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Occurrence of meiotic abnormalities and a new report of tetraploid cytotype (4*x*) of *Artemisia nilagirica* (C.B. Clarke) Pamp. from Kullu district in Northwest Himalayas

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ABSTRACT: *Artemisia nilagirica* (Asteraceae), an important medicinal herb, is widely distributed in the hilly regions of India. On accession based analysis from Western Himalayas in Kullu district, Himachal Pradesh, we here report the presence of individuals with different chromosome counts ($2n$ = 36, 54) and two ploidy levels ($2n$ = 4*x*, 6*x*). Both the cytotypes showed allopolyploid meiotic behaviour with perfect 18 and 27 bivalents and equal segregation during anaphase-I. A few meiocytes in these polyploid taxa showed some meiotic irregularities involving chromatin stickiness and abnormal spindle activity leading to laggards, bridges and abnormal sporads and consequently some sterile/unstained pollen grains. Both the polyploid taxa grow under similar ecological conditions but 6*x* plants were observed to be more robust, grow much taller and possessed larger inflorescences. The 6*x* plants also differ from 4*x* individuals in microscopic characters like stomata and pollen grains which are larger sized in 6*x* compared to 4*x*.

KEY WORDS: *Artemisia nilagirica*; Asteraceae; Chromosome; Intraspecific euploidy; Male meiosis; Western Himalays.

INTRODUCTION

Artemisia nilagirica (Clarke) Pamp., popularly known as 'Indian Wormwood', 'Fleabane'or 'Mugwort' is an aromatic shrub grows in Himachal Pradesh, Jammu and Kashmir, Maharashtra, Karnataka, Tamil Nadu, and Kerela states of India, ascending to an altitude of 3600 m. The species which is indigineous to Europe, Asia, North Africa, Alaska and North America is frequently used for the treatment of diabetes, epilepsy, depression, insomnia, anxiety and stress (Walter *et al*., 2003). Various plant parts are used for the treatment of cancer in the hilly regions of Uttarakhand state, India (Sharma *et al*., 2011). Plant is used as an anthelmintic, antiseptic, antispasmodic and as a tonic (Duke *et al*., 2002). Sati *et al*. (2013) reported that *A. nilagirica* oil is very effective against root rot pathogens such as *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Macrophomina phaseolina*. Sonker *et al*., (2015) reported the efficient use of this oil as a mycotoxicant against post harvest mycobiota of table grapes. Recently, Mishra *et al*. (2017) reported its termiticidal activity from south India.

Perusal of existing chromosomal literature reveals that a diploid cytotype with $2n$ = 18 has been reported by Mehra and Ramanandan (1969, 1974) from Kashmir hills and Bala and Gupta (2013) from Kangra district, Himachal Pradesh. From south Indian hills, Mathew and Mathew (1988) detected the presence of 6*x* individuals with $2n$ = 54. Through extensive and intensive explorative surveys from Kullu district, Himachal Pradesh we have detected the existence of two intraspecific euploid cytomorphovariants (4*x*, 6*x*). The aims of the present investigation were to (i) analyze the meiotic course including microsporogenesis and pollen fertility in both the cytotypes, (ii) pinpoint the morphological traits that could be employed for the segregation of these cytotypes.

MATERIALS AND METHODS

Materials for cytological, pollen grains and morphometric traits were collected from the wild individuals growing in Kullu district, Himachal Pradesh during the months of April-September, 2010–2013. For male meiotic analysis, capitula of suitable sizes were fixed in Carnoy's fixative (mixture of ethanol, chloroform and glacial acetic acid in a volume ratio of 6:3:1) for 24 h. Subsequently, the materials were transferred in 70% alcohol and stored in a refrigerator until use. Meiotic preparations were made by squash technique in 1% acetocarmine. Freshly prepared slides were examined to determine the chromosome number. PMCs were also examined for detailed meiotic behaviour at different stages, diakinesis, metaphase-I (M-I), anaphase-I/II (A-I/II), telophases-I/II (T-I/II) and tetrads. In accessions depicting normal meiotic course, 20–30 PMCs were examined for determining the chromosome counts, while those showing meiotic aberrations, 200–300 PMCs prepared from different florets/capitula were analyzed. Pollen fertility was estimated through stainability tests by squashing the mature anthers in glycerol-acetocarmine (1:1) mixture. Well filled pollen with stained nuclei and cytoplasm were taken as apparently fertile while those with partially stained/ unstained cytoplasm and shriveled nature were considered as sterile. For stomatal studies,

Table 1: meiotic abnormalities in one of the accessions of tetraploid cytotype (2*n* = 4*x* = 36) and hexaploid cytotype (2*n* = 6*x* = 56) analyzed from Palchan (PUN 58969)

	2n = 4x = 36		2n = 6x = 56	
Meiotic abnormalities	PMCs analyzed/ number	%	PMCs analyzed/ number	%
PMCs showing chromatin stickiness	659/734	89.78%	329/686	47.95%
PMCs showing scattered/unoriented bivalents			74/686	10.78%
PMCs with chromatin bridges at A-I/A-II	20/ 734	2.72%	71/ 686	10.34%
PMCs with laggards at A-I/T-I, A-II/T-II	15/734	2.04%	72/686	10.49%
PMCs with multipolarity			30/686	4.37%
Dyads			154/405	38.02%
Abnormal sporads	154/305	50.5%		
Sporads with micronuclei	30/305	9.83%	27/405	6.67%
Pollen sterility		31%		17-37%

Table 2: Distribution, habit, habitat, macro-and microscopic characters in the two cytotypes of ***A. nilagirica***.

Characters	Tetraploid (4*x* cytotype)	Hexaploid (6*x* cytotype)
Distribution	Less common	More common
Habitat	Shrubberies, along roadsides	Dry and waste places along roadsides
Appearance	Short and thin	Tall and robust
Plant height (cm)	70–90	120–145
Inflorescence	less branched	Branched
Length (cm)	9–12 (10 ± 1.29)	13–20 (16.25 ± 2.98)
No. of floral heads/spike	20–60 (40.75 ± 16.6)	40-100 (70 ± 25.2)
Stomatal size (µm)	24.87–25.69 × 20.55–22.23 (25.15 ± 10.37 × 21.34 ± 0.74)	26.25–27.31 × 23.11–24.42 (26.84 ± 0.46)
Pollen grains		
Fertility (% age)	69-100	63-83
Size (µm)	Uniform-sized	Heterogenous-sized
Large-sized		31.23–35.18 × 31.13–34.73
Typical-sized	26.53–26.95 × 24.69–26.61	27.60–29.14 × 27.59–30.78
Small-sized		22.02–26.22 × 19.97–24.18
Sample	PUN 58969, 58970	PUN 58971, 58972, 58973

abaxial epidermal peels obtained through KOH treatment were stained in 1% safranin. Best plates of chromosome spreads, meiotic abnormalities, sporads, pollen grains and epidermal peels were photographed from the temporary mounts using Nikon Eclipse 80i microscope. Voucher specimens of the cytologically examined accessions were deposited in the Herbarium (PUN), Department of Botany, Punjabi University, Patiala.

RESULTS

While exploring the cytomorphological diversity in the flowering plants of Kullu district, we have detected the presence of individuals with two chromosome counts (2*n* = 36, 2*n* = 54) with ploidy levels (4*x*, 6*x*) in *Artemisia nilagirica.* Detailed results covering meiotic course including pollen grains and morphometric analysis in two cytotypes is given as under:

Cytology

The tetraploid (2*n* = 36): Accessions collected from moist and shady places around Palchan (2400 m) and Bahang (2450 m) near Manali in Kullu district showed the gametic chromosome count of *n* = 18 as confirmed from the presence of 18 countable bivalents at M-I (Fig. 1a) and 18:18 distribution of chromosomes at M-II (Fig. 1b). While the accession analyzed from Bahang (PUN 58970) showed normal meiotic behaviour resulting into 100% fertile pollen grains. In the accession scored from Palchan (PUN 58969), 89.78% of the 734 analyzed meiocytes showed chromatin stickiness (Fig. 1c) and abnormal spindles resulting into chromatin bridges and laggards (Fig. 1d). Consequently, the accession showed sporads with micronuclei (Fig. 1e). Out of 305 sporads analyzed, 154 sporads (50.5%) were found to be abnormal. The products of such sporads yielded 31% sterile pollen grains (Table 1).

The hexaploid (2*n* = 54): Three accessions gathered from Palchan (2400 m PUN 58973), Vashisht Village (2475 m; PUN 58971) and Solang Valley (2750 m; PUN 58972) showed the gametic chromosome number *n* = 27 ascertained from the presence of 27 bivalents at diakinesis (Fig. 1f) and 27:27 chromosomal distribution at A-I (Fig. 1g). Meiocytes in all the three accessions showed some meiotic irregularties involving chromation stickiness (Fig. 1h), abnormal spindle activity in the form of scattered/unoriented bivalents (Fig. 1i), chromatin bridges (Fig. 1j) laggards (Fig. 1k) and multipolar PMCs (Fig. 1l). Analysis of 686 PMCs at different meiotic stages revealed that 576 PMCs) (83.93%) showed such abnormalities. These meiotic irregulaties in the meiocytes resulted into abnormal sporads such as dyads (Fig. 1m) and sporads with micronuclei (Fig. 1n). Out of the 405 sporads analyzed, 181 (44.69) were observed to be abnormal. The products of such sporads yielded heterogeneous sized fertile and sterile (17–37%) pollen grains (Table 1)

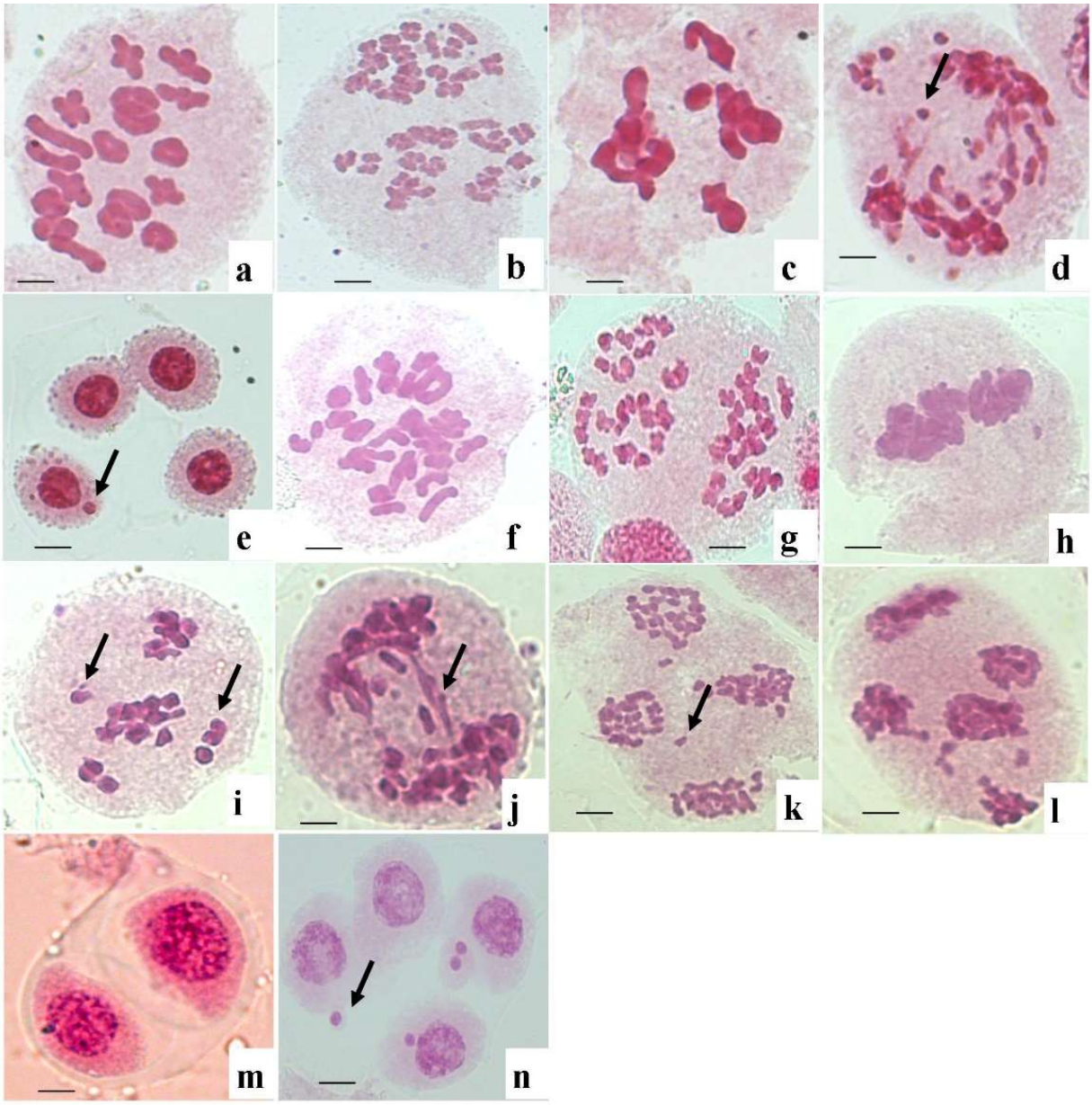

Fig. 1. Male meiosis of ***Artemisia nilagirica*** (**a**) a PMC showing 18 bivalents at M-I; (**b**) a PMC showing 18:18 chromosomes at M-II; (**c**) a PMC showing chromatin stickiness; (**d**) a PMC showing laggards and chromatin bridges at A-II; (arrowed) (**e**) a tetrad with one unit showing micronuclei (arrowed); (**f**) a PMC showing 27 bivalents at diakinesis (**g**) a PMC showing 27:27 distribution of chromosomes at A-I (**h**) a PMC showing chromatin stickiness; (**i**) a PMC showing unoriented bivalents at M-I (arrowed); (**j**) a PMC showing chromatin bridges at A-I(arrowed); (**k**) a PMC showing laggards at A-II (arrowed); (**l**) a multipolar PMC at A-II; (**m**) a dyad; (**n**) a tetrad with micronuclei (arrowed). Scale bar = 10μm.

Morphometric analysis

Plants of 4*x* and 6*x* cytotypes showed random distribution in the area but differ significantly in various morphological traits related to general appearance, size of inflorescence, number of capitula per panicle and in microscopic parameters like stomatal size and pollen diameter. (Table 2). The hexaploid individuals which grow taller (120–145 cm) are more robust compared to the 4*x* which are thin and smaller in size (70–90 cm; Figs. 2a, 2b). The 6*x* plants also possessed larger sized and much branched panicles with higher number of floral heads, 40–100 (70 ± 25.2) compared to the 4*x,* 20–60 (40.75 ± 16.6). The 6*x* plants could also be segregated on the basis of stomata and pollen grains. Stomata of the 6*x* individuals were larger (26.25–27.31 × 23.11–24.42 μm) than those of 4*x* (24.87–25.69 μm × 20.55–22.23 μm) (Figs. 2c, 2d). Fertile pollen grains of 4*x* were uniform sized (Fig. 2e) and measure (26.53–26.95 μm × 24.69–26.61 μm) while those in 6*x* are heterogeneous sized (Fig. 2f). Accordingly, the pollen grains in 6*x* individuals were

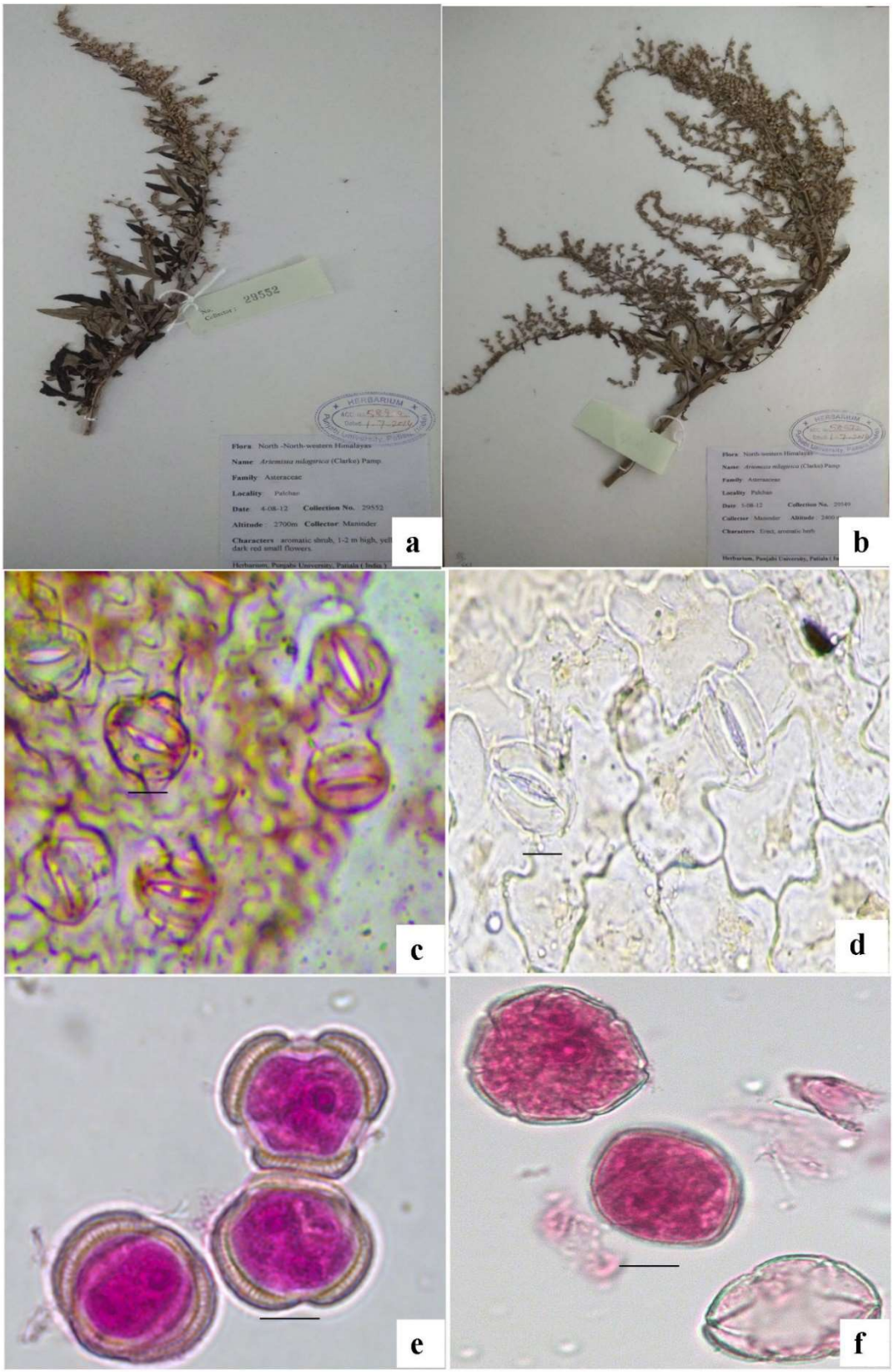

Fig. 2. ***Artemisia nilagirica*** (**a**) A photograph of accession of 4*x*; (**b**) A photograph of accession of 6*x*; (**c**) Stomata of 4*x* plant; (**d**) Stomata of 6*x* plant; (**e**) Pollen grains of 4*x*; (**f**) Pollen grains of 6*x*. Scale bar = 10µm

categorized as large (31.23–35.18 µm × 31.13–34.73 µm), typical (27.59–30.78 µm × 27.60–29.14 µm) and small- sized (22.02–26.22 µm × 19.97–24.18 µm).

DISCUSSION

Accession based study on *A. nilagirica* from Kullu district, Himachal Pradesh report the presence of two intraspecific euploid (4*x*, 6*x*) cytotypes in the studied area. Earlier, Mehra and Remanandan (1969, 1974) have reported the existence of 2*x* plants from KashmirHimalayas. Bala and Gupta (2013) have reported 2*x* individuals from Kangra district (Himachal Pradesh). Bala and Gupta (2013) have also reported the presence of up to 4 B-chromosomes in 2*x* individuals. Mathew and Mathew (1988) while analyzing the cytomorphological diversity in the members of Asteraceae from South India have reported the presence of 6*x* cytotype ($2n = 54$). The present report of 4*x* cytotype is the first record and indicates that the species in India exhibits the existence of intraspecific euploid cytotypes at three ploidy levels (2*x*, 4*x*, 6*x*).

Polyploids in nature originate either between species through interspecific or intergeneric hybridization or within a species when genetically differentiated sub-populations of that species hybridize among themselves. Accordingly, polyploids are classified as allo- or autopolyploids. The polyploids are also categorized as auto- or allopolyploids on the basis of chromosome pairing. The presently detected 4*x* and 6*x* plants of *A.* nilagirica showed typical allopolyploid meiotic behaviour depicting regular chromosome pairing into bivalents and their equal segregation during anaphases. There are numerous reports available in literature where polyploid plants show diploid like chromosomal pairing. Some pollen sterility in these polyploid individuals seem to be resulted due to chromatin stickiness and abnormal spindle activity as already reported in a number of species by cytologists (Caetano-Pereira and Pagliarini, 2001; Mendes-Bonato *et al*., 2002; Kumar and Singhal, 2011; Rana *et al*., 2013; Kumar *et al*., 2014, 2016). However, presence of only bivalents in a polyploid must not be confirmation of its allopolyploid nature. There are many examples of naturally/artificially produced autotetraploids which have very low frequency of multivalent formation (Morrisson and Rajathy 1960; Gottschalk 1978) Several factors such as preferential pairing (Watanabe, 1983), chromosome size (Santos *et al*. 2003), chromosome morphology (Feldman, 1966) , limitation of chiasmata formation (Timmis and Rees, 1971) and specific genes *Ph1* in wheat (Vega and Feldman, 1998) are reported for suppressing multivalent formation in polyploids, so the present species could be tentatively considered as allopolyploid on meiotic analysis, till confirmation through genomic analysis. Such allopolyploids are considered to be more common than autopolyploids mainly due to their well adoptive superiority involving perfect chromosome pairing and segregation and high seed set. Genome duplication (mainly through allopolyploidy) seems to have played an important part in speciation through shaping phenotypic and ecological diversity altering habitat use, life histories, competitive abilities and interaction with herbivores and pathogens and pollinator mediated reproduction (Thompson *et al*., 2004; Oswald and Nuismer, 2007; Thompson and Merg 2008; Arvanitis *et al*., 2010; Boalt *et al*., 2010; Lavania, 2015; Ramsey and Ramsey, 2014; Segraves and Anneberg 2016). And it has often been observed that changes in morphological characters in higher polyploids having multiple set of chromosomes are associated with plant vigour and ability to grow in different environments. All the factors could enable higher polyploids to be more competitive and potentially more invasive in a new environment (Pandit *et al*., 2011, 2014; Laport *et al*., 2016).

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