observed in *A. mexicana* (Fig. 4i). Bivalents were either X-shaped/rod-shaped or circular where biggest bivalents were measured to 1.88 μ and smallest 0.94 μ. The average number of chiasmata per cell in *A. mexicana* was recorded to be 18.6 (Table 3). In *A. filiculoides*, bivalents (20 numbers) were much reduced in size, remained X to rod shaped (Figs. 4 ii, iii, 5 vi) and in some cases univalents were also noticed (Fig. 5 v). In a microsporangium, some spore mother cells had the normal meiosis, while others had the abnormal meiosis where 1 to 2 small, circular and dot like univalents were observed. Some-

Table 1. Karyological data of *Azolla* species

Species	Parameters	Sets													
		A	B	C	D	E	F	G	H	I	J	K	L	M	N
A. mexicana	AL in μ	—	—	—	1.74	—	1.57	1.43	1.38	1.21	—	1.03	0.92	—	—
(USA)	SAL in μ	—	—	—	6.96	—	6.28	5.72	16.56	12.10	—	8.24	5.52	—	—
	RL %	—	—	—	2.83	—	2.56	2.33	2.25	1.97	—	1.68	1.49	—	—
A. filiculoides	AL in μ	—	—	—	—	—	—	1.45	1.37	1.22	1.12	1.05	—	0.88	0.73
(USA)	SAL in μ	—	—	—	—	—	—	5.80	16.44	2.44	2.24	6.30	—	5.28	5.84
	RL %	—	—	—	—	—	—	3.27	3.09	2.75	2.53	2.37	—	1.98	1.65
A. pinnata	AL in μ	2.31	2.17	1.87	—	1.65	—	1.45	—	1.27	1.13	1.06	—	—	—
(India)	SAL in μ	4.62	8.68	11.22	—	13.20	—	17.40	—	7.62	4.52	2.12	—	—	—
	RL %	3.33	3.13	2.70	—	2.38	—	2.09	—	1.83	1.63	1.53	—	—	—
A. pinnata	AL in μ	2.33	2.18	1.87	—	1.66	—	1.48	—	1.27	1.15	1.06	—	—	—
(Vietnam)	SAL in μ	6.99	13.08	16.83	—	19.92	—	26.64	—	11.43	6.90	3.18	—	—	—
	RL %	2.22	2.08	1.78	—	1.58	—	1.41	—	1.21	1.10	1.01	—	—	—
A. pinnata	AL in μ	—	—	1.82	—	1.67	—	1.48	1.33	1.20	—	1.08	0.95	0.85	—
(Africa)	SAL in μ	—	—	3.64	—	3.34	—	5.92	10.64	7.20	—	10.80	7.60	3.40	—
	RL %	—	—	3.46	—	3.18	—	2.82	2.53	2.28	—	2.05	1.81	1.62	—

AL=Absolute length, SAL=Sum total of absolute length, RL=Relative length.

Table 2. Karyological data of *Azolla* species

Species	Somatic chromosome number	Mean length of longest set in μ	Mean length of shortest set in μ	Longest & shortest ratio	Total chromatin length TCL in μ	Average length in μ	Relative length (%)	Karyotypic formula K ($2n$)
A. mexicana	48	1.74	0.92	1.89	61.38	1.28	2.16	4D+4F+4G+12H+10I+8K+6L
A. filiculoides	40	1.45	0.73	1.99	44.34	1.11	2.52	4G+12H+2I+2J+6K+6M+8N
A. pinnata (India)	44	2.31	1.06	2.18	69.38	1.58	2.33	2A+4B+6C+8E+12G+6I+4J+2K
A. pinnata (Vietnam)	66	2.33	1.06	2.20	104.97	1.59	1.55	3A+6B+9C+12E+18G+9I+6J+3K
A. pinnata (Africa)	44	1.82	0.85	2.14	52.54	1.19	2.27	2C+2E+4G+8H+6I+10K+8L+4M

times streched bivalents were also observed. The average size of longest bivalents was 1.57 μ and that of smallest one was 0.79 μ. The average number of chiasmata per cell was 15.5. In species of *A. pinnata* (Indian and African isolates) an association of 22 bivalents was observed. The chiasmata frequencies per cell were 14.3 and 16.3 respectively in two isolates (Table 3).

In *A. pinnata*, Vietnam isolate, the meiotic irregularities were noticed in almost all SMC. Univalents and trivalents were frequently associated with bivalents (Figs. 4 v, 5 i, ii, iii). Univalents were short, elliptical to spherical in certain cases; bivalents were either X-shaped, or oval or rod shaped. The trivalent configurations were mostly inverted or elongated Y-shaped. During late diakinesis to early metaphase I more number of univalents were observed. However, the frequency of bivalents was alsways more. The average number of bivalents at late diakinesis was 12.43 whereas of trivalents was 11.71.

Duringg early part of metaphase I, the cytoplasm appeared granular in all cases. In *A. filiculoides*, bivalents were semi-mitotic in appearance (Fig. 4 iii). Univalents were also observed in very low frequency in some abnormal cases. In *A. pinnata* Vietnam isolate, the

Table 3. Types of chromosomal associations at early metaphase I of microspore mother cells in *Azolla* species

Species	Cytotype	Number of univalents (Is)	Number of bivalents (IIs)	Number of trivalents (IIIs)	Average number of chiasmata per cell	Average number of chiasmata per bivalents
A. mexicana	Diploid	—	24.0	—	18.6	1.15
A. filiculoides	Diploid	0	20.0	—	15.5	1.07
		1.0	19.0	—		
		8.0	16.0	—		
	Average	3.0	18.33	—		
A. pinnata (India)	Diploid	0	22.0	0	14.3	0.90
A. pinnata (Vietnam)	Triploid	4.0	16.0	10.0	26.5	1.8
		8.0	11.0	12.0		
		9.0	18.0	7.0		
		9.0	6.0	15.0		
		4.0	10.0	14.0		
		3.0	6.0	17.0		
		5.0	20.0	7.0		
	Average	6.0	12.43	11.71		
A. pinnata (Africa)	Diploid	0	22.0	0	16.3	1.0

more number of free univalents or loosely attached univalents or loosely paired bivalents or streched bivalents and trivalents were frequently observed (Fig. 5 iii). Occasionally, bivalents used to undergo premature disjunction. Increase of univalents and decrease of both bivalents and trivalents were noticed during early metaphase I. The maximum number of univalents was 9 in one of the SMC of *A. pinnata*, Vietnam isolate (Fig. 5 iii).

Disjunction of exchanged chromosomes was normal in *A. mexicana* and *A. pinnata* (both Indian and African isolates) with 24: 24 and 22: 22 distribution of chromosomes respectively forming two regular diads in each. In *A. pinnata*, Vietnam isolate, the disjunction at anaphase I was irregular where diads were of unequal size.

In *A. filiculoides*, precocious movement of 1 to 2 chromosomes was marked in certain SMC (Fig. 6 iv). In majority of SMC studied, the spindle was normal bow-shaped like other species while in few cases (2 to 3 cells out of 100 cells), the abnormal spindles (curved spindles) with non-visibility of polar regions were observed (Fig. 6 i). The nuclear membrane, nucleolus and cell plate between the diads were not observed. It has been observed that while one

diad was at metaphase I, the other one was still in prophase II (Fig. 6 iii). The separation of diads into tetrads was simultaneous in *A. mexicana* and *A. pinnata* (both Indian and African isolates). In *A. filiculoides* and *A. pinata* (Vietnam) precocious movement of 1 to 2 chromosomes was observed at anaphase II. The formation of cell plates between the daughter nuclei was simultaneous to produce tetrahedral tetrads in majority cases (Fig. 7 iii).

The mature microspores of *A. mexicana*, *A. pinnata* (India) and *A. pinnata* (Africa) were normal (circular and smooth) attending diameters of average 20.03 μ, 20.43 μ and 18.7 μ respectively. In *A. filiculoides*, apart from normal type (21.47 μ in diameter), abnormal ones (of less than 12 μ and above 3 μ diameters) were observed (Fig. 6 vi). In *A. pinnata* (Vietnam) normal type (21.27 μ in diameter) was occasionally observed, but the frequency of abnormal microspores was more (Fig. 6 vii). Even in a sporangium, all microspores were abnormal ranging from 5 to 15 μ in diameter (Table 4).

Table 4. Microspores in *Azolla* species

Species	Average diameter of normal microspores in μ	Diameter of abnormal microspores in μ	Thickness of wall layer in μ	Number of abnormal microspores per microsporangium	Shape of abnormal microspores
A. mexicana	20.03	—	1.0 to 1.2	0	—
A. filiculoides	21.47	3 to 12	1.0 to 1.3	Very few abnormal microspores seen	Small, circular to wrinkled, thin walled to thicker wall
A. pinnata (India)	20.43	—	1.0 to 1.3	0	—
A. pinnata (Vietnam)	21.27	4.5 to 15.0	1.0 to 1.3	Almost all microsporangia seen with abnormal microspores	Small, circular with thin walled or bigger, wrinkled with thicker wall
A. pinnata (Africa)	18.70	12 to 15.0	1.0 to 1.3	Abnormal microspores also seen in less numbers	Bigger in size than above two

Discussion

The problem involved in comparative karyotype study of different species of *Azolla* is of complex nature. The extreme smallness of chromosomes in the complement offered a great handicap for critical analysis. Loyal (1972) has established the fact that *Azolla* has the smallest chromosomes among the ferns. The conventional method of karyotypic analysis from somatic metaphase chromosomes during present investigation showed that the chromosomes were not only small but also appeared to be simple. The centric gap and satellite or the knob could not be observed for which the karyotypes in terms of measurement of 'total-form', 'arm-ratio' and 'satellite-index' were not analysed. Therefore, total chromatin length (TCL) has been employed as an index of species differentiation.

In agreement with Loyal (1972) the karyotype of *Azolla* is chraracterized by a greater proportion of asymmetrical chromosomes. Stebbins (1959) expressed that evolved species are more specialized and have more asymmetrical karyotypes. On the basis of asymmetrical karyotypes, *Azolla* may be considered as one of the advanced members of the pteridophytes. Among three species of *Azolla*, asymmetry was more pronounced in *A. pinnata* for which this species might be considered as advanced over *A. mexicana* and *A. filiculoides*.

The centromere could not be observed in any of the chromosomes of *Azolla* probably due to its extreme smallness. In certain organisms, the chromosomes do not seem to have any localized centromeres. Either the whole chromosome or a long region of each chromosomes is attached to the spindle and behaves as if endowed with centromeric properties; chromosomes

of this kind have been studied by La Cour (1947) in *Luzula*. Whether the *Luzula*-system of centromere is operating in *Azolla* is yet to be confirmed.

In *Azolla* only one type of chromosomes was observed. In terms of absolute length, chromosomes were grouped into different sets. The repeatation of sets within the genomes suggested that species of *Azolla* were phylogenetically related.

While studying the meiotic behaviour of microspore mother cells, the eccentric position of the chromatic materials had been recorded in its allied genera *Marsilea* (Loyal and Kumar 1979) and *Salvinia* (Loyal and Grewal 1966). The absence of nuclear membrane, vacuolization of cytoplasm and gradual granulation were also marked in these genera. In this respect, *Azolla* has the close affinity to other heterosporous genera.

Chromosome number often provides a clue in determining the status of a species (Sharma 1976). In *A. mexicana* and *A. filiculoides*, the chromosome numbers varied confirming different species status. Unsally a low chromosome number indicates the primitiveness, a higher number suggests that it may be derived from a lower basic number in the course of evolution.

Table 5. Comparison of morphological and ecological parameters among the cytotypes of *A. pinnata* grown under field condition in identical circumstances

Vegetative and reproductive parameters	*A. pinnata* (India) diploid cytotype 2n=44	*A. pinnata* (Vietnam) triploid cytotype 2n=66	*A. pinnata* (Africa) diploid cytotype 2n=44
Length of main axis of rhizome in mm	16.02	16.69	12.82
Frond-area (mm^2)	219.17	409.83	193.73
Root-length (mm)	48.87	80.90	28.40
Upper lobe (mm)	1.34/0.63	1.47/0.70	1.10/0.44
Length of shoot-trichome (μ)	97.13	128.33	95.37
Length of root-trichome (mm)	1.84	2.72	2.25
Length of leaf-trichome (μ) (Upper lobe)	91.57	104.60	96.27
Stomatal size on dorsal lobe upper surface (μ)	105.80/45.7	167.67/53.27	106.67/38.43
Size of microsporocarp (mm)	1.82/1.42	1.87/1.52	1.13/0.83
Length of megasporocarps (mm)	0.81	Not seen (Female sterile)	0.77
Massular trichomes (Glochidia) μ	83.23	96.07	72.97
Ecological tolerance	Susceptible to high temperature in summer	Tolerance to high temperature in summer	Susceptible to high temperature in summer

The formation of regular bivalents and normal disjunction indicates true diploid nature (Sharma 1976). In *A. pinnata* (Indian and African isolates) occurrence of normal disjunction of 22: 22 and regular bivalents indicate true diploid nature. In this respect, *A. mexicana* having normal 24: 24 disjunction was also true diploid. But in *A. pinnata* (Vietnam), the green isolate, having irregular meiotic events and higher chromosome number suggested polyploid nature, a triploid one. Bir (1973) has noted that polyploidy is of general occurrence in the pteridophytes but very small percentage of Leptosporangiate ferns attain chromosome number beyond the tetraploid level.

In respect of frond area, length of rhizome, root, leaf, size of shoot-trichomes, root-trichomes, leaf trichomes and stomatal size the triploid (*A. pinnata*, Vietnam isolate) exhibited an over all gigas appearance over the diploid local (Cuttack, India) isolate and African isolate of *A. pinnata*. The stomatal frequency was also found to be less in leaf lobes of *A. pinnata* Vietnam isolate, confirming its ploid nature.

It might be that *A. pinnata* could occur in two cytological races: (i) a diploid cytotype 2n=

44 and (ii) a triploid cytotype 2n=66. The triploid cytotype could be the green isolate from Hanoi (Vietnam). In the diploid cytotype *A. pinnata* from Cuttack (India), there is a regular formation of both male and female sporocarps with normal microspore in microsporangia. In triploid cytotype *A. pinnata* from Hanoi (Vietnam), there was a great deal of fluctuation in the formation of megaporocaprs. The matured megasporocarps were not seen; the sexual phase was dominated by the formation of microsporcarps and even in sporophyte presence of highest 18 microsporcarps was recorded. It is well known that in *Azolla* a megasporangium is always first to be formed and if a megaspore persists, the initials of microsporangia are suppressed and a megasporocarp develops (Konar and Kapoor 1974). In the triploid cytotype *A. pinnata* (Hanoi, Vietnam), it could be that due to lack of meiotic regularities, no functional megaspores developed and for which the initial cells of microsporangia were proliferated. This might be the reason for which only microsporocarps were abundantly observed. The microsporangia also did not set fertile microspores and instead of which abnormal microspores of different size (mostly sporelets) were formed.

While discussing the biology of life system in two cytotypes of *Marsilea minuta* through normal meiosis and sexual reproduction, the diploid cytotype is able to create genetic varibility which is conserved in the population (Loyal and Kumar 1979). During suppression of meiosis, the race is able to extend its vegetative phase and therefore competes favourably with its more aggressive triploid descendant. The triploid population has the efficient means of perennation. Through the present investigation similar analogies could be met which could be focussed in light of their findings as follows: The vegetative multiplication of Vietnam isolate is higher than other isolates of *A. pinnata* and secondly it is noticed at Cuttack condition that the green isolate of Vietnam is tolerant to high temperature (Singh 1979a). Thus, it has the ability to survive under unfavourable conditions. Reports on the International Network on soil Fertility and Fertilizer Evaluation for Rice (INSFFER) of study-tour on *Luzula* in Vietnam (Anonymous 1982) indicated that in natural condition the 'green-*Azolla*' was characterized by high yield, good quality and resistant ability to high temperature that made it wide-spread. The higher adaptive value of allopolyploids possessing environmental tolerance has also been proved (Dobzhansky 1951).

As regards the possible ancestor, the diploid cytotype of India and triploid cytotype of Vietnam are allopatric in their distribution, the former may not be the possible ancestor of triploid cytotype. However, the karyomorphological similarities of somatic chromosomes of these two cytotypes make it certain that the diploid cytotype of India must have resemblances with a similar cytotype occurring in Vietnam. There are 4 strains of *A. pinnata* in Vietnam, (i) purple, (ii) red, (iii) wild and (iv) green (Anonymous 1982). It is quite likely that the ancestor of triploid might be present in Vietnam and there must be karyomorphological resemblances among diploid cytotypes, one belonging to Cuttack (India) and another belonging to Vietnam. The cytology of other strains from Vietnam will reveal the fact. Recently, Le Duy Thanh (1983) has reported the chromosome number of *A. pinnata* in Vietnam as 2n=66 without mentioning the strain utilized, although chromosome number of *A. pinnata* (Vietnam, green strain) was earlier reported 2n=66 (Nayak and Singh 1981, 1982).

In Africa isolate of *A. pinnata*, the meiosis was found to be normal. The cause of presence of unequal sized microspores in some cases could not be explained satisfactorily. This isolate was also differing in various morphological parameters as regards the leaf branch pattern, leaf size, trichomes and stomata. The karyomorphological data suggested this isolate quite apart from those of Cuttack (India) isolate. Although Sweet and Hills (1971) basing on morphological features have included. *A. african* under *A. pinnata* var. *pinnata*, but taking into account of karyological and morphological dissimilarities, *A. pinnata*, Africa isolate could be given a species status such as *A. africana*.

Le Duy Thanh (1983) has reported the chromosome number of *A. mexicana* as 2n=40 to which the present investigation differed. The vigorous production of sporocarps in *A. mexicana* suggested that it was not sensitive to environmental variation since meiosis was not suppressed in this species. In comparison to *A. mexicana* other species were observed to be temperature sensitive where meiosis was supressed at higher temperature.

In *A. filiculoides*, the meiosis was normal but in some of SMC abnormal meiosis was recorded. Mehera and Singh (1957) have observed curved-spindle among some members of pteriodophytes. Mehera and Loyal (1959) have observed defective anaphase in *Marsilea*. According to them, the occurrence of both normal and abnormal SMC in same sporangium was not due to environment but perhaps due to lack of rigid genic control governing the proper formation and functioning of spindle. Meiotic irregularities in megaspore mother cells in *A. filiculoides* resulted in increase of microsporocarps. Le Duy Thanh (1983) has reported 2n=44 in *A. filiculoides* but present report was confirmity with that of Duncan (1940). Loyal (1958, 1972) has pointed out the basic number of *Azolla* as x=11 and established *A. pinnata* at tetraploid level. Basing on that report Singh *et al.* (1982) established *A. pinnata* (Vietnam isolate) to be a hexaploid. But it has been observed that by counting the secondary association of chromosomes from somatic metaphase, an association of 22 chromosomes occurred in maximum frequency in all isolates of *A. pinnata*. In *A. mexicana* and *A. filiculoides*, the secondary association comprised groups of 24 and 20 chromosomes respectively. Therefore, the basic number suggested for species as x=20 for *A. filiculoides*, x=22 for *A. pinnata* and x=24 for *A. mexicana*. The basic number x=20 existed in *Marsilea* (Mehera and Loyal 1959). Therefore, the nearest ally of *Azolla* would be *Marsilea*. The heterosporous genus *Regnellidium* has also basic number x=19 (Abraham *et al.* 1962, Loyal 1962). The basic number x=8 as reported by Mahabale (1963) in *A. pinnata* is cnfusing. The possible phylogram for *Azolla* is represented here by correlating the works of others (Fig. 8).

Summary

The somatic chromosome numbers (2n) were 48, 40, 44, 44 and 66 respectively in *A. mexicana*, *A. filiculoides*, *A. pinnata* Aftrica, *A. pinnata* Cuttack, India and *A. pinnata* (green isolate, Vietnam). The karyotype study revealed that chromosomes were small and asymmetrical. The total chromatin length (TLC) was employed as an index of species delimitation. Basing on absolute length (AL) the chromosomes were grouped into different sets and karyotypic formula was established for each species.

Meiotic behaviour of microspore mother cells (SMC) of all species was almost similar except *A. pinnata* (Vietnam) and *A. filiculoides*. In all cases microspore mother cells (SMC) exhibited many common features. Usually quarterets of SMC were observed on pressing. Vacuolisation put the cytoplasm more clear during early prophase and gradual granulation brought more stainability in later stages. Marginal orientation of chromatic materials was noticed. Multivalents were observed in *A. pinnata* (Vietnam). In *A. filiculoides*, deformed spindle and unequal spore formation were noticed in some of the SMC. In *A. pinnata* (Vietnam) almost all spores were much smaller in size. *A. mexicana*, *A. pinnata* (India), *A. filiculoides* and *A. pinnata* (Africa) were considered to be true diploids whereas *A. pinnata* (green isolate, Vietnam) was a triploid.

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References

Abraham, A., Ninan, C. A. and Mathew, P. N. 1962. Studies on cytology and phylogeny of the ptridophytes VII. Observations on one hundred species of South India-ferns. J. Indian Bot. Soc. **41**: 339–421.

Anonymous 1982. INSFFER-*Azolla*-Report. Inter. Rice Res. Inst., Los Banos, Manila, Philippines.

Bir, S. S. 1973. Cytology of Indian pteridophytes. In 'Glimpses in Plant Research' **1**: 28–119.

Dobzhansky, T. 1951. Genetics and the Origin of Species. Columbia University Press.

Duncan, R. E. 1940. The cytology of sporangium development in *Azolla filiculoides*. Bull. Torrey Bot. Club **67**: 391–412.

Konar, R. N. and Kapoor, R. K. 1972. Anatomical studies on *Azolla pinnata*. Phytomorphology **22**: 211–223.

La Cour, L. F. 1947. Improvement in plant cytological techniques II. Bot. Rev. **13**: 216–240.

Le Duy Thanh, 1983. Some cytogenetical features of *Azolla*. In XV International Genetics Congress. Dec. 12–21. New Delhi.

Loyal, D. S. 1958. Cytology of two species of Salviniaceae. Curr. Sci. **27**: 357–358.

— 1962. Abnormal sporelings of *Regnellidium diphyllum*, Lyndm. and chromosome number of the genus. Res. Bull. Punjap University (Science) **13**: 25–30.

— 1972. Chromosome size and structure in some heterosporous ferns with a bearing on evolutionary problems. Advancing Frontiers in Cytogenetics and Improvement of Plants (P. N. Kachroo, Ed.) pp. 293–299. Hindustan Publishing Corpn., New Delhi.

— and Grewal, R. K. 1966. Cytological study on sterility in *Salvinia auriculata* Aubelt, with a bearing on its reproductive mechanisms. Cytologia **31**: 330–338.

— and Kumar, K. 1979. The cytological observations on some natural population of *Marsilea minuta*, their reproductive mechanisms and evolutionary features. In Recent Researches in Plant Sciences. (S. S. Bir Ed.). Kalyani Publishers, New Delhi.

Lumpkin, T. A. and Plucknett, D. L. 1982. *Azolla* as a green manure: Westview Press Inc., USA.

Mahabale, T. S. 1963. Evolutionary tendencies in the genus *Azolla*. J. Indian Bot. Soc. **42**: 51–54.

Mehera, P. N. 1961. Cytological evolution of ferns with particular reference to Himalayan forms. Presidential address, Botany Section, 48th Indian Science Congress Association. Roorkee. pp. 1–24.

— and Loyal, D. S. 1959. Cytological studies in *Marsilea* with particular reference to *Marsilea minuta*. Research Bulletin of Punjab University (NS) **10** (III-IV): 357–374.

— and Singh, G. 1957. Cytology of Hymenophyllaceae. Journal Genetics **55**: 379–389.

Moore, A. W. 1969. *Azolla*: Biology and agronomical significance. Bot. Rev. **35**: 17–35.

Nayak, S. K. and Singh, P. K. 1981. Cytological studies on *Azolla* sps. In 69th Session of the Indian Science Congress Association. Mysore.

— and — 1982. Karyological studies on three species of *Azolla*. In Symposium 'Recent Trends in Botanical Research'. Department of Botany, Patna University, Patna.

Sharma, A. K. 1976. The Chromosomes. Oxford and IBH Publishing Co., New Delhi.

Singh, P. K. 1979a. Use of *Azolla* in rice production in India. In Nitrogen and Rice. pp. 407–418. International Rice Research Institute, Los Banos, Manila, Philippines.

— 1979b. Symbiotic algal N_2-fixation, and crop productivity. In C. P. Mallick (Ed.). Annual Review of Plant Sciences. pp. 37–58. Kalyani Publishers, New Delhi.

—, Patra, R. N. and Nayak, S. K. 1984. Sporocarp germination, cytology and mineral nutrition of *Azolla*. In Practical Application of *Azolla* for Rice Production. W. S. Silver and E. C. Schroder (Ed). Martinus Nijhoff. Pub. pp. 55–72. Netherlands.

Stebbins, G. L. 1959. Genes, Chromosomes and Evolution. W. B. Turrill, Ed. Pergamon, London.

Sweet, A. R. and Hills, L. V. 1971. A study of *Azolla pinnata* R. Br. American Fern Journal **61**(1): 129–172.