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Nucleotide polymorphism pattern and multiple maternal origin in *Thinopyrum intermedium* inferred by *trn*H-*psb*A sequences

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Abstract

Thinopyrum intermedium is an important species with potential utilization value in breeding of wheat. In this study, the non-coding intergenic region of *trn*H-*psb*A was investigated to assess the genetic diversity and infer the maternal origin within *T. intermedium* accessions. Eleven haplotypes were distinguished among the thirty-five accessions of *T. intermedium*. They showed a relatively low nucleotide diversity (π) of 0.00473 ± 0.00037 and a moderately high haplotype diversity (H_d) of 0.733 ± 0.061. In the phylogenetic analysis, all accessions of *T. intermedium* were positioned into two clades, which corresponded to the different diploid donors. These results suggested that there were two phylogenetically divergent maternal donors in *T. intermedium*.

Additional key words: haplotypes, maternal donor, nucleotide diversity.

Introduction

Genetic diversity is essential to the long-term survival of any species, especially in environments which are subject to climate changes or the introduction of new pests, pathogens or competitors (Rajora and Mosseler 2001). The ongoing genetic advancement of crops is dependent on a continuing supply of genetic variability. The tribe *Triticeae* in *Poaceae* offers a vast gene pool from which traits of agronomic interest can be extracted to enrich the genetic diversity of wheat for disease resistance and quality improvement (Maxted *et al.* 2008, Mirzaghaderi *et al.* 2010). *Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey [syn. *Elytrigia intermedium* (Host) Nevski, *Agropyron intermedium* (Host) Beauvoir] is a perennial allohexaploid species in *Triticeae*. Although its exact genomic constitution has not been settled, *T. intermedium* is thought to compose of St from *Pseudoroegneria* (Nevski) A. Löve), E (=E^e) and J (=E^b) genomes from *Lophoyrum elongatum* (Host) D.R. Dewey and *Thinopyrum bessarabicum* (Savul. & Rayss) A. Löve (Liu and Wang 1993, Wang *et al.* 1994, Xu and Conner 1994). The native range of this species extends from central and southeastern Europe to Asia (Barkworth and Dewey 1985). *T. intermedium* has not only ecological value from heavy root production that holds the soil in place and restores its natural fertility (Alderson and Sharp 1994) but also significant grain production with nutritional qualities similar to wheat (Cox *et al.* 2002). Therefore, it has been utilized in wheat breeding programs. So far, the disease resistance genes *Lr38*, *Sr43*, *Sr44*, *Pm40*, *Pm43*, *Bdv2*, *Bdv3* and *Wsm1* from *T. intermedium* have been transferred into wheat (Li and Wang 2009). Previously, the studies of *T. intermedium* were focused on the genomic constitution (Liu and Wang 1993, Xu and Conner 1994), the attempt of alien gene transfer for wheat improvement (Fedak and Han 2005, Xu *et al.* 2009), and the mapping of resistant gene from *T. intermedium* in derivatives of wheat (He *et al.* 2009). However, the genetic diversity and the plastid donor of *T. intermedium* remained unclear and needed to be studied.

Genetic markers have contributed to the study of plant evolutionary ecology by providing methods to detect genetic variability among individuals or populations

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Abbreviations: CTAB - cetyltrimethylammonium bromide; PCR - polymerase chain reaction; bp - base pair.
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(Cruzan 1998). A number of techniques have been developed to investigate genetic diversity, *e.g.*, random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and inter-simple sequence repeats (ISSR) markers (Zietkiewicz *et al*. 1994, Vos *et al*. 1995, Sikdar *et al.* 2010, Sciacca *et al*. 2010). With the development of sequencing technology, DNA sequence variation provides a further method to assess genetic diversity (Morrell *et al*. 2003). In plants, chloroplast genomes are thought to evolve more slowly relative to the nucleus genome, but faster than mitochondria genome. It has a fast mutation rate and a low recombination rate (Clegg and Zurawski 1992), which represents a significant resource for investigating molecular diversity, genetic relatedness and phylogenetic relationships in plants (Redinbaugh *et al*. 2000, Mason-Gamer *et al*. 2002). The intergenic region of *trn*H-*psb*A is among the most variable regions in the angiosperm chloroplast genome. This region contains two parts differing in their evolutionary conservation, the *psb*A 3'UTR and the *trn*H-*psb*A intergenic non-transcribed spacer. It is a valuable instrument for plant population genetics and phylogenetic (Shaw *et al*. 2005, Ford *et al*. 2007, Štorchová and Olsom 2007). In the study, the objectives were to quantify nucleotide diversity at the intergenic region of *trn*H-*psb*A in *T. intermedium* and discuss the possible maternal origin of *T. intermedium*.

Materials and methods

Thirty-five accessions of *T. intermedium* were used in this study. In order to infer the possible maternal origin of *T. intermedium*, ten accessions of diploid *Lophopyrum elongatum* (E^e), two accessions of *Thinopyrum bessarabicum* (E^b) and three diploid *Pseudoroegneria* species (*P. strigosa*, *P. spicata* and *P. libanotica*, St) were also included in this study (Table 1). *Bromus inermis* L. was used as outgroup. All the accessions were kindly provided by American National Plant Germplasm System (Pullmam, Washington, USA) and deposited at the perennial garden and herbarium of Triticeae Research Institute, Sichuan Agricultural University, China.

DNA was extracted from young leaf tissue of each accession using a modified CTAB protocol (Doyle and Doyle 1987). The *trn*H-*psb*A sequences were amplified by primers as described by Shaw *et al*. (2005). The amplification was conducted in a 0.05 cm^3 reaction volume including 0.005 cm^3 10× PCR buffer, 2 mM $MgCl_2$, 0.25 mM each of dNTPs, 1.5 U Taq DNA polymerase, 20 μM of each primer and 20 ~ 50 ng template DNA. Cycling parameters were 94 ºC for 4 min, then 30 cycles of 94 ºC for 1 min, 55 ºC for 30 s, 72 ºC for 1.5 min, and a final extension at 72 ºC for 7 min. The PCR products were purified and cloned into the *pMD19-T* vector (*TaKaRa*, Dalian, China) according to the manufacture's protocol. At least three random clones for each accession were chosen to sequence. All clones were sequenced in both directions in *Beijing BGI Company,* Beijing, China.

All the sequences from *T. intermedium* and the closely related diploid taxa were aligned by using *Clustal X* (Thompson *et al*. 1999) with manual rectification. Nucleotide polymorphosim was measured by θ_w (Watterson *et al*. 1975), diversity by π and haplotype diversity by H_d (Nei and Li 1979). Values for the polymorphic sites and the mean numbers of pair-wise differences among sequences were also estimated. The locus of *trn*H-*psb*A was tested for departure from neutrality by D (Tajima 1989) and D* and F* (Fu and Li 1993) statistics. All the above estimations were performed by using the software *DnaSP v. 5.10* (Rozas *et al*. 2003).

Phylogenetic analysis was conducted in *PAUP v. 4.0 beta 10 Win* (Swofford 2003). All the characters were equally weighted and the gaps were treated as missing data. For maximum parsimony (MP), heuristic searches were carried out with the following options: heuristic search mode with 100 random additional sequence replicates, tree-bisection-reconnection (TBR) branch swapping, ACCTRAN optimization and MULTREES option on for the data set. The MP trees were combined to generate the 50 % majority-rule tree. In complement to parsimony analysis, the Neighbor-Joining (NJ) method was used for phylogenetic reconstruction as implemented by the software of *MEGA v. 4.0.2* (Tamura *et al*. 2007) based on the Kimura two-parameter genetic distance (Kimura 1980). Topological robustness analysis was assessed by bootstrap analysis with 1000 replicates.

Results

The length of *trn*H-*psb*A sequences in 35 *T. intermedium* individuals was 549 bp after alignment. There were 14 variable nucleotide sites (seven transitions and seven transversions sites) and no insertion or deletions in any of sequences. The average nucleotide composition for all individuals was the highest for A (0.358), followed by T (0.289), G (0.178) and C (0.173). The small inversion was observed at sites 482 - 487 in all accessions of *T. intermedium* and the potential diploid donors. The features of the reversion were a central region of 6 nt (TTCTAG), which could mutate to its reverse complement sequence (CTAGAA), and two flanking sequences of 26 nt. The two flanking sequences could be reversely complemented to each other.

Eleven different haplotypes were detected in 35 *T. intermedium* samples analyzed. The most common

Table 1. Plant materials used in this study.

Genus	Species	Accession No.	Genomic constitution	Origin	GenBank No.
Bromus	*B. inermis*	PI618974	--	Xinjiang, China	HQ221767
Lophopyrum	*L. elongatum*	PI179162	E^e	Turkey	HQ221809
	L. elongatum	PI238222	E^e	Belgium	HQ221812
	L. elongatum	PI547326	E^e	France	HQ221810
	L. elongatum	PI578680	E^e	Colorado, USA	HQ221811
	L. elongatum	PI578682	E^e	Nebraska, USA	HQ221807
	L. elongatum	PI578683	E^e	Nebraska, USA	HQ221815
	L. elongatum	PI578684	E^e	Nebraska, USA	HQ221808
	L. elongatum	PI578686	E^e	Saskatchewan, Canada	HQ221817
	L. elongatum	PI595139	E^e	Xinjiang, China	HQ221816
	L. elongatum	W621859	E^e	Krym, Ukraine	HQ221814
Thinopyrum	*T. bessarabicum*	W610232	E^b	Russian Federation	HQ221789
	T. bessarabicum	W621890	E^b	Ukraine	HQ221813
	T. intermedium	PI172688	E^eE^bSt	Turkey	HQ221797
	T. intermedium	PI210992	E^eE^bSt	Afghanistan	HQ221792
	T. intermedium	PI249145	E^eE^bSt	Portugal	HQ221796
	T. intermedium	PI286118	E^eE^bSt	Denmark	HQ221804
	T. intermedium	PI317406	E^eE^bSt	Afghanistan	HQ221779
	T. intermedium	PI401135	E^eE^bSt	Iran	HQ221781
	T. intermedium	PI401171	E^eE^bSt	Iran	HQ221775
	T. intermedium	PI494618	E^eE^bSt	Romania	HQ221772
	T. intermedium	PI494686	E^eE^bSt	Romania	HQ221803
	T. intermedium	PI531723	E^eE^bSt	Canada	HQ221778
	T. intermedium	PI531725	E^eE^bSt	Germany	HQ221786
	T. intermedium	PI531726	E^eE^bSt	Australia	HQ221788
	T. intermedium	PI547319	E^eE^bSt	Russian Federation	HQ221782
	T. intermedium	PI547332	E^eE^bSt	Kars, Turkey	HQ221801
	T. intermedium	PI547333	E^eE^bSt	China	HQ221795
	T. intermedium	PI547334	E^eE^bSt	Poland	HQ221783
	T. intermedium	PI547335	E^eE^bSt	Poland	HQ221793
	T. intermedium	PI547336	E^eE^bSt	France	HQ221787
	T. intermedium	PI547337	E^eE^bSt	France	HQ221774
	T. intermedium	PI547338	E^eE^bSt	France	HQ221780
	T. intermedium	PI556987	E^eE^bSt	North Dakota, USA	HQ221800
	T. intermedium	PI574517	E^eE^bSt	South Dakota, USA	HQ221802
	T. intermedium	PI574518	E^eE^bSt	South Carolina, USA	HQ221799
	T. intermedium	PI578688	E^eE^bSt	Missouri, USA	HQ221805
	T. intermedium	PI578690	E^eE^bSt	New Mexico, USA	HQ221784
	T. intermedium	PI578692	E^eE^bSt	Idaho, USA	HQ221798
	T. intermedium	PI598480	E^eE^bSt	Moldova	HQ221806
	T. intermedium	PI598738	E^eE^bSt	Russian Federation	HQ221794
	T. intermedium	PI619581	E^eE^bSt	Russian Federation	HQ221790
	T. intermedium	PI109219	E^eE^bSt	Columbia, USA	HQ221771
	T. intermedium	PI249146	E^eE^bSt	Portugal	HQ221777
	T. intermedium	PI401228	E^eE^bSt	Iran	HQ221773
	T. intermedium	PI401237	E^eE^bSt	Iran	HQ221785
	T. intermedium	PI440053	E^eE^bSt	Kazakhstan	HQ221776
	T. intermedium	PI469214	E^eE^bSt	Maryland, USA	HQ221791
Pseudoroegneria	*P. strigosa*	PI499493	St	Xinjiang, China	HQ221768
	P. spicata	PI232131	St	Nevada, USA	HQ221770
	P. libanotica	PI228392	St	Iran	HQ221769

haplotypes found in 16 samples occupied 45.71 % of all the samples. The following common haplotype was detected in 28.57 % of all the samples. The other nine haplotypes were restricted to a single sample, respectively. The haplotype frequency distribution was strongly skewed, with the vast majority of haplotypes found only once. The polymorphic sites were 14 and the average number of pair-wise differences among sequences was 2.6151. Molecular diversity indices (mean ± SD) were calculated and the values of θ_w and π were 0.00615 ± 0.00028 and 0.00473 ± 0.00037, respectively. The value of H_d was 0.733 ± 0.061. These results showed relatively low nucleotide diversity and a moderately high haplotype diversity in the *T. intermedium* accessions studied here.

Tajima's D, Fu and Li's D* and F* tests were used to detect the departure from neutrality at the *trn*H-*ps*bA locus. The value of D was -0.74666 ($P > 0.1$). D* gave a similar trend as F*. Significant D* and F* values were detected at this locus, which were -2.83929 ($P < 0.05$) and -2.54988 ($P < 0.05$).

To reveal the possible maternal donor origin of *T. intermedium*, all the 35 accessions of *T. intermedium* were implemented by MP phylogenetic reconstruction together with 15 accessions of potential diploid donors. MP analysis generated 8 maximally parsimonious trees with the tree length of 40 steps, consistency index of 0.9500 and retention index of 0.9775. The 50 % majority rule tree was constructed from these trees (Fig. 1). The MP tree showed that all the accessions of *T. intermedium* were clustered into two clades together with its potential parental donors. The clade A with 70 % bootstrap support comprised of sixteen accessions of *T. intermedium* and its potential diploid donors, three species of *Pseudoroegneria* with St genome (*P. spicata*, *P. libanotica*, *P. strigosa*), eight accessions of diploid *L. elongatum* (E^e), and two accessions of diploid *T. bessarabicum* (E^b). In clade B, it contained only two accessions of *L. elongatum* and the remaining nineteen accessions of *T. intermedium*. Although the topology of the NJ tree was different from that of the MP tree, the phylogenetic relationship was relatively stable among the accessions of *T. intermedium* (Fig. 2). The two major stable branches in both trees were identified.

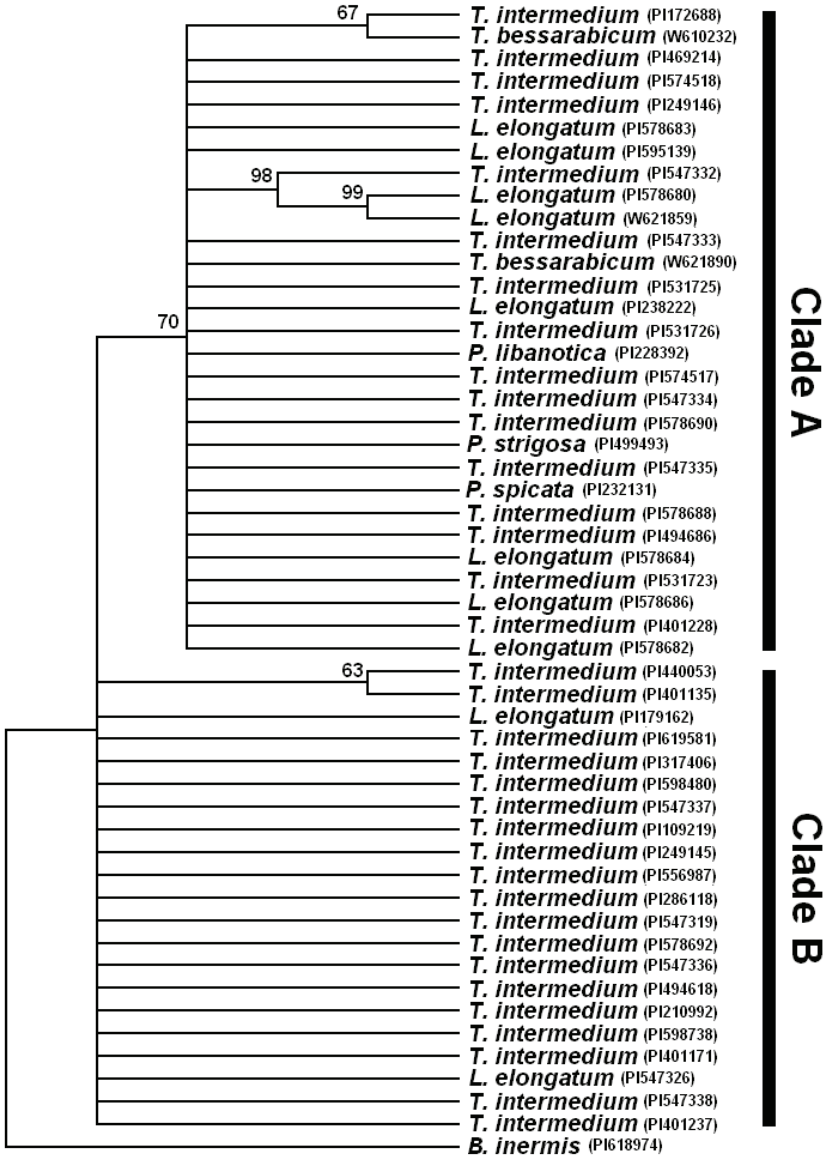

Fig. 1. Fifty-percent majority-rule MP tree inferred from *trn*H-*psb*A intergenic spacer of *T. intermedium* and its affinitive diploid donors. Numbers above branches are bootstrap values > 50 %.

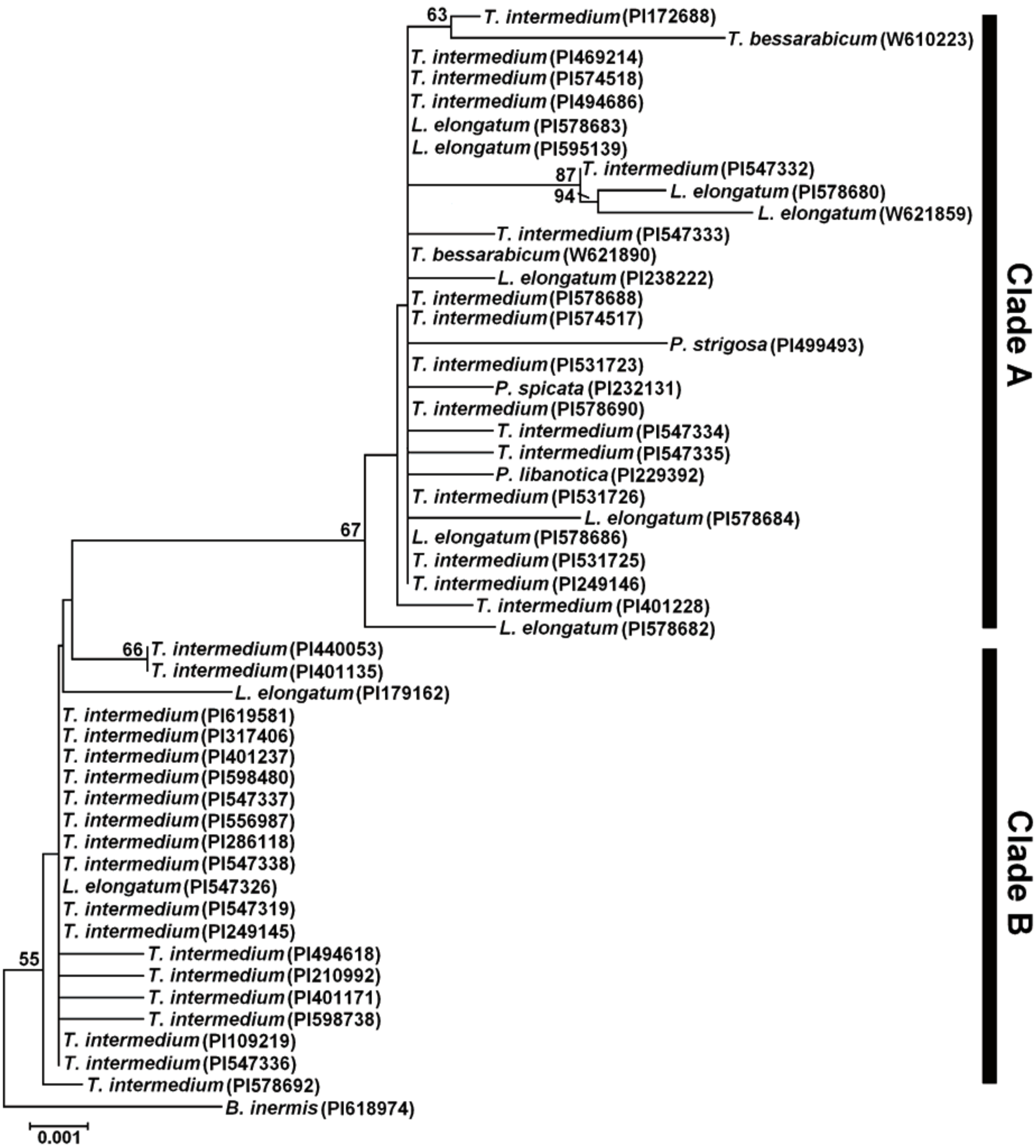

Fig. 2. NJ tree based on the *trn*H-*psb*A sequence of *T. intermedium* and its affinitive diploid donors. *Bar* at the left bottom indicates scale value. Numbers above branches indicate bootstrap values > 50 %.

Discussion

An increasing number of attempts with regard to transfer of valuable traits from *T. intermedium* into wheat have been successfully made (Dewey 1984, Fedak and Han 2005, Li and Wang 2009). However, there are negligible reports on the genetic diversity of *T. intermedium*. In this study, the results revealed a relatively low level of nucleotide polymorphism ($\theta_w = 0.00615 \pm 0.00028$ and $\pi = 0.00473 \pm 0.00037$) and a moderately high haplotype diversity ($H_d = 0.733 \pm 0.061$) in *T. intermedium*. Negative Tajima's D and Fu and Li's D* and F* were observed at the *trn*H-*psb*A locus. Negative Tajima's D and Fu and Li's D* and F* may result from either some form of selective pressure or form population expansion. Thus, it can provide some implications with regard to demographic history of *T. intermedium*. However, it is weak to infer the population history by a single locus. More detailed studies referring multiple loci are needed to better explain the population history.

In this study, an inversion with the length of 26 bp was detected in *trn*H-*psb*A region of *T. intermedium*. The accessions with the inversion were scattered on clades A and B in both the NJ and MP trees. However, inversion in *trn*H-*psb*A was not unique to *T. intermedium*. A number of previous studies have reported that inversions widely existed in angiosperms (Doyle *et al*. 1992, Bain and Jansen 2006) and were often revealed in phylogenetic analysis (Scheen *et al*. 2004, Bain and Jansen 2006). The widespread occurrence of small inversions in the chloroplast genome including *rpl*16, *psb*C-*trn*S, *trn*L-F is well documented (Kim and Lee 2005, Catalano *et al*. 2009). The mechanism responsible for the inversion is not clear, but it might have originated several times during the population expansion process (Yin *et al.* 2010). In this case, the same inversion in *L. elongatum*, *T. bessarabicum* and three *Pseudoroegneria* species acting as potential diploid donors was identified, suggesting that the inversion of *T. intermedium* was probably derived from the diploid progenitors.

On basis of sequence data of the chloroplast *ndh*F gene, Redingbaugh *et al*. (2000) suggested a strong preference for cpDNA inheritance from the St genome-containing parent in hybridization event between different species. As described by Mason-Gamer *et al*. (2002), *Pseudoroegneria* is the maternal genome donor to tetraploid *Elymus* (StY), which is further supported by the data from the PCR-RFLP analysis of four chloroplast gene regions and the *trn*L-F chloroplast sequences (McMillan and Sun 2004, Liu *et al.* 2006). *T. intermedium* is an allohexaploid species whose genomic constitution is considered as E^eE^bSt **(**Liu and Wang 1993, Xu and Conner 1994**)**. Therefore, it was suggested that *Pseudoroegneria* as maternal genome donor contributed to *T. intermedium*. In the phylogenetic analysis of the present study, the cpDNA sequences of *T. intermedium* were grouped into two clades corresponding to its potential parental donors. Clade A included sixteen accessions of *T. intermedium*, three *Pseudoroegneria* species, eight *L. elongatum* accessions and two *T. bessarabicum* accessions. The grouping (clade B) of the remaining nineteen accessions of *T. intermedium* and two accessions of *L. elongatum* was sister to clade A. These divergent clades suggested that there were at least two species that acted as maternal donor contributing to the formation of *T. intermedium* or that the polyploidy even took place more than once. In terms of the inference of plastid donor origin, the similar conclusion that both *Pseudoroegneria* and *Agropyron* species acted as maternal donors has also been suggested in the formation of *Kengyilia* species (Zhang *et al.* 2009, Zeng *et al.* 2010).

Only two of ten accessions *L. elongatum* PI179162, PI547326 belonged to clade B, while the other eight accessions belonged to clade A in both MP and NJ trees. This result suggested that there has been genetic differentiation within *L. elongatum* in terms of the cytoplasmic genome.

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