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Maternal origin, genome constitution and evolutionary relationships of polyploid *Elymus* species and *Hordelymus europaeus*

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Abstract

The *trn*S/*psb*C region of chloroplast DNA (cpDNA) was sequenced for 18 *Elymus* polyploid species, *Hordelymus europaeus* and their putative diploid ancestors. The objective was to determine the maternal origin and evolutionary relationships of these polyploid taxa. Phylogenetic analysis showed that *Elymus* and *Pseudoroegneria* species formed a highly supported monophyletic group (100 % bootstrap values), suggesting that *Pseudoroegneria* is the maternal genome donor to polyploid *Elymus* species studied here. The phylogenetic tree based on cpDNA sequence data indicates that *E. submuticus* contains a St-genome. Taking into consideration of our previously published *RPB*2 data, we can conclude that hexaploid *E. submuticus* contains at least one copy of St and Y genomes. Our Neighor-joining analysis of cpDNA data put *Psathyrostachys juncea*, *Hordeum bogdanii* and *Hordelymus europaeus* into one group, suggesting a close relationship among them.

Additional key words: *Hordeum*, *Psathyrostachys*, *Pseudoroegneria*, RAPD, *Taeniatherum.*

Introduction

According to the genomic classification of Löve (1984) and Dewey (1984), *Elymus* is the largest genus of the tribe *Triticeae*, with more than 150 perennial species. These species inhabit grasslands, semi-desert, forests and forest edges in all continents, except Africa and Antarctica (Helfgott and Mason-Gamer 2004). As an exclusively allopolyploid genus, *Elymus* has its origin from a few related genera in the *Triticeae* through natural hybridization (Dewey 1984). Based on meiotic chromosome pairing results, five basic genomes among the entire genus have been found. All species contain the St genome which was donated by *Pseudoroegneria* (Dewey 1984) in combination with one or more of genomes H, Y, P or W. So far, the origin of Y has not been identified, although it is mostly found in Asia, such as China, Japan and Pakistan (Lu 1993). The H, P and W genomes are derived from *Hordeum*, *Agropyron*, and *Australopyrum*, respectively (Dewey 1984, Helfgott and Mason-Gamer 2004). However, the genomic constitution of approximately 40 % of the species within the genus is still unknown and there are many *Elymus* species whose genomic constitution could be questioned (Svitashev *et al*. 1998).

Recently, chloroplast DNA data indicated that *Pseudoroegneria* is the maternal genome donor to some *Elymus* species (Redinbaugh *et al*. 2000, Mason-Gamer 2001, Mason-Gamer *et al*. 2002, McMillan and Sun 2004, Xu and Ban 2004, Liu *et al*. 2006). The nuclear gene sequence analyses confirmed that North American *Elymus* allotetraploids have a St and H genomic content (Helfgott and Mason-Gamer 2004). Phylogenetic analysis of Asian *Elymus* species confirmed the cytogenetic results that the St, H, P and W genome in polyploid *Elymus* species were donated by *Pseudoroegneria*, *Hordeum*, *Agropyron* and *Australopyrum*, respectively (Liu *et al*. 2006, Sun *et al*. 2008, Zeng *et al.* 2008). Although the chloroplast DNA data indicated that *Pseudoroegneria* is the maternal donor to the studied *Elymus* species, maternal origin of other species in this genus is unknown.

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Abbreviations: *Ag*. - *Agropyron*; cpDNA - chloroplast DNA; *E*. - *Elymus*; *H*. - *Hordeum*; *Hor*. - *Hordelymus*; *Lop*. - *Lophopyrum*; *P*. - *Pseudoroegneria*; *Psa*. - *Psathyrostachys;* RAPD - random amplified polymorphic DNA; *T*. -*Taeniatherum*; *Th*. -*Thinopyrum.*
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Hordelymus is a monotypic genus comprising only the tetraploid species *Hor. europaeus* (L.) Harz. It is commonly found in rich woodlands and occurs in Europe, northern Africa, and western Asia. Von Bothmer and Jacobsen (1989) based on cytogenetic studies suggested T and H genome in this species. Von Bothmer *et al*. (1994) based on another cytogenetic study suggested that it contains the T and Ns genome. The presence of the Ns genome was confirmed by Svitashev *et al*. (1998). However, genomic hybridization suggested presences of Ns genome and absence of T genome in *Hor. europaeus* (Ellneskog-Staam *et al*. 2006). Recently, Petersen and Seberg (2008) reported *Psathyrostachys* as female genome donor of *Hordelymus*.

In this study, we sequenced and analyzed the *trn*S/*psb*C region of cpDNA for 18 *Elymus* polyploid species, *Hordelymus europaeus*, and their putative ancestral diploid species. The objective was to determine the maternal origin and evolutionary relationships of polyploid *Elymus* species, and *Hordelymus europaeus.*

Materials and methods

A total of 30 *Triticeae* accessions were used in this study (Table 1), including 18 *Elymus* species with different genome combinations (StH, StY, StHY, StPY), one *Hordelymus* species, and 10 accessions of diploid *Triticeae* species from genus *Pseudoroegneria* (St genome), *Hordeum* (H), *Agropyron* (P), *Lophopyrum* (E^e), *Thinopyrum* (E^b), *Psathyrostachys* (Ns) and *Taeniatherum* (T) (Table 1). *Bromus catharticus* was used as the outgroup based on previous phylogenetic analysis of *Poaceae* (Hsiao *et al*. 1995). Seeds were germinated on filter paper in Petri dishes. Germinated seeds were transplanted to a sand-peat mixture, and the plants maintained in a greenhouse. DNA was extracted from freeze-dried leaf tissue collected from 5 - 10 plants (30-d-old) of each accession using the method of Junghans and Metzlaff (1990).

The spacer between *trn*S gene [tRNA - Ser (UGA)] and the adjacent *psb*C gene (PS II 44 kDa) were amplified using primer pair *trnS* (GGT TCG AAT CCC TCT CTC TC) and *Psb*C (GGT CGT GAC CAA GAA ACC AC) (Demesure *et al*. 1995). Amplification of DNA was carried out in 0.02 cm^3 reaction mixture containing 30 ng template DNA, 0.2 µM of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of *Taq* DNA polymerase (*New England Biolabs*, Pickering, Canada) and distilled deionized water to the final volume. The reaction mixture was amplified using *Techne Genius* (Cambridge, UK) thermal cycler with following protocol: one cycle of 4 min at 94 °C, 35 cycles of 1 min at 94 °C , 2 min at 62 °C, 2 min at 72 °C, followed by 10 min at 72 °C.

The PCR products were purified using *QIAquick*™ PCR purification kit (*Qiagen*, Mississauga, USA) according to the manufacturer's instruction. The purified PCR products were used either for direct sequencing or cloning using the *pGEM-T Easy Vector System II* kit (*Promega*, Madison, WI, USA) according to the manufacturer's protocol. For the cloning product, six to eight white colonies were randomly selected for screening using *TrnS-PsbC* primers to check if the DNA fragment is successfully insert into plasmid. The colony that was confirmed to contain the insert, were incubated in 4 cm^3 LB broth with 100 µg cm^{-3} ampicillin at 37 °C water bath overnight.

Plasmid DNA was isolated using *GenElute TM Plasmid Miniprep* kit (*Sigma*, St. Louis, MO, USA) following the manufacture's instructions. Both PCR product and plasmid DNA were sequenced commercially at *Macrogen* (Seoul, Korea). To increase quality of the data, three clones from each cloned PCR amplicon were sequenced from both forward and reverse strands independently.

Automated sequence outputs were corrected visually by comparison with chromatographs. *BLAST 2* sequences and *All-in-One Seq* analyzer (http://www-personal.umich.edu/~ino/blast.html) were used to generate full sequence of each clone.

Multiple nucleotide sequence alignments were carried out using *ClustalX* (Thompson *et al*. 1997) and checked manually. Phylogenetic analysis was performed using the program *PAUP** version *4 beta 10 Win* (Swofford 2003). The most parsimonious trees were obtained by performing a heuristic search using the Tree Bisection-Reconnection (TBR) option with MulTrees on, and ten replications of random addition sequence with the stepwise addition option. All characters were specified as unweighted. Gaps were treated as missing data and multistate taxa interpreted as uncertainty. Multiple parsimonious trees were combined to form a strict consensus tree. Overall character congruence was estimated by the consistency index (CI), rescaled consistency index (RC) and then the retention index (RI). Bootstrap values based on 1000 replications (Felsenstein 1985) were calculated by performing a heuristic search using the TBR option with MulTrees off. To complement the parsimonious analysis, cluster analysis based on pairwise Tajima-Nei distances (tajima and Nei 1984) was performed.

Results

Of the sequences presented here, some were generated from cloned PCR products, while others were generated from directly sequenced PCR product. The latter sequences were shorter than the sequences from the cloned product since both ends of the sequences from direct sequencing were not well read. The length of sequences in *trnS[tRNA-ser (UGA)]-psbC[spII44kd protein]* region was approximately 1.5 kb in the sequences from cloned product. After alignment, a variable polyA repeat was detected between 1490 bp to 1530 bp. The flanking region of the microsatellite $(A)_n$ was highly conserved with sequence (TGAAAGAAA) in the species sequences from cloned product.

A total of 169 sequence mutations, including 154 single-base substitutions, 7 single base insertion/ deletions and 8 two-base substitutions were detected among the alignment with information from all taxa which was 1348 nucleotides. Of the 154 single-base

Table 1. Species used in this study. The genome designations are according to Wang *et al*. (1994).

Species	Accession No.	Genome	Origin
Agropyron cristatum (L.) Gaertn.	PI 383534	P	Kars, Turkey
Elymus abolinii (Drobow) Tzvelev	PI 531554	StY	Xinjiang, China
Elymus antarcticus Hook. f.	PI 636671	StH	Chile
Elymus batalinii (Krasn.) Nevski	PI 314623	StPY	Alma Ata, USSR
Elymus caninus (L.) L.	H 3169	StH	Västmanland, Sweden
Elymus ciliaris (Trin.) Tzvelev	PI 564917	StY	Vladivostok, USSR
Elymus dahuricus Turcz. Ex Griseb.	PI 628674	StHY	Xinjiang, China
Elymus dentatus (Hook. f.) Tzvelev	PI 628702	StH	Altay, USSR
Elymus hystrix L.	H 5495	StH	Canada
Elymus interuptus Buckley	PI 531617	StH	Manitoba, Canada
Elymus sibiricus L.	PI 499461	StH	Lanzhou, China
Elymus submuticus (Keng) Á. Löve	PI 499480	??	Lanzhou, China
Elymus trachycaulus (Link) Gould *ex* Shinners	H 3526	StH	Nerungri, Russia
	PI 537323	StH	Utah, USA
Elymus tsukushiensis Honda	PI 531698	StHY	Osaka, Japan
Elymus villosus Muhl. *ex* Willd.	PI 531703StH	StH	Missouri, USA
Elymus violaceus (Hornem.) Feilberg	H 10588	StH	Julianehåb, Greenland
Elymus wawawaiensis J. Carlson *ex* Barkworth	PI 506262	StH	Washington, USA
Elymus wiegandii Fernald	PI 531708	StH	Aylwin, Quebec, Canada
Elymus coreanus (Honda) Jensen *et* Wang	PI 531578	XmNs/??	Russia
Hordeum bogdanii Wilensky	PI 499645	H	Xinjiang, China
Hordeum stenostachys Godr.	H 6439	H	Argentina
Hordelymus europaeus (L.) Harz	NGB9218	??	Denmark
Lophopyrum elongatum (Host) Á. Löve	PI 142012	E^e	Odessa, Russia
Psathyrostachys juncea (Fisch.) Nevski	PI 406469	Ns	USSR
Pseudoroegneria libanotica (Hack.) D.R. Dewey	PI 330687	St	Kandavan Pass, Iran
Pseudoroegneria spicata (Pursh) Á. Löve	PI 506273	St	Washington, USA
Taeniatherum caput-medusae (L.) Nevski subsp. *caput-medusae*	PI 222048	T	Kabul, Afghanistan
Taeniatherum caput-medusae (L.) Nevski subsp. *asperum* Melderis	PI 561091	T	Siirt, Turkey
Thinopyrum bessarabicum (Savul. & Rayss) Á. Löve	PI 531712	E^b	Estonia
Bromus catharticus Vahl	CN32048		Canada

Table 2. Tajima-Nei distance among *Hordelymus*, *Hordeum*, *Taeniatherum* and *Psathyrostachys* species.

		NGB9218	H6439	PI 499645	PI 406469	PI 561091	PI 222048
Hordelymus	NGB9218 (??)	-	-	-	-	-	-
Hordeum	H6439 (H)	0.03193	-	-	-	-	-
Hordeum	PI 499645 (H)	0.03817	0.003118	-	-	-	-
Psathyrostachys	PI 406469 (Ns)	0.03990	0.05330	0.04693	-	-	-
Taeniatherum	PI 561091 (T)	0.02423	0.02196	0.04055	0.05657	-	-
Taeniatherum	PI 222048 (T)	0.01961	0.02651	0.03114	0.04532	0.01429	-

substitutions, 47 nucleotides were T/C substitutions. G/T, G/C, A/C, A/G and A/T substitutions were detected at 19, 22, 23, 25 and 18 positions, respectively. The occurrence of transition and transversion at the 154 positions are 46.75 and 53.25 %, respectively.

The total of 1348 characters from each sequence was included for further analysis, of which 1187 characters are constant. 79 variable characters were parsimony-uninformative, and 82 were parsimony informative. Parsimony analysis produced 230 equally parsimonious trees with a consistency index (CI) of 0.702, a retention index (RI) of 0.725, and rescaled consistency index (RCI) of 0.509. The strict consensus tree based on maximum parsimony analysis is shown in Fig. 1. The bootstrap (1000 replicates) values are shown on each branch. All *Elymus* species were grouped together with *P. spicata* (accession PI 506273) and *P. libanotica* (PI 330687) with 100 % bootstrap support with exception of one accession of *E. trachycaulus* (PI 537323). Included in this clade are *Ag. cristatum* (P) and *E. coreanus*. *Lop. elongatum* (E^e) is sister to the St clade with 67 % bootstrap value. Two *Taeniatherum* subspecies, *T. caput-medusae* subsp. *asperum* and *T. caput-medusae* subsp. *caput-medusae*, were not grouped together. The tetraploid species *Hordelymus europaeus* is part of a polytomy at the basis of the strict consensus tree. The Neighbor-joining tree (Fig. 2) showed a similar topology as the maximum parsimony tree. However, the Neighbor-joining tree grouped the *Hor. europaeus* with *T. caput-medusae* subsp. *caput-medusae* (T), *Psa. juncea* (Ns) and *H. bogdanii* (H) together. Within this clade, *Psa. juncea* and *H. bogdanii* are sisters to *Hor. europaeus* with moderate support (70 % bootstrap value).

The genetic distances among the T, Ns, H genome species and *Hor. europaeus* were analyzed (Table 2). A smallest genetic distance was found between *Hor. europaeus* and *T. caput-medusae* subsp. *caput-medusae* (T genome) (0.01961), followed by 0.02423 between *Hor. europaeus* and *T. caput-medusae* subsp. *asperum* (T), and 0.03193 between *Hor. europaeus* and *H. stenostachys*. The genetic distance between *Hor. europaeus* and *Psa. juncea* was 0.0399.

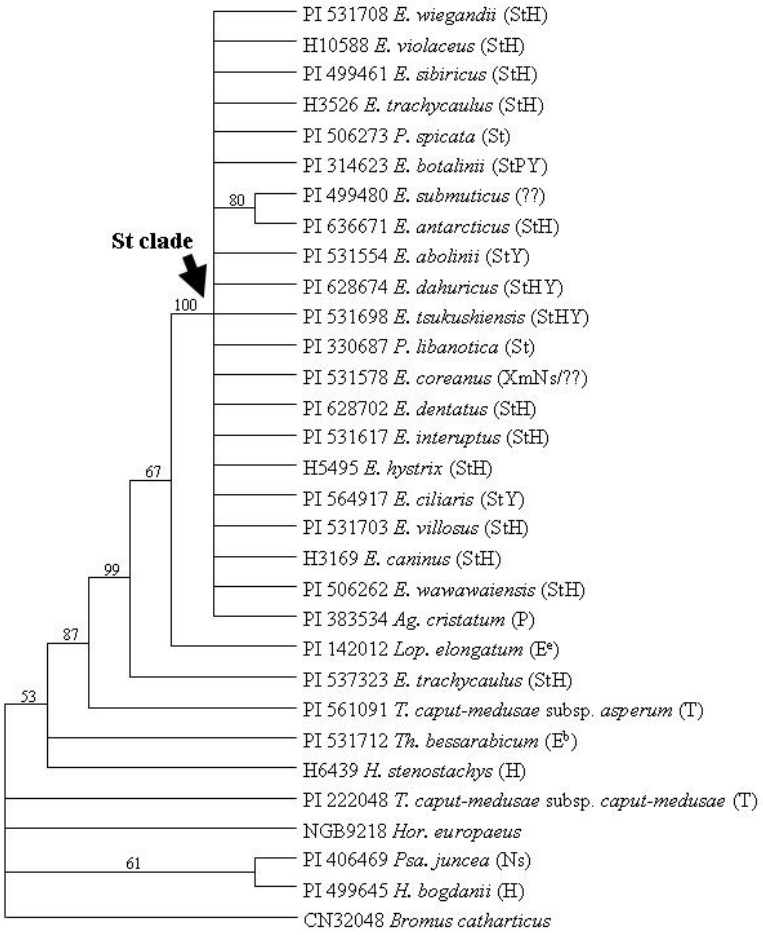

Fig. 1. Strict consensus tree of 230 most parsimonious trees inferred from the *Trnt-PsbC* sequence using heuristic search with *TBR* branch swapping. Numbers on branches are bootstrap values. *Bromus catharticus* was used as an outgroup. Tree length = 242, consistency index (CI) = 0.702, retention index (RI) = 0.725, rescaled consistency index (RCI) = 0.509.

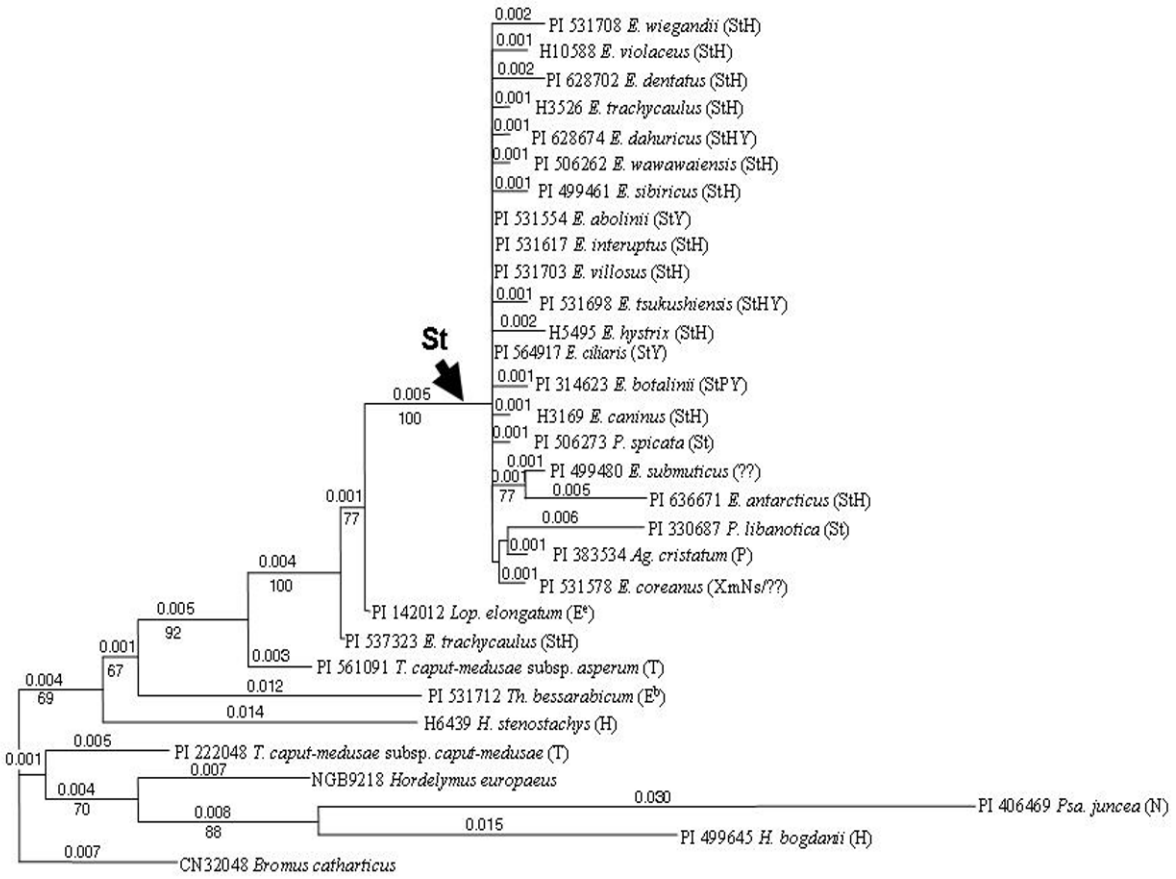

Fig. 2. The NJ tree of the 31 taxa based on Tajima-Nei distances. *Bromus catharticus* was used as an outgroup. The values above the branches show the branch lengths. Bootstrap support above 50 % with 1000 replicates are given below the branches.

Discussion

Comparison of cpDNA sequences is a useful way to identify the female parent in interspecies hybridization. Chloroplast genome is generally uniparentally inherited in monocotyledones and thus can be used to identify the maternal genome donor of a given polyploid. cpDNA sequence analysis of the *ndh*F region indicated that the *Pseudoroegneria* chloroplast genome was preferred in the speciation of *Elymus* species (Jones *et al*. 2000, Redinbaugh *et al*. 2000). The cpDNA data used in phylogenetic analysis of North American *Elymus* indicated that *Pseudoroegneria* is the potential chloroplast donor to *Elymus* species (Mason-Gamer *et al*. 2002). Studies of cpDNA in tetraploid and hexaploid *Elymus* species containing StH, StHY or StY genome obtained the same conclusion (McMillan and Sun 2004, Xu and Ban 2004). A recent study based on analysis of *trn*L-F sequences on tetraploid and hexaploid *Elymus* species by Liu *et al*. (2006) also come to a similar conclusion. In this study, *Elymus* species and *Pseudoroegneria* species formed a highly supported monophyletic group (100 % bootstrap values). This suggests that *Pseudoroegneria* also is the maternal genome donor to polyploid *Elymus* species studied here. Although the biological reason for the fact that the *Pseudoroegneria* (St) was the maternal parent for all St-genome containing polyploid species studied so far remains unknown, a similar selection for cpDNA inheritance from progenitors containing a specific nuclear genome was noted among some *Triticum* and *Aegilops* species (Wang *et al*. 1997).

Elymus submuticus is a hexaploid *Elymus* species with an unknown genome. The RPB2 nuclear gene phylogeny by Sun *et al*. (2008) suggested that *E. submuticus* contains a Y-genome. The phylogenetic tree based on cpDNA sequence data indicated that *E. submuticus* contains a St-genome (Figs. 1 and 2). Based on *RPB*2 (Sun *et al*. 2008) and cpDNA data presented here, we can conclude that *E. submuticus* belongs to the StY-genome group.

Intraspecific cpDNA sequence divergence in

E. trachycaulus was found to be extensive. Two accessions of this species were analyzed and, as expected, accession H3526 was grouped into the clade containing *Pseudoroegneria*. Unexpectedly, accession PI 537323 was grouped outside of the St-clade. As the plastids are generally inherited uniparentally their evolutionary history need not reflect the whole history of the organisms, especially in a tribe in which hybridization is so common. It has been shown that the same morphologically defined polyploid taxon may arise several times (Soltis and Soltis 1993). Possibility of lateral gene transfer between the monogenomic diploid species mediated through the heterogenomic, polyploidy species can not be ruled out in *Triticeae* (Kellogg *et al*. 1996). Grouping *E. trachycaulus* accession PI 537323 outside of the St-clade might be attributed to lateral transfer of plastids.

The polyploid species with St-genome combination formed a highly supported clade (100 % bootstrap support) which is an unresolved polytomy with *Pseudoroegneria*. The lack of resolution among the *Elymus* cpDNA sequences associated with the St genome is similar to the results of Mason-Gamer *et al*. (2002). *Ag. cristatum* (P) was included in the St clade, suggesting a close relationship between St genome and P genome. This was not unexpected since a close affinity between the St and P genomes was also reported in the cytological investigations (Wang *et al*. 1985).

Studies of genome-specific RAPD markers for *E. coreanus* and chromosome pairing in *E. coreanus* and various F_1 hybridizations have demonstrated that *E. coreanus* possess the Ns genome from the genus *Psathyrostachys* and the Xm genome of unidentified origin (Jensen and Wang 1997, Svitashev *et al*. 1998). Phylogenetic analysis based on cpDNA data here grouped *E. coreanus* into St clade, and separated it from *Psathyrostachys juncea* (Ns). Previous *ndh*F DNA sequences analyses grouped the allotetraploid *Leymus* (NsXm) species with the *Hordeum* species, and separated allotetraploid NsXm genome species from the Ns-containing diploid (Jones *et al*. 2000; Redinbaugh *et al*. 2000). Our data is consistent with the finding of separation between NsXm species and Ns-containing diploid, but did not support the grouping of allotetraploid *Leymus* (NsXm) species with the *Hordeum* species reported in previous studies (Jones *et al*. 2000, Redinbaugh *et al*. 2000) Our result suggests that *E. coreanus* may be originated from St genome species, or some degree lateral transfer of plastids from a species with an St-like genome might possibly have occurred. This suggestion needs to be further verified with additional sampling and sequencing.

Cytogenetic evidence suggested that *Hor. europaeus* is an allopolyploid species with two distantly related genomes (Von Bothmer *et al*. 1994). Löve (1984) suggested that this species contains the H genome from *Hordeum* and the T genome from *Taeniatherum*. However, the presence of the H genome was refuted by cytogenetic data (Von Bothmer and Jacobsen 1989, Von Bothmer *et al*. 1994) and by storage protein data (Pelger 1993). On the basis of cytogenetic evidence, Von Bothmer *et al*. (1994) indicated that *Taeniatherum* (T genome) and *Psathyrostachys* species (Ns genome) as the possible closest relatives of *Hordelymus*. Svitashev *et al*. (1998) confirmed the presence of the Ns genome in *Hor. europaeus* using RAPD method. Recently, genomic southern hybridization data indicated the presence of an Ns genome highly homologous to that of *Psathyrostachys* and *Leymus* and rejected the presence of H, T, E or St genomes in *Hor. europaeus* (Ellneskog-Staam *et al*. 2006). Phylogenetic analyses of sequence data from two plastid genes (*rbc*L and *ndh*F) provided substantial support for the progenitor of *Psathyrostachys* as female genome donor of *Hordelymus* (Petersen and Seberg 2008). Our cpDNA sequence showed a high similarity between *T. caput-medusae* subsp. *caput-medusae* (T genome) and *Hor. europaeus*. The genetic distances between *Hor. europaeus* and two *Hordeum* species were 0.03193 (*H. stenostachys*) and 0.03817 (*H. bogdanii*). Genetic distance was 0.0399 between *Hor. europaeus* and *Psa. juncea*. Parsimonious analysis placed *Hor. europaeus, Psa. juncea, H. bogdanii,* and *T. caput-medusae* subsp. *caput-medusae* in a polytomy at the basis of the strict consensus tree. Neighbor-joining method suggested a close relationship among *Psa. juncea* and *H. bogdanii* and *Hor. europaeus* (Fig. 2).

In summary, our study suggests that *Pseudoroegneria* also is the maternal genome donor to these polyploid *Elymus* species studied here, and demonstrates that *E. submuticus* belongs to the StY-genome group. Our cpDNA data suggested a close relationship among *Psa. juncea* and *H. bogdanii* and *Hor. europaeus.*

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