Adiantum lunulatum Burm.—Naturalized Triploid Apomicts from Kangra Valley of Himachal Pradesh

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Received June 15, 2017; accepted August 17, 2017

Summary *Adiantum lunulatum* Burm. of family Adiantaceae locally known as Hansraj is commonly found only in district Kangra rather than other parts of Himachal Pradesh. The entire plant is utilized for medicinal purposes. The focus of the current study was to elucidate the cytological status of this fern but its occurrence was not as common compared to its other family members such as *Adiantum capillus-veneris* L. or *Adiantum caudatum* L. from Himachal Pradesh. Specimens from 16 different populations of the species from Kangra valley were investigated for detailed meiosis. The apogamous mode of reproduction is an established phenomenon in fern. Two types of sporangia were found one with normal meiotic course (32 spores) and other one with abnormal (64 spores). Meiotic analysis revealed that some accessions with 32 well developed spores showed $n=90$ bivalents while others showed meiotic abnormalities such as stickiness, unoriented bivalents, laggards, bridges, micronuclei, and polyads with 64 spores per sporangia. Presence of univalents and trivalents at M-I were reported. Thus, failure of the species to produce viable spores due to meiotic abnormalities is an obvious consequence. Heterogenous, empty, and shriveled spores of different shapes and sizes were also present.

Key words Black maidenhair, Meiosis, Abnormality, Apomixis, Spore fertility.

“Apomixis: A sexual fusion takes no part in the initiation of the new sporophyte which is formed directly from the central tissue of the prothallus at a stage of its development which, in a sexual gametophyte, would just precede the thickening of the central cushion. The only difference that can be detected is that sporangia from which the functional spores are derived contain 32 spores instead of the customary 64” (Manton 1950).

Apomixis in ferns is a peculiar type of asexual reproduction with nearly 10% globally to be known as apomictic (Liu *et al.* 2012). Dryopteridaceae and Pteridaceae, accounts for approximately 70% of apomictic taxa. Since its discovery by Farlow (1874) and de Bary (1878), many satisfactory accounts can be consulted for cytological studies by Dopp 1932, Allen 1914, Steil 1919, Manton 1950, Mehra and Singh 1957, Braithwaite 1964, and Klekowski 1973 on apomixis.

District Kangra lies in the western Himalayas between 31°2 to 32°5N and 75° to 77°45 E transversing Shivaliks, Dhauladhar, and the Himalayas from north-west to south-east. The altitude ranges from 500 to 5000m AMSL. *Adiantum lunulatum* Burm. has been used as medicine for a long time. In western India, it is used extensively in treatment of fevers of children, the rootstock is considered good for fever and elephantiasis (Kirtikar and Basu 1975). Rhizome is short, sub-erect and apex covered with linear scales that are a little

broader at the base. Stipe is slender, chest nut brown, bare and sheeny (Mehra 1938, 1944). The frond varies from 15–45cm in population growing under similar environmental conditions. A lot of variation in size and shape of pinnae also exists. The pinnae may be lobed, the large as well as the small ones, or more or less entire (Mehra and Khullar 1977). Cytology has revealed this fern to be a great species complex in India. From the eastern Himalayas four types have been reported, *viz.* diploid sexual, diploid apomict, triploid apomict, and tetraploid sexual (Mehra and Verma 1963). From south India too, such types have also been reported (Abraham *et al.* 1962, Fabbri 1965). The triploid apomicts are the most widespread polyploidy type and formerly only this type was recorded from western Himalayas (Mehra 1938, 1944). Chromosomal reports were in accordance with previous reports by some distinguished workers (Ghatak 1960, Roy and Sinha 1961, Mehra and Khullar 1977, Bir and Verma 2010) *i.e.*, presence of 90 bivalents at M-I confirming its triploid apomictic status but large numbers of chromosomal abnormalities were detected for the first time. The normal meiotic course corroborates with spore viability while abnormalities were known to cause partial sterility in plants.

Materials and methods

Meiotic studies were carried out from only wild plant materials collected from different localities of district Kangra. The explorations were done in the rainy season

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DOI: 10.1508/cytologia.82.507

Figs. 1–20. 1) *Adiantum lunulatum* Burm. in wild. SMC from 16-celled sporangia: 2) SMC with 90_{II} at diakinesis (DG). 3) Interchromosomal connections at diakinesis (SM). 4) 90_{II} at M-I (RK). 5) Normal separation of chromosomes at A-I (GK). 6) SMC at A-II (TP). 7) SMC at T-I (DG). 8) SMC at T-II (GK). 9) Chromosomal stickiness at M-I (S). 10) SMC with unoriented bivalents at M-I (KK). SMC from 32-celled sporangia: 11) SMC at M-I showing interchromosomal connections (GP). 12) SMC showing trivalents at M-I (GP). 13) Scattered univalents at M-I (D). 14) Unoriented bivalents (two to three) at M-I (MG). 15) Few (six) unoriented bivalents at M-I (L). 16) Large number unoriented bivalents at M-I (D). 17) SMC with early disjunction at A-I (GP). 18) SMC with late disjunction at A-I (CP). 19) Mild chromatin stickiness at M-I (DS). 20) Intense chromatin stickiness at M-I (GP). DG-Dera Gopipur, SM-Samela, RK-Rajiana Khas, GK-Ghurkari, TP-Thanpuri, GK-Ghurkari, KK- Khas Kachiari, GP-Gopalpur, D-Dugh, L-Lahnga, KK-Khas Kachiari, MG-MacLeod Ganj, S-Sunehar, DS-Dharmshala, CP-Chandpur. Scale bar=10 μ m.

Figs. 21–36. 21) Chromatin fragments at A-I (MG). 22) SMC with laggaris at A-I (L). 23) SMC with laggaris at A-II (MG). 24) Chromatin thread stretched between poles at T-I (D). 25) Single chromatin bridge at A-I (DS) 26) Multiple bridges at A-II (CP). 27) Single chromatin bridge at T-I (L). 28) SMC showing tripolar spindle apparatus (D). 29) SMC showing Chromatin mass or Pcynotic nuclei (GP). 30) Chromatin mass at T-II (D). 31) SMC showing polyad with micronuclei (D). 32) Another polyad with micronuclei (MG). 33) Heterogenous spores at 10× (D). 34) Heterogenous spores at 40× with sporangium (MG). 35) Sterile and apparently fertile spores at 40×. 36) Trilete heterogenous spores at 100×. DG-Dera Gopipur, SM-Samela, RK-Rajiana Khas, GK-Ghurkari, TP-Thampur, GK-Ghurkari, KK- Khas Kachiari, GP-Gopalpur, D-Dugh, L-Lahnga, KK-Khas Kachiari, MG-MacLeod Ganj, S-Sunehar, DS-Dharmshala, CP-Chandpur. Scale bar=10 μm.

(July to September) when the temperature ranges from 10–24°C. Voucher specimens were deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN). Pinnae with young sporangia were fixed in Carnoy's fixative (6:3:1, alcohol:chloroform:glacial acetic acid, v/v) for 24h and then transferred to 70% alcohol and refrigerated until use. To study meiotic course, young sporangia were squashed in 1% acetocarmine. A few sori were dealt at a time and larger lumps such as indusia were removed. Chromosome abnormalities were recorded from freshly prepared slides. Spore fertility was estimated by squashing mature sporangia in glyceracetocarmine (1:1) mixture. Well filled spores

with stained cytoplasm were considered to be apparently fertile while shriveled and unstained spores were regarded as sterile, along with heterogenous spores with variable size differences. Photomicrographs were taken from freshly prepared slides with the Magnus MLX microscope.

Results

Excursion trips were taken to District Kangra for the collection of study material between 2014 and 2016. *Adiantum lunulatum* Burm. is found to be more prevalent in Kangra valley as compared to the rest of

Table 1. Locality with map coordinates, spore fertility, and spore size along with status of meiotic course.

S. No	Locality	Map coordinates	Meiosis	Spore fertility %	Spore size
1	Dehra Gopipur (446 m)	31°52'54.3216"N76°12'52.722"E	Normal	90.56	53.22×48.75 μm
2	Samela (666 m)	32°4'55.9128"N76°15'21.1608"E	N	90.38	53.35×48.70 μm
3	Kangra bypass (679 m)	32°6'8.0316"N76°17'17.5056"E	N	97.56	53.40×48.64 μm
4	Rajiana Khas (689 m)	32°0'11.07"N76°15'8.1252"E	N	88.46	53.23×48.86 μm
5	Lalehar (712 m)	31°41'36.3336"N76°15'2.196"E	N	95.45	53.46×48.76 μm
6	53 Mile (739 m)	32°6'11.4984"N76°19'1.3872"E	N	97.95	53.27×49.02 μm
7	Ghurkari (752 m)	32°7'38.4276"N76°17'34.0908"E	N	97.05	53.35×48.58 μm
8	Khas Kachiari (760 m)	32°7'45.858"N76°17'43.026"E	N	98.24	53.16×48.48 μm
9	Sunehar (766 m)	32°5'27.942"N76°20'13.038"E	N	91.42	53.40×48.67 μm
10	Thanpuri (789 m)	32°5'55.2408"N76°20'10.4316"E	N	98.96	53.07×48.86 μm
11	Gopalpur (1258 m)	32°8'24.2628"N76°26'51.4752"E	Abnormal	72.00	53.20×48.66 μm 33.70×32.91 μm
12	Chandpur (1445 m)	32°7'17.2452"N76°33'55.584"E	AN	78.72	53.33×48.63 μm 33.57×32.92 μm
13	Dharmshala (1448 m)	32°13'8.5512"N76°19'24.2544"E	AN	65.38	53.28×48.77 μm 33.68×32.54 μm
14	Lahnga (1570 m)	32°7'47.3448"N76°33'55.584"E	AN	58.69	53.35×48.62 μm 33.54×32.74 μm
15	Dugh (1660 m)	31°53'37.7952"N76°46'0.606"E	AN	64.58	53.18×48.68 μm 33.66×32.68 μm
16	Bhagsunag road (1896 m)	32°14'38.8392"N76°19'58.8612"E	AN	55.76	53.35×48.79 μm 33.89×32.10 μm

Table 2. Locality with altitude, voucher specimen, percentage of meiotic abnormalities and spore fertility in six populations of *Adiantum philipense* L. of family Adiantaceae.

Localities	PUN*	Stickiness	Unoriented bivalents	Laggards	Bridges	Micronuclei	Polyad	Spore fertility
HP: Kangra, Gopalpur (1258 m)	4843	93.10	12.06	5.17	8.62	6.89	—	72%
HP: Kangra, Chandpur (1445 m)	4844	76.92	3.84	15.38	20.51	10.25	—	78.72%
HP: Kangra, Dharmshala (1448 m)	4845	93.81	9.27	14.43	12.37	7.21	—	65.38%
HP: Kangra, Lahnga (1570 m)	4846	91.34	18.26	16.34	19.23	9.61	—	58.69%
HP: Kangra, Dugh (1660 m)	4847	83.18	21.23	22.12	19.46	14.15	7.07	64.58%
HP: McLeodganj, Bhagsunag road (1896 m)	4848	82.22	21.48	23.70	26.66	18.51	8.14	55.76%

PUN*=Accession number Herbarium code of Department of Botany, Punjabi University, Patiala, HP=Himachal Pradesh.

Himachal Pradesh. The accessions were found to be at perfect stages for the study of detailed meiotic course in the month of September. Two types of plants were observed—10 accessions with 32 well-developed spores per sporangia showed 90 bivalents with regular pairing *i.e.*, normal meiosis in Samela, Dehra Gopipur, Kangra bypass, Sunehar, Lalehar, Khas Kachiari, Rajiana Khas, 53 Mile, Ghurkari, and Thanpuri populations (Fig. 37). All these individuals were collected from locations at a lower altitude while in six accessions, 64 spores per sporangia showed meiotic abnormalities and consequently empty, shriveled spores of different shapes and sizes.

Stipes were 10–15 cm long, tufted, wiry, naked, polished dark chestnut brown; fronds were 15–30 cm long and 7.5 cm broad, simply pinnate, often elongated and rooting at the apex; pinnae subdimidiate, the lower edge nearly in a line or oblique with the petiole, the upper edge was rounded and like the bluntly rounded sides usually more or less lobed; petioles of the lower ones spreading one-fourth to half an inch long, texture herbaceous; the rachis and both surfaces naked; sori in continuous lines along the edge (Beddome 1883).

The incidence of chromatin stickiness was found at early as well as late meiotic stages ranging from mild to intense, causing difficulty in separating bivalents at anaphase in all populations irrespective of its reproductive behavior (Figs. 19, 20). Among six abnormal accessions, 76.92% stickiness was the lowest recorded percentage in the accession collected from Chandpur (Table 2). Chromosomal stickiness further leads to formation of laggards at A-I (Fig. 22), A-II (Fig. 23) and as well as T-I stage. Unoriented bivalents were seen at metaphase-I along with some univalents. Incidence of unoriented univalents was higher in the accession collected from Dugh and Bhagsunag road (Fig. 13). Extra chromatin mass or chromatin filaments were also seen at anaphase-I and telophase-I accompanied by chromatin stickiness (Figs. 29, 30). Laggards were observed at anaphase-I (Fig. 22) and anaphase-II (Fig. 23). Along with single chromatin bridges at T-I (Fig. 25), multiple bridges were more frequent anaphase-II (Fig. 26). Spindle apparatus was also found to be distorted in a few cells in the accession collected from Dugh (Fig. 28). Micronuclei were present in every accession and in some cases along polyads with

Fig. 37. Map depicts the places visited in Kangra valley during collection surveys.

the maximum percentage observed in the Bhagsunag (1896m) accession (Figs. 31, 32). As an obvious consequence of abnormal meiotic course, spore fertility was also found to be low (Fig. 35). In the present study, two types of sporangia were found *i.e.*, one with 32 well-developed spores and other with 64 spores per sporangia. In the latter, heterogenous spores were found with comparable size differences in all the accessions (Figs. 33, 34, Table 1).

Discussion

There exists great variability in morphology (diploid apomicts, diploid sexual, triploid apomict, and tetraploid sexual), frond size, and pinnae size and shape in *Adiantum lunulatum* Burm. (Mehra and Khullar 1977). In pteridophytes we have a treasure of apomictic species in abundance. The most common among them are triploid apomicts throughout the western Himalayas. Triploid apomicts are characterized by large fronds and pinnae. All the populations from District Kangra share the same cytological situation ($n=90$) according to Ghatak 1960, Roy and Sinha 1961, Mehra 1961, and Mehra and Khullar 1977, thereby confirming its triploid apomictic status. Recent findings elucidate the abnormal meiotic course step by step with solid documentation supported by microphotographs.

The detailed cytological examination of *Adiantum lunulatum* Burm. revealed irregularities like unoriented bivalents, laggard chromosomes, chromosome bridges, irregular spindle fibre orientation, micronuclei at quartet stage, and polyads. Sterile spores with contracted cytoplasm were formed due to malformation during chromosome segregation. Heterogenous spores were present

with comparable size differences.

The term “chromatin stickiness” was first used by Beadle in 1933 in maize cells affected by mutation. Univalents are enormously scattered at M-I while bivalents are distributed on the metaphasic plate. Weak pairing of some chromosome pairs results in formation of univalents along with clumping of chromosomes, trivalents, and irregular grouping of chromosomes. As a result, laggards at anaphase-I were observed due to delayed terminalization or stickiness of the ends of chromosomes. Chromatin stickiness prevails in every stage of the cell cycle (Koernicke 1905). Chromatin stickiness ranges from mild to intense where chromosomes lose their individuality.

The sticky surface of sticky chromosomes causes chromosome agglomeration, possibly due to effects on chromatin and chromosome organization. When spindle fibres pulled these bridges with an inability to separate they broke into fragments, which either form laggards or micronuclei at a later stage. Chromatin stickiness together with abnormal spindles, laggards, and micronuclei formation is the probable cause of low fertility (Babich *et al.* 1997). Chromatin bridges were formed due to the sticky nature of chromosomes. Thick sticky bridges either single or multiple were developed at anaphase stage from asymmetrical length of chromatids or chromosome interchanges (Dixit and Dubey 1986). SMC from eight-celled sporangium showed a normal distribution of chromosomes at the equatorial plate at M-I with normal segregation at A-I and 32 well-developed spores. SMC from 16-celled sporangium showed bivalents at the equatorial plate while univalents scattered away from the plate. Separation at anaphase stages was abnormal due to formation of laggards, which finally lead to shriv-

eled, empty spores of variable sizes. The presence of univalents at metaphase leads to the formation of micronuclei at the end of the division because many of these univalents remain as laggards at anaphase (Roy and Sinha 1961).

Bridge formation may result from interlocking of bivalent chromosomes (Bhattacharjee 1953), failure of chiasmata in a bivalent to terminalize, and chromosomes getting stretched between the poles (Saylor and Smith 1966) or due to paracentric inversion (Sinha and Godward 1972). Laggards that failed to reach poles often constituted of micronuclei during microsporogenesis (Jiang *et al.* 2011). Random subgrouping of unoriented bivalents may result from distortion or breakage in spindles. The spindle apparatus is normally bipolar and acts as a single unit, playing a crucial role in chromosome alignment during metaphase (Nirmala and Rao 1996). Pollen fertility is greatly reduced by formation of monads, dyads, triads, or polyads with or without micronuclei due to meiotic abnormalities in many flowering plants (Saggoo *et al.* 2011). Similar consequences in pteridophytes may lead to reduced spore fertility. These cytological studies in combination with modern techniques can expand the horizons for understanding apomictic lineages in pteridophytes.

Acknowledgements

Authors are grateful to the University Grants Commission, New Delhi for providing facilities under the DRS SAP III and ASIST programme. Financial assistance to one of us (Mandeep Kaur) by UGC–BSR, Government of India, New Delhi is gratefully acknowledged. Thanks are also due to Head, Department of Botany for providing all the necessary laboratory facilities.

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