

Phylogenetic analysis of North American *Elymus* and the monogenomic Triticeae (Poaceae) using three chloroplast DNA data sets

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Abstract: Although the monogenomic genera of the Triticeae have been analyzed in numerous biosystematic studies, the allopolyploid genera have not been as extensively studied within a phylogenetic framework. We focus on North American species of *Elymus*, which, under the current genomic system of classification, are almost all allotetraploid, combining the **St** genome of *Pseudoroegneria* with the **H** genome of *Hordeum*. We analyze new and previously published chloroplast DNA data from *Elymus* and from most of the monogenomic genera of the Triticeae in an attempt to identify the maternal genome donor of *Elymus*. We also present a cpDNA phylogeny for the monogenomic genera that includes more data than, and thus builds on, those previously published. The chloroplast DNA data indicate that *Pseudoroegneria* is the maternal genome donor to all but one of the *Elymus* individuals. There is little divergence among the *Elymus* and *Pseudoroegneria* chloroplast genomes, and as a group, they show little divergence from the rest of the Triticeae. Within the monogenomic Triticeae, the problematic group *Thinopyrum* is resolved as monophyletic on the chloroplast DNA tree. At the intergeneric level, the data reveal several deeper-level relationships that were not resolved by previous cpDNA trees.

Key words: chloroplast genome, phylogeny, polyploidy, systematics.

Résumé : Tandis que les genres monogénomiques chez les hordées ont été analysés dans de nombreuses études biosystématiques, les genres allopolyploïdes n'ont pas été étudiés autant dans un cadre d'analyse phylogénétique. Les auteurs ont choisi d'étudier les espèces nord-américaines du genre *Elymus*, lesquelles sont presque toutes allotétraploïdes, d'après le système actuel de classification génomique, combinant le génome **St** du genre *Pseudoroegneria* et le génome **H** du genre *Hordeum*. Les auteurs ont analysé des séquences d'ADN chloroplastique (certaines nouvelles, d'autres rapportées antérieurement) provenant du genre *Elymus* et de la plupart des genres monogénomiques chez les hordées afin d'identifier le parent maternel du genre *Elymus*. Les auteurs présentent également une phylogénie basée sur l'ADNcp pour les genres monogénomiques, une phylogénie qui s'appuie sur plus de données que les phylogénies produites antérieurement. Les données tirées de l'analyse de l'ADN chloroplastique indiquent que le genre *Pseudoroegneria* constitue le parent maternel de tous les *Elymus* sauf un. Il y a peu de divergence entre les génomes chloroplastiques des genres *Elymus* et *Pseudoroegneria* qui, à leur tour, présentent peu de divergence par rapport aux autres hordées. Parmi les hordées monogénomiques, le groupe problématique des *Thinopyrum* apparaît monophylétique au sein de l'arbre basé sur l'ADN chloroplastique. Au niveau intergénérique, les données révèlent plusieurs relations complexes qui n'avaient pas été mises en évidence dans les études antérieures.

Mots clés : génome chloroplastique, phylogénie, polyploidie, systématique.

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Introduction

Cytogenetic studies of genome-pairing data have had profound effects on the classification of the Triticeae (e.g., Dewey 1982, 1984; Löve 1982, 1984; Barkworth and Dewey

1985). In cytogenetic analyses, the degree of chromosome pairing at meiosis in microspore mother cells is assumed to indicate the overall degree of similarity (and thus relatedness) among genomes. In the Triticeae, cytogenetic data have been especially widely used in the delimitation of gen-

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era and in the determination of the origins of the many polyploids in the tribe, and have had enormous influence on classification within the tribe. In North America, the genomic system of classification exists side by side with the traditional system of Hitchcock (1951). Both systems are commonly used, but they are very different in terms of generic delimitation, and this duality can lead to confusion and miscommunication. Even if few botanists believe that the traditional system accurately reflects the phylogenetic history of the Triticeae, it is based on clearly visible characters that are easy to recognize in the field. The genomic system, on the other hand, is intended to reflect evolutionary history, but its genera can be difficult to distinguish on morphological grounds. Furthermore, the very ability of cytogenetic data to uncover phylogenetic history remains controversial, and has been questioned on philosophical and methodological grounds (reviewed in Seberg and Petersen 1998).

In spite of such concerns about the utility of genome-pairing data for classification, the data do appear to be useful for the delimitation of monophyletic groups. Most molecular phylogenetic studies of the monogenomic Triticeae agree that many of the genomically defined genera are monophyletic (Kellogg and Appels 1995; Hsiao et al. 1995; Mason-Gamer and Kellogg 1996a, 2000; Petersen and Seberg 1997, 2000), in spite of extensive disagreement regarding relationships among genera (Kellogg et al. 1996; Mason-Gamer and Kellogg 1996b).

Molecular phylogenetic studies addressing the allopolyploid genera in the Triticeae are far fewer in number, and most of the current hypotheses about the origins of the allopolyploid taxa are based on morphology and on patterns of genome pairing. Cytogenetic techniques have been used extensively in *Elymus* to clarify its evolutionary origin and generic limits, and the genus has thus been defined as the collection of all allopolyploid Triticeae species that include an **St** (*Pseudoroegneria*) genome. The genus is explicitly heterogeneous under this definition, because the **St** genome can be found in allopolyploid combinations with **H** (derived from *Hordeum*), **Y** (from an unknown donor), **P** (from *Agropyron*), and **W** (from *Australopyrum*), or with a combination of these genomes in various tetraploid, hexaploid, and (rarely) octoploid configurations (e.g., Dewey 1984; Jensen 1990a, 1990b; Torabinejad and Mueller 1993; Jensen et al. 1994; Jensen and Salomon 1995). However, genome-pairing data suggest that nearly all of the native North American species of *Elymus* are **StStHH** allotetraploids (e.g., Dewey 1982, 1983a, 1983b, 1984). The single possible exception is *Elymus californicus*, an allo-octoploid that lacks an RAPD marker thought to be specific to the **St** genome (Jensen and Wang 1997). This species has not been subject to genome-pairing or other phylogenetic analyses, and its genomic complement is poorly understood. Genome-pairing data from *Elymus* have received some support from studies using repetitive DNA markers and (or) presumed genome-specific random-amplified polymorphic DNA (RAPD) markers (e.g., Svitashv et al. 1996, 1998; Sun et al. 1997). A recent phylogenetic analysis of North American *Elymus* species using nuclear starch synthase (GBSSI) gene sequences further suggests that these individuals contain both **St** and **H** nuclear genomes (Mason-Gamer 2001).

This study addresses two main goals. The first is to identify the maternal genome donor to North American *Elymus*. Given our current understanding of these species, the cpDNA donor is expected to be *Pseudoroegneria*, *Hordeum*, or both. The results presented here complement those of Mason-Gamer and Kellogg (1996a), which included a broad sampling from throughout Triticeae, but few **St**-genome polyploids, as well as those of Redinbaugh et al. (2000), which included a wide sampling of **St**-containing polyploids, but did not sample extensively from throughout the tribe. The second goal is to generate a well-supported cpDNA phylogeny for the monogenomic members of the tribe. Two previous studies have addressed this goal (Mason-Gamer and Kellogg 1996a; Petersen and Seberg 1997). Trees from both studies resolved some of the intergeneric relationships, but in both cases, the trees suffered from a partial lack of resolution and (or) support. This study, by combining data and adding an additional data set, yields trees with greater resolution and support.

Materials and methods

Between one and three individuals representing each of nine native North American *Elymus* species and one Asian species (*E. ciliaris*) were included (Table 1). All of these species are presumed to be **StStHH** allotetraploids, except for *E. ciliaris*, which has an **StStYY** complement, and *E. californicus*, an octoploid whose genomic constitution is not well understood. Additional taxa were sampled from throughout the monogenomic Triticeae (Table 1).

The chloroplast tRNA genes trnT, trnL-3', trnL-5', and trnF, along with their intervening non-coding regions, were amplified and sequenced using the primers a, b, c, d, e, and f described in Taberlet et al. (1991). Amplifications were done using primers a and f (CATTACAAATGCGATGCTCT; ATTTGAACTGGTGACACGAG). Some PCR products were cleaned using a Qiaquick gel extraction kit (Qiagen, Valencia, Calif.); others were cleaned with 10 U exonuclease I (#70073Z, USB Corp., Cleveland, Ohio), 1 U shrimp alkaline phosphatase (SAP; #70092Y, USB Corp.), and a 1/10 dilution of SAP reaction buffer, followed by 15 min at 37°C and 15 min at 80°C. The cleaned products were sequenced using ABI BigDye terminators according to product instructions, and reactions were run on an ABI Prism 377 sequencer (PE Applied Biosystems, Foster City, Calif.). Sequencing primers included the amplification primers a and f, along with primers b, c, d, and e (TCTACCGAT-TTCGCCATATC, CGAAATCGGTAGACGCTACG, GGG-GATAGAGGGACTTGAAC, and GGTTC AAGTCCCTCT ATCCC, respectively).

The *rpoA* gene was amplified and sequenced using primers rpoA1, rpoA2, rpoA4, rpoA5, rpoA8, and rpoA9 designed by Petersen and Seberg (1997). We generated new *rpoA* sequences rather than using those generated by Petersen and Seberg (1997) so that we could have a close match among data sets in terms of the individuals used. Amplifications were done using primers rpoA1 and rpoA2 (ACACCTATGCCGCATAATGG and GTTAGGTATTGG-AGCAACAT, respectively). PCR products were cleaned and sequenced as described above. Sequencing primers included

the amplification primers *rpoA1* and *rpoA2*, along with primers *rpoA4*, *rpoA5*, *rpoA8*, and *rpoA9* (AGATCTTCT-TCAGTCTTACT, GAAGTTATCCTATAGATGCT, TCA-AGTTAGCTATAGGTTGT, and GGACACTCTAGAGAA GCATC, respectively).

Restriction site data from the monogenomic genera of the Triticeae and from two species of *Elymus* (*E. lanceolatus* and *E. ciliaris*) are described in Mason-Gamer and Kellogg (1996a). Because restriction site data were not collected from additional *Elymus* representatives, these data contribute mainly to the phylogeny of the monogenomic genera, within which the allopolyploid *Elymus* species are interpreted.

The *rpoA* and tRNA sequences were easily aligned by eye. All analyses of separate and combined data sets were carried out using cladistic parsimony with all characters weighted equally with PAUP* version 4.0 (Swofford 2002). Heuristic searches were run using tree bisection and reconnection (TBR) branch swapping, stepwise addition, and simple addition sequence. Gaps in the sequence alignments were treated as missing data, and 14 insertion–deletion (indel) events were coded as binary characters (10 from the tRNA alignment and 4 from the *rpoA* alignment). Trees from each analysis were used to generate strict consensus trees. Nodal support was estimated using 1000 fast bootstrap replicates, as implemented in PAUP* 4.0 (Swofford 2002). The consistency index (CI), retention index (RI), and rescaled consistency index (RCI) were calculated with uninformative characters excluded. Trees were rooted using *Psathyrostachys*. Both of the earlier cpDNA analyses (Mason-Gamer and Kellogg 1996a; Petersen and Seberg 1997) used *Bromus* (tribe Bromeae) as an outgroup, and both studies provided strong evidence (100% bootstrap in both cases) that *Psathyrostachys* is at the base of the Triticeae tree. In the present study, *Bromus* was excluded from the analyses used to generate our final figures because of regions of ambiguous alignment in the tRNA spacer data set. However, when *Bromus* was included, *Psathyrostachys* remained at the base of the Triticeae tree with 96% bootstrap support, and the tree topology was unaffected (results not shown).

The chloroplast genome is clonally inherited, so there is virtually no chance that the different cpDNA data sets track different histories. However, because different processes of sequence evolution may lead to apparent phylogenetic incongruence (e.g., Bull et al. 1993), the data sets were analyzed separately and compared. First, data sets were reduced to include the 37 individuals that were shared by all three, so that direct comparisons could be made (see Table 1). The reduced data sets were analyzed separately and in combination using unweighted cladistic parsimony. The separate and combined data sets were compared in terms of variable and informative characters, CI, RI, and RCI. The trees from the separate analyses were then examined for reciprocally well-supported conflict. Each individual data set and the combined data set were constrained using strict consensus trees from each of the other data sets. In the constrained analyses, weakly supported nodes (in this case, those with bootstrap support lower than 50%) were collapsed on the constraint trees (i.e., the constraint trees are the 50% majority rule bootstrap trees from each data set). As discussed earlier (e.g., Mason-Gamer and Kellogg 1996b), examining

phylogenetic conflict using constraint trees with weakly supported nodes is likely to indicate conflict where little exists in terms of actual character state distribution. The unresolved parts of the constraint topologies were treated as soft polytomies. Based on the results of the comparisons, the three data sets were combined for the remaining analyses. Parsimony analyses, with equally weighted characters, were done as described above for all taxa including *Elymus*, and for the monogenomic taxa only.

Results

Data sets

The lengths of the PCR products from the tRNA genes and spacers ranged from 1627 to 1767 bp. Of the 2088 aligned sites, 186 were variable and 101 were potentially parsimony informative, and 10 indel characters were coded. In spite of the large difference between the absolute and aligned sequence lengths, the sequences were easily aligned; the increase in the length of the alignment was largely due to taxon-specific insertions. The lengths of the PCR products from the *rpoA* gene ranged from 1347 to 1368 bp. Of the 1374 aligned positions, 66 characters were variable and 37 were potentially parsimony informative, and 4 indel characters were coded. The restriction site characters were presented in detail in Mason-Gamer and Kellogg (1996a). This data set yielded 129 variable sites, 82 of which were potentially parsimony informative, and 2 length changes.

Data set comparisons

Results of analyses of the three separate data sets are summarized in Table 2 and Fig. 1. The three strict consensus trees (Figs. 1A–1C) show few topological differences. The only resolved differences involve the position of *Heterantherium piliferum* and *Pseudoroegneria strigosa* 1, and in neither case are the differences reciprocally well supported. *Heterantherium piliferum* is grouped with *Aegilops*, *Secale*, and *Taeniatherum* with 85% support on the tRNA tree, and its position is unresolved on the restriction site tree. Its conflicting placement with *Thinopyrum* on the *rpoA* gene tree is weak (36% bootstrap support), and is supported by a single G→A change at position 796 of the *rpoA* alignment. However, there is another single *rpoA* change, T→A at position 1192, that groups *Heterantherium* with *Aegilops*, *Secale*, and *Taeniatherum* in complete agreement with the tRNA tree. Two other characters in the *rpoA* data set, at positions 302 and 1315, group *Heterantherium* with *Dasypyrum villosum* 1 and *Hordeum*, respectively. *Pseudoroegneria strigosa* 1 is in a clade with *Thinopyrum*, *Dasypyrum*, *Elymus*, and the rest of *Pseudoroegneria* on both the tRNA spacer and the restriction site trees, with bootstrap support of 50 and 84%, respectively. On the *rpoA* tree, it is at the base of the *Aegilops*, *Taeniatherum*, and *Secale* clade with 47% bootstrap support. This apparently conflicting position is supported by the shared deletion of one of two duplicate copies of the short sequence AAAGAAA from positions 1178–1184 in the *rpoA* alignment. No other *rpoA* characters provide specific information on the placement of *P. aegilopoides* 1.

Table 1. List of taxa.

Taxon ^a	No. ^b	Collection No.	<i>rpoA</i> GenBank accession No.	tRNA GenBank accession No.
<i>Elymus</i>				
<i>Elymus californicus</i> ^c (Bolander) Gould	—	Barkworth s.n.	AY115927	AF519130
<i>Elymus canadensis</i> L.	1	Barkworth 97–86	AY115928	AF519131
<i>Elymus canadensis</i> ^c	2	PI578675	AY115929	AF519132
<i>Elymus canadensis</i> ^c	4	PI531568	AY115930	AF519133
<i>Elymus ciliaris</i> (Trin.) Tzvelev	—	D2811	AY115931	AF519134
<i>Elymus elymoides</i> ^c (Rafin.) Swezey	—	PI531606	AY115932	AF519135
<i>Elymus glaucus</i> ^c Buckley	4	RJMG130	AY115933	AF519136
<i>Elymus glaucus</i> ^c	6	W6–10215	AY115934	AF519137
<i>Elymus glaucus</i> ^c	7	PI593652	AY115935	AF519138
<i>Elymushystrix</i> ^c L.	—	Barkworth 97–87	AY115936	AF519139
<i>Elymus lanceolatus</i> (Scribn. & Smith) Gould	1	R-13–6–10	AY115937	—
<i>Elymus lanceolatus</i>	3	PI531623	AY115938	AF519140
<i>Elymus trachycaulus</i> ^c (Link) Gould ex Shinnars	1	PI372500	AY115939	AF519141
<i>Elymus trachycaulus</i> ^c	3	PI452446	AY115940	AF519142
<i>Elymus virginicus</i> ^c L.	1	PI436945	AY115941	AF519143
<i>Elymus virginicus</i> ^c	2	PI490361	AY115942	AF519144
<i>Elymus virginicus</i> ^c	6	RJMG163	AY115943	AF519145
<i>Elymus wawawaiensis</i> ^c J.Carlson ex Barkworth	1	PI285272	AY115944	AF519146
<i>Elymus wawawaiensis</i> ^c	3	PI598812	AY115945	AF519147
Monogenomic Triticeae				
<i>Aegilops caudata</i> L.	—	G758	AY115908	AF519111
<i>Aegilops speltoides</i> Tausch	—	Morrison s.n.	AY115909	AF519112
<i>Aegilops tauschii</i> Coss.	—	Morrison s.n.	AY115910	AF519113
<i>Aegilops uniaristata</i> Vis.	—	G1297	AY115911	AF519114
<i>Agropyron cristatum</i> (L.) Gaertn.	1	C-3–6–10	AY115912	AF519115
<i>Agropyron cristatum</i>	2	PI281862	AY115913	AF519116
<i>Agropyron mongolicum</i> Keng	—	D2774	AY115914	AF519117
<i>Australopyrum retrofractum</i> (Vickery) Á.Löve	—	Crane 86146	AY115915	AF519118
<i>Australopyrum velutinum</i> (Nees) B.K.Simon	—	D2873–2878	AY115916	AF519119
<i>Dasypyrum villosum</i> (L.) Candargy	1	PI251478	AY115925	AF519128
<i>Dasypyrum villosum</i>	2	PI470279	AY115926	AF519129
<i>Eremopyrum bonaepartis</i> (Spreng.) Nevski	1	H5554	AY115946	AF519148
<i>Eremopyrum bonaepartis</i>	2	H5569	AY115947	AF519149
<i>Eremopyrum distans</i> (C.Koch) Nevski	—	H5552	AY115948	AF519150
<i>Eremopyrum orientale</i> (L.) Jaub. & Spach	—	H5555	AY115949	AF519151
<i>Henrardia persica</i> (Boiss.) C.E.Hubb.	—	H5556	AY115950	AF519152
<i>Heterantherium piliferum</i> (Banks & Sol.) Hochst.	—	PI402352	AY115951	AF519153
<i>Hordeum brachyantherum</i> ^c Nevski	—	PI531764	AY115917	AF519120
<i>Hordeum brevisubulatum</i> (Trin.) Link	—	PI401387	AY115918	AF519121
<i>Hordeum bulbosum</i> L.	—	PI440417	AY115919	AF519122
<i>Hordeum jubatum</i> ^c L.	—	RJMG106	AY115920	AF519123
<i>Hordeum marinum</i> ^c Hudson	—	PI304346	AY115921	AF519124
<i>Hordeum murinum</i> ^c L.	1	PI247054	AY115922	AF519125
<i>Hordeum murinum</i> ^c	2	CIho15683	AY115923	AF519126
<i>Hordeum pusillum</i> ^c Nuttall	—	CIho15654	AY115924	AF519127
<i>Peridictyon sanctum</i> (Janka) Seberg, Fred., & Baden	—	KJ248	AY115952	AF519154
<i>Psathyrostachys fragilis</i> (Boiss.) Nevski	—	C-46–6–15	AY115967	AF519169
<i>Psathyrostachys juncea</i> (Fisch.) Nevski	—	PI206684	AY115968	AF519170
<i>Pseudoroegneria strigosa</i> ssp. <i>aegilopoides</i> (Drobov) Á.Löve	1	MA-109–31–50	AY115953	AF519155
<i>Pseudoroegneria strigosa</i> ssp. <i>aegilopoides</i>	2	PI531755	AY115954	—
<i>Pseudoroegneria libanotica</i> (Hackel) D.R.Dewey	—	PI228391	AY115955	AF519156
<i>Pseudoroegneria spicata</i> subsp. <i>inermis</i> (Scribn., and J.G.Smith) Á.Löve	2	PI236681	AY115956	AF519157
<i>Pseudoroegneria spicata</i> (Pursh) Á.Löve subsp. <i>spicata</i>	3	PI610986	AY115957	AF519158

Table 1. (concluded).

Taxon ^a	No. ^b	Collection No.	<i>rpoA</i> GenBank accession No.	tRNA GenBank accession No.
<i>Pseudoroegneria spicata</i> subsp. <i>spicata</i>	4	D2844	AY115958	AF519159
<i>Pseudoroegneria spicata</i> subsp. <i>inermis</i>	8	D2839	AY115959	AF519160
<i>Secale cereale</i> L.	—	Kellogg s.n.	AY115960	AF519162
<i>Secale montanum</i> Guss.	1	PI440654	AY115961	AF519161
<i>Secale montanum</i> subsp. <i>anatolicum</i> (Boiss.) Tzelev	2	PI206991	AY115962	AF519163
<i>Taeniatherum caput-medusae</i> (L.) Nevski	—	MB-106-41-79	AY115963	AF519164
<i>Thinopyrum bessarabicum</i> (Savul. & Rayss) Á.Löve	—	PI531711	AY115964	AF519165
<i>Thinopyrum elongatum</i> (Host) D.R.Dewey	—	PI531719	AY115965	AF519166
<i>Thinopyrum scirpeum</i> (C.Presl) D.R.Dewey	—	C-15-21-25	AY115966	AF519167
<i>Triticum baeoticum</i> Boiss.	—	Morrison s.n.	—	AF519168

^aVouchers stored at Grey Herbarium or Stillingher Herbarium of the University of Idaho.

^bNumbers identify specific individuals for species represented more than once, and correspond to numbers in Figs. 1 and 2.

^cTaxa without cpDNA restriction site data

Table 2. Features of the three matched data sets, separate and combined, and their trees.

Data set	Variable characters	Informative characters	Tree statistics				
			Trees	Length	CI	RI	RCI
tRNA spacers	152	81	120	194	0.752	0.920	0.778
<i>rpoA</i> gene	58	30	2	66	0.816	0.923	0.825
Restriction sites	126	81	38	157	0.723	0.905	0.726
Combined	336	192	14	421	0.738	0.919	0.754

Tree lengths increase by between 0 and 2 steps when data sets are constrained by the 50% majority rule bootstrap trees derived from the other data sets (Table 3). The tRNA data showed no character conflict with the *rpoA*, restriction site, or combined data constraint trees; i.e., no characters increase or decrease in steps in the constrained analyses. Similarly, no characters from the combined data set require more or fewer steps on the tRNA, *rpoA*, or restriction site trees. The *rpoA* data set shows an overall increase in one step on each of the constraint trees. Not surprisingly, the length change involves the same three characters described above: the AAAGAAA deletion and nucleotide position 796 require two steps rather than one, and nucleotide position 1192 requires one step rather than two. The restriction site data showed no conflict with the *rpoA* tree, and a two-step net increase on the tRNA and combined trees. Depending on which unconstrained and constrained restriction site trees are used for analyzing character steps, the two-step length change can involve sites 34 (+1 step) and 109 (–1 step) and the placement of *Peridictyon*; site 124 (+1 step) and *Heteranthelium*; and (or) site 139 (+1 step) and *Aegilops speltoides*.

Analysis of Triticeae with *Elymus*

The cladistic parsimony search of the three combined data sets yielded more than 325 000 trees of length 501, with a CI of 0.703, RI of 0.899, and RCI of 0.718. The large number of trees (which represents the limit of computer memory with PAUP* 4.0 running on 250 megabytes of allocated

RAM) reflects the fact that some of the *Elymus* and *Pseudoroegneria* sequences are very similar, and that there is little hierarchical structure among them. All but one of the *Elymus* sequences (*E. californicus*) are placed in a *Pseudoroegneria* + *Thinopyrum* + *Dasypyrum* clade (Fig. 2), although the support for this clade is lower than when the monogenomic taxa are analyzed alone (66% vs. 88%).

Elymus californicus is the only representative of *Elymus* that is not in the *Pseudoroegneria* + *Thinopyrum* + *Dasypyrum* clade. A phylogram representing one of the shortest trees (Fig. 2) shows *E. californicus* weakly associated with *Hordeum*, but this node is not present on all of the shortest trees. Therefore, in the strict consensus tree (not shown), the node supporting *E. californicus* + *Hordeum* collapses, and the position of *E. californicus* is unresolved.

The close relationships among the chloroplast genomes of *Elymus* (except *E. californicus*) suggest that their chloroplast is derived from a single monogenomic taxon. A Wilcoxon signed-ranks test (Siegel 1956; Templeton 1983) was carried out to compare our two a priori hypotheses about the origin of the chloroplast (*Pseudoroegneria* vs. *Hordeum*). This amounts to a comparison of trees constrained to include an *Elymus* + *Pseudoroegneria* clade vs. those with an *Elymus* + *Hordeum* clade. The test was carried out as described in Mason-Gamer and Kellogg (1996b), and shows the latter hypothesis to be significantly worse than the former ($P < 0.0001$; $n = 29$; test statistic = 45). The trees constrained to include *Elymus* + *Hordeum* required 23 additional steps relative to those constrained to include *Elymus* + *Pseudoroegneria*

Fig. 1. Strict consensus trees of the separate (A–C) and combined (D) data sets. Before the analyses, the data sets were reduced to include only the taxa in common. Numbers above the branches show bootstrap frequencies based on 1000 fast-bootstrap replicates. Weakly supported nodes with less than 50% bootstrap support and indicated by dotted lines were excluded from the trees used as constraints in the data set comparisons.

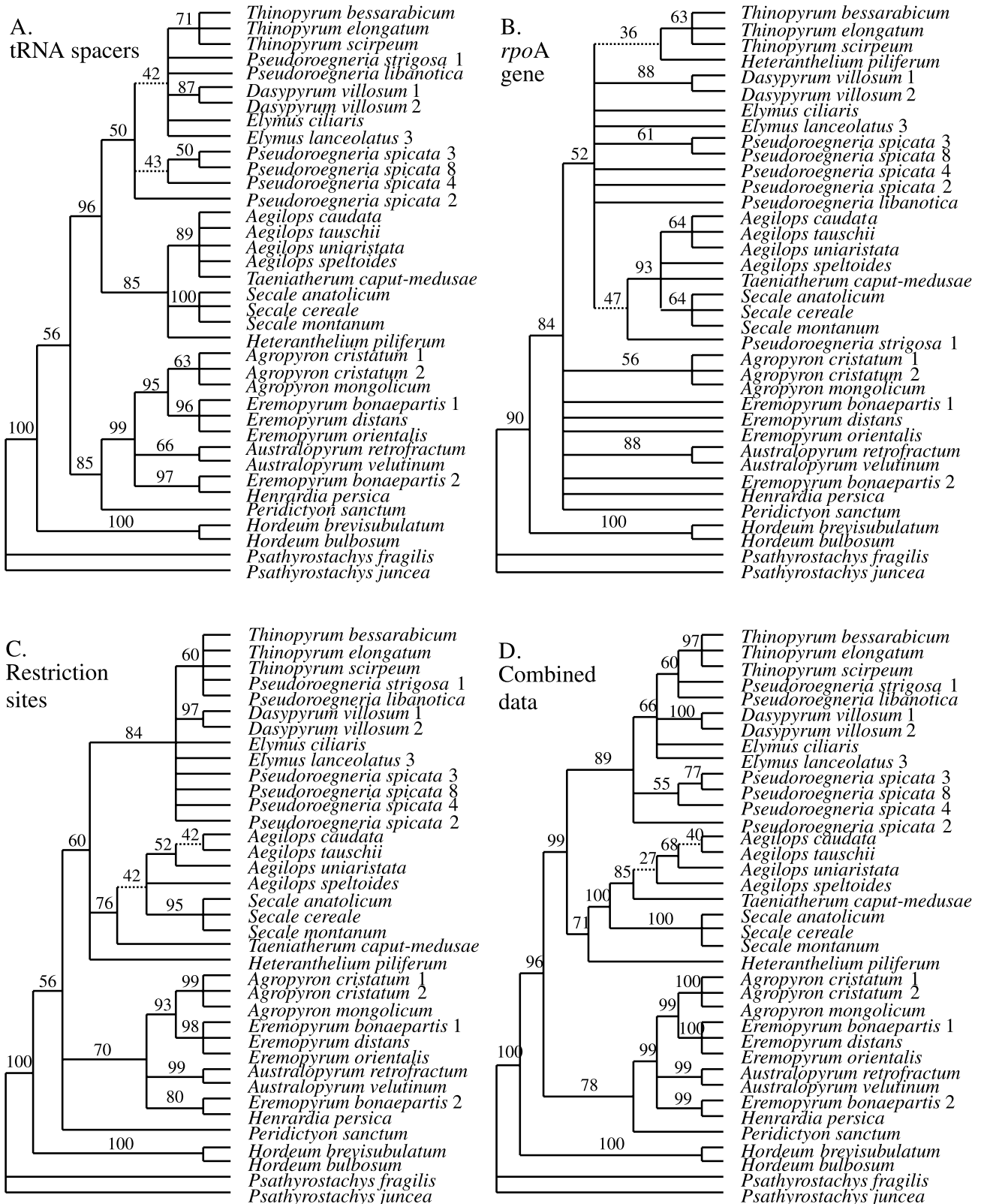


Table 3. Results of constrained analyses: tree length / increase in length.

Data set	Constraint trees			
	tRNA spacers	<i>rpoA</i> gene	Restriction sites	Combined
tRNA spacers	194 ^a	194/0	194/0	194/0
<i>rpoA</i> gene	67/1	66 ^a	67/1	67/1
Restriction sites	159/2	157/0	157 ^a	159/2
Combined	421/0	421/0	421/0	421 ^a

^aNumbers on the diagonal represent lengths of most parsimonious unconstrained trees.

(i.e., the *Elymus* + *Pseudoroegneria* trees are two steps longer than the shortest cladistic parsimony trees).

Analyses of monogenomic genera

The parsimony search of the combined data sets resulted in 321 most-parsimonious trees of length 454 (one of which is shown in Fig. 3), with a CI of 0.736, RI of 0.917, and RCI of 0.751. Not surprisingly, the present analysis of three cpDNA data sets is consistent with, but better resolved and supported than, either of the earlier published analyses of individual cpDNA data sets (restriction sites in Mason-Gamer and Kellogg 1996a; *rpoA* sequences in Petersen and Seberg 1997). As in the earlier analyses, most of the genera (as far as they have been sampled) are monophyletic, but in contrast to the earlier analyses, *Thinopyrum* forms a well-supported monophyletic group in the present study (Fig. 3). Our tree also highlights the polyphyly of *Eremopyrum* and the paraphyly of *Pseudoroegneria*, as in Mason-Gamer and Kellogg (1996a). As in both of the earlier cpDNA analyses, the monophyly of the *Triticum*–*Aegilops* group is in question. The present analysis is in agreement with one or both of the previous cpDNA studies with regard to several intergeneric relationships that include the following: (i) the close relationship between *Secale*, *Taeniatherum*, and *Triticum*–*Aegilops*; (ii) a *Pseudoroegneria* + *Thinopyrum* + *Dasyphyrum* clade; (iii) an *Agropyron* + *Eremopyrum* + *Australopyrum* + *Henrardia* clade; (iv) the basal placement of *Psathyrostachys*; and (v) the placement of *Hordeum* near the base of the tree, inside of *Psathyrostachys*. Further, the present analysis provides moderate support for some additional relationships that were unresolved or very weakly supported (< 35%) in the previous cpDNA studies. These include (i) the sister relationship between *Taeniatherum* and *Triticum*–*Aegilops*; (ii) the placement of *Heterantherium* with a *Secale* + *Taeniatherum* + *Triticum*–*Aegilops* clade; (iii) the placement of *Peridictyon* at the base of the *Agropyron* + *Eremopyrum* + *Australopyrum* + *Henrardia* clade; and (iv) the placement of the *Pseudoroegneria* + *Thinopyrum* + *Dasyphyrum* clade with the *Secale* + *Taeniatherum* + *Aegilops*–*Triticum* + *Heterantherium* clade.

Discussion

Comparisons among data sets

Although different segments of the clonal chloroplast genome are expected to track identical histories, analyses of separate cpDNA data sets have been known to yield trees with numerous topological differences (e.g., Olmstead and Sweere 1994). In the present case, however, the results were

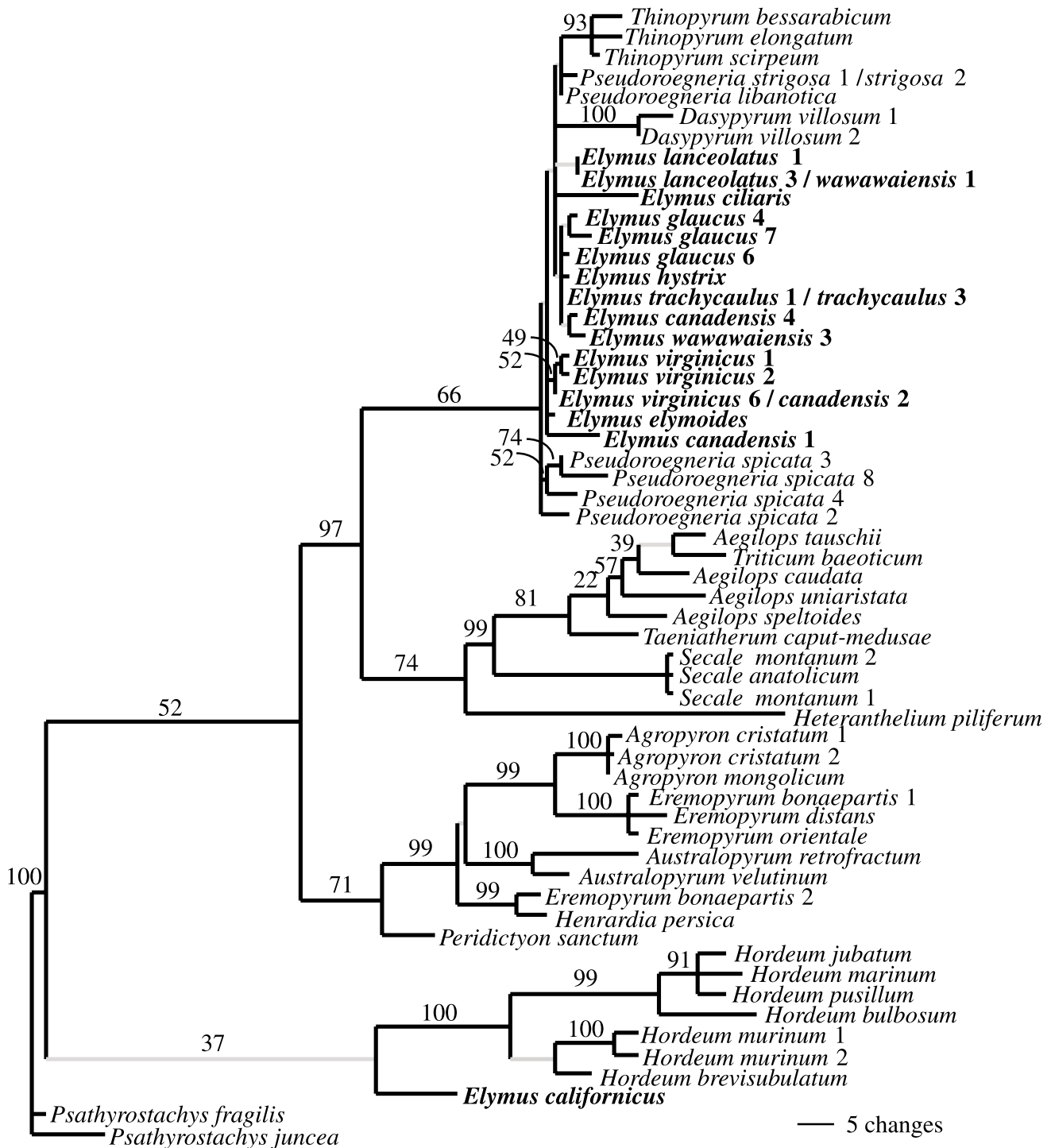
predictable, and their interpretation straightforward. The trees were topologically similar overall, there were no well-supported differences among the trees, and the constrained analyses revealed very little character conflict between any data set and its opposing trees. The combined data set yielded trees that were better resolved and supported than did any of the data sets analyzed alone. Together, these observations support the decision to combine the data sets.

Origin of the *Elymus* chloroplast

Studies of genomic complement, based on patterns of genome pairing, have suggested that North American species of *Elymus* combine the **St** genome of *Pseudoroegneria* with the **H** genome of *Hordeum* in an **StStHH** allotetraploid configuration. Recent molecular phylogenetic analyses using the granule-bound starch synthase gene (Mason-Gamer 2001) support this result. Unlike the starch synthase gene, however, the chloroplast genome is generally uniparentally inherited, and thus can be used to identify the maternal genome donor of a given polyploid. For example, several now-classic studies identified cpDNA donors to polyploid species of *Glycine* (Doyle et al. 1999), *Heuchera* (Soltis et al. 1989), and *Tragopogon* (Soltis and Soltis 1989). More recent examples include studies of *Oryza* (Ge et al. 1999), *Argyranthemum* (Brochmann et al. 2000), and *Stylosanthes* (Liu and Musial 2001).

Our data strongly suggest that *Pseudoroegneria* is the chloroplast genome donor to North American *Elymus*, but the results are complicated by the paraphyly of *Pseudoroegneria* on the cpDNA trees. It is difficult to rule out either *Thinopyrum* or *Dasyphyrum* as potential chloroplast donors, although no other data of which we are aware identify either one as a potential progenitor to *Elymus*. A *Pseudoroegneria* donor is consistent with genome-pairing data (e.g., Dewey 1984; Jensen 1990a, 1990b; Torabinejad and Mueller 1993; Jensen et al. 1994; Jensen and Salomon 1995) and starch synthase gene sequences (Mason-Gamer 2001). In contrast to the cpDNA results, neither *Dasyphyrum* nor *Thinopyrum* are associated with *Pseudoroegneria* on the starch synthase tree. The other hypothesized cpDNA donor to *Elymus*, based on genome-pairing data (e.g., Dewey 1984; Jensen 1990a, 1990b; Torabinejad and Mueller 1993; Jensen et al. 1994; Jensen and Salomon 1995) and starch synthase sequence analyses (Mason-Gamer 2001), was *Hordeum*. On our trees, *Hordeum* forms a well-supported clade that excludes *Elymus* (except *E. californicus*; discussed below). A Wilcoxon signed-ranks test comparing *Elymus* + *Hordeum* with *Elymus* + *Pseudoroegneria* significantly favors *Pseudoroegneria* as the more likely cpDNA genome donor. The apparent

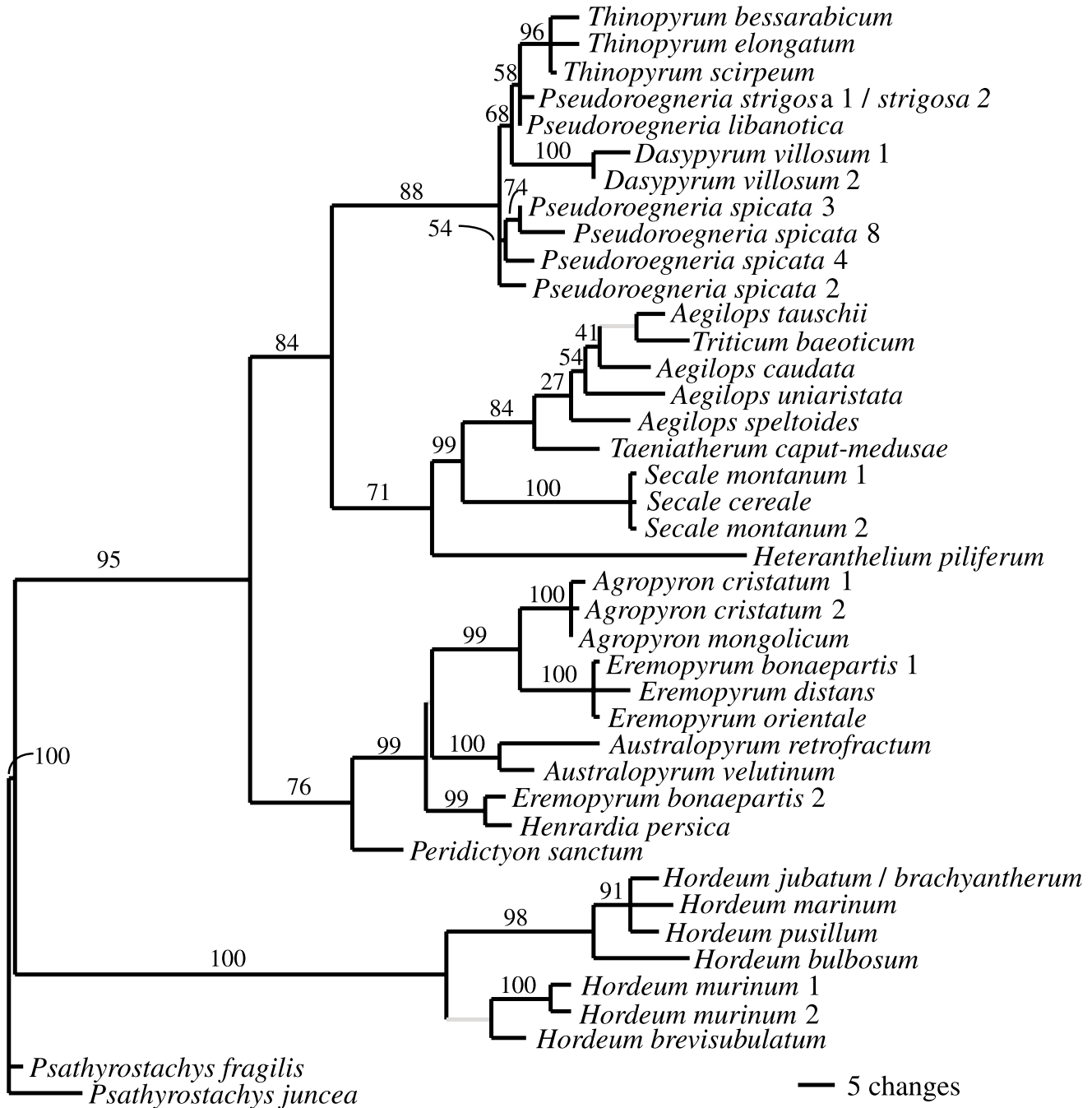
Fig. 2. One of 325 000 most parsimonious trees based on combined cpDNA restriction sites, *rpoA* sequences, and tRNA spacer sequences. *Elymus* individuals are highlighted with bold text. Numbers following species names differentiate individuals within species and correspond to the numbers in the second column in Table 1. Bootstrap support = 50% is shown above nodes; one weak node (37%) is further discussed in the text. Nodes that collapse in the strict consensus tree are drawn in gray.



predominance of the **St**-genome donor as the maternal parent appears to extend beyond North American tetraploid *Elymus* (Redinbaugh et al. 2000). In their analyses of a portion of the *ndhF* gene, they included a wider variety of putative **St**-containing polyploids, all of which show a high level of sequence similarity to *Pseudoroegneria*.

The placement of *E. californicus* in *Elymus* has been questioned (Jensen and Wang 1997; M. Barkworth, Utah State University, Logan, Utah, personal communication), but has not been addressed using genome-pairing data. Jensen and Wang (1997) surveyed *E. californicus* for an RAPD marker thought to be specific to the **St** (*Pseudoroegneria*)

Fig. 3. One of 321 most-parsimonious trees based on combined cpDNA restriction sites, *rpoA* sequences, and tRNA spacer sequences. Only the monogenomic taxa were included in this analysis. Numbers following species names differentiate individuals within species, and correspond to the numbers in second column in Table 1. Bootstrap support = 50% is shown above nodes. Nodes that collapse in the strict consensus tree are drawn in gray.



genome and did not find it (they did not address the presence of the **H** genome of *Hordeum*). Furthermore, they found an RAPD marker thought to be specific to the **Ns** (*Psathyrostachys*) genome. In the genomic system of classification, polyploids with an **Ns** genome characterize the genus *Leymus*, and Jensen and Wang (1997) therefore suggest that *E. californicus* should be transferred to *Leymus*. The cpDNA data support Jensen and Wang's (1997) conclusion that *E. californicus* does not belong in *Elymus*, but are equivocal with respect to its placement within the Triticeae.

The tree in Fig. 2 shows *E. californicus* to be weakly grouped with *Hordeum*, but this is not the case on all of the shortest trees. Although it is near the base of the tree, it does not group with *Psathyrostachys* on any of the shortest trees, as would be expected if it were an **Ns** polyploid. Data from the starch synthase gene (Mason-Gamer 2001) do place *E. californicus* at the base of the maximum-likelihood Triticeae tree along with *Psathyrostachys*, but the relationship is very weakly supported and is not seen on all of the shortest trees from a cladistic parsimony analysis of the

same data. So far, molecular phylogenetic data fail to resolve the placement of this problematic species. Its clarification awaits a more detailed analysis of *Leymus* and *Psathyrostachys*, which itself promises to be a complex and challenging problem (Anamthawat-Jónsson and Bödvarsdóttir 2001).

Effect of missing data

In the combined data set, the restriction site data are scored as missing for all but two of the *Elymus* individuals (Table 1). A major a priori concern is that the placement of taxa with missing data will be unstable, leading to an increase in the number of most parsimonious trees, and subsequent loss of resolution in the strict consensus tree (e.g., Wilkinson 1995; Wiens 1998; Kearney 2002). In this case, missing data may be affecting the ability to resolve relationships among the *Elymus* individuals, but, based on the topology of the strict consensus tree, the placement of *Elymus* within the Triticeae appears to be stable. Furthermore, the removal of all taxa missing any one data set yielded trees very similar to those with the full set of taxa (Fig. 1D vs. Fig. 2). Following the reasoning and observations of Wiens (1998), this data set may be less likely than some to suffer from the effects of the missing data because (i) the taxa in question fall within a restricted monophyletic subset of the tree and (ii) the individuals with missing entries (and therefore artificially short branches) are not distributed among individuals with long branches, in which case long-branch attraction could give misleading results.

Triticeae cpDNA phylogeny

Phylogenetic studies of the Triticeae have used not only cpDNA data, but also several nuclear markers, including two distinct 5S rDNA spacer regions (Kellogg and Appels 1995), the internal transcribed spacer (ITS) of the nuclear rDNA repeat (Hsiao et al. 1995), and the genes for granule-bound starch synthase (GBSSI; Mason-Gamer and Kellogg 2000; Mason-Gamer 2001) and disrupted meiotic cDNA1 (DMC1; Petersen and Seberg 2000). Among many of the published gene trees, extensive phylogenetic conflict has been demonstrated (Kellogg et al. 1996; Mason-Gamer and Kellogg 1996b), suggesting a reticulate evolutionary history. In analyses of conflict, the cpDNA data showed the highest level of conflict with other data sets in all of the tested pairwise combinations. More recently, a rigorous analysis of morphological variation in the tribe yielded trees that differ from each of the molecular trees (Seberg and Frederiksen 2001). Therefore, it is important to recognize that the trees presented here are cpDNA genome trees; it would be misleading to view them in a broader sense. Because the history of the group may be highly reticulate, its phylogeny must be interpreted in the context of all available trees.

The Triticeae have been broadly sampled in two earlier cpDNA-based phylogenetic studies (Mason-Gamer and Kellogg 1996a; Petersen and Seberg 1997). Each of those studies introduced new information and their results were consistent with one another, but the phylogenetic trees in both suffered from a lack of resolution and (or) support. The present analysis builds upon the previous two by adding more data from the chloroplast genome, resulting in in-

creased resolution and support. Here we focus on conclusions that are new or better supported relative to those of the earlier cpDNA analyses. Relationships shown here that were also supported by the earlier studies have already been discussed (Mason-Gamer and Kellogg 1996a; Kellogg et al. 1996; Petersen and Seberg 1997).

In terms of generic delimitation, the present tree clarifies the monophyly of *Thinopyrum*, placing it as a well-supported clade within a paraphyletic *Pseudoroegneria* (Fig. 3), whereas the earlier cpDNA-based analyses left the question of its monophyly unresolved. The possible monophyly of *Thinopyrum* has, based on cytogenetic data, been the subject of intense debate (e.g., Jauhar 1988, 1990; Wang 1985; Wang and Hsiao 1989). Jauhar (1988, 1990) follows Löve (1982, 1984) in recognizing two genera on the basis of morphological and genome-pairing data (such that in our sample, *Thinopyrum bessarabicum* would remain in a narrower *Thinopyrum*, whereas *T. elongatum* and *T. scirpeum* would be in a separate genus, *Lophopyrum*). Wang (1985) and Wang and Hsiao (1989), on the other hand, concluded that Löve's *Lophopyrum* and *Thinopyrum* have the same genome, supporting Dewey (1984) who combined them into a broader *Thinopyrum* (the convention we follow). Although our cpDNA results clearly support the monophyly of the broader *Thinopyrum*, they hardly close the door on the question. Gene trees from nuclear 5S rDNA short spacers (Kellogg and Appels 1995) and from the nuclear DMC1 gene (Petersen and Seberg 2000), as well as the morphological cladogram of Seberg and Frederiksen (2001), suggest that the broader *Thinopyrum* is polyphyletic. The nuclear GBSSI gene (Mason-Gamer and Kellogg 2000; Mason-Gamer 2001) and the nuclear ITS sequences (Hsiao et al. 1995) are equivocal, with results depending on the method of analysis. Therefore, the clear cpDNA-based evidence for the monophyly of the broader *Thinopyrum* only serves to increase the conflict among data sets with regard to this question. As suggested earlier (Kellogg et al. 1996), conflict among gene trees suggests that disagreement among cytogeneticists over *Thinopyrum*'s monophyly reflects the group's complex phylogenetic history rather than simply differences of opinion or interpretation.

In terms of intergeneric relationships, the present cpDNA results are consistent with those previously published (Mason-Gamer and Kellogg 1996a; Petersen and Seberg 1997), but in some cases provide more resolution or stronger support. Given the demonstrated conflict between cpDNA and nuclear gene trees, it is not surprising that few of our new-found relationships reflect those seen in morphological or nuclear gene trees. In a rare example of partial agreement, the cpDNA clade containing *Taeniatherum* and *Triticum-Aegilops* is also seen on the 5S short spacer data tree, but (in contrast to the cpDNA tree) only if the narrowly defined *Thinopyrum* is included in the clade (Fig. 2A in Kellogg et al. 1996). However, a close relationship between *Taeniatherum* and *Triticum-Aegilops* is completely at odds with the DMC1 tree (Petersen and Seberg 2000) and the morphology-based cladogram (Seberg and Frederiksen 2001). The relationship is unresolved by the ITS data (Hsiao et al. 1995), and *Taeniatherum* was not included in the 5S long spacer data set (Kellogg and Appels 1995).

The other intergeneric relationships noted in the results (the placement of *Heterantherium* with a *Secale* + *Taeniatherum* + *Triticum*–*Aegilops* clade; the placement of *Peridictyon* at the base of the *Agropyron* + *Eremopyrum* + *Australopyrum* + *Hennardia* clade; and the grouping of the *Pseudoroegneria* + *Thinopyrum* + *Dasypyrum* clade with the *Secale* + *Taeniatherum* + *Aegilops*–*Triticum* + *Heterantherium* clade) involve numerous genera. Because of the high levels of conflict between the cpDNA trees and those derived from other data sets, these relationships can not even be directly compared with those seen in the other trees.

In conclusion, the combined cpDNA data suggest, in agreement with Redinbaugh et al. (2000), that *Pseudoroegneria* is the maternal genome donor to North American *Elymus*. In addition, as was previously suspected, *E. californicus* probably does not belong in *Elymus*, although its placement remains unresolved. The analysis of monogenomic genera provides a cpDNA tree that is consistent with but more resolved than earlier cpDNA-based trees, thus further emphasizing the degree of phylogenetic conflict between cpDNA trees and those derived from morphological characteristics and nuclear genetic markers.

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