Weed Science 2012 60:583–588

Genetic Relationship between Cultivated and Feral Creeping Bentgrass (*Agrostis stolonifera*) in a Cultural Landscape

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Gene flow is an important consideration in the adoption of crops with novel traits or transgenes when sexually compatible relatives occur in the landscape. Unfortunately, gene flow and its long-term environmental impacts are very difficult to predict without releasing and studying the novel genotype. This project uses a retrospective population genetics approach to characterize the relationship between cultivated creeping bentgrass (CB) on a golf course and the same species in five feral populations nearby. CB plants were collected from an 8-yr-old golf course, five weedy populations up to 1,020 m from the golf course, and four modern CB cultivars. Using microsatellite markers and Bayesian inference, two major genetic clusters were distinguished: (1) CB cultivars and individuals from the golf course (cultivar genotype), and (2) the majority of individuals (62%) from the five feral populations (feral genotype). Two feral CB individuals (3.3% of all feral plants) were partially assigned to the cultivar genotype. Principal coordinates analysis agreed with this assignment, suggesting that an intraspecific hybridization event may have occurred. Plants in four feral populations showed a high degree of genetic similarity, but one feral population (Reservoir) was heterogeneous indicating that genetically complex CB populations can develop in cultural landscapes. While recognizing the limitations inherent in a single study of CB population genetics, these results add to the relevant knowledge for predictive ecological risk assessment.

Nomenclature: Creeping bentgrass, *Agrostis stolonifera* L.

Key words: Microsatellite markers, population genetics, gene flow, ecological risk assessment.

Biotechnology and modern breeding methods have created many novel turfgrass traits, but the potential for gene flow and ecological impacts in cultural, agricultural, and natural landscapes is largely unknown. This uncertainty has affected commercialization of glyphosate-resistant creeping bentgrass (GRCB) and glyphosate-resistant Kentucky bluegrass (*Poa pratensis* L.) in the United States (APHIS 2012; Waltz 2011; Wang and Brummer 2012). The glyphosate-resistance (GR) trait is due to a single copy of the *cp4 epsps* transgene and offers golf course managers the ability to remove weeds (e.g., annual bluegrass, *Poa annua* L.) by spraying glyphosate (Fei and Nelson 2003, 2004; Gardner et al. 2004, 2006). However, intraspecific and interspecific transgene flow and dispersal of GRCB in Oregon has raised questions about ecological impacts, including invasion in some plant communities (Bollman et al. 2012; Watrud et al. 2004). Thus, empirical research is needed to address questions about negative impacts on native plant communities, critical habitat for endangered species, agricultural systems, and urban landscapes (Ahrens et al. 2011a; Snow et al. 2005; Watrud et al. 2004; Wipff and Fricker 2000). Research could also help land managers who might need to respond to consumer preferences (e.g., organic lawn care), regional laws (e.g., moratoriums on genetically engineered crops), coexistence strategies, and other situations in the future.

CB is a wind-pollinated, C_3, allotetraploid, cool-season perennial grass species native to Eurasia. Anecdotal evidence suggests that naturalization began in North America before the 1750s (MacBryde 2006), although some authors have suggested that CB could be native to salt marshes and lakes in small parts of northern North America (Barkworth et al. 2007). CB and some closely related species are recognized as perennial weeds in the United States and other countries (Behrendt and Hanf 1979; IPA 2011; MacBryde 2006). CB is a phenotypically plastic species that grows in diverse habitats including marshes, dunes, stream banks, grasslands, ditches, urban areas, wastelands, and roadsides (Ahrens et al. 2011a,b; Barkworth et al. 2007; Bollman et al. 2012; Hart et al. 2009; Hubbard 1984; Kik et al. 1993; MacBryde 2006). In the northeastern United States, it is most common in habitats with intermediate levels of human management or disturbance such as roadsides, wastelands, and powerline rights-of-way (Ahrens et al. 2011b). Habitat suitability in the northeastern United States was postively correlated with herbaceous plant communities and negatively correlated with tree canopy cover and wetland soils (Ahrens et al. 2011a).

CB gene flow and dispersal has been demonstrated through controlled experiments and environmental release (Belanger et al. 2003; MacBryde 2006; Watrud et al. 2004). For example, long-distance, pollen-mediated transgene flow and seed dispersal were documented from GRCB fields in Oregon (Reichman et al. 2006; Watrud et al 2004; Zapiola et al. 2008). Since this environmental release, the GR trait has persisted and GR *Agrostis* have become difficult to control along irrigation ditches (Bollman et al. 2012; Charles 2011). However, there is little information about gene flow and dispersal in other climates, plant communities, and complex cultural landscapes (properties representing the combined work of nature and man; United Nations Educational, Scientific, and Cultural Organization [UNESCO] 2010). A theoretical framework for CB gene flow in cultural landscapes in the northeastern United States is shown in Figure 1. The framework expresses the potential for intraspecific and interspecific hybridization between populations, as well as backcrossing in established cultivated and feral populations. In a specific location, the model would be affected by the native and introduced *Agrostis* species present, size and abundance of feral and cultivated populations, human management activities (e.g., mowing, herbicides), distribution of favorable habitats (e.g., roadsides), reproductive features (e.g., time of flowering), factors affecting pollen movement (e.g., wind direction), factors affecting seed and stolon dispersal, and other factors. This theoretical framework could be applied to

DOI: 10.1614/WS-D-12-00041.1

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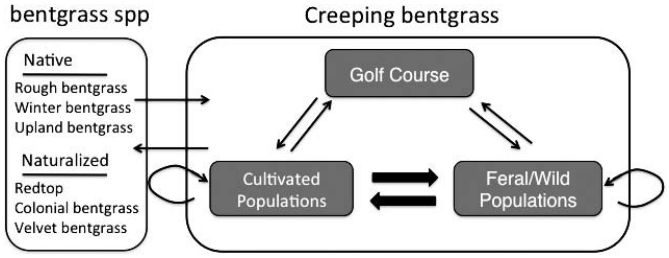

Figure 1. Theoretical framework indicating potential intraspecific and interspecific gene flow for creeping bentgrass (*A. stolonifera*). Bentgrass species listed are typical of plant communities in the northeastern United States including rough bentgrass (*A. scabra* Willd.), winter bentgrass [*A. hyemalis* (Walter) Britton et al.], upland bentgrass [*A. perennans* (Walter) Tuck], redtop (*A. gigantea*), colonial bentgrass (*A. capillaris* L.), and velvet bentgrass (*A. canina* L.). Hybridization between these species has been demonstrated experimentally.

other cool-season turfgrass species that coexist with weedy populations such as Kentucky bluegrass, tall fescue (*Festuca arundinacea* Schreb.), or red fescue (*Festuca rubra* L.) (http://www.invasiveplantatlas.org; http://issg.org; McCarty 2005).

Over the past 15 yr, various tools have been developed to study *Agrostis* genetics including isozymes, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and microsatellites (single sequence repeats [SSR]) (Golembiewski et al. 1997; Kubik et al. 2009; Reichman et al. 2011; Rotter et al. 2010; Scheef et al. 2003; Vergara and Bughrara 2003; Warnke et al. 1997; Yamamoto and Duich 1994; Zapiola 2009; Zhao et al. 2006). Because RFLP and AFLP are difficult to use with polyploid species (loci may be present in more than one genome), SSR markers have been more effective for studying genetic diversity in CB cultivars (Kubik et al. 2009), hybridization events in *Agrostis* and *Polypogon* species (Zapiola 2009), and the evolutionary history of CB (Reichman et al. 2011; Rotter et al. 2010).

In this study, microsatellite molecular markers were used to examine the genetic relationship between cultivated CB on an 8-yr-old golf course and feral CB populations in the receiving environment at distances up to 1,020 m. This study builds upon previous work that examined *Agrostis* distribution and spatial organization of suitable habitat around the same golf course (Ahrens et al. 2011a). The expectation was that feral CB plants close to the golf course would be most similar to cultivars on the golf course, or descendents of hybridization events, while more distant feral populations would consist of individuals from the weedy metapopulation. To our knowledge, this is the first study to look at CB population genetics in a complex cultural landscape.

Methods and Materials

CB plants were collected from a golf course and from five feral populations in Bloomfield, CT, USA. Botanical surveys, aerial maps, habitat suitability models, and GIS coordinates for the study site have been published (Ahrens et al. 2011a). The study site was a highly fragmented landscape containing homes, light industry, recreational land, second growth forest, and various rights-of-way (roads, railroads, and electric power lines). The golf course was built in 2003 and CB was planted in fairways and greens (cultivar not recorded). For this study, CB plants were collected from the following locations: golf course putting green (11 individuals), golf course edge (six

Figure 2. Aerial photograph showing the golf course and five feral populations identified as: (a) Golf Course Edge, (b) Powerline Right-of-way, (c) Reservoir area, (d) Historic Site, and (e) Meadow. More information about the study site can be found in Ahrens et al. (2011).

feral individuals), reservoir recreation area (27 feral individuals), power line cut (12 feral individuals), meadow (10 feral individuals), and historic site (six feral individuals) (Figure 2). Due to the stoloniferous habit of CB, individuals were collected at least 1 m from each other to minimize the chance of taking two samples from the same genet. The total number of collected individuals for each population was determined by the size of the population and the number of CB individuals that could be clearly identified by leaf morphology and flowers. The distance from the golf course edge to each feral population was calculated in ArcGIS (ESRI, Redlands, CA). All collected plants were transferred to the greenhouse, planted in Fafard 3B soil mixture (Agawam, MA), grown in 20 to 30 C (daytime temperature) under natural light, and fertilized every 2 wk with 20–10–10 fertilizer (Lesco Fertilizer, Troy, MI). Observation of leaf and flower morphology in the greenhouse confirmed that the collected plants were *A. stolonifera*. After 4 wk of growth, young leaf tissue was clipped for DNA extraction (four to eight leaves). A small number of individuals from the modern cultivars A4, Crenshaw, Penncross, and Seaside were grown from seed in the greenhouse. A4, Crenshaw, and Penncross seed was obtained from Des Moines Forage & Turf Seed (Ankeny, IA) and the Seaside cultivar was obtained from University of Connecticut Plant Science Farm (Storrs, CT).

Genomic DNA was extracted from leaf tissue (90 to 100 mg) using the DNeasy Mini plant kits (Qiagen, Valencia, CA). The amount of DNA was measured using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). DNA samples were run on a 1.5% agarose gel to confirm integrity and the DNA was stored at −20 C. Polymerase chain reaction (PCR) was conducted with primer pairs for eight microsatellite markers using the protocol developed by Reichman et al. (2011). Forward primers were labeled with FAM 6′ or HEX 5′ fluorescent tags. For fragment analysis, PCR products were diluted 10-fold with nuclease-free water. The diluted PCR

https://doi.org/10.1614/WS-D-12-00041.1 Published online by Cambridge University Press

product and nondiluted PCR product were added to a loading mixture with HiDye formamide (ABI, Applied Biosystems, Carlsbad, CA) and fluorescently tagged molecular weight markers ROX 400HD (ABI), ROX 500 (ABI), and GENEFLO 1000 (ChimerX, Milwaukee, WI). The PCR products, HiDye, and molecular weight markers were combined in an ABI 96 well plate and sent to Cornell University Life Sciences Core Laboratories Center (CLC). CLC conducted fragment analysis using capillary electrophoresis on an ABI 3730xl machine using a four-color dye set. The results were imported to GeneMarker (Softgenetics LLC, State College, PA) to confirm fragment sizes, bin alleles, and analyze data.

Amplified PCR products from all eight SSR primer pairs were used for estimating genetic parameters. The number of amplified fragments, unique amplified fragments, average number of amplified fragments per individual, and polymorphism information content (PIC) were determined. PIC was calculated with the equation: $PIC = 1 - \Sigma f^2_i$, where f is the frequency of the ith fragment. PIC was described by Botstein et al. (1980) to describe how informative the primer markers are based on the frequency of the amplified alleles.

Individuals were conceptually clustered based on their genotype using a Bayesian classification scheme in STRUCTURE 2.0 (Pritchard et al. 2000). STRUCTURE was run 10 times for each K using a burn-in period of 50,000 and a sample of 100,000 in the Markov chain Monte Carlo simulations (Pritchard et al. 2000). The analysis considered numbers of conceptual populations from 1 to 10. Using Structure Harvester (Earl and von Holdt 2011) the data were extracted from Structure and the methods described in Evanno et al. (2005) were employed to choose the optimal K. CLUMPP v1.1.2 (Jakobsson and Rosenberg 2007) was used to make a consensus of genotype clusters of all 10 runs within the optimal K. DISTRUCT v1.1 (Rosenberg 2004) was used to control visualization variables of the CLUMPP output. Principle coordinate analysis was performed using NTSYSpc 2.2 (Exeter Software, Setauket, New York) (Rohlf 2005). For NTSYS, the allele table was transformed to a binary matrix of presence or absence of each allele at all loci for all individuals. Roger's modified distance coefficient was employed to convert the binary data into allele frequencies (Rohlf 2005; Wright 1978) as previously developed for *A. stolonifera* population genetics (Reichman et al. 2011).

Results and Discussion

CB is a valuable turfgrass for golf courses and other sites, but its history as a naturalized weed and the recent escape of transgenic CB raises questions about future patterns of gene flow, dispersal, fitness, selection pressures (e.g., herbicides, salinity), habitat expansion, weediness, and impacts on plant communities. This project used a retrospective approach to characterize genetic relationships between CB on an 8-yr-old golf course and five feral populations at distances of up to 1,020 m. The expectation was that feral CB plants close to the golf course would be most similar to cultivars, while more distant feral populations would consist of individuals from a weedy metapopulation.

Interactions between cultivated and weedy plants have been studied in other species with respect to the implications for environmental impact and coexistence policies (Arnaud et al. 2003; Guadagnuolo et al. 2001; Jenczewski et al. 1999; Leak-Garcia 2009; Sahoo et al. 2010; Schmidt 2011; Wegier et al. 2011). Results from these studies are generally consistent with theoretical models suggesting that recurrent gene flow leads to assimilation of novel alleles (Haygood et al. 2003). Research also suggest that transgenic traits such as GR can persist without ongoing selection pressure from glyphosate (Warwick et al. 2008).

Table 1. Creeping bentgrass accessions and SSR molecular marker performance.[a]

Site	*n*	D	Amplified fragments	Unique fragments	N_a	PIC
Total	86	—	130	41	16.3	0.72
Cultivars (Cult)	14	—	64	15	8.0	0.79
Golf course (GC)	11	—	52	8	6.5	0.82
GC Edge (Edge)	6	2	15	1	1.9	0.65
Reservoir (Res)	27	150	62	13	7.8	0.63
Powerline (PL)	12	280	20	2	2.5	0.71
Meadow (Mead)	9	466	23	2	2.9	0.76
Historic (His)	6	1,020	12	0	1.5	0.49

[a] Abbreviations: *n*, number of individuals collected from the population (sample size); D, distance in meters from the golf course to collected populations; N_a, average number of amplified fragments per locus; PIC, polymorphism information content.

Using genomic DNA from 86 CB accessions, the SSR markers amplified 130 DNA fragments with 41 unique fragments (Table 1). An average of 16.3 fragments were amplified per locus. The high PIC scores indicate that the markers were appropriate for the individuals collected, and the individuals were correctly identified with regard to species. For further validation, a principle coordinates analysis (PCA) was performed with individuals from the golf course and four modern cultivars (data not shown). The four modern cultivars separated from one another, but A4 and Penncross grouped close to each other. This close relationship was expected because A4 is a recent, direct descendent of Penncross (Kubik et al. 2009). PCA revealed that golf course individuals were most similar to A4 and Penncross individuals, indicating that the golf course used A4 or Penncross cultivars for their putting greens and tees. Unfortunately, a loss of Seaside plants in the greenhouse restricted analysis to just two individuals and they were each assigned to a different genetic cluster.

Individuals from the golf course, modern cultivars, and the weedy Reservoir population produced the highest average number of amplified fragments per locus (N_a) (Table 1). Our results showed 20% private alleles in the cultivar and golf course individuals, a result in close agreement with the 19% reported by Reichman et al. (2011). In contrast, individuals collected from four weedy populations had fewer unique alleles (zero to two) and a low N_a (1.5 to 2.9) (Table 1). Thus, these four feral populations had low genetic diversity compared to the golf course, the modern cultivars, and one feral population (Reservoir). These results contrasted with a previous study in Oregon showing higher allelic richness and percent private alleles in feral CB populations compared to CB cultivars (Reichman et al. 2011). These conflicting results might be explained by the difference in spatial scales at which the feral populations were collected, the history of CB introduction, patterns of escape and dispersal over time, and climatic differences between the northeastern and northwestern United States.

AMOVA results indicated that there was more molecular variation in individuals within populations (86%) than

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Table 2. Analysis of molecular variance (AMOVA). A permutation test gave an F_{ST} of 0.136 showing that variation among populations was significant.

Source of variation	df	Sum of squares	Variance components	% Variation
Among populations	6	91.3	0.812	14
Within population	79	424.2	5.369	86
Total	85	515.5	6.37	100

variation among populations (14%) (Table 2). The permutation test in Arlequin gave an F_{ST} (0.136) significantly different than the null distribution ($P < 0.01$) indicating that there were significant differences among populations. The results of the permutation test were similar to Reichman et al. (2011) where most variation occurred within CB populations.

STRUCTURE 2.0 software was used to perform analysis on 10 cluster scenarios (k = 1 to 10). Using the method described by Evanno et al. (2005), the optimal number of genetic clusters determined to be k = 5 with a delta K of 5.83 (Figure 3). The LnPD of the optimal cluster grouping (k = 5) ranged from −1514 to −1543. Using a Bayesian inference approach, the CB accessions were grouped into two major genetic clusters: (1) CB cultivars and individuals collected from the golf course (hereafter called the cultivar genotype), and (2) the majority of individuals collected from feral populations (hereafter called the feral genotype) (Figure 3). These results was similar to the study in Oregon where three wild CB populations were shown to be genetically distinct from three modern cultivars (Reichman et al. 2011). To our knowledge, no other studies have been done to compare modern turfgrass cultivars (e.g., Kentucky bluegrass, fescues) with their conspecific feral populations. However, a study of invasive pear trees (*Pyrus calleryana* Decne.) showed a distinction between the genetics of invasive and cultivated trees growing in proximity despite the relatively recent introduction of this nonnative pear in the 1960s (Culley and Hardiman 2009).

Four feral populations (Golf Course Edge, Powerline, Historic, Meadow) showed a largely homogeneous genetic structure carrying the feral genotype (Figure 3). In contrast, the Reservoir population showed a heterogeneous structure with individual assignment to the feral genotype, cultivar genotype, and other unspecified genotypic clusters. PCA was performed on the Reservoir plants plus the modern cultivars and the golf course individuals (Figure 4). The Reservoir population fragmented into three distinct regions in the ordination plot; the green genotypic assignment in Figure 3 clustered on the left side of the ordination plot; red, pink, and yellow genetic assignments in Figure 3 were clustered at the bottom and middle sections. Of special interest was the individual that showed contributions from the feral and cultivar genotypes (see arrow in Figure 4); it clustered in the upper right with the modern cultivars and golf course plants. The PCA analysis strengthens the identification of a putative crop–weed hybrid in the Reservoir site (150 m from the golf course).

Bayesian inference (k = 5) showed that two feral individuals (3.3% of all feral accessions) were partially assigned to the cultivar genotype (Figure 3). These feral plants were located approximately 150 m and 280 m away from the edge of the golf course and could represent crop–weed hybridization events. However, it is impossible to strictly assign the partial cultivar cluster in these putative hybrids to

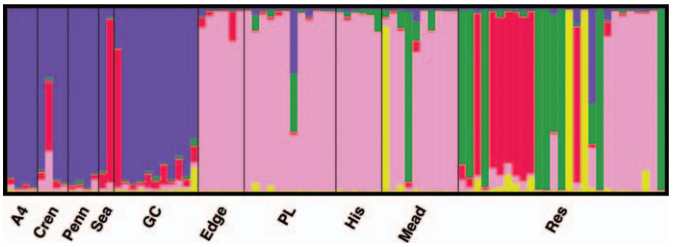

Figure 3. Assignment of individual genotypes using consensus of Bayesian inference through STRUCTURE 2.0 software for k = 5. Modern cultivars are labeled as: A4; Crenshaw (Cren); Penncross (Penn); Seaside (Sea). Populations collected in the study site are labeled as: Golf course (GC); Golf course edge (Edge); Powerline right-of-way (PL); Historic site (His); Meadow (Mead); Reservoir area (Res).

the golf course source population because cultivars could exist at unidentified sites in the cultural landscape, and viable CB pollen can travel long distances (Watrud et al. 2004). Many factors affect gene flow and the success of hybrid progeny in forming new populations (Chandler and Dunwell 2009; Ellstrand 2003). CB gene flow and propagule dispersal from this golf course has not been studied, but it might be relatively low because frequent mowing removes inflorescences. Unlike Oregon where rivers and irrigation ditches have distributed propagules over long distances, the movement of CB stolons is probably a rare event in this cultural landscape (Charles 2011; Zapiola et al. 2008). A second factor affecting population genetics could be that modern cultivars and crop–weed hybrids are less fit in unmanaged or minimally managed habitats compared to CB carrying the feral genotype. A 3-yr field experiment that introduced the A4 cultivar to established agricultural or natural grasslands showed that competition from established grassland vegetation greatly reduced the survival of young CB plants (Ahrens and Auer, unpublished data). Thus, while our previous study at this golf course suggested that 34% of the surrounding cultural landscape was highly suitable habitat for CB, the existing vegetation may be competing successfully against the introduction of CB cultivars and crop–weed hybrids (Ahrens et al. 2011a). In addition, the suitable habitat may already be colonized by the feral CB genotype that is adapted to local environmental conditions. Another relevant factor might be that hybrid crop–weed offspring experience outbreeding depression with substantial changes in fitness as demonstrated in *Ipomopsis aggregata* (Pursh) V. E. Grant (Ellstrand 1992; Waser et al. 2000). Also, if CB cultivars and their offspring become established in feral populations at very low frequency, backcrossing and/or the increased fitness of the feral genotype could lead to genetic drift and loss of cultivar alleles.

Microsatellite analysis showed that four weedy populations (Powerline Right-of-way, Golf Course Edge, Historic Site, and Meadow) were genetically homogenous (pink color; Figure 3) and distinct from modern cultivars. Future work should determine if these feral populations are part of a larger metapopulation of weedy CB that has naturalized over the past 250 yr in the northeastern United States. In contrast, the Reservoir population with 27 individuals tested showed a heterogenic pattern in the STRUCTURE and NTSYS outputs (Figures 3 and 4). Seven individuals clustered with the feral metapopulation, while the remaining 20 individuals showed some similarity to the cultivar genotype and other unspecified clusters. Land use history and vegetation patterns could also provide some explanation for the observed

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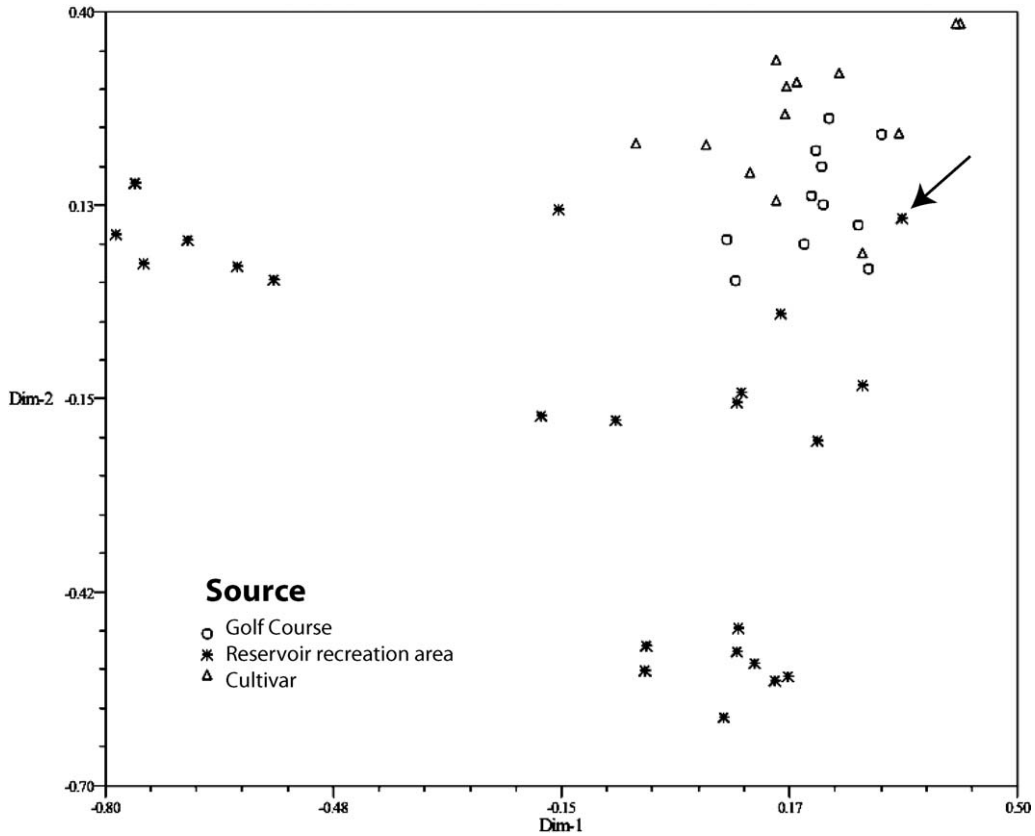

Figure 4. Principle coordinate analysis of feral CB from the Reservoir area compared with accessions from the golf course and modern cultivars. Arrow indicates the feral individual partially assigned to the cultivar genotype.

heterogeneity (Figure 2). The site was cleared in 1967 for the construction of the reservoir and planted with a seed mix including redtop (*Agrostis gigantea* Roth), Kentucky bluegrass, and creeping red fescue (Dave Malesko, personal communication). Today, redtop is one of the dominant species in this minimally managed grassland, perhaps due to its tolerance of wet soils and summer flooding (CWA, personal observation). The site is open on both the western and southern edges, allowing the prevailing winds (from the south and southwest in June) to potentially carry CB pollen or seeds to the field (http://www.utk.edu, accessed December 2011). The relationship between one individual from the Seaside cultivar and eight individuals from the weedy Reservoir population was difficult to interpret, but we cannot rule out the possibility that these individuals may be the result of previous plantings (Figure 3). Overall, it seems likely that opportunities for CB gene flow and various anthropogenic forces may have contributed to the genetic variation observed in the Reservoir population.

The Reservoir population indicates that genetically complex CB populations exist in cultural landscapes and that cultivated genotypes can coexist with weedy populations. Thus, this project and others provide evidence that CB with novel traits and transgenes could become part of the theoretical framework of gene flow (Figure 1), plant dispersal patterns, and established populations in cultural landscapes over time. While recognizing the limitations inherent in a single study of CB population genetics, our results add to the relevant knowledge for predictive ecological risk assessments, coexistence policies, containment guidelines, and land management strategies.

Acknowledgments

The authors gratefully acknowledge the assistance of Kathryn Theiss (University of Connecticut) in data analysis and Lidia Watrud (Western Ecology Division, U.S. Environmental Protection Agency) for providing access to microsatellite markers prior to publication. This project was supported by the Biotechnology Risk Assessment Program Competitive Grant no. 2008-39211-19566 from the USDA National Institute of Food and Agriculture to Carol Auer and Thomas Meyer. Funding was also provided by the Storrs Agricultural Experiment Station and the University of Connecticut.

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Received March 22, 2012, and approved May 29, 2012.