Agrostis Species Relationships Based on *trnL-trnF* and *atpI-atpH* Intergenic Spacer Regions

Keenan Amundsen¹

Department of Agronomy and Horticulture, University of Nebraska, 377H Plant Science Hall, Lincoln, NE 68583-0915

Scott Warnke

Floral and Nursery Plants Research Unit, U.S. Department of Agriculture, Beltsville, MD 20705

Additional index words. turfgrass, *Agrostis*, chloroplast, phylogeny

Abstract. The bentgrasses (*Agrostis* spp.) are among the most important species to the turfgrass industry. They have complex genomes resulting from polyploidization and high rates of interspecific hybridization. An understanding of species relationships would improve the efficiency of developing improved bentgrass cultivars. To elucidate the evolutionary relationships among *Agrostis* species, phylogenetic analyses were performed on sequences of two chloroplast-encoded intergenic spacer regions. A 298-bp region of the *trnL-trnF* intergenic spacer and a 451-bp region of the *atpI-atpH* intergenic spacer were included in the analyses. A total of 47 *Agrostis* accessions were included with both cultivated and unimproved material from the National Plant Germplasm System. Of these 47 *Agrostis* collections, there were 10 unique *trnL-trnF* haplotypes and eight distinct *atpI-atpH* haplotypes, indicating a high degree of shared sequence identity within these chloroplast intergenic regions. These findings suggest that the chloroplast genomes of *A. canina* and *A. stolonifera* are more closely related to each other than either species is to *A. capillaris*, incongruent with our previous understanding of genome relationships in the genus.

The bentgrasses (*Agrostis* spp.) are important species to the turfgrass industry because of their ability to form a dense, high-quality turf at low mowing heights. There are between 150 and 200 species of *Agrostis* (Harvey, 2007), and the evolutionary relationships between species are not clearly understood. Deducing these relationships is complicated by a number of factors. The major turf-type *Agrostis* species are highly outcrossing and exhibit high rates of heterozygosity (Warnke et al., 1998). Several species are polyploid causing homoeologous loci. Homologous genes often have high levels of sequence identity and may not be informative. Intermediate morphologies exist between taxa making taxonomic classification difficult. Interspecific hybridization is common and these reticulation events are difficult to represent in bifurcating phylograms.

Jones (1956a, 1956b, 1956c) described the chromosome pairing behavior during metaphase I of meiosis among *Agrostis stolonifera*, *A. canina*, *A. capillaris*, *A. vinealis*, and *A. gigantea*. *Agrostis vinealis* is an autotetraploidization of *A. canina* (Jones, 1956a) and these species have the genome designations $A_1A_1A_1A_1$ and A_1A_1 , respectively

(Brilman, 2001). The tetraploids *A. capillaris* ($A_1A_1A_2A_2$) and *A. stolonifera* ($A_2A_2A_3A_3$) share one sub-genome in common (A_2A_2) and the hexaploid *A. gigantea* ($A_1A_1A_2A_2A_3A_3$) has two sub-genomes in common with both *A. capillaris* and *A. stolonifera* (Jones, 1956b, 1956c). The experiments conducted by Jones are among the most comprehensive describing the relationships between these common turf-type *Agrostis* species.

Evidence of *Agrostis* species relationships based on contemporary molecular marker technologies is lacking. Molecular markers have been used in several studies to explore *Agrostis* genetic diversity. For example, Vergara and Bughrara (2003) examined 400 polymorphic amplified fragment length polymorphism (AFLP) makers in a set of 40 *Agrostis* accessions representing 14 species. They reported that *A. transaspica* is diploid and based on clustering of AFLP markers, this species may have contributed the A_3A_3 genome to the evolution of *A. stolonifera* and *A. gigantea*. Amundsen and Warnke (2011) explored genetic diversity among 75 *Agrostis* accessions along with six closely related species based on 1309 miniature inverted-repeat transposable element (MITE) display markers. The modified AFLP technique, MITE display, anchors the amplified products to MITEs. The *Agrostis* accessions were divided into six clades based on marker clustering (Amundsen and Warnke, 2011). Species relationships can be partially inferred

from these *Agrostis* diversity studies, but there have been no studies designed to specifically explore these relationships.

Species relationships can be deduced from nuclear and plastid DNA sequences. Reichman et al. (2006) sequenced the chloroplast *matK* gene and nuclear internal transcribed spacer (ITS) region from 19 *Agrostis* and three *Polypogon* accessions. Phylogenetic analysis of the DNA sequences was used to determine if transgenic *A. stolonifera* from a controlled area was capable of pollinating wild populations of related species growing at a distance. The individual phylogenies were used to distinguish *Agrostis* accessions by species. Certain *Agrostis* species have been included in phylogenies of the Aveneae and Poeae tribes based on DNA sequence data, placing *Agrostis* species sister to *Calamagrostis* and *Polypogon* species within the tribe Aveneae (Döring et al., 2007; Quintanar et al., 2007; Soreng et al., 2007).

Inference of *Agrostis* evolution, a highly outcrossing polyploid, based on ITS or other high copy-repetitive sequence elements, might not provide an accurate depiction of species evolution (Feliner and Rosselló, 2007) and single or low-copy nuclear genes might be more informative (Feliner and Rosselló, 2007; Sang, 2002). One drawback of using low copy genes is that they can be difficult to identify in non-model organisms such as *Agrostis*. Rotter et al. (2007) sequenced expressed sequence tag DNA libraries of the two *Agrostis* tetraploid species *A. stolonifera* and *A. capillaris* covering $\approx 10\%$ of the predicted gene space. This sequencing effort led to the identification of 177 and 161 *Oryza* Conserved Ortholog Set (COS; Fulton et al., 2002) genes conserved in the *A. capillaris* and *A. stolonifera* DNA sequence libraries, respectively. Fulton et al. (2002) describe a reference set of 1025 COS genes that are single or low copy genes and conserved across diverse genomes. Seven of the 161 *A. stolonifera* and 11 of the 177 *A. capillaris* *Oryza* COS gene homologs were found to have conserved sequences in other grasses. These sequences were used in phylogenetic analyses that placed the *Agrostis* species closer to *Festuca arundinacea* than to *Avena sativa*, which is interesting because *Agrostis* is in the same tribe as *A. sativa*, whereas *F. arundinacea* is in the Poeae tribe. *Agrostis* was placed in the Aveneae tribe based on morphological characteristics and enzyme restriction patterns of chloroplast DNA (Soreng and Davis, 1998; Watson, 1990). The two tribes are closely related (Kellogg, 1998; Soreng et al., 2007) and share many common chloroplast DNA restriction sites (Soreng et al., 1990).

Chloroplast sequences would be more informative for deducing *Agrostis* relationships because chloroplasts are maternally inherited in *Agrostis* and have simple genomes (Warnke, 2003). Soreng et al. (2007) performed a phylogenetic analysis of the tribe Poeae using morphological characteristics and

Received for publication 26 Aug. 2011. Accepted for publication 10 Nov. 2011.

¹To whom reprint requests should be addressed; e-mail kamundsen2@unl.edu.

sequence data of three chloroplast-encoded genes, maturase K (*matK*), NADH dehydrogenase subunit F (*ndhF*), and ATP synthase beta subunit (*atpB*). The sequence data for this project are available through the National Center for Biotechnology Information databases and the analysis included the species *Agrostis tenerrima* Trin., *Calamagrostis arundinacea* (L.) Roth., *C. canadensis* (Michx.) P. Beauv., *Polypogon monspeliensis*, *Festuca rubra* L., and *F. subverticillata* (Pers.) E.B. Alexeev. Soreng et al. (2007) found that molecular character data were better than morphological data at describing a well-resolved set of species relationships. Similarly, Döring et al. (2007) sequenced the *matK* gene from a number of specimens from the Aveneae/Poeae complex including *A. capillaris*, *C. rivalis* (Torges) H. Scholz., *C. macrolepis* Litv., *Apera spica-venti* (L.) P. Beauv., *P. monspeliensis*, *Avena sativa* L., *F. altissima* All., and *F. gigantea* (L.) Vill. *Agrostis* can hybridize with *Polypogon* spp. and shares morphological characteristics in common with *Apera* and *Calamagrostis* spp. (Harvey, 2007). The results of Döring et al. (2007) were consistent with Soreng et al. (2007) and it is interesting that both phylogenies of chloroplast-encoded gene sequence data placed *Agrostis* more closely to *Avena* than *Festuca* in contrast to the COS phylogenetic analysis performed by Rotter et al. (2007).

Different homologous DNA segments from related taxa often have inconsistent evolutionary histories and analyzing the DNA segments separately will often yield trees of differing topologies (McBreen and Lockhart, 2006). These inconsistencies may arise from reticulation events such as horizontal gene transfer, recombination, and hybridization that are not well represented by bifurcating phylogenetic trees (Huson and Bryant, 2006; Huson et al., 2005; Linder and Rieseberg, 2004). A phylogenetic network might be the best representation of the evolution of *Agrostis*, because a number of *Agrostis* species are polyploids and thought to have evolved from naturally occurring interspecific hybrids (Casler and Duncan, 2003). The software program SplitsTree4 can render phylogenetic networks from individual gene trees (Huson and Bryant, 2006).

Since the 1956 cytological experiments of Jones, there have been a number of advancements for investigating relationships between *Agrostis* species, particularly with the use of molecular markers, sequence data, and phylogenetic analyses. Genetic relationships in *Agrostis* have been studied using nuclear markers (Amundsen and Warnke, 2011; Vergara and Bughara, 2003), but the focus of these studies was to resolve genetic diversity. *Agrostis* has been included in other phylogenetic studies of the tribe Poeae and Aveneae (Döring et al., 2007; Quintanar et al., 2007; Soreng et al., 2007), but to date, there are no studies focused on resolving evolutionary relationships within

Agrostis based on chloroplast sequences. In the present study, the chloroplast encoded *trnL-trnF* and *atpI-atpH* intergenic spacer regions were sequenced from a diverse set of *Agrostis* germplasm to study the relationships between accessions. Phylogenies of both cultivated and non-cultivated National Plant Germplasm System *Agrostis* accessions were compared to identify any incongruence and infer species relationships. Species relationships are complex in *Agrostis*, but a better understanding will assist plant breeders in maximizing wide cross-breeding, identify bridging species to introgress important traits, identify evolutionary events that have led to important turf characteristics, and resolve polyploidization events in the genus.

Materials and Methods

Plant material and DNA extraction. Accessions of *Agrostis* were chosen that represent cultivated and non-cultivated accessions, various ploidy levels as predicted by flow cytometry, different species, and different geographic regions (Table 1). Seeds of each accession were obtained from the National Plant Germplasm System, Dr. Doug Johnson (Forage and Range Research Laboratory, U.S. Department of Agriculture), or from the National Turfgrass Evaluation Program. Seeds were planted in Premier Pro-Mix BX soil (Premier Horticulture Inc., Quakertown, PA) in 2.5-cm-diameter Cone-tainers (Stuewe and Sons, Tangent, OR) and maintained in a temperature-controlled greenhouse on

Table 1. Accessions used in phylogenetic analyses.

Study ID	Accession	Taxon	Cultivar ^a	Origin
aint-1	PI203444	<i>Apera interrupta</i>		Turkey
aint-2	PI204389	<i>Apera interrupta</i>		Turkey
pchi-1	PI372227	<i>Polypogon chilensis</i>		Uruguay
pvir-1	PI204395	<i>Polypogon viridis</i>		Turkey
pvir-2	PI223241	<i>Polypogon viridis</i>		Afghanistan
acan-10	PI189141	<i>Agrostis canina</i>	Novobent	Netherlands
acan-11	PI290707	<i>Agrostis canina</i>		United Kingdom
acap-7	PI237717	<i>Agrostis capillaris</i>	Odenwalder	Germany
acap-25	PI578528	<i>Agrostis capillaris</i>	Exeter	United States
acap-26	PI600936	<i>Agrostis capillaris</i>	Duchess	United Kingdom
acas-11	PI240132	<i>Agrostis castellana</i>		Portugal
acas-3	PI318928	<i>Agrostis castellana</i>		Spain
acas-8	PI469217	<i>Agrostis castellana</i>	Highland	United States
acla-2	PI632584	<i>Agrostis clavata</i>		Mongolia
acla-5	W621240	<i>Agrostis clavata</i>		Mongolia
agig-77	PI251099	<i>Agrostis gigantea</i>		Yugoslavia
alim-1	W623620	<i>Agrostis limprichtii</i>		China
alya-1	PI636652	<i>Agrostis lyallii</i>		New Zealand
amon-3	PI632549	<i>Agrostis mongolica</i>		Mongolia
amon-5	W619706	<i>Agrostis mongolica</i>		Mongolia
amun-7	PI230236	<i>Agrostis munroana</i>		Iran
apal-1	PI238226	<i>Agrostis pallida</i>		Spain
asp-4	PI502279	<i>Agrostis</i> sp.		Russian
asto-36	PI578529	<i>Agrostis stolonifera</i>	Seaside	United States
asto-40	PI632687	<i>Agrostis stolonifera</i>	Penncross	United States
asto-19	PI204390	<i>Agrostis stolonifera</i>		Turkey
asto-23	PI251945	<i>Agrostis stolonifera</i>		Austria
asto-29	PI302902	<i>Agrostis stolonifera</i>		Spain
asto-30	PI318934	<i>Agrostis stolonifera</i>		Spain
asto-34	PI531251	<i>Agrostis stolonifera</i>	Keszthelyi4	Hungary
atri-1	PI598462	<i>Agrostis trinii</i>		Russian
atri-2	PI632559	<i>Agrostis trinii</i>		Mongolia
atri-3	PI636572	<i>Agrostis trinii</i>		Mongolia
atri-4	W619572	<i>Agrostis trinii</i>		Mongolia
avin-1	PI440110	<i>Agrostis vinealis</i>		Russian
agig-1	RUS-07-01-002	<i>Agrostis gigantea</i>		Russia
agig-5	RUS-07-12-041	<i>Agrostis gigantea</i>		Russia
agig-12	RUS-07-32-131	<i>Agrostis gigantea</i>		Russia
asto-1	KGZ-06-08-051	<i>Agrostis stolonifera</i>		Kyrgyzstan
agig-47	PRC-06-IN/118-402	<i>Agrostis gigantea</i>		China
asto-3	NT-1998-26	<i>Agrostis stolonifera</i>	Penn G-1	United States
asto-4	NT-1998-27	<i>Agrostis stolonifera</i>	Penn A-4	United States
asto-5	NT-1998-29	<i>Agrostis stolonifera</i>	Penn A-2	United States
asto-6	NT-2003-2	<i>Agrostis stolonifera</i>	Penn A-1	United States
asto-7	NT-2003-6	<i>Agrostis stolonifera</i>	Alpha	United States
asto-9	NT-2003-10	<i>Agrostis stolonifera</i>	Declaration	United States
asto-12	NT-2003-15	<i>Agrostis stolonifera</i>	Kingpin	United States
asto-13	NT-2003-21	<i>Agrostis stolonifera</i>	007	United States
acan-2	NT-1998-11	<i>Agrostis canina</i>	Vesper (Pick MVB)	United States
acan-6	NT-2003-16	<i>Agrostis canina</i>	Villa (IS-AC 1)	United States
acan-7	NT-2003-18	<i>Agrostis canina</i>	Venus (EFD)	United States
acan-8	NT-2003-19	<i>Agrostis canina</i>	Vesper	United States

^aCultivar = cultivated accessions are indicated by their cultivar name.

a 12-h photoperiod with 24 °C day and 21 °C night temperatures. Total DNA was extracted from a single genotype of each accession by either the DNeasy 96 Plant kit (Qiagen, Valencia, CA) or by a modified CTAB method (Amundsen et al., 2011).

Conserved chloroplast primer screen. The *Agrostis stolonifera* L. cv. Penn-A4 chloroplast genome (EF115543) was obtained from GenBank. Chloroplast-specific primer sequences reported by Shaw et al. (2005, 2007) and Small et al. (1998) were computationally screened against the *A. stolonifera* chloroplast genome in LaserGene (DNASTAR Inc., Madison, WI). Conserved primer pairs are shown in Table 2. A polymerase chain reaction (PCR) protocol consisting of denaturing the sample at 95.0 °C for 5.0 min followed by 35 cycles of 95.0 °C for 1.0 min, 45.0 °C for 45.0 s, and 72.0 °C for 1.5 min followed by holding the sample at 72.0 °C for 5.0 min was used to test the conserved primer pairs against a panel of *Agrostis* germplasm. Each PCR reaction contained 1.0 units recombinant *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA), 20 mM Tris pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM each dNTP (Promega, Madison, WI), 0.5 µM each primer, and 20 ng template DNA.

The software program DNASTAR LaserGene Version 6 was used to identify conserved chloroplast-specific primer pairs reported by Shaw et al. (2005, 2007) and Small et al. (1998) in an *Agrostis stolonifera* cv. Penn-A4 chloroplast genome (GenBank Accession EF115543). Seventeen conserved primer pairs were identified and their relative base pair position on the chloroplast genome was determined (Table 2). A subset of conserved primer pairs was screened on a diverse panel of *Agrostis* DNA and successfully amplified products sequenced. The primer pairs amplifying the *trnL-trnF* and *atpI-atpH* intergenic spacer regions of the chloroplast genome amplified well in all accessions tried and were subsequently sequenced from the 52 accessions composed of one *Apera* accession, three *Polypogon* accessions, and 48 *Agrostis* accessions (Table 1).

DNA sequencing and phylogenetic analysis. All sequencing reactions of *atpI-atpH* and *trnL-trnF* intergenic spacer regions were performed using the BigDye3.1 sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions were run separately for each primer specified by the A and K primer group identifier in Table 2 and on a GeneAmp2700 ThermalCycler (Applied Biosystems). Sequences were aligned with ClustalW2.0.9 (Thompson et al., 1994) and manually adjusted to further optimize alignments. Different methods were tested for encoding insertions and deletions in the alignments, including the complete omission of the gapped regions, the replacement of gap characters by a fifth base, the treatment of gaps as missing data, and the Barriol method (Barriol, 1994). The Barriol method encodes gapped regions with multistate characters to account for informative gaps in the data matrix.

Table 2. Primers conserved in the *Agrostis stolonifera* chloroplast genome.

ID ^z	Name ^y	Position ^x	Sequence 5'-3'	Tm ^w	Ref ^v
A	<i>trnL-trnF</i> spacer:E	47467	GGTTCAAGTCCCTCTATCCC	49.1	THI
	<i>trnL-trnF</i> spacer:F	47888	ATTTGAAGTGGTGACACGAG	47.1	THI
B	<i>trnT-trnL</i> spacer:A	46123	CATTACAAATGCGATGCTCT	48.0	THI
	<i>trnT-trnL</i> spacer:B	47003	TCTACCGATTTCGCCATATC	49.4	THI
C	rpS16R	5218	AACATCWATTGCAASGATTTCGATA	55.3	THII
	rpS16F	6105	AAACGATGTGGTARAAAGCAAC	50.5	THII
D	3' <i>trnG</i> ^{UUC}	13397	GTAGCGGGAATCGAACCCGCATC	64.2	THII
	5' <i>trnG</i> ^{2G}	14137	GCGGGTATAGTTTAGTGGTAAAA	49.7	THII
E	<i>trnC</i> ^{GCA} R	18642	CACCCRGATTYGAAGTGGGG	54.1	THII
	rpob	19952	CKACAAAAYCCYTCRAATTG	46.7	THII
F	psbMR	17235	ATGGAAGTAAATATTCTYGCA	49.7	THII
			TTTATTGCT		
	<i>trnD</i> ^{GUC} R	16464	GGGATTGTAGYTCAATTGGT	45.5	THII
	ycf6R	17690	GCCCAAGCRAGACTTACTATATCCAT	57.3	THII
	<i>trnC</i> ^{GCA} F	18659	CCAGTTCRAATCYGGGTG	45.2	THII
G	ycf6F	17715	ATGGATATAGTAAGTCTYGCTTGGGC	57.3	THII
	<i>trnE</i> ^{UUC}	15972	AGGACATCTCTCTTTCAAGGAG	48.6	THII
	<i>trnT</i> ^{GGU}	15369	CTACCACTGAGTTAAAAGGG	43.4	THII
	<i>trnD</i> ^{GUC} F	16483	ACCAATTGAACTACAATCCC	45.5	THII
	<i>trnY</i> ^{GUA}	16164	CCGAGCTGGATTGTAACCA	53.3	THII
H	rpS4R2	45605	CTGTNAGWCCRTAATGAAAACG	49.2	THII
	<i>trnT</i> ^{UGU} R	46147	AGGTTAGAGCATCGCATTG	49.7	THII
I	<i>trnL</i> ^{5' UAA} (TabC)	46991	CGAAATCGGTAGACGCTACG	52.7	THII
	3' <i>trnL</i> ^{UAA} (TabD)	47487	GGGGATAGAGGGACTTGAAC	49.1	THII
J	psbB	70842	TCCAAAAANKKGGAGATCCAAC	54.5	THII
	psbH	71468	TCAAYRGTYTGTGTAGCCAT	50.4	THII
K	atpI	31735	TATTTACAAGYGGTATTCAAGCT	50.2	THIII
	atpH	32393	CCAAYCCAGCAGCAATAAC	48.0	THIII
L	ndhC	50106	TATTATTAGAAATGYCCARA	53.8	THIII
			AAATATCATATTC		
M	<i>trnV</i> ^(UAC) x2	51106	GTCTACGGTTCGARTCCGTA	49.3	THIII
N	psbE	62673	TATCGAATACTGGTAATAATATCAGC	46.6	THIII
	petL	64048	AGTAGAAAACCGAAATAACTAGTTA	45.9	THIII
O	rpL32-F	105950	CAGTTCCAAAAAACGTAAGTTC	48.2	THIII
	<i>trnL</i> ^(UAG)	106833	CTGCTTCCTAAGAGCAGCGT	51.7	THIII
P	rpS16x1	6084	GTTGCTTTTATACCACATCGTTT	50.5	THIII
	<i>trnQ</i> ^(UUG)	6978	GCGTGGCCAAAGYGGTAAGGC	59.1	THIII
Q	TabE	47467	GGTTCAAGTCCCTCTATCCC	49.1	THIII
	ndhJ	48565	ATGCCYGAAGTTGGATAGG	48.5	THIII
R	<i>trnT</i> ^(GGU) -R	15350	CCCTTTTAACTCAGTGGTAG	43.4	THIII
	psbD	9263	CTCCGTARCCAGTCATCCATA	49.2	THIII

^zID = primer group identifier.

^yName = name of the polymerase chain reaction primer.

^xPosition = primer location on the *Agrostis stolonifera* Penn A-4 chloroplast genome.

^wTm = The DNASTAR Lasergene Version 6 predicted melting temperature of the polymerase chain reaction primer.

^vRef = Reference source for the primers THI (Small et al., 1998), THII (Shaw et al., 2005), and THIII (Shaw et al., 2007).

Apera accessions *aint-1* and *aint-2* defined the outgroup in all phylogenetic analyses.

Maximum parsimony and maximum likelihood analyses were performed using the software program PAUP* Version 4.0b10 (Swofford, 2002) and Bayesian analysis was done using MrBayes Version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Maximum parsimony

analysis was performed using a heuristic search with random addition of sequences. The most likely model of evolution was determined using ModelTest3.7 (Posada and Crandall, 1998) and implemented in both maximum likelihood and Bayesian phylogenetic analyses. Bayesian analysis was run for 1,000,000 MCMC generations and sampled after every 100. The average SD of split

frequencies between the independent analyses for each of the chloroplast DNA regions was below 0.01, suggesting high confidence of convergence. Bootstrap analysis was run on both the maximum parsimony (10,000 replicates) and maximum likelihood (1,000 replicates) analyses. SplitsTree4 was used to build consensus networks from multiple gene trees to identify discordances between phylogenies with default threshold and edge weight parameters. The split graph was drawn as a rectangular cladogram using the cluster network split transformation.

Results

The alignment of a 298-bp conserved region of the *trnL-trnF* intergenic spacer region was used in the phylogenetic analyses. This region contains two gaps and 38 variable sites, 32 of which are parsimony-informative (10.7% of bps). Thirteen unique haplotypes were identified in the set of 52 accessions (Table 3). Similarly, alignment of a 451-bp conserved region of the *atpI-atpH* intergenic spacer region had 12 gaps and 58 variable sites, 48 of which are parsimony-informative (10.6% of bps). There were 11 distinct haplotypes (Table 4) of the *atpI-atpH* intergenic spacer region.

A phylogenetic analysis of the *atpI-atpH* and *trnL-trnF* intergenic spacer regions was performed separately on the cultivated *Agrostis* accessions from Table 1 along with the *Polypogon* and *Apera* accessions or on the full set of 52 accessions listed in Table 1. Of the four gap-encoding methods, complete omission of the gapped regions reduced the number of *atpI-atpH* haplotypes from 11 to nine and *trnL-trnF* haplotypes from 13 to 11. The other three methods: new state, missing, and the Barriol method, resulted in similar phylogenies so the Barriol method was chosen because it effectively represents informative gaps. The predicted model of evolution for both the *trnL-trnF* and *atpI-atpH* regions was a general time reversible model with equal base frequencies.

Maximum parsimony analysis of the *trnL-trnF* region for the set of 52 accessions gave a single most parsimonious tree with a length of 45, consistency index of 0.9778, and homoplasy index of 0.0222 (Fig. 1). Maximum parsimony analysis of the *atpI-atpH* region for the same taxa gave a single most parsimonious tree with length of 79, a consistency index of 0.9367, and homoplasy index of 0.0633 (Fig. 2). All three phylogenetic analysis methods, maximum parsimony, maximum likelihood, and Bayesian inference, gave topologically similar trees for both intergenic spacer regions.

A maximum parsimony analysis of the *trnL-trnF* region from cultivated *Agrostis* accessions (Table 1) had a single best tree of length of 30, consistency index of 1.0, and homoplasy index of 0.0 (Fig. 3). The maximum parsimony phylogeny of the *atpI-atpH* region had a length of 42, consistency index of 1.0, and homoplasy index of 0.0 (Fig. 4). Maximum parsimony, maximum likelihood,

Table 3. Unique *trnL-trnF* intergenic spacer haplotype.

Representative group ID	Accessions with identical haplotypes
<i>aint-1</i>	<i>aint-1</i> , <i>aint-2</i>
<i>pchi-1</i>	<i>pchi-1</i>
<i>pvir-1</i>	<i>pvir-1</i> , <i>pvir-2</i>
<i>acan-10</i>	<i>acan-10</i> , <i>acan-11</i> , <i>agig-1</i> , <i>acan-2</i> , <i>acan-6</i> , <i>acan-7</i> , <i>acan-8</i>
<i>acap-7</i>	<i>acap-7</i> , <i>acap-25</i> , <i>acap-26</i> , <i>acas-11</i> , <i>acas-3</i> , <i>acas-8</i> , <i>apal-1</i> , <i>asto-29</i> , <i>asto-30</i> , <i>atri-1</i> , <i>agig-5</i> , <i>agig-12</i>
<i>acla-2</i>	<i>acla-2</i> , <i>amon-3</i> , <i>amon-5</i> , <i>atri-2</i> , <i>atri-4</i>
<i>acla-5</i>	<i>acla-5</i> , <i>agig-77</i> , <i>asp-4</i> , <i>atri-3</i> , <i>avin-1</i> , <i>asto-1</i> , <i>agig-47</i>
<i>alim-1</i>	<i>alim-1</i>
<i>alya-1</i>	<i>alya-1</i>
<i>amun-7</i>	<i>amun-7</i>
<i>asto-36</i>	<i>asto-36</i> , <i>asto-40</i> , <i>asto-19</i> , <i>asto-34</i> , <i>asto-3</i> , <i>asto-4</i> , <i>asto-5</i> , <i>asto-6</i> , <i>asto-7</i> , <i>asto-9</i> , <i>asto-13</i>
<i>asto-23</i>	<i>asto-23</i>
<i>asto-12</i>	<i>asto-12</i>

Table 4. Unique *atpI-atpH* intergenic spacer haplotypes.

Representative group ID	Accessions with identical haplotypes
<i>aint-1</i>	<i>aint-1</i> , <i>aint-2</i>
<i>pchi-1</i>	<i>pchi-1</i>
<i>pvir-1</i>	<i>pvir-1</i> , <i>pvir-2</i>
<i>acan-10</i>	<i>acan-10</i> , <i>acan-11</i> , <i>acla-5</i> , <i>agig-77</i> , <i>asp-4</i> , <i>asto-40</i> , <i>asto-23</i> , <i>asto-34</i> , <i>atri-3</i> , <i>avin-1</i> , <i>agig-1</i> , <i>asto-1</i> , <i>agig-47</i> , <i>asto-4</i> , <i>asto-5</i> , <i>asto-6</i> , <i>asto-7</i> , <i>asto-12</i> , <i>asto-13</i> , <i>acan-2</i> , <i>acan-6</i> , <i>acan-7</i> , <i>acan-8</i>
<i>acap-7</i>	<i>acap-7</i> , <i>acap-25</i> , <i>acap-26</i> , <i>acas-11</i> , <i>acas-8</i> , <i>apal-1</i> , <i>asto-29</i> , <i>asto-30</i> , <i>atri-1</i> , <i>agig-5</i> , <i>agig-12</i>
<i>acas-3</i>	<i>acas-3</i>
<i>acla-2</i>	<i>acla-2</i> , <i>amon-3</i> , <i>amon-5</i> , <i>atri-2</i> , <i>atri-4</i>
<i>alim-1</i>	<i>alim-1</i>
<i>alya-1</i>	<i>alya-1</i>
<i>amun-7</i>	<i>amun-7</i>
<i>asto-36</i>	<i>asto-36</i> , <i>asto-19</i> , <i>asto-3</i> , <i>asto-9</i>

Fig. 1. Maximum parsimony phylogenetic analysis of the *trnL-trnF* intergenic spacer. The most parsimonious tree from the maximum parsimony analysis. The bootstrap support values greater than 50 from the parsimony analysis are indicated.

Fig. 2. Maximum parsimony phylogenetic analysis of the *atpI-atpH* intergenic spacer. The most parsimonious tree from the maximum parsimony analysis. The bootstrap support values greater than 50 from the parsimony analysis are indicated.

Fig. 3. Maximum parsimony phylogenetic analysis of the *trnL-trnF* intergenic spacer from cultivated *Agrostis* spp. The most parsimonious tree from the maximum parsimony analysis. The bootstrap support values greater than 50 from the parsimony analysis are indicated.

and Bayesian inference gave topologically similar trees for both intergenic spacer regions. Both the *trnL-trnF* and *atpI-atpH* intergenic spacer regions divided the accessions into five distinct clades. The *Polypogon* accessions formed a single clade that is closely related to the clade representing the *A. capillaris* and *A. castellana* accessions. The remaining *A. stolonifera* and *A. canina* accessions formed two more groups. The *atpI-atpH* intergenic spacer region was not successful at distinguishing the *A. canina* accessions from the *A. stolonifera* accessions. The *A. stolonifera* accessions *asto-3*, *asto-9*, and *asto-36* formed a distinct subgroup within the *A. stolonifera* and *A. canina*

accessions. The *trnL-trnF* spacer region was able to distinguish the *A. canina* accessions from the *A. stolonifera* accessions forming two distinct clades.

A Splitstree4 consensus network of the chloroplast-encoded intergenic spacer regions showed one disagreement; the placement of *amun-7* was inconsistent between the individual sequence phylogenies (Fig. 5). One difference between the individual chloroplast DNA sequence analyses of the placement of *amun-7* is in its relationship to the *A. stolonifera* and *A. canina* accessions. The analysis of the *trnL-trnF* region supports a closer relationship of *amun-7* to the *A. stolonifera* and *A. canina* accessions com-

pared with the same relationships from the *atpI-atpH* phylogeny.

Discussion

Polyploidization events, origins of turf-type species, sources of novel germplasm, and targets for developing interspecific hybrids may be inferred from the evolution of *Agrostis* species. Chloroplast DNA sequence regions have been used to resolve phylogenies of several grass species (Fortune et al., 2008; Ge et al., 2002; Gillespie et al., 2006; Guo and Ge, 2005). There were only a few distinct haplotypes of the *trnL-trnF* and *atpI-atpH* intergenic spacer regions within this set of *Agrostis*, and therefore these chloroplast regions might not provide a detailed view of evolutionary processes in *Agrostis*. Another possibility is that the chloroplast genomes among *Agrostis* share a high degree of sequence identity, which would make it difficult to resolve species relationships using chloroplast-based sequence data.

The lack of cohesion of species in the individual chloroplast sequence-based phylogenies calls into question the ability to resolve species relationships using these chloroplast-encoded DNA sequences. For example, *asto-1*, *asto-29*, and *asto-30* do not fall into or near the same clade as the rest of the *A. stolonifera* accessions. Similarly, the four *A. trinii* accessions and the five *A. gigantea* accessions are distributed throughout the phylogenetic tree and do not appear to be closely related. There are several possible reasons for this inability to distinguish species based on chloroplast sequence data, including incorrect accession identification in the National Plant Germplasm System. Additionally, multiple origins of a single species could result in different maternal chloroplast ancestral genomes. The possibility also exists that the chloroplast regions examined do not accurately reflect chloroplast evolution.

The phylogenetic analysis of the *atpI-atpH* and *trnL-trnF* intergenic spacer regions of cultivated *Agrostis* accessions included four *Agrostis* species along with the *Apera* and *Polypogon* accessions. It is interesting that the *Polypogon* accessions are closely related to the *A. capillaris* accessions. Because *Polypogon* species are known to form intergeneric hybrids with *Agrostis* species (Barkworth, 2007), they may have evolved from a common ancestor. Furthermore, it is believed that the genome of the diploid *A. canina* contributed to the evolution of *A. capillaris* but not *A. stolonifera*. The data presented by these chloroplast phylogenies suggest that *A. canina* is more closely related to *A. stolonifera* than *A. capillaris*. Further experiments examining other chloroplast genome regions or nuclear COS DNA sequences may help to further resolve species relationships in *Agrostis*. An understanding of these relationships is important to turfgrass breeders because they can incorporate related material directly into breeding programs, recreate evolutionary events that led to the

Fig. 4. Maximum parsimony phylogenetic analysis of the *atpI-atpH* intergenic spacer from cultivated *Agrostis* spp. The most parsimonious tree from the maximum parsimony analysis. The bootstrap support values greater than 50 from the parsimony analysis are indicated.

Fig. 5. Consensus network of the *atpI-atpH* and *trnL-trnF* intergenic spacer regions. Splits are indicated with the bold dashed line.

high-quality turf-type *Agrostis*, or introduce novel stress tolerance genes through wide crossbreeding. This study provides valuable information to *Agrostis* breeders on species relationships and potential sources of novel

germplasm. Ultimately this information will guide strategies aimed at better use of *Agrostis* germplasm resources with the goal of developing new cultivars that require fewer inputs and have high turf quality.

Literature Cited

- Amundsen, K., D. Rotter, H.M. Li, J. Messing, G. Jung, F. Belanger, and S. Warnke. 2011. Miniature inverted-repeat transposable element identification and genetic marker development in *Agrostis*. *Crop Sci.* 52:854–861.
- Amundsen, K. and S. Warnke. 2011. Species relationships in the genus *Agrostis* based on flow cytometry and MITE-display molecular markers. *Crop Sci.* 51:1224–1231.
- Barkworth, M.E. 2007. Polypogon Desf. In: Barkworth, M.E., L.K. Anderton, K.M. Capels, S. Long, and M.B. Piep (eds.). *Manual of grasses for North America*. Utah State University Press, Logan, UT.
- Barriel, V. 1994. Phylogénies moléculaires et insertions-délétions de nucléotides. *C. R. Acad. Sci. Paris ser. 3* 317:693–701.
- Brilman, L.A. 2001. Utilization of interspecific crosses for turfgrass improvement. *Intl. Turfgrass Soc. Res. J.* 9:157–161.
- Casler, M.D. and R.R. Duncan. 2003. Introduction, p. 3–26. In: Casler, M.D. and Duncan, R.R. (eds.). *Turfgrass biology, genetics, and breeding*. John Wiley & Sons, Hoboken, NJ.
- Döring, E., J. Schneider, K.W. Hilu, and M. Roser. 2007. Phylogenetic relationships in the Aveneae/Poeae complex (Pooideae, Poaceae). *Kew Bull.* 62:407–424.
- Feliner, G.N. and J.A. Rosselló. 2007. Better the devil you know? Guidelines for insightful utilization of nrDNA ITS in species-level evolutionary studies in plants. *Mol. Phylogenet. Evol.* 44:911–919.
- Fortune, P.M., N. Pourtau, N. Viron, and M.L. Ainouche. 2008. Molecular phylogeny and reticulate origins of the polyploid *Bromus* species from section *Genea* (Poaceae). *Amer. J. Bot.* 95:454–464.
- Fulton, T.M., R. Van der Hoeven, N.T. Eannetta, and S.D. Tanksley. 2002. Identification, analysis, and utilization of conserved ortholog set markers for comparative genomics in higher plants. *Plant Cell* 14:1457–1467.
- Ge, S., A. Li, B.-R. Lu, S.-Z. Zhang, and D.-Y. Hong. 2002. A phylogeny of the rice tribe Oryzeae (Poaceae) based on matK sequence data. *Amer. J. Bot.* 89:1967–1972.
- Gillespie, L.J., A. Archambault, and R.J. Soreng. 2006. Phylogeny of *Poa* (Poaceae) based on trnT-trnF sequence data: Major clades and basal relationships. *Aliso* 23:420–434.
- Guo, Y.-L. and S. Ge. 2005. Molecular phylogeny of Oryzeae (Poaceae) based on DNA sequences from chloroplast, mitochondrial, and nuclear genomes. *Amer. J. Bot.* 92:1548–1558.
- Harvey, M.J. 2007. *Agrostis*, p. 148–154. In: Barkworth, M.E., L.K. Anderton, K.M. Capels, S. Long, and M.B. Piep (eds.). *Manual of grasses for North America*. Utah State University Press, Logan, UT.
- Huelsenbeck, J.P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754–755.
- Huson, D.H. and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* 23:254–267.
- Huson, D.H., T. Kloepper, P.J. Lockhart, and M.A. Steel. 2005. Reconstruction of reticulate networks from gene trees. *Proc. of RECOMB*, Springer, Heidelberg, 2005, LNBI 3500. p. 233–249.
- Jones, K. 1956a. Species determination in *Agrostis*. Part I. Cytological relationships in *Agrostis canina* L. *J. Genet.* 54:370–376.
- Jones, K. 1956b. Species differentiation in *Agrostis* II. The significance of chromosome pairing in the tetraploid hybrids of *Agrostis canina*

- subsp. *montana* Hartm., *A. tenuis* Sibth. and *A. stolonifera* L. J. Genet. 54:377–393.
- Jones, K. 1956c. Species differentiation in *Agrostis* III. *Agrostis gigantea* Roth. and its hybrids with *A. tenuis* Sibth. and *A. stolonifera* L. J. Genet. 54:394–399.
- Kellogg, E.A. 1998. Relationships of cereal crops and other grasses. Proc. Natl. Acad. Sci. USA 95:2005–2010.
- Linder, C.R. and L.H. Rieseberg. 2004. Reconstructing patterns of reticulate evolution in plants. Amer. J. Bot. 91:1700–1708.
- McBreen, K. and P.J. Lockhart. 2006. Reconstructing reticulate evolutionary histories of plants. Trends Plant Sci. 11:398–404.
- Posada, D. and K.A. Crandall. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817–818.
- Quintanar, A., S. Castroviejo, and P. Catalán. 2007. Phylogeny of the tribe Aveneae (Pooideae, Poaceae) inferred from plastid *trnT-F* and nuclear ITS sequences. Amer. J. Bot. 94:1554–1569.
- Reichman, J.R., L.S. Watrud, E.H. Lee, C.A. Burdick, M.A. Bollman, M.J. Storm, G.A. King, and C. Mallory-Smith. 2006. Establishment of transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) in nonagronomic habitats. Mol. Ecol. 15:4243–4255.
- Ronquist, F. and J.P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.
- Rotter, D., A.K. Bharti, H.M. Li, C. Luo, S.A. Bonos, S. Bughrara, G. Jung, J. Messing, W.A. Meyer, S. Rudd, S.E. Warnke, and F.C. Belanger. 2007. Analysis of EST sequences suggests recent origin of allotetraploid colonial and creeping bentgrasses. Mol. Genet. Genomics 278:197–209.
- Sang, T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. Crit. Rev. Biochem. Mol. Biol. 37:121–147.
- Shaw, J., L. Edgar, S. Edward, and S. Randall. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. Amer. J. Bot. 94:275–288.
- Shaw, J., E.B. Lickey, J.T. Beck, S.B. Farmer, W. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling, and R.L. Small. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Amer. J. Bot. 92:142–166.
- Small, R.L., J.A. Ryburn, R.C. Cronn, T. Seelanan, and J.F. Wendel. 1998. The tortoise and the hare: Choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. Amer. J. Bot. 85:1301–1315.
- Soreng, R.J. and J.I. Davis. 1998. Phylogenetics and character evolution in the grass family (Poaceae): Simultaneous analysis of morphological and chloroplast DNA restriction site character sets. Bot. Rev. 64:1–85.
- Soreng, R.J., J.I. Davis, and J.J. Doyle. 1990. A phylogenetic analysis of chloroplast DNA restriction site variation in Poaceae subfam. Pooideae. Pl. Syst. Evol. 172:83–97.
- Soreng, R.J., J.I. Davis, and M.A. Voionmaa. 2007. A phylogenetic analysis of Poaceae tribe Poeae sensu lato based on morphological characters and sequence data from three plastid-encoded genes: Evidence for reticulation, and a new classification for the tribe. Kew Bull. 62:425–454.
- Swofford, D.L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, MA.
- Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties, and weighted matrix choice. Nucleic Acids Res. 22:4673–4680.
- Vergara, G.V. and S.S. Bughrara. 2003. AFLP analyses of genetic diversity in bentgrass. Crop Sci. 43:2162–2171.
- Warnke, S. 2003. Creeping bentgrass (*Agrostis stolonifera* L.), p. 175–185. In: Casler, M.D. and R.R. Duncan (eds.). Turfgrass biology, genetics, and breeding. John Wiley & Sons, Hoboken, NJ.
- Warnke, S.E., D.S. Douches, and B.E. Branham. 1998. Isozyme analysis supports allotetraploid inheritance in tetraploid creeping bentgrass (*Agrostis palustris* Huds.). Crop Sci. 38:801–805.
- Watson, L. 1990. The grass family. Poaceae, p. 1–31. In: Chapman, G.P. (ed.). Reproductive versatility in the grasses. Cambridge University Press, Cambridge, MA.