

## Chromosome numbers and taxonomic implications in the fern genus *Azolla* (*Azollaceae*)

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**Abstract:** Investigation of chromosome numbers of all *Azolla* species, and for the first time of hybrids, has been undertaken. Removal of wax from the leaf surface proved invaluable in achieving clear cytological preparations and providing unambiguous chromosome numbers. In contrast to previous records, the species *A. pinnata*, *A. filiculoides*, *A. filiculoides* var. *rubra*, *A. caroliniana*, *A. microphylla*, and *A. mexicana* were found to be  $2n=44$ , and *A. nilotica* to be  $2n=52$ . Several triploids ( $2n=66$ ) and one tetraploid ( $2n=88$ ) were identified. No geographical pattern could be observed in the distribution of triploids which probably derive from the function of unreduced gametes. The chromosome number of hybrids occasionally deviates from the diploid chromosome number ( $2n=44$ ). The small chromosome size limits karyotypic analysis and only differences in overall chromosome size can be observed. Taxonomic implications of chromosome numbers and sizes are discussed.

*Azolla* is a heterosporous aquatic fern which grows in symbiotic association with the cyanobacterium *Anabaena azollae* STRAS. which is able to fix atmospheric nitrogen. As a result, *Azolla* is an economically important fern, having long been used in south-east Asia as a green manure associated with wetland rice cultivation. This use of biological nitrogen-fixation to maintain fertility in paddy soils by means of *Azolla* and its endosymbiont has stimulated much research interest during the last decade. *Azolla* clones collected worldwide are cultured at the International Rice Research Institute (IRRI) and other research centres, where they are evaluated for nitrogen-fixing potential under different environmental conditions, and hybridization programmes to select potentially useful clones are in operation. Previous work by one of the authors has already drawn attention to a serious lack of basic knowledge in *Azolla*, together with a need for clarification of species delimitation and taxonomic relationships (FOWLER & STENNETT-WILLSON 1978, DUNHAM & FOWLER 1987 a). Despite recent published work employing morphological methods (TAN & al. 1986, DUNHAM & FOWLER 1987 b) available information is still inadequate for accurate identification of plants even at species level, and a stable

taxonomic framework with more precise species recognition has not yet emerged. This is mainly due to the small size of plants, the pattern of morphological variation and the phenotypic plasticity of the vegetative characters.

Based primarily on reproductive structures, seven extant species are currently described belonging to two sections. Taxonomic problems mainly involve the five closely related species of section *Azolla*: *A. filiculoides* LAM. which is indigenous to the American continent and has been introduced to Europe, S. Africa, China, Japan and southern Australia; *A. rubra* R. BR. which is usually regarded as a variety of *A. filiculoides*, is recorded only in Australia and New Zealand; *A. caroliniana* WILLD. which is indigenous to eastern United States but found elsewhere in N., C. and S. America, and Europe; *A. mexicana* PRESL which is distributed from northern S. America into N. America extending to British Columbia; and *A. microphylla* KAULF. which is reported from western and northern S. America, C. America and the West Indies. Taxonomic difficulties also exist in sect. *Rhizosperma* (MEY.) METT. where two species are currently recognized: *A. nilotica* DECNE. ex METT. which is found only in Africa from Egypt to S. Africa; and *A. pinnata* R. BR. which is distributed widely in tropical Africa, Australasia and SE. Asia. *A. pinnata* includes two varieties: var. *pinnata* and var. *imbricata* (ROXB. ex GRIFF.) BONAP. which NAKAI (1925) elevated to specific level.

Consequently, attention has been focused on the cytology of the genus with the hope of resolving the complex taxonomic problems. With the exception of *A. nilotica* and *A. rubra*, chromosome counts are already available for all other species, but the chromosome numbers recorded are contradictory, poorly illustrated and often based on the examination of only a single clone (LITARDIÈRE 1921; TSCHERMAK-WOESS & DOLEŽAL-JANISCH 1959; LOYAL 1958, 1975; LOYAL & al. 1982; SINGH & al. 1984; THÀNH 1984; LIN & SLEEP 1988; NAYAK & SINGH 1989). The variety of chromosome numbers recorded in the literature can only be explained by the technical difficulties that *Azolla* presents as cytological material. All the above studies reveal several problems which cause considerable difficulty in the establishment of unequivocal counts and in the successful undertaking of a wide cytological investigation. These problems include the minute and numerous chromosomes compounded by the delicate nature of the roots.

The differences in chromosome numbers previously recorded has indicated the need for a careful cytological survey of *Azolla*. This information would not only improve taxonomic knowledge of the genus, but would also provide data for selection of clones suitable for use in hybridization programmes. Preliminary results of this investigation have been published (STERGIANOU & FOWLER 1989). The purpose of the present paper is to document in greater detail the chromosome numbers of *Azolla* clones, and for the first time of artificially obtained hybrids, and to interpret the patterns of morphological and chromosomal variation.

### Materials and methods

The complete list of specimens examined is given in Tables 1 and 2, with details of origin. With a few exceptions, all clones listed originate from *Azolla* germplasm collections maintained at the International Rice Research Institute, Philippines, and the Biological Nitrogen Fixation (BNF) Resource Center for South and Southeast Asia, Thailand. All hybrids listed in Table 2 were obtained from the *Azolla* Research Laboratory, University of the Philippines at Los Baños (UPLB) and from IRRI. The specimens have also been established

Table 1. List of *Azolla* specimens studied, origins and chromosome numbers. *BNF* Biological Nitrogen Fixation Center for South and Southeast Asia, Thailand; *CIAT* International Centre for Tropical Agriculture, Colombia; *CRRI* Central Rice Research Institute, India; *DA NEPAL* Department of Agriculture, Nepal; *DA VIETNAM* Department of Agriculture, Vietnam; *FUJIAN AAS [FAAS]* Fujian Academy of Agricultural Sciences, China; *HUNAN AAS* Hunan Academy of Agricultural Sciences, China; *IB CHINA* Institute of Botany, Academia Sinica, Beijing, China; *IITA* International Institute for Tropical Agriculture, Nigeria; *IRRI* International Rice Research Institute, Philippines; *NIAB* Nuclear Institute for Agriculture and Biology, Pakistan; *ORSTOM* Office de la Recherche Scientifique et Technique Outre-Mer, Senegal; *PPBS* School of Biological Sciences, Portsmouth Polytechnic, U.K.; *UCL* Université Catholique de Louvain, Belgium; *UPLB* University of the Philippines at Los Baños

Accession no.	Origin	Collector & date	Chromosome no.
<b><i>A. pinnata</i> var. <i>imbricata</i> (as recognized in the IRRI collection)</b>			
IRRI 2	Malaysia: Butterworth	IRRI, 1977	2n = 44
IRRI 4	Philippines: Ifugao	IRRI, 1977	2n = 44
IRRI 5	Thailand: Bangkok	LUMPKIN TA, 1977	2n = 66
IRRI 13	Nepal: Lalitpur	DA NEPAL, 1978	2n = 44
IRRI 20	Vietnam: La Van, Thai Vinh	DA VIETNAM, 1979	2n = 44
IRRI 22	China: Tancheng, Shandong Prov.	FUJIAN AAS, 1979	2n = 44
IRRI 23	India: Cuttack, Orissa	CRRI, 1978	2n = 44
IRRI 29	China: Changsha, Hunan Prov.	HUNAN AAS, 1980	2n = 66
IRRI 39	Australia: Griffith, NSW	IRRI, 1980	2n = 44
IRRI 49	China: Fuzhou, Fujian Prov.	FUJIAN AAS, 1982	2n = 44
IRRI 56	Pakistan	NIAB, 1982	2n = 44
IRRI 64	Sri Lanka: Hunnasgiriya	KULASOORIYA S, 1984	2n = 66
IRRI 75	Indonesia: Cupak, Barut Sumatra	LUMPKIN TA, 1984	2n = 44
IRRI 76	Indonesia: Loana, Selabam Sulawesi	LUMPKIN TA, 1984	2n = 44
IRRI 80	Nigeria: IITA	LUMPKIN TA, 1984	2n = 66
IRRI 84	Japan: Okinawa	LUMPKIN TA, 1984	2n = 44
IRRI 501	Zaire: Ndjili	VINCKE PP, 1977	2n = 44
PPBS 1	Thailand: NE. of Bangkok	BOGNER J, 1971	2n = 88
BNF NE 5	Thailand: Sakolnakorn, Muang	BNF	2n = 44
BNF NE 13	Thailand: Nakornrachasima, Muang	BNF	2n = 66
BNF NE 14	Thailand: Nakornrachasima, Jor-hor	BNF	2n = 66
BNF NE 8/16	Thailand: Ubon, Warinchamrab	BNF	2n = 44
BNF NE 17/19	Thailand: Chacherng sao, Muang	BNF	2n = 44
BNF NE 1001	Thailand: Ubon, Kerng nai	BNF	2n = 66
BNF NE 1002	Thailand: Yasothon, Muang	BNF	2n = 44
BNF N 1001	Thailand: Prae, Muang	BNF	2n = 44
BNF BK 5	Thailand: Bangkok	BNF	2n = 66
BNF C 1001	Thailand: Patumthani, Lamlookka	BNF	2n = 44
BNF C 1002	Thailand: Saraburi, Muang	BNF	2n = 44
BNF C 1003	Thailand: Ayuthaya, Maharach	BNF	2n = 44
BNF C 1004	Thailand: Anghong, Pamoek	BNF	2n = 44
BNF C 1005	Thailand: Ayuthaya, Muang	BNF	2n = 44

Table 1 (continued)

Accession no.	Origin	Collector & date	Chromosome no.
BNF C 1006	Thailand: Patumthani, Muang	BNF	2 n = 44
BNF C 1007	Thailand: Ayuthaya, Muang	BNF	2 n = 44
BNF C 1009	Thailand: Singburi, Intraburi	BNF	2 n = 44
BNF C 1010	Thailand: Nakornpathom, Watbanglan	BNF	2 n = 44
BNF C 1011	Thailand: Nakornpathom, Klongraimai	BNF	2 n = 66
BNF C 1015	Thailand: Chainat, Muang	BNF	2 n = 66
BNF C 1016	Thailand: Bangkok, Facult. Educ.	FOWLER K, 1989	2 n = 66
BNF C 1017	Thailand: Bangkok, Wat Tava	FOWLER K, 1989	2 n = 66
BNF C 1018	Thailand: Bangkok, Airport Hotel	FOWLER K, 1989	2 n = 66
BNF SKI	Sri Lanka	BNF	2 n = 66
<b>A. pinnata var. pinnata (as recognized in the IRRI collection)</b>			
IRRI 7001	Australia: Kakadu Natl. Park, N.T.	YATAZAWE, 1982	2 n = 66
IRRI 7006	Ivory Coast	IITA, 1980	2 n = 44
IRRI 7007	Senegal	ORSTOM, 1980	2 n = 44
IRRI 7015	Madagascar: Maharirana	SCHRAMM, 1986	2 n = 44
IRRI 7503	Niger: Niamey	BOUDOURESQUE, 1980	2 n = 44
IRRI 7517	Botswana: Okavango	SMITH PA, 1984	2 n = 44
IRRI 7526	Rwanda: Kirirambogo	VAN HOVE C, 1985	2 n = 44
PPBS 2	Kenya: Eldoret, Rift Valley Prov.	MUGWERU RN, 1988	2 n = 44
<b>A. nilotica</b>			
IRRI 5001	Sudan: Kosti	LUMPKIN TA, 1982	2 n = 52
IRRI 5002	Sudan: Kosti	LUMPKIN TA, 1984	2 n = 52
IRRI 5501	Burundi: Bujumbura	BOUHARMONT J, 1978	2 n = 52
<b>A. filiculoides</b>			
IRRI 1001	E. Germany	IB China, 1979	2 n = 44
IRRI 1007	U.S.A.: Walker Lake, Nevada	RAINS DW, 1981	2 n = 44
IRRI 1010	Peru: Lima	CIAT, 1982	2 n = 44
IRRI 1510	Mexico: Hidalgo	FERRERA-CERRATO R, 1984	2 n = 44
IRRI 1536	Bolivia: Titicaca	CHARLIER S, 1985	2 n = 66
IRRI 1540	Uruguay: Montevideo	ESKEW DL, 1985	2 n = 44
PPBS 101	U.K.: Garden Centre, Hants.	LOVERIDGE R	2 n = 44
PPBS 102	U.K.: Fordingbridge, Hants.	FOWLER K, 1982	2 n = 44
PPBS 103	U.K.: Romsey, Hants.	DUNHAM DG, 1983	2 n = 44
PPBS 104	U.K.: Univ. Coll. N. Wales, Bangor	DUNHAM DG, 1983	2 n = 44
IRRI 6003	Japan: Tanabe, Tsuzuki, Kyoto	LUMPKIN TA, 1984	2 n = 44
IRRI 6007	Japan: Chikugo, Furuoka	WATANABE I, 1986	2 n = 44
IRRI 6008	Japan: Matsue	WATANABE I, 1986	2 n = 44
<b>A. rubra</b>			
IRRI 6502	Australia: Victoria (37°40" S/144°40" E)	CORRICK MG, 1985	2 n = 44
IRRI 6503	New Zealand: near Kingston, S. Island	VAN HOVE C, 1986	2 n = 44
IRRI 6504	New Zealand: Hunterville, N. Island	VAN HOVE C, 1986	2 n = 44

Table 1 (continued)

Accession no.	Origin	Collector & date	Chromosome no.
<b><i>A. mexicana</i></b>			
IRRI 2001	U.S.A.: Graylodge, California	RAINS DW, 1978	2 n = 44
IRRI 2003	Guyana	RAINS DW, 1981	2 n = 44
IRRI 2007	U.S.A.	WATANABE I, 1987	2 n = 44
IRRI 2008	Colombia: Cali	CIAT, 1982	2 n = 44
IRRI 2009	Brazil: Parana	LUMPKIN TA, 1984	2 n = 44
IRRI 2011	Japan: Osaka	LUMPKIN TA, 1984	2 n = 44
IRRI 2012	Colombia: Monteria	ZIMMERMAN WJ, 1985	2 n = 44
IRRI 2019	Colombia: Letioia	ZIMMERMAN WJ, 1985	2 n = 44
IRRI 2021	U.S.A.: Lake Alice, Florida	ZIMMERMAN WJ, 1985	2 n = 44
<b><i>A. caroliniana</i></b>			
IRRI 3001	U.S.A.: Ohio	RAINS DW, 1978	2 n = 44
IRRI 3002	U.S.A.: Madison, Wisconsin	RAINS DW, 1981	2 n = 44
IRRI 3004	Uruguay: Treinta y tres	RAINS DW, 1982	2 n = 44
IRRI 3006	Brazil: Solimoes River, Manaus	LUMPKIN TA, 1984	2 n = 44
IRRI 3012	Brazil: Guaiba, Rio Grande do Sul	LUMPKIN TA, 1984	2 n = 66
IRRI 3014	Brazil: Negro River, Catalao, Amazonas	LUMPKIN TA, 1984	2 n = 44
IRRI 3017	Brazil: Rio Grande do Sul	WATANABE I, 1987	2 n = 44
IRRI 3506	Brazil: Marajo Island	VAN HOVE C, 1983	2 n = 44
IRRI 3510	Mexico: Tabasco	FERRERA-CERRATO R, 1984	2 n = 66
IRRI 3512	Peru: Zana	RAMIREZ N, 1984	2 n = 44
IRRI 3516	U.S.A.: Township	SCHMITT S, 1984	2 n = 44
IRRI 3522	Guyana: Burma	VAN HOVE C, 1987	2 n = 44
IRRI 3525	Rwanda: Cyili	UCL, 1985	2 n = 44
PPBS 301	Netherlands: Heerenveen, Friesland	FOWLER K, 1987	2 n = 44
PPBS 302	Netherlands: Heerenveen, Friesland	FOWLER K, 1987	2 n = 44
PPBS 304	Netherlands: Wolvega, Friesland	FOWLER K, 1987	2 n = 44
<b><i>A. microphylla</i></b>			
IRRI 4001	Paraguay	RAINS DW, 1981	2 n = 44
IRRI 4014	Paraguay	RAINS DW, 1981	2 n = 44
IRRI 4018	Paraguay	RAINS DW, 1981	2 n = 44
IRRI 4021	Ecuador: Santa Cruz, Galapagos Islands	LUMPKIN TA, 1982	2 n = 44
IRRI 4024	Ecuador: Galapagos Islands	GUNAPALA, 1985	2 n = 44
IRRI 4054	Brazil: Baia	WATANABE I, 1987	2 n = 44
IRRI 4059	Philippines: Koronadal, South Cotobato	OLIVEROS, 1988	2 n = 44
IRRI 4069	Paraguay: Campo Esperanza, Roma Plata	WATANABE I, 1988	2 n = 44
IRRI 4075	Paraguay: Logo Canada, Nuevo Italia	WATANABE I, 1988	2 n = 44
IRRI 4501	Brazil: Paraiso do Norte	FOIRE MF, 1983	2 n = 44
IRRI 4504	Ecuador: Galapagos Islands	LUMPKIN TA, 1983	2 n = 44
IRRI 4506	Mexico: Sinaloa	FERRERA-CERRATO R, 1984	2 n = 66
IRRI 4507	Guyana: Corriverton	VAN HOVE C, 1987	2 n = 44

Table 2. List of artificial *Azolla* hybrids studied, origins and chromosome numbers

Accession no.	Parentage	Origin/date	Chromosome no.
IRRI 2024	<i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)	UPLB —	2 n = 44
IRRI 2027	<i>A. mexicana</i> (IRRI no. 2002) × (IRRI no. 2024-3) [ <i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)]	UPLB —	2 n = 45
IRRI 4028	<i>A. filiculoides</i> × <i>A. microphylla</i>	IRRI, 1985	2 n = 44
IRRI 4030	<i>A. filiculoides</i> × <i>A. microphylla</i>	IRRI, 1985	2 n = 44
IRRI 4052	<i>A. microphylla</i> × <i>A. filiculoides</i>	FAAS, 1987	2 n = 44
IRRI 4083	<i>A. microphylla</i> (IRRI no. 4018) × <i>A. filiculoides</i> (IRRI no. 1009-3)	UPLB —	2 n = 45
IRRI 4084	<i>A. microphylla</i> (IRRI no. 4018) × <i>A. caroliniana</i> (IRRI no. 3004-1)	UPLB —	2 n = 46
IRRI 4085	<i>A. microphylla</i> (IRRI no. 4003) × (IRRI no. 2024-3) [ <i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)]	UPLB —	2 n = 46
IRRI 4086	<i>A. microphylla</i> (IRRI no. 4003) × <i>A. caroliniana</i> (IRRI no. 3004-1)	UPLB —	2 n = 45
— —	<i>A. microphylla</i> (IRRI no. 4018) × <i>A. caroliniana</i> (IRRI no. 3004-3)	UPLB —	2 n = 46
— —	<i>A. mexicana</i> (IRRI no. 2002) × (IRRI no. 2024-6) [ <i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)]	UPLB —	2 n = 44
— —	<i>A. mexicana</i> (IRRI no. 2002) × (IRRI no. 2024-7) [ <i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)]	UPLB —	2 n = 47
— —	IRRI no. 2024-1 [ <i>A. microphylla</i> (IRRI no. 4018) × <i>A. mexicana</i> (IRRI no. 2001)] × <i>A. microphylla</i> (IRRI no. 4018)	UPLB —	2 n = 45

at Portsmouth Polytechnic since September 1988 as two duplicate collections: one is maintained in tapwater-soil culture in a greenhouse and the other in nitrogen-free IRRI medium (WATANABE & al. 1977) within a Fisons Fi-totron 600 H growth cabinet. This medium is changed every three weeks and maintained with the following selected conditions: 12 h day/12 h night cycle at 26 °C/18 °C; light intensity 18 klux; relative humidity 70–80%. Herbarium specimens of all plants examined are deposited in the Cryptogamic Herbarium, Natural History Museum, London.

As a result of the adaptation of *Azolla* to a floating aquatic life, problems were encountered in addition to those recorded in the literature. These problems were the slow growth of the roots with a low rate of cell division, and the impermeability of leaves to water solutions. To overcome these difficulties, the shoot tip, which includes the apical meristem enclosed by overlapping leaf lobes, was used in preference to root tips because it has a larger meristem and higher meristematic activity. The impermeability of the leaf resulted in the failure of spindle disruption using the normal 3–4 h chemical pretreatment in either colchicine, hydroxyquinoline or α-bromonaphthalene. This failure was found to be due to the presence of wax on the leaf surface which was acting as an antiwetting agent, preventing chemical absorption. A method for the removal of the wax was developed using detergents: several anionic, cationic and non-ionic detergents were tested in various combinations of concentration, duration of treatment and temperatures, but the most successful treatment was found to be 1% Tween 80 (polyoxyethylene sorbitan mono-oleate). The leaves were incubated three times in an aqueous solution of 1% Tween 80 for 5 min in a shaker (1 cycle/sec) at 20–25 °C. After each incubation they were thoroughly rinsed with distilled water. Effective treatment varies according to the *Azolla* species, possibly due to variation in the amount of wax on their leaves. *A. rubra* requires four washes with Tween 80 at 20 °C, whereas *A. filiculoides*, *A. caroliniana*, *A. mexicana*, and *A. microphylla* require three washes and *A. pinnata* and *A. nilotica* only two. Following the above treatment, the leaves were incubated in 0.002 M hydroxyquinoline with 0.1% Tween 80 for 6 h at 18 °C, and shaken (1 cycle/sec). Although the treatment with Tween 80 solves the problem of chemical penetration, it is very critical: a slight deviation from the optimum Tween 80 concentration, incubation time, temperature or inadequate rinsing can be either detrimental to the tissue or can result in inadequate chemical penetration. However, when the optimum conditions of treatment for each species were selected, a high number of well spread c-metaphases was observed in squash preparations enabling a successful cytological investigation of *Azolla*.

After the Tween 80 treatment the usual procedures for chromosome squash preparations were followed. The leaves were fixed in 1 : 3 glacial acetic acid : absolute ethanol. Following at least 12 h fixation, the material was hydrolysed in 1 M HCl at 60 °C for 6 min, stained in Feulgen for 1 h, and squashed in acetocarmine. The staining of *Azolla* chromosomes even after using the combination of Feulgen and acetocarmine squash is not ideal. The slides were occasionally warmed in a flame with more drops of acetocarmine along the edges of the coverslip until a fairly acceptable staining was achieved.

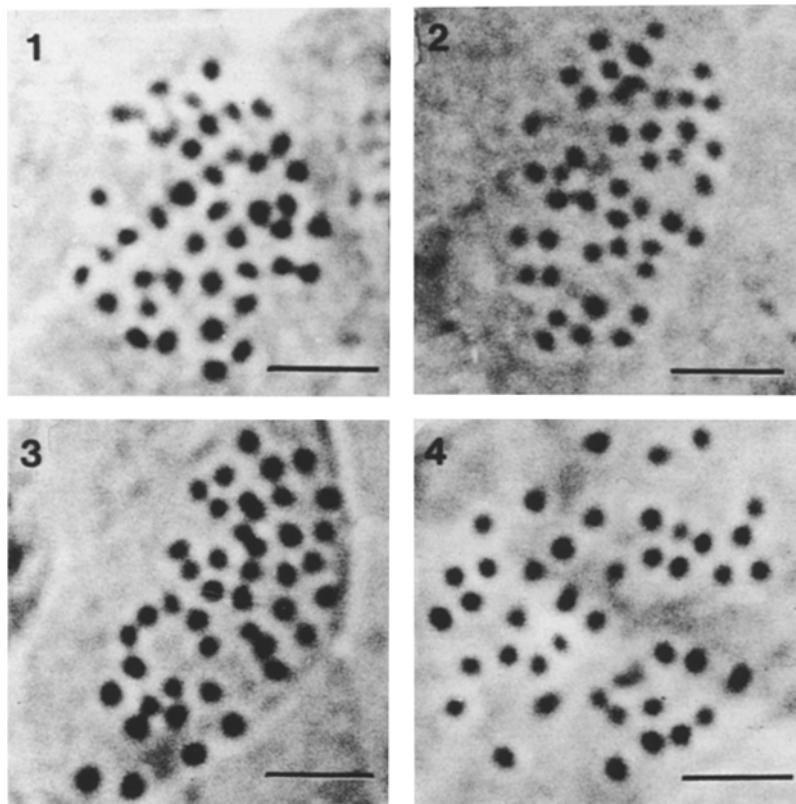
The results were repeatedly verified by examining ten or more cells from each plant. Photographs of c-metaphases of species and hybrids are presented in Figs. 1–15 and the chromosome numbers are presented in Tables 1 and 2.

## Results

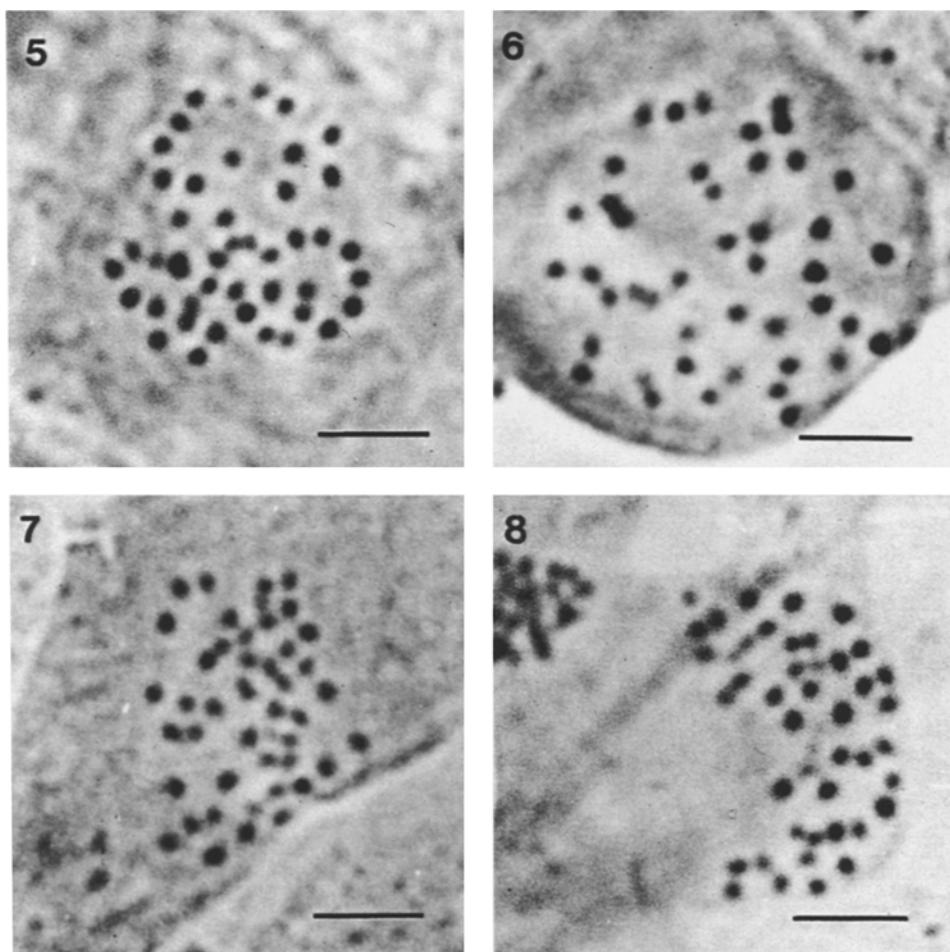
All plants of natural origin examined in this study were euploid, with a base number of  $n=22$  for *A. filiculoides*, *A. rubra*, *A. caroliniana*, *A. microphylla*, *A. mexicana*, and *A. pinnata*, and  $n=26$  for *A. nilotica*. These results contradict most previous publications which record differences in the chromosome number between species within sect. *Azolla*. To investigate if the cause of recorded differences in chromosome number was a result of intraspecific variation, 108 collections from representative locations were chromosome counted. With the exception of 19 triploids and one tetraploid, no other intraspecific variation was found. The chromosome number of hybrids, however, was found to deviate from the diploid chromosome number  $2n=44$ . Using the normal pretreatment methods it is extremely difficult to obtain unambiguous chromosome counts in *Azolla* and this is probably the cause of the

differences recorded by previous authors. The triploids occurred mostly in *A. pinnata*, although *A. filiculoides*, *A. microphylla*, and *A. caroliniana* were also represented. A single tetraploid specimen of *A. pinnata* was also identified. The chromosome number of hybrids was often found to be different from the diploid number ( $2n=44$ ).

**Karyotypes.** The minute size of the chromosomes limits the use of karyological studies in *Azolla*. Arm ratios cannot be calculated and only the gross chromosome morphology can be used for karyotype distinction. The size of all chromosomes is less than 1  $\mu\text{m}$  with the largest around 0.8  $\mu\text{m}$  in sect. *Azolla* and 0.5  $\mu\text{m}$  in *A. pinnata*. With the exception of *A. rubra* which has the largest chromosomes, all other species of sect. *Azolla*, namely *A. filiculoides*, *A. caroliniana*, *A. microphylla*, and *A. mexicana*, appear cytologically uniform in having the same chromosome number and similar chromosome size (Figs. 1–6). In sect. *Rhizosperma*, *A. pinnata* var. *imbricata* and *A. pinnata* var. *pinnata* have very similar karyotypes; both have very small chromosomes which are much smaller than in all other *Azolla* spp. (Figs. 9, 11, and 12). The only distinct karyotype was observed in *A. pinnata* var. *pinnata* (specimen IRRI 7001) from Australia. This plant was found to be triploid, with one pair of chromosomes considerably larger than the rest (Figs. 14 and 15); this



Figs. 1–4. Mitotic metaphases in *Azolla* sect. *Azolla* ( $2n=44$ ). Bars: 3  $\mu\text{m}$ . — Fig. 1. *A. rubra* from New Zealand (IRRI accession no. 6504). — Fig. 2. *A. filiculoides* from Japan (IRRI 6007). — Fig. 3. *A. filiculoides* from U.S.A. (IRRI 1007). — Fig. 4. *A. caroliniana* from Uruguay (IRRI 3004).



Figs. 5—8. Mitotic metaphases of species and hybrids in *Azolla* sect. *Azolla*. Bars: 3  $\mu\text{m}$ . — Fig. 5. *A. microphylla* ( $2n=44$ ) from Paraguay (IRRI accession no. 4018). — Fig. 6. *A. mexicana* ( $2n=44$ ) from U.S.A. (IRRI 2007). — Fig. 7. *A. mexicana* (IRRI 2002)  $\times$  [ *A. microphylla* (IRRI 4018)  $\times$  *A. mexicana* (IRRI 2001) ] ( $2n=47$ ) raised in UPLB (University of the Philippines at Los Baños). — Fig. 8. *A. mexicana* (IRRI 2002)  $\times$  [ *A. microphylla* (IRRI 4018)  $\times$  *A. mexicana* (IRRI 2001) ] ( $2n=45$ ) raised in UPLB (IRRI 2027)

bimodal karyotype appears to be unique for the genus. With the exception of *A. pinnata* var. *pinnata* (IRRI 7001), there is a continuous range in the chromosome size of the karyotypes of all species, with the largest chromosome being almost twice the size of the smallest (Figs. 1—6, 9, and 10).

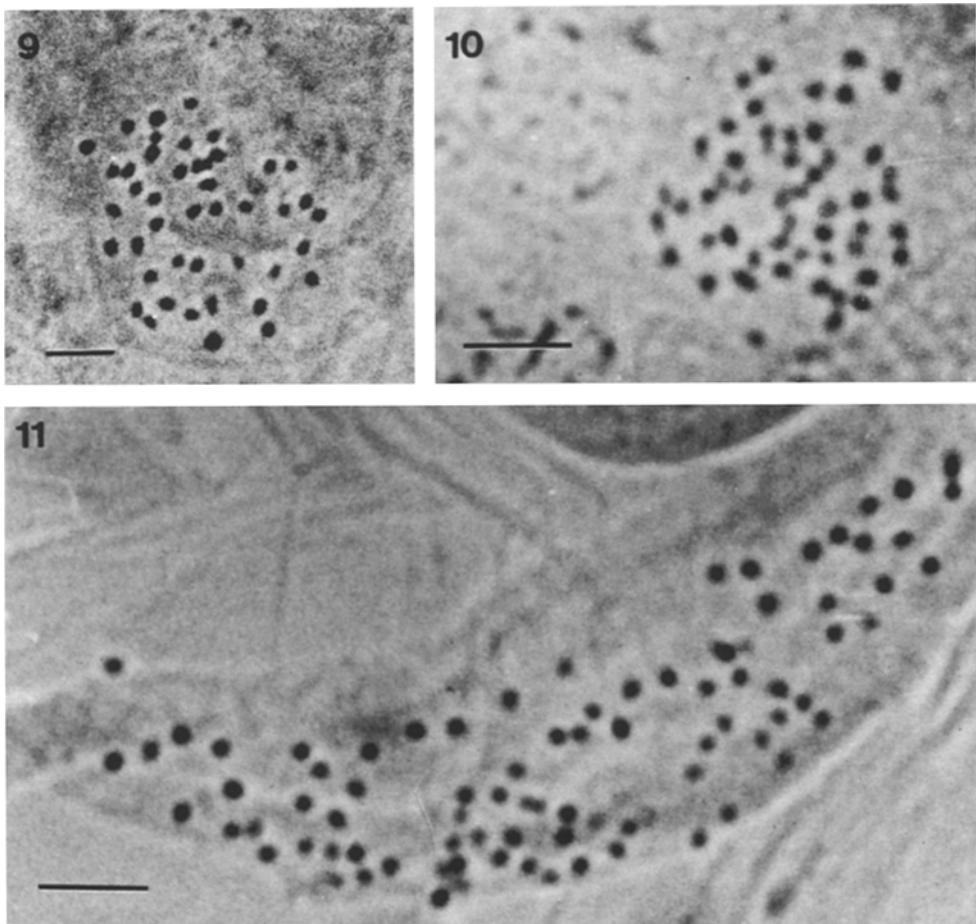
NAYAK & SINGH (1989) grouped the chromosomes into sets according to size and recorded differences between species of sect. *Azolla* in both the number of sets and in the "shape" of chromosomes. It is known that the shape of chromosomes can vary between preparations of the same plant; this depends only on the mitotic stage the chromosomes had reached at the time of fixation. Our experience indicates that the grouping of such small chromosomes into sets based on size can only lead to error, since there is always differential contraction of chromosomes. Furthermore,

as already mentioned, *Azolla* exhibits more or less continuous variation in chromosome size.

**Chromosome counts.** In a preliminary report (STERGIANOU & FOWLER 1989) the authors gave chromosome numbers for the seven species of *Azolla* currently recognized; such information on *A. nilotica* and *A. rubra* had not previously been published.

*A. nilotica*. Three clones from C. Africa were examined and all were found to be  $2n=52$  (Fig. 10). This chromosome number is unique for the genus and no other records have previously been published.

*A. pinnata* var. *imbricata*. LOYAL (1958) reported  $n=22$ , later confirming this using vegetative material from roots (LOYAL 1975) and from shoot tips (LOYAL & al. 1982). THÀNH (1984) indicated  $2n=66$ ; SINGH & al. (1984) and NAYAK & SINGH (1989) reported  $2n=44$  and  $2n=66$ . STERGIANOU & FOWLER (1989) reported  $2n=44$  for *A. pinnata* var. *imbricata* together with the occurrence of triploids ( $2n=66$ ) and one tetraploid ( $2n=88$ ). Since tetraploid and triploid clones were

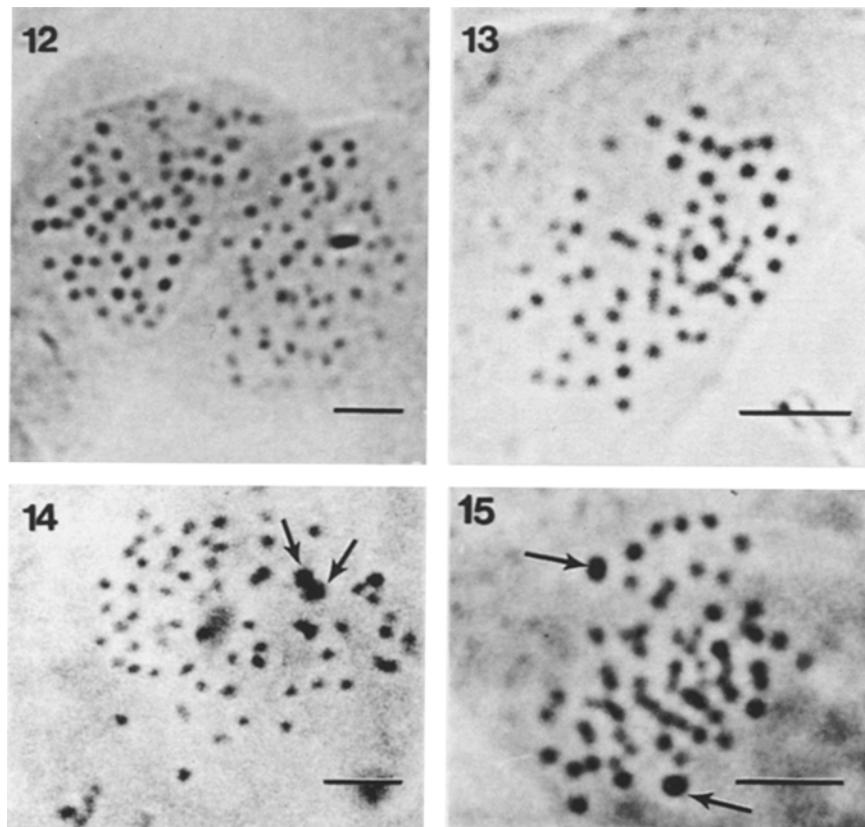


Figs. 9–11. Mitotic metaphases in *Azolla* sect. *Rhizosperma*. Bars: 3  $\mu\text{m}$ . – Fig. 9. *A. pinnata* var. *imbricata* ( $2n=44$ ) from Malaysia (IRRI accession no. 2). – Fig. 10. *A. nilotica* ( $2n=52$ ) from Sudan (IRRI 5001). – Fig. 11. Tetraploid *A. pinnata* var. *imbricata* ( $2n=88$ ) from Thailand (PPBS accession no. 1).

found in the Bangkok region of Thailand, more plants from this area were chromosome-counted in order to determine the origin of triploids and to ascertain whether more tetraploids occurred. 23 populations were sampled from which 14 diploids and nine triploids were identified; no further tetraploid populations were found. In the present study a total of 27 diploids ( $2n=44$ ), 14 triploids ( $2n=66$ ) and one tetraploid ( $2n=88$ ) were identified as *A. pinnata* var. *imbricata* (Figs. 9, 11, and 12). Triploids were mainly found in the Bangkok region where more plants were examined, but also occurred in China, Sri Lanka, and Nigeria.

*A. pinnata* var. *pinnata*. Seven diploids ( $2n=44$ ) and one triploid ( $2n=66$ ) (Figs. 14 and 15) are included in the present study. No counts for this variety of *A. pinnata* are previously reported in the literature.

*A. filiculoides*. 13 clones from N. and S. America, Europe, and Japan were chromosome counted in the present study. These include 12 diploids ( $2n=44$ ) (Figs. 2 and 3) and one triploid ( $2n=66$ ). The  $2n=44$  count for *A. filiculoides* presented here agrees with a mitotic count of  $2n=44$  reported by THÀNH (1984) and a meiotic count of  $n=22$  from a Chinese clone which is well illustrated with a photograph (LIN & SLEEP 1988). The above report differs from the  $2n=40$



Figs. 12–15. Triploids in *Azolla* ( $2n=66$ ). Bars: 3 µm. — Fig. 12, *A. pinnata* var. *imbricata* from Thailand (BNF accession no. NE 1001). — Fig. 13, *A. caroliniana* from Mexico (IRRI 3510). — Figs. 14 and 15, *A. pinnata* var. *pinnata* from Australia (IRRI 7001). Arrows indicate the pair of relatively large chromosomes which characterises this karyotype

reported by SINGH & al. (1984), THÀNH & HANG (1988) and NAYAK & SINGH (1989); however, these results are not illustrated with photographic evidence.

*A. rubra*. The chromosome number of three clones from Australia and New Zealand was  $2n=44$  (Fig. 1). There is no previous published record of a chromosome count for this species.

*A. microphylla*. 13 plants were found to be diploids with  $2n=44$  (Fig. 5), and one triploid of Mexican origin with  $2n=66$ . THÀNH & HANG (1988) recorded  $2n=48$  for this species.

*A. caroliniana*. 16 plants from N. and S. America and Europe were examined. 14 were found to be diploids with  $2n=44$ , and two triploids with  $2n=66$  from Brazil and Mexico were identified (Figs. 4 and 13). This report disagrees with LITARDIÈRE (1921) who recorded  $2n=48$  based on sectioned material. TSCHERMAK-WOESS & DOLEŽAL-JANISCH (1959) confirmed the count of LITARDIÈRE, but do not provide any illustration. A count of  $2n=42$  has been recorded by THÀNH & HANG (1988).

*A. mexicana*. All nine plants examined in the present study were diploids with  $2n=44$  (Fig. 6). This chromosome record agrees with a report by THÀNH & HANG (1988) but differs from previous reports of  $2n=48$  (SINGH & al. 1984, NAYAK & SINGH 1989) and  $2n=40$  by THÀNH (1984).

**Hybrids.** The chromosome numbers of artificially obtained hybrids in sect. *Azolla* were investigated for the first time. Four out of eight first generation hybrids and three out of four second generation hybrids show deviation from the normal diploid chromosome number of  $2n=44$ . These include aneuploids with  $2n=45$ ,  $2n=46$ , and  $2n=47$  (Figs. 7 and 8).

## Discussion

**Taxonomic implications.** Extensive investigation of the chromosome numbers of the genus *Azolla* has provided considerable data for discussion. The basic number in all species of sect. *Azolla* and *A. pinnata* of sect. *Rhizosperma* is  $n=22$ , whereas the only different count of the genus is  $n=26$  recorded in *A. nilotica*. This finding is particularly interesting with respect to the grouping of *A. pinnata* with *A. nilotica* in sect. *Rhizosperma*. The chromosome number clearly separates *A. nilotica* from all other species, and indicates that *A. pinnata* might have closer phylogenetic affinities with species of sect. *Azolla* than with *A. nilotica*. Classification proposals based solely on cytological evidence are untenable, but there is additional morphological and other evidence to support the information from chromosome numbers.

Due to its distinctive morphological features, *A. nilotica* has never been confused with other taxa or been given any other name. In comparison with other members of the genus, it has a very large sub-erect frond, often reaching 40 cm in length with long internodes, a complex amphiphloic siphonostelic vascular system consonant with its large size, and large roots growing in fascicles. Our own observations on the above features support those of LUMPKIN & PLUCKNETT (1982). Distinctive features of the reproductive structures include sporocarps occurring in groups of four, rather than in pairs as in all other species, sporoderm sculpture and structure and the nature of massula processes. METTENIUS (1867) gives a very comprehensive protologue for this species; DEMALSY (1953) and LUMPKIN (1981) provide morpho-

anatomical accounts. The morphology of the megasporangium apparatus and features of the sporoderm are described by FOWLER & STENNETT-WILLSON (1978). *Azolla* possesses a unique megasporangium with a number of pseudocellular structures called floats attached proximally. The floats, which do not endow buoyancy, are structurally similar to the massulae which contain the microspores. Species in sect. *Azolla* have three floats whereas those in sect. *Rhizosperma* have nine floats. It is on the basis of float number and type of massula process that *A. nilotica* and *A. pinnata* are grouped together in sect. *Rhizosperma* (METTENIUS 1847, 1867).

*Azolla* spp. often appear to grow just as well on a stable substrate, such as wet mud, as free floating on water. However, whereas most species seem well adapted to a floating existence, *A. nilotica* prefers a more stable environment since any physical disturbance soon leads to fragmentation and death. This vulnerability and differing ecological preference is due to its size and more complex morphology resulting from its phylogenetic history. Although the genus has a long evolutionary history, the geological record is based mainly on reproductive structures, well preserved vegetative remains being relatively scarce. However, vegetative remains of the extinct *A. schoppii* DIJK., dating from the Cretaceous to Palaeocene of N. America (SWEET & CHANDRASEKHARAM 1973), appears to show closer morphological resemblance to *A. nilotica* than to any other extant species. This tends to support the view that the sub-erect habit of *A. nilotica* may possibly be a primitive character and the evolutionary trend is towards a reduction in the size of plants which are better adapted to an aquatic existence. This trend might also be correlated with the general concept of chromosome evolution where the small aquatic plants of sect. *Azolla* and *A. pinnata* of sect. *Rhizosperma* have reduced the haploid chromosome number from  $n=26$  to  $n=22$ . Cytological and morphological evidence, together with some support from habitat preference, suggests that *A. nilotica* is distinct from all other *Azolla* spp. and should be placed in a separate section of its own.

Although the chromosome number of *A. pinnata* indicates certain affinities with the species of sect. *Azolla*, morphological and cytological differences clearly separate it as a distinct species. It has alternative branching pattern, nine floats rather than three in the megasporangium apparatus and the smallest chromosomes to be found in the genus. However, as indicated previously, *A. pinnata* has a very wide distribution and two varieties are currently recognized. The chromosomes of *A. pinnata* var. *imbricata* and *A. pinnata* var. *pinnata* from Africa are found to be very similar. The only Australian *A. pinnata* var. *pinnata* which was examined was found to be triploid (IRRI 7001), with a pair of very large chromosomes (Figs. 14 and 15). It remains to be discovered whether this plant with the bimodal karyotype derives from or represents Australian *A. pinnata* var. *pinnata* in general and whether this chromosomal variant corresponds with sufficient morphological distinction to merit recognition as a separate taxon.

Despite morphological taxonomic work by SVENSON (1944), TAN & al. (1986) and DUNHAM & FOWLER (1987 b), the relationships of the species of sect. *Azolla* are still somewhat obscure. The predominance of the diploid number  $2n=44$  in these species provides only limited information in understanding the taxonomic complexities of this section.

A possible exception to this is *A. rubra* which has the largest chromosomes in the genus. Apart from its recognition as a distinct species by METTENIUS (1847),

it has since been regarded by most workers as either synonymous with *A. filiculoides* or has been accorded varietal status (METTENIUS 1867, STRASBURGER 1873, SVENSON 1944, TAN & al. 1986). An exception to this was its acceptance as a distinct species by LUMPKIN & PLUCKNETT (1982) but who also included populations from Japan formerly called *A. japonica* FRANCH. & SAV. During the present investigation, clones attributed to *A. rubra* from Japan and collected by LUMPKIN (IRRI 6003) and WATANABE (IRRI 6007, 6008), showed chromosomes indistinguishable from *A. filiculoides* and quite unlike those of *A. rubra* from Australasia. This not only emphasises the very close resemblance between *A. filiculoides* and *A. rubra*, but also provides further confirmation that *A. japonica* and *A. filiculoides* are conspecific. The variety *rubra* is indigenous to Australia and New Zealand, with a record of its presence in that region since the Quaternary (DUIGAN & COOKSON 1956). It has most probably diversified from a form similar to *A. filiculoides* as a result of prolonged geographical isolation but it has not yet been established if it is genetically isolated from this species. Morphological differences in degree of leaf imbrication, number of glochidial septa and megasporoderm structure were not considered sufficient to merit full specific status by DUNHAM & FOWLER (1987 b). Although the cytological evidence presented here provides a clearer distinction between *A. filiculoides* from the Americas and Australia, the authors support the view of DUNHAM & FOWLER (1987 b) that the indigenous Australian specimens should be regarded as a separate subspecies rather than as a distinct species.

The taxonomic treatment of the other taxa of sect. *Azolla*, which do not exhibit variation in chromosome size and number, and do not show discontinuous macro-morphological distinctions, is very difficult.

The only evidence which supports the differences between the chromosome complements of these taxa is derived from the cytological examination of the second generation hybrids. The chromosome numbers of these plants often deviate from the diploid number, indicating that some abnormalities in meiosis have produced aneuploid gametes. These results, however, must be treated with caution because similar deviation occasionally appears in first generation hybrids. Although the ability of aneuploid gametes to function only in vitro cannot be precluded, it is likely that the first generation hybrids which exhibit aneuploid chromosome numbers were not maintained in water culture by vegetative reproduction, but have been self-fertilized. Furthermore, hybrids which are known to have grown strictly vegetatively in water culture such as IRRI nos. 2024, 4028, 4030, and 4052, all have  $2n=44$ . If the suggestion that some differences in chromosome structure exist between the taxa of sect. *Azolla* is correct, the examination of meiosis might evaluate their significance. With the exception of *A. filiculoides* var. *rubra*, in which hybrids have not yet been raised, it is significant that all interspecific hybrids of sect. *Azolla* were partly fertile, producing large numbers of spores and some functional aneuploid gametes. This suggestion is particularly interesting considering the partially sympatric distribution of *A. microphylla*, *A. mexicana*, and *A. caroliniana*, as it is possible that introgression of genes has occurred between *A. caroliniana* and either *A. mexicana* or *A. microphylla*.

**Chromosome numbers and triploidy.** It is interesting to note that in more than 100 clones of natural origin examined, no deviation from the diploid numbers  $2n=44$  and  $2n=52$  or triploid number  $2n=66$  have been observed. That aneuploidy is extremely rare in *Azolla* can probably be explained by the nature of

gametes: selection against gametic aneuploidy is likely to occur in the unprotected gametes of *Azolla*, where fertilization occurs in water. This behaviour resembles that of the male gametes of flowering plants which differ from the behaviour of relatively protected female gametes (LEVAN 1936, BRANDHAM 1982).

Table 1 shows that the number of triploids found was proportional to the number of plants examined for each species, and no apparent geographical pattern can be seen in the distribution of triploids. It seems that triploidy has arisen independently in most species by means of a similar mechanism. The investigation of polyploidy in the Bangkok area where the tetraploid clone originated (STERGIANOU & FOWLER 1989), did not reveal more tetraploids but a high incidence of triploidy was recorded. This suggests that tetraploid clones are rare and that triploidy is more likely to result from the occasional function of unreduced gametes rather than the hybridization of tetraploid and diploid clones. It is also possible that unreduced gametes are produced and function more frequently in polluted waters such as those of Bangkok. In most plants, including *Azolla*, polyploid clones are larger than their diploid ancestors. The superiority of triploid *Azolla* over the diploids may explain the frequent occurrence of triploids in the rivers of Bangkok. In this connection it is interesting to note that triploids were found only in areas where water levels are maintained throughout the year. A reason for this could be that triploids are expected to be infertile in natural conditions and only micro- and megasporangia survive in drier conditions.

KLEKOWSKI (1976) observed that polyploidy in heterosporous ferns is not as common as in homosporous ferns and hypothesized that polyploidy developed in homosporous ferns to restore variability. Whereas high polyploid levels do not appear to have developed in *Azolla*, other causes might indirectly result in the occurrence of low polyploid levels. Evolutionary trends in *Azolla* represent a reduction in the size of plants and chromosomes and an increase in growth rate (capable of doubling their size within seven days). Negative correlations have been demonstrated in higher plants between DNA amount per diploid genome and the duration of mitotic cycle in meristematic tissues, and consequently growth rate, duration of meiosis as well as with minimum generation time (VAN'T HOF & SPARROW 1963, EVANS & REES 1971, VAN'T HOF 1974). Low ploidy levels in *Azolla* might therefore be better explained by the overall reduction of DNA amounts.

The present work indicates that changes in chromosome size and DNA values is a major element in karyotype evolution in *Azolla*. It is repeatedly suggested that such differences can influence fundamental characteristics such as cell cycle, generation time and differential development and consequently can affect adaptation to different altitudes and climates (BENNETT 1972, 1973, 1976). Species with high DNA amounts tend to be localized in temperate latitudes and vice versa. In this respect it is interesting to note that *A. pinnata*, which has the smallest chromosomes, has a tropical distribution whereas *A. rubra*, with the largest chromosomes, occurs in higher latitudes; this aspect deserves further investigation.

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