Accelerating wheat–alien introgression breeding and genome analysis using genome-wide markers

(ゲノムワイドマーカーを用いたコムギの異種遺伝子導入およびゲノム分析の促進)

Edet, Offiong Ukpong

2019

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A Thesis submitted to the United Graduate School of Agricultural Sciences, Tottori University in partial fulfilment of the requirements for the award of Doctoral (PhD) degree in Agricultural Sciences (Plant Molecular Breeding)

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2019

Dedication

This thesis is dedicated to the glory of God and the memory of my late parents, Mr. and Mrs. Ukpong Edet.

Acknowledgements

The invaluable roles of my chief supervisor, Prof. Dr. H. Tsujimoto, deserve special mention. His kind disposition, dogged supervision and scholarly guide gave me a safe ride to success. I will ever remain grateful to Prof. Benjamin E. Ubi, Dean, Faculty of Science, Ebonyi State University, Nigeria, for his rare contributions.

The inputs of my co-supervisors, Prof. Dr. M. Shigyo of Faculty of Agriculture, Yamaguchi University, Japan and Dr. K. Akashi of Faculty of Agriculture, Tottori University, are highly acknowledged. I appreciate the support of my collaborators, Dr. J-S. Kim of RIKEN Center for Sustainable Resource Science, Tsukuba, Japan, Dr. M. Okamoto of Center for Bioscience Research and Education, Utsunomiya University, Japan, Dr. K. Hanada and Mr. Takeda of Department of Bioscience and Bioinformatics, Kyushu Institute of Technology, Japan, Dr. M. Kishii of International Maize and Wheat Improvement Center, Mexico and Dr. S. Cho of Department of Crop Science and Biotechnology, Chonbuk National University, Republic of Korea. The friendly members of my Laboratory and my colleagues will always be remembered for their immeasurable assistance. My sincere appreciation also goes to Japan Student Services Organization and the management of Tottori University for respectively providing financial support for my upkeep and granting me tuition fee waivers.

I thank my younger brother, Francis, uncle, ASP Tony and fiancée, Gina for always keeping in touch. Their warm words of encouragement did not only inspire me but bridged the distance between Japan and Nigeria. My greatest gratitude goes to the Almighty God, Great Architect of the Universe for enabling me to get to this stage.

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CHAPTER ONE

INTRODUCTION

Breeding of wheat for improved adaptability and stability of yield under adverse environmental conditions is hindered by narrow genetic diversity in wheat landraces. Therefore, to keep pace with the high global dependence on wheat-rated diets and the attendant growing demand for wheat (FAO, 2017, 2018; Reeves, 2016; Shiferaw *et al*., 2013), mining of useful genes from wheat's wild relatives, through distant hybridization, to broaden diversity in wheat germplasm, would continue to play a significant role in improved cultivar development. As these wild relatives are resistant to various biotic and abiotic stresses (Gong *et al*., 2017; Sha *et al*., 2017; Stoyanov, 2014; Zhang *et al*., 2017), their chromosome segments in the genetic background of wheat would aid to combat the current trend in global climatic change which results in reduced yield of crops (Lesk *et al*., 2016; Tack *et al*., 2015; Zhang *et al*., 2017; Zhao *et al*., 2017). This breeding approach is, however, largely hindered by linkage drag (Zhang et al., 2017) and low rate of success in distant hybridization. With the current status of genome sequencing technology and improved interspecific hybridization techniques, these hindrances can effectively be managed. While *in vitro* culture techniques, example embryo rescue (Cisneros and Tel-Zur, 2010) and induction of homoeologous chromosome recombination (Liu *et al*., 2014) have been employed to achieve successful distant hybridization and useful gene recombination, integration of appropriate molecular markers into breeding programs to conduct marker-assisted backcrossing and selection can immensely assist in selecting against deleterious genes. However, although the genomes of important cultivated Triticeae species have been extensively analyzed, the current status of genomic resources of wheat's wild relatives is inadequate, culminating in a poor understanding of

their genomics (Ceoloni *et al*., 2015; Rey *et al*., 2015). This has resulted in underutilization of the readily available wild genetic resources, as the success of wheat–alien introgression breeding relies on the availability of appropriate cytological and/or DNA markers to identify the alien chromatins in wheat genetic background. This research, therefore, sought to address this gap by developing and validating genome-wide markers of two distant relatives of wheat, *Leymus racemosus* and *L. mollis*, to further wheat–*Leymus* introgression breeding. Also, although the application of *in situ* hybridization (ISH) techniques for alien identification have aided production and characterization of wheat–alien chromosome introgression lines (CILs) (Ali *et al*., 2016; Bao *et al*., 2012; Ceoloni *et al*., 2017; Kishii, 2011; Kishii *et al*., 2010; Kishii *et al*., 2004), some plant species, *L. mollis* for instance, lack variable cytological markers to enable the differentiation of their chromosomes in wheat genetic background (Kishii *et al*., 1999; Kishii *et al*., 2002).

Wheat breeders, in various attempts to deal with the aforesaid, have had to resort to applying available expressed sequence tags (ESTs) from a few perennial grasses and heterologous markers from annual cereals, example barley, to aid their work (Hagras *et al*., 2005; Wang *et al*., 2017). The outcomes from these alternatives are hardly satisfactory because of increased species divergence arising from mutations and other genetic events during speciation. For instance, low transferability and polymorphism was reported when EST-derived markers were applied to identify *Lophopyrum elongatum* specific loci in wheat background (Mullan *et al*., 2005). Thus, to effectively harness useful genes from these wild genetic resources, their genomic information base should be continually enriched to at least include data on outstanding species that can serve as representatives for their evolutionary close relatives. Efforts to achieve this have generated enormous

molecular cytogenetic data, EST-SSR markers, EST linkage maps and other useful information (Bushman *et al*., 2008; Larson *et al*., 2012; Pang *et al*., 2014; Rey and Prieto, 2017; Wang *et al*., 2017; Yang *et al*., 2017). However, before this study, genome-wide molecular markers of *Leymus* species were lacking. This made it difficult to take the advantage of marker-assisted selection to speed up the development of wheat–*Leymus* CILs.

Furthermore, following the overwhelming role of wild species as potential sources of essential alleles for the improvement of their cultivated relatives, especially common wheat, analysis of genomic and evolutionary relationship among these species and their cultivated relatives has received enormous attention and continues to be a key research interest (Badaeva, 2002; Badaeva *et al*., 2004; Badaeva *et al*., 2018; Friebe *et al*., 1996; Molnár-Láng *et al*., 2015). This analysis has mostly been conducted using cytogenetic approaches that rely on meiotic chromosome pairing in hybrids of wide crosses. Chromosome pairing is affected by diverse factors; hence, the reliability of failed chromosome pairing as an indicator of genome dissimilarity has been questioned (Baum *et al*., 1987; Farooq *et al*., 1990; Seberg and Petersen, 1998). Chromosome banding and molecular cytogenetic techniques have also helped to generate useful information on genome differences and phylogenetic relationships among important Triticeae species (Badaeva, 2002; Badaeva *et al*., 2004; Badaeva *et al*., 2018; Coriton *et al*., 2009; Molnar *et al*., 2015). Other studies have explained the origin and differentiation of *Aegilops* species and *Aegilops-Triticum* relationships (Goryunova *et al*., 2010; Goryunova *et al*., 2004; Mizuno *et al*., 2010; Wang *et al*., 2011). However, the inclusion of *Ae. speltoides* in section Sitopsis, exact progenitors of some *Aegilops* species and the B genome donor of hexaploid wheat and other polyploid *Triticum* species are still in dispute (Badaeva *et*

al., 2004; Badaeva *et al*., 1998; J. Dvorak, 1998 ; Feldman and Levy, 2015; Goryunova *et al*., 2008; Goryunova *et al*., 2004; Resta *et al*., 1996). Therefore, to validate the results so far obtained and fill remaining gaps where possible, molecular data, especially those with a wide genomic coverage, are indispensable.

Given the background enumerated above, this research was designed to achieve the following objectives:

1. To develop and validate *L. racemosus* genome-wide polymorphic markers
2. To analyze the transferability of *L. racemosus* markers to related species whose genomes have not been sequenced
3. To device and validate a molecular marker-based strategy to produce and characterize wheat–*L. mollis* chromosome addition lines (CALs), without necessarily applying ISH for alien identification
4. To assess the applicability of DArTseq genotyping in characterizing wheat–alien CILs and analyzing genomic relationships among Triticeae species

As contained in the results and discussion chapters of this thesis, the four objectives were successfully attained, birthing three research articles published in *BMC Genetics* and *Scientific Reports*.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introgression breeding of wheat

Selection pressure during domestication and subsequent breeding for superior yield characteristics unfortunately narrowed diversity in cultivated wheat germplasm, necessitating introgression of useful genes from wheat's primary, secondary and tertiary gene pools to broaden its genetic diversity and improve adaptability. Essentially, introgression breeding is achieved by artificially crossing two distant species and doubling the chromosomes of the hybrid to produce amphidiploids, and subsequent production of stable genomes carrying whole or segments of alien chromosomes (Liu *et al*., 2014). Because of genetic incompatibility, in most cases, embryo rescue is applied to raise hybrids of distant crosses and where spontaneous chromosome doubling does not occur, fertility is achieved by artificially doubling chromosomes by colchicine or any other suitable treatment. Noteworthy is that apart from Triticale, which has been developed and commercialized mostly as a forage crop (Ayalew *et al*., 2018; McGoverin *et al*., 2011), amphiploids are not released as wheat cultivars. They are rather used as intermediary materials to generate wheat–alien chromosome introgression lines: addition, substitution, translocation and recombination lines (Liu *et al*., 2014; Molnár-Láng *et al*., 2015).

The most common strategy adopted to harness genes from the direct progenitors of bread wheat is using synthetic hexaploid wheats (SHWs), which are artificially resynthesized hexaploid wheat lines through hybridization of tetraploid wheat (*Triticum turgidum* L., AB genome) and *Aegilops tauschii* Coss., the D genome donor. These lines, which combine diverse genes from the AB and D genomes were massively produced by

the International Center for Wheat and Maize Improvement (CIMMYT) between 1988 and 2010 (Börner *et al*., 2015). Gene introgression through SHWs have resulted in the identification of many useful genes and development of wheat lines with resistance to leaf, stem and stripe rusts, cereal cyst and root lesion nematodes, Fusarium crown rot, yellow leaf spot, Septoria tritici blotch (STB), and Septoria nodorum blotch (SNB) (Börner *et al*., 2015; Mulki *et al*., 2013; Ogbonnaya *et al*., 2013; Olson *et al*., 2013). Boron (B) toxicity has also been successfully managed with SHWs. Sixteen SHW lines derived from a susceptible tetraploid parent were reported to be tolerant to B toxicity (Ilyas *et al*., 2015). Also, genomic regions believed to contain novel genetic loci for tolerance to B toxicity were identified in SHWs, using genome-wide scan and DArT markers (Emebiri & Ogbonnaya, 2015).

Wild *Aegilops* and *Triticum* species, including bread wheat's direct progenitors and non-progenitors, have also been directly exploited to enhance improved performance of elite cultivars (Fedak, 2015; Zhang *et al*., 2015). Being the closest genus to *Triticum*, genes from *Aegilops* species have extensively been introgressed into wheat to breed for resistance to biotic and abiotic stresses in cultivars of wheat. Notable among these stresses are cereal cyst nematode, root-knot nematode, Hessian fly, greenbug, powdery mildew, rusts, drought, cold, heat and salinity (Schneider *et al*., 2008; Sharma and Gill, 1983; Stoyanov, 2014; Zhang *et al*., 2015). Similarly, wild *Triticum* species have enormously assisted the development of wheat lines with proven resistance to different rust diseases (Chhuneja *et al*., 2008; Kolmer *et al*., 2010; Kuraparthy *et al*., 2007; Singh *et al*., 2007), Fusarium head blight (Cai *et al*., 2005; Fedak, 2015), Karnal bunt (Vasu *et al*., 2000) and powdery mildew (Chhuneja *et al*., 2012; Hua *et al*., 2009; Mohler *et al*., 2011; Mohler *et al*., 2013). Improved storage proteins, cold hardiness and resistance to Hessian fly have

also been recorded through wild *Triticum* species introgression (Fedak, 2015).

Leymus species (Hochst.) and other distant relatives of wheat in the tribe Triticeae have proven to be potential gene sources for improvement of bread wheat (Ali *et al*., 2016; Anamthawat-Jonsson, 2001; Anamthawat-Jonsson *et al*., 2009; Chen *et al*., 2005; Hajjar and Hodgkin, 2007; King *et al*., 2013; Kishii *et al*., 2010; Kishii *et al*., 2004; Niu *et al*., 2011; Olson *et al*., 2013; Pradhan and Prasad, 2015; Rahmatov *et al*., 2016; Zhang *et al*., 2017). Tetraploid *Leymus* species, including *L. racemosus and L. mollis* analyzed in this study, are believed to have two distinct basic genomes, Ns and Xm (2n = 4x = 28, NsNsXmXm), from *Psathyrostachys* and an unknown source, respectively, (Fan *et al*., 2009), but more recent analyses suggest that the two genomes are from *Psathyrostachys*, presenting them as segmental polyploids (2n = 4x = 28, $Ns_1Ns_1Ns_2Ns_2$) (Anamthawat-Jonsson, 2014; Fan *et al*., 2014). *Leymus* species are known for their high potentials for improving bread wheat's resistance to important abiotic and biotic stresses (Chen *et al*., 2005; McGuire, 1981; Qi *et al*., 2008; Xiao *et al*., 2012; Yang *et al*., 2015). The recognition of the potentials of *Leymus* species as valuable gene sources for the improvement of wheat dates back to the 1960s when Tsitsin (1965) reported the production of different combinations of wheat–*Leymus* amphidiploids. Subsequent studies in this direction have demonstrated high cytogenetic stability in wheat–*L. mollis* octoploids and varying segregation and transmission rates of alien (*L. mollis*) chromosomes in different backcross generations of wheat–*L. mollis* backcross populations (Fu *et al*., 1993; Fu *et al*., 1996; Fu *et al*., 1997; Wang *et al*., 2013). The segregation rates are usually narrower in BC_1F_1 as compared to F_2, while alien transmission rates are higher in disomic lines, especially disomic substitution lines, than monosomic lines (Fu *et al*., 1996; Fu *et al*., 1997). Different types (whole-arm or

Robertsonian, intercalary and distal) of wheat–*Leymus* translocation lines have also been developed (Bao *et al*., 2012; Chen *et al*., 2005; Kishii, 2011; Li *et al*., 2015; Pang *et al*., 2014). Li *et al*. (2015) recorded average translocation frequency of 7.55% for *L. mollis* chromosomes, while Kishii (Kishii, 2011) found that the translocation frequencies of *L. racemosus* chromosomes ranged between 0 and 8%, with higher translocation frequencies in the short arms. Already, wheat lines with introgressed chromosome segments of *Leymus* species have reportedly shown resistance to stripe rust, Fusarium head blight and heat stress (Bao *et al*., 2012; Chen *et al*., 2005; Mohammed *et al*., 2014; Yang *et al*., 2014; Yang *et al*., 2015; Yang *et al*., 2017). Particularly, *L. racemosus* is reported to exhibit biological nitrogen inhibition (BNI) activity, a trait with both agronomic and environmental consequences, and one of its chromosomes, LrN, known to control this activity has been introgressed into wheat to enhance this activity in wheat genome (Kishii *et al*., 2004; Subbarao *et al*., 2007; Subbarao *et al*., 2013; Subbarao *et al*., 2007; Subbarao *et al*., 2015).

2.2 Wheat–alien characterization methods

The successful production of wheat–alien introgression lines is highly dependent on the availability of appropriate cytological and/or DNA markers to identify the alien chromatins in wheat genetic background. Phenotypic markers can also be relied upon to identify lines with different alien chromosomes, but better results can be assured with molecular approaches. Cytogenetic techniques of alien identification progressed from mitotic chromosome count through banding techniques to ISH: fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) (Rey *et al*., 2015). Currently, molecular cytogenetic techniques, such as ISH and flow sorting, and conventional DNA and genome sequence-based markers are extensively applied to

identify and characterize alien chromosomes in wheat–alien lines (Ali *et al*., 2016; Edet *et al*., 2018; Sheikh *et al*., 2018; Tiwari *et al*., 2014; Yang *et al*., 2017; Zhang *et al*., 2017). However, the genomic resources of distant wild relatives of wheat are still far from being adequate, which hinders maximal utilization of available wild genetic resources for wheat improvement (Rey *et al*., 2015). Since sequencing of the genomes of most wild relatives of wheat is unlikely, as they are not regarded as crops, efforts to transfer markers across related species is being exploited to aid characterization of chromatins from species whose genomes have not been sequenced.

2.3 Analysis of genomes of Triticeae species

Attempts to shed light on the evolutionary relationships among various species in tribe Triticeae started many decades ago, and currently still occupy a substantial space in Triticeae research (Badaeva *et al*., 2018; Kihara, 1930). The popular 'genome analyzer' system developed by early researchers relied on meiotic chromosome pairing in hybrids of distant crosses (Dewey, 1984; Love, 1984). This foundational strategy accelerated analysis of genomes of Triticeae species but has been criticized for some reasons. Key among the points of disagreement is that chromosome pairing is controlled by diverse genetic factors and therefore, is not a reliable basis to judge similarity or dissimilarity of genomes (Farooq *et al*., 1990; Moore, 2009; Seberg and Petersen, 1998). Nevertheless, this methodology alongside C-banding and ISH have been applied to reveal the genomic relationships between important species, generating tons of interesting and informative data (Badaeva, 2002; Badaeva *et al*., 2004; Badaeva *et al*., 2018; Coriton *et al.*, 2009; Molnar *et al*., 2015). Other molecular approaches, including isozyme analysis, variations in low-molecular-weight glutenin subunit and DNA marker systems have provided some

explanations on *Aegilops-Triticum* relationships, the origin and differentiation of *Aegilops* species, and intra- and inter-specific variations in the D and U genome clusters of *Aegilops* species (Goryunova *et al*., 2010; Goryunova *et al*., 2004; Mizuno *et al*., 2010; Wang *et al*., 2011). Also, a combination of morphology, organelle and nuclear genes reportedly gave insights into the phylogenetic relationships among diploid taxa in Triticeae (Seberg and Petersen, 2007). Nevertheless, diploid progenitors of *Ae. crassa*, *Ae. vavilovii*, *Ae. juvenalis*, *Ae. columnaris* and *Ae. triaristata*, and the exact progenitor of the B genome of hexaploid wheat and other polyploid *Triticum* species are still in dispute (Badaeva *et al*., 2004; Badaeva *et al*., 1998; Dvorak, 1998 ; Feldman and Levy, 2015; Goryunova *et al*., 2004; Resta *et al*., 1996). Also, there are opposing opinions regarding the donors of A genomes of polyploid species in the Emmer (AB group) and Timopheevi (AG group) lineages of wheat (Chantret *et al*., 2005; Dorofeev *et al*., 1979; Giorgi *et al*., 2003; Golovnina *et al*., 2009; Gornicki *et al*., 2014; Kilian *et al*., 2007; Migushova and Konarev, 1975). In the classification of *Aegilops* species, the justification for including *Ae. speltoides* in section Sitopsis is still under discussion (Giorgi *et al*., 2003; Goryunova *et al*., 2008; Kilian *et al*., 2011; van Slageren, 1994).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials

Twenty-two wheat-*L. racemosus* CILs (Table 1), two cultivars of bread wheat, seven *Triticum* species other than bread wheat, 23 *Aegilops* species, eight distant relatives of wheat (Table 2) and 13 uncharacterized backcross populations of wheat-*L. mollis* chromosome introgression lines (Table 3) were studied. The wheat-*L. racemosus* chromosome addition lines (CALs) and wheat-*L. mollis* backcross populations were obtained from the gene bank of Tottori Alien Chromosome Bank of Wheat (TACBOW), a subsidiary of the National BioResource Project (NBRP)-KOMUGI, Japan (https://shigen.nig.ac.jp/wheat/komugi/), while the translocation and recombination lines were provided by Dr. M. Kishii of the International Maize and Wheat Improvement Center (CIMMYT), Mexico. The Triticeae species were obtained from NBRP-KOMUGI gene bank through Prof. H. Tsujimoto of Arid Land Research Center (ALRC), Tottori University, Japan and Dr. S. Nasuda of the Laboratory of Plant Genetics, Graduate School of Agriculture, Kyoto University, Japan. DNA samples of all the *Aegilops* and *Triticum* species, except *T. aestivum*, were provided by Dr. S. Nasuda. Apart from the *Leymus* and *Elymus* species maintained as perennial plants in ALRC, other experimental plants—excluding samples provided by Dr. S. Nasuda—were raised and maintained in trays until leaf samples were ready for DNA extraction. Between 2–4 weeks after sowing, leaf samples were collected, frozen in liquid nitrogen and stored at –80^{o}C until needed for DNA extraction. DNA samples were isolated and purified using the cetyl trimethyl ammonium bromide method. DNA quality check, quantification and concentration adjustment were done with NanoDrop2000C Spectrophotometer.

Table 1 Wheat–*L. racemosus* CILs

Genotype ID	Description	Alien chromosome ID	Chromosome constitution (2n)	Reference
TACBOW 0001	Disomic addition	LrA	21″ + 1″ [A]	(M. Kishii et al., 2004)
TACBOW 0003	Disomic addition	LrE	21″ + 1″ [E]	(M. Kishii et al., 2004)
TACBOW 0004	Disomic addition	LrF	21″ + 1″ [F]	(M. Kishii et al., 2004)
TACBOW 0005	Disomic addition	LrH	21″ + 1″ [H]	(M. Kishii et al., 2004)
TACBOW 0006	Disomic addition	LrI	21″ + 1″ [I]	(M. Kishii et al., 2004)
TACBOW 0007	Disomic addition	LrJ	21″ + 1″ [J]	(M. Kishii et al., 2004)
TACBOW 0008	Disomic addition	LrK	21″ + 1″ [K]	(M. Kishii et al., 2004)
TACBOW 0009	Disomic addition	LrL	21″ + 1″ [L]	(M. Kishii et al., 2004)
TACBOW 0010	Disomic addition	LrN	21″ + 1″ [N]	(M. Kishii et al., 2004)
I short	I short arm translocation	LrIS	42	(M Kishii, 2011)
I long	I long arm translocation	LrIL	42	(M Kishii, 2011)
J short	J short arm translocation	LrJS	42	(M Kishii, 2011)
J long	J long arm translocation	LrJL	42	(M Kishii, 2011)
N short	N short arm translocation	LrNS	42	(M Kishii, 2011)
N long	N long arm translocation	LrNL	42	(M Kishii, 2011)
N recomb #1	N recombination No. 1	LrNR1	42	(O. U. Edet et al., 2018)
N recomb #2	N recombination No. 2	LrNR2	42	(O. U. Edet et al., 2018)
N recomb #3	N recombination No. 3	LrNR3	42	(O. U. Edet et al., 2018)
N recomb #4	N recombination No. 4	LrNR4	42	(O. U. Edet et al., 2018)
N recomb #5	N recombination No. 5	LrNR5	42	(O. U. Edet et al., 2018)
N recomb #6	N recombination No. 6	LrNR6	42	(O. U. Edet et al., 2018)
N recomb #7	N recombination No. 7	LrNR7	42	(O. U. Edet et al., 2018)

TACBOW, Tottori Alien Chromosome Bank of Wheat; Lr, *Leymus racemosus*; A–N, Arbitrary numbering of *L. racemosus* chromosomes; IS, I-short arm; IL, I-long arm; JS, J-short arm; JL, J-short arm; NS, N-short arm; NL, N-long arm; NR, N-recombination

Table 2 Species of tribe Triticeae studied

ID	Species	Subspecies/cultivar	Ploidy	Genome	Source
KU–12007	*Aegilops mutica*	-	2x	T	NBRP
KU–2–5	*Ae. Speltoides*	*typica*	2x	S	NBRP
KU–4–1	*Ae. longissima*	*typica*	2x	S^{l}	NBRP
KU–5–3	*Ae. sharonensis*	*typica*	2x	S^{sh}	NBRP
KU–4–6	*Ae. searsii*	-	2x	S^{s}	NBRP
KU–3–1	*Ae. bicornis*	*typica*	2x	S^{b}	NBRP
KU–2159	*Ae. tauschii*	*typica*	2x	D	NBRP
KU–5860	*Ae. caudata*	*polyathera*	2x	C	NBRP
KU–17–1	*Ae. comosa*	*comosa*	2x	M	NBRP
KU–19–3	*Ae. uniaristata*	*typica*	2x	N	NBRP
KU–8–2	*Ae. umbellulata*	*typica*	2x	U	NBRP
KU–7–1	*Ae. cylindrical*	*typica*	4x	CD	NBRP
KU–22–1	*Ae. ventricosa*	*comosa*	4x	$D^{v}N^{v}$	NBRP
KU–9–1	*Ae. ovata*	*vulgaris*	4x	$U^{g}M^{g}$	NBRP
KU–11–1	*Ae. Columnaris*	*tpica*	4x	$U^{c}M^{c}$	NBRP
KU–13–6	*Ae. kotschyi*	*leptostachya*	4x	$S^{k}U^{k}$	NBRP
KU–15–1	*Ae. Triuncialis*	*typica*	4x	$C^{t}U^{t}$	NBRP
KU–13–1	*Ae. variabilis*	*intermedia*	4x	$S^{p}U^{p}$	NBRP
KU–12–1	*Ae. biuncialis*	*typica*	4x	$U^{b}M^{b}$	NBRP
KU–10–1	*Ae. triaristata*	*vulgaris*	4x, 6x	$U^{n}M^{n}$,$U^{n}M^{n}N^{n}$	NBRP
KU–21–1	*Ae. crassa*	*typica*	4x, 6x	$D^{c}M^{c}$, $D^{c}D^{c}M^{c}$	NBRP
KU–21–7	*Ae. Vavilovii*	*palaestina*	6x	$D^{v}M^{v}S^{v}$	NBRP
KU–23–3	*Ae. Juvenalis*	*typica*	6x	$D^{j}M^{j}U^{j}$	NBRP
KU–199–11	*Triticum urartu*	*nigrum*	2x	A^{u}	NBRP
KT001–001	*T. boeoticum*	*boeoticum*	2x	A^{b}	NBRP
KT009-017	*T. durum*	Langdon KU	4x	AB	NBRP
KU–491	*T. dicoccum*	-	4x	AB	NBRP

Table 2 continued

KU–108–1	*T. dicoccoides*	*kotschyanum*	4x	AB	NBRP
KU–196–1	*T. araraticum*	*tumaniani*	4x	AG	NBRP
KT020-003	*T. aestivum*	Chinese Spring	6x	ABD	NBRP
KU-260	*T. aestivum*	Norin 61	6x	ABD	NBRP
KT018–002	*T. macha*	*palaeo-imereticum*	6x	ABD	NBRP
TACBOW0071	*Secale cereal*	Pektos	2x	R	NBRP
TACBOW0119	*Dasypyrum villosum*	-	2x	V	NBRP
TACBOW0116	*Hordeum vulgare*	Betzes	2x	H	NBRP
TACBOW0117	*H. bulbosum*	-	2x	H	NBRP
TACBOW0122	*Elymus ciliaris*	-	4x	SY	NBRP
TACBOW0121	*Psathyrostachys huashanica*	-	2x	Ns	NBRP
TACBOW0112	*L. racemosus*	-	4x	N_1N_2	NBRP
TACBOW0113	*L. mollis*	-	4x	N_1N_2	NBRP

Names and genome symbols of *Aegilops* and *Triticum* species are as reviewed by Kilian *et al*. (2011), Bernhardt (2015) and Zhang *et al*. (2015). TACBOW: Tottori Alien Chromosome Bank of Wheat. NBRP: National BioResource Project (https://shigen.nig.ac.jp/wheat/komugi/). Additional information on the wild distant relatives of wheat can be found in Hagras *et al*. (2005)

Table 3 Uncharacterized wheat–*L. mollis* backcross populations

Sample ID	2n	Tentative description
CS–LmA	42 – 44	LmA addition
CS–LmC	42 – 44	LmC addition
CS–LmE	42 – 44	LmE addition
CS–LmF	42 – 44	LmF addition
CS–LmG	42 – 44	LmG addition
CS–LmH	42 – 44	LmH addition
CS–LmI	42 – 44	LmI addition
CS–LmJ	42– 44	LmJ addition
CS–LmK	42– 44	LmK addition
CS–LmL	42 – 44	LmL addition
CS–LmM	42 – 44	LmM addition
CS–LmN	42 – 44	LmN addition
Lm BC2-1 (2n = 50) x CS	42 – 50	Mixed Lm chromosomes

CS, Chinese Spring; Lm, *L. mollis*; A–N, Arbitrary tags of *L. mollis* chromosomes

3.2 Designing of primers and development of markers from *L. racemosus* sequence information

Leymus racemosus genome scaffolds polymorphisms against wheat reference genome (v1.1) (IWGSC, 2014) were evaluated by BLASTN, and primers sensitive to relatively large (>3-nt) gaps and mismatches were designed by Primer3 software (version 4.0). More primers designed from *L. racemosus* RNA-seq by collaborators were obtained and screened alongside those designed from genomic sequence.

Utilizing genomic DNA samples from bread wheat and *L. racemosus,* 294 randomly selected primer sets—150 from genomic sequence and 144 from RNA-seq. Each 20 µL reaction volume contained 10 µL KAPA Taq Extra HotStart ReadyMix with dye (KapaBiosystems), 1 µL (10 μM) each of forward and reverse primers, 2 µL (50 ng) DNA template and 6 µL PCR grade water. With BIORAD T100 Thermal Cycler, the samples, in a 96-well plate, were subjected to touchdown PCR: 95^{o}C initialization for 3 minutes, 5 cycles of 95^{o}C denaturation for 30 s, 65^{o}C to 61^{o}C (-1^{o}C/cycle) annealing for 30 s and 72^{o}C extension for 30 s; 30 cycles of 95^{o}C denaturation (30 s), 60^{o}C annealing (30 s) and 72^{o}C extension (30 s) and final extension at 72^{o}C for 10 seconds. Because the average melting temperature (Tm) of primers designed from DNA sequence was about 8^{o}C higher than the average Tm of primers designed from RNA-seq, the annealing range of 65–60^{o}C in the cycling program was substituted with 57–52^{o}C for the primers designed from the RNA-seq. All PCR products were electrophoresed for 30 minutes on 1.5% agarose S gel in Tris-acetate-EDTA (1X TAE) buffer, stained in ethidium bromide solution for 10 minutes and photographed with AE-6932GXCF transilluminator.

Leymus racemosus polymorphic markers were applied to genotype nine wheat–*L. racemosus* chromosome addition lines and markers specific to *L. racemosus*

chromosomes I, J and N were deployed to genotype two each of I-, J- and N-translocation lines. Markers specific to chromosome N were further used to genotype seven N-recombination lines. Bands of dominant PCR markers were scored in a binary fashion, "1" and "0" for presence and absence, respectively, while the few codominant markers were differentiated using 1 to designate band size in wheat and 2 for band size in an introgression line or another Triticeae species, depending on the case. The scores were analyzed using simple proportion to determine the percentage of screened primers amplified in *L. racemosus* genome as well as the proportion of the amplified *Leymus* markers polymorphic in wheat. Also, the frequency of amplification of alien chromosomes in the CILs was computed, in order to determine the proportion of the developed markers in each alien chromosome. Markers specifically amplified by each of the 19 chromosome addition lines were designated chromosome-specific and *L. racemosus* chromosomes I-, J- and N-specific markers specifically located on any of the translocation lines were accordingly considered to be arm-specific. Arm-specific markers of chromosome N specifically amplified by the seven N-recombination lines were applied to map the positions of the recombinant fragments.

3.3 Sequencing and analysis of some PCR products

Sanger sequencing was applied to determine the nucleotide sequence of PCR products generated by one of the markers which amplified all the *L. racemosus* chromosomes added to wheat. All the PCR products were purified with AxyPrep PCR cleanup kit, according to the PCR cleanup spin protocol (AXYGEN Biosciences). The purified products were premixed following Macrogen's recommendation (Macrogen, Japan) and same delivered to the company for sequencing. Each genotype sequence was searched

against nucleotide sequences in NCBI and Ensembl Plants databases using BLASTN. Also, the DNA scaffold from which the marker was developed was searched in like manner. Polymorphisms between the chromosomes were determined by aligning all the sequences using JustBio multiple alignment tool (http://www.justbio.com/hosted-tools.html).

3.4 Analysis of transferability of *L. racemosus* markers to related species

All the markers amplified in *L. racemosus* were applied to genotype 12 species in the tribe Triticeae, including *L. racemosus* as a positive control. This enabled the assessment of the proportion of *L. racemosus* markers that could be utilized to analyze related genomes, with emphasis on determining polymorphism between bread wheat genome and genomes of the other species. For all the amplified markers, the rates of polymorphisms between wheat and the other species were computed. This gave a basis to decide the suitability of *L. racemosus* markers for genotyping of wheat lines carrying chromosomes from these species. The co-amplified markers were used to reconstruct the phylogenetic relationship among the species, to further aid the estimation of the reliability of the markers. In addition, the markers amplified in each species were applied to compare the proportions of polymorphic markers from DNA and RNA sequence information.

3.5 Molecular marker-assisted selection of wheat lines carrying *L. mollis* chromosomes

Leymus racemosus PCR markers transferred to *L. mollis* genome were used to genotype wheat–*L. mollis* backcross populations to enable faster identification and selection of alien carriers. Non-carrier segregants and duplicated carriers were discarded, and the

remaining plants were advanced to BC_3F_4 under a temperature-controlled (22°C day/18°C night) greenhouse. In each generation, disomic plants were distinguished using progeny test. In this test, it was assumed that monosomic addition lines produce non-carrier (2n = 42, AABBDD), monosomic addition (2n = 43, AABBDD + 1′ [Ns]), and disomic addition (2n = 44, AABBDD + 1″ [Ns]) segregants, whereas genetically stable disomic plants do not segregate: they produce only disomic addition lines (2n = 44, AABBDD + 1″ [Ns]). This is because, in meiosis, the monosomic addition lines are expected to produce two different gametes: ABD and ABDNs, while the disomic addition lines should produce one gamete, ABDNs.

3.6 Identification of *L. mollis* chromosomes in wheat–*L. mollis* lines by GISH

The cytological status of each line selected by molecular markers was confirmed by GISH. *Leymus mollis* genomic DNA was labeled with fluorescein-12-dUTP (Thermo Scientific) using Random Primers DNA labeling system (Invitrogen). With the labeled *L. mollis* genomic DNA as probe, GISH was performed for the 10 CALs following a protocol described for Triticeae species (Brammer *et al*., 2013), with slight modifications: steps 3–9 of slide pre-hybridization were skipped and the probe was denatured at 100°C for 5 min instead of 75°C for 10 min. After hybridization, the slides were viewed and photographed with an Olympus BX61 automated fluorescence microscope (Olympus).

3.7 Genotyping-by-sequencing

To increase the number of *Leymus* chromosome-specific markers in the wheat–*L. racemosus* and wheat–*L. mollis* addition lines and further confirm the PCR-based results, DArTseq was applied to genotype the 32 CILs and the parents. Also, DNA samples from

the 34 Triticeae species used for genomic relationships analysis (Table 3) were genotyped by DArTseq. DNA samples, 1 ***µ***g each, were sent to Diversity Arrays Technology Pty Ltd, Australia (http://www.diversityarrays.com/) for sequencing and marker identification. All the genomic representations were sequenced on HiSeq 2500 and the wheat–*Leymus* genomes were aligned to wheat_ChineseSpring04 reference genome and wheat_ConsensusMap_version_4. The 34 Triticeae samples, sent to the Company at a different time, were also sequenced on HiSeq 2500 but aligned to wheat_ChineseSpring10 reference genome and the same consensus map (wheat_ConsensusMap_version_4).

DArTseq is one of the cheap and easy but efficient genotyping-by-sequencing platforms which allow genome-wide marker discovery through restriction enzyme-mediated genome complexity reduction and sequencing of the restriction fragments (Andrews *et al*., 2016; Davey *et al*., 2011; Melville *et al*., 2017). It utilizes Next-Generation-Sequencing platforms to sequence the most informative representations of genomic DNA samples to aid marker discovery. In comparison to the array version of DArT, DArTseq results in higher marker densities (Kilian *et al*., 2012). The high marker number generated by this system gives it an edge over previous molecular marker procedures applied for diversity studies and genomic analysis of Triticeae species (Goryunova *et al*., 2010; Goryunova *et al*., 2004; Mizuno *et al*., 2010; Wang *et al*., 2011). It, therefore, serves as a cheap alternative to genome analysis by whole genome sequencing, where the sequence information of genomes intended to be analyzed are not available. Two types of data are generated by DArTseq: SNP and SilicoDArT. SNP markers are nucleotide polymorphisms present in the restriction fragments, while SilicoDArT markers represent presence/absence variation (PAV) of the restriction

fragments. Therefore, codominant SNP markers are scored “0” (reference allele homozygote), “1” (SNP allele homozygote) and “2” (heterozygote: presence of both reference and SNP alleles), while dominant SilicoDArT markers are scored in a binary fashion, with “1” representing presence of the restriction fragment with the marker sequence and “0” designating its absence. The choice of which data to use depends on the research objective. It should be noted that for the wheat–*Leymus* CILs, a score of “0” in the SNP data means that the genomic representation lacks the *Leymus* allele of the marker (presence of wheat allele only); “1” refers to the absence of the wheat allele (presence of *Leymus* allele only), while “2” represents the presence of the two alleles.

Since the wheat–*L. racemosus* CALs had been previously characterized by FISH (M. Kishii et al., 2004), SNP markers with call rate of 85% and above were used to analyze the lines, while only SNP markers with call rate of 100% (definite scores across all the samples: bread wheat, *L. mollis* and wheat–*L. mollis* genomes) were used for the analysis of wheat–*L. mollis* CALs. Also, a few SNP markers with alternative scores (1 or 2) in wheat and *L. racemosus* genomes were included in the analysis of the wheat–*L. racemosus* CALs, but such markers were excluded in the analysis of the wheat–*L. mollis* CALs. Genetic mapping-related statistics were not considered because the objective was to identify polymorphic markers to differentiate between the wheat, *Leymus* and wheat–*Leymus* genomes. The data were analyzed for polymorphism between the wheat and *Leymus* genomes, and polymorphic markers were used to identify *Leymus* segments in the wheat–*Leymus* lines. Possible substitutions of wheat chromosomes in the wheat–*L. mollis* lines were analyzed by filtering the SNP markers specific to the *L. mollis* segment (markers with score of “1”) in each line. Noteworthy is that wheat DArTseq platform grouped all the markers (including *Leymus*-specific) into the 21 chromosomes of wheat,

hence the presence of *L. mollis* chromosome in a wheat–*L. mollis* line would be indicated by a score of "1" if the wheat homoeolog is substituted, or 2 if the homoeolog is not substituted. The former case indicates a substitution line, while the latter indicates an addition line. In the unlikely case of zero genotyping error and complete absence of segment deletion, the genomes of all the wheat–*Leymus* CALs would be scored "2", since they have both wheat and *Leymus* segments.

Based on the correspondence between the SNP alleles and reference alleles in each CS chromosome provided by DArTseq, the *Leymus* chromosome-specific SNP markers were used to determine the homoeologous groups (HGs) of *Leymus* chromosomes in the lines. Genomic relationship between the genomes of the two *Leymus* species was roughly estimated by marker polymorphism between the genomes, whereas markers consistently called (call rate of 100%) among the two genomes, wheat–*L. mollis and* wheat–*L. racemosus* addition lines were used to estimate the relationship between the chromosomes of the two *Leymus* species. This latter set of markers was used for cluster analysis (http://genomes.urv.cat/UPGMA/) to reveal the associations among the chromosomes of the two *Leymus* species.

Frequently called SNP markers (>90% call rate) were used for phylogenetic tree reconstruction and differentiation of the genomes of polyploid species of *Aegilops*, whereas SilicoDArT markers (>70% call rate) were used for the determination of putative progenitors of the polyploid *Aegilops* and *Triticum* species. This reduction in call rate was made to accommodate more markers, ensure wider genomic coverage and reduce bias. To estimate the phylogenetic relationships among the 11 diploid and 12 polyploid *Aegilops* species, the raw genotypic data of the two sets (diploid and polyploid) were subjected to cluster analysis. Pearson's correlation coefficient (**r**) was used as similarity

index, and the genetic distances among the species were estimated by transforming the **r** values to distance values, using **d = 100(1 – r)** (http://genomes.urv.cat/UPGMA/). Species-specific SilicoDArT markers of the polyploid species of *Aegilops* were used to differentiate their genomes, while species-specific SilicoDArT markers of diploid species of *Aegilops* were used to estimate the diploid-polyploid evolutionary relationships among all the *Aegilops* species. Diploid *Triticum* and *Aegilops* species whose total SilicoDArT markers showed at least 10% homoeology to the total SilicoDArT markers in any of the three genomes of hexaploid wheat were selected as analyzers to determine the putative progenitors of the corresponding genomes of each polyploid *Triticum* species. Species-specific SilicoDArT markers of these selected diploid species were used as analyzers to determine the putative progenitors of each polyploid *Triticum* species. In determining the progenitors of all the polyploid species (*Aegilops* and *Triticum*), the proportions of the species-specific markers of the diploid analyzers retained in the genomes of the polyploid species were used as a basis to draw conclusions on genomic proximity and evolutionary relationships among the species. Species-specific markers of *Ae. speltoides* and *Ae. searsii* were further used to examine the relationship between the seven B/G-genome chromosomes of each of the polyploid *Triticum* species and the chromosomes of the diploid species. The two diploid species were chosen based on the proximity of their genomes to the B/G genomes of the polyploid species.

3.8 Preliminary phenotypic evaluation of wheat–*L. mollis* lines

The 10 wheat–*L. mollis* CALs alongside the background wheat cultivar (CS) were laid out in a completely randomized design (CRD) with six replicates in a greenhouse. Seeds of all the genotypes were sown in petri dishes under the same condition, and uniform

seedlings of each genotype were transplanted to plastic pots, one plant in each pot. DNA samples of seedlings from monosomic lines were genotyped by PCR to ensure that only alien carriers were transplanted. All the plants grew under a temperature-controlled (22^{o}C day/18^{o}C night) condition. Adequate cultural practices necessary for optimum crop performance were observed. Data were taken on number of days to heading and physiological maturity, plant height, spike length, number of spikes per plant, grain yield per spike and grain yield per plant. Two-tailed t-test was applied to compare the mean values of traits between CS and each CAL. At this preliminary stage, differences among the CALs were not considered, as all the lines are intended to be collectively evaluated under different stress conditions for further selection and production of translocation and recombination lines with desired segments of *L. mollis* chromosomes.

CHAPTER FOUR

RESULTS

4.1 *Leymus racemosus* polymorphic markers

From a total of 294 primer sets screened by PCR, 164 sets (~56%) amplified *L. racemosus* genome. Out of the amplified markers, 110 (~67%) were polymorphic in wheat—absence or difference in size of bands in wheat (Fig. 1a; Table 4). Six of the polymorphic markers showed size polymorphism (codominant), while 104 markers were dominant (presence/absence polymorphism). Also, out of 11,570 SNP markers filtered based on high call rate, 8,522 (~74%) were polymorphic in wheat (absence of SNP alleles in wheat)—8,430 SNPs were absent in the wheat cultivar (CS) analyzed in this study, while 92 were present but showed presence of both reference and SNP alleles in *L. racemosus* (Fig. 1c; Table 4). These 92 markers form part of the polymorphisms observed between the CS in TACBOW gene bank and the reference CS genome on DArT platform. Taken together, a total of 8,632 polymorphic markers were developed from *L. racemosus* genome.

4.2 Markers in wheat–*L. racemosus* CALs

About 65% of the polymorphic PCR markers amplified *L. racemosus* chromosomes in nine wheat–*L. racemosus* chromosome addition lines, while approximately 43% of the SNP markers identified the nine alien chromosomes (Table 4; Fig. 1b and d). Only SNP data from DArTseq was used in this analysis, as SilicoDArT data was less informative in analyzing the required polymorphism. This is because SilicoDArT data is binary (dominant), making it impossible to identify codominant polymorphisms (in this case, presence of both reference and SNP alleles), which was mostly utilized to genotype the

chromosome introgression lines, since they have genome representations of wheat (alien chromosome recipient) and *L. racemosus* (alien chromosome donor). A total of 3,551 chromosome-specific markers were developed for the nine *L. racemosus* chromosomes in wheat genetic background, ranging from two in LrE to 533 in LrL (Table 4). The large number of markers on each chromosome, except LrE, enabled reliable differentiation of the nine wheat–*L. racemosus* CALs.

4.3 Confirmation of homoeologous groups of *L. racemosus* chromosomes in CS background

To confirm the validity of the chromosome-specific SNP markers, correspondence of *L. racemosus* chromosome-specific markers with the homoeologous groups of CS chromosomes was exploited to determine the most probable homoeologous group (HG) of each *L. racemosus* chromosome in the chromosome addition lines (Table 5). The results revealed that the alien chromosomes spread between HG2 and 7: LrA and L in HG2, LrH and N in HG3, LrF, I, K and J in HG 4, 5, 6 and 7, respectively. While this result confirmed the HGs of seven of the chromosomes, it also clarified the HGs of LrJ and LrN, which had not been clearly determined in previous studies (Kishii *et al*., 2004; Larson *et al*., 2012)

4.4 Detailed characterization of *L. racemosus* chromosomes I, J, N and their respective translocations

As shown in Table 5, I-, J- and N-specific markers were successfully allocated to their respective arms using their respective translocation lines.

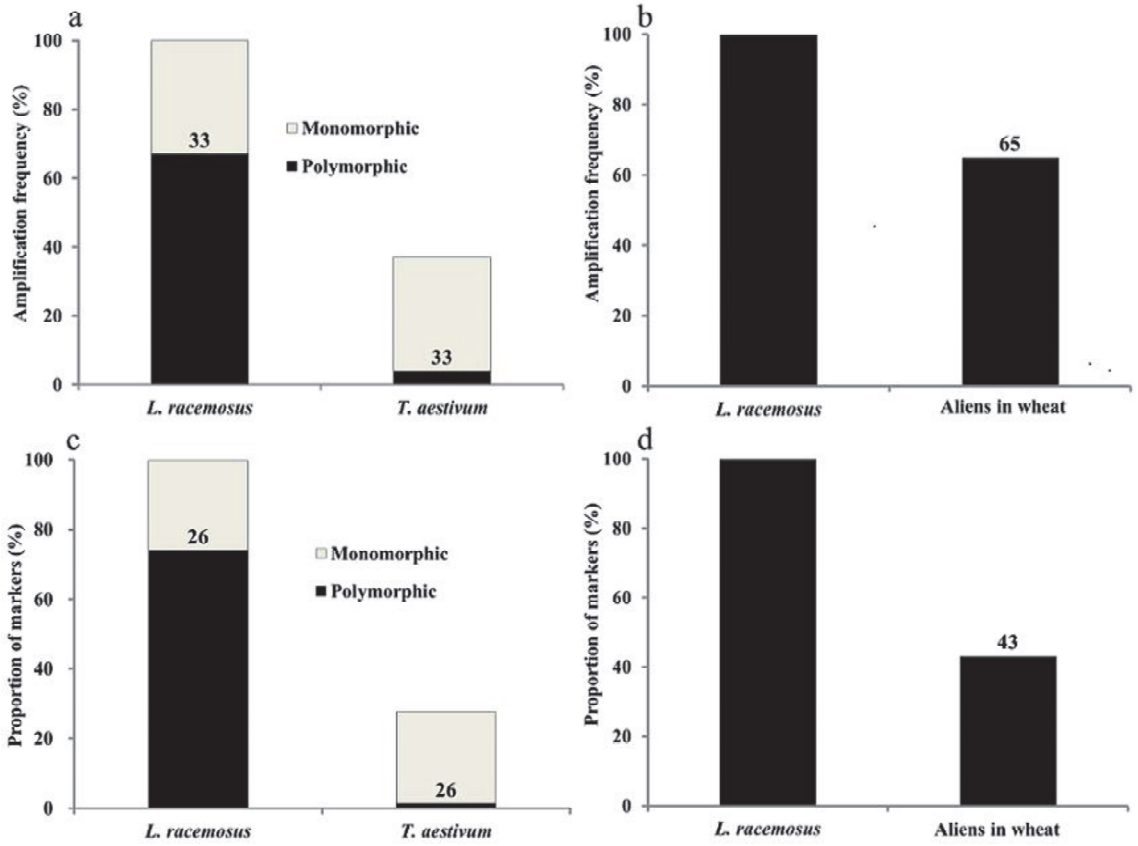

Fig. 1 Analysis of markers in *L. racemosus*, bread wheat and nine wheat–*L. racemosus* CALs. **a** Amplification of 164 pre-screened *L. racemosus* PCR-based markers in *L. racemosus* and bread wheat genomes. **b** Amplification of 110 polymorphic *L. racemosus* PCR-based markers in nine wheat-*L. racemosus* CALs, with *L. racemosus* genome as a positive control. **c** Differentiation of *L. racemosus* and bread wheat genomes using 11,570 SNP markers. **d** Proportion of SNP markers in the genomes of nine wheat-*L. racemosus* CALs

Table 4 Identification of wheat-*L. racemosus* chromosome introgression lines using *L. racemosus* polymorphic markers

Genotype ID	Description	Chromosome constitution (2n)	Total number of markers		Chromosome-specific markers		
			PCR	DArTseq (SNP)	PCR	DArTseq (SNP)	Total
TACBOW 0001	Disomic addition	21″+1″[LrA]	14	434	6	381	387
TACBOW 0003	Disomic addition	21″+1″[LrE]	4	46	1	1	2
TACBOW 0004	Disomic addition	21″+1″[LrF]	10	499	4	344	348
TACBOW 0005	Disomic addition	21″+1″[LrH]	20	528	12	410	422
TACBOW 0006	Disomic addition	21″+1″[LrI]	12	558	4	491	495
TACBOW 0007	Disomic addition	21″+1″[LrJ]	17	511	9	450	459
TACBOW 0008	Disomic addition	21″+1″[LrK]	12	519	7	465	472
TACBOW 0009	Disomic addition	21″+1″[LrL]	18	647	8	525	533
TACBOW 0010	Disomic addition	21″+1″[LrN]	16	549	5	428	433
All aliens	-	-	**72**	**3656**	**56**	**3495**	**3551**
KT020-003 (CS)	*Triticum aestivum*	42	6	92	-	-	-
TACBOW 0112	*Leymus racemosus*	28	110	8522	-	-	-

TACBOW, Tottori Alien Chromosome Bank of Wheat; Lr, *Leymus racemosus*; A–N, Arbitrary numbering of *L. racemosus* chromosomes; ″, bivalent; Bold numbers, total markers in alien chromosomes in wheat background in each category; CS: Chinese Spring

Table 5 Determination of homoeologous groups of *L. racemosus* chromosomes in wheat genetic background using chromosome-specific DArTseq SNP markers

Alien	Number of markers corresponding to each homoeologous group of bread wheat (Chinese Spring) chromosome																				Most probable HG of alien chromosome	
	1A	1B	1D	2A	2B	2D	3A	3B	3D	4A	4B	4D	5A	5B	5D	6A	6B	6D	7A	7B	7D	Previous reports	Current
LrA	3	1	1	**75**	**71**	**198**	3	0	4	0	2	2	1	0	5	1	3	5	2	3	1	2[a,b]	2
LrE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	ND	ND
LrF	4	2	3	1	2	5	0	0	0	**77**	**79**	**141**	2	12	20	0	2	0	3	1	2	4[a,b]	4
LrH	1	2	7	0	2	2	**68**	**94**	**204**	2	2	4	3	1	2	0	6	2	0	4	4	3[a,b]	3
LrI	3	0	5	3	1	4	1	1	5	2	7	9	**74**	**162**	**193**	4	2	4	3	6	2	5[a,b]	5
LrJ	1	0	3	4	2	5	2	2	4	12	2	4	5	7	2	0	3	5	**84**	**105**	**208**	(6,7)[a], (3,7)[b]	7
LrK	4	2	4	1	3	7	1	2	2	0	2	6	2	2	6	**81**	**117**	**205**	5	8	9	6[a,b]	6
LrL	1	0	1	**135**	**158**	**293**	1	6	6	3	3	2	2	7	9	0	3	6	11	5	5	2[a,b]	2
LrN	4	6	3	0	1	4	**77**	**131**	**176**	3	0	0	1	3	3	0	2	1	2	4	5	2[a], (3,7)[b]	3

Lr, *L. racemosus*; HG, homoeologous group; ND, not determined; [a], (Larson *et al*., 2012); [b], (M. Kishii et al., 2004)

A detailed analysis of the homology between each of the three chromosomes and their respective translocated arms gave a clearer picture of the structures of the translocation lines.

For chromosome I, the markers were adequately allocated to the short (IS) and long (IL) arm translocations, revealing the proportions of chromosome I markers that differentiated each of the translocated arms and eight markers located on a segment of chromosome I that may not have been transmitted during the production of the translocation lines (Fig. 2a). However, a few markers specific to the translocation lines but absent in I-addition line were observed. If no genotyping error is assumed, these markers would represent polymorphisms that may have arisen from the interactions between the translocated arms and CS genome.

Most J-chromosome markers were found to be present only in the JS translocation, about half of which were co-located on the JL translocation (Fig. 2b). This result obviously indicates that what was hitherto regarded as JL translocation may be a truncated segment of JS translocation. The 10 unique markers (Fig. 2b) each present in the two translocation lines may have resulted from changes in each genetic background or small chromosomal rearrangements during the various production processes. As observed in chromosome I, 14 markers identified a segment of chromosome J which may not have been transmitted to the translocation lines.

Chromosome N and its translocated arms presented a similar scenario as chromosome I. The markers in both the NS and NL arm translocations were clearly shown (Fig. 2c). However, 27 markers were found to be specific to NL and nine were specific to NS translocation lines (Fig. 2c), indicating unique polymorphisms which may have been acquired from interactions between the background and the translocated arms as observed

for chromosomes I and J. Also, the 13 markers in the whole N-addition line, which are absent in the translocated arms, suggest that the NS and NL translocations lost the region of chromosome N identified by these markers (Fig. 2c).

4.5 Analysis of recombination positions of wheat-LrN recombination lines

The chromosome N arm-specific markers were used to determine locations of the recombinant fragments on LrN and the corresponding CS chromosomes, revealing the probable fraction of CS chromosome replaced in each recombination line (Tables 6, 7; Fig. 3). N recombinant fragments 2, 3, 5 and 6 were mapped on the short arm, while the recombinant fragments 4 and 7 were found in the long arm of each of the lines. Although the two markers that specified recombinant fragment 1 were traced to NL, the number seems too small to confirm the location of this fragment. Recombination lines 6 and 7 were observed to have the largest fragments, with all the markers in the short arm translocation represented in recombination line 6, and all except two markers in long arm translocation represented in recombination line 7 (Table 7; Fig. 3). Other lines were found to have relatively small fragments which can best be described as different sizes of bins represented in recombination lines 6 or 7. With the two markers recorded for recombination line 1, it would appear as though there was no recombination event, although low recombination rates between wheat chromosomes and aliens is not unusual (Lukaszewski, 1999).

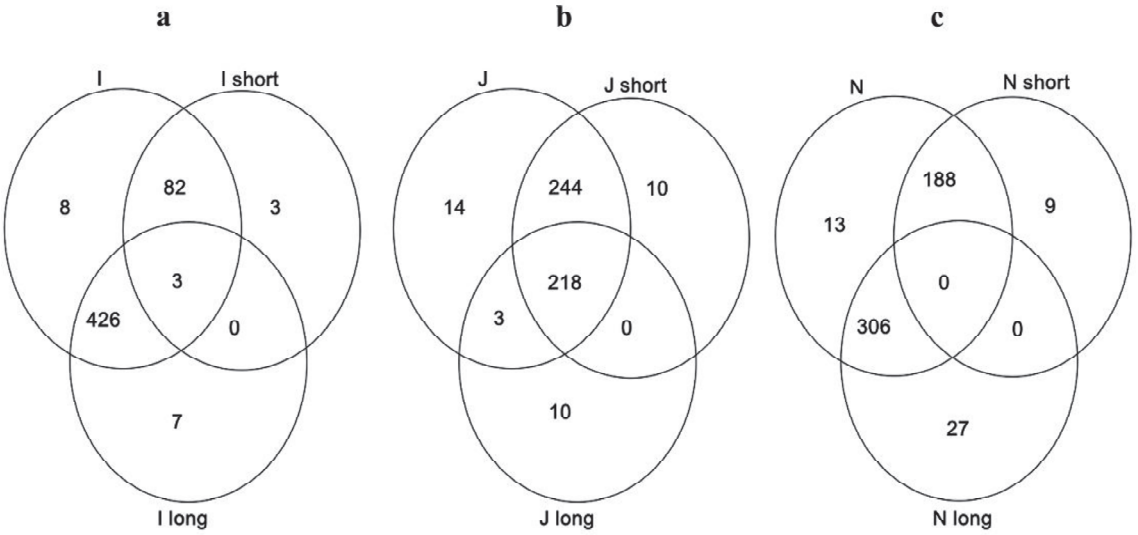

Fig. 2 Venn diagrams showing homology and polymorphism between chromosomes I, J, N and their respective translocation lines. **a** homology and polymorphism between I and its translocated arms. **b** homology and polymorphism between J and its translocated arms. **c** homology and polymorphism between N and its translocated arms

Table 6 Determination of arm-specific markers of chromosomes LrI, LrJ and LrN based on markers specific to their translocated arms

Genotype ID	Description	Chromosome constitution (2n)	Arm-specific markers		
			PCR	DArTseq	Total
I short	I short arm translocation	42	1	80	81
I long	I long arm translocation	42	0	404	404
J short	J short arm translocation	42	5	231	236
J long	J long arm translocation	42	0	2	2
N short	N short arm translocation	42	2	162	164
N long	N long arm translocation	42	3	255	258

Table 7 Determination of arm locations of N-recombinant fragments using arm-specific markers

Genotype	Arm location of amplified markers				Fragment location
	PCR		DArTseq		
	Short	Long	Short	Long	
N recombination No. 1	0	0	0	2	Not certain
N recombination No. 2	2	0	160	0	Short arm
N recombination No. 3	0	0	67	0	Short arm
N recombination No. 4	0	1	0	48	Long arm
N recombination No. 5	2	0	158	0	Short arm
N recombination No. 6	2	0	162	0	Short arm
N recombination No. 7	0	3	0	248	Long arm
N short arm translocation	2	-	162	-	-
N long arm translocation	-	3	-	255	-

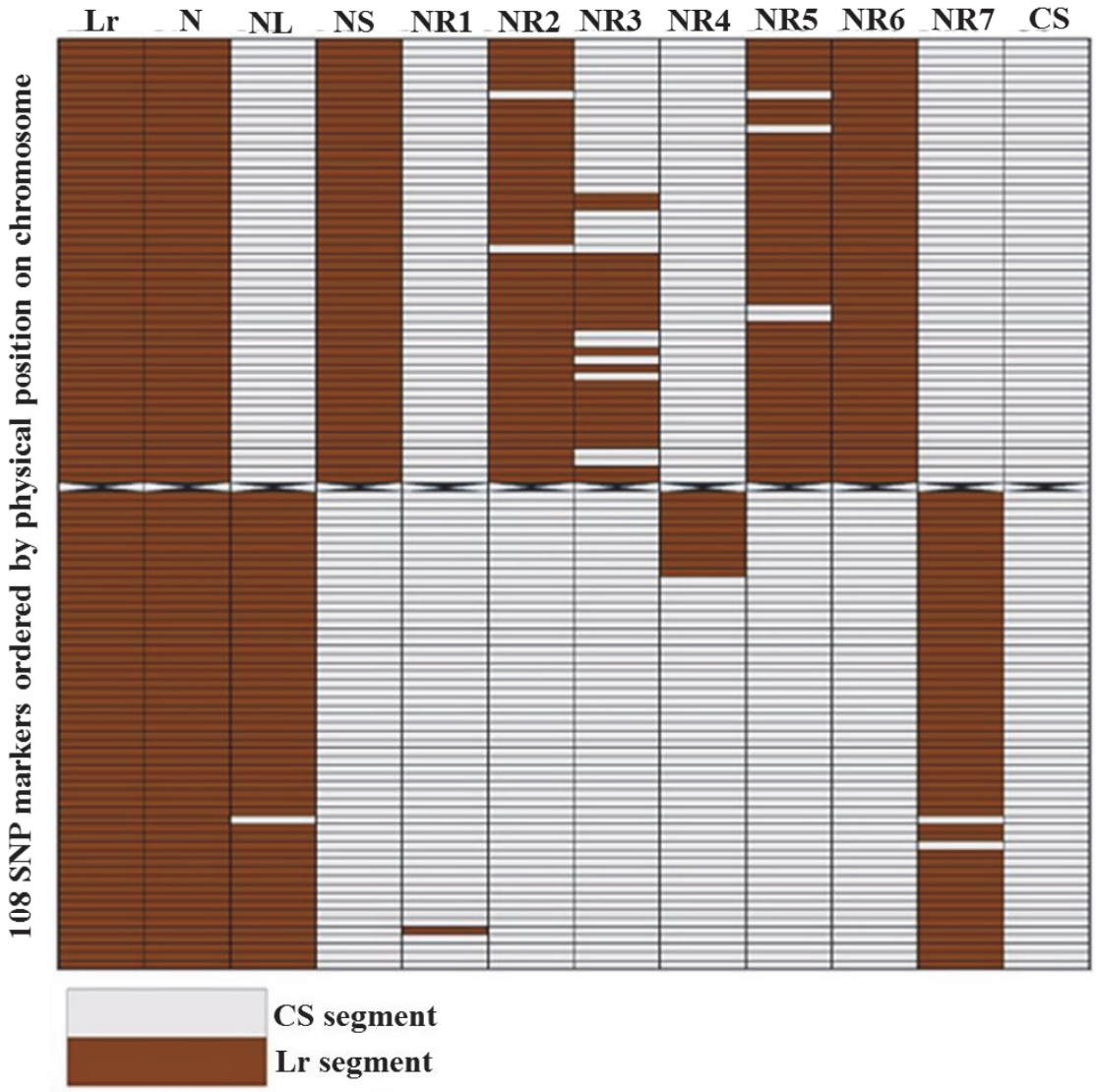

Fig. 3 Graphical genotyping of N-recombination lines using 108 chromosome N-specific SNP markers corresponding to wheat chromosome 3B. Lr, *L. racemosus*, N, N-addition line, NL, N long arm translocation line, NS, N short arm translocation line, NR1–NR7, N recombination lines 1–7, CS, Chinese Spring

4.6 Universal markers of *L. racemosus* chromosomes

Two of the polymorphic markers, *21_s46518* and *333_s46518* (developed from the same DNA sequence scaffold) identified all the *L. racemosus* chromosomes in wheat (Fig. 4a). On sequencing PCR products generated with one of these markers, and conducting BLASTN search, it was observed that 26% of the sequence of the PCR product of *L. racemosus* and 16% of that of wheat–LrN aligned to a section of CACTA-family transposon in *Lolium perenne* (perennial rye-grass), an important and highly researched commercial pasture crop of the grass family, Poaceae. Sequences of other CILs showed no significant alignment to the transposon sequence. This may be an indication of the presence of CACTA-family transposon in *Leymus* species.

4.7 Unique SNP markers in wheat–*L. racemosus* CALs

DArTseq data further revealed additional 1,468 unique SNP markers in the nine wheat–*L. racemosus* addition lines, absent in both parents. One hundred and ninety-seven of these markers were common to the lines, while 1,271 were line-specific, with a range of 38–355 specific markers on each line (Table 8). Like the *L. racemosus* chromosome-specific markers, the additional line-specific markers also facilitated differentiation of the nine addition lines. These additional SNP markers account for polymorphisms acquired from the interactions between the added chromosomes and the background (CS genome), and their effects may be of agronomic significance.

4.8 Transferability of *L. racemosus* markers to related species

The result of marker transferability analysis, utilizing 164 pre-screened *L. racemosus* PCR-based markers, showed that 75% of the markers were transferable, particularly

revealing higher amplification frequencies in three other important perennial Triticeae species (*L. mollis, Psathyrostachys huashanica* and *Elymus ciliaris*) in comparison to wheat and other species studied (Fig. 5a–d; Table 9). More importantly, the amplified markers in each of these species were found to be reasonably polymorphic in wheat, obviously indicating their suitability in genotyping wheat–alien CILs carrying chromosomes from these species.

Interestingly, the two universal markers which identified all *L. racemosus* chromosomes in wheat genetic background were found to be *Leymus*-specific, as they amplified only the two *Leymus* species out of the 12 Triticeae species analyzed, revealing size polymorphism between the two *Leymus* genomes (Fig. 5b). These markers can, therefore, be applied to separate *Leymus* genomes from genomes of other species in the same tribe, and their (*Leymus*) chromosomes, if introgressed into wheat, can easily be sorted out in one PCR. Informative co-amplification between the two *Leymus* species and *Psathyrostachys huashanica* was also observed (Fig. 5c), and a phylogenetic analysis using 123 markers co-amplified among the 12 Triticeae species (Fig. 6) showed an expected close evolutionary relationship between the three species, which agrees with reports suggesting that *Leymus* species are segmental polyploids with variant N-genomes from genus *Psathyrostachys* (Anamthawat-Jonsson, 2001, 2014; Fan *et al*., 2014). Also, one highly conserved marker sequence amplified all the species, showing size polymorphism among them (Fig. 5d).

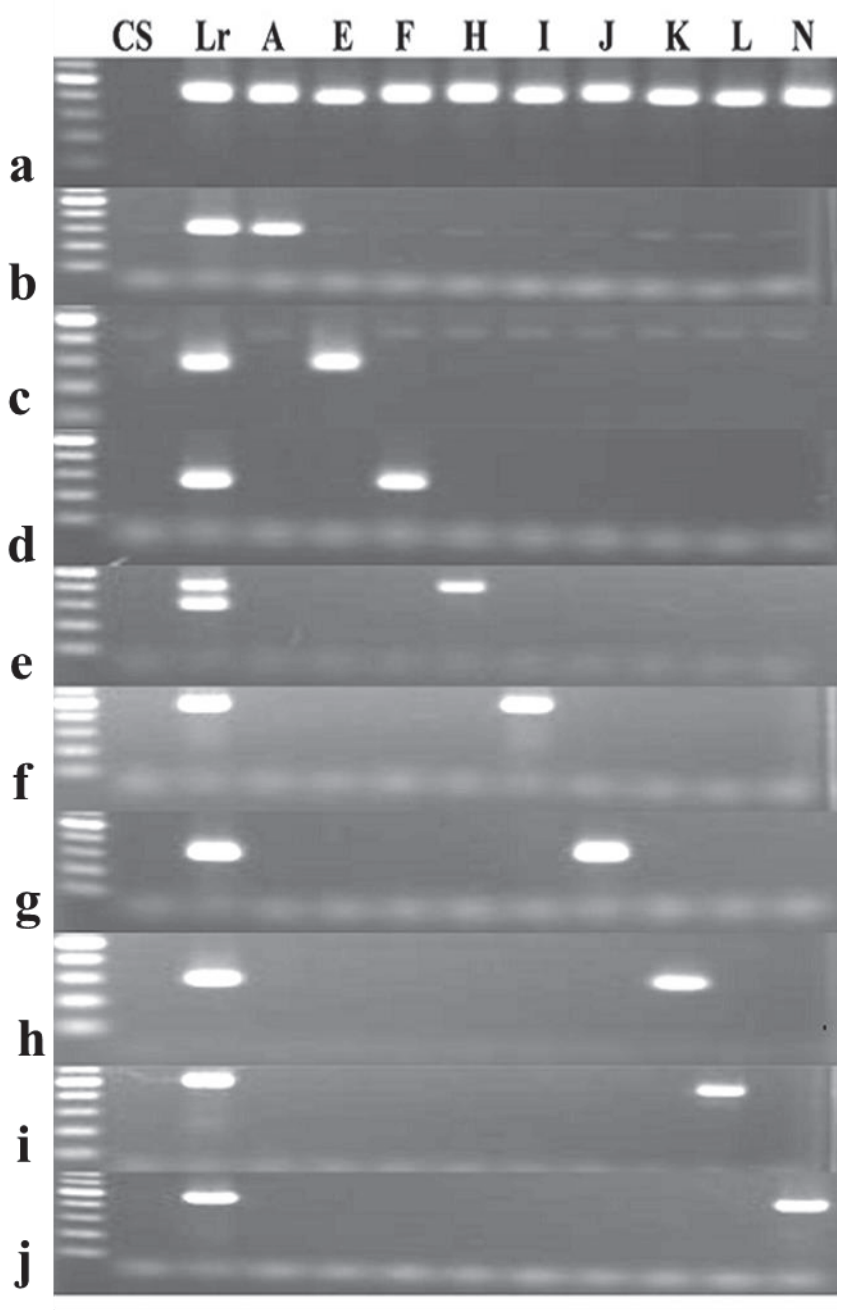

Fig. 4 Representative gels of PCR amplification of wheat, *L. racemosus* and wheat–*L. racemosus* CALs. **a** Amplification of nine *L. racemosus* chromosomes in wheat background by a universal marker (*21_s46518*). **b–j** Amplification of the nine chromosomes by their respective PCR-based chromosome-specific markers. CS, Chinese Spring; Lr, *L. racemosus*; A–N, Nine wheat–*L. racemosus* CALs

Table 8 Additional 1,468 unique genotype-based SNP markers in nine wheat–*L. racemosus* chromosome CALs

Genotype ID	Alien chromosome ID	Common SNP markers on each chromosome	Line-specific SNP markers
TACBOW 0001	LrA	99	355
TACBOW 0003	LrE	79	38
TACBOW 0004	LrF	70	173
TACBOW 0005	LrH	86	109
TACBOW 0006	LrI	74	46
TACBOW 0007	LrJ	68	130
TACBOW 0008	LrK	61	186
TACBOW 0009	LrL	92	124
TACBOW 0010	LrN	81	110
KT020-003 (CS)	-	0	0
TACBOW 0112 (Lr)	-	0	0
Total		**197**	**1271**

TACBOW: Tottori Alien Chromosome Bank of Wheat (Japan); CS: Chinese Spring; Lr: *L. racemosus*;**197** represents the total number of SNP markers common to the nine CALs, without repetition

Table 9 Transferability of 164 pre-screened *L. racemosus* markers to 11 species in tribe Triticeae

Species	Amplified markers			Polymorphism in wheat (%)	Polymorphism based on marker source (%)	
	DNA	RNA	Total		DNA	RNA
L. racemosus	76	88	164 **(100)**	67	83	53
L. mollis	31	64	95 **(58)**	49	74	38
P. huashanica	23	55	78 **(48)**	44	70	33
E. ciliaris	17	49	66 **(40)**	32	42	29
H. vulgare	5	31	36 **(22)**	22	0	16
H. bulbosum	8	31	39 **(24)**	23	25	23
D. villosum	9	39	48 **(29)**	21	33	18
S. cereal	9	38	47 **(29)**	15	11	16
T. urartu	9	36	45 **(27)**	7	0	8
Ae. speltoides	8	39	47 **(29)**	6	13	5
Ae. tauschii	10	39	49 **(30)**	0	0	0
T. aestivum	10	51	61 **(37)**	-	-	-
***L. racemosus*-specific markers**	33	8	41 **(25)**	-	-	-

Bold numbers in brackets, percentages of amplified markers; ***L. racemosus*-specific markers** represent the proportion of the 164 markers not transferred to the other Triticeae species analyzed

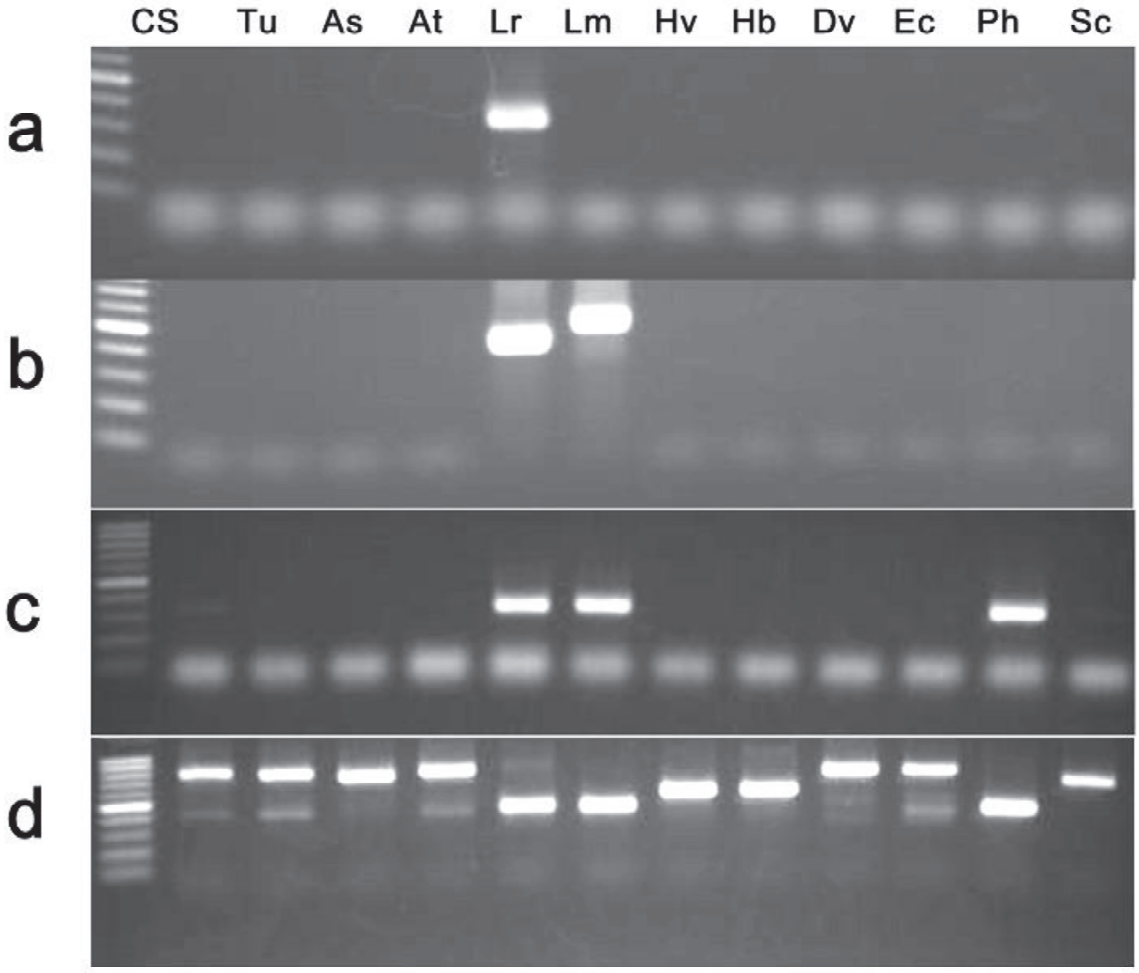

Fig. 5 Representative gels of PCR amplification of 12 Triticeae species. **a** Amplification of *L. racemosus* by its genome-specific marker **b** Amplification of *Leymus* species by a *Leymus*-specific marker. **c** Specific amplification of *Leymus* species and *Psathyrostachys huashanica* (a species of *Leymus* N-genome progenitor genus). **d** Amplification of all the species by a conserved marker sequence, showing size polymorphism between the species.

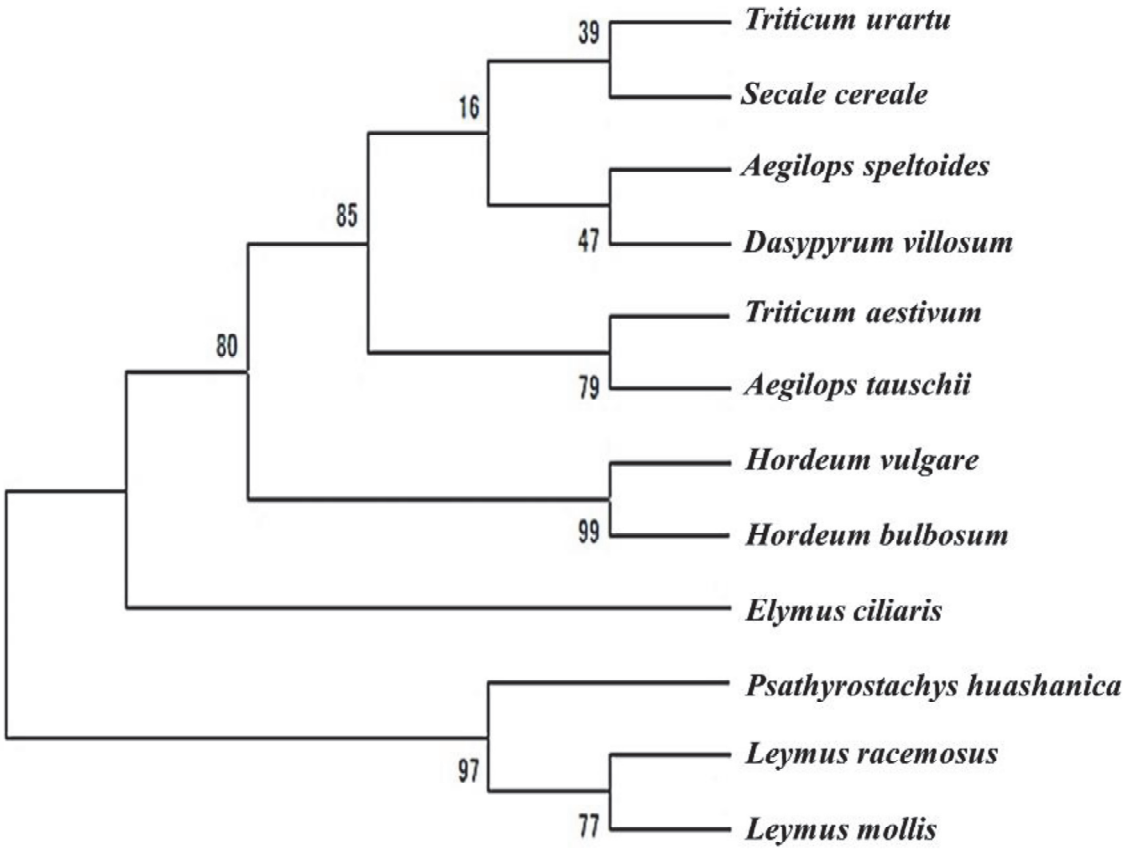

Fig. 6 Phylogenetic tree constructed from the co-amplification of 123 PCR-based *L. racemosus* markers using UPGMA as clustering method

4.9 Comparison of the proportions of polymorphic markers from *L. racemosus* genomic sequence and RNA-seq

In a bid to compare the performance of markers developed from DNA and RNA-seq, the proportions of polymorphic markers from the two sources were compared. As expected, markers from genomic sequence were more polymorphic than those from RNA-seq, indicating that the polymorphisms between hexaploid wheat and the other species studied may be more traceable to the variations in the repetitive sequences of the genomes (Table 9). However, the appreciable polymorphisms recorded from the RNA-seq markers, which account for variations in the genic regions, make the two approaches equally informative.

4.10 Molecular marker-assisted production of 10 wheat–*L. mollis* CALs

With the aid of the integrative approach enumerated in chapter three, 10 distinct wheat-*L. mollis* CALs were developed and characterized (Fig. 7; Table 10). Alien carriers in backcross populations generated from a wheat–*L. mollis* octoploid plant (2n = 8x = 56, AABBDDNs$_1$Ns$_2$) were unmistakably identified using polymorphic PCR markers, and the *L. mollis* chromosomes were differentiated with chromosome-specific markers. Progeny test aided recognition of six disomic and four monosomic lines as the populations were advanced to BC$_3$F$_4$ (Fig. 7). Interestingly, the *Leymus*-specific marker which identified all the *L. racemosus* chromosomes also identified all the *L. mollis* chromosomes analyzed (Figs. 4a, 5b and 8), making it a universal marker for the chromosomes of the two *Leymus* species. From 95 PCR markers transferred from *L. racemosus* to *L. mollis* and 15,426 SNP markers in the genomes of *L. mollis* and CS, 14,577 polymorphic *L. mollis* markers were developed—47 PCR-based and 14,530 SNP markers (Table 10). The PCR markers indicated 49% marker polymorphism between *L. mollis* and CS (Table 9),

whereas the SNP markers revealed a higher polymorphism (~94%) (Offiong U. Edet et al., 2018); online Supplementary Table S1). Chromosomes of *L. mollis* in the wheat background were efficiently identified by 27 PCR markers and 6,317 SNP markers, and a total of 5,957 *L. mollis* chromosome-specific markers, ranging from 185 in LmL to 796 in LmI (Table 10), enabled unambiguous differentiation of the 10 lines. The number of SNP markers with a genotypic score of "1" (representing only the *L. mollis* allele) in each line ranged between 3 (0.05%) and 15 (0.2%), which lie within genotyping error range. All the lines retained almost 100% of the reference (wheat) alleles in addition to the SNP alleles, showing that none of the CS chromosomes was substituted. Therefore, all the introgressions were confirmed to be addition lines.

4.11 HGs of *L. mollis* chromosomes added to CS

As done for *L. racemosus* chromosomes, the HGs of all the *L. mollis* chromosomes were determined from the correspondence of *L. mollis* chromosome-specific SNP markers to the HGs of CS. The 10 *L. mollis* chromosomes fitted well into seven HGs, with six chromosomes falling into three HGs (Table 11). The chromosomes in the same HGs belong to different sub-genomes, granted that *L. mollis* is a tetraploid species.

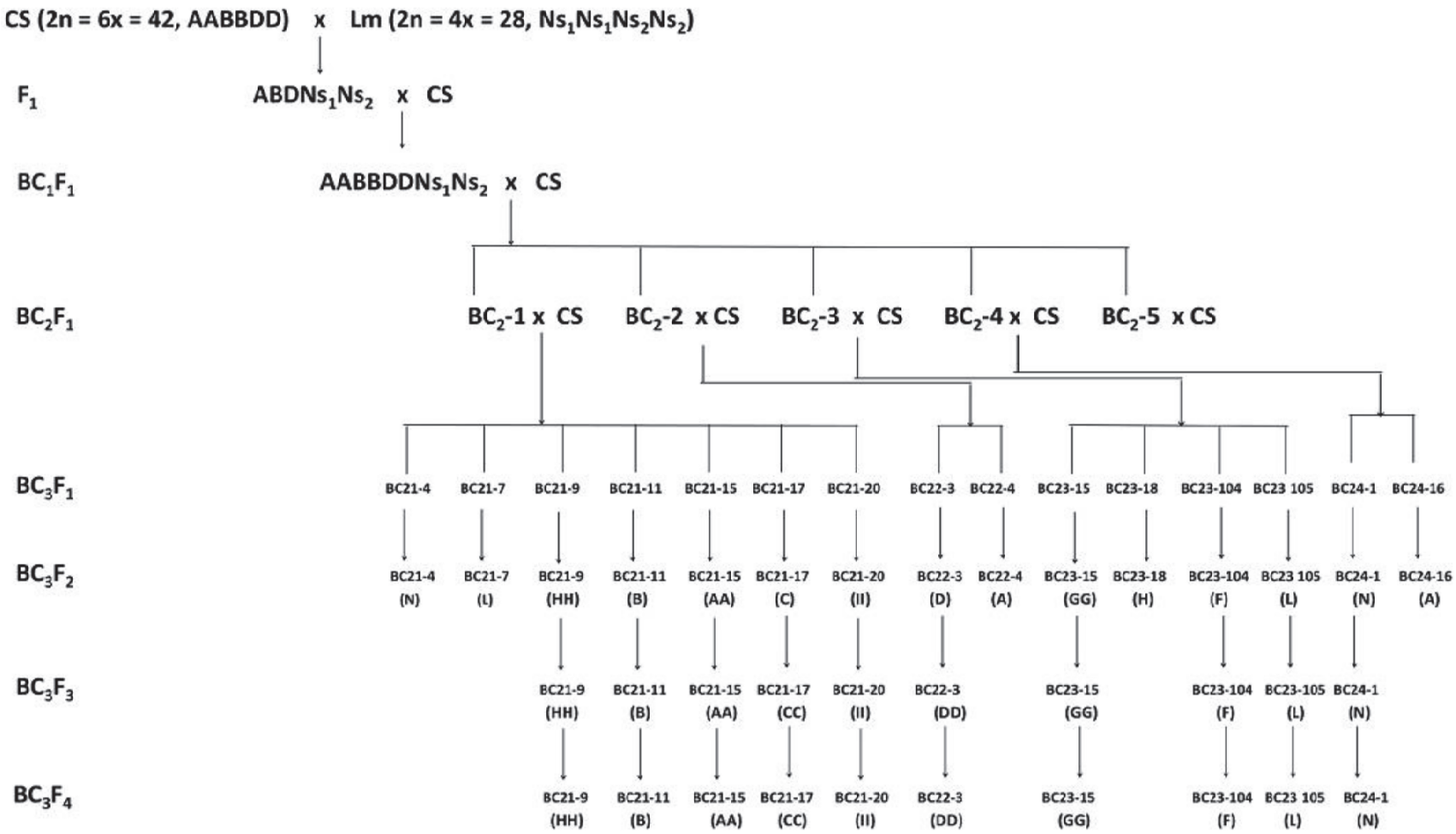

Fig. 7 Pedigree of 10 wheat–*L. mollis* CALs. CS, Chinese Spring; Lm, *L. mollis*; single letters in brackets, monosomic lines; double letters in brackets, disomic lines

Table 10 Identification of wheat–*L. mollis* CALs using PCR and SNP markers

Alien chromosome ID	Description	Chromosome constitution (2n)	Total number of markers		Chromosome-specific markers		
			PCR	SNPs	PCR	SNPs	Total
LmA	Disomic addition	21″+1″	7	810	3	777	780
LmB	Monosomic addition	21″+1′	6	787	1	661	662
LmC	Disomic addition	21″+1″	7	601	3	500	503
LmD	Disomic addition	21″+1″	3	454	1	428	429
LmF	Monosomic addition	21″+1′	7	714	2	603	613
LmG	Disomic addition	21″+1″	6	857	0	757	757
LmH	Disomic addition	21″+1″	7	757	1	641	642
LmI	Disomic addition	21″+1″	10	896	3	796	799
LmL	Monosomic addition	21″+1′	5	209	3	185	188
LmN	Monosomic addition	21″+1′	4	620	0	592	592
All alien chromosomes	**-**	**-**	**27**	**6317**	**17**	**5940**	**5957**
KT020-003 (CS)	*T. aestivum*	42	0	-	-	-	-
TACBOW 0113	*L. mollis*	28	47	14530	-	-	-

TACBOW, Tottori Alien Chromosome Bank of Wheat; Lm, *Leymus mollis*; A–N, Lm chromosomes in the wheat genome; ″, bivalent; ′, univalent; bold, numbers of all the markers that identified Lm chromosomes in the wheat background; CS, Chinese Spring

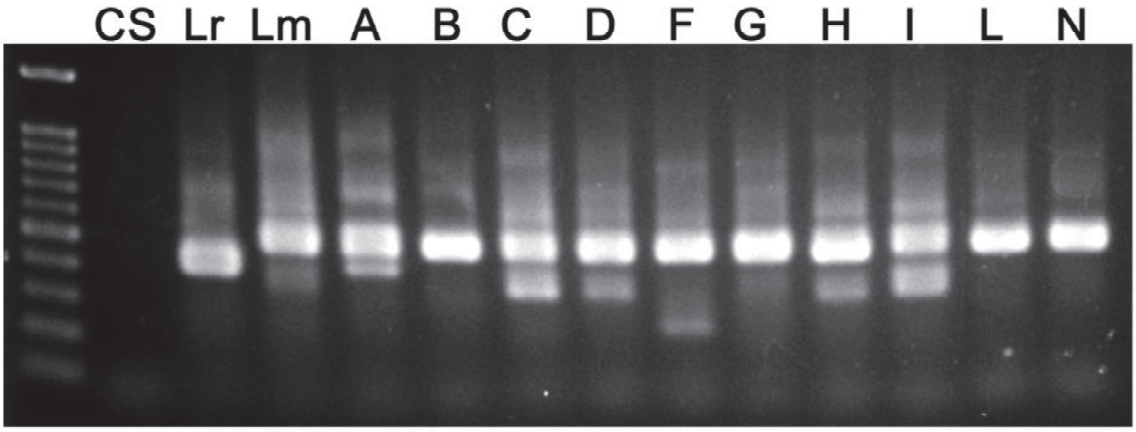

Fig. 8 Gel picture showing amplification of 10 *L. mollis* chromosomes in wheat background by a *Leymus* chromosomes' universal marker. CS, Chinese Spring; Lr, *L. racemosus*; Lm, *L. mollis*; A – N, wheat-*L. mollis* arbitrary tags of *L. mollis* chromosomes

Table 11 Determination of HGs of *L. mollis* chromosomes in wheat genetic background using *L. mollis* chromosome-specific SNP markers

Alien ID	Number of markers corresponding to each homoeologous group of CS chromosome																				HG
	1A	1B	1D	2A	2B	2D	3A	3B	3D	4A	4B	4D	5A	5B	5D	6A	6B	6D	7A	7B	7D	
LmA	2	5	6	**153**	**219**	**343**	2	2	4	3	6	5	1	3	5	6	8	6	3	5	6	2
LmB	4	2	4	1	3	4	2	1	3	1	16	30	**183**	**161**	**254**	1	2	5	4	4	4	5
LmC	5	4	7	3	1	0	4	2	6	13	1	2	2	2	2	1	3	2	**110**	**127**	**225**	7
LmD	1	1	3	3	2	1	2	1	3	1	4	3	1	4	1	**104**	**133**	**167**	5	3	6	6
LmF	4	4	3	3	2	3	**132**	**165**	**271**	3	1	0	3	3	6	1	2	2	2	3	8	3
LmG	0	3	5	0	4	7	2	1	3	29	4	7	2	5	6	3	1	4	**192**	**200**	**315**	7
LmH	6	6	3	2	2	8	**135**	**180**	**298**	2	1	3	2	6	5	0	3	3	4	2	5	3
LmI	2	2	3	3	5	6	6	1	3	14	2	6	**171**	**220**	**334**	5	4	9	8	8	7	5
LmL	**35**	**51**	**72**	2	4	2	1	1	2	2	0	4	1	1	3	0	2	4	2	2	4	1
LmN	2	1	1	4	1	4	2	3	3	**120**	**153**	**240**	25	5	7	3	3	2	5	2	6	4

Lm, *L. mollis*; HG, homoeologous group

4.12 Unique SNP markers in wheat-*L. mollis* CALs

As observed in wheat-*L. racemosus* CALs, 527 unique SNP markers absent in CS and *L. mollis* were found in the 10 wheat-*L. mollis* CALs (Table 12). Out of these markers, 470 markers (~89%) were specific to different lines, which, once again enabled a confirmatory differentiation of the lines and also points to the likelihood of genetic modification in the CALs, which may be responsible for generating additional polymorphisms.

4.13 Confirmation of the cytological status of each *L. mollis* chromosome in the wheat–*L. mollis* CALs by GISH

Since the marker-assisted selection procedure adopted here, especially in differentiating monosomic and disomic lines, is currently not a common practice, the presence and status (monosomic or disomic) of aliens in the 10 CALs were validated by GISH. As shown in Fig. 9, GISH results perfectly agreed with the marker-based results for all the CALs. The lines designated as disomic (LmA, LmC, LmD, LmG, LmH and LmI) or monosomic (LmB, LmF, LmL and LmN) by marker-based analysis were validated by GISH analysis, proving the possibility of producing wheat-alien CALs without applying *in situ* hybridization for alien identification.

Table 12 Additional 527 unique SNP markers in 10 wheat-*L. mollis* CALs

Alien ID	Common SNPs on each chromosome	Line-specific SNPs
LmA	70	40
LmB	57	44
LmC	73	59
LmD	55	40
LmF	49	37
LmG	80	70
LmH	64	52
LmI	82	69
LmL	40	23
LmN	45	36
KT020-003 (CS)	-	-
TACBOW 0113 (Lm)	-	-
Total	**527**	**470**

TACBOW, Tottori Alien Chromosome Bank of Wheat; CS, Chinese Spring; Lm, *L. mollis*

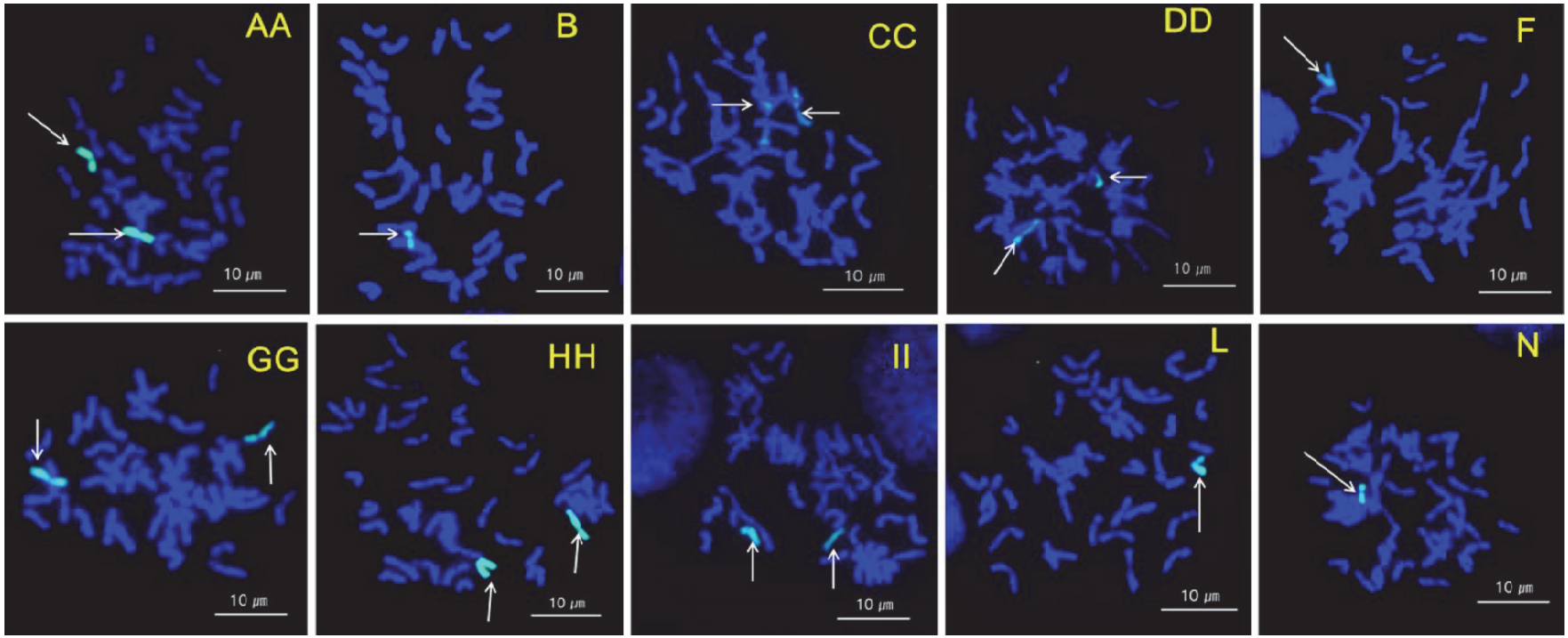

Fig. 9 Identification of *L. mollis* chromosomes added to wheat using GISH. A–N, *L. mollis* chromosomes; double letters, disomic lines; single letters, monosomic lines; arrows point to the added chromosomes detected with fluorescein-12-dUTP (green)

4.14 Relationship between *L. mollis* and *L. racemosus* genomes

Out of 8,653 SNP markers consistently scored in the two *Leymus* genomes, 75% were monomorphic, indicating a close genomic relationship between the species. Also, a cluster analysis using 579 SNP markers shared among the wheat-*L. mollis* CALs and wheat-*L. racemosus* CALs revealed an interesting relationship between the chromosomes of the two species (Fig. 10). Seven chromosomes each of *L. mollis* and *L. racemosus* in six HGs clustered in pairs: *L. mollis* chromosome H (LmH) and *L. racemosus* chromosome N (LrN); *L. mollis* chromosome F (LmF) and *L. racemosus* chromosome H (LrH); *L. mollis* chromosome C (LmC) and *L. racemosus* chromosome J (LrJ); *L. mollis* chromosome D (LmD) and *L. racemosus* chromosome K (LrK); *L. mollis* chromosome N (LmN) and *L. racemosus* chromosome F (LrF); *L. mollis* chromosome I (LmI) and *L. racemosus* chromosome I (LrI); *L. mollis* chromosome A (LmA) and *L. racemosus* chromosome A (LrA) (Fig. 10). In HG3, *L. mollis* chromosomes LmF and LmH associated with *L. racemosus* chromosomes LrH and LrN, respectively. Apart from LmL in HG1, whose homoeolog in *L. racemosus* was not produced, every other HG included at least two chromosomes, one from each *Leymus* species. In each HG, the genomic distance between homoeologous chromosomes of the two species was narrower than that within the same species (Table 13). In HG3 for instance, the distance indices between chromosomes LmF and LrH (18) and LmH and LrN (16) were clearly lower than those between LmF and LmH (90) and LrH and LrN (88). Using the genetic association among the chromosomes (Fig. 10; Table 13), the chromosomes were tentatively named (Table 14). For each of the species, the chromosomes were numbered according to their HGs and each chromosome of a homoeologous pair was arbitrarily assigned to a different sub-genome, designated as Ns_1 or Ns_2. Superscripts “m” (*L. mollis*) and “r” (*L. racemosus*)

were used to differentiate chromosomes of the two species. All non-homoeologous chromosomes in each species were assumed to be in the same sub-genome, as homoeology between two chromosomes should not exist in one sub-genome.

4.15 Phenotypic variation between CS and wheat–*L. mollis* CALs

From the preliminary phenotypic evaluation conducted in this study, all the addition lines were significantly different from CS in at least one of the seven traits measured (Fig. 11), indicating the effects of *L. mollis* chromosomes in the lines. Number of days to heading (DH) was significantly reduced in six of the lines, significantly increased in one, while three lines were not significantly different from CS (Fig. 11a). Five of the six lines with significantly reduced DH also reached physiological maturity significantly earlier than CS, while the other five lines were not significantly different from CS (Fig. 11b). Plant height was significantly reduced in five lines, significantly increased in one line, while four lines were not different from CS (Fig. 11c). All the lines were significantly different from CS in at least one yield component (Fig. 11d–g). Although spikes were significantly longer in some of the lines (Fig. 11d), number of spikes per plant, grain yield per spike and grain yield per plant were significantly lower in all the lines, except LmG and LmI, which were not significantly different from CS in number of spikes per plant and grain yield per spike, respectively.

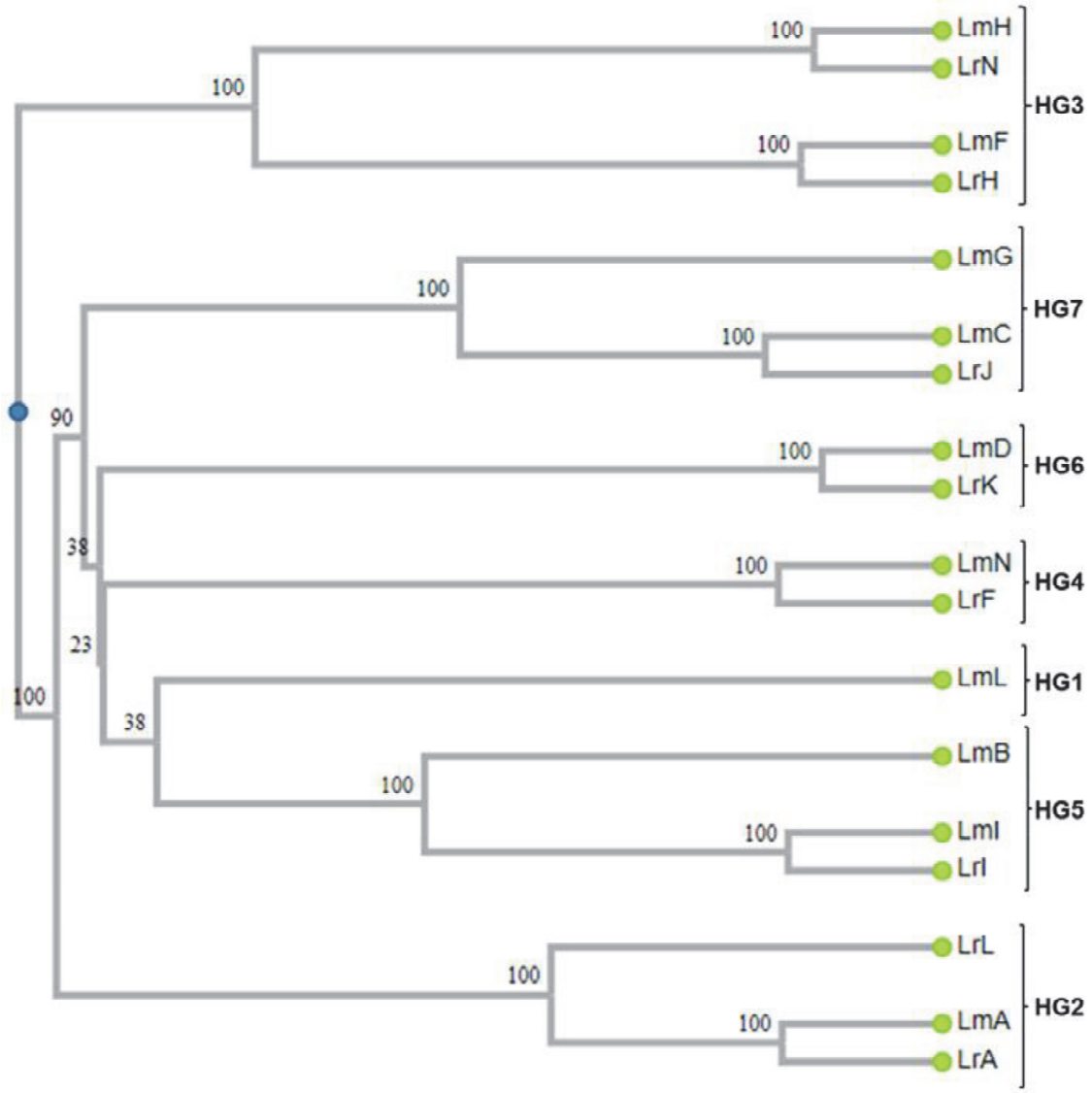

Fig. 10 Relationship between *L. mollis* and *L. racemosus* chromosomes. LmA–N, *L. mollis* chromosomes; LrA–N, *L. racemosus* chromosomes; numbers at the nodes are bootstrap values; clustering method: Unweighted pair group method with arithmetic mean (UPGMA) (http://genomes.urv.cat/UPGMA/)

Table 13 Genomic distance matrix among the chromosomes of *L. mollis* and *L. racemosus*

	LmA	LmB	LmC	LmD	LmF	LmG	LmH	LmI	LmL	LmN	LrA	LrF	LrH	LrI	LrJ	LrK	LrL	LrN
LmA	0	113	116	112	121	114	120	120	105	110	**20**	110	120	121	120	112	**36**	122
LmB		0	110	103	114	104	115	**78**	103	105	109	105	113	**52**	107	101	106	114
LmC			0	108	116	**78**	117	114	104	106	112	108	116	114	**22**	107	111	117
LmD				0	113	105	114	109	103	106	111	106	113	111	106	**15**	109	115
LmF					0	112	**90**	118	106	112	121	112	**18**	121	120	112	115	**81**
LmG						0	114	111	103	107	112	101	112	112	**43**	103	109	113
LmH							0	124	106	113	121	114	**86**	126	122	115	117	**16**
LmI								0	96	111	117	105	119	**19**	116	108	111	124
LmL									0	103	105	96	101	96	100	97	104	99
LmN										0	106	**21**	110	111	108	107	103	114
LrA											0	111	121	116	116	110	**62**	124
LrF												0	112	108	110	107	108	114
LrH													0	121	120	113	116	**88**
LrI														0	113	111	114	127
LrJ															0	111	113	123
LrK																0	107	116
LrL																	0	119
LrN																		0

LmA–N, *L. mollis* chromosomes; LrA–N, *L. racemosus* chromosomes; emboldened numbers, distance indices between homoeologous chromosomes; Distance indices were calculated by transforming Pearson's correlation coefficients (*r*) to distance (d) values using d = (1 – r) × 100 (http://genomes.urv.cat/UPGMA/)

Table 14 Proposed nomenclature system for *Leymus* chromosomes

S/N	*L. mollis* chromosomes				*L. racemosus* chromosomes			
	Arbitrary ID	Status in wheat background	HG	Proposed name	Arbitrary ID	Status in wheat background	HG	Proposed name
1	LmL	Monosomic	1	$1Ns_1^{\mathbf{m}}$	–	–	1	–
2	–	–	1	–	–	–	1	–
3	LmA	Disomic	2	$2Ns_1^{\mathbf{m}}$	LrA	Disomic	2	$2Ns_1^{\mathbf{r}}$
4	–	–	–	–	LrL	Disomic	2	$2Ns_2^{\mathbf{r}}$
5	LmF	Monosomic	3	$3Ns_1^{m}$	LrH	Disomic	3	$3Ns_1^{r}$
6	LmH	Disomic	3	$3Ns_2^{m}$	LrN	Disomic	3	$3Ns_2^{r}$
7	LmN	Monosomic	4	$4Ns_1^{\mathbf{m}}$	LrF	Disomic	4	$4Ns_1^{\mathbf{r}}$
8	–	–	4	–	–	–	4	–
9	LmI	Disomic	5	$5Ns_1^{\mathbf{m}}$	LrI	Disomic	5	$5Ns_1^{\mathbf{r}}$
10	LmB	Monosomic	5	$5Ns_2^{\mathbf{m}}$	–	–	5	–
11	LmD	Disomic	6	$6Ns_1^{\mathbf{m}}$	LrK	Disomic	6	$6Ns_1^{\mathbf{r}}$
12	–	–	6	–	–	–	6	–
13	LmC	Disomic	7	$7Ns_1^{\mathbf{m}}$	LrJ	Disomic	7	$7Ns_1^{\mathbf{r}}$
14	LmG	Disomic	7	$7Ns_2^{\mathbf{m}}$	–	–	7	–

HG, homoeologous group; Lm, *L. mollis*; Lr, *L. racemosus*; Ns_1 and Ns_1, different Ns genomes (Anamthawat-Jonsson, 2014); superscript **m** and **r,** Ns genomes in *L. mollis* and *L. racemosus*, respectively; A–N, *Leymus* chromosomes in the wheat genome; –, chromosomes either not identified or whose HGs have not been clearly established in this study

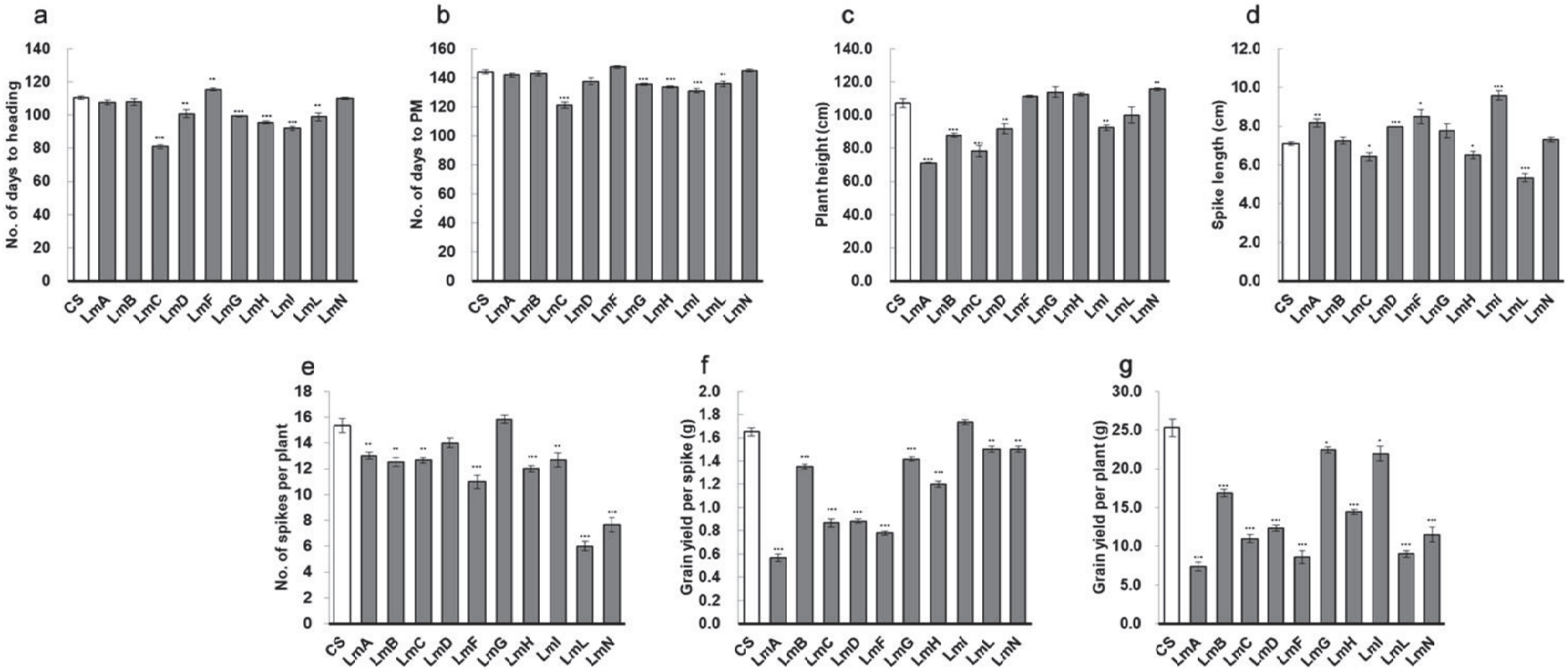

Fig. 11 Variation in agronomic traits between CS and wheat–*L. mollis* CALs. CS, Chinese Spring; LmA–N, *L. mollis* chromosomes; PM, physiological maturity; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; error bars, standard errors of the means. Mean values (n = 6) between CS and each addition line were compared using two-tailed t-test. Differences among the lines were not considered at this preliminary stage, as all the lines will be subjected to different conditions of stress for further selection and production of translocation and recombination lines with desired segments of *L. mollis* chromosomes.

4.16 Diploid analyzers of polyploid *Aegilops* and *Triticum* species

In the preceding result sections, the application of DArTseq genotyping enabled clear differentiation of wheat and *Leymus* genomes in the CILs. It was also possible to identify markers in the sub-genomes of wheat and the corresponding alleles in *Leymus* species (Tables 5 and 11). A similar strategy was therefore applied to select appropriate markers in the diploid genomes for the analysis of genomic relationships among the *Aegilops* and *Triticum* species reported here.

To determine the putative progenitors of each of the polyploid species of *Aegilops*, SilicoDArT markers in the diploid genomes of all the *Aegilops* species were used as genome analyzers (Table 15). For each of the diploid species, species-specific markers were selected by filtering markers present in one species but absent in all the others. This made dominant SilicoDArT markers preferred in this analysis, as codominant SNP markers do not give information on PAVs. The progenitors of the polyploid species were estimated based on the proportions of diploid markers that are retained in each polyploid genome (diploid-polyploid monomorphism). Because the number of the species-specific markers is affected by genetic similarity among the diploid species, especially the Sitopsis species, the genomes of the polyploid species of *Aegilops* were first analyzed with all the markers in each diploid genome of *Aegilops* species (Fig. 12a) before being analyzed with diploid species-specific markers (Fig. 12b). This allowed the determination of the suitability of the species-specific markers in estimating the progenitors of the polyploid species. The use of species-specific markers as analyzers reduced the background noise produced by monomorphic markers among the diploid species (Fig. 12b)

Having confirmed the adequacy of the species-specific markers of the analyzers,

polyploid genomes of *Triticum* species were analyzed with only species-specific diploid analyzers (Table 16). Therefore, the conclusions made regarding the putative progenitors of the polyploid species (*Aegilops* and *Triticum*) are based on the species-specific markers of the analyzers. To select analyzers for the polyploid genomes of *Triticum* species, the genomes of 16 bread wheat-related diploid species were screened to determine the proportions of homoeology of their SilicoDArT markers to the total SilicoDArT markers in each of the three genomes of bread wheat. This homoeology was estimated based on the number of markers of the diploid species assigned to each genome of bread wheat (Table 16). Making the estimation was possible because DArTseq platform optimized for bread wheat was used in this study. A diploid species with at least 10% homoeology to any of the three genomes of hexaploid wheat was selected as an analyzer for the corresponding genomes of each of the six polyploid *Triticum* species. With this criterion, a total of 13 analyzers were selected. Species-specific markers of the 13 selected diploid analyzers were then filtered for the analysis of the putative progenitors of the genomes of the polyploid species (Table 16).

Table 15 SilicoDArT markers of diploid analyzers of polyploid *Aegilops* species

Species	Total No. of markers	Species-specific markers (%)
Ae. mutica	12238	837 (6.84)
Ae. speltoides	9330	699 (7.49)
Ae. longissima	18321	761 (4.15)
Ae. sharonensis	18205	723 (3.97)
Ae. bicornis	16465	598 (3.63)
Ae. searsii	15402	1633 (10.60)
Ae. tauschii	20288	7420 (36.57)
Ae. caudata	19086	6514 (34.13)
Ae. comosa	17377	3941 (22.68)
Ae. uniaristata	16719	4003 (23.94)
Ae. umbellulata	19523	6627 (33.94)

Table 16 Selection of diploid analyzers of polyploid *Triticum* species with respect to homoeology of their SilicoDArT markers to those of A, B and D genomes of bread wheat

Species	No. of markers								
	A genome			B genome			D genome		
	Shared	Specific	Total (%)	Shared	Specific	Total (%)	Shared	Specific	Total (%)
Reference genome (bread wheat)	16901	4688	21,589 (100)	13415	7227	20642 (100)	30187	5167	35354 (100)
T. urartu	6114	3672	9786 **(45.3)**	1249	95	1344 (6.5)	2763	93	2856 (8.1)
T. boeoticum	5492	753	6245 **(28.9)**	1369	71	1436 (7.0)	2870	59	2929 (8.3)
Ae. mutica	1824	1348	3172 **(14.7)**	2331	1966	4297 **(20.8)**	4690	2284	6974 **(19.7)**
Ae. speltoides	1139	1129	2268 **(10.5)**	2507	4405	6912 **(33.5)**	3335	1883	5218 **(14.8)**
Ae. longissima	2301	392	2693 **(12.5)**	3513	755	4268 **(20.7)**	6831	594	7425 **(21.0)**
Ae. sharonensis	2294	336	2630 **(12.2)**	3487	769	4256 **(20.6)**	6799	632	7431 **(21.0)**
Ae. bicornis	2018	379	2397 **(11.1)**	3026	756	3782 **(18.3)**	6258	638	6896 **(19.5)**
Ae. searsii	1915	156	2071 (9.6)	3610	495	3105 **(15.0)**	5558	265	5823 **(16.5)**
Ae. tauschii	817	99	916 (4.2)	934	174	1108 (5.4)	8988	7568	16556 **(46.8)**
Ae. caudata	1849	1120	2969 **(13.8)**	1955	1013	2968 **(14.4)**	5321	1848	7169 **(20.3)**
Ae. comosa	1959	1022	2981 **(13.8)**	2126	1102	3228 **(15.6)**	5545	1904	7449 **(21.1)**
Ae. uniaristata	1805	672	2477 **(11.5)**	2049	724	2773 **(13.4)**	5155	1254	6409 **(18.1)**
Ae. umbellulata	2014	928	2942 **(13.6)**	2055	1017	3072 **(14.9)**	5174	1653	6827 **(19.3)**
S. cereale	699	23	722 (3.3)	959	64	1023 (5.0)	1840	30	1870 (5.3)
D. villosum	566	20	586 (2.7)	793	44	837 (4.1)	1538	16	1554 (4.4)
H. vulgare	291	8	299 (1.4)	431	19	450 (2.2)	824	9	833 (2.4)

A species whose proportion of markers assigned to any of the bread wheat's reference genomes (A, B or D) is not less than 10% was selected **(emboldened)** for the analysis of the corresponding genomes of the polyploid species.

4.17 Genomic differentiation and evolutionary relationships among polyploid and diploid species of *Aegilops*

Before applying the genome analyzers to determine the progenitors of the polyploid species, a total of 28,264 polyploid species-specific SilicoDArT markers, ranging from 187 in *Ae. juvenalis* to 4,759 in *Ae. cylindrica* (Table 17), were used to confirm genomic difference among the 12 polyploid species of *Aegilops*. The polyploid species-specific markers were selected in the same manner as the diploid species-specific markers. The relatively low numbers of specific markers in the genomes of *Ae. crassa* and *Ae. juvenalis* is obviously because large proportions of their genomes (D and U genomes) are shared by the other species (Table 17). With the possibility of genomic adjustments during polyploidization (Feldman and Levy, 2015; Feldman and Levy, 2012) and the assumption that the original progenitors of the polyploid species may be different from the accessions of the diploid species used in this study, only diploid analyzers with considerably higher proportions of monomorphism with the polyploid species were taken as the putative progenitors of the polyploid species. This analysis confirmed the putative diploid progenitors of *Ae. ventricosa* (D^vN^v), *Ae. cylindrica* (C^cD^c), *Ae. kotschyi* (S^kU^k), *Ae. biuncialis* (U^bM^b), *Ae. triuncialis* (U^tC^t), *Ae. ovata* (U^gM^g), and *Ae. variabilis* (S^pU^p) (van Slageren, 1994) (Fig. 12). Noteworthy is that the proportions of the markers of three Sitopsis species (*Ae. bicornis*, *Ae. longissima*, *and Ae. sharonensis*) retained in the genomes of the two polyploid species with S-related genomes (*Ae. kotschyi* and *Ae. variabilis*) were not reasonably different. This made it difficult to decide which of the Sitopsis species donated the S-related genomes to *Ae. kotschyi* and *Ae. variabilis*, although *Ae. bicornis* and *Ae. longissima*, respectively, seemed to be the most likely candidates. This observation confirms the likelihood of a common ancestry of the Sitopsis

species (Kilian *et al*., 2011). Therefore, the original progenitor of the S-related genomes of the polyploid species may have been an ancient relative of the Sitopsis species, which is probably extinct. Although polyploid species-specific markers (Table 17) were used to differentiate *Ae. kotschyi* (S^kU^k) from *Ae. variabilis* (S^pU^p) and *Ae. biuncialis* (U^bM^b) from *Ae. ovata* (U^gM^g), these pairs of species have identical genomic constitutions (same progenitors; Fig. 12), and therefore may be considered as variants/subspecies of the same species in each case.

The unidentified diploid progenitors of five polyploid *Aegilops* species (*Ae. triaristata* [U^nX^n, $U^nX^nN^n$], *Ae. crassa* [$D^{cr}X^{cr}$, $D^{cr1}D^{cr2}X^{cr}$], *Ae. juvenalis* [$X^jD^jU^j$], *Ae. vavilovii* [$X^{va}D^{va}S^{va}$], and *Ae. columnaris* [U^cX^c]) (Badaeva *et al*., 2004; Badaeva *et al*., 1998; Dvorak, 1998 ; Goryunova *et al*., 2004; Resta *et al*., 1996) are most likely to be traced to *Ae. speltoides* or *Ae. mutica*. The competing proportions of monomorphic markers between each of the diploid species (*Ae. speltoides* and *Ae. mutica*) and the genomes of the five polyploid species (Fig. 12) strongly suggest that the unidentified genomes may have been donated by an ancient species closely related to these two diploid species or their direct ancestor(s). Based on these results, modifications in the genomic representations of *Ae. crassa*, *Ae. juvenalis*, *Ae. vavilovii*, *Ae. columnaris,* and *Ae. triaristata* were proposed (Table 18). In this proposal, the genomes of *Ae. speltoides* and *Ae. mutica* are jointly represented as T^s. This does not suggest that the two diploid species are genomically the same but reflects the difficulty encountered in clearly determining which of the two species may have donated the controversial genome to the five polyploid species. Following the diploid-polyploid relationship analysis, the genome of tetraploid *Ae. crassa* is considered as being constituted of *Ae. tauschii* and T^s genomes (DT^s), while the genome of hexaploid *Ae. crassa* is designated as $D^1D^2T^s$ (tetraploid *Ae. crassa* x *Ae.*

tauschii). *Aegilops vavilovii* is assumed to have evolved from the hybridization of tetraploid *Ae. crassa* and *Ae. searsii*, granted that *Ae. crassa* and *Ae. vavilovii* have similar morphological traits and are reported to be sympatric (Cabi *et al*., 2010). The analysis also shows that *Ae. juvenalis* has T^s, D and U genomes; hexaploid *Ae. triaristata* lacks *Ae. comosa* genome but has the genomes of *Ae. umbellulata*, *Ae. uniaristata* and T^s, and *Ae. columnaris* is composed of *Ae. umbellulata* and T^s genomes.

4.18 Cluster analysis of diploid and polyploid *Aegilops* species

A phylogenetic tree (Fig. 13a) constructed with 15,512 frequently called SNP markers separated the diploid *Aegilops* species into already established botanical sections (van Slageren, 1994), except that *Ae. speltoides* did not cluster with other species in the section Sitopsis, an observation which has been reported by other researchers (D. Giorgi et al., 2003; Goryunova et al., 2008; O. Seberg & Petersen, 2007). *Aegilops umbellulata* (section Aegilops) seemed more distant from the others, whereas *Ae. speltoides* (section Sitopsis) appeared closer to *Ae. mutica* (section Amblyopyrum), and relatively distant from other species of section Sitopsis. Among Sitopsis species, *Ae. longissima* and *Ae. sharonensis* appeared genomically more proximal to each other than to others. The polyploid species of *Aegilops* formed two clusters based on the putative common diploid progenitors, *Ae. tauschii* (D cluster) and *Ae. umbellulata* (U cluster) (Fig. 13b). *Aegilops juvenalis*, bearing both D and U genomes, clustered closely with *Ae. crassa* and *Ae. vavilovii* in the D cluster, indicating a possible evolutionary link between its (*Ae. juvenalis*) genome and the two species in the D cluster. This again suggests the likelihood of the presence of a diploid genome, perhaps T^s, common to *Ae. juvenalis*, *Ae. crassa* and *Ae. vavilovii* (Fig. 12).

Table 17 Species-specific SilicoDArT markers of 12 polyploid species of *Aegilops*

Species	Ploidy	Genome	No. of markers
Ae. crassa	6x	$D^{cr1}D^{cr2}X^{cr}$	684
Ae. vavilovii	6x	$D^{va}X^{va}S^{va}$	2153
Ae. ventricosa	4x	$D^{v}N^{v}$	3027
Ae. cylindrica	4x	CD	4759
Ae. juvenalis	6x	$X^{j}D^{j}U^{j}$	187
Ae. kotschyi	4x	$S^{k}U^{k}$	2271
Ae. biuncialis	4x	$U^{b}M^{b}$	2601
Ae. triuncialis	4x	$C^{t}U^{t}$	2051
Ae. ovata L.	4x	$U^{g}M^{g}$	3163
Ae. triaristata	6x	$U^{n}X^{n}N^{n}$	2215
Ae. columnaris	4x	$U^{c1}X^{c2}$	2470
Ae. variabilis	4x	$S^{p}U^{p}$	2683
Total	**-**	**-**	**28264**

References of genomic formulas: (Badaeva et al., 2004; Badaeva et al., 1998; Badaeva et al., 2018; J. Dvorak, 1998 ; Goryunova et al., 2004; Resta et al., 1996); (B. Kilian et al., 2011)

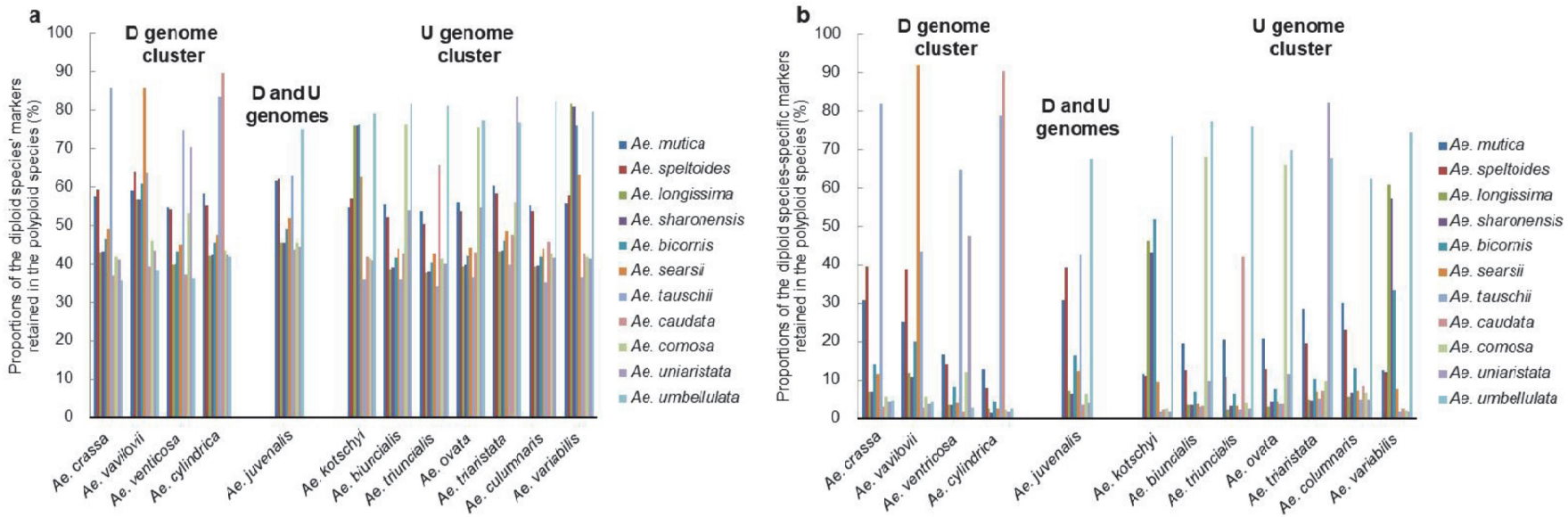

Fig. 12 Estimation of the putative diploid progenitors of 12 polyploid *Aegilops* species based on the proportions of diploid species' SilicoDArT markers retained in the genomes of the polyploid species. **a** Analysis based on all the markers of the diploid species. **b** Analysis based on the species-specific markers of the diploid species

Table 18 Proposed modifications in the genomic representations of five polyploid species of *Aegilop*s

Species	Ploidy	Reported genomic formula	Proposed genomic formula
Ae. crassa	4x, 6x	$D^{cr}X^{cr}$, $D^{cr1}D^{cr2}X^{cr}$	DT^{s}, $D^{1}D^{2}T^{s}$
Ae. vavilovii	6x	$D^{va}X^{va}S^{va}$	$DT^{s}S^{s}$
Ae. Juvenalis	6x	$X^{j}D^{j}U^{j}$	UDT^{s}
Ae. triaristata	4x, 6x	$U^{n}X^{n}$, $U^{n}X^{n}N^{n}$	UN, UNT^{s}
Ae. columnaris	4x	$U^{c1}X^{c2}$	UT^{s}

T^{s}, joint representation of *Ae. speltoides* and *Ae. mutica*; references of genomic formulas:

(Badaeva et al., 2004; Badaeva et al., 1998; Badaeva et al., 2018; J. Dvorak, 1998 ;

Goryunova et al., 2004; Resta et al., 1996)

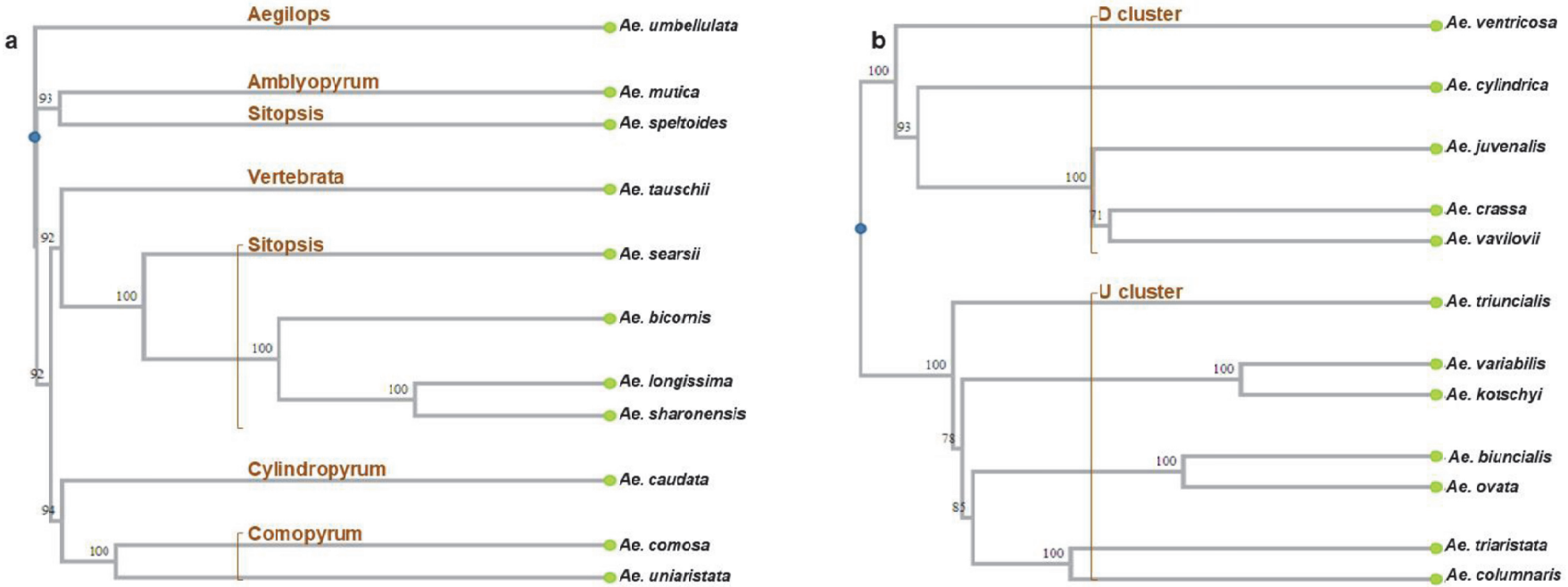

Fig. 13 Reconstruction of the evolutionary relationships among *Aegilops* species using of 15,512 SNP markers. **a** Diploid species. **b** Polyploid species. The sections of the diploids and two main clusters of the polyploids (Badaeva, 2002; Goryunova et al., 2004; B. Kilian et al., 2011) are labelled in brown.

4.19 Genomic and evolutionary relationships in the *Aegilops-Triticum* species

Species-specific SilicoDArT markers of 13 bread wheat-related diploid species (Table 16) were used to determine the elementary donors of the A, B and D genomes in six polyploid *Triticum* species. As described for the estimation of the progenitors of the polyploid species of *Aegilops*, the proportions of species-specific markers of the diploid species' markers shared with the genomes of the polyploid species enabled the determination of the progenitors of the genomes of the polyploid *Triticum* species (Figs. 14–17).

The genome of *T. urartu* was the closest to the A genomes of all the polyploid species analyzed (Fig. 14), suggesting that *T. urartu* is the most likely donor of the A genome in each of them. The considerable similarity between the A genome of each of the polyploids and *T. boeoticum*—another A genome species—suggests a common ancestry of *T. boeoticum* and *T. urartu*. Similarly, *Ae. searsii* seemed to be the most closely related to the B/G genomes of the polyploid species (Fig. 15). However, the proportion of *Ae. speltoides* markers assigned to the reference B genome was higher than those of every other diploid species analyzed (Table 16). This strongly suggests an evolutionary link between the genome of *Ae. speltoides* and the B/G genomes of the polyploids. This is further supported by an almost equal similarity of the genomes of the diploid species to the G genome of *T. araraticum* (Fig. 15). Using the species-specific markers of *Ae. speltoides* and *Ae. searsii* as analyzers, it was observed that chromosome 4B/G of each of the polyploids chromosome showed almost equal similarity to chromosome 4S of the diploid species chromosome (Fig. 16). But chromosomes 2S, 3S and 7S of *Ae. speltoides* appeared more closely related to the corresponding chromosomes of *T. araraticum* than those of *Ae. searsii* were. These observations give the impression that the B/G genomes

of polyploid *Triticum* species are likely to be recombinant genomes with varying contributions from *Ae. speltoides* and *Ae. searsii*. Analysis of the D genomes of the three hexaploid species unambiguously traced them to *Ae. tauschii* as the sole donor (Fig. 17).

A further analysis using 66, 434 SNP markers consistently called in the six polyploid genomes indicated 72% similarity (monomorphism) across their A genomes, B/G genomes and the combined AB/AG genomes. However, higher similarity was observed among the AB genomes: hexaploids, 94%; tetraploids, 90%; hexaploid and tetraploid genomes combined, 84%. The slight differences in the proportions of monomorphic markers in the different groups of the AB genomes suggest that the AB genomes of the hexaploid species originated from the same tetraploid species, whereas those of the tetraploid species may have evolved from different accessions of the elementary A and B genome progenitors (*T. urartu* and *Ae. speltoides/Ae. searsii*, respectively). The lower similarity (84% as compared to 94%) across the hexaploid and tetraploid AB genomes may reflect further modification of AB genomes in hexaploid species resulting from their interaction with the D genome.

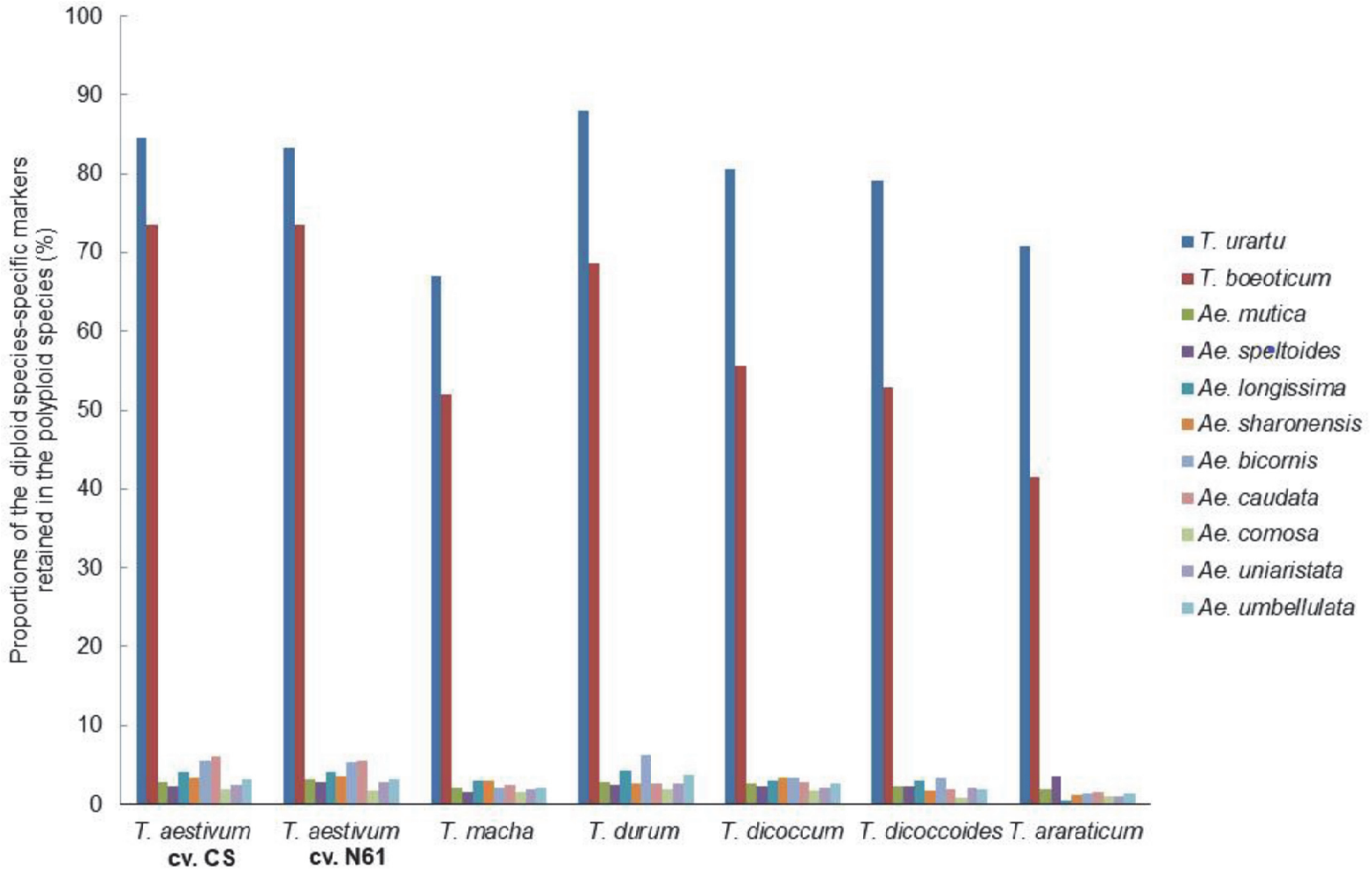

Fig. 14 Determination of the donor of the A genomes of six polyploid *Triticum* species using 11 diploid species-specific SilicoDArT markers assigned to the A genome of bread wheat

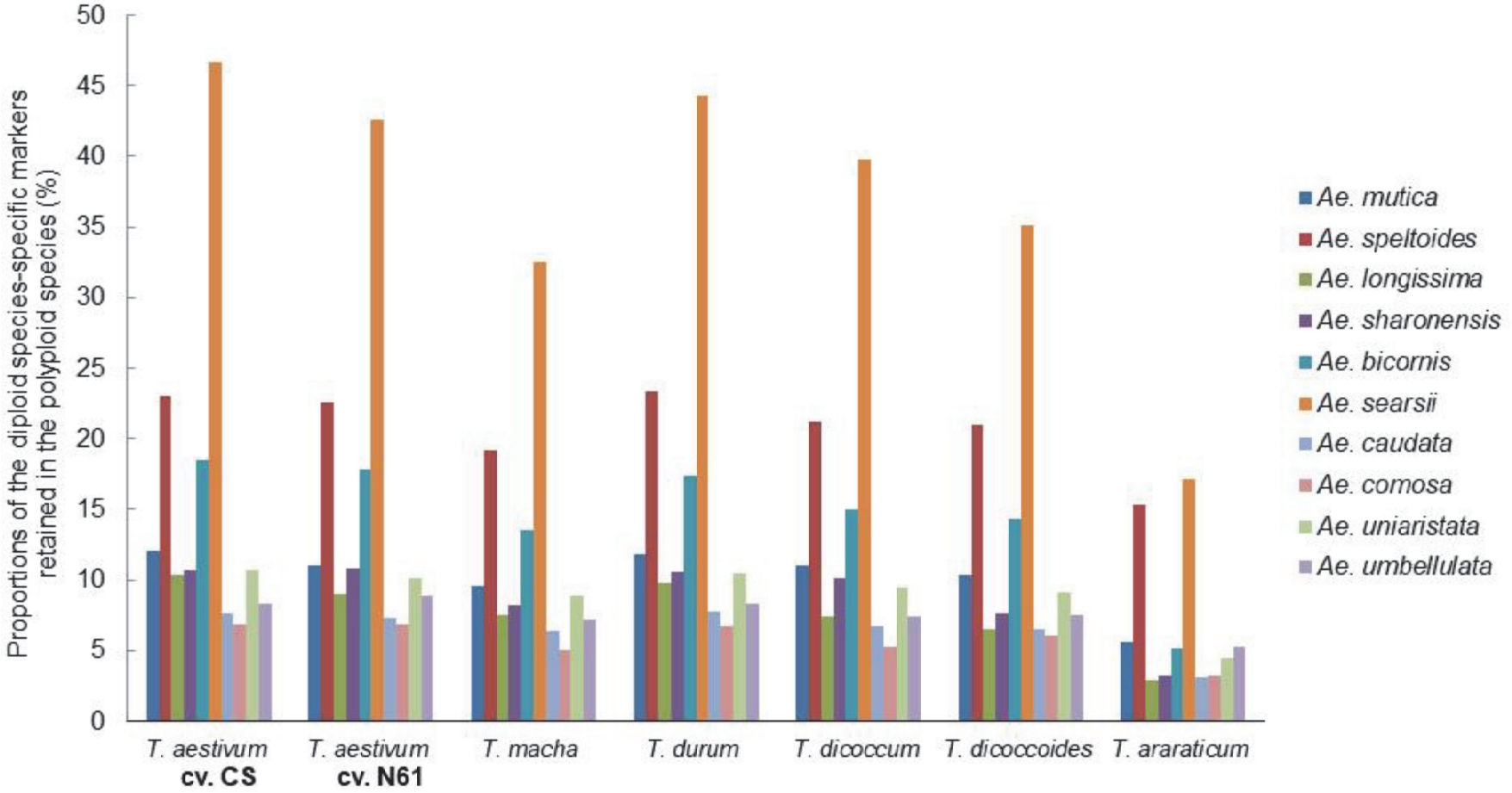

Fig. 15 Determination of the donor of the B/G genomes of six polyploid *Triticum* species using 10 diploid species-specific SilicoDArT markers assigned to the B genome of bread wheat

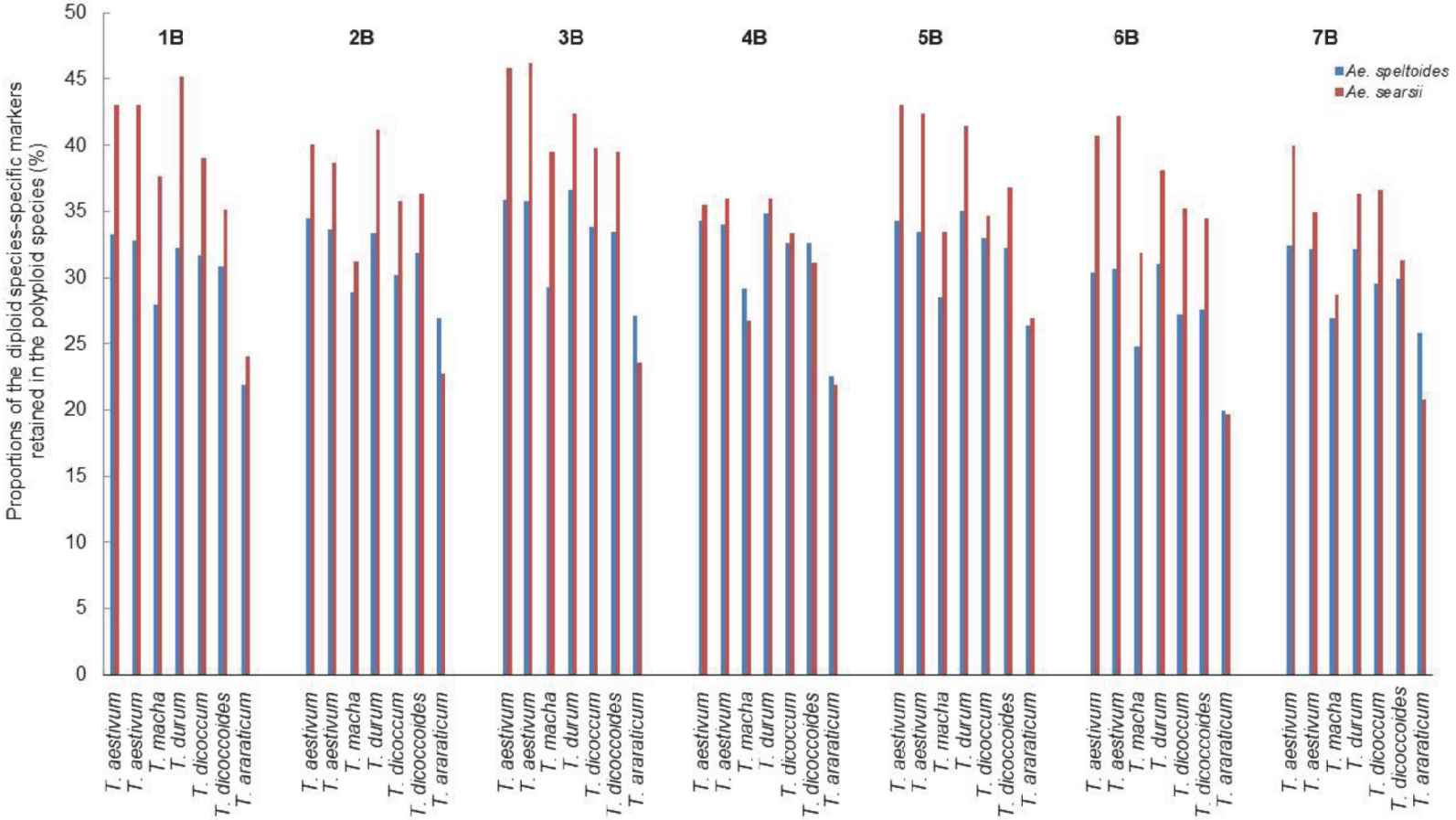

Fig. 16 Comparison of the B/G genome chromosomes of polyploid *Triticum* species and the chromosomes of *Ae. speltoides* and *Ae. searsii.* The B/G chromosomes are numbered 1B – 7B for convenience. This does not change the genomic representation of *T. araraticum*: AAGG.

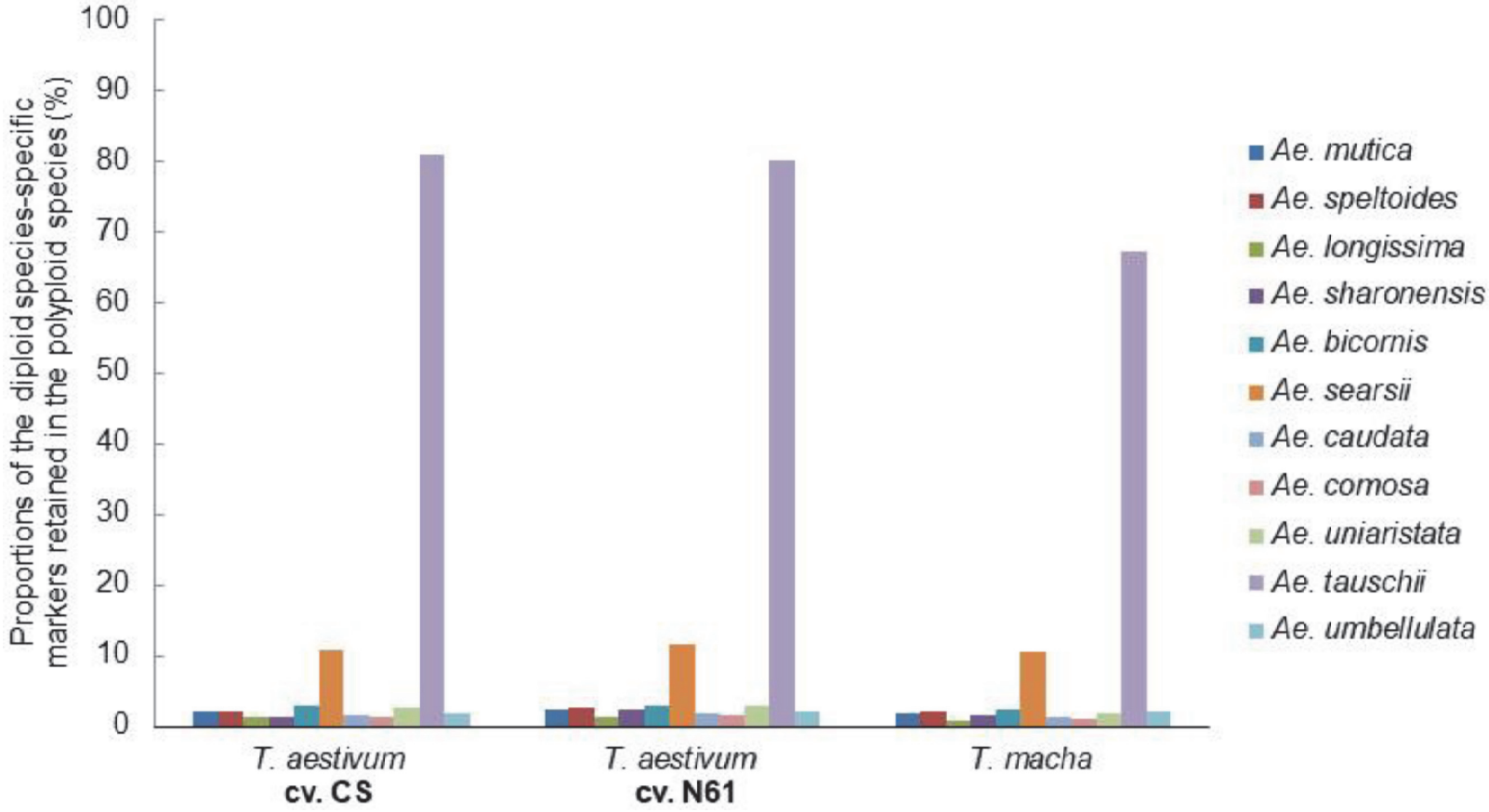

Fig. 17 Determination of the donor of the D genomes of two hexaploid *Triticum* species using 11 diploid species-specific SilicoDArT markers assigned to the D genome of bread wheat

CHAPTER FIVE

DISCUSSION

5.1 Fast-tracking introgression breeding and wheat-alien characterization with appropriate molecular markers

Utilizing introgressive hybridization to mitigate the age-long wheat genetic erosion had since been identified and is currently inevitable, but the achievements are still not satisfactory mostly because of poor understanding of the genomics of important wild relatives of wheat (Alix, 2017; Hajjar and Hodgkin, 2007). To create the necessary platform for successful breeding of wheat through the intermediary of its tertiary gene pool, mobilization of research resources towards genome analysis and development of molecular markers from notable Triticeae perennial species must be intensified. The availability of such markers would help to hasten introgression breeding and boost yields to match the projected high global increase in wheat demand in 2050 (Reeves, 2016). Typically, production of wheat-alien CILs requires a minimum of eight years of laborious cytogenetic and/or phenotypic screening after successful production of F_1 hybrid plants from an intergeneric hybridization. Absence of known chromosome markers, as the case of *L. mollis*, can further frustrate reliable characterization of alien chromosomes by ISH, justifying the need to develop alternative strategies to achieve wide and easy utilization of extant genes in the genomes of wheat's wild relatives for its (wheat) improvement. Based on the results herein, the incorporation of marker-assisted selection can significantly reduce the duration and physical efforts needed for this process. In the case of the 10 wheat-*L. mollis* CALs reported here, the entire processes of marker development, genotyping, selection and confirmation of the status of each alien chromosome in all the lines were completed within three years, a significant time gain of not less than five years.

The marker-based approach of identifying disomic lines is simple, fast and highly efficient, as it did not conflict with GISH results. The use of molecular markers from the genomes of important wild relatives to track their chromatins in wheat can therefore be said to be one of the most promising methods to facilitate speedy expansion of diversity in wheat germplasm (Gong *et al*., 2017; J. Zhang *et al*., 2017). This would, undoubtedly, incentivize plant breeders to develop wheat cultivars with improved and wider adaptability in order to keep pace with the projected global increase in wheat demand.

From the stand point of the results obtained in this study, especially the clear identification of all the recombinant segments of *L. racemosus* chromosome N, it is evident that adequate molecular markers from the genomes of potential gene donors would not only accelerate introgression breeding, but can reliably be deployed to tackle linkage drag, where necessary. The massive chromosome-specific markers developed for each of the chromosome addition lines (except LrE) are, therefore, expected to aid breeders in conducting more stringent screening and selection in their efforts to develop cultivars with only necessary chromosome segments to satisfy specific breeding goals within a reasonable time frame. At the moment, the few chromosome-specific markers developed for LrE is attributed to high homology between wheat genome and the chromosome, having observed about 30% monomorphic markers between *L. racemosus* and *T. aestivum* genomes. However, the possibility of some form of genetic instability, cytochimerism or alien loss for instance, is not completely ruled out. However, since the original stocks of all the addition lines in the TACBOW gene bank, which had earlier been characterized (Kishii *et al*., 1999; Kishii *et al*., 2004) were studied, it is obvious that alien chromosome loss is not responsible for the strange result obtained for LrE.

The difference between the number of markers developed for each *Leymus*

species and the number of markers that identified their chromosomes in wheat background account for the difference between the whole sets (14 each) of chromosomes in their genomes and the number of the chromosomes of each of them that were studied in the nineteen genotypes (nine wheat-*L. racemosus* and 10 wheat-*L. mollis* CALs). Another factor likely to contribute to this difference is the possibility of losing some segments of the chromosomes during production of the lines. However, these excess markers are advantageous, as they can be used to select lines with other *Leymus* chromosomes yet to be introgressed in wheat.

5.2 Overcoming undesired duplication of alien chromosomes in different lines

In addition to the time gain recorded in the production of the 10 wheat–*L. mollis* CALs, spurious duplication of different segments of the same chromosome was completely avoided. Chromosome-specific markers and the additional unique SNP markers found in the CALs clearly differentiated the 10 lines. The duplicates initially selected by chromosome counting were clarified by molecular markers. Identical PCR amplifications and DArTseq genotypic data revealed lines carrying the same *L. mollis* chromosomes. This would not be possible with GISH because GISH uses genomic DNA as probe, which can only identify the alien chromosomes but cannot show differences between them. Also, the unique CALs-specific polymorphisms revealed by DArTseq analysis, absent in either of the parents, would not be captured by ISH techniques, as hybridization probes are usually designed to track alien segments, not polymorphisms which may arise from genome interactions. Furthermore, using phenotypic data to identify plants that carry different segments of the same chromosome is difficult, and such genotypes may be mistaken as carriers of different chromosomes. This can be effectively managed with

appropriate molecular markers. However, although this study has proven the possibility of producing wheat-alien CALs without alien identification by ISH, total replacement of hybridization procedures with molecular markers is not advocated. Rather, the integration of efficient DNA markers with ISH strategies in wheat–alien breeding programs to accelerate the process and improve outcomes is recommended.

5.3 Ease of determining HGs of alien chromosomes

Previous reports have shown that determining HGs of alien chromosomes in wheat with other methods is not only tasking but can result in the assignment of different HGs to the same chromosome (Kishii *et al*., 2004; Larson *et al*., 2012). To determine the HGs of alien chromosomes, their ability to substitute wheat chromosomes in interspecific or intergeneric hybrids and functionally compensate the substituted chromosomes in substitution lines is usually applied (Badaeva *et al*., 1991; Calderon *et al*., 2012; Molnar-Lang *et al*., 2014; Morris and Sears, 1967). This approach is relatively difficult, and its accuracy relies, to a large extent, on the morphological similarities between the alien carrier and corresponding wild type, which is under environmental influence. The clarity of the HGs of *Leymus* chromosomes determined in this study confirms that all the CALs carry distinct alien chromosomes and serves as a guide for producing other wheat–*Leymus* lines carrying the remaining chromosomes, highlighting the accuracy and reliability of the procedure reported here. The simple approach of applying chromosome-specific SNP markers to determine the HG of each *Leymus* chromosome is consistent, as it agrees perfectly with the grouping of the chromosomes revealed by the genetic relationship analysis using shared SNP markers which are completely different from the chromosome-specific markers. Interestingly, the chromosome-specific markers' approach of

determining HGs of alien chromosomes clarified the HGs of chromosomes LrJ (HG 7) and LrN (HG 3), which were previously not reported with certainty (Kishii *et al*., 2004; Larson *et al*., 2012). Classifying alien chromosomes into their correct HGs is essential as it can contribute to an in-depth understanding of the interaction of each chromosome with the background of its carrier (Garg *et al*., 2007; Zhang *et al*., 2005). The failure to determine the HG of LrE chromosome, as was the case with earlier reports cited here, is another indication that the line may be genetically unstable.

5.4 Possibility of genome or alien modification in wheat–alien translocations

Characterization of the wheat–alien CILs by molecular markers brought to light detailed chromosome segments rearrangements, some of which can be likened to "zebra" chromosome (Zhang *et al*., 2005; Zhang *et al*., 2008). These interactions between alien chromosomes and carrier genomes need to be properly dissected. Analysis of the chromosome addition and translocation lines in this study indicated the possibility of genetic modification of either the introgressed chromosomes, background (wheat genome) or both. These modifications, capable of generating additional polymorphisms, may result from small chromosomal rearrangements, activation of transposable elements or any other interactive genetic event between alien materials with the genome of wheat (Garg *et al*., 2007; Zhang *et al*., 2005). Also, by graphically genotyping the recombination lines, different patterns of recombination events in each line were uncovered. This observation indicates that the interaction of the same *L. racemosus* chromosome (LrN) produced different genotypes, which are likely to result in diversity in agronomic traits. Of more importance is the potential effect of these interactions on the overall performance of the genotypes (Gorafi *et al*., 2016), necessitating detailed studies to clarify the

underlying mechanism of such genetic events and their agronomic implications. Such studies would be greatly enhanced by the availability of adequate molecular markers to track aliens and unique polymorphisms which may result from genome interactions.

5.5 Association of universal markers of *Leymus* chromosomes with CACTA-family transposon

The universal markers developed in this study are particularly valuable since they can be applied to easily track the transmission of alien chromosomes over generations, given the possibility of alien chromosome elimination in the course of cultivar multiplication and maintenance (Gernand *et al*., 2005; Ishii *et al*., 2010; Sanei *et al*., 2011). Following the alignment of the sequence of one of these markers to CACTA-family transposon in *L. perenne*, it may be safe to speculate that this marker sequence is part of a possible CACTA-family transposon in *Leymus*. CACTA-family transposons, one of the most abundant superfamilies of class II transposons exclusively found in plants, have been reported to play significant roles in genome variation in Triticeae and other plants (Fedoroff, 2013; Langdon *et al*., 2003; Miura *et al*., 2004; Sergeeva *et al*., 2010; Wicker *et al*., 2003).

5.6 *Leymus racemosus* chromosome N-specific markers and biological nitrification inhibition (BNI) activity

Biological nitrification inhibition (BNI) activity in *L. racemosus,* a highly desirable trait with agronomic and environmental consequences, had previously been reported to be chiefly controlled by chromosome LrN (Subbarao *et al*., 2007). The N-specific markers are, therefore, particularly of high value, as they can easily be applied to identify genotypes with BNI activity, avoiding the cumbersome and expensive process of root

exudates analysis, requiring expertise which an ordinary plant breeder may not possess. Intriguingly, only DNA sequences of the PCR products of *L. racemosus* and wheat–LrN generated with one of the universal markers aligned to the CACTA-family transposon in *L. perenne,* one of the forage grasses reported to have endogenous BNI activity (Subbarao *et al*., 2007). However, whether BNI activity is linked with actions of mobile genetic elements, transposons in this case, could not be ascertained in this study.

5.7 Transferability of markers between *L. racemosus* and related species

Sequencing of all the potential gene sources for wheat breeding does seem feasible, since many of the species are wild and do not have direct benefits. Hence, transferability of markers between useful species of this gene pool, as a compensational approach of analysis, is highly desired (Almeida *et al*., 2014; da Silva *et al*., 2017; Xiao *et al*., 2016; Zeng *et al*., 2016). This study has proven that markers from *L. racemosus* can be successfully transferred to *L. mollis, P. huashanica* and *E. ciliaris*, three other important species in the tribe Triticeae. Also, the transferred markers were found to be reasonably polymorphic in wheat, suggesting their suitability for characterizing wheat genotypes with alien chromosomes from the three genomes. The genera of these species, because of their status as profitable forage grasses and gene mines for hexaploid breeding, have received fair research attention (Anamthawat-Jonsson, 2001, 2014; Anamthawat-Jonsson and Bodvarsdottir, 2001; Baum *et al*., 2016; Wang *et al*., 2006; Wu *et al*., 2003; Yang *et al*., 2017). However, their genomes have not yet been sequenced, leaving breeders with the option of transferring markers from evolutionary closely related species to analyze their genomes and wheat genotypes carrying their chromatins. This has already been demonstrated for *L. mollis* in this study.

5.8 High similarity between *L. mollis* and *L. racemosus* genomes

The high proportion of monomorphic markers in the two genomes of *Leymus* species strongly indicates high genomic similarity between the genomes, and the association of their chromosomes suggests that the genomic distance between the sub-genomes of the two species is narrower than the distance between the sub-genomes within each of the species. This gives the impression that the two *Leymus* species may have evolved from independent hybridization events of the same diploid species with partially differentiated genomes, for instance, Ns_1 and Ns_2. However, a convincing conclusion could not be drawn on the evolutionary relationship between the two *Leymus* species or their sub-genomes, as this would require the application of the genomic resources of their diploid progenitors as analyzers of the polyploid genomes. Diploid analyzers would also enable reliable discrimination of the sub-genomes and help to clarify the sources of the elementary genomes of the *Leymus* species. Worthy of note is that the chromosomes of hybrids from *Leymus* species form complete meiotic pairs (Dewey, 1970; Dewey, 1972; Dewey, 1984; Kishii *et al*., 2003), which is a rare phenomenon in interspecific hybrids. This and the high transferability of DNA markers between *L. racemosus* and *L. mollis* suggest that the genomic resources of any of the two species should be interchangeably deployed to analyze their genomes. But the chromosomes of *L. mollis* could not be characterized with *L. racemosus* cytological markers, so the genomic difference between these species still needs to be clarified. The tentative nomenclature for *Leymus* chromosomes proposed here can easily accommodate all the chromosomes of *Leymus* species, irrespective of ploidy level. Considering the consistency of HGs determined independently using the chromosome-specific and common SNP markers, we are certain

about the HGs of the *L. mollis* chromosomes, but the accuracy of the assignment of the chromosomes to the sub-genomes needs to be confirmed using diploid analyzers.

5.9 Potential of *L. mollis* chromosomes in developing improved wheat lines

The preliminary phenotypic results obtained in this study have shown that *L. mollis* chromosome segments can be used to develop early maturing cultivars which can be cultivated in areas with short periods of favorable wheat growing conditions. However, the exact segment of the chromosome(s) should be identified and used to produce translocation or recombination lines, to reduce the effects of deleterious genes which may have caused the reduction in the yield components observed in the addition lines. Since all the genotypes were evaluated under normal growth conditions, it cannot be concluded that the relative superior yield components of CS will be sustained under stress conditions. Therefore, all the genotypes should be evaluated under different simulated and/or actual stress conditions to explore the possibility of selecting lines with the sturdy traits of *Leymus* species (Bao *et al*., 2012; McGuire, 1981; Xiao *et al*., 2012; Yang *et al*., 2015; Zhang *et al*., 2017).

5.10 Successful reconstruction of the phylogeny of *Aegilops* species and marker polymorphisms among polyploid species of *Aegilops*

The clustering patterns of *Aegilops* species were largely consistent with the established classifications (Dvorak and Zhang, 1992; Golovnina *et al*., 2007; Golovnina *et al*., 2009). Diploid species separated on the basis of their known botanical sections in the genus, while polyploid species were delineated following the presence of common diploid progenitor genomes (D and U genomes) among them (Badaeva, 2002; Goryunova *et al*.,

2004; van Slageren, 1994). However, consistent with previous reports (Giorgi *et al*., 2003; Goryunova *et al*., 2008; Seberg and Petersen, 2007), *Ae. speltoides* appeared distant from other species in the section Sitopsis; hence, its inclusion in the section needs to be reconsidered.

Markers specific to each of the 12 polyploid species clearly showed considerable polymorphisms among the genomes, including the genomes of species which arose from the same diploid progenitors. This suggests that genetic modifications, such as chromosomal alterations (Badaeva *et al*., 2004), may have occurred during independent evolution events of those species with identical progenitors. Without these specific markers, it would be difficult to genomically differentiate *Ae. kotschyi* (S^kU^k) from *Ae. variabilis* (S^pU^p) and *Ae. biuncialis* (U^bM^b) from *Ae. ovata* (U^gM^g) because, from the stand point of this study and previous studies (Kilian *et al*., 2011; van Slageren, 1994), the species in each pair evolved from the same progenitors. Although each of the species in these two sets is recognized as independent, based on differences in cytoplasm progenitors and/or nuclear genome variation (Goryunova *et al*., 2017; Kilian *et al*., 2011), this classification does not seem to be justified. Therefore, it may be appropriate to regard each pair as variants/subspecies of the same species.

5.11 Clarification of the speculated unidentified diploid progenitors of five polyploid *Aegilops* species

The reported unidentified diploid genomes initially represented as modified M (*Ae. comosa*) genome and later changed to unknown (X) in the genomes of *Ae. triaristata*, *Ae. crassa*, *Ae. juvenalis*, *Ae. vavilovii,* and *Ae. columnaris* (Badaeva *et al*., 2004; Badaeva *et al*., 1998; Dvorak, 1998 ; Goryunova *et al*., 2004; Resta *et al*., 1996; van Slageren, 1994)

is traceable to *Ae. mutica* or *Ae. speltoides*. The small proportions (<10%) of *Ae. comosa*-specific markers shared with the five polyploid species is insufficient to infer the existence of remnants of *Ae. comosa* genome in the polyploid genomes. Assuming *Ae. comosa* was originally involved in the evolution of the polyploids, species-specific elements from other progenitors may have spread and eventually masked *Ae. comosa*-specific elements (Anamthawat-Jonsson, 2014). The results obtained from this study suggest that ancient or ancestral forms of *Ae. speltoides* or *Ae. mutica*, which are probably extinct, donated the unidentified genomes to the five polyploid species. It can also be said, following the results, that all the polyploid species originally had a genome of such an ancient species. This observation agrees with the hypothesis that *Ae. mutica* (syn. *Amblyopyrum muticum*) and *Ae. speltoides*, both allogamous species with ancestral traits, diverged earlier than other *Aegilops s*pecies and may therefore be the ancestors of the other *Aegilops* species (Feldman and Levy, 2015). Therefore, each diploid *Aegilops* species may have retained a substantial portion of the common ancestral genome (*Ae. speltoides/Ae. mutica* or their ancestor). The difference in the representation of the common progenitor in each of the polyploid species may have resulted from the peculiar evolutionary event(s) of each species. Polyploids that arose from the hybridization of the common diploid ancestor with other diploid species should have larger portions of the genome of the common ancestor than those that did not directly evolve from the common ancestor.

5.12 Clarification of the complex nature of B genomes of *Aegilops–Triticum* polyploids

While validating the putative diploid progenitors of the A and D genomes of polyploid

Triticum species—*T. urartu* and *Ae. tauschii*, respectively—(Daud and Gustafson, 1996; Lelley *et al*., 2000; Ling *et al*., 2013; Luo *et al*., 2017; Odintsova *et al*., 2008), this study has provided information that may help to explain the complex nature of the B/G genomes. The genomes of both *Ae. speltoides* and *Ae. searsii* were found to be similar to the B/G genomes of the polyploid species analyzed, especially *T. araraticum*, a relatively less advanced tetraploid species (Miyashita *et al*., 1994; Tsunewaki *et al*., 1993); thus, the B/G genomes of polyploid *Triticum* species may have evolved from an ancestral genome that later differentiated into those of *Ae. speltoides* and *Ae. searsii*. Alternatively, the B/G genome may have arisen from the hybridization of *Ae. speltoides* and *Ae. searsii* before the emergence of the AB/AG genome at different times. The above considerations support earlier postulations that the B genome is the most modified of the three genomes of hexaploid wheat, whereas the A and D genomes still retain substantial genomic similarity to their respective progenitors, *T. urartu* and *Ae. tauschii* (Feldman and Levy, 2015). The previously suggested origin of the A genome of *T. araraticum* from *T. boeoticum* (Dorofeev *et al*., 1979; Migushova and Konarev, 1975) is probably invalid. The results from this study agree with the hypothesis that both Emmer and Timopheevi lineages of polyploid wheats have the same sources of elementary A and B/G genomes (Chantret *et al*., 2005; Giorgi *et al*., 2003; Golovnina *et al*., 2009; Gornicki *et al*., 2014; Kilian *et al*., 2007). However, a common ancestry of the A-genome species cannot be ruled out and the A genomes of polyploid *Triticum* species may have evolved from a common ancestor of *T. urartu* and *T. boeoticum* before the differentiation of the two species. Although no karyotypic differences have been detected between these diploid A-genome species (Giorgi and Bozzini, 1969; Golovnina *et al*., 2009), low fertility of interspecific F_1 hybrid plants of these two species has been reported (Fricano *et al*., 2014). The latter study,

consistent with the results reported here, suggests that the two species are genomically different. More importantly, this study, in agreement with previous similar studies (Miyashita *et al*., 1994; Tsunewaki *et al*., 1993), indicates that the A and G genomes of *T. araraticum* (Timopheevi lineage) are less modified than the A and B genomes of the Emmer lineage.

CHAPTER SIX

CONCLUSION

The molecular markers developed in this study are expected to play valuable roles in hexaploid wheat breeding, especially in developing wheat–alien CILs. Their application to characterize 32 wheat-*Leymus* CILs reported herein validates their usefulness. Specifically, the universal and LrN-specific markers are of great breeding importance. The universal markers can readily be applied to monitor and confirm alien presence and transmission, and the LrN-specific markers can find application in mapping of chromosome segments associated with biological nitrification inhibition (BNI) activity. The additional SNP markers found in the nineteen CALs would be useful in identifying and analyzing unique polymorphisms which may result from alien interaction with the background, while the *Leymus* markers not mapped on any of the nineteen chromosomes would aid production of other wheat-*Leymus* CILs carrying other chromosomes of the two *Leymus* species**.** Also, the remarkable transferability of the PCR-based markers to three other notable perennial Triticeae species is an added advantage, as they can be used to characterize wheat–alien CILs bearing chromosomes from these genomes. This has already been proven by the results obtained in this study for the 10 wheat–*L. mollis* CALs.

This study has proven that wheat–alien addition lines can be rapidly developed and reliably characterized using DNA markers for effective selection of carriers and recognition of disomic and monosomic lines. This approach requires the development of chromosome-specific DNA markers from the genomes of potential gene donors or closely related sequenced genomes. The integration of DArTseq genotyping allows confirmation of the PCR results, development of more chromosome-specific markers, and further characterization of the lines. Allocation of all the markers on the DArTseq platform to

wheat chromosomes enables effective analysis of wheat–alien complexes. The application of genotyping-by-sequencing approaches, including DArTseq, in the analysis of germplasm of wheat and other plants has gained reasonable popularity (Andrews *et al*., 2016; Baloch *et al*., 2017; Davey *et al*., 2011; Li *et al*., 2015). However, this is not the case with the characterization of wheat–alien introgression lines involving distant relatives of wheat, possibly because it may be thought that wide genetic distance and differences in the ploidy levels of bread wheat and these relatives would not allow identification of homoeology between their genomes. The results of this study have shown clearly that alien segment-specific markers can easily be isolated and the correspondence of alien chromosome-specific SNP markers with bread wheat reference alleles can be used to determine the HGs of the alien chromosomes. This study has demonstrated that DArTseq SNP markers can be integrated with PCR markers to produce and characterize wheat–alien addition lines without necessarily applying ISH for alien chromosome identification. Given its reliability and savings in time and efforts, this simple methodology is recommended to accelerate introgression breeding of wheat.

It has also been shown that DArTseq genotyping can be applied to conduct a large-scale analysis of evolutionary relationships among plant genomes because shared and species-specific markers can be easily identified. It also ensures a wide genomic coverage and is not subject to the criticism associated with the factors that affect meiotic chromosome pairing in hybrids of distant crosses, which forms the main anchor of cytogenetic systems of genome analysis (Baum *et al*., 1987; Dewey, 1984; Farooq *et al*., 1990; Kihara, 1930; Love, 1984; Moore, 2009; Seberg and Petersen, 1998). Also, the number of informative markers generated by DArTseq outstrips what is possible with conventional DNA marker procedures (Goryunova *et al*., 2010; Goryunova *et al*., 2004;

Mizuno *et al*., 2010; Wang *et al*., 2011), making it more robust and reliable. Genotyping of all the available accessions of species in tribe Triticeae using this platform would clarify the genomic relationships between the cultivated and wild species. This information would make the use of the available gene pools for breeding much more precise and would also help to clarify Triticeae taxonomy. As polyploidy and interspecific hybridization are key events in the evolution of higher plants (Alix, 2017), this genome analysis approach would be useful in other groups of plant species, especially polyploids whose phylogeny are still unclear. This method is, therefore, recommended as an efficient and cost-effective alternative to whole genome sequence-based analysis, as the genomes of most important wild species are yet to be sequenced.

SUMMARY

Over time, wild relatives of wheat have played important roles in enhancing improved yield and adaptability of wheat. Regardless of their value as potential gene sources for wheat breeding, the current status of their genomic resources is not enough to aid proper understanding of their genome structures and support maximal utilization of their genes to broaden genetic diversity in wheat germplasm. This inadequate genomic information base needs to be continually enriched to enhance aggressive utilization of wild genetic resources to manage biotic and abiotic stresses which pose threats to the general performance of elite cultivars of wheat.

The species in the genus *Leymus* are known to be resistant to salinity and economically important diseases of wheat. Additionally, *Leymus racemosus* is particularly reported to exhibit biological nitrogen inhibition (BNI) activity, a valuable trait of agronomic and environmental importance. To optimally harness these genes for the improvement of wheat, there should be efficient cytological and molecular markers to unmistakably map the alien chromatins in the genetic background of wheat. Before this study, variable cytological markers enabled molecular cytogenetic characterization of *L. racemosus* chromosomes in wheat, but there were no DNA markers to conduct detailed characterization of wheat–*L. racemosus* translocation and recombination lines. Worse still, the lack of *L. mollis* cytological and DNA markers greatly delayed the production of wheat–*L. mollis* chromosome addition lines (CALs). Earlier attempts to apply *L. racemosus* cytological markers to characterize the chromosomes of *L. mollis* in wheat failed to produce satisfactory hybridization signals, even though chromosomes of hybrids of the two *Leymus* species are known to form complete meiotic pairs. This necessitated the development of an alternative strategy to characterize wheat–*L. mollis* lines without

necessarily applying *in situ* hybridization for alien identification.

Furthermore, although there is an appreciable volume of data aiming at elucidating the phylogeny of Triticeae species, there are still some controversies surrounding the genomic relationships among *Aegilops* and *Triticum* species. Among such controversial discussions are the opposing views regarding the primary donor(s) of B/G genomes of polyploid *Triticum* species, including bread wheat, and the speculated unidentified diploid genomes believed to have participated in the evolution of *Ae. crassa*, *Ae. vavilovii*, *Ae. juvenalis*, *Ae. columnaris* and *Ae. triaristata*. Resolving these issues using the 'genome analyzer' method, which relies on meiotic chromosome pairing in hybrids of distant crosses, although quite informative, had since come under heavy criticism. Therefore, the application of molecular methods to accumulate useful data that would aid to clarify the evolutionary relationships among these species remains the focus of contemporary studies. Such markers have helped to provide some explanations on *Aegilops-Triticum* relationships, the origin and differentiation of *Aegilops* species, and intra- and inter-specific variations in the D and U genome clusters of *Aegilops* species. However, the numbers of markers in most of the cases are hardly enough to satisfactorily assure genomic coverage and convincing conclusions.

Consequently, this study was basically designed to develop and validate DNA markers from the genomes of *L. racemosus* and *L. mollis*, develop a molecular marker-based strategy for production of wheat–*L. mollis* CALs and assess the suitability of DArTseq genotyping, an efficient genotyping-by-sequencing platform, in wheat–alien characterization and analysis of the genomes of selected species in tribe Triticeae.

Using genome sequence information of *L. racemosus* and DArTseq genotyping, thousands of polymorphic markers were developed from the genomes of *L. racemosus*

and *L. mollis*. Unique SNP markers, absent in the genomes of the parents, were also identified in the genomes of 19 wheat-*Leymus* CALs. Polymorphic *L. racemosus* PCR markers were successfully transferred to other distant relatives of wheat, indicating their suitability for mapping alien chromatins from other wild relatives of wheat. A good number of *L. racemosus* markers were applied to characterize 22 wheat–*L. racemosus* chromosome introgression lines, while the PCR markers transferred to *L. mollis* genome, in combination with *L. mollis* genome-based SNP markers, aided selection and characterization of 10 new wheat–*L. mollis* CALs. Genomic *in situ* hybridization confirmed the presence of the alien chromosomes in the 10 CALs. This study has therefore demonstrated that wheat–*L. mollis* CALs can be speedily produced without completely relying on *in situ* hybridization for alien identification. DArTseq genotyping particularly aided identification of the homoeologous groups of all the *Leymus* chromosomes introgressed into wheat, and comparison of the chromosomes of *L. racemosus* and *L. mollis*. The similarity between the two species and the association of their chromosomes were applied to propose, for the first time, a nomenclature system for *Leymus* chromosomes.

Similarly, with DArTseq analysis, the genomes of 34 species in tribe Triticeae were clearly differentiated, and the phylogenetic relationships among the diploid and polyploid *Aegilops* and *Triticum* species were estimated. The SNP markers among *Aegilops* species enabled reliable reconstruction of their phylogeny: diploid species clustered according to known botanical sections, while the polyploid species formed two main clusters following the presence of two common diploid genomes, *Ae. tauschii* or *Ae. umbellulata*. Also, using species-specific SilicoDArT markers in diploid species as ‘analyzers’, the putative diploid progenitors of the polyploid species were elucidated.

While confirming the genomic constitutions of seven polyploid species of *Aegilops*, this analysis traced the so-called unidentified diploid progenitors of five polyploid *Aegilops* species to two genomically proximal diploid species, *Ae. speltoides* and *Ae. mutica*. The analysis also enhanced a satisfactory determination of the primary donors of A, B/G and D genomes in polyploid *Triticum* species and provided information that helped to clarify the complex and controversial nature of the B/G genomes of polyploid *Triticum* species. The findings suggest that the B/G genomes either evolved from the hybridization of *Ae. speltoides* and *Ae. searsii* or from a common ancestral species which later differentiated into the present day *Ae. speltoides* and *Ae. searsii*. On the other hand, the A and D genomes substantially matched the genomes of *T. urartu* and *Ae. tauschii*, respectively. However, the significant homology between the A genomes of the polyploid species and the genome of *T. boeoticum*, another A genome species, is an indication of the likelihood of common ancestry of the two A genome species. Therefore, like the B/G genomes, the A genomes of polyploid *Triticum* species may have arisen from an ancestral species that later differentiated into *T. urartu* and *T. boeoticum* after the evolution of the A genomes of the polyploid species. The A genomes of all the polyploid *Triticum* species were also proven to derive from the same primary A genome species, most likely *T. urartu*, invalidating earlier claims that the A genomes of polyploid wheats in Emmer and Timopheevi lineages were donated by different A genome species, *T. urartu* and *T. boeoticum*, respectively.

This study has shown the efficacy of applying genome-wide markers for speedy introgression breeding of wheat and analysis of evolutionary relationships among plant species. To improve speed and ease determination of homoeologous groups of alien chromosomes in similar studies, DArTseq genotyping is highly recommended.

摘　要

これまで、コムギ近縁遠縁野生種は、コムギ品種の収量と適応性向上のために、重要な役割を果たしてきた。しかし、これらはコムギ育種の潜在的な遺伝資源としての価値があるにも関わらず、これらの「ゲノム資源」は、ゲノムを科学し、それらの遺伝子を最大限利用するには不十分である。これは特に、第三次遺伝子プールとして分類される遠縁種において顕著である。現在のコムギの優良品種に脅威を与える生物的および非生物的ストレスを回避するために、野生の遺伝資源の積極的利用が必要であり、そのためにも野生種の「ゲノム資源」を絶えず高度化する必要がある。

ハマニンニク(*Leymus*)属に属する種は、塩性土壌および経済的に重要な病害に耐性であることが知られている。また、オオハマニンニク(*L. racemosus*)は、生物学的硝化抑制（BNI）活性を示すと報告されており、これは農業および環境に特に重要である。コムギの改良のためにこれらの遺伝子を適切に利用するためには、効果的な細胞学的および分子マーカーが必要であり、これらのマーカーによってコムギの遺伝的背景においてそれらの染色体部位を間違いなく位置付けることができる。本研究の前には、様々な細胞学的マーカーを用いて、コムギの遺伝的背景に存在するオオハマニンニクの分子細胞遺伝学的分析が可能であったが、コムギーオオハマニンニク転座および組換え系統の詳細な解析を行うための DNA マーカーは開発されていなかった。染色体の分子細胞学的特徴付けが可能になったが、添加系統の詳細な解析を行うための DNA マーカーは開発されていない。また、ハマニンニク(*L. mollis*)については、細胞学的マ

ーカーも DNA マーカーもないために、コムギーハマニンニク添加系統の開発が非常に遅れていた。これまで、オオハマニンニクの細胞学的マーカーをハマニンニクに適用する試みがなされたが、満足のいくシグナルが得られなかった。

一方で、コムギに近縁なエギロプス(*Aegilops*)属とコムギ(*Triticum*)属の種のゲノムと系統関係を解明することを目的として多量のデータがあるが、これらの種間のゲノム関係に関してはなお不明な点がある。そのため、これら豊富な遺伝子プールをコムギ育種のために開拓するための架け橋が必要である。本研究で扱っている未解明な課題としては、パンコムギを含む倍数性コムギ属ゲノムの B および G ゲノムの供与親に関する事や、*Ae. crassa*、*Ae. vavilovii*、*Ae. juvenalis*、*Ae. columnaris* および *Ae. triaristata* の進化において関与したと信じられている二倍性ゲノムに関する事などである。種間雑種の減数分裂での染色体対合に基づく、ゲノム分析は非常に有用な情報を与えたが、後に大きい批判を浴びることになり、これら種間の進化関係を明らかにするために分子手法が必要とされた。

従って、本研究は、①オオハマニンニクとハマニンニクのゲノムから DNA マーカーの開発・検証、②分子マーカーによるコムギーハマニンニク染色体添加系統開発法の提案と、DArTseq によるジェノタイピングによるその系統の異種染色体の解析、そして、③コムギ連のいくつかの種のゲノムの分析を実施するよう、設計された。

オオハマニンニクのゲノム配列情報および DArTseq ジェノタイピングを用

いて、数千の多型マーカーを、オオハマニンニクとハマニンニクのゲノムで、開発した。19 のコムギ－*Leymus* の染色体添加系統において、両親種のゲノムにおいて存在しない、ユニークな SNP マーカーを見いだされた。オオハマニンニクの PCR マーカーは、他のコムギ遠縁種にも適用され、これらがコムギの遺伝的背景に存在する遠縁種のクロマチンをマッピングするために有用であることが示された。オオハマニンニクのマーカーは 22 のコムギ－オオハマニンニク染色体導入系統を詳細に分析するために用いられた。また、ハマニンニクに適用された PCR マーカーとハマニンニクのゲノムの SNP マーカーは 10 種類のハマニンニク染色体添加コムギ系統の選抜と分析に利用された。GISH によって、この 10 種類の染色体添加系統において異種染色体が存在することを確認した。この研究は、*in situ* ハイブリダイゼーションによる異種染色体の確認することなく、染色体添加系統を作ることができることを示し、時間と努力を節約できることを示した。DArTseq によるジェノタイピングは特に、コムギに導入されたハマニンニク属の全染色体の同祖群の決定およびオオハマニンニクとハマニンニクのゲノムを比較するための助けとなった。両種間での類縁性とそれらの染色体の関連性によって、初めてハマニンニク属染色体の命名法を提案した。

同様にして、DArT 分析を用いて、コムギ連の 34 種のゲノムを区別し、2 倍性及び倍数性のエギロプス属・コムギ属の種の系統関係を明らかした。エギロプス属の SNP はこれらの系統関係を再構成することを可能した：2 倍性の種は既知の節に属し、倍数性種は、共通の 2 倍性ゲノム、つまり、*Ae. tauschii* と

Ae. umbellulata のゲノムの存在によって、2 つの群に分類された。また、2 倍性の種特異的 SilicoDArT マーカーを「アナライザー」として用いることによって、倍数性種のゲノムにおける仮想的な 2 倍性種を分析した。7 種の倍数性種のゲノム構成を確認する中で、5 種においてこれまで未同定であった 2 倍体親が、ゲノムレベルで似た 2 つの種、つまり *Ae. speltoides* と *Ae. mutica* に求めることができることを明らかにした。本研究では未同定ゲノムを T^s というゲノムで置き換え、5 種の倍数性種のゲノム構成を変更することを提案した。

この分析によって、倍数性コムギ属種の A、B/G、D ゲノムの供与親の決定にも満足のいく結果が得られ、倍数性コムギ属種における B/G ゲノムの複雑な構成を明らかにするための証拠に基づく情報を与えた。この結果は、B ゲノムは、*Ae. speltoides* と *Ae. searsii* の雑種に起源するか、その後 *Ae. speltoides* および *Ae. searsii* に分化する共通の祖先種の一つから進化したことを示した。一方、A および D ゲノムはそれぞれ、*T. urartu* および *Ae. tauschii* に実質的に一致した。しかし、倍数性種の B/G ゲノムと別の A ゲノム種である *T. boeoticum* のゲノム間に有意な相同性があり、2 つの A ゲノム種が共通の祖先であったとする説得力のある証拠を示唆している。従って、B/G ゲノムのように、倍数性コムギ種の A ゲノムは *T. urartu* と *T. boeoticum* が分化した前に、それらの共通の祖先から由来したと考えられる。全倍数性コムギ種の A ゲノムは、同じ A ゲノム種、おそらく *T. urartu* に由来することが明らかとなり、Emmer と Timopheevi コムギ系譜の倍数性コムギ種の A ゲノム異なる種、つまり、それぞれ *T. urartu* および *T. boeoticum* から来たとする、これまでの説を支持してい

ない。

この研究によってコムギの迅速な染色体導入育種のため、近縁野生種遺伝資源のゲノム基盤マーカーの利用が有効であることが明らかとなった。また、コムギの異種委染色体導入系統の分析およびその植物種の進化的関係を分析するために、**DArTseq** によるジェノタイピングが有効であることが証明された。

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LIST OF PUBLISHED PAPERS

First article: Results section (4.1 – 4.9)

Title: Efficient anchoring of alien chromosome segments introgressed into bread wheat by new *Leymus racemosus* genome-based markers

Authors: Offiong Ukpong Edet, Jun-Sik Kim, Masanori Okamoto, Kousuke Hanada, Tomoyuki Takeda, Masahiro Kishii, Yasir Serag Alnor Gorafi and Hisashi Tsujimoto

Journal: BMC Genetics (2018) 19: 18

Published online: March, 2018

Second article: Results section (4.10 – 4.15)

Title: Novel molecular marker-assisted strategy for production of wheat–*Leymus mollis* chromosome addition lines

Authors: Offiong Ukpong Edet, Yasir Serag Alnor Gorafi, Seon-woo Cho, Masahiro Kishii and Hisashi Tsujimoto

Journal: Scientific Reports (2018) 8: 16117

Published online: October, 2018

Third article: Results section (4.16 – 4.19)

Title: DArTseq-based analysis of genomic relationships among species of tribe Triticeae

Authors: Offiong Ukpong Edet, Yasir Serag Alnor Gorafi, Shuhei Nasuda and Hisashi Tsujimoto

Journal: Scientific Reports (2018) 8: 16397

Published online: November, 2018