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Relationships among Creeping Bentgrass Cultivars Based on Isozyme Polymorphisms

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ABSTRACT

An understanding of the genetic variability within a crop species is essential to its improvement. The objectives of this research were to study the utility of isozyme patterns for creeping bentgrass (*Agrostis palustris* Huds.) cultivar identification and to estimate the relationships between creeping bentgrass cultivars based on isozyme patterns. Seventy to 73 plants from each of 18 creeping bentgrass cultivars and 25 plants from one plant introduction were scored for 24 isozyme polymorphisms representing six loci. All cultivars except a small group containing the cultivars Pennlinks, Pro/Cup, Southshore, and Lopez were uniquely characterized based on a 20% or greater band frequency in one cultivar versus absence of the band in the most closely clustered cultivar. The isozyme patterns from each plant were used to calculate the genetic distance within a cultivar, and the average band frequency within a cultivar was used to calculate genetic distances between cultivars. The cultivars Pennlinks, Pro/Cup, Southshore, and Lopez had the highest average within-cultivar genetic distances indicating that additional marker loci will be needed to distinguish these cultivars. The unweighted pair group method with arithmetic average (UPGMA) cluster analysis generated from the between-cultivar genetic distance matrix divided the cultivars into two groups. One group contains 10 cultivars including the variety Seaside which may have provided initial germplasm for this group. The second group contains the cultivars Pennlinks, Southshore, Pro/Cup, Lopez, and four cultivars with unique allozymes. The plant introduction PI251945 was distantly related to the cultivated U.S. germplasm indicating that European material can be a source of genetic diversity to broaden U.S. bentgrass germplasm.

THE BENTGRASSES are native to Western Europe (Harlan, 1992) with the genus *Agrostis* consisting of approximately 200 species (Hitchcock, 1951). The four species commonly used as turfgrasses are *A. palustris* Huds. – creeping bentgrass ($2n=4x=28$), *A. canina* L. – velvet bentgrass ($2n=2x=14$), *A. tenuis* Sibth. – colonial bentgrass ($2n=4x=28$), and *A. gigantea* Roth. – redtop bentgrass ($2n=6x=42$). Creeping bentgrass is the most widely utilized of the turf-type bentgrasses because it has excellent tolerance of low mowing heights and a strong stoloniferous growth habit making it ideally suited for the establishment of golf course greens and fairways in temperate and subarctic climate zones.

An understanding of the genetic diversity present in the cultivated germplasm of a species as well as the location of new sources of genetic variability is important for the optimal utilization of genetic resources. Plant breeders must have an understanding of the genetic variability of elite germplasm because continued reselection within this germplasm can narrow the genetic base of elite material and ultimately increase the potential vulnerability to pests and abiotic stresses. Information about the location of new sources of genetic variability can help broaden the genetic base of elite material and maintain long-term improvement.

Information about the genetic relationships of creeping bentgrass cultivars is limited because it is a cross pollinated species, and in many cases the parental clones of synthetic cultivars are of unknown origin. However, in other species, genetic similarity between cultivars have been estimated based on molecular markers such as isozymes in *Glycine max* L. – soybean (Cox et al., 1985) and *Solanum tuberosum* L. – potato (Douches and Ludlam, 1991); RFLPs in *Hordeum vulgare* L. – barley (Melchinger et al., 1994), *Zea mays* L. – corn (Messmer et al., 1993), and *Festuca arundinacea* Schreb. – tall fescue (Xu et al., 1994); and RAPD markers in [*Buchloe dactyloides* (Nutt.) Engelm.] – buffalograss (Huff et al., 1993; Wu and Lin, 1994). Isozyme markers are not as numerous as RFLP or RAPD markers; however, they are polymorphic in creeping bentgrass populations (Warnke et al., 1997) and technically simpler to apply with large population sizes. The objectives of this research were to (i) assess isozyme patterns for creeping bentgrass cultivar identification, (ii) determine the optimum sample size for estimating allozyme frequencies of creeping bentgrass populations, and (iii) estimate the genetic relationship between creeping bentgrass cultivars based on allozyme frequencies.

MATERIALS AND METHODS

Eighteen major creeping bentgrass cultivars commercially available in the USA and one plant introduction were assayed for isozyme polymorphisms. Seed was obtained from the 1993 National Turfgrass Evaluation Programs (NTEP) bentgrass cultivar trial and directly from the proprietary seed companies. In a few cases seed from the 1989 NTEP trial was used (Table 1).

Individual plants at least 60 d old were used for isozyme analysis. Plants were maintained in the greenhouse and fertilized every 2 wk with a (20:8.7:16.6 N-P-K) water soluble fertilizer solution and trimmed regularly to promote tiller production. Healthy plants free from disease and insect pressure were found to provide much higher enzyme activity. Furthermore, electrophoretic consistency was improved by utilizing etiolated tissue from plants maintained at low light levels and

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Abbreviations: UPGMA, unweighted pair group method with arithmetic averages; AWGD, average within-cultivar genetic distance; PGM, phosphoglucomutase; PGI, phosphoglucose isomerase; TPI, triosphosphate isomerase; GOT, glutanate oxaloacetate transaminase; NTEP, National Turfgrass Evaluation Program.

Table 1. Creeping bentgrass cultivars studied, the number of plants scored in each, and the average-within cultivar genetic distance (AWGD).

Code	Cultivar	Year†	Source	Sponsor	Plants scored	AWGD‡
1	Penneagle	1978	1993 NTEP bentgrass trial	Tee-2-Green Corp.	72	0.233
2	Penncross	1955	1993 NTEP bentgrass trial	Tee-2-Green Corp.	73	0.210
3	Trueline	1995	1993 NTEP bentgrass trial	Turf Merchants	73	0.267
4	Crenshaw	1993	1993 NTEP bentgrass trial	Loft's Seed	73	0.203
5	Southshore	1992	1993 NTEP bentgrass trial	Loft's Seed	73	0.326
6	Providence	1988	1993 NTEP bentgrass trial	Seed Research	70	0.230
7	National	1988	1989 NTEP bentgrass trial	Pickseed West	70	0.312
8	Viper	1995	International Seeds	International Seeds	72	0.173
9	18th Green	1995	1993 NTEP bentgrass trial	Johnson Seeds	73	0.257
10	Cobra	1988	1989 NTEP bentgrass trial	International Seeds	73	0.180
11	Emerald	1973	1989 NTEP bentgrass trial	International Seeds	73	0.138
12	Pennlinks	1987	1993 NTEP bentgrass trial	Tee-2-Green Corp.	73	0.387
13	SR1020	1987	Seed Research	Seed Research	73	0.265
14	Putter	1988	Jacklin Seed	Jacklin Seed	73	0.161
15	Seaside	1924	1993 NTEP bentgrass trial	Standard	73	0.258
16	Lopez	1994	1993 NTEP bentgrass trial	Finelawn Research	72	0.325
17	Pro/Cup	1994	1993 NTEP bentgrass trial	Forbes Seed and Grain	73	0.340
18	Cato	1993	1993 NTEP bentgrass trial	Pickseed West	70	0.152
19	PI251945	1958§	Plant Intro. Station Pullman, WA		25	0.104

† Year of release.
‡ Average within-cultivar genetic distance calculated by Nei's (1972) distance formula.
§ Year collected.

constant temperature for 1 wk prior to analysis. A four- to five-leaf sample of newly expanded leaves was collected from each plant and crushed with a plexiglass rod, rounded on one end, in 80 µL of an extraction buffer composed of 75 m*M* Tris-HCL buffer, pH 7.5, 50 g L^{-1} polyvinylpyrrolidone-40, and 14 m*M* mercaptoethanol (0.2% v/v). The extraction was conducted in chilled 12-sample porcelain color plates. The crude extracts were absorbed onto 3- by 8-mm Whatman 3MM wicks and stored over night at −20°C, or at −80°C when longer-term storage was needed.

A tris-citrate–lithium borate pH 8.3 buffer system was utilized (Weeden and Wendel, 1989). Gel slabs consisted of 100 g L^{-1} potato starch obtained from Starch Art, (Smithville, TX). Gel trays that provided for a direct contact between the gel tray and buffer were used. The wicks containing the crude protein extract were inserted into a slice approximately 1 cm from the anodal end of the gel. The gels were subject to approximately 4 h of electric current at 50 to 60 mA. The enzymes phosphoglucose isomerase (PGI, E.C.5.3.1.9), phosphoglucomutase (PGM, E.C.5.4.2.2), glutamate oxaloacetate transaminase (GOT, E.C.2.6.1.1.), and triosphosphate isomerase (TPI, E.C.5.3.1.1) were resolved by means of stains prepared according to the methods of Vallejos (1983). PGM activity was improved by the inclusion of 1.5 mL of 5 mg mL^{-1} (w/v in H_2O) glucose 1-6 diphosphate to 100 mL of staining solution.

Each gel contained 25 plants from a cultivar and two additional creeping bentgrass check plants. The check plants were part of an earlier genetics study so their allelic makeup was known. Locus designation and allele numbering is described in Warnke et al. (1997). Allelic bands were scored as 1, present, or 0, absent, for each plant. A total of 75 plants from each cultivar were analyzed; however, because of enzyme degradation in some samples, fewer plants were used to establish band frequencies (Table 1). The band frequency in the population was obtained by dividing the number of plants containing the band by the total plants examined. Genetic distances between and within each cultivar were calculated using Nei's distance formula (Nei, 1972). A dendrogram based on the distance matrix was constructed by applying the unweighted pair group method with arithmetic averages (UPGMA) cluster analysis. The distance matrix and dendrogram were both constructed using NTSYS-pc version 1.7 (Rohlf, 1992). Between-variety genetic distances were calculated from random samples of 4, 8, 12, 16, 20, 25, and 70 plants to establish the minimum sample size sufficient to estimate genetic diversity. The mean genetic distances of the seven sample sizes were compared with a *t*-test.

RESULTS AND DISCUSSION

A total of 24 allozymes were scored for 70 plants in each of the 18 cultivars. Segregation data is available for 14 of the 24 allozymes scored (Warnke et al., 1996). The assignment of additional allozymes was based on mobility differences compared with the check plants run with each gel. The *Pgi-2* locus in creeping bentgrass is highly variable with seven scorable alleles present; however, the dimeric structure of this enzyme and close mobilities of many alleles does not allow for the accurate classification of all alleles present in some genotypes without progeny testing. Therefore, only the fastest and slowest migrating alleles for each plant, i.e., those that were easily detected, were scored. Average allozyme frequencies ranged from 0.01 for $Pgm\text{-}1^4$ and $Pgm\text{-}2^4$ to 1.00 for $Pgm\text{-}1^1$ and $Got\text{-}2^1$ (Table 2).

Genetic distances between cultivars ranged from 0.007 for Southshore and Pennlinks to 0.277 for Cato and PI251945 (Table 3). The UPGMA cluster analysis separates the 18 U.S. cultivars into two main groups (Fig. 1). The first group includes 10 cultivars (Penneagle, Putter, Penncross, Trueline, Viper, Emerald, 18th Green, Cobra, Crenshaw, and Seaside). With the exception of Crenshaw these are strongly creeping cultivars having a prostrate to semi-erect growth habit. The cultivar Seaside is the oldest variety in this group and may have provided germplasm used in the development of some of the creeping bentgrasses in this grouping. Seaside originated as a naturalized population growing in tidal flats near Coos Bay, OR, and was the only widely available seeded bentgrass in the U.S. from the 1920s until 1955 when Penncross was released (Duich, 1985). The second cluster contains eight cultivars that can be divided into two groups. Four of them (Southshore,

Table 2. Cultivar allele frequencies for loci that were polymorphic within *A. palustris*.

	Cultivars																			
Allele	1†	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Average
Tpi-1[1]‡	0.01	0.29	0.16	0.01	0.28	0.07	0.04	0.00	0.00	0.03	0.00	0.41	0.03	0.00	0.20	0.09	0.35	0.04	0.08	0.11
Tpi-1[2]	0.96	0.92	0.99	0.95	0.97	0.98	1.00	1.00	0.96	1.00	1.00	1.00	1.00	0.99	0.95	1.00	1.00	1.00	0.84	0.97
Tpi-1[3]	0.77	0.83	0.87	0.80	0.72	0.85	0.81	1.00	0.96	1.00	0.95	0.71	0.72	0.92	0.87	0.77	0.77	0.85	1.00	0.85
Tpi-2[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	1.00	1.00	0.99
Tpi-2[2]	0.99	0.95	0.75	0.99	0.89	0.96	0.98	1.00	1.00	1.00	1.00	0.87	1.00	0.96	0.99	0.85	0.88	0.88	1.00	0.94
Tpi-2[3]	0.39	0.23	0.40	0.01	0.36	0.32	0.31	0.00	0.00	0.03	0.05	0.40	0.05	0.39	0.00	0.37	0.48	0.63	0.16	0.24
Got-2[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Got-2[2]	0.96	0.97	0.96	1.00	0.93	0.99	0.94	0.93	0.79	0.83	1.00	0.97	0.99	1.00	0.71	0.97	0.93	1.00	1.00	0.94
Got-2[3]	0.12	0.00	0.10	0.01	0.29	0.00	0.11	0.43	0.01	0.37	0.00	0.20	0.00	0.00	0.33	0.01	0.16	0.00	0.04	0.12
Pgm-1[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pgm-1[2]	0.00	0.00	0.00	0.19	0.63	0.31	0.56	0.00	0.00	0.00	0.00	0.57	0.47	0.00	0.00	0.71	0.67	1.00	0.00	0.27
Pgm-1[3]	1.00	1.00	1.00	0.81	0.31	0.45	0.43	0.96	1.00	1.00	1.00	0.37	0.15	0.63	1.00	0.29	0.36	0.00	1.00	0.67
Pgm-1[4]	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Pgm-2[1]	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.44	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.01	0.36	0.00	0.07
Pgm-2[2]	1.00	1.00	1.00	1.00	1.00	0.97	1.00	1.00	0.92	1.00	1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00	0.99
Pgm-2[3]	0.20	0.21	0.17	0.36	0.38	0.45	0.62	0.19	0.65	0.15	0.39	0.44	0.48	0.00	0.81	0.36	0.47	0.08	0.24	0.35
Pgm-2[4]	0.00	0.00	0.00	0.00	0.00	0.04	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Pgi-2[1]	0.08	0.00	0.44	0.20	0.09	0.00	0.01	0.83	0.61	0.21	0.60	0.33	0.23	0.00	0.00	0.40	0.14	0.00	0.00	0.22
Pgi-2[2]	0.32	0.00	0.00	0.48	0.09	0.17	0.08	0.00	0.04	0.24	0.25	0.09	0.03	0.15	0.49	0.37	0.31	0.00	1.00	0.22
Pgi-2[3]	0.57	0.63	0.32	0.23	0.60	0.41	0.10	0.13	0.31	0.19	0.07	0.49	0.54	0.65	0.39	0.08	0.33	0.37	0.00	0.34
Pgi-2[4]	0.34	0.48	0.47	0.24	0.48	0.79	0.79	0.31	0.43	0.63	0.31	0.36	0.85	0.62	0.31	0.36	0.40	0.96	0.04	0.48
Pgi-2[5]	0.42	0.81	0.57	0.60	0.52	0.37	0.19	0.64	0.60	0.29	0.71	0.60	0.00	0.50	0.51	0.69	0.60	0.05	0.04	0.46
Pgi-2[6]	0.26	0.00	0.11	0.00	0.15	0.09	0.06	0.08	0.00	0.29	0.07	0.11	0.23	0.00	0.28	0.07	0.14	0.03	0.76	0.14
Pgi-2[7]	0.05	0.00	0.00	0.24	0.01	0.00	0.63	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05

† Cultivar code described in Table 1.
‡ Allele numbering described in Warnke et al. (1996).

Pennlinks, Pro/Cup, and Lopez) cluster quite closely and are difficult to distinguish from one another with the isozyme polymorphisms studied. One reason for the tight clustering of these four cultivars is that they possess most of the allozymes observed in this study. Therefore, the only differences between the cultivars are allele frequency differences rather than unique allele differences and it is the presence of unique alleles that increases genetic distance. The high average within-cultivar genetic distances (AWGD) of these four cultivars is an indication of their high genetic diversity (Table 1). The cultivar Southshore is a very broad-based cultivar derived from the progenies of 203 selected clones (Hurley et al., 1994), which may explain its high AWGD. The other four cultivars in the second group (Providence, SR1020, National, and Cato) do not group closely with any of the other cultivars, and, in fact, these cultivars all possess some unique allozymes. The plant introduction PI251945 was included in the study to gain some insights into the European bentgrass germplasm. PI251945 was collected in 1958 from Austria and it is quite distantly related to the U.S. cultivars examined, suggesting that European germplasm can be used to broaden the genetic diversity of U.S. germplasm. The cultivars SR1020 (five clone synthetic) and Crenshaw (six clone synthetic) share three parental clones in common (Engelke et al. 1995). Despite this common genetic base, they have very different isozyme profiles and do not cluster closely in the dendrogram. These cultivars are synthetics, and differences in the initial parent clones that make up these cultivars can result in large genetic distances between the cultivars because of numerous allozyme differences.

Table 3. Matrix of Nei's coefficients of genetic distance† for 19 creeping bentgrass cultivars.

	Cultivar code																	
Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	0.025																	
3	0.024	0.024																
4	0.030	0.043	0.043															
5	0.063	0.062	0.069	0.067														
6	0.044	0.048	0.052	0.048	0.026													
7	0.098	0.114	0.102	0.073	0.059	0.036												
8	0.065	0.070	0.030	0.053	0.108	0.099	0.137											
9	0.062	0.058	0.040	0.047	0.105	0.071	0.115	0.036										
10	0.029	0.052	0.032	0.042	0.084	0.051	0.090	0.037	0.054									
11	0.044	0.049	0.024	0.021	0.100	0.071	0.113	0.018	0.023	0.037								
12	0.064	0.059	0.054	0.059	0.007	0.036	0.069	0.085	0.085	0.087	0.076							
13	0.105	0.122	0.115	0.105	0.052	0.031	0.060	0.145	0.104	0.098	0.132	0.066						
14	0.022	0.023	0.033	0.050	0.051	0.026	0.090	0.084	0.081	0.046	0.067	0.060	0.080					
15	0.040	0.058	0.068	0.039	0.085	0.068	0.108	0.081	0.055	0.043	0.053	0.083	0.129	0.084				
16	0.086	0.096	0.074	0.048	0.035	0.046	0.069	0.091	0.096	0.098	0.066	0.024	0.084	0.081	0.107			
17	0.066	0.071	0.068	0.054	0.010	0.030	0.058	0.108	0.101	0.089	0.084	0.008	0.071	0.064	0.078	0.015		
18	0.164	0.178	0.164	0.174	0.065	0.061	0.078	0.232	0.208	0.169	0.219	0.088	0.052	0.109	0.241	0.086	0.075	
19	0.073	0.152	0.126	0.079	0.175	0.135	0.176	0.147	0.153	0.076	0.103	0.171	0.195	0.137	0.077	0.149	0.143	0.277

† Genetic distance based on Nei (1972).
‡ Cultivar code described in Table 1.

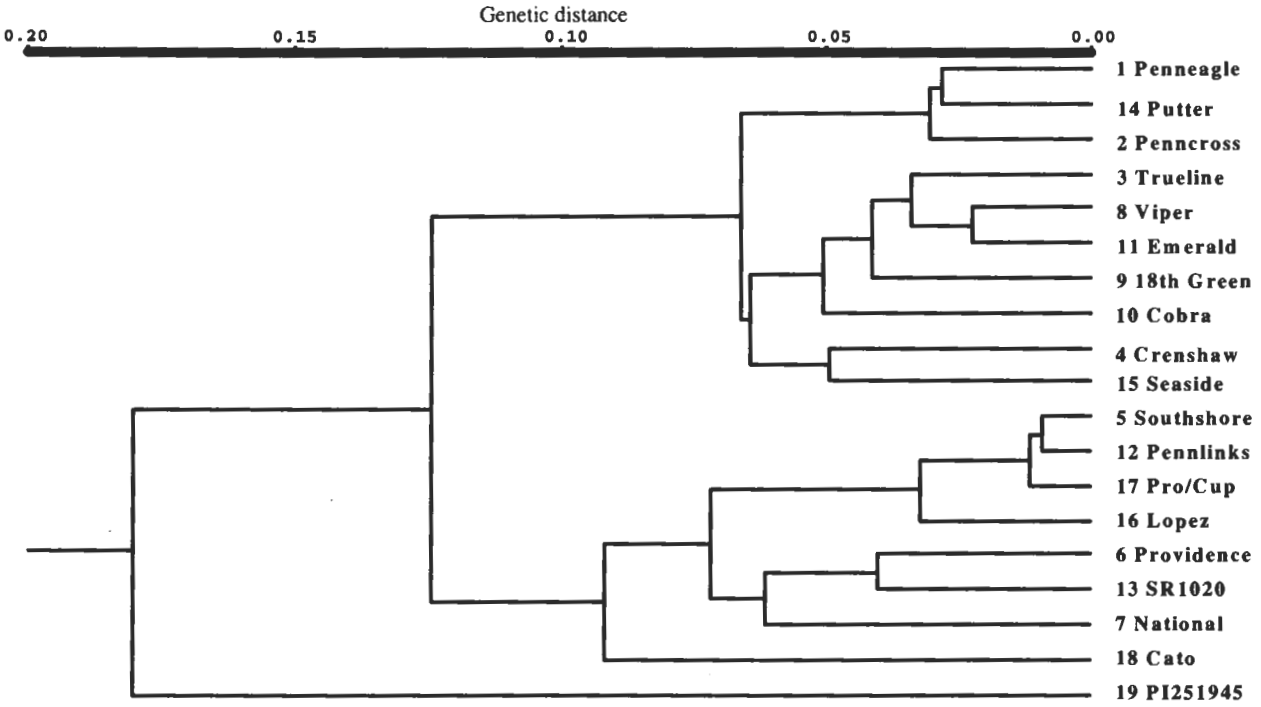

Fig. 1. Dendrogram of 18 creeping bentgrass cultivars generated by UPGMA cluster analysis.

Allozyme differences between the cultivars could also arise due to genetic drift because of the small population sizes or to differences in the strength and direction of selection imposed by breeders.

Creeping bentgrass cultivar identification via isozymes is complicated by the fact that cultivars are synthetics and considerable within-cultivar variation exists. The establishment of isozymes as a reliable characteristic for fingerprinting requires that allozyme frequencies in a given cultivar be stable in different environments and generations. In this study, all cultivars except Southshore, Pennlinks, Pro/Cup, and Lopez were distinguished by the presence of an allozyme in one cultivar at a frequency greater than 20% and its absence from the most closely-related cultivar. The cultivar Lopez is close to meeting this requirement at the *Got-2*^{3} allele. However, further research needs to be done on seed lot variability and post-establishment effects on allozyme frequencies to determine the overall utility of isozymes for creeping bentgrass cultivar discrimination. Additionally, more enzyme systems will need to be evaluated to reliably distinguish Pennlinks, Pro/Cup, and Southshore.

Yamamoto and Duich (1994) examined the utility of isozyme analysis with bulk plant leaf sampling and were able to distinguish 12 creeping bentgrass cultivars using only the *Pgi-2* locus. Østergaard and Nielsen (1981) investigated the effectiveness of the *Pgi-2* locus for cultivar identification in tetraploid ryegrass and found that allelic frequencies did not differ among seed lots. Additionally, seed size differences and low germination did not lead to significant within-cultivar allozyme frequency differences. Hayward et al. (1978) demonstrated *Pgi-2*

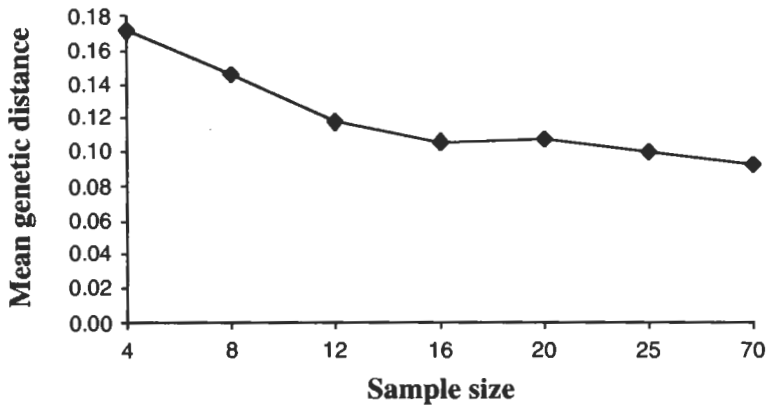

Fig. 2 Effects of sample size on the mean genetic distances among creeping bentgrass cultivars. The difference between population sizes of 16 and 20 was not significant; however, the difference between sizes 16 and 25 and 20 and 25 is significant, while the difference between sizes 25 and 70 is not significant based on a *t*-test ($\alpha = 0.05$).

isozyme profiles in perennial ryegrass did not differ significantly between seed stocks and samples from 3-yr old swards. Similar studies must be conducted for creeping bentgrass to establish the utility of isozyme analysis for creeping bentgrass cultivar discrimination in the turf industry.

Genetic diversity studies of cross-pollinated species require adequate sampling to ensure the accuracy of allele frequency estimates. One method for determining optimum sample sizes was described by Xu et al. (1994) in an RFLP analysis of tall fescue cultivars. Average genetic distances were calculated for different sample sizes and the point at which there was no longer a significant difference between the means of two sample sizes was considered the most efficient sample size. In their study, the largest sample size examined was 20 and there was no significant difference between sample sizes of 16 and 20 plants. In our study, average genetic distances between cultivars were calculated using data from 4, 8, 12, 16, 20, 25, and 70 plants. The mean distance between cultivars decreased and became consistent as sample size increased (Fig. 2). The difference between means was not significant ($\alpha = 0.05$) for sample sizes of 16 and 20, or 16 and 25; however, there was a significant difference between sample sizes of 16 and 70, and 20 and 70, but not between 25 and 70. The results suggested that a random sample of 25 plants is the minimum number of plants needed to estimate accurately the isozyme variation within these creeping bentgrass cultivars.

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