Genet Resour Crop Evol (2010) 57:1065–1077
DOI 10.1007/s10722-010-9548-6

RESEARCH ARTICLE

Velvet bentgrass (*Agrostis canina* L.) is the likely ancestral diploid maternal parent of allotetraploid creeping bentgrass (*Agrostis stolonifera* L.)

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Received: 18 December 2009 / Accepted: 26 February 2010 / Published online: 13 March 2010

Abstract Understanding genetic relationships among the three most important *Agrostis* species will be important in breeding and genomic studies aimed at cultivar improvement. Creeping, colonial, and velvet bentgrasses (*Agrostis stolonifera* L., *A. capillaris* L., and *A. canina* L., respectively) are commercially important turfgrass species often used on golf courses. Velvet bentgrass is a diploid and creeping and colonial bentgrasses are both allotetraploids. A model for the genomic relationships among these species was previously developed from cytological evidence. The genome designations were A_1A_1 for velvet bentgrass, $A_1A_1A_2A_2$ for colonial bentgrass, and $A_2A_2A_3A_3$ for creeping bentgrass. Here we used phylogenetic analysis based on DNA sequences of nuclear ITS and protein coding genes and the plastid *trnK* intron and *matK* gene to reexamine these relationships. In contrast to the previous model, the DNA sequence analysis suggested that velvet bentgrass was closely related to creeping bentgrass and it is likely the maternal parent of creeping bentgrass. Phylogenetic analysis of some conserved nuclear genes revealed a close relationship of the velvet bentgrass sequences with the A_2 subgenome sequences of creeping bentgrass. We therefore propose that velvet bentgrass be designated as having the A_2 genome, rather than the A_1 genome as in the previous model.

Keywords *Agrostis* · Bentgrass · Phylogeny · Polyploidy

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Introduction

Three species from the genus *Agrostis* are commercially important turfgrasses, often used on golf courses in temperate regions throughout the world. The most widely used species is creeping bentgrass (*Agrostis stolonifera* L.) (Warnke 2003). Colonial bentgrass (*A. capillaris* L.) and velvet bentgrass (*A. canina* L.) are also used, particularly in the northeastern regions of the United States (Brilman 2003; Ruemmele 2003). The bentgrasses are crops of significant importance in the US agricultural economy. Seed production occurs primarily in Oregon. In 2007, 3.4 million pounds of creeping bentgrass seed and 1.4 million pounds of colonial bentgrass seed were produced in Oregon (Oregon State University Extension Service, http://oregonstate.edu.oain/Econinfo/grnhaygras.htm). Academic and private breeding programs are developing new cultivars of all three species. Genetic linkage maps of creeping and colonial bentgrasses have been published and EST resources have been generated (Chakraborty et al. 2005; Rotter et al. 2007, 2009). Additional genomic

Table 1 Genome organization, as proposed by Jones (1956a, b, c), of some *Agrostis* spp.

Species	Common name	Chromosome number	2C DNA, pg[a]	Genome composition
A. canina	Velvet bentgrass	14	3.42	A_1A_1
A. vinealis	Brown bentgrass	28	6.31	$A_1A_1A_1A_1$
A. capillaris	Colonial bentgrass	28	5.87	$A_1A_1A_2A_2$
A. stolonifera	Creeping bentgrass	28	5.27	$A_2A_2A_3A_3$
A. gigantea	Redtop	42	8.18	$A_1A_1A_2A_2A_3A_3$

[a] Bonos et al. (2002)

studies of these species are underway. Better understanding of the genetic relationships among the bentgrasses will be useful for future efforts aimed at cultivar improvement.

The genetic relationships of creeping, colonial, and velvet bentgrasses were examined by Jones (1956a, b, c) based on cytological evidence from metaphase I of meiosis in the three species and their interspecific hybrids. Table 1 summarizes the genome organization of the species as proposed by Jones. He proposed that creeping and colonial bentgrasses were each allotetraploids with one genome in common, designated the A_2 genome. The diploid species velvet bentgrass was considered to be related to the colonial bentgrass A_1 genome. Brown bentgrass (*A. vinealis* Schreb., formerly considered *A. canina* L. var. *montana* Hartmann) was considered to be an autotetraploid of the A_1 genome (Jones 1956a). Brown bentgrass is not used commercially but is a common grassland species (Ridgway et al. 2003). The diploid origins of the A_2 and A_3 genomes were unknown. The hexaploid species *A. gigantea* Roth. was considered to possess the A_1, A_2, and A_3 genomes. Nuclear DNA content of these *Agrostis* spp. reflected their ploidy levels (Bonos et al. 2002).

More recent data on the disomic inheritance of polymorphic isozymes in progeny from controlled crosses, as well as analysis of molecular marker segregation support the genomic allotetraploid nature of creeping bentgrass (Warnke et al. 1998; Chakraborty et al. 2005). Similarly, genetic linkage mapping of distinct homoeologous genes in colonial bentgrass supports the allotetraploid nature of this species (Rotter et al. 2009).

We previously used EST sequence data to estimate the divergence times of the subgenomes of creeping and colonial bentgrass (Rotter et al. 2007). Estimates of the divergence times between the A_1 and A_2 subgenomes of colonial bentgrass and the A_2 and A_3 subgenomes of creeping bentgrass were similar, at 8.9 and 10.6 million years ago, respectively (Rotter et al. 2007). The shared A_2 subgenomes of colonial and creeping bentgrass were similar to each other, with an estimated divergence time from a common ancestor of 2.2 million years ago. In carrying out the estimates of divergence times between the subgenomes of creeping and colonial bentgrasses efforts were made to use oligonucleotides designed to be specific for the A_1 subgenome of colonial bentgrass to amplify fragments from the velvet bentgrass genome (Rotter et al. 2007). These attempts were unsuccessful, raising the possibility that the velvet bentgrass genome may not be closely related to the A_1 subgenome of colonial bentgrass.

Recently, there has been increased interest in interspecific hybridization between *Agrostis* spp., both from the perspective of outcrossing of transgenic creeping bentgrass (Banks et al. 2004; MacBryde 2005), and from a consideration of the potential usefulness of interspecific hybridization in breeding improved cultivars (Belanger et al. 2003, 2004). More information on the phylogenetic relationships of these species would clearly be helpful. The objective of this project was therefore to use nuclear and chloroplast DNA sequence data to analyze the relationships among creeping, colonial, and velvet bentgrasses. Based on the phylogenetic results obtained we propose a new model for the genome relationships among these species.

Materials and methods

Plant materials

An single individual genotype from each of the following sources was used in this study for the ITS

Table 2 Sources of additional *Agrostis* spp. accessions

A. canina (velvet bentgrass)
PI 189141 (The Netherlands, cv. 'Novobent')
PI 230234 (Iran)
PI 290707 (UK)
PI 578526 (USA, cv. 'Kingstown')
ISC 8 (Rutgers breeding program)
ISC12 (Rutgers breeding program)
A. capillaris (colonial bentgrass)
PI 420235 (UK, cv. 'Goginan')
PI 494120 (Germany, cv. 'Tendenz')
PI 494121 (The Netherlands, cv. 'Bardot')
A. stolonifera (creeping bentgrass)
PI 601363 (USA, cv. 'Pennlinks')
W6 6602 (USA, cv. 'Emerald')

and *trnK/matK* sequencing: creeping bentgrass 'L93' (Agribiotech, Inc., Somerset, New Jersey), colonial bentgrass 'SR7100' and velvet bentgrass 'SR7200' (Seed Research, Corvallis, Oregon), and brown bentgrass PI440110 (Western Region Plant Introduction Station, Pullman, Washington). Voucher specimens of the plants used have been deposited in the Chrysler Herbarium, Rutgers University, New Brunswick, NJ under accessions A. Koroch 18–21.

Additional *Agrostis* spp. accessions, as well as those above, were used for the analysis presented in Fig. 3. The additional accessions are listed in Table 2.

PCR amplification, cloning, and DNA sequencing

Genomic DNA from individual plants of each species was isolated by using a commercial kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma–Aldrich, St. Louis, Missouri). The primers used for amplification of the nuclear 5.8S rDNA gene and the flanking internal transcribed spacer (ITS) regions were ITSL (5′-TCGTAACAAGGTTTCCGTAGGTG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) (Hsiao et al. 1995). Approximately 0.5 μg of genomic DNA was used per 50 μl PCR reaction, which contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM each dNTP, 20 pmol each primer, and 1 μl of Taq polymerase (Applied Biosystems Inc., Foster City, California). PCR was carried out in a GeneAmp 9700 thermocycler (Applied Biosystems Inc.). The initial denaturation was conducted at 94°C for 4 min, followed by 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 50°C, and 1 min extension at 72°C, followed by a final extension at 72°C for 10 min. The PCR products were separated on a 1% (w/v) agarose gel. The amplified DNA fragments were excised and purified by using a commercial kit (QIAquick Gel Extraction Kit, Qiagen USA, Valencia, California). The purified fragments were ligated into the pGEM-T Easy vector (Promega, Madison, Wisconsin) and transformed by electroporation into XL1-Blue MRF *Escherichia coli* cells (Stratagene, La Jolla, California). Plasmids from individual transformed colonies were sequenced (Genewiz Inc., South Plainfield, New Jersey). GenBank accession numbers for the ITS clones generated in this study are FJ042801 to FJ042872.

Primers for amplification of the plastid *matK* gene and flanking *trnK* intron sequences were *trnK*F (5′GGGGTTGCTAACTCAACGG-3′) and *trnK*R (5′AACTAGTCGGATGGAGTAG-3′) (Cronn et al. 2002). For the *matK* gene amplifications, the PCR reactions were carried out with the Elongase Amplification System (Invitrogen, Carlsbad, California). The 50 μl reactions contained 0.5 μg of genomic DNA, 60 mM Tris-SO_4, pH 9.1, 18 mM $(NH4)_2SO_4$, 1.5 mM $MgSO_4$, 0.25 mM each dNTP, 20 pmol of each primer, and 1 μl of Elongase enzyme mix. The initial denaturation was conducted at 94°C for 2 min, followed by 30 cycles of 1 min denaturation at 94°C, 1.5 min annealing at 50°C, and 2 min extension at 68°C, followed by a final extension at 68°C for 10 min. The PCR products were sequenced directly. For each sequencing reaction a 5 μl aliquot of the PCR product was treated with 2 μl of ExoSAP-IT (USB, Cleveland, Ohio) to remove unincorporated primers and excess dNTPs. The ExoSAP-IT reaction was carried out at 37°C for 15 min followed by 80°C for 15 min to inactivate the enzymes. Sequencing was done using the amplification primers and 10 additional primers. Additional sequencing primers for the *matK* region were (5′CAGTAGCGGGAACTATGGTATCG3′), (5′TAATTGGGTAGAAAAGGAACG3′), (5′AAGCATTTCTCGGTTTATCG3′), (5′GCATTTTTCATTGCACACGAC3′), (5′GAGCAACAAATTCGTCCAGA3′), (5′TCCCATTCTCGCTACGAGAA3′), (5′CTTTATGGATCCTCTGATGC3′), (5′CATGCGCTAGAACTTTAGCT3′), (5′CTAAAGTTCTAGCGCATGAA3′), (5′AGTGGGTAGACAAAGTATTG3′). GenBank accession numbers for the *trnK/matK* sequences generated in this study are FJ231112 to FJ231115.

The 11 additional *Agrostis* spp. accessions listed in Table 2 were analyzed for the presence of the 18 bp duplication in the *trnK* upstream intron region by sequencing of PCR fragments amplified from tissue samples from a single plant prepared as described by Klimyuk et al. (1993). The *trnK*F primer was used as the forward primer and 5′AAGCATTTCTCGGTTTA TCG3′ was used as the reverse primer for the amplification.

Primers for amplification of the nuclear protein coding genes were based on the EST sequences previously reported (Rotter et al. 2007). The creeping and colonial bentgrass ESTs had been sequenced from the 3′ ends to capture the variation in the untranslated region that can be used for molecular marker development. To avoid attempting to amplify large fragments due to the presence of introns, the forward primers for amplification of the velvet and brown bentgrass sequences were designed to be in the coding sequence of the most 3′ exon, based on the positions of introns in the most similar rice gene. The reverse primers were from the 3′ untranslated region. Primers for the amplification of the nuclear photosystem I, subunit N conserved orthologous sequence gene were (5′CCCGTTCATCTCCGACGAC3′) and (5′GGTTA TGCCAGAGCAAATACG3′). Primers for the nuclear unknown conserved orthologous sequence gene were (5′AGCATTGCACGGCGCTCAG3′) and (5′TAAGA TTTCATAACTGGAAACAC3′). PCR reactions and sequencing for these nuclear genes were carried out with the Elongase Amplification System as described above for the *trnK*/*matK* gene amplifications except that the annealing temperature was 52°C.

Phylogenetic analysis

The CLUSTAL-X (Thompson et al. 1997) program was used to align DNA sequences. For the ITS analysis the sequences included the ITS-1, 5.8S rDNA and ITS-2 regions. For the *trnK*/*matK* analysis, the sequences included the *matK* gene as well as portions of the flanking *trnK* intron. The *matK* gene is located within the intron in the *trnK* gene and in most phylogenetic analyses only the *matK* gene sequences are used for comparison. Since we were comparing closely related sequences, which could be unambiguously aligned, we carried out the analysis including portions of the flanking intron sequences to increase the number of informative polymorphic sites. For the ITS and *trnK*/*matK* phylogenetic analyses the sequences were trimmed to include only the regions of sequence overlap for all the sequences in the analysis. For analysis of the nuclear protein coding genes, for each gene the sequences were aligned and trimmed to include only the region of overlap for all the species and the trimmed sequences for both genes were combined. The combined sequences consisted of 45 bases of coding sequence and 282 bases of 3′ untranslated sequence.

Simple indel coding as binary characters was used to characterize gaps within the aligned sequences (Simmons and Ochoterena 2000). Phylogenetic analyses were performed with the PAUP* program (version 4.0b10 for Macintosh; Swofford 2002). The phylogenetic trees for the ITS and *trnK*/*matK* sequences were generated by using the maximum parsimony full heuristic search option set to random sequence addition, tree-bisection-reconnection (TBR) branch swapping, and Multrees on, with 1,000 bootstrap replications. Since there were fewer taxa in the comparison, the phylogenetic tree of the conserved nuclear protein-coding genes was generated from an exhaustive maximum parsimony analysis. The *Brachypodium distachyon* sequences were chosen to root the trees since the tribe Brachypodieae is basal to the Aveneae/Poeae complex (Doring et al. 2007). For the nuclear protein coding genes, trees generated from the individual gene sequences also had a similar topology (not shown).

The sequences were also analyzed by the neighbor-joining and maximum likelihood methods in the PAUP* program, which generated trees of similar topology to those of the maximum parsimony analysis (not shown). For the neighbor-joining analyses the trees were generated using the Hasegawa-Kishino-Yano (HKY85) model of sequence evolution, and 1,000 bootstrap replications. For the maximum likelihood analyses, the trees were generated with a fast heuristic search using the HKY85 model of sequence evolution, and 100 bootstrap replications.

Results

ITS phylogeny

The high copy nuclear ITS region is often used in phylogenetic analyses because it is easily amplifiable

and, as a result of concerted evolution, the PCR products can often be sequenced directly (Baldwin et al. 1995; Hsiao et al. 1995). However, since we were interested in examining the subgenome relationships of creeping, colonial, velvet, and brown bentgrasses we cloned the ITS PCR products and sequenced 11–25 clones from each species.

Maximum parsimony phylogenetic analysis of the *Agrostis* spp. ITS sequences is shown in Fig. 1. The tree was based upon 579 total characters, of which 445 were constant, 84 variable characters were parsimony uninformative, and 50 characters were parsimony informative. Figure 1 includes 32 previously reported ITS sequences, which were generated

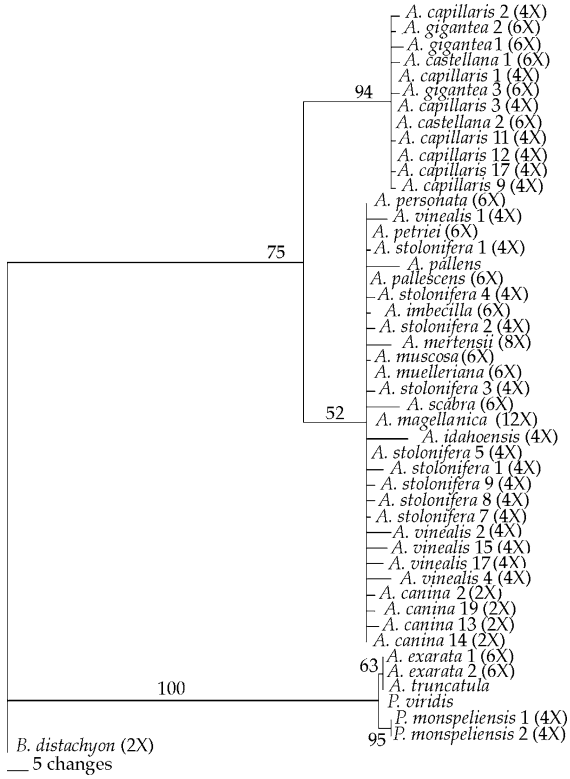

Fig. 1 Rooted 50% majority rule consensus maximum parsimony phylogenetic tree of nuclear ITS sequences. The tree length is 213, the consistency index is 0.690, and the retention index is 0.794. The *B. distachyon* sequence was designated as the outgroup for rooting the tree. The numbers at the nodes are the bootstrap percentages based on 1,000 replications. Accession numbers of the sequences are given in Table 3. When known, the ploidy level of the species is indicated in parenthesis. The ploidy values were obtained from Heiser and Whitaker (1948), Jones (1956a, b, c), Reeder (1977), Brede and Sellmann (2003), and Murray et al. (2005)

by direct sequencing of PCR products, and the sequences of 4 clones each from creeping, colonial, velvet, and brown bentgrasses generated in this study. The accession numbers of the sequences used in the analysis presented in Fig. 1 are listed in Table 3. The phylogenetic tree produced when all the clones generated from this study were included (25 velvet bentgrass, 23 colonial bentgrass, 13 creeping bentgrass, and 11 brown bentgrass) had the identical topology (not shown). Where known, the ploidy level of the species is indicated in Fig. 1. The ploidy levels of *A. pallens*, *A. truncatula,* and *Polypogon viridis* have not been reported.

In this analysis the colonial and creeping bentgrass sequences grouped into separate clades. The colonial bentgrass clade also included the hexaploid species *A. castellana* and *A. gigantea*. The creeping bentgrass clade also included velvet bentgrass, brown bentgrass, *A. petriei*, *A. pallescens*, *A. personata*, *A. magellanica*., *A. mertensii*, *A. muelleriana*, *A. imbecilla*, *A. muscosa*, *A. scabra*, *A. pallens*, and *A. idahoensis*. *A. exarata* and *A. truncatula* grouped in a separate clade along with the *Polypogon* spp.

trnK/matK phylogeny

The rapidly evolving maternally inherited plastid *matK* gene has been widely used in angiosperm phylogenetic analyses (Hilu et al. 2003). Figure 2 shows the maximum parsimony analysis of the *matK* gene and portions of the flanking *trnK* intron sequences, which included 18 previously reported sequences in addition to those generated in this study. The tree was based upon 2,293 total characters, of which 2,090 were constant, 158 variable characters were parsimony uninformative, and 45 characters were parsimony informative. When the flanking intron sequences were removed and the *matK* gene only analyzed, the tree topology was the same (not shown).

The close relationship of velvet bentgrass with creeping bentgrass is further supported by the presence of a shared eighteen bp tandem repeat in the upstream *trnK* intron region (Fig. 3). Since this repeat was so distinct, additional accessions of creeping, colonial, and velvet bentgrass were also analyzed for the presence or absence of the 18 bp repeat and are included in Fig. 3. The repeat was found in a total of seven and six velvet and creeping bentgrass

Table 3 Accession numbers of DNA sequences used in the phylogenetic analyses and sequence comparison presented in Figs. 1, 2, 3 and 4

Sequence name	Accession number	Source
ITS		
A. canina L. 2	FJ042856	This study
A. canina 13	FJ042859	This study
A. canina 14	FJ042866	This study
A. canina 19	FJ042855	This study
A. capillaris L. 1	AY520820	Unpublished
A. capillaris 2	DQ146767	Reichman et al. (2006)
A. capillaris 3	AF498395	Subbotin et al. (2004)
A. capillaris 9	FJ042831	This study
A. capillaris 11	FJ042825	This study
A. capillaris 12	FJ042838	This study
A. capillaris 17	FJ042828	This study
A. castellana Boiss. & Reut. 1	DQ146768	Reichman et al. (2006)
A. castellana 2	DQ539591	Quintanar et al. (2007)
A. exarata Trin. 1	DQ146769	Reichman et al. (2006)
A. exarata Roth. 2	DQ146770	Reichman et al. (2006)
A. gigantea 1	DQ146772	Reichman et al. (2006)
A. gigantea 2	DQ146771	Reichman et al. (2006)
A. gigantea 3	EF565133	Unpublished
A. idahoensis Nash.	DQ146773	Reichman et al. (2006)
A. imbecilla Zotov	AY705881	Unpublished
A. magellanica Lam.	AY705883	Unpublished
A. mertensii Trin.	DQ146774	Reichman et al. (2006)
A. muelleriana Vickery	AY705880	Unpublished
A. muscosa Kirk	AY705884	Unpublished
A. pallens Trin.	DQ146775	Reichman et al. (2006)
A. pallescens Cheeseman	AY705879	Unpublished
A. personata Edgar	AY705881	Unpublished
A. petriei Hack.	AY705882	Unpublished
A. scabra Willd.	DQ146776	Reichman et al. (2006)
A. stolonifera L. 1	DQ146777	Reichman et al. (2006)
A. stolonifera 2	DQ146778	Reichman et al. (2006)
A. stolonifera 3	DQ146781	Reichman et al. (2006)
A. stolonifera 4	DQ146780	Reichman et al. (2006)
A. stolonifera 5	EF541167	Unpublished
A. stolonifera 6	FJ042826	This study
A. stolonifera 7	FJ042858	This study
A. stolonifera 8	FJ042845	This study
A. stolonifera 9	FJ042843	This study
A. vinealis Schreb. 1	DQ146791	Reichman et al. (2006)
A. vinealis 2	FJ042802	This study
A. vinealis 4	FJ042871	This study
A. vinealis 15	FJ042805	This study
A. vinealis 17	FJ042814	This study
A. truncatula Castrov. et Charpin	DQ539592	Quintanar et al. (2007)
P. monspeliensis (L.) Desf. 1	DQ146795	Reichman et al. (2006)
P. monspeliensis 2	DQ146794	Reichman et al. (2006)
P. viridis (Gouan) Breistr.	DQ146796	Reichman et al. (2006)
B. distachyon (L.) Beauv.	AF303399	Torrecilla and Catalan (2002)
trnK/matK		
A. canina	FJ231115	This study
A. capillaris 1	FJ231112	This study
A. capillaris 2	DQ146798	Reichman et al. (2006)
A. castellana	DQ146799	Reichman et al. (2006)
A. exarata 1	DQ146800	Reichman et al. (2006)
A. exarata 2	DQ146801	Reichman et al. (2006)
A. gigantea 1	DQ146802	Reichman et al. (2006)
A. gigantea 2	DQ146803	Reichman et al. (2006)
A. idahoensis	DQ146804	Reichman et al. (2006)
A. mertensii	DQ146805	Reichman et al. (2006)
A. pallens	DQ146806	Reichman et al. (2006)
A. scabra	DQ146807	Reichman et al. (2006)
A. stolonifera 1	FJ231114	This study
A. stolonifera 2	DQ146811	Reichman et al. (2006)
A. stolonifera 3	DQ146810	Reichman et al. (2006)
A. stolonifera 4	DQ146812	Reichman et al. (2006)
A. vinealis 1	DQ146812	Reichman et al. (2006)
A. vinealis 2	FJ231113	This study
P. monspeliensis 1	DQ146823	Reichman et al. (2006)
P. monspeliensis 2	DQ146822	Reichman et al. (2006)
P. viridis	DQ146824	Reichman et al. (2006)
B. distachyon	EU325680	Bortiri et al. (2008)
O. sativa L.	AY522329	Tang et al. (2004)
Photosystem I, N subunit		
A. canina	FJ514815	This study
A. capillaris, A_1	DV854551	Rotter et al. (2007)
A. capillaris, A_2	DV854140	Rotter et al. (2007)
A. stolonifera, A_2	DV865983	Rotter et al. (2007)
A. stolonifera, A_3	DV864532	Rotter et al. (2007)

Table 3 continued

Sequence name	Accession number	Source
A. vinealis	FJ514816	This study
B. distachyon	DV477931	Vogel et al. (2006)
Unknown protein		
A. canina	FJ514817	This study
A. capillaris, A_1	DV858879	Rotter et al. (2007)
A. capillaris, A_2	DV858126	Rotter et al. (2007)
A. stolonifera, A_2	DV865689	Rotter et al. (2007)
A. stolonifera, A_3	DV867060	Rotter et al. (2007)
A. vinealis	FJ514818	This study
B. distachyon	No accession	http://blast.brachybase.org

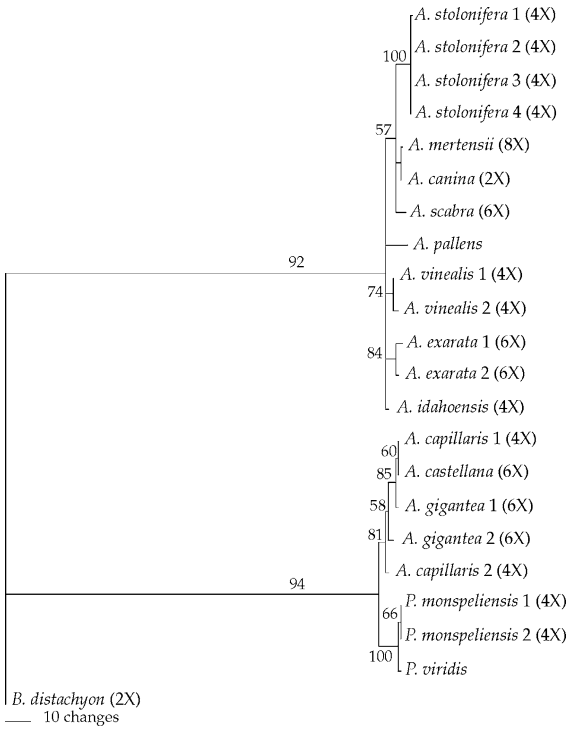

Fig. 2 Rooted 50% majority rule maximum parsimony phylogenetic tree of plastid *matK* and flanking *trnK* intron sequences. The tree length is 224, the consistency index is 0.915, and the retention index is 0.909. The *B. distachyon* sequence was designated as the outgroup for rooting the tree. The numbers at the nodes are the bootstrap percentages based on 1,000 replications. Accession numbers of the sequences are given in Table 3. When known, the ploidy level of the species is indicated in parenthesis

accessions, respectively. One velvet bentgrass accession (PI 189141) had an additional 17 bp tandem repeat in this region. The 18 bp repeat was also found in the *A. mertensii* and *A. scabra* sequences. Figure 3 also includes the sequences of *B. distachyon* and *Oryza sativa* (rice), species that are basal to the tribe Aveneae (Catalan et al. 1997). Neither of these two more ancient species has the duplication. The duplication is also not found in *Festuca arundinacea* Schreber (tall fescue) or *Deschampsia antarctica* Desv. (Antarctic hairgrass), both members of the sister tribe Poeae. Since the duplication is found in only some of the *Agrostis* spp. it must have occurred after the divergence of *Agrostis*.

Nuclear conserved orthologous set gene phylogeny

Since the *trnK/matK* analysis indicated velvet bentgrass was the likely maternal parent of creeping bentgrass, phylogenetic analysis of nuclear conserved orthologous set genes (Fulton et al. 2002) was used to determine whether velvet bentgrass was closer to the A_2 or A_3 subgenome of creeping bentgrass. The analysis of the combined dataset was based upon 353 total characters, of which 258 were constant, 90 variable characters were parsimony uninformative, and 5 characters were parsimony informative. The single most parsimonious phylogenetic tree recovered from an exhaustive search is presented in Fig. 4.

Discussion

Creeping and colonial bentgrasses have previously been included in ITS and chloroplast DNA-based phylogenetic studies of the Poaceae (Doring et al. 2007; Quintanar et al. 2007; Bouchenak-Khelladi et al. 2008) but there has not been a DNA-based study that specifically examined the phylogenetic relationships of the commercially important *Agrostis* turfgrass species to each other. Reichman et al. (2006) analyzed *matK* and ITS sequences of creeping, colonial, velvet, and brown bentgrasses in the context of identification of intraspecific or interspecific hybrids generated from pollination from herbicide resistant creeping bentgrass plants. Their reported

Fig. 3 Sequence alignment of the region of the 18 bp duplication in the upstream *trnK* intron seen in some *Agrostis* spp. The region shown corresponds to positions 130–178 in the *A. canina* sequence

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A. canina                TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina PI290707       TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina PI578526       TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina PI230234       TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina ISC8           TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina ISC12          TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. canina PI189141       TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAATTTGAATGGAATAAAAATTACGAT
A. stolonifera 1         TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. stolonifera 2         TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. stolonifera 3         TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. stolonifera 4         TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. stolonifera PI601363  TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. stolonifera W6 6602   TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. mertensii             TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. scabra                TTTCTTTTTTTTGAATGGAATAAAAATTTTGAATGGAATAAAAA----------------TTACGAT
A. capillaris 1          TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. capillaris 2          TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. capillaris PI42023    TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. capillaris PI494120   TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. capillaris PI494121   TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. castellana            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. exarata 1             TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. exarata 2             TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. idahoensis            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. gigantea 1            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. gigantea 2            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. pallens               TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. vinealis 1            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
A. vinealis 2            TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
P. monspeliensis 1       TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
P. monspeliensis 2       TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTACGAT
P. viridis               TTTCTTTTTTTTGAATGGAATAAAAA-----------------------------------TTTCGAT
F. arundinacea           TTTCTTTTTTTTTATGGAATAAAAA------------------------------------TTACGAT
D. antarctica            TTCTTTTTTTTTTAATGGAATATAAA-----------------------------------TTACGAT
B. distachyon            TTTTATTTTTGGAATGGAATAGAAAAT----------------------------------TTACTAT
O. sativa                TTGGATTTTTGGAATGGAATAAAAAAA----------------------------------TTCTGAT
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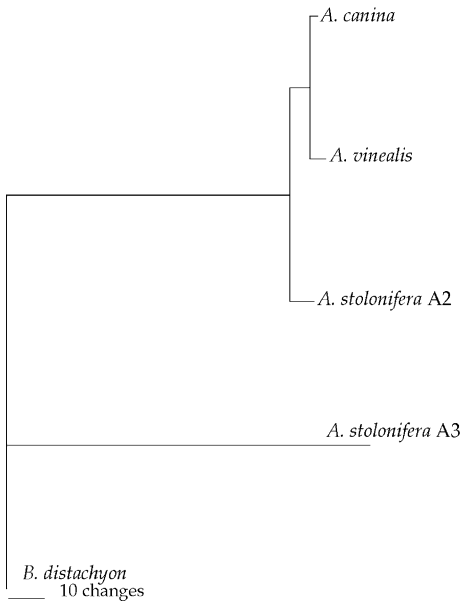

Fig. 4 The single most parsimonious phylogenetic tree recovered from an exhaustive search of the nuclear conserved orthologous set gene sequences. The *B. distachyon* sequence was designated the outgroup for rooting the tree. Accession numbers of the gene sequences are given in Table 3

phylogeny did not consider the allotetraploid nature of creeping and colonial bentgrasses and the velvet bentgrass sample in their analysis was misidentified since the reported ITS sequence is more similar to those of *Poa* spp. than to *Agrostis* spp.

To better understand the phylogenetic relationships of creeping, colonial, and velvet bentgrasses, we used plastid *trnK*/*matK* and nuclear ITS and protein-coding DNA sequences. ITS sequences are commonly used in phylogenetic analysis (Alvarez and Wendel 2003), so sequences from several additional *Agrostis* spp. were available for inclusion in this analysis. For the allotetraploid creeping and colonial bentgrasses it was a possibility that distinct subgenome ITS sequence types would be found. Allopolyploid species may have distinct ITS sequence variants originating from their parental species. Phylogenetic placement of such divergent sequences into different clades can be used to infer the identities of the subgenome donors. For example, ITS sequences from several individuals of the allohexaploid species tall fescue grouped into two clades, suggesting one subgenome donor was *F. pratensis* Huds. and another donor was from a *Festuca* species within section Bovinae (Gaut et al. 2000). Similarly, additivity of polymorphic ITS sequences was used to identify the parents of five allotetraploid *Paeonia* spp. (Sang et al. 1995).

Alternatively, in some *Gossypium*, *Aegilops*, *Nicotiana* and *Bromus* allotetraploid species concerted evolution has also occurred between homoeologous loci and has resulted in homogenization of the ITS regions to one of the parental types (Wendel et al. 1995; Wang et al. 2000; Kovarik et al. 2004; Fortune et al. 2008). Our data on allotetraploid creeping and

colonial bentgrasses suggests that concerted evolution has also resulted in homogenization of the homoeologous ITS regions in these species since all of the sequences from each species grouped into the same clade. That there was no grouping comprised of both creeping and colonial bentgrass ITS sequences indicates the ITS sequences in each species have been homogenized to different subgenomes. Based on conclusions from the plastid *trnK*/*matK* and nuclear protein coding gene phylogenies (discussed below) we can conclude that the colonial bentgrass ITS sequences have been homogenized to the A_1 subgenome and the creeping bentgrass ITS sequences to the A_2 subgenome. The new ITS sequences generated here are from cloned PCR products, whereas those sequences from previous reports are from direct sequencing of PCR products. It is possible that multiple ITS sequence types exist in the other polyploid *Agrostis* spp. but were not seen from direct sequencing of the PCR products.

The ITS phylogenetic analysis also revealed close relationships of some of the other *Agrostis* spp. to creeping and colonial bentgrasses. Interspecific hybridization among *Agrostis* spp. is known to occur, and is certainly the origin of the polyploid species. Wipff and Fricker (2001) have presented a comprehensive summary of the older literature on interspecific and intergeneric hybridization among *Agrostis* spp. Phylogenetic analyses can be used to deduce some of the ancestral relationships. The close relationship of the hexaploid species *A. gigantea* and *A. castellana* with colonial bentgrass suggests these species all have a shared subgenome, as proposed by Jones (1956c). Similarly, the close relationship of *A. idahoensis*, *A. imbecilla*, *A. magellanica, A. mertensii, A. muelleriana, A. muscosa*, *A. pallens*, *A. pallescens*, *A. personata*, *A. petriei*, and *A. scabra* with creeping bentgrass suggests these species may all share a subgenome. Unexpectedly, velvet bentgrass and brown bentgrass were included in the creeping bentgrass clade, suggesting creeping bentgrass also shares a subgenome with these species. The close relationship of velvet bentgrass with brown bentgrass was proposed by Jones (1956a), but both species were considered to be closely related to colonial bentgrass, not to creeping bentgrass. The ITS sequence data presented here implies that velvet bentgrass may have been one of the diploid parental species of creeping bentgrass.

The close relationship of velvet bentgrass to creeping bentgrass is also supported by the *trnK*/*matK* phylogenetic analysis. The sequences grouped into two main clades. One branched into two subclades, one of which contained the colonial bentgrass, *A. castellana*, and *A. gigantea* sequences. The other subclade contained the *Polypogon* spp. sequences. The second main clade contained the creeping, velvet, and brown bentgrasses as well as *A. exarata*, *A. idahoensis*, *A. mertensii*, *A. pallens*, and *A. scabra*. That the colonial bentgrass and creeping bentgrass sequences grouped in different clades suggests they do not have the same diploid maternal parent, whereas the close relationship of the colonial bentgrass with *A. castellana* and *A. gigantea* does suggest a shared plastid genome donor among these species. The creeping, velvet, and brown bentgrass sequences as well as those of *A. pallens*, *A. exarata*, *A. idahoensis*, *A. mertensii*, and *A. scabra* were closely grouped together suggesting these species may share a common plastid genome donor.

Sequence alignment revealed an 18 bp duplication in the *trnK* intron region shared by creeping and velvet bentgrass, *A. mertensii* and *A. scabra*, further supporting the hypothesis that these species had the same plastid genome donor. Shared duplications or deletions in plastid DNA sequences have previously been used to infer evolutionary relationships (Wolfson et al. 1991; Saski et al. 2007).

The close relationship of the *matK* gene and the shared 18 bp duplication in the *trnK* intron imply that the diploid species velvet bentgrass was the maternal parent of allotetraploid creeping bentgrass. Since *A. mertensii* and *A. scabra* are higher-level polyploids, an octoploid and hexaploid, respectively (Reeder 1977; Brede and Sellmann 2003), it is possible that either velvet bentgrass or creeping bentgrass may have been the ancestral maternal parent of these species. Similarly, the close relationship of the colonial bentgrass *trnK*/*matK* sequences to those of *A. castellana* and *A. gigantea* suggests these species may share the same plastid genome donor.

Quintanar et al. (2007) noted that in their phylogenetic analysis of the tribe Aveneae, *Agrostis* was paraphyletic. This was also seen in this study where some *Polypogon* spp. were embedded in the *Agrostis* clades. Intergeneric hybridization between *Polypogon* spp. and *Agrostis* spp. has been reported (summarized by Wipf and Fricker 2001) so it is possible

that past intergeneric hybridization events have contributed to the genomes of these species.

The shared 18 bp duplication in the plastid *trnK* intron sequence suggested that velvet bentgrass was likely the diploid maternal parent of allotetraploid creeping bentgrass. If so, then the velvet bentgrass genome designation should be revised to either A_2 or A_3, corresponding to one of the creeping bentgrass subgenomes. Although the ITS sequences of creeping and colonial bentgrass appeared to be homogenized, analysis of single copy conserved nuclear genes revealed homoeologous subgenome specific sequences (Rotter et al. 2007). A similar situation has been found in some cotton allotetraploids where the ITS sequences were homogenized within the species but subgenome-specific gene sequences could be identified (Wendel et al. 1995; Senchina et al. 2003). Low copy nuclear genes in allopolyploids are less likely to be subjected to concerted evolution and may be more useful for identification of subgenome donor species (Small et al. 2004). Previously we used phylogenetic analysis of conserved orthologous set genes (Fulton et al. 2002) to make subgenome assignments of some homoeologous creeping and colonial bentgrass genes (Rotter et al. 2007). To determine which genome designation is most appropriate for velvet bentgrass we used oligonucleotide primers designed from two of the conserved orthologous set genes to amplify the orthologous sequences from velvet and brown bentgrasses. Phylogenetic analysis of velvet and brown bentgrass nuclear conserved orthologous set genes indicated they were closely related to the creeping bentgrass A_2 subgenome gene sequences.

Overall, the data presented here suggests that velvet bentgrass was the maternal parent of creeping bentgrass. Based on this new information some of the *Agrostis* spp. genome models developed by Jones (1956a, b, c) should be revised. We propose that the $A_1A_1A_2A_2$ and $A_2A_2A_3A_3$ genome designations for colonial and creeping bentgrasses, respectively should be retained. Cytology, DNA content, and genetic linkage mapping clearly indicate these species are each allotetraploid and subgenome assignments of the colonial bentgrass linkage groups have been made (Jones 1956b, c; Bonos et al. 2002; Chakraborty et al. 2005; Rotter et al. 2009). The close relationship of the shared A_2 subgenomes was supported by estimation of a recent divergence from a common ancestral genome (Rotter et al. 2007).

The close relationship of the autotetraploid brown bentgrass with the diploid velvet bentgrass suggested by Jones (1956a) is supported in the ITS, *trnK/matK*, and nuclear gene phylogenetic trees. However, the phylogenetic data indicate that velvet and brown bentgrasses are closely related to creeping bentgrass, contrary to the models suggested by Jones. Sequence analysis of some conserved orthologous set genes from both velvet and brown bentgrass indicated they are both closely related to the A_2 subgenome sequences of creeping bentgrass. We therefore propose that both velvet and brown bentgrass be assigned the A_2 genome designation. That brown bentgrass did not have the 18 bp duplication in the *trnK* intron that was shared by velvet and creeping bentgrass suggests that the sequence duplication occurred in velvet bentgrass after the autopolyploidy event that generated brown bentgrass. A model for the proposed genomic relationships is presented in Fig. 5. Based on the shared A_2 subgenomes of creeping and colonial bentgrasses, velvet bentgrass is proposed as the paternal diploid parent of colonial bentgrass.

Ultimately, identification of the ancestral diploid species that generated the allotetraploid creeping and colonial bentgrass will be important in understanding the origin of some of the phenotypic differences between the species. For example, creeping bentrass is stoloniferous whereas velvet and colonial bentgrasses are not. In the future, identification of the A_3 diploid parental species of creeping bentgrass will help answer the question of whether the stoloniferous growth habit of creeping bentgrass originates from the A_3 parental genome or from the interaction of both parental genomes.

In this work we focused on the three *Agrostis* spp. important as turfgrasses. Our analysis included only one confirmed diploid species, velvet bentgrass. In the future, identification of additional diploid *Agrostis* spp. combined with DNA-based phylogenetic studies could be carried out to possibly identify the other diploid species that hybridized to form the numerous polyploid *Agrostis* spp.

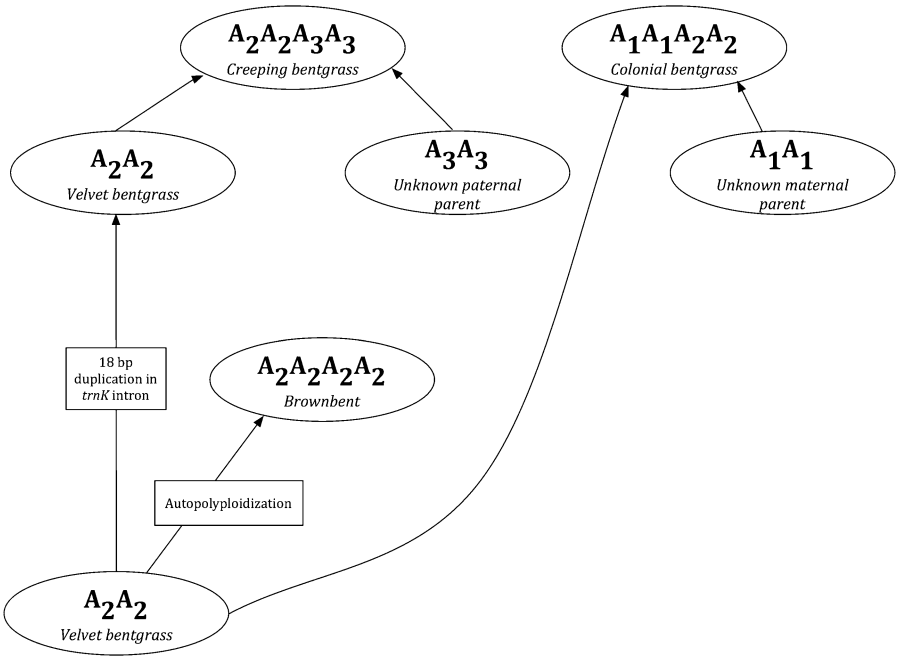

Fig. 5 A new model of genome relationships among velvet, creeping, colonial and brown bentgrasses

Acknowledgments This research was supported with funds provided by the United States Golf Association and the Rutgers Center for Turfgrass Science. We thank Dr. A. Koroch for preparing the voucher specimens.

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