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Chromosome count, male meiotic behaviour and pollen fertility analysis in *Agropyron thomsonii* Hook.f. and *Elymus nutans* Griseb. (Triticeae: Poaceae) from Western Himalaya, India

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Abstract. Present cytological study records existing chromosome number diversity, their male meiotic course and pollen fertility analysis in the two wheatgrass species of tribe Triticeae Dumort. (Poaceae) from Western Himalaya, India. *Agropyron thomsonii* Hook. f. is an endemic grass of alpine zones of Western Himalaya and *Elymus nutans* Griseb., a widely distributed grass in sub-alpine to glacial regions of Himalaya. The gametophytic chromosome number count of *n*=21 (Jadh Ganga Valley, Uttarkashi) is a pioneer count for *A. thomsonii.* During the male meiotic course of *A. thomsonii,* 14.04-16.29% and 2.97-4.17% pollen mother cells, respectively at prophase-I and metaphase-I, observed to be involved in phenomenon of cytomixis. Seven accessions of *E. nutans* collected from Bhagirathi Valley and Jadh Ganga Valley of Uttarkashi district, and Pangi Valley of Chamba district, recorded with gametophytic chromosome number count of *n*=21 and record of 1B-chromosome in PUN61958 is a new record for the species. In three accessions 5.56-9.41% and 2.5% pollen mother cells at prophase-I and metaphase-I, respectively were also noted with phenomenon of cytomixis. In addition to phenomenon of cytomixis, during meiotic course of both species pollen mother cells also depicted associated meiotic course irregularities *viz.* non-synchronous disjunction of bivalents, chromatin bridges, laggards, micronuclei in sporads and shrivelled microspores. These species are growing in cold climatic condition habitats. So, cold stress seems to be a preferential inductor for cytomixis and associated meiotic abnormalities in the gametic cells of stamens of *A. thomsonii* and *E. nutans* that ultimately leads to reduction in pollen fertility.

Keywords: Himalayan grasses, cold stress, polyploidy, male meiosis, cytomixis and meiotic abnormalities, pollen sterility.

INTRODUCTION

Tribe Triticeae Dumort. (Poaceae) includes annual and perennial grass taxa, having large-sized chromosomes, comprising a polyploid complex of

2*x*, 4*x*, 6*x*, 8*x*, 10*x* and 12*x* ploidy levels with a uniform base number of *x*=7 (Dewey 1984). The Members of tribe Triticeae have distribution to almost all floristic regions of the globe. In Himalaya, *Agropyron* Gaertn. and *Elymus* L. are prominent grass genera of temperate to alpine zone habitats, and an inflorescence is a spike that has one to many spikelets at each rachis node. Earlier, the genus *Agropyron* was represented by more than 33 species in Himalaya along with adjoining regions of similar terrain (Bor 1960), and the modern concept of *Agropyron* restricts it to only those species having keeled glumes and/or pectinately arranged spikelets (Cope 1982). So, the most of the species of *Agropyron* are now assigned to genus *Elymus* (Melderis 1978; Singh 1983; Karthikeyan et al. 1989). At present in Himalayan regions of India genus *Agropyron* is represented by a single species, *i.e. A. thomsonii* Hook. f. and genus *Elymus* by 31 species (Singhal *et al.* 2018b). *A. thomsonii* Hook.f. [= *E. thomsonii* (Hook. F.) Melederis; = *E. nayarii* Karthik.] and *Elymus nutans* Griseb. are perennial and caespitose wild grasses growing in the high altitudinal regions of Himalaya (above 3000 m). Former is an endemic grass to Western Himalaya (Pusalkar and Singh 2012) and later species have wider in distribution that is growing in different ecological habitats of Bhutan, China, India (Himalaya), Iran, Japan, Mongolia, Nepal, Pakistan and Russia (Bor 1960; Lu 1993; Murti 2001; Chen and Zhu 2006; Pusalkar and Singh 2012; Dvorský *et al.* 2018). Scrutiny of published cytological data reveals that *A. thomsonii* is still unrecorded for its chromosome number. As germplasm of grasses remained in a central position towards the breeding programs and germplasm enhancement to ensure the food and fodder demands. So to make their programs successful, there is a need to have complete knowledge and understanding about the genetic diversity of available germplasm (Kawano 2018). The present study is in a line of endeavour to explore the morphological diversity in the grasses from phytogeographically distinct and unexplored regions of Western Himalaya. So, this study aimed to record the exact chromosome number in *A. thomsonii* and *E. nutans* along with to comment on the behaviour of pollen mother cells during the male meiotic course, and pollen fertility. We also try to correlate prevalence of cold conditions in natural habitat and the possible cause of cytomixis, associated meiotic abnormalities and reduction in pollen fertility in the currently studied species.

MATERIALS AND METHODS

Wild plant accessions of *A. thomsonii* and *E. nutans* were collected for detailed male meiotic and pollen fertility studies from Bhagirathi and Jadh Ganga valley, Uttarkashi district, Uttarakhand and Pangi valley, Chamba, Himachal Pradesh. Young and unopened spikes, fixed in Carnoy's fixative (Ethanol: Chloroform: Glacial acetic acid= 6: 3: 1). After 48 h, the materials were transferred to 70% ethanol and stored in a refrigerator. Meiocyte preparations were made by squashing the developing anthers from the unopened florets in 1% acetocarmine. Chromosome counts and meiotic course was studied from freshly prepared slides having pollen mother cells (PMCs)/meiocytes at diakinesis, metaphase-I (M-I), anaphase-I (A-I) and telophases (T-I, T-II). Apparent pollen fertility was estimated through stainability tests by squashing the mature anthers in glycerol and 1% acetocarmine (1:1) mixture. Well-filled pollen grains with completely stained nuclei and cytoplasm were scored as fertile/viable, while partially stained and shrivelled ones as sterile/non-viable. Good preparations of PMCs with well-spread bivalents/chromosomes, meiotic irregularities, and pollen grains selected for photomicrographs using Nikon 80i Eclipse and Leica Qwin Digital Imaging System. Pollen size was measured through microscopy. Meiotically analyzed accessions were identified by studying, in detail the floral characters and by consulting the floras *viz.* Grasses of Burma, Ceylon, India and Pakistan (Bor 1960), Flora of Pakistan-Poaceae (Cope 1982), Flora of Cold Deserts of Western Himalaya-Monocotyledons (Murti 2001) and Flora of Gangotri National Park, Western Himalaya (Pusalkar and Singh 2012). Identifications revalidated by comparing the specimens with the vouchers already submitted by taxonomists in the Herbaria, Botanical Survey of India, Northern Circle, Dehra Dun (BSD) and available specimens, facilitated by other herbaria as online resource (Global Plants[1]). Voucher specimens of cytologically examined accessions deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN) and Herbarium Botanical Survey of India, Northern Circle, Dehra Dun (BSD).

RESULTS

In the present exploration study, cytological investigations made on two accessions of *A. thomsonii* gathered from the alpine meadows of Jadh Ganga Valley and seven accessions of *E. nutans* from Bhagirathi Valley, Jadh Ganga Valley and Pangi Valley. Data regarding the name of taxon, sites of the collection with altitude, accession number (BSD, PUN), gametic chromosome number, ploidy level, pollen fertility percentage tabulated in Table 1.

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[1] Global plants (https://plants.jstor.org/).

Table 1. Information on taxon, locality of collection with altitude, accession number/s, meiotic chromosome number, ploidy level, pollen fertility percentage of the cytologically investigated taxa.

Sr. No	Taxon	Locality with altitude (m)	Accession number/s	Meiotic chromosome number (*n*)	Ploidy level	Pollen fertility (%)
1.	*Agropyron thomsonii* Hook. f. (= *Elymus nayarii* Karthik.)	Nelong I, Uttarkashi, Uttrakhand, 3450	PUN 61593	21	6*x*	60
		Nelong CP, Uttarkashi, Uttrakhand, 3500	PUN 61594	21	6*x*	65
2.	*Elymus nutans* Griseb.	Sural, Chamba, HInmachal Pradesh, 3008	BSD 1181196	21	6*x*	97
		Nelong I, Uttarkashi, Uttrakhand, 3450	PUN 61955	21	6*x*	95
		Nelong CP, Uttarkashi, Uttrakhand, 3500	PUN 61956	21	6*x*	90
		Bhojwasa, Uttarkashi, Uttrakhand, 3700	PUN 61022	21	6*x*	98
		Bhojwasa, Uttarkashi, Uttrakhand, 3750	PUN 61015	21	6*x*	98
		Bhojwasa, Uttarkashi, Uttrakhand, 3800	PUN 61957	21	6*x*	95
		Gaumukh, Uttarkashi, Uttrakhand, 3900	PUN 61958	21+0-1B	6*x*	95

Table 2. Data on the percentage of PMCs involved in chromatin transfer, abnormal sporads and pollen size of *Agropyron thomsonii* and *Elymus nutans.*

Taxon	Accession number (PUN)	PMCs involved in cytomixis (%)		Out of the plate bivalents/ chromosomes (%)		Laggards/ Chromatin bridges (%)		Sporads (%)			Pollen Size (μm)
								Dyads with	Tetrads with		
		Prophase-I	M-I	M-I	A-I	A-I/T-I	A-II/T-II	Micronuclei	Micronuclei	1-2 Shrivelled microspores	
Agropyron thomsonii Hook. f.	61593	14.04 (40/285)	2.97 (4/135)	–	–	12.30 (8/65)	15.00 (6/40)	10.00 (2/20)	10.29 (7/68)	4.41 (3/68)	Small: 29.31 x 29.31 Medium: 36.80 x 36.80 Large: 42.68 x 42.68
	61594	16.29 (57/350)	4.17 (10/240)	6.25 (15/240)	8.57 (6/70)	17.14 (12/70)	14.29 (8/56)	13.64 (3/22)	8.89 (8/90)	6.67 (6/90)	
Elymus nutans Griseb.	61955	9.41 (8/85)	-	-	-	6.67 (4/60)	-	-	-	-	Small: 27.86 x 27.86 Medium: 37.47 x 37.47 Large: 41.32 x 41.32
	61956	8.00 (6/75)	2.50 (2/80)	12.50 (10/80)	-	16.00 (8/50)	-	-	4.00 (2/50)	-	
	61958	5.56 (5/90)	-	-	-	11.43 (4/35)	-	-	-	-	

Two wild accessions of *A. thomsonii* collected from the glacial floristic area of Nelong, Jadh Ganga Valley, an eastern part of cold deserts of India. Current meiotically analyzed accessions of *A. thomsonii* are of dwarf habit (plant height 28 cm; spike length 9.5 cm; spikelet length 1.5 cm), which are hexaploid (6*x*; *x*=7) in nature with gametophytic chromosome number count of *n*=21, that confirmed from the presence of 21 bivalents in PMCs at M-I (Fig. 1A) and 21:21 chromosomes at A-I poles (Fig. 1B). During the meiotic course, 14.04-16.29% prophase-I PMCs (Fig. 1D, E) and 2.97-4.17% metaphase-I PMCs (Fig. 1F) observed with the phenomenon of cytomixis. In the majority of PMCs, chromatin migration occurred as deformed mass or partially deformed bivalents through cytoplasmic channels. Partial/complete migration of chromatin material among neighbouring PMCs leads to formation of hypoploid, hyperploid and enucleated PMCs (Fig. 1D, E). Some PMCs observed in a state of pycnosis (Fig. 1H), and in few instances nucleolus pioneered migration of chromatin material occurs that noted as a presence of additional nucleolus in diakinesis PMCs (Fig. 1C). Further during the meiotic course considerable number of PMCs at M-I, A-I/II and T-I/II observed with associated meiotic abnormalities like,

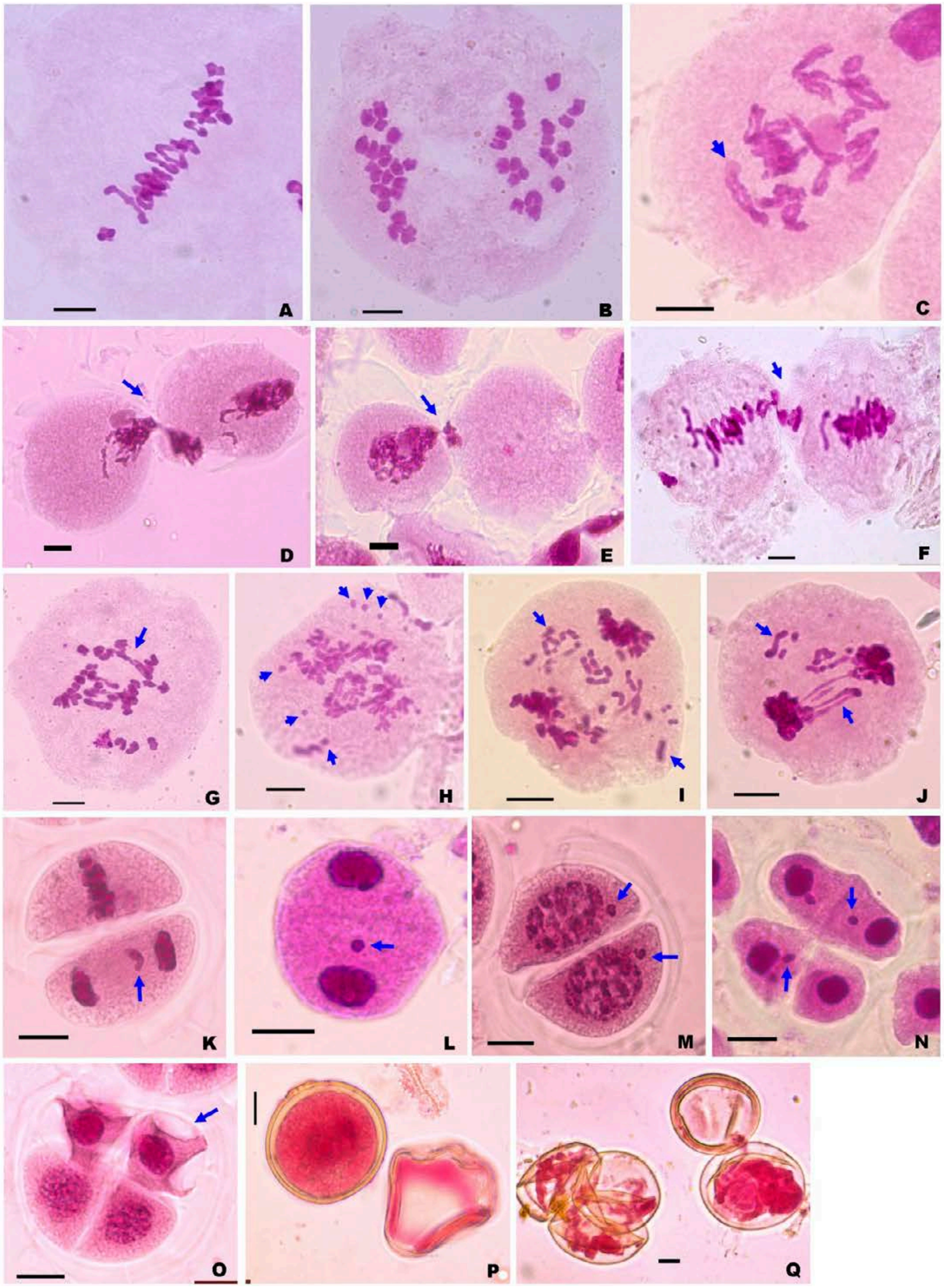

Figure 1. Male meiosis in *Agropyron thomsonii*: (A) M-I PMCs with 21 bivalents (B) A-I PMC with 21:21 chromosomes at each pole; (B) PMCs involved in chromatin migration at P I (arrowed); (C) a diakinesis PMC possessing relatively small sized additional nucleolus PMCs; (D) prophase-I PMCs depicting phenomenon of cytomixis (arrowed); (E) diakinesis PMCs with chromatin migration and depicting formation of hyperploid and enucleated PMC (arrowed); (F) M-I PMC involved in chromatin migration (arrowed); (G) A-I PMC with non-synchronous dysjunction of bivalents depicted by univalents and bivalent bridges (arrowed); (H) a PMC depicting abnormal spindle and state of pycnosis with fragments (arrowed); (I) A-I PMC with several laggards (arrowed); (J) early T-I PMC with dicentric bridge, fragments and laggards (arrowed); (K) T-II PMC as dyad with on subunit with laggard (arrowed); (L) T-I PMCs with miocronuclei at one pole (arrowed); (M) dyad subunits with micronuclei (arrowed); (N) tetrad microspore subunits with micronuclei (arrowed); (O) A tetrad with two shrivelled microspore units (arrowed); (P, Q) fully stained fertile, shrivelled and unstained as sterile pollen grains. Scale bar=10μm.

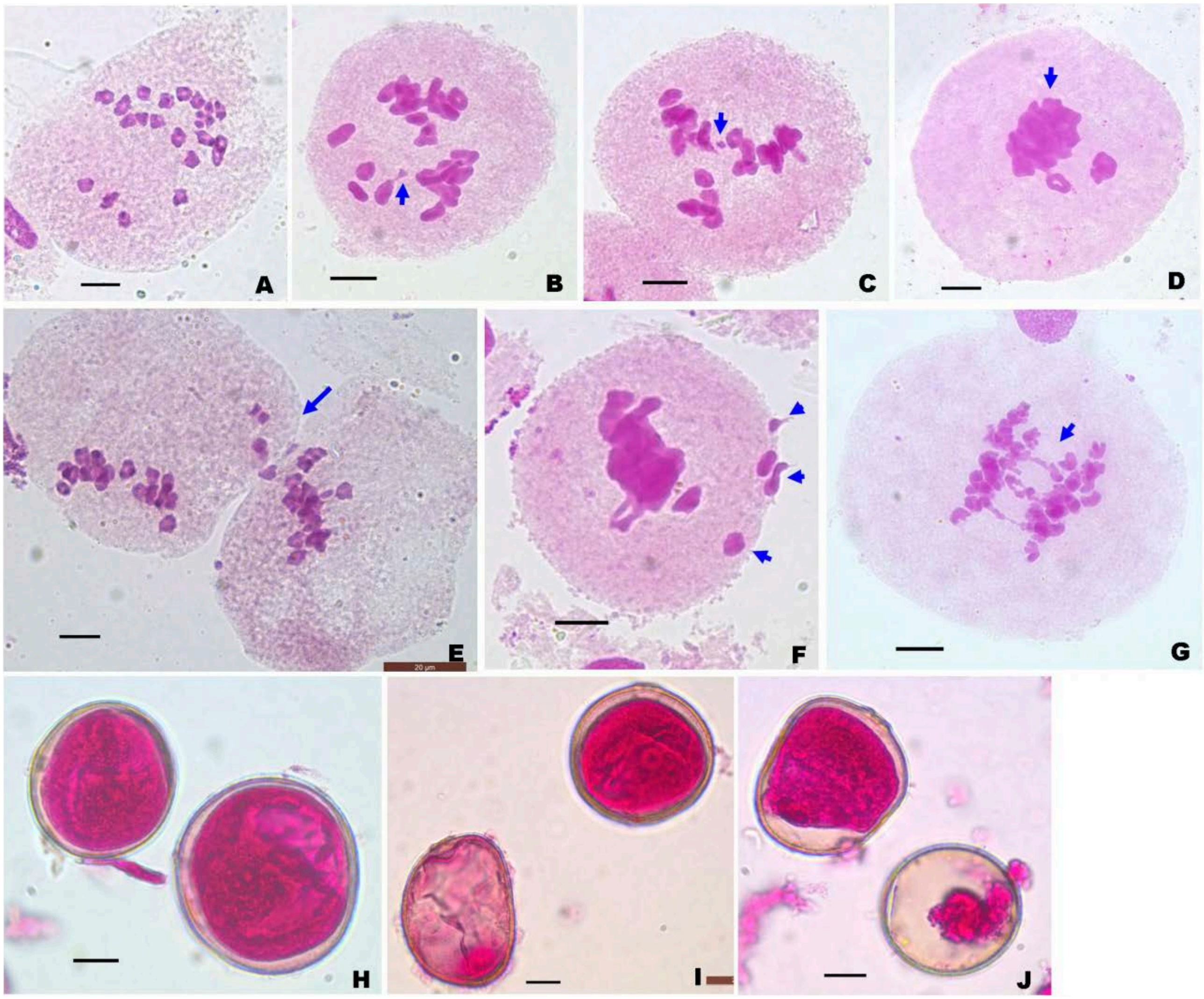

Figure 2. Male meiosis in *Elymus nutans*: (A) M-I PMC with equal-sized 21 bivalents; (B) M-I PMC with 1B-chromosome associated with A-bivalent (arrowed); (C) M-I PMC with independent 1B-chromosome (arrowed); (D) M-I PMC with chromatin stickiness; (E) M-I PMC depicting the phenomenon of cytomixis (arrowed); (F) M-I PMC with migrated chromatin material as pycnotic mass (arrowed); (G) A-I PMC with non-synchronous dysjunction of bivalents (arrowed); (H) heterogeneous sized pollen grains (arrowed); (I, J) fully stained fertile, partially stained and unstained as sterile pollen grains. Scale bar=10μm.

chromatin bridges (Fig. 1G) and laggards/ fragments (Fig. 1I, J, K, L), and micronuclei in sporads (Figs. 1M, N), collectively depicting a syndrome of errors occurred during the meiotic course (Table 2). During, the microsporogenesis two types of sporads were noted, first having all normal microspores and another type with shrivelled microspores (Fig. 1O). These meiotic abnormalities during meiotic course have resulted in low pollen fertility (60-65%) and formation of heterogeneous sized pollen grains in these accessions (Fig. 1P, Q).

Seven accessions of *E. nutans* collected from subalpine and glacial vegetation zones of three valleys noted to have variable plant height in their natural habitat, *viz.* dwarf (PUN 60482, 61956: plant height, 9-15 cm; spike length, 3-5.5 cm; spikelet length, 10-12.5 mm; awn length, 7-10 mm), intermediate (PUN 61955: plant height, 25 cm; spike length, 8-8.5 cm; spikelet length, 9-10 mm; awn length, 15-17.5 mm) and tall (PUN 61022, 61957-58, BSD 1181196: plant height, 50-65 cm; spike length, 15-18 cm; spikelet length 30-45 mm; awn length, 25-45 mm). These accessions unequivocally have a gametic chromosome number count of *n*=21, confirmed from the presence of 21 bivalents in PMCs at M-I (Fig. 2A). 1B-chromosome was also observed in few pollen mother cells at metaphase-I (Fig. 2B, C) of an accession scored from Gaumukh. Majority of the pollen mother cells during meiotic course observed with normal meiotic behaviour except some percentage of pollen mother cells of three accessions (PUN 61955, 61956, 61958) noted with the phenomenon of cytomixis at diakinesis and M-I (Fig. 2E), chromatin stickiness (Fig. 2D), with migrated chromatin material masses (Fig. 2F), late dysjunction of bivalents, chromatin bridges (Fig. 2G), and formation of variable-sized pollen grains (Fig. 2H) in these accessions (Table 2). Due to a low ratio of meiotically abnormal pollen mother cells to normal pollen mother cells in the studied accessions, recorded high percentage of pollen fertility (90-98%) (Fig. 2I, J).

DISCUSSION

Chromosome number and ploidy

A. thomsonii is an endemic grass to Western Himalaya, and the analyzed gametophytic chromosome number count of $2n=42$ is a first record for the species. In the case of *E. nutans* record of 1B-chromosome is also a first record in the species. Earlier, the first chromosome number count of $2n=42$ for *E. nutans* has recorded by Gohil and Koul (1985) from Fotula, a cold desert region of Ladakh, Western Himalaya, India and from Sichuan and Qinghai regions of China by Liu (1985). Reports of chromosome number, $2n=42$ are also known from Pakistan Himalaya (Salomon *et al.* 1988) and other distant regions of China (Lu *et al.* 1990; Lu 1993, 1994; Lu and Bothmer 1993; Chen *et al.* 2009, 2013; Dou *et al.* 2009, 2017; Yan *et al.* 2009, 2010). As tribe Triteceae has basic chromosome number, $x=7$ and species of genus *Agropyron* have three ploidy levels ($2n=2x$, $4x$, $6x$) with only P genome, but the species of genus *Elymus* have four ploidy levels ($2n=2x$, $4x$, $6x$, $8x$) with genome constitution of 'S/St', 'H' and 'Y' in different combinations (Dewey 1984). So, *A. thomsonii* chromosomally exists at hexaploid ploidy level, and the genome is autopolyploid in nature. Similarly, *E. nutans* is also a hexaploid species, but its genome constitution is of strict allopolyploid in nature (Lu 1993, 1994). Both the species are hexaploid and during the meiotic course in pollen mother cells, noted with formation of regular bivalents without any indication of multivalents, displaying the diploid like meiosis. The diploid like meiosis is probably the result of the selection of mutations in *loci* involved in chromosome pairing and chiasma formation facilitated by parental genome chromosomes (McGuire and Dvořák 1982). Analysis of synaptonemal-complex in allopolyploid grasses reveals the existence of diplodizing genetic system as in *Festuca* spp. (Thomas and Humphreys 1991, Thomas and Thomas 1993), *Triticum* and *Aegilops* spp. (Holm 1986, Holm and Wang 1988, Cuñado *et al.* 1996 a, b, c, Cuñado and Santos 1999) that works through the restriction of synapsis to homologous chromosomes and suppression of crossing over among non-homologous chromosomes.

Cytomixis, meiotic abnormalities, and its consequent effects

The abnormal meiotic course often leads to disturbances in microsporogenesis henceforth resulting in pollen malformation or sterility and furthermore negatively influence the reproductive success of the species in the wild (Lattoo *et al.* 2006; Kumar and Singhal 2008; Singhal and Kumar 2008; Kumar 2010). Meiotic abnormalities in natural conditions act as agents of polyploidy in plants (Mason and Pires 2015). The deviation from the normal meiotic course may result in unreduced gamete formation. Such male meiotic studies in wheat grasses (*Agropyron* and *Elymus*) can be of great importance in discovering wild relatives of cultivated crops. In the current study the phenomenon of cytomixis observed predominantly during the first meiotic division in both species. Cytomixis is a natural phenomenon, involving transfer of chromatin material mainly in proximate meiocytes/cells of plants through cytomictic channels (Mursalimov and Deineko 2017). Körnicke (1901) was the first one to observe cytomixis in meiocytes of *Crocus sativus.* However it was Gates (1911) who coined the term '*Cytomyxis*' (nowadays '*Cytomixis*') and was defined it as the chromatin extrusion process which is a natural part of meiosis. Nowadays, cytologists based on the observations with modern tools employed in plant sciences consider it, as cell to cell communication (Kravets *et al.* 2017); a biological process (Sidorchuk *et al.* 2016) that takes place without any damage to migrated chromatin material (Mursalimov *et al.* 2018) and as an additional putative genetic recombination process (Mursalimov and Deineko 2017). So, cytomixis is a natural meiotic aberration of potential evolutionary significance (Singhal *et al.* 2018a). In present study, during male meiotic course of *A. thomsonii*, 14.04-16.29% and 2.97-4.17% PMCs at prophase-I and M-I, respectively depicted the phenomenon of chromatin transfer.

Similarly, 5.56-9.41% and 2.5% PMCs of *E. nutans* at prophase-I and M-I, respectively showed the phenomenon of chromatin transfer among proximate pollen mother cells. Cytomixis was observed in the early stages of the first meiotic division only. High frequency of cytomixis during the first meiotic division results in high sterility and formation of heterogeneous sized pollen grains (De and Sharma 1983; Consolaro and Pagliarini 1995; de Souza and Pagliarini 1997; Pierozzi and Benatti 1998; Singhal and Kumar 2008). The chromatin transfer involves partial or complete migration of nuclear contents, which results into origin of hyper, hypoploid and enucleated meiocytes. Also, few PMCs during chromatin transfer along with/without migrated chromatin material also acquire additional nucleoli (Kumar 2010). Kumar and Singhal (2016) while enlisting 31 species possessing additional nucleoli suggested that in majority of the species with an additional nucleoli are resulted due to phenomenon of cytomixis. PMC's at M-I, A-I/II and T-I/II observed with other meiotic abnormalities, depict a syndrome of errors occurred during the meiotic course. Differential extent and intensity of pairing/crossing over among non-sister chromatids of homologous chromosomes results to non-synchronous dysjunction of bivalents during meiosis-I, and formation of chromatin bridges occurs (Kumar 2010). Crossing over within paracentric inversion pairing loops or U-type exchange between non-sister chromatids during paring creates dicentric bivalent bridges and acentric fragments configurations observed at early T-I. Formation of dicentric bridge and laggards/fragment as meiotic configurations during meiotic course appears as a meiotic syndrome that depicts reduced control over meiotic course (Jones and Brumpton 1971). Presence of large number of laggards, possibly due to abnormal spindle, disturbed cytoskeleton and other cellular changes. These chromatin fragments/laggards lead to formation of micronuclei in sporads. As, abnormal chromosome segregation, presence of micronuclei and reduced pollen fertility results due to formation of multiple spindles at meiosis-I (Vasek 1962). In Jadh Ganga Valley, cold conditions are prevalent and both the accessions of *A. thomsonii* growing in this region are prone to extreme temperature fluctuations *i.e.* short warm days and long cold nights. Male reproductive organs and their development are extremely sensitive to cold stress (Liu *et al.* 2019). So, during the microsporogenesis, formation of shrivelled microspores noted in tetrads that may be due to cold stress-induced abnormal development mediated through malnutrition. Cold stress disrupts stamen development and prominently interferes with tapetum programmed cell death, which is crucial for progression of normal meiotic course and development of microspores to pollens (Oliver *et al.* 2005, 2007; Sharma and Nayyar 2014; Liu *et al.* 2019).

The phenomenon of cytomixis and associated meiotic abnormalities in *A. thomsonii* and *E. nutans* seems to be due to low-temperature stress conditions that are prevalent in the region and have potential to alter the expression of certain alleles controlling the vital steps of meiosis. Previous cytological studies of Bedi (1990), Bellucci *et al.* (2003), Malallah and Attia (2003), Kumar *et al.* (2010, 2011, 2014, 2017), Singhal and Kumar (2008, 2010) and Mandal *et al.* (2013) are also in the view that cytomixis is under direct control of genetic factors.

Pollen development is a complex process regulated at different genetic levels. Mutants showing abnormal pollen development can be of beneficial help in understanding the process of pollen development (Sheila 1993). Cytomixis, coupled with associated meiotic abnormalities, leads to the formation of genetically variable pollen grains, affecting pollen size and fertility. Pollen size variation depends upon the extent of chromatin material, or amount of DNA (Stebbins 1971) possessed/lost by meiocytes during chromatin transfer, and presently somewhat pollen size variation was observed. Effects of cytomixis on meiotic course, pollen size, and pollen fertility have previously been reported in grasses *viz. Agropyron cristatum* (Bauchan *et al.* 1987), *Alopecurus arundinaceus* (Koul 1990), *Brachiaria humidicola* (Boldrini *et al.* 2006), *Elymus semicostatus* (Singhal *et al.* 2018c), *Urochloa panicoides* (Basavaiah and Murthy 1987), and many other flowering plants *viz. Vicia faba* (Bhat *et al.* 2006), *Nicotiana tabacum* (Mursalimov and Deineko 2011), *Chlorophytum borivilianum* (Mandal and Nandi 2017), *Anchusa* spp. (Keshavarzi et al. 2017), *Thalictrum cultratum* (Kumar et al. 2017) and *Clematis ladakhiana* (Khan et al. 2018).

In the end, it may be summarized that individuals of *A. thomsonii* and *E. nutans* of cold desert habitat are of dwarf habit and whereas of other alpine regions of western Himalaya are taller. Respectively, both the species unequivocally noted with chromosome number count of $2n=42$ and $2n=42+0\text{-}1B$ with $6x$ ploidy level are pioneer counts for the species. In natural habitats of these species cold climatic conditions are prevalent and seem these species are differentially affected by cold stress, which is a potential inducer for abnormal meiotic course and sporads. The phenomenon of cytomixis and associated meiotic abnormalities observed in pollen mother cells of *A. thomsonii* and *E. nutans* affects the pollen size and pollen fertility in the species.

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