Theor Appl Genet (2004) 108:535–542
DOI 10.1007/s00122-003-1453-3

ORIGINAL PAPER

Erin McMillan · Genlou Sun

Genetic relationships of tetraploid *Elymus* species and their genomic donor species inferred from polymerase chain reaction-restriction length polymorphism analysis of chloroplast gene regions

Received: 1 July 2003 / Accepted: 28 August 2003 / Published online: 26 September 2003

Abstract The genetic relationships of 38 individuals from 13 *Elymus* tetraploid species, two *Pseudoroegneria* species and one *Hordeum* species were examined using polymerase chain reaction-restriction length polymorphism analysis of chloroplast gene regions. The 13 *Elymus* species contain SH and SY genomes with either a single spikelet or multiple spikelets per rachis node. The *Pseudoroegneria* and *Hordeum* species contain an S genome with single spikelet per rachis node and an H genome with multiple spikelets per rachis node, respectively. Four chloroplast gene regions, *trnD-trnT* intron, *trnK* [tRNA-Lys (UUU) exon1]–*trnK* [tRNA-Lys (UUU) exon2], *trnC-trnD*, and *rbcL* were amplified with specific primers and subsequently digested with up to 16 different restriction enzymes. Interspecific variation was detected in the four regions. A dendrogram based on similarity matrices using the unweighted pair group method with arithmetic average algorithm separated the 38 individuals into two distinct groups: the *Elymus* and *Pseudoroegneria* species as one group and *Horduem* as a second group. This result corresponded well with previous findings, and strongly suggested that a *Pseudoroegneria* species is the maternal donor to tetraploid *Elymus* species. Unlike previous studies using nuclear genes, the chloroplast DNA used in this study could not clearly separate the SY-genome species from SH-genome species. No clear separation between the species with a single spikelet per rachis node and the species with multiple spikelets per rachis node was found. Intra-specific variation was detected for the species studied. These observations provide molecular evidence for the highly diverse nature of the *Elymus* gene pool based on morphological characteristics.

Communicated by H.F. Linskens

E. McMillan · G. Sun (✉)
Biology Department, Saint Mary's University,
Halifax, Nova Scotia, B3H 3C3, Canada
e-mail: genlou.sun@smu.ca
Tel.: +1-902-4205642
Fax: +1-902-4968104

Introduction

Elymus L. is the largest and most widely distributed genus in the grass tribe *Triticeae*. The genus encompasses approximately 150 species occurring worldwide. Although predominately a northern temperate genus, *Elymus* species occur from the Arctic and temperate to subtropical regions. These species inhabit various ecological niches, including grasslands, semi-desert, mountain slopes and valleys among bushes, and inside or along the edge of forests. Asia is an important center for the origin and diversity of the perennial species in the *Triticeae*, including *Elymus*, where more than half, approximately 80, of the known *Elymus* species originated. North America is host to the second most abundant number of *Elymus* species, approximately 50, which are thought to have low barriers to interspecific hybridization.

As an exclusively allopolyploid genus, *Elymus* has its origin in other groups, and thus it has close relationships with other genera in the *Triticeae*. Cytologically, five basic genomes, namely, S, H, Y, P and W in different combinations, have so far been found in this genus (Jensen 1990). The S genome is so far the most important composition of the genus. The S genome donor, *Pseudoroegneria*, is a perennial genus containing approximately 15 species of diploids (S) and tetraploids (SS, S_1S_2 or SP) (Jensen et al. 1992; Lu 1994). Many intergeneric hybrids between diploid *Pseudoroegneria* and tetraploid *Elymus* species with the SH and SY genomes have been generated, and these hybrids were relatively easy to produce, even without the assistance of embryo rescue (Lu 1994). Meiotic pairing data indicates that the S genomes in the two genera have very high homology (up to 7 bivalents per cell), although with slight modifications in some species (Löve and Connor 1982; Lu 1991, 1994).

The taxonomy of *Elymus* is extremely complex because of the huge morphological variation within and between species (both environment and developmental stage can affect morphometric markers), the polyploid origin of the genus and the frequent spontaneous

hybridizations between species. An additional factor that adds to the complexity is the wide divergence of classification concepts among taxonomists. The main arguments relating to the circumscription of *Elymus* proposed by various authors revolve around whether to employ single versus multiple spikelets per rachis node as a key characteristic in the taxonomic circumscription. Some taxonomists delimited *Elymus* by including all species with multiple spikelets per rachis node, and thus placed those species with single spikelets per node into other genera, such as *Agropyron* or *Roegneria*; the treatments used, for example, by Hitchcock (1951), Keng (1959) and Baum (1983). In these treatments, *Elymus* had rather narrow circumscription, and encompassed few species. However, other taxonomists, such as Tzvelev (1976), Melderis et al. (1980) and Löve (1984), did not consider this morphological feature significant in generic circumscription. They included species in *Elymus* regardless of the number of spikelets per rachis node, and their circumscription was therefore much wider and included many species.

Today, there is no complete agreement on the circumscription of the genus. The reason, apart from the aforementioned differences in criteria used, is that various taxonomists deal with *Elymus* species from different geographical and historical perspectives. The various taxonomist classifications are based on material from different geographic regions, and users tend to follow the classification systems established by their own taxonomists. As an example, in North America taxonomists tend to follow Hitchcock's (1951) definition of *Elymus*, Chinese agrostologists essentially follow Keng's (1959) treatment of the *Triticeae*, whereas in Russia and Europe classification systems of Tzvelev's (1976) and Melderis et al. (1980), respectively, are followed to a great extent.

Traditionally, species of *Elymus* are classified into approximately ten different taxonomic sections based on their morphological similarities. Analysis of meiotic chromosome pairing has been widely applied in interspecific and inter-generic hybrids to elucidate homology or homoeology between the parental genomes in *Elymus* (e.g., Lu and Salomon 1992). In cytogenetic analyses, the degree of chromosome pairing at meiosis in microspore mother cells is assumed to indicate the overall degree of similarity (an thus relatedness) among species. The data given by meiotic studies together with other sources of information, such as morphology, distribution and isozyme variation, can facilitate a better understanding of phylogenetic relationships within a group of organisms (Bothmer et al. 1986).

DNA molecular markers represent a significant resource for investigating genetic relatedness and phylogenetic relationships. Molecular phylogenetic studies addressing the *Elymus* genus in the *Triticeae* are far fewer in number (Svitashev et al. 1996, 1998; Sun et al. 1997; Redinbaugh et al. 2000; Mason-Gamer 2001; Mason-Gamer et al. 2002). Most of these studies were based on nuclear genome data (Svitashev et al. 1996, 1998; Sun et al. 1997; Mason-Gamer 2001) or only involved North American *Elymus* species (Mason-Gamer et al. 2002). Our investigation was designed to study the molecular relationships of North American and Asian *Elymus* tetraploid species using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of chloroplast gene regions. The objectives of this study were (1) to investigate the maternal genome donor to North American and Asian SH- and SY-genome tetraploid species, (2) to test whether this approach enables us to discriminate between the SY and SH genomes species as in previous studies using nuclear genes and (3) to evaluate whether or not the multiple versus single spikelets per rachis node is a key component reflecting the genomic similarity of the species.

Materials and methods

Plant materials

A total of 38 individuals from 16 species representing SH and SY genomes with either a single spikelet or multiple spikelets per rachis node, and S and H genome donor species were used in this study. Thirteen species of the genus *Elymus*, two species belonging to the genus *Pseudoroegneria* and one species of the genus *Hordeum* were chosen for the study. The genomic constitutions, accession numbers, spikelet characteristic and origin of individuals used in this investigation are given in Table 1.

DNA extraction

Leaf tissue samples were frozen in liquid nitrogen, and stored at −80°C. DNA extraction followed the procedure of Junghan and Metzlaff (1990) with modifications according to Sun et al. (1997). The frozen leaf tissue (300 mg) was pulverized in liquid nitrogen. The powder was suspended in 800 μl of lysis buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 50 mM EDTA, 0.5% SDS, 10 mM β-mercaptoethanol) for 15 min at room temperature. Then 600 μl (Phenol-Tris)-chloroform (pH 7.5) was added, the mixture shaken vigorously, incubated for 2 min at room temperature and then centrifuged for 4 min at 1,200 rpm. The supernatant (with DNA) was transferred to a new tube and one volume of chloroform was added. The DNA was precipitated with 95% cold ethanol. After one hour of incubation at −20°C, the DNA was pelleted by centrifugation for 30 min. The DNA was washed twice with cold 70% ethanol, re-suspended in 400 μl TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, pH 8.0) and then digested with 50 μg/ml RNase at 37°C for 30 min. The RNase was removed by one extraction with 400 μl (Phenol-Tris)-chloroform (pH 7.5) and another extraction with 40 μl chloroform. The DNA was precipitated with cold absolute ethanol. The precipitated DNA was washed twice with cold 70% ethanol, air-dried and re-suspended in 50–100 μl TE buffer. The isolated genomic DNA was stored at −20°C until further use.

PCR amplification and restriction enzyme digestion

Several pairs of primers designed from chloroplast genes were used in this study (Sun 2002). The primer name and sequences are given in Table 2. Amplification of DNA was carried out in 20 μl reaction mixtures containing 20 ng template DNA, 0.2 μM of each primer, 2.0 mM $MgCl_2$, 0.2 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 1 unit of *Taq* DNA polymerase (Rose Scientific, Edmonto, Alberta, Canada) and water to the final volume. The mixture was amplified using the Techne "Genius" Thermo-cycler. PCR was used to amplify the chloroplast *trnD-trnT* intron, *trnC-*

Table 1 *Elymus*, *Pseudoroegneria* and *Hordeum* species used in this study together with their genomic constitution, accession numbers, spikelet characteristic and origin

No.	Species	Genome	Spikelet/node	Accession[a]	Origin
1	*E. hystrix* L.	SSHH	Multiple	H5495	Canada
2	*E. hystrix* L.	SSHH	Multiple	H3997	USA
3	E. *hystrix* L.	SSHH	Multiple	PI531616	USA
4	*E. komarovii* (Roshev.) Löve	?	Multiple	H3618	URS
5	*E. caninus* (L.) L.	SSHH	Single	PI314210	Former Soviet Union
6	*E. caninus* (L.) L.	SSHH	Single	PI314205	Former Soviet Union
7	*E. caninus* (L.) L.	SSHH	Single	PI314612	Former Soviet Union
8	*E. confusus* (Roshev.) Tzvel.	SSHH	Single	PI598463	Russian Federation
9	*E. confusus* (Roshev.) Tzvel.	SSHH	Single	PI10312	Russian Federation
10	*E. ciliaris* (Trin.) Tzvel.	SSYY	Single	PI564917	Russian Federation
11	*E. ciliaris* (Trin.) Tzvel.	SSYY	Single	PI531575	China
12	*E. pendulinus* (Nevski) Tzvel.	SSYY	Single	PI531650	China
13	*E. pendulinus* (Nevski) Tzvel.	SSYY	Single	PI531649	China
14	*E. abolinii* (Drob.) Tzvel.	SSYY	Single	PI531554	China
15	*E. abolinii* (Drob.) Tzvel.	SSYY	Single	PI531556	Estonia
16	*E. abolinii* (Drob.) Tzvel.	SSYY	Single	PI531557	Estonia
17	*E. sibiricus* L.	SSHH	Multiple	PI499456	China
18	*E. sibiricus* L.	SSHH	Multiple	PI499460	China
19	*E. sibiricus* L.	SSHH	Multiple	PI499461	China
20	*E. virginicus* L.	SSHH	Multiple	PI436945	USA
21	*E. virginicus* L.	SSHH	Multiple	PI436950	USA
22	*E. canadensis* L.	SSHH	Multiple	PI531569	Uzbekistan
23	*E. canadensis* L.	SSHH	Multiple	PI315864	Slovakia
24	*E. canadensis* L.	SSHH	Multiple	PI232250	USA
25	*E. canadensis* L.	SSHH	Multiple	PI564908	USA
26	*E. mutablis* (Drob.) Tzvel.	SSHH	Single	PI315491	Former Soviet Union
27	*E. mutablis* (Drob.) Tzvel.	SSHH	Single	PI499449	China
28	*E. alaskanus* (Scrib. & Merr.) Löve	SSHH	Single	H10596	Greenland
29	*E. alaskanus* (Scrib. & Merr.) Löve	SSHH	Single	H10619	Canada
30	*E. trachycaulus* (Link) Gould ex Shinn.	SSHH	Single	PI236722	Maryland, USA
31	*E. trachycaulus* (Link) Gould ex Shinn.	SSHH	Single	PI440097	Kazakhstan
32	*P. spicata* (Pursh) A. Löve	SS	Single	PI286198	USA
33	*P. spicata* (Pursh) A. Löve	SS	Single	PI232140	USA
34	*P. tauri* (Boiss. & Balansa) A. Löve	SSPP	Single	PI401330	Iran
35	*P. tauri* (Boiss. & Balansa) A. Löve	SSPP	Single	PI380650	Iran
36	*H. bogdanii* Wilensky	HH	Multiple	PI499498	China
37	*H. bogdanii* Wilensky	HH	Multiple	PI499645	China
38	*H. bogdanii* Wilensky	HH	Multiple	PI531762	Tajikistan

[a] PI refers to the Plant Introduction number of the USDA; H refers to the collection number from the Swedish University of Agricultural Sciences.

Table 2 DNA sequences of the primer pairs used in this study

Primer pair	5′→3′ sequence	Annealing temp. (°C)	Approximate size (bp)
*trn*T	CCC TTT TAA CTC AGT GGT AG	54	1200
*trn*D	ACC AAT TGA ACT ACA ATC CC	–	–
*trn*K	CAA CGG TAG AGT ACT CGG CTT TTA	54	1100
*trn*K	GGG TTG CCC GGG ACT CGA AC	–	–
*trn*C	TCC CCA GTT CAA ATC TGG GT	48	2300
*trn*D	GGG ATT GTA GTT CAA TTG GT	–	–
*rbc*L	TGT CAC CAA AAA CAG AGA CT	55	1400
*rbc*L	TTC CAT ACT TCA CAA GCA GC	–	–

trnD and *trnK* [tRNA-Lys (UUU) exon1]–*trnK* [tRNA-Lys (UUU) exon2] as follows: one cycle of 4 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 54°C (or 48°C; see Table 2), 1 min 45 s at 72°C, followed by 10 min at 72°C. PCR conditions for *rbcL* were as follows: 4 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min 30 s at 72°C, followed by 10 min at 72°C.

Amplified PCR products were digested according to the conditions recommended by the supplier (MBI Fermentas) using 5–10 units of enzyme per 10 μl of PCR product and incubating the reaction overnight. PCR products from two or three successfully amplified DNA samples were initially digested with a multitude of restriction endonucleases to determine which enzymes could cleave the amplified DNA region. The digestion reaction as well as a marker and several undigested PCR products were run on 2% agarose gels. The restriction fragments from complete sample set digestions, along with a 1 kb ladder-marker DNA were separated using acrylamide (6%) gel electrophoresis in 1× Tris-borate buffer, stained with ethidium bromide and photographed under UV light. The restriction enzymes that were used in this study included: *Alu*I,

*Hinf*I, *Dra*I, *Hae*III, *Msp*I, *Bme*1390I, *Mva*I, *Xho*I, *Bcl*I, *Hin* 6I, *Bgl*I, *Pst*I, *Taq*I, *Hpa*II, *Rsa*I and *Nmu*CI.

Data analysis

Data was scored as 1 for the presence and 0 for the absence of a DNA band for each digestion with each PCR fragment. A data matrix was entered into the NTSYS (Numerical Taxonomy System) program (Rohlf 1993). The data were analyzed using the qualitative routine to generate Jaccard's (J) similarity coefficients. Similarity coefficients were used to construct a dendrogram using the UPGMA (unweighted pair group method with arithmetic average) and the SHAN (sequential, hierarchical and nested clustering) routine in the NTSYS program. A principal coordinate analysis (PCA) was performed with the same program using the DCENTER and EIGEN procedures. The multivariate approach was selected to supplement the cluster analysis because it is more sensitive to closely related individuals, whereas the PCA is more informative regarding distances among major groups (Hauser and Crovello 1982).

Results

PCR amplification

The PCR-RFLP analysis of the four chloroplast gene regions, i.e., *trnD-trnT* intron, *trnK* [tRNA-Lys (UUU) exon1]–*trnK* [tRNA-Lys (UUU) exon2], *trnC-trnD* and *rbcL* was carried out for 38 accessions containing 13 tetraploid *Elymus* species, two *Pesudoroegneria* species and one wild *Hordeum* species. The *trnD-trnT*, *trnK* and *rbcL* primer pairs successfully amplified all 38 accessions with a single fragment for each of the specific primers pairs used. The size of the amplified *rbcL* fragment was 1.4 kb (Fig. 1), that of *trnD-trnT* was 1.2 kb and the size of the amplified *trnK* [tRNA-Lys (UUU) exon1]–*trnK* [tRNA-Lys (UUU) exon2] fragment was 1.1 kb. Primers *trnC-trnD* amplified a 2.3 kb product from 35 *Elymus* and *Pseudoroegneria* species accessions but failed to amplify the DNA from the three *Hordeum* species accessions.

PCR-RFLP analysis

PCR-amplified chloroplast gene regions from the 38 accessions were digested with restriction enzymes for interspecific PCR-RFLP studies. The fragment amplified with the *trnT-trnD* primers from 38 accessions was digested with *Alu*I, *Hinf*I, *Dra*I, *Hae*III, *Msp*I and *Pst*I, since a previous study found that these restriction enzymes recognized at least one restriction site within the *trnT-trnD* region from *Elymus* species (Sun 2002). Interspecific variation was observed for these enzymes (Fig. 2). PCR products from the *trnK* [tRNA-Lys (UUU) exon1]–*trnK* [tRNA-Lys (UUU) exon2] gene region were digested with seven restriction enzymes, i.e., *Dra*I, *Hinf*I, *Alu*I, *Msp*I, *Pst*I, *Hae*III and *Taq*I. Interspecific polymorphism was observed for all enzymes except *Hinf* I (Fig. 3). PCR-RFLP analysis of the *trnC-trnD* region was initially screened for primer-enzyme restriction site

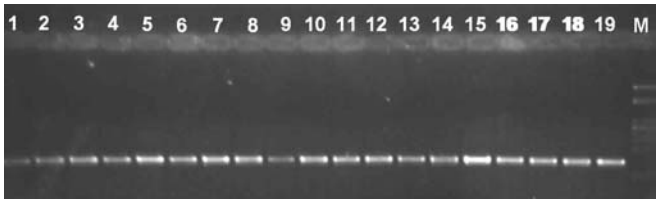

Fig. 1 Amplification of the chloroplast(cp) DNA *rbcL* region in *Elymus* accessions. The DNA fragments were separated on a 1.2% agarose gel and stained with ethidium bromide. *Lanes 1–19* refer to the accession numbers in Table 1. *Lane M* is a lambda DNA/*Pst*I marker

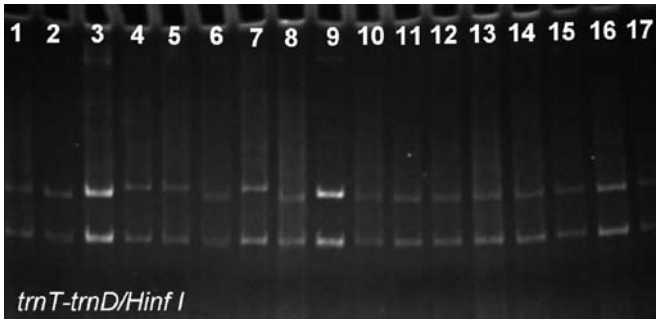

Fig. 2 Restriction fragment patterns of cpDNA from 17 accessions detected by the fragment/enzyme combination *trnT-trnD/Hinf*I. The DNA fragments were separated on a 6% polyacrylamide gel and stained with ethidium bromide. *Lanes 1–11* Accession numbers 1–11, *lanes 12–17* accession numbers 14–19 in Table 1

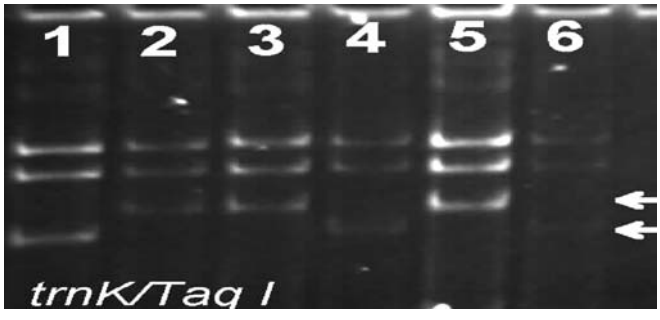

Fig. 3 Restriction fragment patterns of cpDNA from six accessions detected by the fragment/enzyme combination *trnK/Taq*I. The DNA fragments were separated on a 6% polyacrylamide gel and stained with ethidium bromide. *Lanes 1–2 E. virginicus* (accession numbers 20–21), *lanes 3–6 E. canadensis* (accession numbers 22–25). *Arrowheads* show the polymorphic fragments

combinations. A total of 16 restriction enzymes were tested on the *trn*C-*trn*D region. Nine of them (*Msp*I, *Hinf*I, *Hpa*II, *Rsa*I, *Alu*I, *Nmu*CI, *Taq*I, *Pst*I and *Hae*III) recognized restriction sites within this region. No restriction site was found in this region for *Bme*1390I, *Mva*I, *Bgl*I, *Xho*I, *Hinf*I, *Dra*I or *Bcl*I. Interspecific polymorphism was detected for *Msp*I, *Hinf*I, *HpaII*, *Alu*I, and *Hae*III (Fig. 4). The PCR products from the *rbcL* region from three random accessions were screened for recognition sites for ten restriction enzymes: *Bgl*I, *Pst*I, *Hinf*I,

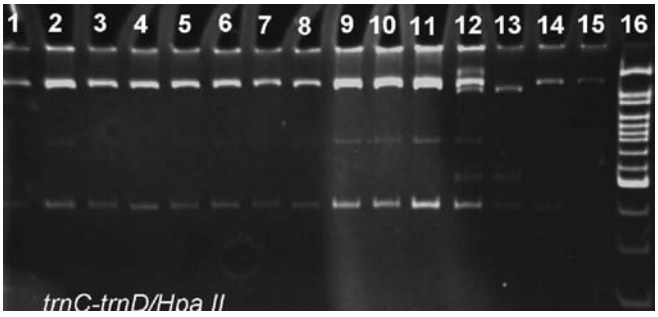

Fig. 4 Restriction fragment patterns of cpDNA from 15 accessions detected by the fragment/enzyme combination *trn*C-*trn*D/*Hpa*II. The DNA fragments were separated on a 6% polyacrylamide gel and stained with ethidium bromide. *Lane 1 E. virginicus* (accession number 20), *lanes 2–15* refer to accession numbers 22–25 in Table 1. *Lane 16* 100 base pair ladder

*Alu*I, *Hae*III, *Rsa*I, *Msp*I, *Xho*I, *Hpa*II and *Dra*I. Restriction sites in the *rbcL* region were found for all tested restriction enzymes except *Bgl*I and *Xho*I. Three restriction enzymes, *Dra*I, *Hinf*I and *Hae*III were used to digest the PCR products from all 38 accessions of *Elymus*, *Pseudoroegneria* and *Hordeum*. Polymorphism was detected with these three enzymes within the *rbcL* region.

Genetic analysis of *Elymus*, *Pseudoroegneria* and *Hordeum* species

The genetic similarity among species was estimated on the basis of the presence or absence of the bands detected in all 38 accessions. The genetic similarity matrix was produced for the PCR-RFLP data using Jaccard's algorithm. Genetic similarity coefficients ranged from 0.35 between *P. spicata* (232140) and *H. bogdanii* (499645) to 1.00 between two *H. bogdanii* accessions (499498 and 531762) and between *E. ciliaris* (531575) and *E. sibiricus* (499456). Genetic similarity values were used for the cluster analysis through UPGMA, resulting in a phenogram (Fig. 5). Cluster analysis divided the species into two main groups, one (I) containing H genome species, and the other (II) either the S genome or the S genome combined with another genome species. Group II was divided into several subgroups. II-1 contained four species with SH genomes. II-2 subgroup contained SY, SH and SP and S genome species. No clear separation between SH genome species and SY genome species was found. Three *E. canadensis* accessions were clustered together. The different accessions of other species were well separated from each other. No variation was detected between one *E. ciliaris* accession and one *E. sibiricus* accession. Associations among the 38 genotypes were also examined with PCA (Fig. 6). Principal coordinates 1, 2 and 3 explained 21%, 12% and 9%, respectively, of the associations. The first coordinate (PC1) clearly separated the *Hordeum* species from other species. The second coordinate (PC2) separated the *Elymus* species and *Pseudoroegneria* into two groups. All SY-genome species, except for one accession of *E. pendulinus,* were grouped into group II.

Discussion

Polyploid evolution has received great attention from botanists in recent decades because of its ubiquity in plants (Grant 1981; Masterson 1994) and because poly-

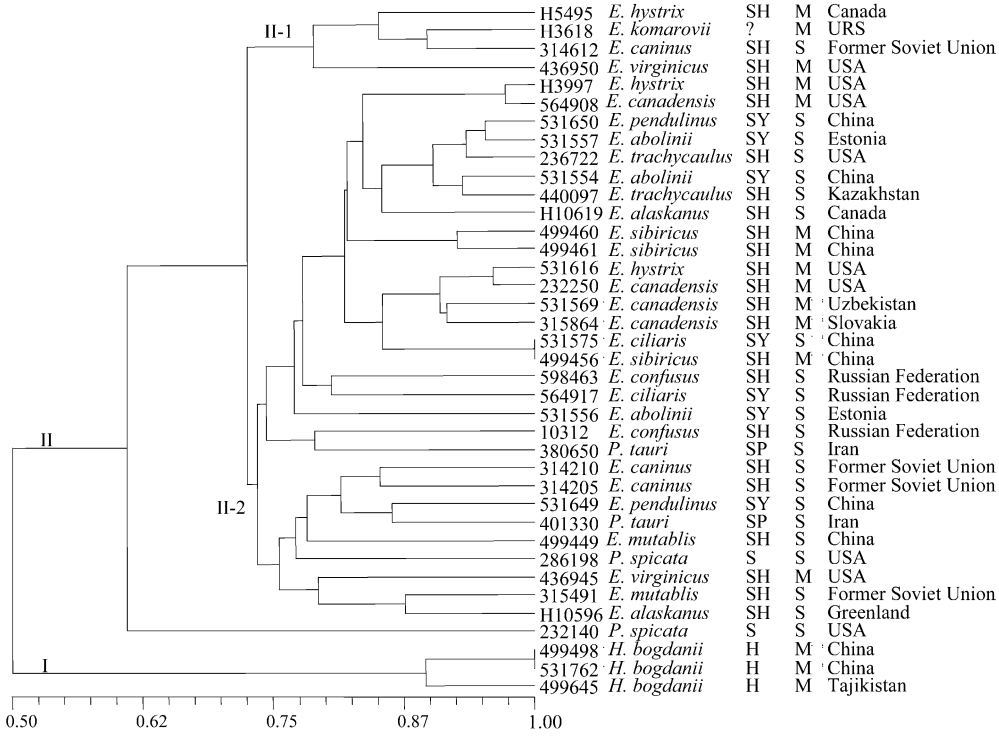

Fig. 5 Phenogram of polymerase chain reaction-restriction length polymorphism (PCR-RFLP) data based on Jaccard's coefficient. *M* and *S* represent multiple and single spikelet per node, respectively

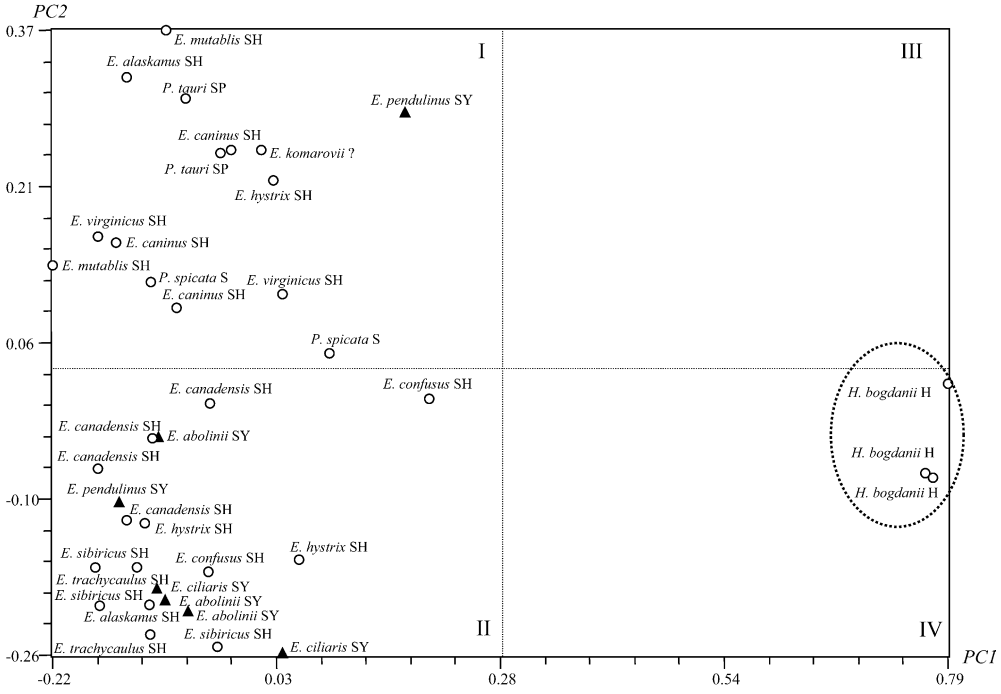

Fig. 6 Association among 38 accessions on the basis of the first two principal coordinates (*PC1*, *PC2*) obtained from a principal coordinate analysis of Jaccard similarity coefficients based on PCR-RFLP data

ploidization may be a significant means of speciation (Leitch and Bennett 1998). Cytogenetic techniques have been used extensively in *Elymus* to clarify its evolutionary origin and genetic limits, and the genus has thus been defined as the collection of all allopolyploid *Triticeae* species that contain an S (*Pseudoroegneria*) genome combined with H (derived from *Hordeum*), Y (from an unknown donor), P (from *Agropyron*) or W (from *Australopyrum*) (e.g., Dewey 1984; Jensen 1990; Salomon and Lu 1992; Jensen and Salomon 1995). Studies of genomic complement, based on patterns of genome pairing, have suggested that tetraploid SH genome *Elymus* species originated from diploid *Pseudoroegneria* (S) species and diploid *Hordeum* (H) species, and SY genome *Elymus* species were derived from diploid *Pseudoroegneria* (S) species and an unknown diploid species (Y). Recent molecular phylogenetic analyses of North American species using the granule-bound starch synthase gene support the idea that North American *Elymus* species combine the S genome of *Pseudoroegneria* with the H genome of *Hordeum* in an SSHH allotetraploid configuration (Mason-Gamer 2001). Unlike nuclear genes, the chloroplast genome is maternally inherited in grasses, provides a mechanism by which to determine the direction of hybridization in polyploid evolution and can thus be used to identify the maternal genome donor of a given polyploidy. Chloroplast (cp) DNA sequence analysis of the *ndhF* region indicated that the *Pseudoroegneria* chloroplast genome was preferred in the speciation of *Elymus* species and *Pascopyrum smithii* (Rydb.) A. Löve (Jones et al. 2000; Redinbaugh et al. 2000). Phylogenetic analysis of North American *Elymus* and the monogenomic *Triticeae* using three cpDNA data sets suggest that *Pseudoroegneria* is the chloroplast genome donor to North American *Elymus* (Mason-Gamer et al. 2002). Our data corresponds well with previous findings and strongly suggests that a *Pseudoroegneria* species is the maternal donor to tetraploid *Elymus* species.

Cytogenetic studies of genome-pairing data have shown that tetraploid *Elymus* species are separated into two groups with different genome compositions: the SH- and SY-genome tetraploids (e.g., Dewey 1980; Lu and Bothmer 1990; Lu 1993). It has been shown that the species groups in *Elymus,* distinguished on the basis of isoenzyme data (Jaaska 1992), fit well with the genomic groups established by cytogenetic studies. Recently, molecular studies using repetitive DNA markers and randomly amplified polymorphic DNA markers clearly separated SH-genome species from SY-species (Svitashev et al. 1996; Sun et al. 1997). In contrast to genome-pairing results and repetitive DNA and randomly amplified polymorphic DNA results, this study, based on PCR-RFLP analysis of cpDNA, did not clearly show a separation between SH-genome species and SY-species. This finding suggests that there is no chloroplast differentiation between these two groups; both *Elymus* SY-genome and SH-genome tetraploid species originated from the same maternal donor, a *Pseudoroegneria* species. That our cpDNA results contradict nuclear gene data is not exceptional for *Triticeae* species. In the past few years, the relationships among diploid genera of *Triticeae* have been repeatedly addressed using molecular data. These include two chloroplast DNA data sets, one based on restriction site variation (Mason-Gamer and Kellogg 1996a) and the other on nucleotide sequences of the gene encoding the α-subunit of the RNA polymerase (*rpoA*; Petersen and Seberg 1997). Published nuclear gene molecular data sets include sequences from the internally

transcribed spacer (ITS) region (Hsiao et al. 1995), sequences from intergenic spacer regions from two independently evolving 5S rDNAs (Kellogg and Appels 1995) and sequences from the starch synthase gene (Mason-Gamer and Kellogg 2000). The most extensive differences are seen in comparisons between cpDNA and nuclear gene data (Kellogg et al. 1996; Mason-Gamer and Kellogg 1996b; Petersen and Seberg 1997). The main reason is probably that the nuclear genome and the plastid genome have had different evolutionary histories. Since the plastids are generally inherited uni-parentally, their evolutionary history may not reflect the history of the organisms, especially in a tribe in which hybridization is so common. This problem is most serious at the polyploid level, especially since it has been shown that the same morphologically defined polyploid taxon may arise several times (Soltis and Soltis 1993).

As mentioned in the introduction, morphological characters such as single versus multiple spikelets at each rachis node have been adopted by some taxonomists, for example, Nevski (1934), Keng (1959), and Baum (1983) as the most important key character used to distinguish the genus *Elymus* (with multiple spikelets) from *Roegneria* (with single spikelets). Many tetraploid species, e.g., *E. caninus*, *E. confuses*, *E. mutablis*, *E. cilaris*, *E. pendulinus*, and *E. abolinii*, have all been placed in the genus *Roegneria* by the above-mentioned taxonomists because of the single spikelets per rachis node, where *E. sibiricus*, *E. virginicus*, and *E. canadensis* have always been kept in *Elymus* because of their multiple spikelets per rachis node. Cluster analysis in this study showed no clear separation between the species with the single spikelets per rachis node and the species with multiple spikelet per rachis node. This result is consistent with cytogenetic evidence, and isozyme electrophoresis data, suggesting that the number of spikelets per rachis node has little or no biological relevance to the phylogenetic relationships among the species (Lu 1994).

The present study was based on PCR-RFLP analysis of four cpDNA fragments. The PCR-RFLP method is convenient for surveying many samples and for investigating many cpDNA regions simultaneously. Compared with the conventional restriction fragment analysis that employs DNA hybridization, PCR-RFLP analysis on MetaPhor agarose gels or polyacrylamide gels is more sensitive for the detection of small-length differences (5–200 bp) among digested fragments. PCR-RFLP polymorphism was detected among the 38 accessions for all four tested primer pairs. Genetic similarity values ranged from 0.35 between *P. spicata* (232140) and *H. bogdanii* (499645) and 1.00 between two *H. bogdanii* accessions (499498 and 531762) and between *E. ciliaris* (531575) and *E. sibiricus* (499456). Intraspecific variation was detected for all species studied. These observations provided molecular evidence for the highly diverse nature of the *Elymus* gene pool based on morphological characteristics. This result suggests that, at least in these genera, phylogenetic studies would require an analysis of a much larger number of individuals, and that the choice of species for phylogenetic standards could have influenced the interpretation of cpDNA sequence data.

Acknowledgements We thank Dr. B. Salomon at The Swedish University of Agricultural Sciences and the Regional Plant Introduction Station, USDA, for kindly supplying the seeds used in this study. This research was supported with grants from NSERC, a Senate Research Grant and the Student Employment Experience Program (SEEP) at Saint Mary's University.

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