CHAPTER I

AFLP Analysis of Genetic Diversity in Bentgrass (*Agrostis* spp.)

ABSTRACT

Bentgrasses (*Agrostis* spp.) are widely occurring temperate grasses with more than 220 species that represent a vast resource for genetic improvement of turfgrass cultivars. Bentgrasses are normally outcrossing species and exhibit many ploidy levels. Difficulties in morphological characterization, which are largely subjected to environmental influences, have resulted in many synonymous species and uncertainties in phylogenetic relationships. To study the genetic diversity and relationships between bentgrass species, forty accessions from the USDA's germplasm collection representing fourteen species of *Agrostis* from twenty countries were investigated using fluorescence-labeled AFLP analysis. Four hundred AFLP markers from five chosen primer combinations were used to differentiate between bentgrass accessions using a bulk of 25 genotypes per accession. Genetic similarities between accessions ranged from 0.62 to 0.98 showing no duplication in the collection and a high level of diversity in *Agrostis*. Both principal component analysis and UPGMA dendrogram clearly distinguished seven groups. Genetic relationships between diploids and other polyploids were revealed in the cluster groupings.

Key words: bentgrass, *Agrostis* spp., genetic diversity, AFLP

INTRODUCTION

Bentgrass, *Agrostis* sp. derivation from Greek: grass, forage, is distributed throughout the world and belongs to the Poaceae family (Watson and Dallwitz, 1992). It is a perennial or annual outcrossing polyploid (2n=14, 28, 42 etc.) which is widely used for putting greens, golf courses, parks and forage. Some species were used for erosion control and revegetation programs in disturbed areas where grazing, mining and flooding had occurred (Brown and Johnston, 1979; Cornely et al., 1983; Winterhalder, 1990). Most bentgrass species are widely adapted to temperate climates and occur in a variety of habitats. Variation in *Agrostis* species denotes potential for genetic improvement of the species for use as turf.

Much of the work in classification of *Agrostis* is predominantly based on morphological and cytological features (Bjorkman, 1960). Linnaeaus grouped *Agrostis* based on the presence or absence of awns, panicle shape and color, and orientation of culms and roots. The basic chromosome number of *Agrostis* is x=7 and differences in ploidy level often determine species boundaries. Diploid species (2n=2x=14) are *A. alpina* Leyss., *A. elegans* Walt., *A. canina* L., *A. flaccida* Hack., and *A. nebulosa* Boiss. & Reut. Most bentgrass species such as *A. capillaris* L., *A. castellana* Boiss. & Reut., *A. palutris* Huds. and *A. stolonifera* L. are tetraploids with 2n=4x=28 (Brede and Sellman, 2001). Species such as *A. castellana*, *A. gigantea* R., *A. alba* L., *A. exarata* Trin., *A. clavata* Trin., *A. diegoensis* Vasey and *A. hallii* Vasey are known to exist in tetraploid and hexaploid forms (2n=6x=42). In a wide collection of *A. gigantea,* Jones (1955a) only found hexaploids. Because of the outcrossing nature of bentgrass, ploidy levels need to

be identified. Bonos et al. (1999) used laser flow cytometry as a rapid option to validate the number of chromosomes of certain species of *Agrostis.*

Current significant species in the USA are velvet bentgrass (*A. canina* L.); colonial bentgrass (*A. capillaris* L. or *A. tenuis* Sibth.); dryland or highland bentgrass (*A. castellana* Boiss. & Reut.); creeping bentgrass (*A. palustris* Huds. or *A. stolonifera* L.); red top bentgrass (*A. gigantea* L.); and Idaho bentgrass (*A. idahoensis* Nash). Creeping bentgrass is the premier turfgrass species for closely mowed golf course putting greens (Funk, 1998).

Intra- and interspecific hybridization is possible in *Agrostis*, however, very few genetic studies have explained the contribution of diploids to polyploids and their relationship. Hybridization experiments by Davies (1953), using germplasm from the UK, showed that hybrids of *A. stolonifera* x *A. gigantea* were among the easiest to produce and indicated some degree of homology between the two polyploid species. Bivalent formation during meiosis in hybrids between *A. capillaris* L. x *A. vinealis* Schreb., established *A. capillaris* to be a segmental allotetraploid and from hybrids of *A. stolonifera* x *A. canina* that *A. stolonifera* was a strict allotetraploid (Jones, 1955a). The use of interspecific hybrids of *A. tenuis, A. stolonifera* and *A. gigantea* and their offspring helped decode the genome constitution in *Agrostis.* Jones (1955b) suggested that if *A. tenuis* or *A. capillaris* was $A_1A_1A_2A_2$ and *A. stolonifera* was $A_2A_2A_3A_3$, then *A. gigantea* would be $A_1A_1A_2A_2A_3A_3$. The A_2 genomes of the two species were not confirmed to be absolutely identical. Creeping bentgrass (*A. stolonifera*) was also found to hybridize with ticklegrass (*A. scabra* Willd.) and spike bentgrass (*A. exarata* Trin.)(Welsh et al., 1987). *A. scabra* was found to hybridize with *A. trinii* (Probatova and Kharkevich, 1983).

Warnke et al. (1998) used isozyme analysis to study allotetraploid inheritance in creeping bentgrass and found strong genetic evidence for disomic rather than tetrasomic inheritance.

Little is known about bentgrass genetic relationships and there is much confusion regarding assignment to groups. Early efforts to use morphological characters in classification resulted in many synonymous species. *A. stolonifera* L. was listed as being synonymous with *A. alba* var. *palustris* Huds., *A. alba* var. *stolonifera* (L.) Sm., *A. stolonifera* var. *compacta* Hart., *A. stolonifera* var. *palustris* (Huds.) Farw. and *A. maritima* Lam. (Biota of North America Program, BONAP Poaceae Listing; Plant Gene Resources of Canada (GRIN-CA). *A. gigantea* R. is synonymous with three other species (BONAP Poaceae Listing) and may include subspecies on the basis of rhizome and shoot development and the morphology of the spikelet (Dihoru, 1980). Species *A. trinii* Turcz. was found synonymous with *A. vinealis* ssp. *trinii* (Tzvelev, 1971) and *A. flacidda* ssp. *trinii* (Koyama, 1987) while Soreng et al.(2002) taxonomy listed *A. vinealis* Schreb. as being synonymous with *A. canina* ssp. *vinealis* (Turcz.) Hult. Kurchenko (1979) listed *A. trinii* and *A. canina* both from Russia as different species based on morpho-analytical characters and behavior in their natural environments. Differences in phenotypic expressions are oftentimes the result of environmental fluctuations; therefore, DNA fingerprinting is considered a more stable and reliable technique to explore genetic diversity and relationships.

A wide variety of DNA marker technologies have been used to differentiate bentgrass cultivars and species such as isozyme (Yamamoto and Duich, 1994; Warnke et al., 1997), RAPD (Golembiewski et al., 1997, Scheef et al., 2003) and RFLPs (Caceres et

al., 2000). These techniques are limited by the low levels of polymorphism at the intra- and interspecific levels. A more powerful tool in fingerprinting for biodiversity studies over other PCR based techniques is by amplified fragment length polymorphism (AFLP) analysis (Zabeau and Vos, 1993; Vos et al. 1995). AFLPs have been used successfully in order to study genetic diversity in crops like rice (*Oryza sativa* L.)(Zhu et al., 1998), cotton (*Gossypium* sp.)(Abdalla et al., 2001) and common bean (*Phaseolus vulgaris*)(Tohme et al., 1996). The high frequency of identifiable polymorphism is useful for distinguishing among genotypes, detecting linkages and for mapping loci in turfgrasses. Zhang et al. (1999) used AFLPs to differentiate bermudagrass (*Cynodon* spp.) genotypes and determine genetic relationships among genotypes. In addition, they showed that the use of fluorescence-based detection of AFLPs has improved both fragment scoring and data handling. Ebina et al. (1999) constructed an AFLP-based genome map of zoysiagrass (*Zoysia* spp.) and developed a linkage map of QTLs associated with some major traits. AFLPs have not been used to study bentgrass. Understanding bentgrass diversity would facilitate the efficient use of germplasm accessions to combine the favorable agronomic and disease resistance traits to produce superior cultivars.

The objectives of this research were to study the genetic diversity between forty Plant Introduction (PI) accessions comprising fourteen species of *Agrostis* from twenty countries and determine the genetic relationships among the species based on AFLP profiles.

MATERIALS AND METHODS

Plant materials and DNA extraction

Bulked leaf samples from 25 plants each of 40 Plant Introduction (PI) accessions (source: USDA, Regional Plant Introduction Station, Pullman, Washington, USA) of *Agrostis* species from different countries were used (Table 1.1, Figure 1.1). Warnke et al. (1997) compared different number of samples per bulk from the same accession in bentgrass and suggested the least variation using 25 plants per bulk for analysis to minimize against sampling errors. Tissues were ground in liquid nitrogen (Extraction buffer: Tris-HCl, SDS, NaCl) and precipitated using chloroform, sodium acetate and ethanol. All samples were treated with RNAse and twice reprecipitated. Extracted DNA was stored in 1% TE buffer. DNA quality was checked by running 5 μl of the undigested samples in 1% agarose gel containing TBE buffer and compared to EcoR1 digested samples. DNA quantification was performed using DyNA Quant 200 Fluorometer.

AFLP analysis

Approximately 150-200 ng of DNA was used to do AFLP analysis for each accession. Digestion was conducted using two restriction enzymes, EcoR1, a six base pair cutter and Mse-1, a four base pair cutter. The AFLP procedure used in this study was as described by Vos et al. (1995) with modifications. Pre-amplification was done on PTC-100 thermal cycler (MJ Research) using 72°C for 2 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min, followed by elongation at 72°C 5 min and 4°C hold. PCR products from initial ligation of adapters were checked on 1.5% TBE agarose gel. Combinations of fluorescent(*) dye labeled E and M primers each with 3 selective

nucleotides at the 3' ends were used. The following E* primers were used: E*-ACA and E*-AGC. The following M primers were tested: M-CAT, M-CAG, M-CGG, M-CTT, M-CCT. The 15 μl selective amplification mixture consisted of 15 pmol E*-primer, 75 pmol M-primer , 2 mM dNTP, 1.5 mM PCR buffer, 37.5 mM $MgCl_2$, 1.0 unit Taq Polymerase and 1 μl pre-amplified product in deionized distilled water. The PCR cycling sequence used for selective amplification was as recommended by Vos et al. (1995). Products from selective amplification were checked initially on 1.5% agarose and diluted four times with 0.1 TE buffer. For separation on acrylamide gels, samples consisted of 0.5 μl of the amplified product and 0.5 μl loading dye. The samples were denatured at 95 °C for 5 minutes prior to loading and ran on a 6% Long Ranger polyacrylamide gel with 0.7X TBE buffer using a Licor Gel Analyzer at a constant 800 v for 6 hours at 50°C.

Chromosome analysis

Root tips of species with an unknown number of chromosomes were collected and fixed in 3:1 ethanol (95%), acetic acid (Farmer's fixative). The root tip was rinsed and placed in 3N HCl for 10 to 20 minutes to soften. On a glass slide, the meristematic region was cut and squashed with a blunt end of the needle and a drop of acetocarmine dye was added. Slides were viewed under a phase contrast microscope to determine the number of chromosomes from a several mitotic cells. Chromosome numbers were determined for PI195197 and PI299461, *A. lachnantha* Nees; PI230236, *A. munroana* Aitch. & Hemsl.; PI283174, *A. transcaspica* Litv.; PI477045, *A. hygrometrica* Litv. and PI362190, *A. mongolica* Roshev.

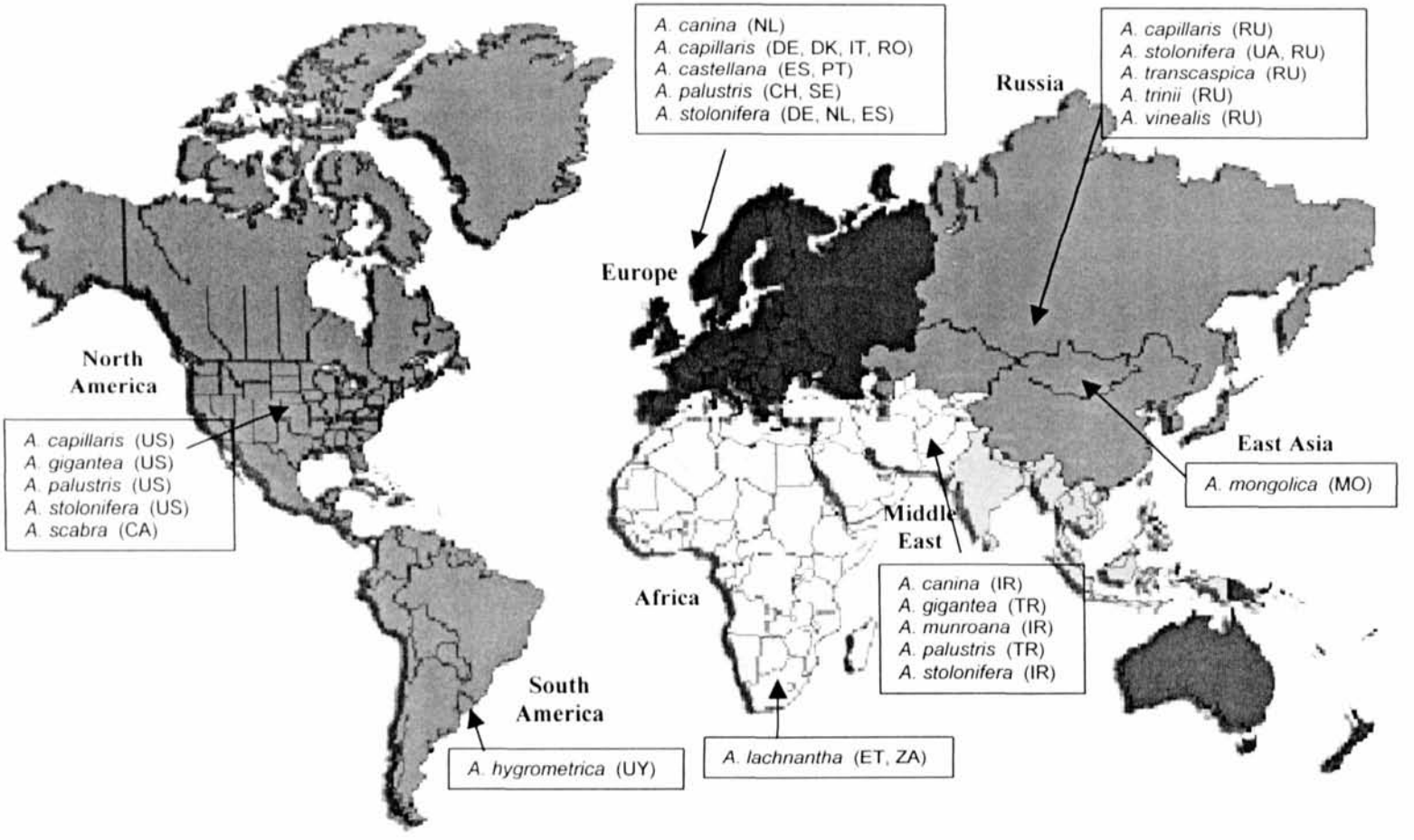

Figure 1.1. World regional sources of 40 accessions of *Agrostis* species.

Legend: CA = Canada; CH = Switzerland; DE = Germany; DK = Denmark; ES = Spain; ET = Ethiopia; IR = Iran; IT = Italy; MO = Mongolia; NL = Netherlands; PT = Portugal; RO = Romania; RU = Russia and former USSR; SE = Sweden; TR = Turkey; UA = Ukraine; US = America; UY = Uruguay; ZA = South Africa

Table 1.1. List of plant introductions (PI), species, number of chromosomes and geographic origin of bentgrass (*Agrostis* spp.) accessions.

AFLP No.**	PI No.	Species	Chromosome No.(2n)	Geographic Source (Code)
1	194 697	*A. canina*	14	Netherlands (NL)
2	230 233	*A. canina*	14	Iran (IR)
3	237 717	*A. capillaris*	28	Germany (DE)
4	252 045	*A. capillaris*	28	Italy (IT)
5	469 217	*A. capillaris*	42	USA 'Highland' (USH)
6	311 011	*A. capillaris*	28	Romania (RO)
7	392 338	*A. capillaris*	28	Former Soviet Union (RU)
8	234 685	*A. capillaris*	28	Denmark (DK)
9	578 528	*A. capillaris*	28	USA 'Exeter' (USE)
10	302 830	*A. castellana*	42	Spain (ES1)
11	287 741	*A. castellana*	42	Spain (ES2)
12	240 135	*A. castellana*	42	Portugal (PT1)
13	240 139	*A. castellana*	42	Portugal (PT2)
14	240 133	*A. castellana*	42	Portugal (PT3)
15	240 131	*A. castellana*	42	Portugal (PT4)
16	287 744	*A. castellana*	42	Spain (ES3)
17	240 142	*A. castellana*	42	Portugal (PT5)
18	318 928	*A. castellana*	42	Spain (ES4)
19	240 132	*A. castellana*	28	Portugal (PT6)
20	235 440	*A. palustris*	28	Switzerland (CH)
21	235 541	*A. palustris*	28	Sweden (SE)
22	204 390	*A. palustris*	28	Turkey (TR)
23	578 530	*A. palustris*	28	America (US)
24	269 838	*A. stolonifera*	28	Germany (DE)
25	494 119	*A. stolonifera*	28	Netherlands (NL)
26	494 118	*A. stolonifera*	28	Ukraine (UA)
27	230 235	*A. stolonifera*	28	Iran (IR)
28	318 934	*A. stolonifera*	28	Spain (ES)
29	439 027	*A. stolonifera*	28	Former Soviet Union (RU)
30	195 917	*A. lachnantha*	21*	Ethiopia (ET)
31	299 461	*A. lachnantha*	21*	South Africa (ZA)
32	362 190	*A. mongolica*	28*	Mongolia (MO)
33	477 045	*A. hygrometrica*	28*	Uruguay (UY)
34	230 236	*A. munroana*	21*	Iran (IR)
35	234 681	*A. scabra*	42	Canada (CA)
36	598 462	*A. trinii*	28	Russian Federation (RU)
37	440 110	*A. vinealis*	28	Russian Federation (RU)
38	283 174	*A. transcaspica*	14*	Former Soviet Union (RU)
39	383 584	*A. gigantea*	42	Turkey (TR)
40	443 051	*A. gigantea*	42	America (US)

*Number of chromosomes were determined in this study.

**AFLP No. = designated sample number for accession in the AFLP analysis.

Data Analyses

Gels were visualized using Gene ImagIR 4.0 (Scanalytics, Inc.,VA, USA). Each informative polymorphic band was scored manually as 1 for presence and 0 for absence. Analyses were done using Numerical Taxonomy and Multivariate Analysis System, NTSYS v.2.1 (Rohlf, 2000). Genetic similarities based on Jaccard's coefficients (Jaccard, 1908) were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. The similarity matrix was run on Sequential, Agglomerative, Hierarchical and Nested clustering, SAHN (Sneath and Sokal, 1973) using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) as an option (Sokal and Michener, 1958). Cophenetic correlation was calculated to measure goodness of fit. Principal component analysis (PCA) was run using SYSTAT to identify the number of groups based on Eigen vectors. The TREE module of NTSYS v.2.1 was used to produce the dendrogram and cluster groupings (Rohlf, 2000).

RESULTS AND DISCUSSION

AFLP fingerprinting and polymorphism level in bentgrass accessions

Selective amplifications were made using different primer combinations from the final products of EcoRI/Mse digestion, adapter ligation and pre-amplification. From the initial eight selective primer combinations, five combinations were chosen for clarity, repetitiveness in duplicated gel runs and high levels of polymorphisms. Four hundred polymorphic markers were scored from the five chosen selective primer combinations. The average number of polymorphic bands across the accessions ranged from 100 to 180 markers per primer combination and 20 to 145 polymorphic bands per individual lane.

Data in Table 1.2 showed the selected primers and number of polymorphic markers scored. The high level of polymorphism revealed the wide genetic diversity between the *Agrostis* accessions. Monte et al (1993) found that if extensive diversity exists among taxa or genus, fewer probes or primer combinations showing high polymorphism may be sufficient to distinguish genotypes. None of the bentgrass accessions shared an identical DNA fingerprint. AFLP profiles between accessions indicated that the collection does not contain duplications. The high level of polymorphism has facilitated analysis of the genetic diversity among bentgrass genotypes.

The most robust primer combination with the highest number of polymorphism was E-ACA/M-CAG, whereas the lowest number was observed with E-AGC/M-CGG. Approximately 12% of the markers were specific for individual *Agrostis* species. Specific AFLP markers were found for *A. lachnantha*, *A. vinealis*, *A. scabra*, *A. munroana*, *A. stolonifera/A.palustris*, *A. transcaspica* and *A. hygrometrica*, whereas no specific markers were detected for *A. canina*, *A. capillaris*, *A. castellana*, *A. mongolica* and *A. gigantea*. Data suggest the possibility of developing probes from specific markers to effectively discriminate between some species in the future.

Diversity between accessions and species

Gaps in phylogenetic information of *Agrostis* have resulted from too few hybridization studies, difficulty in obtaining old botanical records and inconsistencies in morphological characterizations i.e. synonyms, subspecies, and re-classification of some species. New phenotypes with different number of chromosomes and distinct characteristics have been given new species names (Probatova and Karkevich, 1983).

Table 1.2. Number of polymorphic bands obtained from different primer combinations.

Primer Pair	No. Polymorphic Bands
E-ACA/M-CAT	138
E-ACA/M-CAG	142
E-AGC/M-CAT	29
E-AGC/M-CAG	69
E-ACA/M-CGG	21

Adapter and primer sequences were adapted from Vos et al. (1995).

E=5'-GACTGCGTACCAATTC-3', M=5'-GATGAGTCCTGAGTAA-3'.

Another possible source of uncertainty results from spontaneous natural hybrids where lineage and ploidy levels are not known. In this study, assessment of number of chromosomes of the fourteen species of *Agrostis* from the literature and conducted chromosome analyses confirmed the wide range in ploidy levels (2n=14, 21, 42) (Table 1.1). Physical and cytological examinations of the different species showed that ploidy level may not be indicative of plant size in bentgrass. In the germplasm collection, diploid *A. canina* was much smaller compared to another diploid *A. transcaspica*, which in turn had wider and longer leaves and was larger than a triploid, *A. munroana.* Investigations at the molecular level may provide a clearer understanding of the diversity and relationships in bentgrasses.

In this research, four hundred AFLP markers from five selective primer combinations were used to compare 40 bentgrass accessions. Pairwise similarity coefficients (**sc**) were computed based on shared and unique amplification products using UPGMA (Table 1.3). Based on similarity coefficients, the closest pair would be PI235440 (Switzerland) and PI235541 (Sweden) at 0.98. Both accessions belonged to *A. palustris* (creeping bentgrass), had the same number of chromosomes and were found in Europe. The difference between the two PI lines may be due to minimal within-species allelic variation probably resulting from recombination events. Data in Table 1.3 also showed that the most dissimilar pair would be PI230235, *A. stolonifera* from Iran and PI477045, *A. hygrometrica* from Uruguay. The similarity coefficient was only 0.58 indicating large variability in the genomic constitution. Iran and Uruguay are found geographically in distant hemispheres that separated *A. stolonifera* and *A. hygrometrica*

Table 1.3.1. Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20 (..cont. next page)
1	1.00																			
2	0.90	1.00																		
3	0.75	0.72	1.00																	
4	0.79	0.74	0.83	1.00																
5	0.72	0.72	0.84	0.84	1.00															
6	0.74	0.72	0.86	0.86	0.88	1.00														
7	0.73	0.72	0.85	0.83	0.87	0.90	1.00													
8	0.75	0.73	0.87	0.84	0.86	0.89	0.90	1.00												
9	0.75	0.75	0.86	0.86	0.86	0.90	0.88	0.94	1.00											
10	0.74	0.70	0.81	0.84	0.87	0.86	0.87	0.87	0.91	1.00										
11	0.74	0.72	0.80	0.80	0.84	0.84	0.84	0.84	0.88	0.89	1.00									
12	0.77	0.73	0.82	0.84	0.88	0.85	0.85	0.88	0.90	0.92	0.89	1.00								
13	0.76	0.73	0.81	0.84	0.87	0.86	0.85	0.87	0.91	0.92	0.89	0.96	1.00							
14	0.75	0.72	0.82	0.83	0.88	0.85	0.86	0.87	0.89	0.92	0.87	0.95	0.95	1.00						
15	0.73	0.71	0.81	0.81	0.85	0.80	0.82	0.83	0.85	0.87	0.85	0.91	0.90	0.91	1.00					
16	0.72	0.68	0.80	0.80	0.85	0.82	0.83	0.84	0.85	0.89	0.86	0.91	0.90	0.90	0.89	1.00				
17	0.76	0.73	0.83	0.82	0.87	0.86	0.85	0.87	0.90	0.92	0.88	0.96	0.95	0.93	0.90	0.93	1.00			
18	0.76	0.72	0.83	0.84	0.88	0.87	0.86	0.88	0.90	0.90	0.86	0.93	0.94	0.92	0.88	0.89	0.94	1.00		
19	0.76	0.72	0.82	0.82	0.87	0.86	0.86	0.87	0.89	0.89	0.86	0.91	0.92	0.90	0.86	0.88	0.92	0.94	1.00	
20	0.72	0.72	0.68	0.71	0.69	0.72	0.75	0.72	0.72	0.70	0.71	0.72	0.73	0.73	0.69	0.69	0.72	0.72	0.72	1.00
..																				
..																				

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

Table 1.3.2. (Continuation). Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20 (..cont next page)
21	0.73	0.72	0.68	0.72	0.69	0.71	0.75	0.71	0.72	0.70	0.71	0.73	0.72	0.73	0.69	0.69	0.72	0.72	0.72	0.98
22	0.71	0.71	0.70	0.71	0.70	0.73	0.76	0.72	0.72	0.72	0.71	0.71	0.71	0.72	0.69	0.70	0.72	0.72	0.73	0.89
23	0.75	0.74	0.69	0.75	0.71	0.71	0.75	0.71	0.73	0.72	0.73	0.73	0.73	0.73	0.71	0.70	0.73	0.73	0.73	0.92
24	0.71	0.70	0.71	0.71	0.70	0.72	0.76	0.72	0.72	0.71	0.71	0.73	0.72	0.73	0.70	0.69	0.72	0.71	0.72	0.91
25	0.70	0.69	0.67	0.69	0.67	0.69	0.72	0.69	0.69	0.69	0.70	0.70	0.69	0.69	0.67	0.67	0.69	0.69	0.69	0.93
26	0.71	0.71	0.70	0.70	0.68	0.69	0.73	0.70	0.70	0.69	0.69	0.71	0.70	0.71	0.68	0.67	0.70	0.70	0.71	0.92
27	0.69	0.68	0.70	0.71	0.70	0.74	0.76	0.71	0.70	0.70	0.69	0.69	0.69	0.69	0.67	0.68	0.70	0.70	0.71	0.81
28	0.72	0.70	0.68	0.71	0.69	0.70	0.73	0.69	0.70	0.71	0.72	0.72	0.71	0.72	0.68	0.70	0.72	0.72	0.72	0.91
29	0.70	0.68	0.75	0.76	0.74	0.74	0.79	0.75	0.75	0.77	0.76	0.76	0.76	0.76	0.76	0.75	0.75	0.74	0.74	0.77
30	0.67	0.64	0.64	0.65	0.62	0.62	0.62	0.62	0.64	0.62	0.65	0.63	0.62	0.63	0.62	0.63	0.63	0.62	0.64	0.65
31	0.66	0.64	0.65	0.66	0.60	0.62	0.63	0.62	0.64	0.63	0.64	0.62	0.62	0.62	0.62	0.63	0.63	0.61	0.63	0.65
32	0.70	0.69	0.74	0.73	0.74	0.74	0.78	0.73	0.73	0.74	0.74	0.74	0.73	0.75	0.71	0.71	0.73	0.72	0.74	0.77
33	0.68	0.60	0.61	0.63	0.60	0.61	0.61	0.61	0.61	0.63	0.64	0.63	0.62	0.61	0.60	0.60	0.61	0.62	0.61	0.62
34	0.73	0.66	0.63	0.66	0.59	0.62	0.62	0.61	0.63	0.63	0.65	0.66	0.65	0.65	0.62	0.64	0.65	0.63	0.65	0.66
35	0.76	0.71	0.69	0.70	0.66	0.66	0.67	0.68	0.67	0.66	0.69	0.70	0.70	0.69	0.69	0.66	0.68	0.68	0.69	0.68
36	0.73	0.70	0.79	0.79	0.77	0.77	0.78	0.81	0.80	0.77	0.75	0.78	0.77	0.76	0.76	0.75	0.77	0.76	0.77	0.65
37	0.75	0.76	0.65	0.68	0.63	0.63	0.63	0.64	0.65	0.63	0.67	0.68	0.66	0.65	0.66	0.63	0.66	0.65	0.65	0.65
38	0.74	0.71	0.73	0.72	0.71	0.73	0.76	0.73	0.73	0.73	0.74	0.73	0.74	0.73	0.69	0.70	0.73	0.72	0.72	0.75
39	0.75	0.71	0.71	0.73	0.70	0.71	0.76	0.71	0.72	0.72	0.75	0.74	0.74	0.73	0.71	0.70	0.73	0.73	0.73	0.79
40	0.76	0.72	0.78	0.82	0.76	0.78	0.80	0.80	0.81	0.78	0.78	0.80	0.79	0.79	0.78	0.76	0.78	0.78	0.80	0.72

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

Table 1.3.3 (Continuation). Genetic similarity coefficients for 40 bentgrass (*Agrostis* spp.) accessions from data of five primer combinations using fluorescence-labeled AFLP.

No.**	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
21	1.00																			
22	0.90	1.00																		
23	0.93	0.88	1.00																	
24	0.93	0.88	0.91	1.00																
25	0.93	0.87	0.92	0.93	1.00															
26	0.93	0.87	0.91	0.93	0.95	1.00														
27	0.82	0.82	0.81	0.82	0.81	0.80	1.00													
28	0.92	0.86	0.91	0.91	0.93	0.93	0.80	1.00												
29	0.78	0.78	0.80	0.80	0.76	0.77	0.76	0.79	1.00											
30	0.67	0.66	0.69	0.67	0.66	0.67	0.63	0.68	0.62	1.00										
31	0.65	0.65	0.69	0.65	0.65	0.66	0.61	0.67	0.62	0.94	1.00									
32	0.78	0.80	0.79	0.79	0.76	0.77	0.77	0.79	0.87	0.62	0.63	1.00								
33	0.63	0.59	0.63	0.61	0.61	0.60	0.58	0.64	0.59	0.62	0.61	0.61	1.00							
34	0.66	0.64	0.68	0.66	0.65	0.65	0.63	0.66	0.64	0.69	0.69	0.66	0.68	1.00						
35	0.69	0.68	0.71	0.69	0.67	0.67	0.65	0.68	0.68	0.68	0.67	0.69	0.66	0.71	1.00					
36	0.67	0.67	0.69	0.68	0.66	0.67	0.67	0.67	0.73	0.62	0.62	0.70	0.59	0.64	0.67	1.00				
37	0.66	0.63	0.68	0.67	0.65	0.67	0.62	0.67	0.65	0.62	0.61	0.65	0.61	0.67	0.69	0.68	1.00			
38	0.75	0.78	0.77	0.76	0.75	0.75	0.71	0.75	0.73	0.63	0.62	0.74	0.62	0.68	0.71	0.73	0.68	1.00		
39	0.80	0.81	0.80	0.79	0.80	0.79	0.73	0.80	0.74	0.63	0.62	0.75	0.64	0.67	0.72	0.74	0.70	0.86	1.00	
40	0.73	0.72	0.77	0.73	0.72	0.73	0.72	0.75	0.77	0.69	0.69	0.76	0.63	0.70	0.71	0.84	0.71	0.77	0.80	1.00

**AFLP No. = designated sample number for accession in the AFLP analysis and corresponds to the PI No. listed in Table 1.1

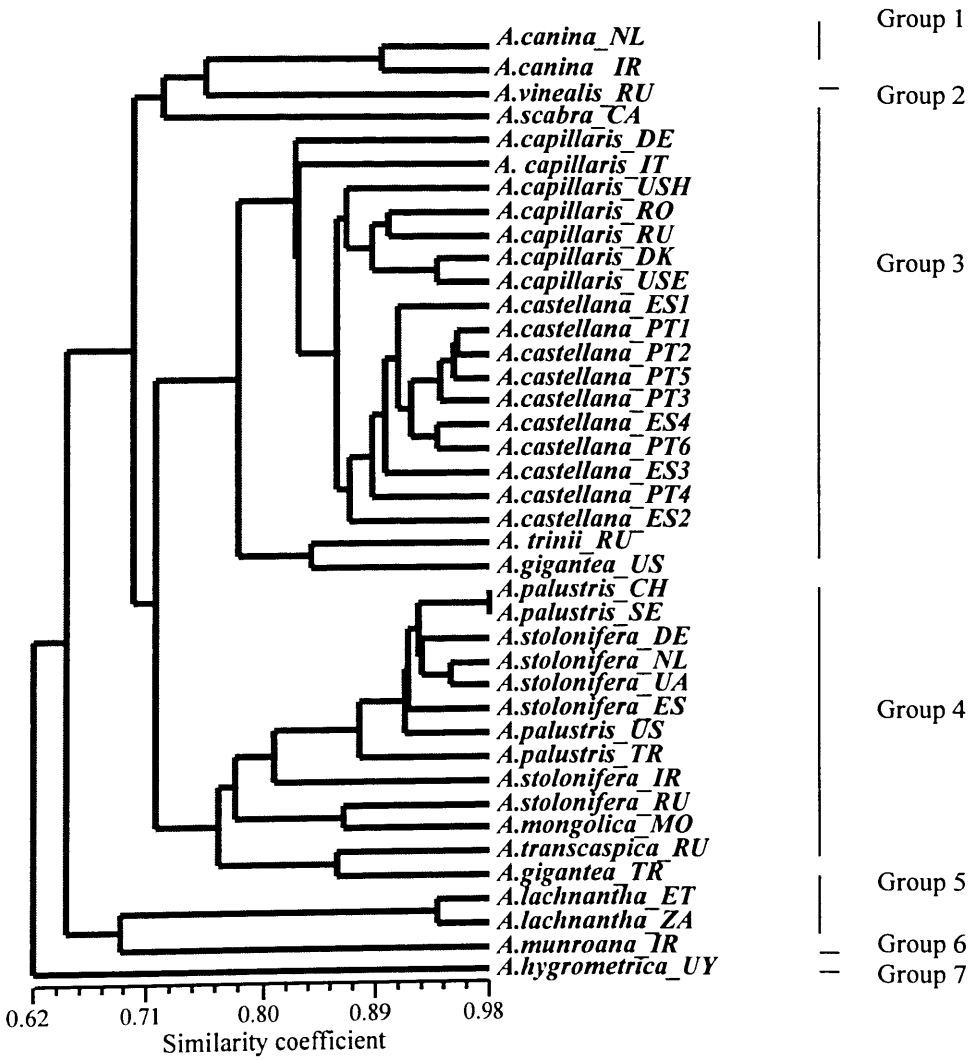

Figure 1.2. UPGMA dendrogram of 40 accessions of 14 *Agrostis* spp. from 20 different countries. PCA analysis distinguishes seven groups based on Eigen values > 1.0.

Legend: CA = Canada; CH = Switzerland; DE = Germany; DK = Denmark; ES = Spain; ET = Ethiopia; IR = Iran; IT = Italy; MO = Mongolia; NL = Netherlands; PT = Portugal; RO = Romania; RU = Russia and former USSR; SE = Sweden; TR = Turkey; UA = Ukraine; US = America; UY = Uruguay; ZA = South Africa

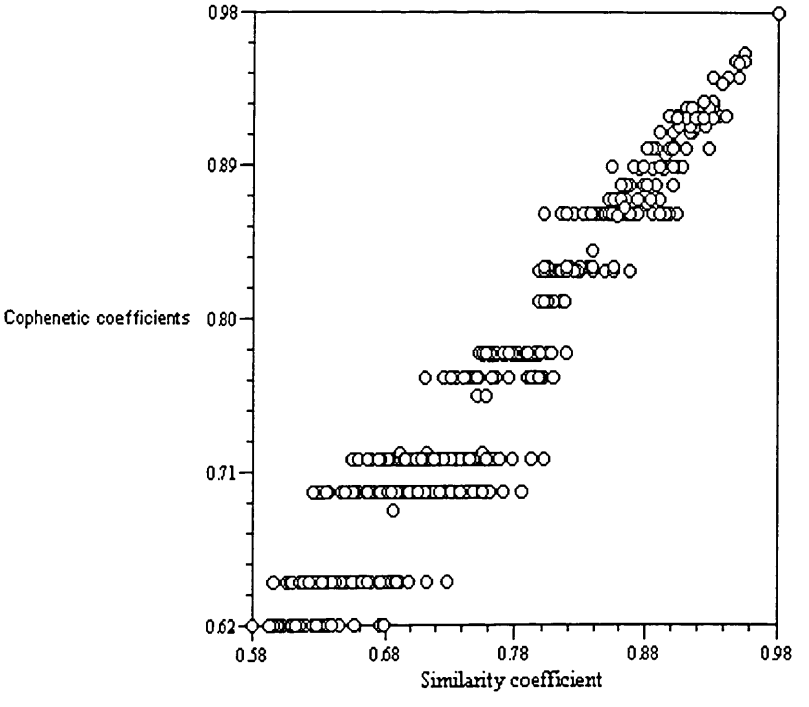

Figure 1.3. Plot analysis of cophenetic correlation and similarity coefficient as a measure of goodness of fit of the similarity indices. r = 0.95951 = normalized Mantel statistic Z; Approximate Mantel t-test: t = 11.9767; P(Z < obs. Z: p = 1.0000).

and AFLP analysis suggested the two species had the least homology, genetic exchange or introduction among the accessions studied.

A single dendogram was generated from the UPGMA cluster analysis with one possible tie found between the closest pair (Figure 1.2). A cophenetic-value (ultrametric) matrix was generated from the coefficients of SAHN's cluster analysis of the distance matrix. The cophenetic correlation was calculated (r = 0.96) as a measure of goodness of fit and the results were plotted in a phenogram (Figure 1.3). Using SYSTAT, a rotated PCA with the bands as observations was used to determine the number of factors or groups based on eigenvalues greater than one. Seven groups were extracted, which explained 72% of the total variance. The dendrogram showed a similarity coefficient of 0.73 for these seven groups.

Group 1 consisted of two accessions of *A. canina* (Netherlands and Iran, **sc** = 0.90) and *A. vinealis* (Russian Federation, **sc** = 0.76 to *A. canina*). Though geographically distant in origin, the two *A. canina* diploids were morphologically similar and AFLP data supported their grouping. In Figure 1, *A. vinealis* grouped closest to *A. canina* than the other twelve species in the study and confirmed their genetic and physical similarity. Species in Group 1 showed very fine, short erect leaves and low growth, but *A. vinealis* was rhizomatous and confirmed earlier descriptions (Hubbard, 1984; Funk, 1998; Brilman, 2001). In catalogues, *A. vinealis* Screb. was listed as being synonymous to eight other species names: *A. canina* ssp. *montana* Hartman, *A. stricta* J.F. Gmel (BONAP Poaceae Listing), *A. syreistschikowii* P.A.S., *A rubra* L., *A. ericetorum* P.B, *A. tenuifolia* M.B., *A. coarctata* E. and *A. pusilla* D. by the Royal Botanic Garden Edinburgh. Jones (1955a) found that *A. canina* ssp. *montana* (*A. vinealis*) also known as

brown velvet bentgrass, was an autotetraploid form of *A. canina* and difficult to distinguish in field specimens. Davies (1953) correlated the morphological difference with ecological preference. Velvet bentgrass occurred in natural wet and damp soils while brown velvet bentgrass was found in heaths and upland ground. Molecular characterization by AFLP confirmed the relationship between *A. canina* and *A. vinealis*. *A. canina* was morphologically distinguished from other species of *Agrostis* physically by having a longer, more pointed ligule and shorter palea than *A. capillaris* or *A. palustris* (Hubbard, 1984). Support that *A. canina* may be grouped separately from the other species was also shown by AFLP data.

Group 2 consisted only of PI234681, *A. scabra* (Canada) and AFLP analysis showed that the similarity with *A. canina* was only 0.74. Morphologically, *A. scabra* was also a non-rhizomatous bunch type grass like *A. canina* but the two species differed in ploidy levels. Dissimilarity may come from the hexaploid nature and more diverse genetic composition of the species in Group 2. Known as ticklegrass or hairgrass, *A. scabra* Willd., a delicate species was listed as being synonymous with winter bentgrass (*A. hyemalis* Tuck.) but differed in flowering time (Lawrence, 1986).

Group 3 consisted of three subgroups. Subgroup A consisted of the seven accessions of *A. capillaris* (colonial bentgrass) from seven countries. This indicated that the seven allotetraploid accessions were similar and/or may have originated from common diploid ancestors. Visual classification between colonial and highland bentgrass was difficult. Hubbard (1984) separated them by flower color, ligule size and growth habit. Two accessions of *A. capillaris* (US) cv. 'Highland' and cv. 'Exeter' were reclassified as *A. castellana* (Hubbard, 1984; Shildrick, 1976; Brilman, 2001). Steiner

and Lupold (1978) supported the suggestion that *A. capillaris* cv. 'Highland' should belong to *A. castellana* based on the percentage distribution of the form of palea apex, the presence and angle of basal hairs and awn types. In this study, AFLP analysis of PI469217 'Highland' and PI578528 'Exeter' showed that though they contained a small number of specific bands present only in *A. castellana*, they clustered more with the *A. capillaris* group. There is no information whether the plant introductions were seeded in open or isolated places during seed increase. Cross contamination at any time may add to the taxonomic variation. Yamamoto and Duich (1994) could not distinguish 'Highland' and 'Exeter' from the other ten colonial bentgrasses using phosphoglucoisomerase, glutamate oxalotransaminase, peroxidase and topoisomerase isozymes. Difference at the Pox-2 locus was observed (Yamamoto and Duich, 1994) but peroxidase isozymes are oftentimes unstable and dependent on stress conditions. Subgroup B consisted of all PI accessions of *A. castellana* from Spain and Portugal with no distinction as to groupings based on origin. The two subgroups, A and B, may share common diploid species progenitors and may be related with *A. capillaris* from Germany and Italy.

Subgroup C consisted of PI598462 *A. trinii* (Russian Federation) and PI443051 *A. gigantea* (USA). Classification of *A. trinii* Turcz. has been unclear and listed as being synonymous to *A. canina* ssp. *trinii* (Turcz.) Hulten (Soreng et al., 2003). Cluster analysis however showed *A. trinii* to be dissimilar to *A. canina* (**sc** = 0.70 to 0.73). Skolovskaya (1938) described *A. trinii* in Siberia and Orient as having both diploid and tetraploid forms. The diploid form of *A. trinii* may have been recognized as subspecies of *A. canina* (Brilman, 2002). PI598462 *A. trinii* was known to be a tetraploid and appeared different phenotypically from *A. vinealis*, an autotetraploid form of *A. canina*.

Agrostis trinii (Russian Federation) could be an allopolyploid with one chromosome set different from *A. canina* and *A. vinealis*, thus forming separate groups. The other member of this subgroup, *A. gigantea* (US) surprisingly was not grouped with the *A. gigantea* (Turkey). Hexaploid *A. gigantea* genomic constitution of $A_1A_1A_2A_2A_3A_3$ may have consisted of A_1A_1 from *A. canina*, with the $A_2A_2A_3A_3$ probably from *A. stolonifera* or $A_1A_1A_2A_2$ from *A. capillaris* (Jones, 1955b). AFLP data supported both possibilities. The two PI lines of *A. gigantea* may possibly have different chromosome sets but share the common A_1A_1 genome. Based on the clustering, PI443051 (USA) may have the $A_1A_1A_2A_2$ from *A. capillaris* while PI383584 (Turkey) may have the $A_2A_2A_3A_3$ from *A. stolonifera*. *A. gigantea* (Turkey) may have a second genomic constitution originating from another species. In this study, *A. transcaspica* (Former USSR) was found to be diploid and clustered closest with the *A. gigantea* (Turkey). This indicated the possibility that *A. transcaspica* was closely related to this species and may be the source for the A_3A_3 genome in *A. gigantea* (Turkey). This relationship would be interesting to examine in future interspecific hybridization and cytogenetic studies.

Group 4 consisted mainly of the creeping bentgrass (*A. palustris* and *A. stolonifera)*, *A. gigantea* (Turkey), *A. mongolica* and *A. transcaspica.* There has been considerable taxonomic confusion regarding creeping bentgrass and whether they should also be *A. stolonifera* with subspecies *palustris*, ssp. *stolonifera* or ssp. *gigantea*. The genetic similarity coefficient between PI accessions in this group ranged from 0.82 to 0.95. Genetic dissimilarity computed from 1- **sc** x 100% (Zhang et al., 1999) ranged from 5 to 18%. Turf breeders believe *A. palustris* (USA) have originated from and frequently outcrossed with materials from Europe. AFLP data supported this idea and results

showed that USA creeping bentgrasses may share some genetic similarity with those from Switzerland and Sweden. The most divergent in *A. stolonifera* would be from Turkey, Iran and the former USSR as opposed to those originating from other parts of Europe. No separation of groups for *A. palustris* and *A. stolonifera* was observed based on AFLP, but they differed slightly from *A. gigantea.* The difference may be due to the third chromosome set of hexaploid *A. gigantea.* Caceres et al.(2000) used RFLP markers to distinguish four creeping bentgrass *A. stolonifera* cultivars from *A. capillaris* 'Highland'. AFLP data supported that PI accessions of *A. stolonifera* from different parts of the globe were in one group and differed from *A. capillaris* (Group 3, Subgroup A). Species *A. stolonifera* also shared slight similarities with *A. mongolica* (**sc** = 0.76 to 0.87*)* and *A. transcaspica* (**sc** = 0.73 to 0.80*)*. PI362190 *A. mongolica* plant type was also stoloniferous and chromosome analysis showed that the bentgrass from Eastern Europe was also a tetraploid. UPGMA analysis grouped *A. mongolica* (Mongolia) closest to *A. stolonifera* (Russia). Because Mongolia and Russia are geographically adjacent, these countries may have similar environmental conditions favorable to both species. The fourth species in Group 4, which comprised mostly of stoloniferous bentgrasses, was *A. transcaspica*, PI283174 (Former USSR). Physical examination showed *A. transcaspica* also to be creeping but differed in leaf characteristics. The species has wider (6 to18 mm) dark green, thick leaves with pointed tips. Soreng et al. (2003) has listed *A. transcaspica* Litv. as being synonymous to *A. stolonifera* ssp. *transcaspica* (Litv.) Tzvelev. AFLP clustering and similarity coefficients indicated that *A. transcaspica*, a diploid may have contributed to the tetraploid or hexaploid creeping bentgrass genome.

Group 5 consisted of *A. lachnantha* N. (Ethiopia and South Africa). The African bentgrasses were morphologically distinct from the other bentgrass species. PI195917 (Ethiopia) bentgrasses were shorter (4 to 10 inches) than PI299461 (South Africa, >10 inches). The latter also has fewer leaves, harder stalks, and produced very tall flowering panicles (>2 feet) in the greenhouse but were highly sterile. The chromosome number reported here differed from the listing of the Index to Plant Chromosome number (IPCN) with gametophytic count (n=21) and sporophytic 2n=28. Chromosome analysis of 2n=21 may suggest intra- or intercrossing variation during seed increase. The two PI accessions of *A. lachnantha* has **sc**=0.94 based on AFLP analysis. Eleven specific AFLP markers were found that could distinguish *A. lachnantha* species from the other thirteen species.

Group 6 consisted only of PI230236 *A. munroana* (Iran). Background information about *A. munroana* Aitch. & Hemsl. was minimal and was earlier referred to as *Calamagrostis munroana* (Aitch. & Hemsl.) Boiss. in 1884 (Soreng et al., 2003). Chromosome analysis of plants of PI230236 showed 2n=21 which confirmed earlier reports (Gohil and Koul, 1986; Mouinuddin et al., 1994). Physical analysis of the mature triploid plant showed a short plant stature (2 to 6 inches) with fine (2 to 3 mm width), flat, soft, normal green leaves. Species *A. munroana* was observed to be a bunch type bentgrass and early flowering with the florets openly branched. *A. munroana* plants were morphologically distinct from triploids of *A. lachnantha.* Their triploid genome constitutions may be largely unlike and AFLP results showed **sc** = 0.61, thus forming separate groups.

Group 7 was the most genetically distant from the thirteen other species studied and included *A. hygrometrica* (Uruguay). Seven specific AFLP markers were found for this species. *A. hygrometrica* Nees. has nine synonyms in genus *Agrostis* or *Bromidium* (Soreng et al., 2003). Plant materials from PI477045 were low growing, bunch-type grasses with hard, lengthy flowering panicles. AFLP analysis showed that bentgrass germplasm from Uruguay (South America) formed a separate group from other bentgrasses of Europe, Asia or North America. Distinct ecological conditions among continents and unique germplasm pools from which the *A. hygrometrica* may intercross would differentiate the accessions.

Assessment of genetic diversity in germplasm collections from several geographic locations using AFLP markers has been conducted for *Morus* germplasm (Sharma et al., 2000) and the Triticeae tribe (Monte et al., 1993). Positive correlations were found for cluster groupings and geographic distances. Results in this study indicated that geographically adjacent countries like Spain and Portugal have bentgrass accessions which also clustered together as in Group 3, Subgroup B and bentgrass accessions from distant locations (Iran vs. Uruguay) were in separate groups with low similarity coefficients. Possibilities of genetic introductions may have occurred with migration, selection and breeding among the colonial, highland and creeping bentgrasses from Europe and USA. Local environmental adaptation may play a significant role in *Agrostis* diversity.

AFLP analysis revealed its usefulness for assessing germplasm collection for possible duplications. It may also indicate where incorrect species determinations would be in the GRIN system or germplasm collection. The four hundred polymorphic markers

from five chosen primer combinations showed the high level of diversity between the *Agrostis* germplasm and distinguished the seven groups. Ploidy level differences did not give ambiguous results in scoring as highly polymorphic, repetitive and specific bands were found. The high cophenetic correlation showed the goodness of fit of the similarity indices. AFLP analyses supported the hybridization paths between species (Figure 1.4). The dendogram showed the relationships between *A. canina* with *A. vinealis* but not with *A. trinii.* Cluster analysis also showed that two hexaploid *A. gigantea* from different geographic sources (USA and Turkey) were not grouped together, possibly due to different chromosome sets. A possible diploid progenitor would be *A. transcaspica.* The percentage genetic dissimilarity among creeping bentgrasses indicated considerable potential for the improvement of turf. Turfgrass breeders may develop superior cultivars either by crosses with germplasm accessions from the same species or among varying species. Important traits from other *Agrostis* species can be introduced to cultivated bentgrasses and AFLP analysis would be a useful tool to monitor introgression and molecular tagging. Using specific amplified products, sequence characterized amplified primers may be developed to genetically distinguish the different bentgrass species in the future. AFLP analysis may be used in identifying bentgrass genotypes and clusters, constructing core collections and screening for duplicate or misclassified accessions in germplasm collections.

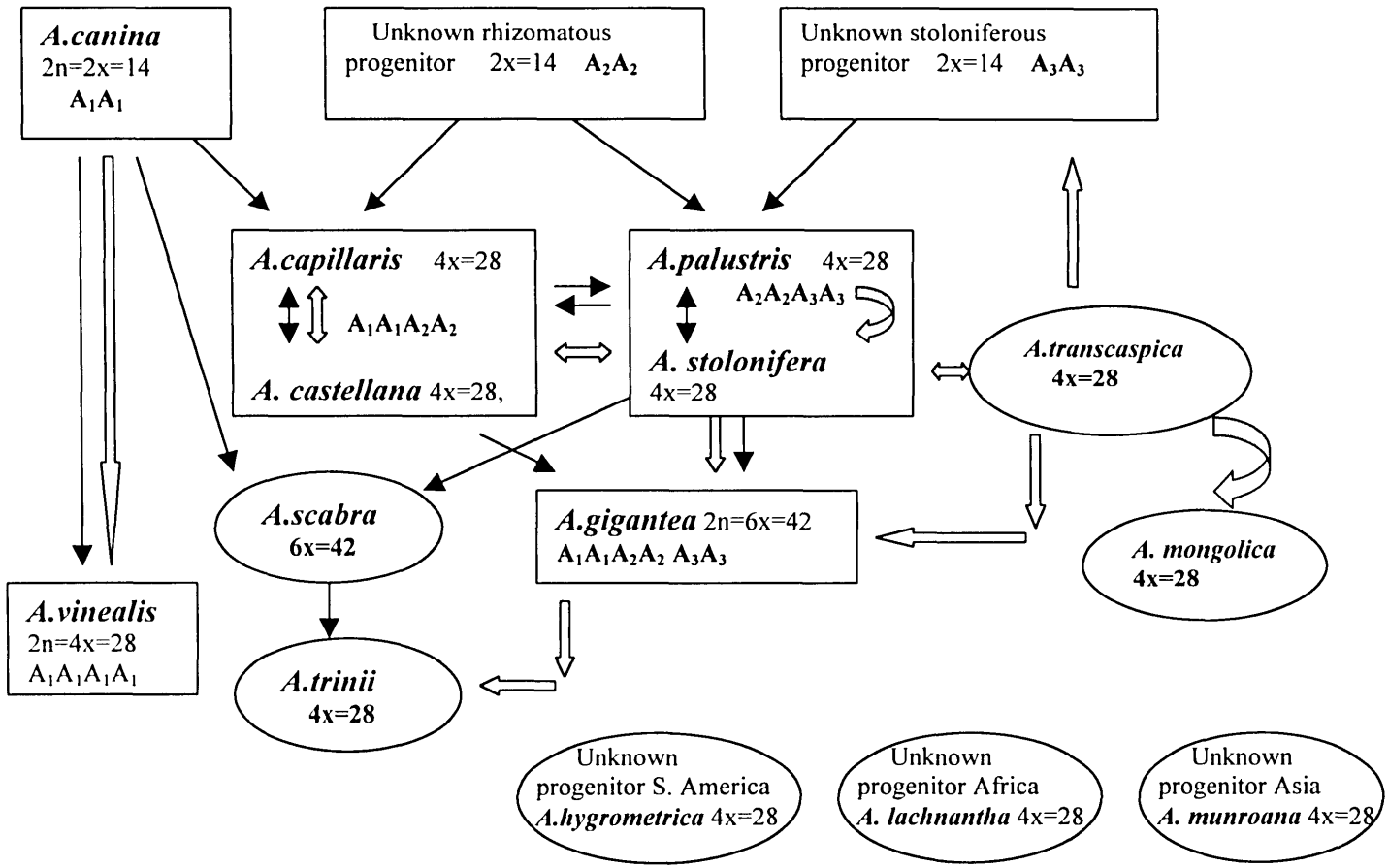

Figure 1.4. Diagram of hybridization pathways of *Agrostis* species (indicated by solid arrows) and relationships supported by AFLP analyses (indicated by unfilled arrows).

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