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Maternal donors of polyploids in *Pseudoroegneria* (Poaceae: Triticeae) and related genera inferred from chloroplast *trnL-F* sequences

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Abstract: To investigate the maternal donors and phylogenetic relationships of polyploids in *Pseudoroegneria* and related genera, the chloroplast *trnL-F* sequences of 31 Triticeae accessions were analyzed. The substitution saturation analysis of *trnL-F* sequences in this study suggested that they are suitable for phylogenetic analysis. In the *trnL-F* sequences tree, 4 major clades were formed: (a) the St/E clade comprised all of the *Pseudoroegneria* species together with species in *Roegneria*, *Elytrigia*, *Douglasdeweya* and *Lophopyrum*; (b) *A. cristatum*, *A. mongolicum*, and *A. pectinatum* subsp. *retrofractum* formed the P/W clade; (c) the Ns clade included species in *Psathyrostachys*; and (d) the H clade consisted of *Hordeum* species. The results suggested that: (a) diploid *Pseudoroegneria* species were the maternal donors of St-containing polyploid species in *Pseudoroegneria*, *Roegneria*, *Douglasdeweya*, and *Elytrigia*, and the *trnL-F* sequences were highly similar among them; (b) the *trnL-F* sequences of *Agropyron* species and *Australopyrum* species were similar, and the P genome was closely related to the W genome; and (c) the *trnL-F* sequences of species with the H or Ns genomes diverged greatly from that of species with the St, E, P, or W genomes.

Key words: *Pseudoroegneria*, *Douglasdeweya*, *Roegneria*, *Elytrigia*, chloroplast *trnL-F*

Pseudoroegneria (Poaceae: Triticeae) ve ilişkili cinslerde kloroplast *trnL-F* dizilerinden elde edilen poliploitlerin maternal vericileri

Özet: *Pseudoroegneria* ve ilişkili cinslerde, poliploidlerin filogenetik ilişkilerini ve maternal vericilerini araştırmak için, 31 Triticeae örneğinin kloroplast *trnL-F* dizileri analiz edildi. Bu çalışmada *trnL-F* dizilerinin yer değişim doygunluk analizleri filogenetik analizler için uygunluğu önerdi. *trnL-F* dizilerine ait ağaçda, dört büyük dal şekillendi. (a) St/E dalı *Roegneria*, *Elytrigia*, *Douglasdeweya* ve *Lophopyrum* ile birlikte tüm *Pseudoroegneria* türlerini içerdi; (b) *A. cristatum*, *A. mongolicum* ve *A. pectinatum* subsp. *retrofractum* türleri P/W dalını şekillendirdi; (c) Ns dalı *Psathyrostachys* türlerini içerdi; (d) H dalı *Hordeum* türlerinden oluştu. Sonuçlar (a) *trnL-F* dizileri yüksek oranda benzerlik gösteren *Pseudoroegneria*, *Roegneria*, *Douglasdeweya* ve *Elytrigia* cinsleri içinde yer alan St içeren poliploid türlerin maternal vericilerinin diploid *Pseudoroegneria* türleri olduğunu; (b) P ve W genomlarının çok yakın ilişkili, *Agropyron* ve *Australopyrum* türlerinin *trnL-F* dizilerinin benzer olduğunu; (c) H veya Ns genomlu türlerin *trnL-F* dizilerinin büyük ölçüde St, E, P veya W genomlu türlerden farklılaştığını önerdi.

Anahtar sözcükler: *Pseudoroegneria*, *Douglasdeweya*, *Roegneria*, *Elytrigia*, kloroplast *trnL-F*

Introduction

Pseudoroegneria is a genus in Triticeae (Poaceae) with *Pseudoroegneria strigosa* (M. Bieb.) Á. Löve as the type species (1). It consists of about 15 species that are built around 1 genome designated St, which is the donor genome of the species in *Douglasdeweya* (StP), *Roegneria* (StY), *Elytrigia* (ESt), *Elymus* (StH), *Kengyilia* (StYP), and *Pascopyrum* (StHNsXm) (2-6). The genus is distributed in the northern hemisphere, with its species occurring on open rocky hillsides from the Middle East and Transcaucasia across Central Asia and northern China to western North America. *Pseudoroegneria* grasses are exceptionally drought tolerant and have excellent forage quality (3).

Morphologically, *Pseudoroegneria* species are caespitose, long-anthered, cross-pollinating perennials (1). However, similar morphological characters are also found in several related genera, such as *Elytrigia* and *Douglasdeweya* (3,5). Cytologically, *Pseudoroegneria* species are diploids or tetraploids, and possess St, StSt, StP, or E^eSt genomes. Based on the ITS data analysis, *Pseudoroegneria* species are closely related to those in 3 Triticeae genera, namely *Peridictyon*, *Heteranthelium*, and *Dasypyrum* (7). Löve and Dewey suggested that the taxonomic treatment for Triticeae species should be based on genomic constitutions, and this view has been widely accepted (3,8-10). Yen et al. (5) established a new genus, *Douglasdeweya* C. Yen, J.L. Yang & B.R. Baum, and the species with StP genomes in *Pseudoroegneria* were transferred to *Douglasdeweya*. Wang et al. (8) indicated *Agropyron mongolicum* (L.) Keng as the maternal donor of *Douglasdeweya wangii* C. Yen, J.L. Yang & B.R. Baum, based on morphological analysis. Jensen et al. (9) suggested that *Pseudoroegneria stipifolia* (Czern. ex Nevski) Á. Löve is most likely the maternal donor of *Douglasdeweya deweyi* C. Yen, J.L. Yang & B.R. Baum.

Species in *Roegneria* C. Koch are characterized by the StY genome (3,6,11,12). *Roegneria alashanica* Keng, *Roegneria elytrigioides* C. Yen et J.L. Yang, *Roegneria magnicaespes* (D.F. Cui) L.B. Cai, and *Roegneria grandis* Keng are similar in morphology (13-16). Lu (17) reported that *R. elytrigioides* contains the StSt genome and transferred it into the genus *Pseudoroegneria*. Considering the morphological similarity among *R. elytrigioides*, *R. alashanica*, *R. magnicaespes*, and *R. grandis*, they are most likely the *Pseudoroegneria* species distributed in China (18-20).

Morphologically, *Pseudoroegneria geniculata* subsp. *scythica* (Nevski) Á. Löve, *Elytrigia caespitosa* (C. Koch) Nevski, *Elytrigia caespitosa* subsp. *nodosa* (Nevski) Tzvelev, and *Elytrigia intermedia* (Host) Nevski are grouped into different genera (2). Cytologically, they contain E^eSt genomes and were suggested to be combined in a new genus, *Trichopyrum* Á. Löve (5,10). In addition, the genomic constitutions of hexaploid *Pseudoroegneria geniculata* subsp. *pruinifera* (Nevski) Á. Löve and octoploid *Pseudoroegneria kosaninii* (Nabelek) Á. Löve are obscure. Therefore, the maternal donors and phylogenetic relationships of several species related to *Pseudoroegneria* are still in dispute now.

Several phylogenies of Triticeae species have been established that were based on sequence data, including ITS, 5S rDNA, chloroplast *rpoA* sequences, chloroplast *trnL-F* sequences, etc. (7,21-24). The chloroplast DNA (cpDNA) sequences, particularly the noncoding regions such as the intron of *trnL* (UAA) and the intergenic spacer of *trnL* (UAA)-*trnF* (GAA), are valuable sources of markers for identifying the maternal donors of polyploids, with additional capacity to reveal phylogenetic relationships of related species (23,25). The chloroplast *trnL-F* sequence has successfully demonstrated the maternal origin of polyploids in *Elymus* (25,26), while it is not estimated among polyploids in *Pseudoroegneria* and related genera. To understand the maternal donors and phylogenetic relationships of polyploids in *Pseudoroegneria* and related genera, the chloroplast *trnL-F* sequences were sequenced and analyzed. The aims were: (a) to investigate the maternal donors of polyploids in *Pseudoroegneria* and related genera; and (b) to evaluate the phylogenetic relationships of species among *Pseudoroegneria*, *Douglasdeweya*, *Roegneria*, *Elytrigia*, and *Lophopyrum*.

Materials and methods

Plant materials

A total of 31 Triticeae accessions, including 13 *Pseudoroegneria* (St), 4 *Roegneria* (StY), 3 *Elytrigia* (E^eSt), 2 *Douglasdeweya* (PSt), 2 *Lophopyrum* (E^e and E^b), 2 *Agropyron* (P), 1 *Australopyrum* (W), 2

Psathyrostachys (Ns), and 2 *Hordeum* (H) accessions, were used in this study. *Bromus catharticus* Vahl was used as an outgroup. All of the seed materials were kindly provided by the American National Plant Germplasm System (Pullman, Washington, USA) and the Triticeae Research Institute, Sichuan Agricultural University, China. These seeds were germinated and grown in the perennial nursery. The mature plants were carefully identified by Professors Chi Yen, Junliang Yang, and Yonghong Zhou. The taxa, accession numbers, genomic constitutions, geographic origins, and GenBank accession numbers are listed in the Table. The nomenclature and genome symbols of the species used in this study follow the opinions of Löve (2), Dewey (3), Wang et al. (12), and Yen et al. (5).

DNA extraction and purification

The leaf samples for each material were collected from mature plants in the perennial nursery of the Triticeae Research Institute and ground in liquid nitrogen in a 1.5 mL microfuge tube. DNA was extracted and purified with a slight modification of the cetyltrimethylammonium bromide (CTAB) procedure outlined by Doyle and Doyle (27).

trnL-F amplification and sequencing.

The amplification of *trnL-F* regions was done using primers r (5'-CGAAATCGGTAGACGCTACG-3') and f (5'-ATTTGAACTGGTGACACGAG-3') (28). The PCR reaction was carried out in a total volume of 25 μL, containing 1× reaction buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM of each dNTP (TakaRa Biotechnology (Dalian) Co., Ltd., Dalian, China), 0.5 units of Ex*Taq* Polymerase (TakaRa), and sterile water to the final volume. The thermocycling profile consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 40 s at 94 °C, 50 s at 60 °C, 2 min at 72 °C, and a final extension of 8 min at 72 °C. PCR reactions of each accession were carried out in an ABI 9700 thermal cycler (Applied Biosystems, CA, USA). Amplification products were purified using the Gel Extraction Kit (50) (OMEGA, GA, USA). The purified products were directly sequenced in both directions by Sunbiotech Company (Beijing, China). The sequences used in this study have been submitted to NCBI (http://www.ncbi.nlm.nih.gov).

Sequence alignment and phylogenetic analysis

The boundaries of the *trnL-F* regions were determined by comparison with the *trnL-F* sequence of *Pseudoroegneria libanotica* (Hackel) D.R. Dewey (GenBank accession number AY730567) (25). The *trnL-F* sequence alignment was executed with the Clustal X program and adjusted manually where necessary (29). Gaps were coded as binary characters by their presence/absence and were used for the phylogenetic analyses. DAMBE 4.5.67 (30) was used to test substitution saturation.

PAUP* 4.0b10 (31) was used to find the most parsimonious trees by performing a heuristic search with tree-bisection-reconnection (TBR) branch swapping, the MULTREES option, ACCTRAN optimization, and 100 random addition replicates. Topological robustness was assessed by bootstrap analysis with 1000 replications using simple taxon addition.

Results

Sequence analysis of *trnL-F* regions

The *trnL-F* fragment sequenced in this study included 4 regions: (1) the partial *trnL* intron, (2) the *trnL* 3' exon, (3) the *trnL-trnF* intergenic spacer, and (4) the partial *trnF* exon with 40 base pairs (bp). The length of the sequenced chloroplast *trnL-F* varied from 859 to 882 bp in all accessions. The average of the G + C content was 29%. Using the parsimony criterion, 47 of the 108 variable sites were parsimoniously informative, including the polymorphisms introduced by insertions/deletions. The analysis of substitution saturation for the *trnL-F* sequence was conducted and is shown in Figure 1.

Phylogenetic analysis of the chloroplast *trnL-F* sequences

The chloroplast *trnL-F* sequences of polyploids related to *Pseudoroegneria* and their putative diploid donor species were included for phylogenetic analysis. Maximum parsimony (MP) analysis resulted in 154 equally most parsimonious trees with 134 steps, a consistency index of 0.8358, and a retention index of 0.7982. The strict consensus tree constructed by MP is shown in Figure 2. The percentage of bootstrap values is indicated above the branches.

Table. Materials used in the *trnL-F* analysis.

No.	Species	Accession No.	Genome	Geographic Origin	GenBank Accession No.
Pseudoroegneria (Nevski) Á. Löve					
1	*P. cognata* (Hack.) Á. Löve	PI 531720	St	Estonia, Russian Federation	EU139482
2	*P. geniculata* (Trin.) Á. Löve	PI 565009	StSt	Russian Federation	EU139485
3	*P. geniculata* subsp. *pruinifera* (Nevski) Á. Löve	PI 547374	—-	Ural, Russian Federation	EU139483
4	*P. geniculata* subsp. *scythica* (Nevski) Á. Löve	PI 502271	E^eSt	Russian Federation	EU139484
5	*P. gracillima* (Nevski) Á. Löve	PI 440000	St	Stavropol, Russian Federation	EU139486
6	*P. kosaninii* (Nabelek) Á. Löve	PI 237636	——	Turkey	EU139487
7	*P. libanotica* (Hackel) D.R. Dewey	PI 228389	St	Iran	AY730567*
8	*P. spicata* (Pursh) Á. Löve	PI 610986	St	Utah, United States	AF519158*
9	*P. spicata* (Pursh) Á. Löve	PI 232124	StSt	Washington, United States	EU139488
10	*P. stipifolia* (Czern. ex Nevski) Á. Löve	PI 325181	St	Stavropol, Russian Federation	EF396989
11	*P. strigosa* subsp. *aegilopoides* (Drobov) Á. Löve	PI 595164	St	Xinjiang, China	EF396990
12	*P. strigosa* (M. Bieb.) Á. Löve	PI 531752	StSt	Estonia, Russian Federation	EU139489
13	*P. tauri* (Boiss. & Balansa) Á. Löve *Roegneria* C. Koch	PI 401323	St	Iran	EF396991
14	*R. alashanica* Keng	Z2006	St-	Yinchuan, Ningxia, China	AY730569*
15	*R. elytrigioides* (C. Yen et J.L. Yang) B.R. Lu	Z2005	StSt	Changdu, Tibet, China	AY730568*
16	*R. grandis* Keng	H3879	StY	Lintong, Shanxi, China	AY730572*
17	*R. magnicaespes* (D.F. Cui) L.B. Cai *Elytrigia* Desvaux	Y0756	St-	Kuqua, Xinjiang, China	EU139490
18	*E. caespitosa* (C. Koch) Nevski	PI 547311	E^eSt	Russian Federation	EU139480
19	*E. caespitosa* subsp. *nodosa* (Nevski) Tzvelev	PI 547345	E^eSt	Ukraine	EU139481
20	*E. intermedia* (Host) Nevski *Lophopyrum* Á. Löve	-	E^bE^eSt	-	DQ912408
21	*L. bessarabicum* (Savul & Rayss) C. Yen, J.L. Yang & Y. Yen	PI 531711	E^b	Estonia, Russian Federation	AF519165*
22	*L. elongatum* (Host) Á. Löve *Douglasdeweya* C. Yen, J.L. Yang & B.R. Baum	PI 531719	E^e	France	AF519166*
23	*D. deweyi* (K.B. Jensen, S.L. Hatch & J.K. Wipff) C. Yen, J.L. Yang & B. R. Baum	PI 531756	PSt	Caucasus, Russian Federation	EU139478
24	*D. wangii* C. Yen, J.L. Yang & B. R. Baum *Agropyron* Gaertner	PI 380645	PSt	Iran	EU139479
25	*A. cristatum* (L.) Gaertner	H10154	P	Altai, Xinjiang, China	AY740791*
26	*A. mongolicum* (L.) Keng *Australopyrum* (Tzvelev) Á. Löve	D2774	P	China	AF519117*
27	*A. pectinatum* subsp. *retrofractum* (J.W. Vickery) Á. Löve *Hordeum* L.	Crane 86146	W	Australia	AF519118*
28	*H. bogdanii* Wilensky	PI 531761	H	China	AY740789*
29	*H. brevisubulatum* (Trin.) Link *Psathyrostachys* (Nevski) Á. Löve	Y1604	H	Fuyun, Xinjiang, China	AY740790*
30	*P. fragilis* (Boiss.) Nevski	C-46-6-15	Ns	-	AF519169*
31	*P. juncea* (Fisch.) Nevski *Bromus* L.	PI 206684	Ns	Turkey	AF519170*
32	*B. catharticus* Vahl	S20004	-	Kunming, Yunnan, China	AY829228*

*GenBank accession number was published previously by GenBank (http://www.ncbi.nlm.nih.gov).

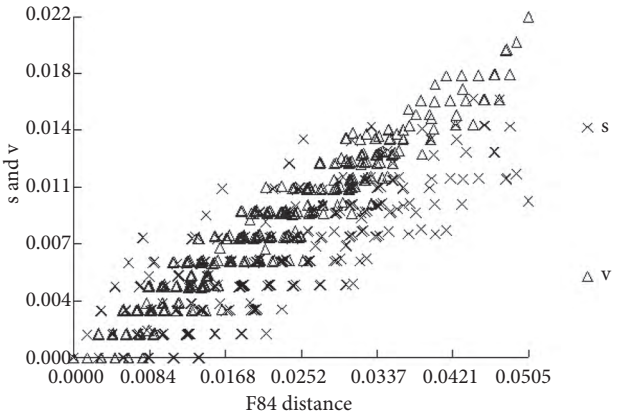

Figure 1. Substitution saturation analysis of *trnL-F* sequences.

Four major clades (St/E, P/W, H, and Ns) were formed. The H clade consisted of *Hordeum* species. The Ns clade included species in *Psathyrostachys*. *Agropyron cristatum* (L.) Gaertner, *A. mongolicum*, and *Australopyrum pectinatum* subsp. *retrofractum* (J.W. Vickery) Á. Löve formed the P/W clade. The St/E clade, the largest, comprised all of the *Pseudoroegneria* species (*Pseudoroegneria cognata* (Hack.) Á. Löve, *Pseudoroegneria gracillima* (Nevski) Á. Löve, *Pseudoroegneria stipifolia* (Czern. ex Nevski) Á. Löve, *Pseudoroegneria strigosa* subsp. *aegilopoides* (Drobow) Á. Löve, *Pseudoroegneria tauri* (Boiss. & Balansa) Á. Löve, *P. libanotica*, *Pseudoroegneria spicata* (Pursh) Á. Löve (2x, 4x), *P. strigosa*, *Pseudoroegneria geniculata* (Trin.) Á. Löve, *P. geniculata* subsp. *scythica*, *P. geniculata* subsp. *pruinifera*, and *P. kosaninii*), together with species in *Roegneria* (*R. alashanica*, *R. elytrigioides*, *R. magnicaespes*, and *R. grandis*), *Elytrigia* (*E. caespitosa*, *E. caespitosa* subsp. *nodosa*, and *E. intermedia*), *Douglasdeweya* (*D. wangii* and *D. deweyi*), and *Lophopyrum* (*Lophopyrum elongatum* (Host) Á. Löve and *Lophopyrum bessarabicum* (Á. Löve) C. Yen, J.L. Yang et Y. Yen). In the St/E clade, *D. deweyi* and *E. caespitosa* subsp. *nodosa* clustered together.

Discussion

Cytologically, genome analysis and GISH (genome in situ hybridization) are commonly used to demonstrate the genome constitution and origin of polyploid taxa in the tribe Triticeae (3,8,9,17,32-35). The molecular data are also successfully utilized to trace the origin of polyploid Triticeae species and their parents (7,21,22,24-26).

The chloroplast genome is maternally inherited in wheat grasses, and the *trnL-F* gene tree represents a maternal genealogy of the polyploid species (23,25). Therefore, it offers an opportunity to identify the maternal parents of polyploid species in *Pseudoroegneria* and related genera.

In the present study, polyploid species *P. spicata* (4x), *P. strigosa*, *P. geniculata*, *P. geniculata* subsp. *scythica*, *E. caespitosa*, *E. caespitosa* subsp. *nodosa*, *E. intermedia*, *R. alashanica*, *R. elytrigioides*, *R. magnicaespes*, *R. grandis*, *D. wangii*, and *D. deweyi* contained the St genome (3,8-10,17,36). They were clustered together with diploid *Pseudoroegneria* species (*P. spicata*, *P. libanotica*, *P. tauri*, *P. gracillima*, *P. stipifolia*, *P. cognata*, and *P. strigosa* subsp. *aegilopoides*). This indicated that the *trnL-F* sequences were highly similar among species in *Pseudoroegneria*, *Roegneria*, *Douglasdeweya*, and *Elytrigia*, and diploid *Pseudoroegneria* species were suggested as their chloroplast genome donors. The genomic constitutions of *P. geniculata* subsp. *pruinifera* and *P. kosaninii* are unknown, and they were also in one clade together with diploid *Pseudoroegneria* species. This indicated that they have the St genome, originated from diploid *Pseudoroegneria* species. Based on the *ndhF* data, Redinbaugh et al. (37) suggested that there is a strong preference for cpDNA inheritance from the St-containing parent in hybridizations between Triticeae species, and the St-containing parent as the female may be more successful. Similar results were also obtained in this study, and all of the polyploid species were clustered together with diploid *Pseudoroegneria* species.

A. mongolicum is indicated as the maternal donor of *D. wangii* based on morphological comparison (8). In the present analysis, *D. wangii* and *A. mongolicum* were in different clades, and *D. wangii* was closely related to *Pseudoroegneria* species. This provided strong evidence that the maternal donor of *D. wangii* was derived from diploid *Pseudoroegneria* species, and that the *Agropyron* species was the paternal donor of *D. wangii*. Jensen et al. (9) suggested that *P. stipifolia* is most likely the maternal donor of *D. deweyi*. In this study, the St genome served as the

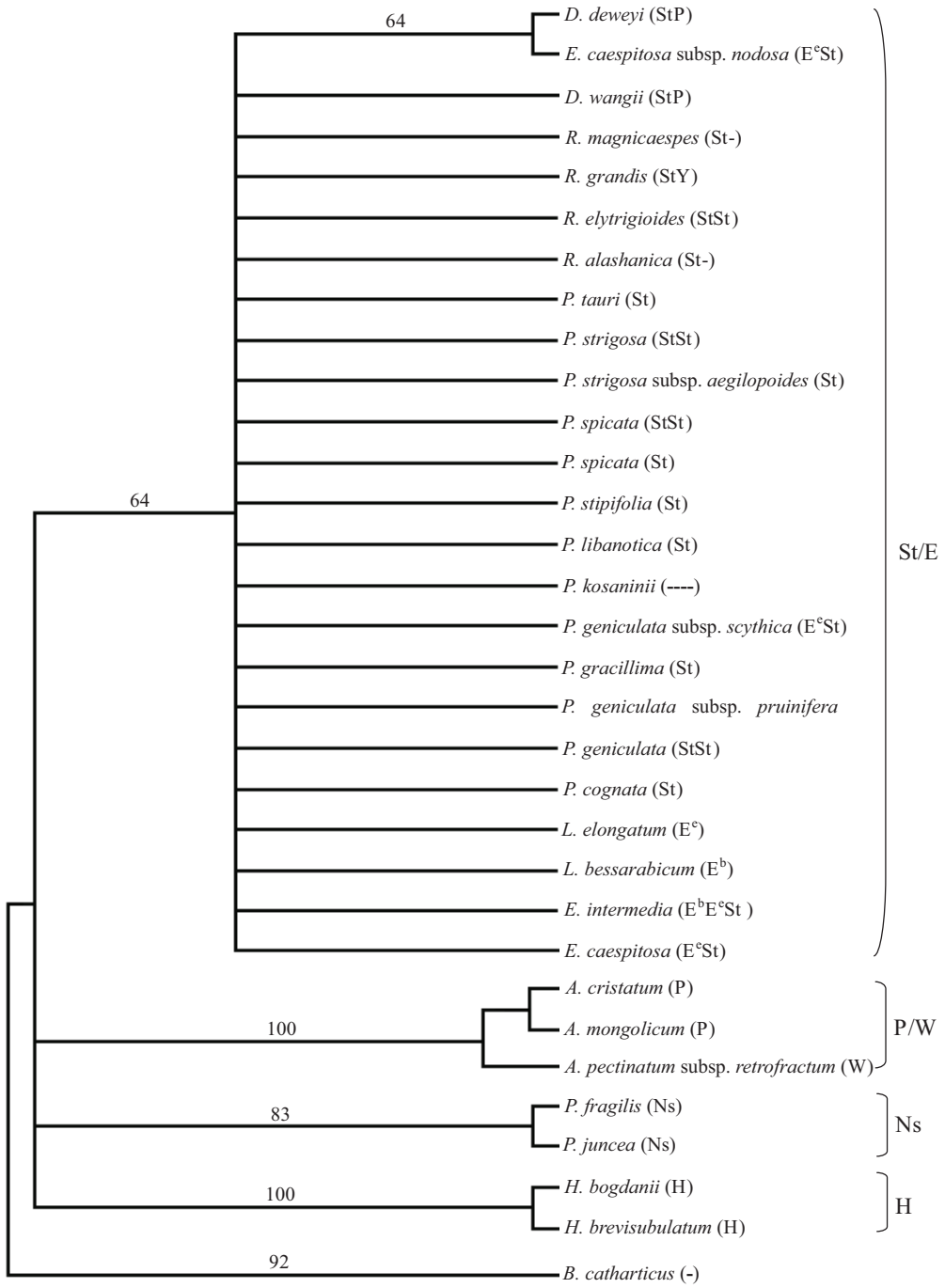

Figure 2. The strict consensus tree of 154 most parsimonious trees inferred from *trnL-F* sequences of all the accessions used in this study. Numbers above the branches indicate bootstrap values >50% by maximum parsimony (MP) analyses. Capital letters in parentheses indicate the genome type of the species. The genome type (St/E, P/W, Ns, or H) of a monophyletic group is given to the right.

maternal genome donor of *D. deweyi*. However, it is not known whether *P. stipifolia* is the maternal donor of *D. deweyi*.

Molecular studies have successfully revealed the evolutionary history of polyploids and phylogenetic relationships in plants (23-25). The cpDNA sequence

region has recently proven to be of great significance at high taxonomic levels for inferring the maternal parents of polyploid species (38). The substitution saturation analysis of *trnL-F* sequences in this study suggested that it is suitable for phylogenetic analysis. *L. elongatum* and *L. bessarabicum* have E^e and E^b genomes, respectively. They were clustered together with diploid *Pseudoroegneria* species. This indicated that their *trnL-F* sequences were highly similar, and the E genome (E^e and E^b) was closely related to the St genome. *Agropyron* species and *Australopyrum* species were in one clade. This indicated that their *trnL-F* sequences were similar, and the P genome was closely related to the W genome. These results were consistent with previous molecular and cytological studies (23-25,39). *Hordeum* species and *Psathyrostachys* species were clustered into different clades, respectively, which revealed that the *trnL-F* sequences of species with H or Ns genomes diverged greatly from those of species with St, E, P or W genomes. Mason-Gamer et al. (23) reported similar results based on molecular evidence from *rpoA*, tRNA spacers, restriction sites, and their combined data.

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