Biochemical Systematics and Ecology 57 (2014) 420–426

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Phylogenetic relationships and Y genome origin in Chinese *Elymus* (Triticeae: Poaceae) based on single copy gene *DMC1*

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ARTICLE INFO

Article history:
Received 4 August 2014
Accepted 28 September 2014
Available online 17 October 2014

Keywords:
Elymus
DMC1
Phylogeny
Polyploid

ABSTRACT

To investigate the phylogenetic relationships among Chinese *Elymus* and related diploid genera, the genome donor of *Elymus*, and the evolutionary history of polyploid *Elymus* species, disrupted meiotic cDNA1 (*DMC1*) sequences were analyzed for 10 *Elymus* species, together with 34 diploid taxa from 13 monogenomic genera. The phylogenetic analyses (Neighbor-Joining) supported three major clades (St, Y and H). Sequence diversity and genealogical analysis suggested that (1) *Elymus* species are unambiguously closely related to *Pseudoroegeria* and *Hordeum*. *Pseudoroegeria* and *Hordeum* might be serve as the St genome and H genome donor of polyploid *Elymus* species; (2) Phylogenetic analyses separated the Y sequences from the St sequences, it confirmed that St and Y genome in *Elymus* species have originated from different donors; (3) the St genome of *Elymus* had several origins and diverse species of *Pseudoroegneria* might have taken part in the formation of polyploid species of *Elymus*; (4) the *DMC1* sequences of *Elymus* are evolutionarily distinct, and it can clarify parental lineages and phylogenetic relationships of genera *Elymus*.

1. Introduction

Polyploidization is a major mechanism in plant evolution and speciation (Soltis et al., 2003; Otto, 2007). Recent studies using genetic markers in many genera suggested that multiple origins (including independent origin) of polyploid species are the rule rather than the exception (Soltis and Soltis, 2000; Symonds et al., 2010; Fan et al., 2012). Polyploidization and chromosome doubling can stimulate changes in genome size, cell size, genomic repatterning, gene expression, retrotransposon activation and epigenetic effect (Soltis et al., 2003; Otto, 2007). These changes may result in full fertility and stabilization of the hybrid condition and assist in establishing the phenotype in nature, which allows polyploids to adapt to new ecological niches or to be competitively superior to the parental donor (Otto, 2007; Fan et al., 2009; Yan and Sun, 2012). However, a clear and appropriate identification of phylogenetic relationships among taxa and genes, as well as genomic elements is needed (Yan et al., 2011).

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http://dx.doi.org/10.1016/j.bse.2014.09.019
0305-1978/

The wheat tribe (Poaceae: Triticeae), an important gene pool for genetic improvement of cereal crops, includes many autopolyploid and allopolyploid taxa (Liu et al., 2006). Data from extensive cytogenetic analyses have been used to illustrate systematic relationships of the tribe and to clarify the ancestry of many polyploidy species. One complex group of polyploids within Triticeae is the genus *Elymus*, following the taxonomic delimitation by Löve (1984) based essentially on genomic constitutions, includes approx 150 perennial species distributed in a wide range of ecological habitats over the temperate and subtropic regions. In Flora of China (Chinese version), *Elymus* included 12 species and 1 subspecies which widely distributed in north of China (Guo et al., 1987). *Elymus* has its origin through a typical allopolylpoidy process (Dewey, 1984; Löve, 1984). Cytological studies suggest that five basic genomes, namely, the St, Y, H, P and W in various combinations constitute *Elymus* species (Lu, 1994). The St genome is a fundamental genome that exists in all *Elymus* species and is donated by the genus *Pseudoroegneria* (Dewey, 1967). The H, P and W genomes are derived from the genera *Hordeum*, *Agropyron* and *Australopyrum* of Triticeae, respectively (Dewey, 1971; Jensen, 1990; Torabinejad and Mueller, 1993). However, the donor of the Y genome that is present in the majority of the Asiatic *Elymus* species has not yet been identified, although extensive investigations have been carried out (Lu, 1994).

Molecular phylogenetic studies have successfully revealed the origins and evolutionary history of polyploids in plants, clarified the nature of different polyploids, and identified their parental lineages and the hybridization events involved in their formation (Soltis and Soltis, 1993; Wendel, 2000; Soltis et al., 2003). Comparative phylogenies between nuclear and chloroplast/mitochondrial sequences have become a powerful tool to identify the mode of polyploidization in particular groups (Mason-Gamer, 2001; Liu et al., 2006; Fan et al., 2013). Among the available nuclear sequences, single copy gene *DMC1* (disrupted meiotic cDNA) sequences have been used successfully in studying phylogenetic and genomic relationships of wheat tribe plants (Gitte and Ole, 2000; Sha et al., 2010; Sun and Sun, 2012).

In this study, we sequenced and analysed the *DMC1* fragments for 10 Chinese *Elymus* polyploids and their putative diploid donors to explore the origin and relationships of the polyploid *Elymus* species. The objectives of this study were (1) to identify the possible origin of the genome, especially the Y genome; (2) to elucidate the phylogenetic relationships of the *Elymus* with related diploid genera.

2. Materials and methods

2.1. Plant material

Forty four species were sampled, including 10 *Elymus* species, 34 diploid species from 14 monogenomic genera in Triticeae and *Bromus sterilis* was used as the outgroup. The taxa names, accession numbers, ploidy level, genome constitution and GenBank accession numbers are listed in Table 1. All seed materials with PI were kindly provided by American National Plant Germplasm System (Pullman, Washington, USA) and the Triticeae Research Institute of Sichuan Agriculture University. The plants and voucher specimens of all the materials have been deposited at the perennial nursery and Herbarium of the Triticeae Research Institute, Sichuan Agriculture University, China (SAUTI).

2.2. DNA amplification and sequencing

The total genomic DNA was extracted and purified from fresh leaf tissue of each accession followed a standard CTAB (cetyltrimethylammonium bromide) procedure (Doyle and Doyle, 1990). The *DMC1* gene was amplified with the universal primers: *DMC1*F (5′-TGCCAATTGCTGAGAGATTTG-3′) and *DMC1*R (5′-AGCCACCTGTTGTAATCTGG-3′). The PCR (Polymerase Chain Reaction) were conducted in 50 μL reaction volume, containing 2.0 μL template DNA at the concentration of 20 ng/μL, 25.0 μL 2 × Taq PCR MasterMiX (4 mmol/L $MgCl_2$, 0.4 mmol/L dNTPs of each nucleotide, 0.05 units/μL Taq DNA polymerase), 0.01 mmol/L primer1.5 μL and with an addition of ddH_2O to the final volume. The PCR amplification protocols were performed with an initial denaturing step at 94 °C for 4 min, followed by 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at 52 °C, 1 min extension at 72 °C, and a final extension step at 72 °C for 10 min on BIO-RAD S1000™ Thermal cycler. PCR products were cloned into the pMD19-T vector according to the manufacturer's instruction (TaKaRa, Dalian, China). For each of the *Elymus* species, 8 cloned PCR products were sequenced to include all the possible *DMC1* sequences from the donor species. Sequencing was conducted by BGI Company (Peking, China).

2.3. Data analysis

DNA sequences were confirmed through BLAST nucleotide alignment on NCBI database. Multiple sequences were aligned using the Clustal W algorithm, followed by manual adjustment by the software MegAlign (DNA Star Inc., USA) (Thompson et al., 1994). Following The homogeneity of the base composition with the Id-test, nucleotide substitutions, transition/transversion ratio, and variability in different regions of the sequences were calculated with MEGA software, version 5.0 (Tamura et al., 2011).

To assess the divergence and relationships among polyploids and its diploid progenitors, nucleotide diversity based on the average number of pairwise comparisons in a sample was estimated using haplotype diversity by Hd (Nei and Li, 1979), Tajima's π (Tajima, 1989) and Watterson's θ_w (Watterson, 1975). Testing of neutrality was also performed by Tajima's and Fu and Li's D statistic (Tajima, 1989; Fu and Li, 1993). Significance of D-values was estimated with the simulated distribution of

Table 1
Eymus species and closely related species used in this study.

Species	Accession No.	Genome	Origin	GenBank No.
Elymus atratus (Nevski) Hand.-Mazz.	ZY3023	StYH	Sichuan,China	KJ746762
				KJ746763
				KJ746764
Elymus breviaristatus Keng ex P. C. Keng	Y3063	StYH	Sichuan,China	KJ746765
				KJ746766
				KJ746767
Elymus canadensis L.	PI499412	StH	Neimenggu, China	KJ746768
				KJ746769
Elymus cyclindricus (Franch.) Honda	Y0552	StYH	Xinjiang, China	KJ746770
				KJ746771
				KJ746772
Elymus dahuricus Trucz.	per97	StYH	Sichuan, China	KJ746773
				KJ746774
				KJ746775
Elymus excelsus Trucz.	ZY11034	StYH	Neimenggu, China	KJ746776
				KJ746777
				KJ746778
Elymus nutans Griseb.	ZY3060	StYH	Gansu, China	KJ746779
				KJ746780
Elymus purpuraristatus C. P. Wang et H. L. Yang	ZY11039	StYH	Neimenggu, China	KJ746781
				KJ746782
				KJ746783
Elymus sibiricus L.	PI619579	StH	Xinjiang, China	EU366409*
				GQ855198*
Elymus tangutorum (Nevski) Hand.-Mazz.	ZY2008	StYH	Tibet, China	FJ695170*
				FJ695171*
Aegilops bicornis Jaub. & Spach.	H6602	S^b		DQ247822*
Aegilops longissima Schweinf. & Muschi.	H6679	S^l		DQ247830*
Aegilops searsii Feldman & Kislev ex Hammer	H6605	S^S		DQ247823*
Aegilops sharonensis Eig.	H6680	S^{Sh}		DQ247831*
Aegilops speltoides Tausch	H6779	B		DQ247833*
Aegilops tauschii Coss.	H6668	D		AF277235*
Agropyron cristatus (L.) Gaertn	H4349	P		AF277241*
Australopyrum retrofractum (Vickery) Á. Löve	H6723	W		AF277251*
Eremopyrum villosum (K. Koch) Nevski	H5552	Q		AF277236*
Eremopyrum tririceum (Gaertn.) Nevski	H5553	Q		AF277237*
Hordeum arizonicum Covas.		H		GU734676*
Hordeum brachyantherum Nevski		H		GU734678*
Hordeum tetraplodum Covas.		H		GU734680*
Hordeum depressum (Scribn. & J.G. Sm.) Rydb.		H		GU734670*
Hordeum chilense Roem. & Schult.	PI531781	H	Chile	FJ695173*
Hordeum stenostachys Godr.	H1783	H		AY137407*
Hordeum bogdanii Wilensky	PI531761	H	China	FJ695172*
Hordeum jubatum L.		H		GU734672*
Lophopyrum elongatum (Host) A. Löve	H66692	E^e		AF277246*
Peridictyon sanctum (Boiss.) Nevski	H5575	Xp		AF277244*
Psathyrostachys huashanica Keng ex P.C.Kuo	PI531823	Ns	Shaanxi, China	GU165826*
Psathyrostachys juncea (Fisch.) Nevski	PI314521	Ns	Former Soviet Union	EU366427*
Psathyrostachys fragilis (Boiss.) Nevski	PI243190	Ns	Iran	EU366426*
Psathyrostachys fragilis ssp. *villosus* Baden		Ns		AF277263*
Psathyrostachys lanuginose (Trin.) Nevski	Y1567	Ns	Xinjiang, China	GU165827*
Psathyrostachys stoloniformis Baden	H9182	Ns	Gansu, China	AF277264*
Pseudoroegneria libanotica (Hackel) D. R. Dewey	PI228389	St	Iran	FJ695174*
Pseudoroegneria spicata (Pursh) Á. Löve	PI547161	St	United States	FJ695175*
Pseudoroegneria stipifolia (Czern. ex Nevski) Á. Löve	PI325181	St	Stavropol, Russian	FJ695176*
Pseudoroegneria strigosa (M. Bieb.) Á. Löve	PI595164	St	Xinjiang, China	FJ695177*
Secale strictum L.	H4342	R		AF277248
Taeniatherum cop-medusae (L.) Nevski	H10254	Ta		AF277249*
Thinopyrum bessarabicum (Savul. & Rayss) A. Löve	H6725	E^b		AF277254*
Triticum monococcum L.	H4547	A^m		AF277250*
Triticum urartum Tum.	H6664	A		DQ247826*
Bromus sterilis L.	OSA420			AF277234*

Note: *Data from published sequences in the GenBank (http://www.ncbi.nlm.nih.gov).

random samples (1000 steps) using a coalescence algorithm assuming neutrality and population equilibrium (Hudson, 1990). These parameters were implemented by DnaSP version 5.10 (Rozas et al., 2003).

The arrays of phylogenetic reconstruction and molecular evolutionary analyses were performed by the Neighbor-Joining (NJ) approach based on the Maximum Composite Likelihood model, using MEGA version 5.0 (Tamura et al., 2011). Topological robustness NJ analysis was assessed by bootstrap analysis with 1000 replicates.

Table 2
Features of the matched data matrix.

	Variable characters	Conserved characters	Informative characters	Identical pairs	Transitionsal pairs	Transversional pairs
DMC1	401	712	189	944	24	14

3. Results

3.1. DMC1 sequences analysis

The length of the of 61 *DMC1* sequences varied from 982 bp to 1061 bp. The data matrix contains 1203 characters. The average of G + C content was 44.78%. Of 401 variable site, 189 were parsimoniously informative (Table 2). Estimates of nucleotide diversity of *DMC1* sequences including the total number of sites (n), the number of polymorphic sites (s), Haplotype diversity (Hd), the average pairwise diversity (π) and the diversity based on the number of segregating sites (θ_w). Neutrality tests such as Tajima's, Fu and Li's D gave negative values for all accessions were -1.70783 ($P < 0.05$) and -2.35677 ($P < 0.05$) respectively (Table 3).

3.2. Phylogenetic analyses

To reveal the phylogenetic relationship among *Elymus* and related diploid *Triticeae* species, all the 61 accessions were implemented by NJ phylogenetic reconstruction based on *DMC1* sequences. NJ analysis of the *DMC1* data yielded a strict consensus phylogenetic tree (-Log likelihood = 3696.801), with the following estimated NJ parameters: the assumed nucleotide frequencies A: 0.2470, C: 0.2274, G: 0.2204, T: 0.3052. The NJ phylogenetic tree was supported by the bootstrap test ($\geq$50%) and bootstrap support (BS) above the branches (Fig. 1). The NJ tree was generally resolved, all the accessions of *Elymus* and their diploid donors were formed three clades (65% BS value), which corresponding to the three genomic types St, Y and H. The H clade consisted of 13 accessions of *Elymus* and 7 accessions of *Hordeum* species. Meanwhile, The St clade were comprised of 5 accessions of *Elymus* and 4 accessions of *Pseudoroegneria* species and the Y clade were comprised 8 accessions of *Elymus*.

4. Discussion

4.1. Nucleotide diversity

The present estimates of nucleotide diversity of Hd, π and θ_w for detecting *Elymus* species and its closely related diploid Triticeae species revealed a high level diversity of *DMC1* sequence in the *Elymus* species. These estimates indicating that the single copy gene *DMC1* sequence has a high evolutionary rate and would therefore provide potentially useful phylogenetic analysis in the *Elymus* species. The Tajima's D, Fu and Li's D of the *Elymus* species showed dramaticlly negative estimates, suggesting that the variations departured from neutrality, and the *Elymus* species might be affected by selective elimination or suffered from a genetic bottleneck created by polyploidization.

4.2. Phylogenetic relationships of Elymus with its proposed diploid ancestors and possible origin of the Y genome

The genus *Elymus* consists of polyploids that are widely distributed over different continents and includes a large number of endemic species. Only a few molecular studies addressing phylogenetic relationships of the StH and StYH genome *Elymus* species are reported. Little is known about phylogeny of the Chinese *Elymus* species at molecular level. Analyses of *DMC1* sequences collected from a wide range of polyploidy *Elymus* species and their related genera will provide opportunities for understanding their phylogenetic relationships, ancestral donors and polyploidization events in the speciation processes.

In the diploid and polyploid *DMC1* tree, St, H, Ns, P, Q, A and Y genomes presented formed distinct clades. There was obvious Y genome clade. These results indicate that *DMC1* sequences of all the genomes derived from the diploid ancestor have remained clearly differentiated in the polyploidy *Elymus*. This can be reflected by the fact that all *Elymus* species contained two or three distinct types of *DMC1* sequences, with one type in the St clade and the others in H clade or Y clade. This strongly suggests that *DMC1* sequences in different *Elymus* species showed a clear linkage with those in their diploids ancestors. This is illustrated by the fact that the StH and StYH genome *Elymus* species were simultaneously clustered in both of their ancestral group, indicating that three distinct types of *DMC1* sequences exist in these polyplois *Elymus*. This provides

Table 3
Estimates of nucleotide diversity and test statistics for *DMC1* data sets.

	n	s	π	Hd	θ_w	Fu & Li's D	Tajima's D
DMC1	804	235	0.03576	0.995	0.05903	-2.35677 ($P < 0.05$)	-1.70783 ($P < 0.05$)

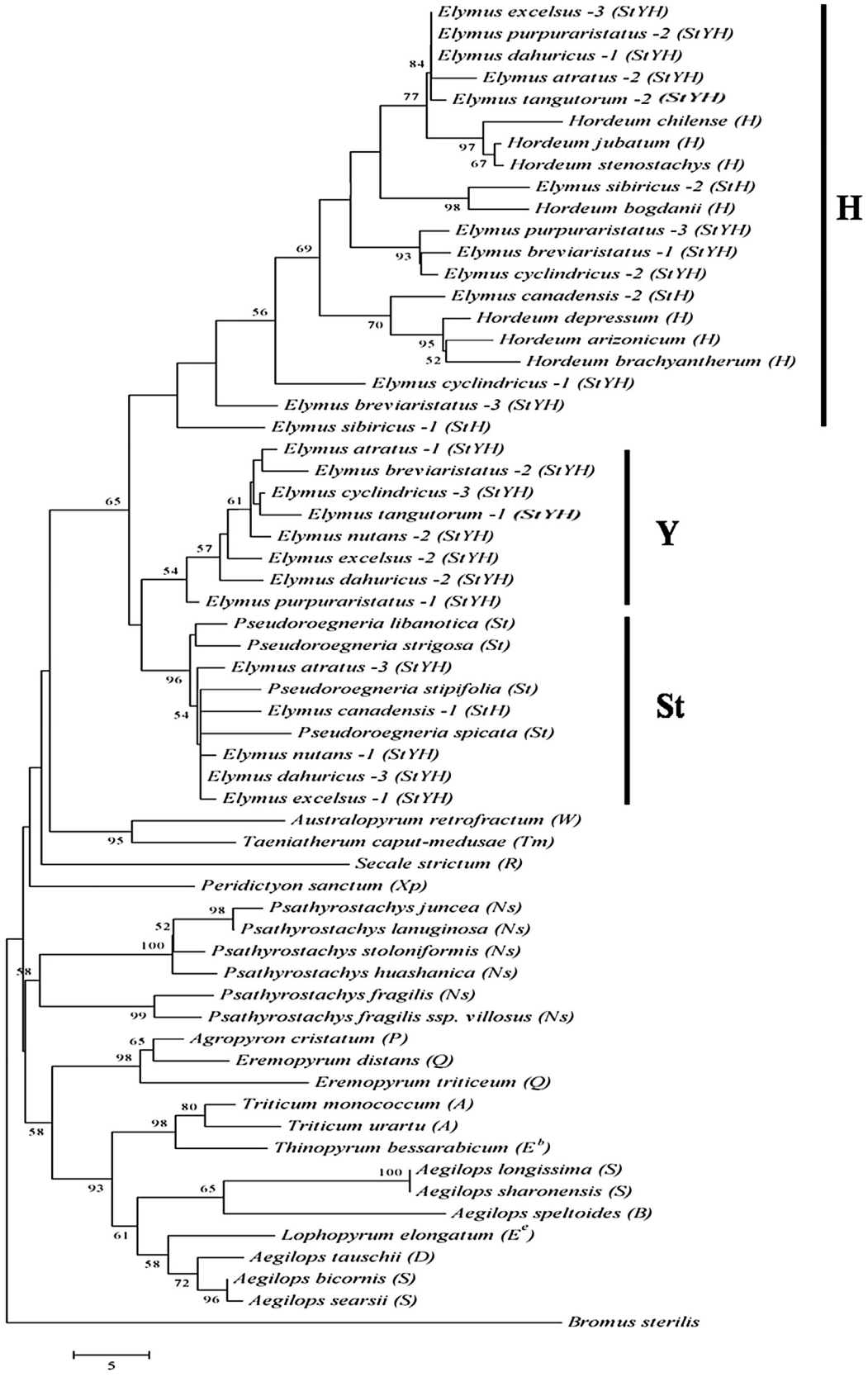

Fig. 1. Neighbor-Joining (NJ) tree of *Elymus* and its related diploid species inferred from the *DMC1* sequences. Numbers at nodes indicate bootstrap values ≥50%. Bar at the left bottom indicates scale value.

strong evidence that the polyploidy *Elymus* species are derived from polyploidization through hybridization between different ancestral genera, as indicated by cytological analyses (Dewey, 1967; Lu, 1994).

The phylogenetic analyses of *Elymus* and as many as 34 accessions from 13 diploid genera, in the present study, provide support for the distinct origin of the Y genome in polyploid StYH species. *DMC1* phylogenetic tree have well separated the Y genome from the St genome. These results are in accordance with the previous findings by Sun et al. (2008) and Mason-Gamer et al. (2010), and support Dewey's hypothesis that there is a Y diploid species from which the Y genome originated. The data do not support the idea that the St and Y genomes have the same origin, which was based on ITS data by Liu et al. (2006). Okito et al. (2009) suggested that *Pse. spicata* might be the donor of the Y genome and a prime candidate for the origin of the Y genome to *E. longearistatus* (StY). In our study, the *Pse. spicata* was included but *DMC1* phylogenetic tree placed this accession in the St genome together with other *Pseudoroegneria* species. This indicates that there is not a close link between *Pse. spicata* and the Y genome. Since the Y genome grouped with the St genome sequences in *DMC1* trees, it implies that the St genome is closely related to the Y genome.

4.3. Differentiation of St genome in polyploid Elymus species

The St genome present in all species of *Elymus*, and is an important genome for this genus. The St genome donor genus is *Pseudoroegneria* which contains approximately 15 diploid (StSt) or tetraploid (StStStSt, or $St_1St_1St_2St_2$ or StStPP) species distributed in the Middle East, central Asia, northern China and western North America (Löve, 1984; Wang et al., 1986). Cytological data suggested that genome differentiation exists among the *Pseudoroegneria* species (Wang et al., 1986). Mason-Gamer et al. (2002) have demonstrated that *Pseudoroegneria* may be paraphyletic. Phylogenetic analysis of *DMC1*sequences grouped the St copy of *Elymus* with *Pseudoroegneria* species together with bootstrap support (96%), suggesting a high degree of similarity among the St genome in *Pseudoroegneria* species and the St genome in *Elymus* species. The tree suggests at least two phylogenetically distinct St genome donors to the *Elymus* species. The St genome in *E. atratus* and *Elymus canadensis* originated from *Pse. stipifolia*, while the St genome in *E. nutans*, *E. dahuricus* and *E. excelsus* more likely originated from *Pse. spicata*. The phylogenetic tree clearly shows St genome differentiation in *Kengyilia* species.

4.4. Application of DMC1 in the phylogeny of Elymus

The most widely used source of data in plant molecular phylogenetic analyses has been cpDNA. As in other plant species, cpDNA have been used to study the phylogeny of *Elymus* species (Liu et al., 2006; Yan and Sun, 2012). Recent studies indicated that low-copy and single copy genes are less frequently subject to concerted evolution, thus making them ideal candidates for identifying parental donors of polyploids (Fan et al., 2009, 2012; Yan and Sun, 2012). We examined the utility of *DMC1* for the phylogenetic studies in *Elymus*. Highly nucleotide variation was found in the amplified region. The 189 parsimony informative characters were obtained for matrix, respectively. The number of phylogenetically informative characters obtained by sequencing approximately 1000 bp of *DMC1* sequences is rather economic character sampling in comparison to the sequencing effort in comparable studies of *Elymus*, indicating that *DMC1* is a good marker for studying the phylogeny of *Elymus*.

The *Elymus* species originated from two or three ancestral genomes. In the study of evolutionary events of genome in the polyploid species and its diploid donor species always encounter the difficulty in distinguishing the orthologous (or homoeologous) copy of the gene in ploypoid species. Given that *DMC1* gene reported here can generate genome-specific amplicons, the availability of genome-specific amplicons together with sequence analysis will provide an excellent opportunity and impetus to investigate the evolutionary dynamics of speciation and the mode of polyploidy formation in *Elymus* species.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31270243). We would like to specially thank the American National Plant Germplasm System for providing some of the seeds.

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