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Milium effusum (Poaceae: Poeae: Miliinae): species of systematic and cytological interest with notes on polymorphic nature and cytogeography

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ABSTRACT

Present communication records polymorphic and polyploid nature of the forest grass species *M. effusum* having wide distribution over different continents and countries. Globally the 4 x plants are widely distributed. The West Himalayan plants, collected at 3000 m alt. and more, are also sporting chromosome number of 2n = 28 based on basic number of x = 7. Further meiotic course analysis of Western Himalayan plants follows diploid like behaviour possibly directed by diploidization. This communication also calls the attention to cytogeography of intraspecific cytotypes of *M. effusum*, where diploid plants (2n = 14) have also been reported, but only 2 times – from the Kola Peninsula, Russia and from the Switzerland mountains. Perhaps these are relict populations, which deserve to be proved, studied and protected.

Keywords: grass cytology, cytogeography, male meiosis, polyploidy, dysploidy, conservation

РЕЗЮМЕ

Сингх Дж., Синхал В.К., Пробатова Н.С. *Milium effusum* (Poaceae: Poeae: Miliinae): вид, представляющий систематический и цитологический интерес, с акцентом на полиморфную природу и цитогеографию. Статья посвящена изучению мейоза и полиморфизму у широко распространенного лесного злака *Milium effusum* на материале, собранном в Западных Гималаях (Индия), на высотах 3 000 м и выше. Кроме цитоморфологии вида, принадлежащего к небольшому уникальному роду *Milium* с разнообразными базовыми числами хромосом (x), обсуждается цитогеография *M. effusum* (соматические числа хромосом); последние были исследованы многими авторами, на разных континентах и в разных странах: 2n = 28 (x = 7). В ходе работы из материнских клеток пыльцы были получены мейотические препараты *M. effusum*. На основании особенностей мейоза сделан вывод о вероятном гибридном происхождении вида, и возможных процессах диплоидизации. Авторы предлагают обратить внимание на 2 местонахождения *M. effusum* с диплоидным числом хромосом 2n = 14: в России (Кольский п-ов) и в горах Швейцарии, которые, вероятно, являются реликтовыми и заслуживают охраны. Особенности *M. effusum*, установленные разными методами, свидетельствуют о его полиморфизме.

Ключевые слова: цитология злаков, цитогеография, мужской мейоз, полиплоидия, дисплоидия, сохранение

Milium is a genus of annual and perennial species, characteristically inflorescence a panicle composed of solitary and 1-flowered alike spikelets. The recent phylogenetic classification system of grass family, corroborated by molecular and morphological studies placed *Milium* under subtribe *Millinae* Dumort., supersubtribe *Poodinae* L.J. Gillespie & Soreng, tribe *Poeae* R. Br., supertribe *Poodae* L. Liu, and subfamily *Pooideae* Benth. (Soreng et al. 2017). In account to world grass flora 6 species and some varieties are assigned to *Milium* (Probatova 1977, 1978, Probatova et al. 2000, Maroofi 2011, WCVP 2021) and a recent revisionary account to 'Grasses of Russia' includes 10 species from 2 sections – *Milium* (perennials) and *Miliellum* Tzvelev (annuals) (Tzvelev & Probatova 2019). To grass flora of India, *Milium* is solely represented by plants of *M. effusum* L. inhabited to Arunachal Pradesh, Himachal Pradesh, Kashmir, Sikkim, West Bengal and Uttarakhand states falling to Indian Himalayan Region (Sinha et al. 2019). *M. effusum* has almost worldwide distribution as to many Temperate, Boreal, Subarctic and Sub-alpine parts of the Northern Hemisphere: from Eastern North America and North Africa across most of Europe to the Urals and the Black Sea, extending eastwards to the Himalaya, China, Korea and Japan, as well as to almost all the Russian Far East, including Kamchatka Peninsula, with exceptions of Magadan Region, Chukotka and North Kurils (Tutin et al. 1950, Cope 1982, Probatova 1985, 2003, Karthikeyan et al. 1989, Probatova et al. 2000, Crins 2007, Kandwal & Gupta 2009, De Frenne et al. 2017, Probatova & Seledets 2018, Sinha et al. 2019, Manikandan et al. 2020). Commonly, the species is known as Wood Millet and diffuse Millet, due to its prevalence along with the woodland plant communities, and its cultivar popularly is known as ‘Aureum’ which is a well-established ornamental grass (Crins 2007, De Frenne et al. 2017). *M. effusum* plants sometimes are aromatic (coumarin like scent), and it is one of scented grass used in the traditional customs in Norway and locally known as ‘Haisennagress' (Alm 2015). However, the fragrant plants of *M. effusum* are rare in Russia and adjacent countries (found once in Ukraine by Probatova). Karyologically, *Milium* is unique to grass family having small to medium sized chromosomes and is a polyploid genus sporting chromosome numbers of 2n = 8, 10, 14, 18, 22, 28 and 42 stemmed on basic numbers of x = 4, 5, 7, 9 and 11 (Sokolovskaya & Probatova 1976, Probatova 2021) and dysploid events are key impetus to karyological evolution. As polyploid and dysploid events are conventional phenomena to grasses also to *Milium*, the cytological status of such taxa of wider distribution remains unconfirmed until extensive to intensive exploration studies to un-accessed regions of biodiversity hotspots. As concerned, the present study is in a line of endeavour to explore phyto-geographically distinct and unexplored regions of Himalaya, India (Kumar et al. 2014, Singhal et al. 2014). This study was aimed to enlist the existing chromosome number diversity in the low to high altitudinal grass species growing in the Uttarkashi district in relation to global distribution status. During the survey we encountered with *M. effusum* which is generally scanty in distribution to global floristic regions and the same is in the forest regions of Uttarkashi where it is restricted over high altitude regions (above 3000 m). So, the present cytological report includes observations of undertaken study to support the cyto-geographic account, analysis of meiotic behaviour, pollen fertility study, and to present a short note to polymorphic and polyploid nature of *M. effusum*.

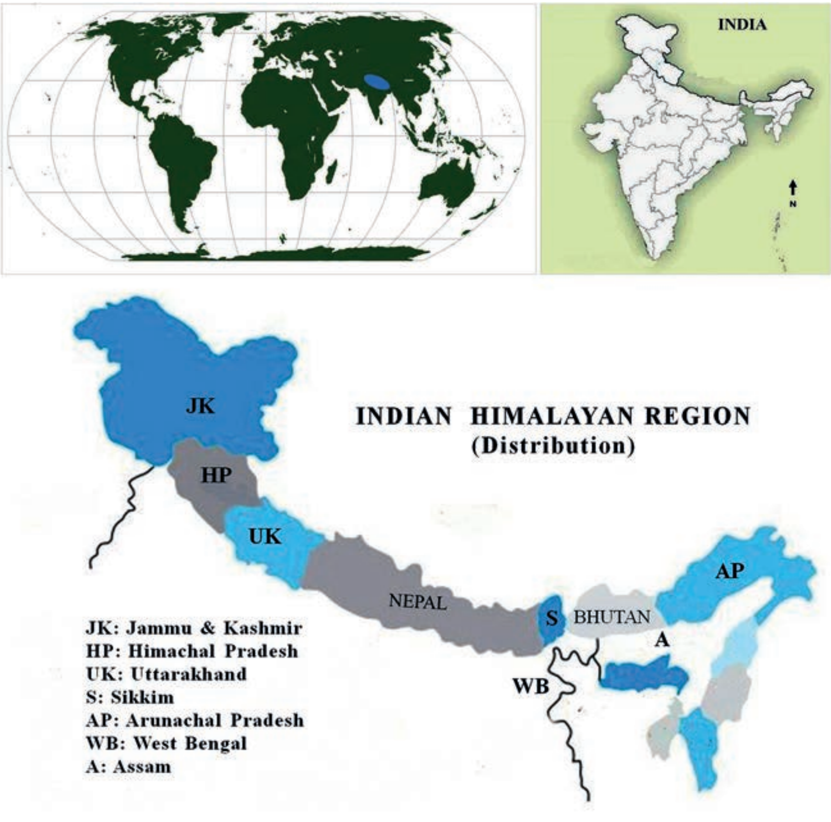

Figure 1 Maps showing geographical location of Indian Himalayan region (IHR), and depicting distribution of plants *Milium effusum* L. to IHR states

MATERIAL AND METHODS

The plants of *M. effusum* were collected from the moist vegetation area around Dodital (3200 m) and on the way to Darwa (3400 m) Mts. above Assi Ganga Valley (30°54'09"N 78°31'09"E),

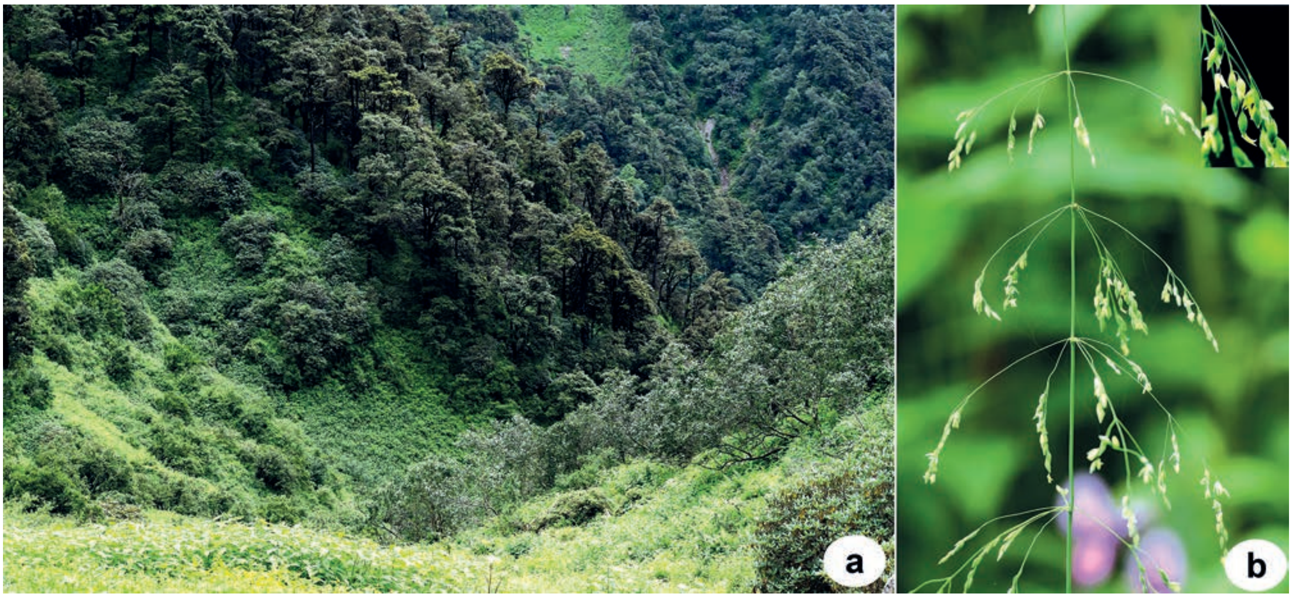

Figure 2 A field photograph showing natural habit of species, mountainous region on way to Darwa (a), and a part of open and spreading panicle, with solitary branch and spikelet sporting yellowish stamens (b)

Uttarakhand state falling to Indian Himalayan Region (Figs 1, 2a). For this young, as flag leaf enclosed panicles were fixed in Carnoy's fixative (ethanol: chloroform:acetic acid = 6:3:1). After 48 h, the materials were transferred to 70 % ethanol and stored in a refrigerator. Meiotic preparations were made by squashing the developing anthers from the unopened florets in 1 % acetocarmine. Pollen fertility was estimated through a stainability test by squashing the mature anthers in a mixture of glycerol and 1 % acetocarmine (1:1). Well-filled pollen grains with completely stained nuclei and cytoplasm were scored as fertile, while partially stained and shrivelled ones as sterile. Preparations of PMCs with well-spread bivalents/ chromosomes, meiotic irregularities, and pollen grains were selected for photomicrographs using a Leica Qwin microscope equipped with a digital imaging system. Vouchers of analyzed plants were deposited in the Herbarium (PUN), Department of Botany, Punjabi University, Patiala.

RESULTS AND DISCUSSION

Polymorphic nature – cryptic species complex

Milium effusum is a perennial herb of polymorphic nature with weak phytocoenotic activity affirmed by morphological variations suggested by previous extensive floristic accounts along with description of analyzed plants. Plants inhabited to North American floristic regions differ from Eurasian origin, primarily in the leaf colour of living plants (glaucous vs. green), longer spikelets, fewer branches at the nodes of the panicle and longer fruits (Haines 2010). Characteristically, North American plants having 2–3 panicle branches at most nodes, spikelets 2.5–5 mm long and fruit 2.5–3 mm are described as *M. effusum* subsp. *cisatlanticum* (Fern.) A. Haines and Eurasian plants with 4–5–6 panicle branches at most nodes, spikelets about 3 mm long and fruits 2.0 mm long belongs to *M. effusum* subsp. *effusum* (Fernald 1950, Crins 2007, Haines 2010). Besides, Eurasian plants also exhibit morphovariants, like plants of Baikal Siberia (Russia) origin have spikelets rose-violet, 3–3.4 mm long, arranged in large spreading panicles as *M. effusum* var. *coloratum* Roshev. (Tzvelev 1976) and plants analyzed from Honsu mountains (Japan) are of pubescent form (leaf blades along margins with accurately ciliated hairs, sheaths of lower stem leaves more or less pubescent) and were described as *M. effusum* f. *ciliatum* Hiyama (Hiyama 1957) and plants of other Eurasian regions including Himalayas as per se the protologue descriptions are associated with *M. effusum* L. subsp. *effusum* (Fig. 2b). *M. effusum* s.l. is almost Holartic species which is defined by degree of bioclimatic continentalization and distribution of species to different tendency gradient zones and association with transformations of ecological ranges, it confirms the supposed polymorphism of *M. effusum* s.l. (Probatova & Seledets 2018). A comparative study of *M. effusum* in Northern and Southern regions of Sweden showed to be highly variable as the levels of genetic diversity and allelic richness were lower in Northern Sweden as compared with Southern Sweden (Tyler 2002). In contrast, different measures of geographic structure all showed higher levels of population differentiation in the northern region of Sweden. On the other hand molecular phylogenetic studies also showed the existence of genetic polymorphism associated with phenotype of species (Kotseruba et al. 2017, 2018). Further genomic sequence analysis of plants collected from different regions of Europe, Caucasus, Siberia, Middle Asia, East Asia, and North America splits plants into two distinct clades with the samples of *M. effusum* from East Asia (Kamchatka, Kuril Islands, Primorskii Krai, Japan, China) clustering into a separate clade than the rest of *M. effusum* samples aggregates together in a second clade with perennials *M. transcaucasicum* Tzvelev and *M. schmidtianum* K. Koch (all from the section *Milium*). Conditionally it forms a representation of two realms, European and East Asian, depicting the possibility of the existence of cryptic species (Kotseruba et al. 2008, 2012, 2013). The plant collections (2014–2017) through the Garhwal Himalayan forest regions (Asi Ganga, Bhagirathi, Chaurangi Khal, Har Ki Dun, Jadh Ganga, Rarhi, Yamuna valleys and their low altitude localities) falling in Uttarkashi district (900–4000 m) were made but the plants were found to growing only in the moist and shady sub-alpine slopes within an altitude range of 3000–3400 m above the Assi Ganga valley (Fig. 2a). This restricted distribution of species possibly may be due to highly specialized nature and adaptive as grass of woodland community (Probatova et al. 2000, De Frenne et al. 2017).

Chromosome number and cytogeography

The male meiotic study of plants of *M. effusum* scored from Dodital (PUN62907) and Darwa (PUN62432) on exploration revealed that individuals of species are unequivocally sporting common gametic chromosome count of n = 14. Chromosome number of n = 14 was confirmed from presence of 14 bivalents in the pollen mother cells at diakinesis (Fig. 3a) and metaphase-I (Fig. 3b). The report of present chromosome count of 2n = 28 in plants inhabited to the Far Eastern region of Western Himalaya and is in line with similar count recorded by Mehra and Sunder (1969) in a plant scored from Far Western region (Kashmir) Western Himalaya, India. Chromosomally *M. effusum* have been extensively explored from different regions of the globe and, like present count to Himalayan plants, chromosome number of 2n = 28 is a widely sported number throughout its distribution (Probatova et al. 2000). Scrutiny of previous known reports of chromosome number/s (2n = 14, 28) associated to *M. effusum* widens the scope of earlier records to blot cytogeography. The most common number of 2n = 28 is known to be recorded in the plants originated from Europe (Iceland, England, Norway, Denmark, Finland, Belgium, Netherlands, France, Germany, Switzerland, Sweden, Austria, Greece, Slovakia, Bulgaria, Poland, Moldova, Belarus, Ukraine (Tischler 1934, Rohweder 1937, Löve & Löve 1944, 1956, 1981, Tutin 1950, Sorsa 1962, 1963, Gadella & Kliphuis 1963, Frey et al. 1977, Majovsky et al. 1978, Skalinska et al. 1978, Strid & Franzen 1981, Strid & Andersson 1985, Vachova 1987, Dmitrieva & Parfenov 1991, Duckert-Henriod 1991, Bennett & Thomas 1991, Bennett & Bennett 1992, Kozuharov & Petrova 1991, Lovkvist & Hultgard 1999, Probatova & Seledets 2008, Korobkov et al. 2012), from Caucasus (Armenia: Ghukasyan 2004a, b), many times from Russia (Probatova & Sokolovskaya 1981, Guzik 1984, Rudyka 1990, Sorokin 1990, 1993, Probatova et al. 2000, 2001,

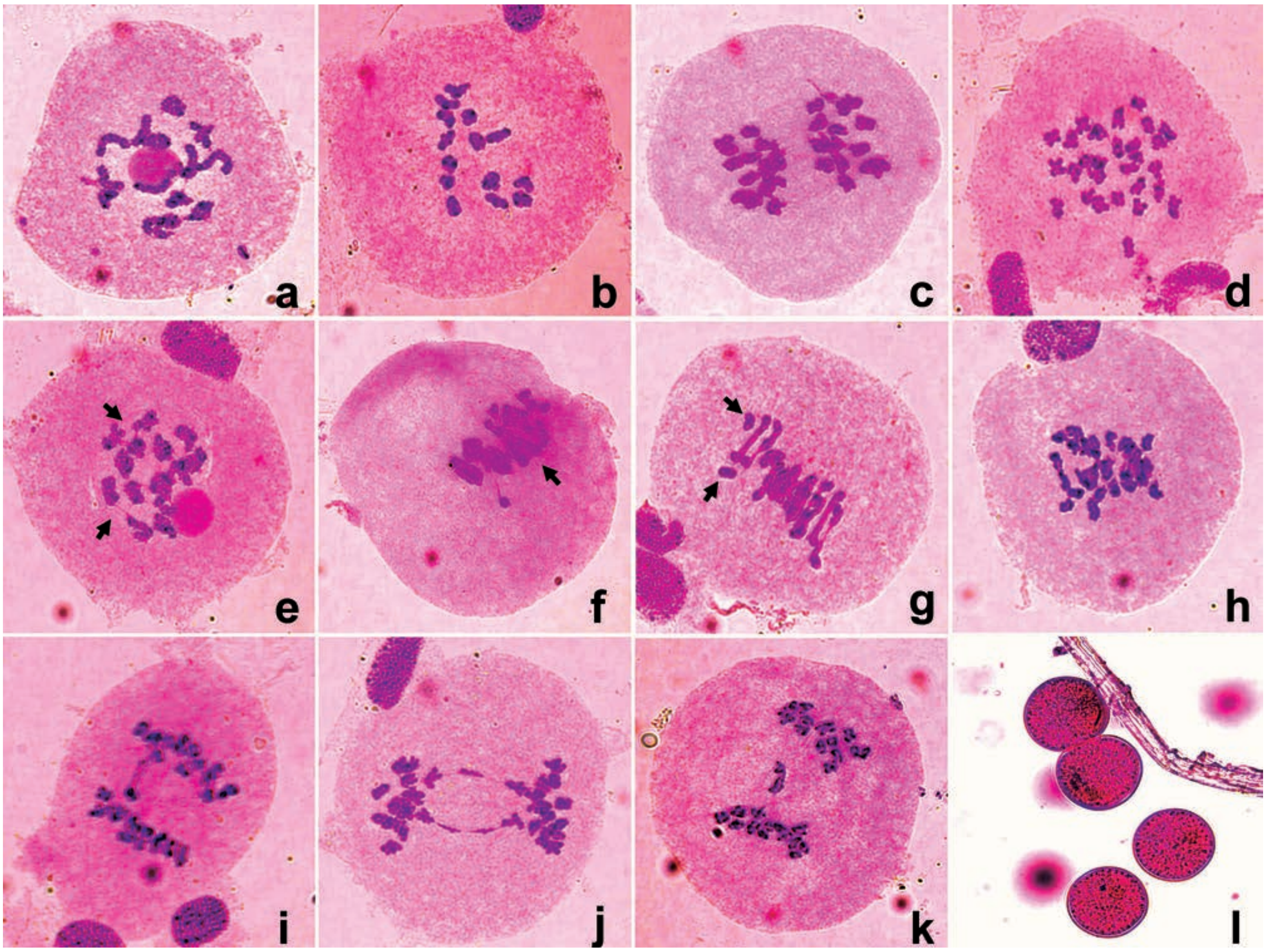

Figure 3 Meiotic course: **a** – a diakinesis pollen mother cells showing 14 bivalents, **b** – a metaphase-I pollen mother cell showing 14 bivalents, **c** – a pollen mother cell showing 14:14 chromosomes at each A-I pole, **d** – an anaphase-I pollen mother cell showing 28 chromosomes, **e** – a diakinesis pollen mother cell depicting inter-bivalent connections between 2–5 bivalents in 1–5 groups (arrowed), **f** – a pollen mother cell depicting chromatin stickiness (arrowed), **g** – a pollen mother cell depicting earlier disjunction of 1–2 bivalents (arrowed), **h** – a pollen mother cell depicting late disjunction of 1–2 bivalents, **i, j** – pollen mother cells depicting late disjunction created 1–2 chromatin bridges, **k** – a pollen mother cell showing lagging bivalent, **l** – stained fertile pollen grains

2004, 2008, 2011, 2012, 2015, Seledets & Probatova 2005, Rodionov et al. 2006, Chepinoga et al. 2012), from Central, South and East Asia (Kazakhstan, India, Japan: Tateoka 1953, Mehra & Sunder 1969, Probatova & Seledets 2008), and from North America (Canada: Bowden 1960). Interestingly, in addition to tetraploid count (2n = 28), the reports of diploid count with chromosome number of 2n = 14 have also been published from plants explored in Kola Peninsula (North-West Russia: Sokolovskaya & Strelkova 1960) and from Appenzell and Valais Canton regions of Switzerland (Duckert-Henriod 1991). The wide distribution of tetraploid cytotype seems facilitated by genomic plasticity in relation to structure and function that imparts adaptability through rapid and successful establishment of polyploids in nature (Ma & Gustafson 2005). The diploid cytotype seems to be a rare (relict?) cytotype which is limited to few individuals further restricted to diminishing habitats. The present cytogeographic streak is an illustrative information on existing chromosomal number diversity in individuals of *M. effusum*.

Besides, tetraploid plants of Sweden origin are also known to possessing a 1B-chromosome (2n = 28+0-1B) (Lovkvist & Hultgard 1999) which possibly associated with buffering of genetic diversity and allelic richness in Sweden plants (Tayler 2002). Commonly Bs are known as accessory or supernumerary chromosomes and considered as non-essential components of the genomes found in a wide variety of plants and possess their own meiotic drive and follow non-mendelian inheritance (Jones & Rees 1982). To origin, Bs arise from the A-chromosomes, but they follow their own pattern of inheritance and accumulation mechanism to future generations. To the constitution of Bs, they are like a "genomic sponge" and accumulate sequences of various origins in a cell (Bednářová et al. 2021).

Meiotic behaviour and polyploid nature

In relation to meiotic behaviour, during the meiotic course most of the pollen mother cells depicted normal course as seen the regular segregation of 14 bivalents into 14:14 and also as 28 chromosomes at A–I (Fig. 3c, d). However, during the meiotic course, most of the pollen mother cells were observed with normal meiotic configurations, but few meiocytes depicted occurrence of interbivalent connections (Fig. 3e), chromatin stickiness as chromatin masses (Fig. 3f), non-synchronous segregation/disjunction of bivalents (Fig. 3g, h), and later phenomenon also noticed in the form of chromatin bridge and laggards at anaphase–I (Fig. 3i-k). Despite meiotic anomalies, the plants were noted with 98–99 % fertile pollen grains of 46.78± 0.86 µm size (Fig. 3l).

The formation of distal interbivalent connections between 2–5 bivalents in 1–5 groups in diakinesis meiocytes seen as loose connections between homoeologs bivalents resulted due to fusion of heterochromatic regions (Thomas & Revell 1946, Bennett & Thomas 1991). During later meiotic stages these structures get demolished through directed restriction to paring by diploidizing genes (Jenkins & Rees 1991). Chromatin stickiness and non-synchronous disjunction in meiocytes depicts some sort of associated genomic changes with spindle apparatus (Kumar & Singhal 2013, Kumar et al. 2017). Chiasma frequency values in meiocytes of analyzed plants depicted variation over pairing to chromosomes having chiasma per bivalent 1.28–1.31 (Table 1). Close observations to paring as ring or rod bivalent, 68.26–71.06 % chromosomes were paring as rod bivalent with single terminal chiasma and 28.39–31.23 % chromosomes as ring bivalent with 2-chiasmata and some PMCs also possessed early disjunction created univalents at metaphase–I. Meiotically the tetraploid plants are typical to allopolyploid nature, favours exclusive bivalent formation and higher frequency of rod bivalents (Bennett & Thomas 1991). This stable diploid like meiotic behaviour in allotetraploid plants is generally due to prior differentiation of homoeologous chromosomes to diploidization pathway which directs pairing of homologous chromosomes as rod-ring bivalents and suppression of pairing of homoeologs (Ladizinsky 1973, Otto & Whitton 2000, Ramsey & Schemske 2002, Comai 2005). In polyploid/allopolyploid plants broadly diploidization occurs at cytological and genic levels (see Li et al. 2021) and in case of *M. effusum* genic diploidization in addition to cytological diploidization seems more implicit force to wide distribution and polymorphic nature. The changes over nuclear content (mean 2C DNA amount: 7.52–10.20 pg) in tetraploid plants were also reported by Bennett & Bennett (1992) among wild and botanical garden managed plants and recorded 35.6 % higher content in managed plants than in plants of natural habitat. Further dominance of tetraploid plants of *M. effusum* over diploid cytotype is facilitated by allopolyploid event, which greatly sprouted to biological diversity associated to *Milium* (Fox et al. 2020) and also to enhanced survival potential of dawning taxa over unfavourable and new habitats (Van de Peer et al. 2021).

CONCLUSION

The present study upholds the widespread status of tetraploid nature plants of *M. effusum*, as also Himalayan origin plants are sporting again the chromosome number of 2n = 28. Besides, a rare diploid cytotype with chromosome number of 2n = 14 suggests intraspecific polyploid nature of *M. effusum* having 2x and 4x cytotypes based on x = 7. Further, to affirm this nature and conservation of minority cytotype needs exploration and re-reporting of 2x plants inhabited to Kola Peninsula in Russia and Valais Canton region of Switzerland.

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Ethics declarations. The authors declare that they have no financial and personal relationships with other people or organizations that can inappropriately influence their work. All data and material can be available from the authors.

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Table: 1. Analysis of meiotic configurations in PMCs (Diakinesis and M-I) depicting chiasma frequency per PMC and per bivalent and percentage of chromosomes associated as ring/rod bivalents.

Accession	PMCs type	No. of PMCs Analyzed	Bivalents		Univalents*	Chiasma per PMC (Mean±SD)	Chiasma per bivalent
			Ring II	Rod II	I		
PUN62907	PMCs with bivalents and univalents	39	155/546 (28.39 %)	388/546 (71.06 %)	6/546 (0.55 %)	16–21 (17.90±1.93)	1.28
Number and % of chromosomes involved in chromosomal associations			310/1098 (28.39 %)	776/1098 (71.06 %)	6/1098 (0.55 %)		
PUN62432	PMCs with bivalents and univalents	42	183/586 (31.23 %)	400/586 68.26 %	6/586 (0.51 %)	15–23 (18.24±2.22)	1.31
Number and % of chromosomes involved in chromosomal associations			366/1172 (31.23 %)	800/1172 (68.26 %)	6/1172 (0.51 %)		

* x univalents = x/2 bivalents

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