Title

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Journal

Crop Science, 60(5)

ISSN

0011-183X

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Publication Date

2020-09-01

DOI

10.1002/csc2.20231

Peer reviewed

Received: 21 February 2020 | Accepted: 19 May 2020

DOI: 10.1002/csc2.20231

Crop Science

ORIGINAL RESEARCH ARTICLE

Turfgrass Science

Kikuyugrass germplasm collections in the United States and Australia show low levels of genetic diversity as revealed by DArTseq genotyping

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Assigned to Associate Editor Bradley Bushman.

Funding information
California Turfgrass & Landscape Foundation (CTLF); USGA; Western Municipal Water District (WMWD); Metropolitan Water District (MWD)

Abstract

Kikuyugrass [*Cenchrus clandestinus* (Hochst. ex Chiov.) Morrone (= *Pennisetum clandestinum* Hochst. ex Chiov.)] is a warm-season grass native to Africa. It was introduced into the United States as forage in Hawaii and for erosion control in California. Kikuyugrass is considered invasive and currently is on the USDA's noxious weed list. Since complete eradication is difficult, it has become the primary species on several golf courses, athletic fields, and lawns. Kikuyugrass possesses exceptional quality with considerable cultural inputs, and little or no winter dormancy compared with other warm-season turfgrasses. With breeding efforts directed specifically at reducing aggressiveness and improving texture, thus reducing inputs, it could become a valuable turf-type species in coastal and inland California. The genetic diversity of kikuyugrass was investigated using single nucleotide polymorphism (SNP) and silicoDArT (presence or absence) markers revealed by the Diversity Arrays Technology sequencing (DArTseq) platform. Accessions were sampled throughout California, Hawaii, and Australia, both from natural stands and various collections. Among the 254 accessions tested, two distinct groups were discovered, and there was no geographic pattern to this differentiation. The overall level of SNP polymorphism was low (polymorphic information content [PIC] average = .33, PIC median = .38). Most (76%) of the observed genetic variation was within populations, whereas 24% was among populations. Average genetic distances within populations ranged from 0.09 to 0.16, whereas distances among populations ranged from 0.13 to 0.36. Accessions from Hawaii and Australia were the most diverse; however, a detectable level of genetic diversity of kikuyugrass also exists in California, mostly because of the past introductions from Australia.

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; CC, country club; DAF, DNA-amplified fingerprint; DArT, Diversity Arrays Technology; MRS, Mealani Research Station; PBI, Plant Breeding Institute; PCoA, principal coordinates analysis; PIC, polymorphic information content; RAPD, randomly amplified polymorphic DNA; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; UCR, University of California, Riverside.

1 | INTRODUCTION

Kikuyugrass [*Cenchrus clandestinus* (Hochst. ex Chiov.) Morrone (= *Pennisetum clandestinum* Hochst. ex Chiov.)] is a warm-season grass species native to the east and central African Highlands. The species is tetraploid ($2n = 4x = 36$), with meiotic behavior indicating allopolyploidy (Hanna et al., 2004; Sujatha, Manga, Rao, & Murty, 1989). It has been introduced in different countries of tropical and subtropical zones and is a valuable forage grass in Australia, New Zealand, and Hawaii. It was introduced into California in the early 20th century for soil erosion control (Mears, 1970; Morris, 2009). Due to its aggressiveness, kikuyugrass colonized various areas throughout coastal and inland California, and it has been placed on the federal noxious weed list (USDA, 2012). Among those areas, it invaded several golf courses and has become the dominant species on fairways and in roughs. When intensively managed with mowing, cultivation practices, and growth regulators, kikuyugrass can provide an exceptional playing surface with lower water requirements than cool-season grasses, and it does not go fully dormant during most winters in southern California. Increasing interest in this species creates a need for new cultivars to better meet the needs of turf managers in California.

Genetic variation is vital for any progress in breeding. Kikuyugrass is predominantly self-pollinating species, and given a low number of the introduction events in California, low genetic diversity is likely (Ingvarsson, 2002; Wright, Kalisz, Slotte, & Wright, 2013). This species produces seeds independent of the growing conditions, and sexual reproduction probably plays a significant role in colonization of new areas. However, once established, populations appear to be maintained mostly clonally (Wilen, Holt, Ellstrand, & Shaw, 1995).

To the best of our knowledge, only one genetic variation study was ever conducted on kikuyugrass in California (Wilen et al., 1995). Using isozymes, very little genetic diversity was observed among subpopulations from three different golf courses representing northern, central, and southern California. A study in Australia used DNA amplified fingerprinting (DAF) and 40 polymorphic loci to identify two main groups among 13 accessions tested (Holton, Skabo, Lowe, & Sinclair, 2007). Another Australian study (Morris, 2009) using 195 randomly amplified polymorphic DNA (RAPD) markers found three main groups among 19 genotypes tested and concluded that significant variation did exist within Australian genotypes. Molecular characterization of germplasm collections is important in understanding the variability of genetic resources. Compared with major crops, genomic and marker information amount for turf species is relatively low. Initially, various types of markers, including protein-based isozyme markers were used, but currently DNA-based markers are widely used in turf species (Baird et al., 2012; Harris-Shultz & Jespersen, 2018). Amplified fragment length polymorphisms (AFLP) have been used as early DNA-based molecular markers in the warm-season grasses—seashore paspalum (*Paspalum vaginatum* Swartz), bermudagrass [*Cynodon dactylon* (L.) Pers.], and zoysiagrass [*Zoysia japonica* Steud, *Zoysia matrella* (L.) Merr., and *Zoysia tenuifolia* Willd. ex Trin.]—to evaluate the genetic diversity within the species (Chen, Wang, Waltz, & Raymer, 2009). Amplified fragment length polymorphism markers were also used to investigate genetic variability among breeder stock and sodded varieties of St. Augustine grass [*Stenotaphrum secundatum* (Walt.) Kuntze] (Kimball, Zuleta, Kenworthy, Lehman, & Milla-Lewis, 2012). Nowadays, sequence specific DNA markers, including simple sequence repeat (SSR) markers and single nucleotide polymorphisms (SNPs), have been widely used. Simple sequence repeat markers have been used in seashore paspalum, zoysiagrass, bahiagrass (*Paspalum notatum* Flueggé), bermudagrass, centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.], St. Augustine grass, and buffalograss [*Buchloë dactyloides* (Nutt.) Engelm]. (Guo, Wu, Anderson, Moss, & Zhu, 2015; Harris-Shultz & Jespersen, 2018; Mulkey, Zuleta, Keebler, Schaff, & Milla-Lewis, 2014; Tan et al., 2014; Wang et al., 2010). Single nucleotide polymorphisms were used to create high-density genetic maps of St. Augustine grass (Yu, Kimball, & Milla-Lewis, 2018). In zoysiagrass, SSR and restriction site associated DNA (RAD) markers are replacing restriction fragment length polymorphism (RFLP) and AFLP markers (Chandra, Milla-Lewis, & Yu, 2017). Isozyme analysis, DAF, and RAPD markers have all been used to determine genetic diversity in kikuyugrass (Holton et al., 2007; Morris, 2009; Wilen et al., 1995).

This study was designed to determine if sufficient genetic variation exists within kikuyugrass in California, including the collection maintained at University of California, Riverside (UCR), to warrant a breeding effort toward new turf-type cultivars. Such breeding efforts must rely on genetic variation available within the state, as the existing prohibition of importation precludes widening the gene pool by standard means. The within-state variation was compared with materials present in collections in Hawaii and Australia. This was accomplished through the generation of silicoDArT and SNP markers via the Diversity Array Technology sequencing (DArTseq) platform. When compared with other molecular marker systems, DArTseq is a relatively new platform. This next-generation sequencing (NGS) method combined with the existing DArT marker platform produced a rapid SNP discovery method (Courtois et al., 2013; Cruz, Kilian,

& Dierig, 2013; Kilian et al., 2012; Raman et al., 2014; Sansaloni et al., 2011). The DArTseq platform represents a new execution of complexity-reduced representations (Altshuler et al., 2000). A principal use for the DArTseq system is its ability for SNP discovery with no prior DNA sequence information. The system is capable of generating thousands of markers for genetic diversity analyses as compared with the hundreds developed with earlier technologies and performs well in polyploid species, thanks to eliminating primer competition from the assay (Kilian et al., 2012). It has already been used to successfully assess genetic diversity in various mono- and dicotyledonous species including rye (*Secale cereale* L.), tetraploid wheat (*Triticum turgidum* L.), tall fescue (*Festuca arundinacea* Schreb.), pigeonpea [*Cajanus cajan* (L.) Millsp], and lesquerella (*Physaria* spp.) (Baird et al., 2012; Cruz et al., 2013; Laido et al., 2013; Targonska-Karasek, Bolibok-Bragoszewska, & Rakoczy-Trojanowska, 2017; White et al., 2008; Yang et al., 2006).

2 | MATERIALS AND METHODS

2.1 | Plant material

Samples from California originated from two sources: collection of accessions originally assembled at UCR by Cockerham, Cudney, Gibeault, Holt, and Shaw (1992) in the early 1990s (16 samples), and samples collected for this study throughout California (86 samples) in 2013. Collection at UCR was a result of intensive selection of seedlings obtained from various sources in California and probably Australia. Among the 86 samples collected in the state for this study, 57 were from golf courses, and the remainder were from parks, beaches, cemeteries, and residential areas, ranging from San Diego in the south to the San Francisco Bay area in the north (Figure 1).

A total of 60 samples were collected from Hawaii. Of these, 48 came from the collection originally created by Dr. Ukio Urata at the University of Hawaii, Agricultural Experiment Station (Fukumoto & Lee, 2003). The original site of the collection remains at the Mealani Research Station (MRS), but individual plots were difficult to recognize. Leaf tissue samples were taken from individual plants in an attempt to identify and sample the original plots based on field location and the original plot map. The remaining 12 samples were collected along roadsides, in residential areas, on a golf course, and in a pasture on the island of Hawaii.

The 90 samples from Australia were selected from the collection at the University of Sydney's Plant Breeding Institute (PBI) at Cobbitty, near Camden, NSW. The collection includes material from an abandoned turf farm at Cobbitty and from the surrounding Camden district, as well as lines from the northern, central and southern areas of New South Wales, Queensland, Victoria, Tasmania, South Australia, and Western Australia. Accessions sampled for DNA included vegetatively propagated lines from naturalized and cultivated populations, seedling selections from commercial cultivars, lines derived from cv. Whittet by chemical mutagenesis, and a number of hybrids from the PBI crossing program.

FIGURE 1 Kikuyugrass collection sites in California

2.2 | Sample collection

Samples in California were collected between June and November 2013. A total of five samples were taken from each kikuyugrass accession in the S.T. Cockerham collection (UCR), two each from the first and second replicate, and one from the third replicate. All samples from golf courses and other locations throughout the state were collected in the stolon or single stem form so that only one genotype was present in each sample. All samples were planted in the greenhouse. All 48 samples from MRS and through the island of Hawaii were collected in 2014. All were dried in situ for shipment to California; no live tissue was imported. Samples from University of Sydney's PBI were placed in plastic bags with 7-g desiccation packets and sent to Diversity Arrays Technology, Australia. The list of samples is provided in Supplemental Table S1.

2.3 | Tissue harvest and DNA analysis

For samples from California and Hawaii, ~100 mg of fresh leaf tissue was collected from each accession, dried over silica gel under vacuum for 3 d, and pulverized with steel pellets and sand in a FastPrep-24 instrument (MP Biomedicals). DNA extraction was performed according to the protocol listed at the Diversity Array Technology website (http://www.diversityarrays.com). DNA extraction from Australian samples was done by DArT using their internal protocols.

DNA was quantified by a NanoDrop 2000c spectrophotometer (Thermo Scientific), and its quality was evaluated on 0.8% agarose gels via electrophoresis. Samples of acceptable quality were diluted to 50–100 ng μl^{-1}, loaded onto three 96-well microtiter plates, and sent to Diversity Arrays Technology, Bruce, ACT, Australia.

All genotyping was done by Diversity Arrays Technology using the DArTseq platform (Kilian et al., 2012). After producing various quality control statistics, the sequences were aligned against the reference created from the tags identified in the sequence reads generated from all samples. In addition, the short sequence tags were aligned against *Zea mays* L., *Setaria italica* (L.) P. Beauv. (foxtail millet), and *Sorghum bicolor* (L.) Moench., all of which are in the same grass subfamily Panicoideae as Pennisetum (Giussani, Cota-Sánchez, Zuloaga, & Kellogg, 2001). The output files from the alignment generated using the Bowtie software were processed using an analytical pipeline developed by DArT (Langmead, Trapnell, Pop, & Salzberg, 2009). DArTseq produces two types of data: SNP markers (nucleotide polymorphisms in restriction fragments) and silicoDArT scores of 0 or 1, which indicate the presence or absence of a restriction fragment.

The silicoDArT 0/1 scores generated by DArTsoft were used to construct the dendrogram and calculate average genetic distances. They were used as input for the RESTDIST and NEIGHBOR programs (PHYLIP 3.6 software package), and a dendrogram was constructed based on the unweighted pair group method with algorithmic mean and Felsenstein's modification of the Nei–Li restriction fragment distance (Felsenstein, 2005). The average genetic distances, which reflect the genetic diversity within the populations and between populations, were calculated in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) based on the distance matrix produced by RESTDIST. The number of clusters in a population was verified using AWclust, which calculates the gap statistic using SNP markers. Both SNP and silicoDArT data were used in GenAlEx version 6.5 (Peakall & Smouse, 2006) to perform the principal coordinates analysis (PCoA) and the analysis of molecular variance (AMOVA). The AMOVA was used to calculate PhiPT, which is a measure of population genetic differentiation. Markers for the analyses were selected with reproducibility = 1, call rate > .95, one ratio > 0.05, and polymorphic information content (PIC) > .4 for silicoDArT-generated 0–1 scores, and reproducibility = 1, call rate > .75, one ratio > 0.05, and PIC > .4 for SNP markers.

3 | RESULTS

3.1 | SilicoDArT and SNP marker quality parameters

The average scoring reproducibility and the call rate of 102,294 identified silicoDArT markers was 99.89 and 93.76%, respectively. To select informative markers, reproducibility at 100% and the call rate at the 95% threshold were applied, and markers with one ratio ≤0.05 were removed. Markers with such low frequency can affect statistical analysis (Tabangin, Woo, & Martin, 2009). Selected markers showed the PIC range .01–.50, with an average of 0.34 and a PIC median of .37. Of the informative markers, 44.27% showed a PIC value >.40. The PIC of 1.78% of markers was <.1, 16.07% of markers ranged between 0.1 and 0.2, 19.91% of markers ranged between 0.2 and 0.3, and 17.97% of markers ranged between 0.3 and 0.4. For further analysis, 1,964 silicoDArT markers were selected that cleared quality parameters.

A total of 19,159 SNP markers were detected with average scoring reproducibility of 98.91% and call rate of 94.33%. Only SNP markers showing 100% scoring reproducibility were included in the analysis. A call rate >75% showed 8,232 SNP markers. Minor frequency markers were removed with one ratio threshold ≤0.05. The PIC range was .00–.50, and the average and median of selected informative markers were 0.33 and 0.38, respectively. Of informative markers, 47.02% showed PIC >.40. PIC of 11.29% of the SNP markers was <0.1, 13.94% of markers ranged between 0.1 and 0.2, 12.74% of markers ranged between 0.2 and 0.3, and 15.01% of markers ranged between 0.3 and 0.4. In total, 2,149 highly polymorphic SNP markers were selected for further analysis.

3.2 | Genetic diversity

The most probable number of clusters was estimated using the gap statistics. The $\log(W_K)$ estimation and gap curve are plotted in Figure 2. The gap curve is plotted in the format of gap (K [optimal number of clusters]) ± standard deviation of $\log(W_K)$. The optimal K is the elbow point in the observed $\log(W_K$ [within-cluster dispersion]) plot, which corresponds to the maximizing point in the gap

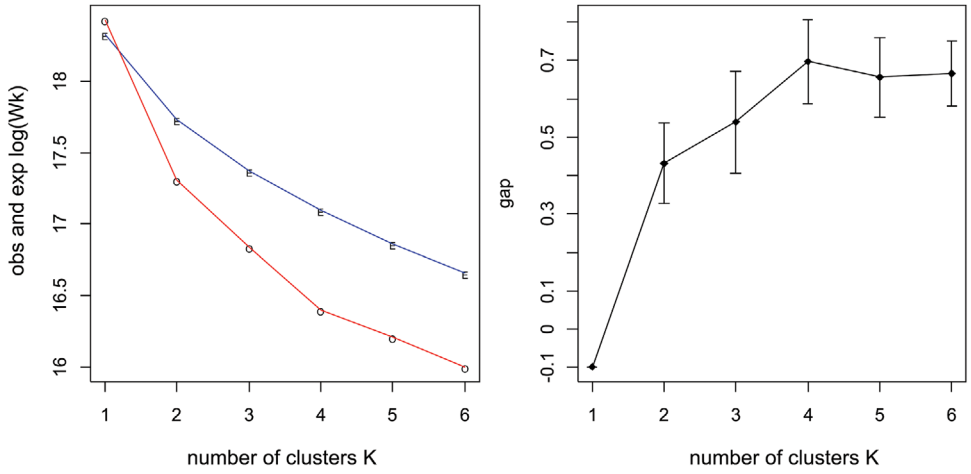

FIGURE 2 Number of clusters of kikuyugrass accessions (K) estimated via the gap statistic. In the left panel, the blue and red curves marked by "E" and "O" are estimated expectations of log(W_k [within-cluster dispersion]) and the observed W_k, respectively. The right panel is the gap statistic plot. The number of clusters is set to range from 1 to 6. The inferred optimal K is the elbow point in the left panel, which is indicated by the maximizing gap on the right panel. The gap curved is plotted in the format of gap (K) ± standard deviation of log(W_k). The gap statistic gives the optimal number of clusters at 2

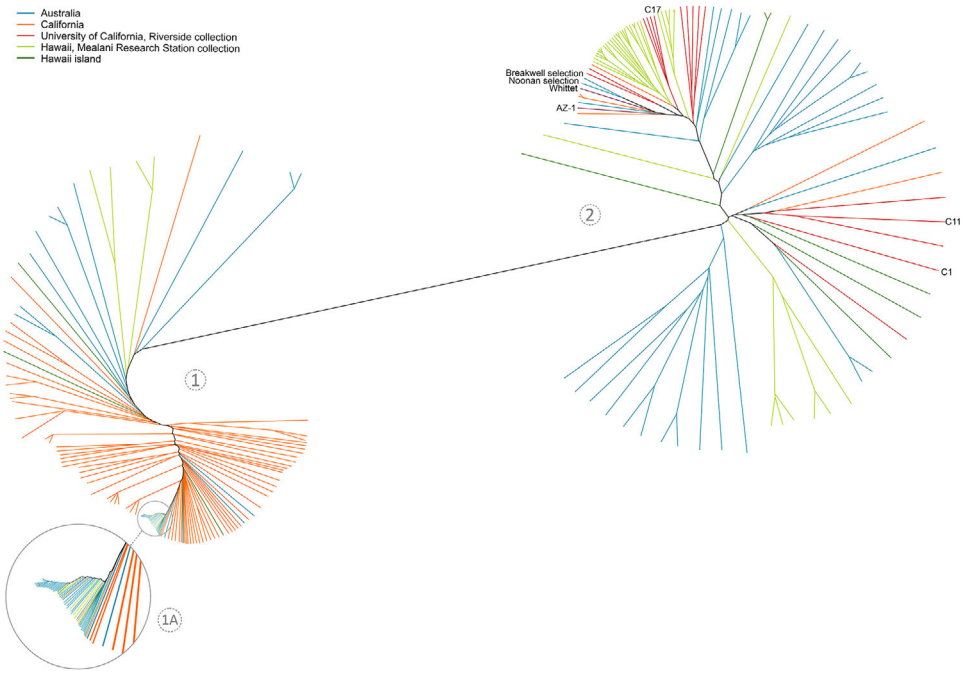

FIGURE 3 Unweighted pair group method with algorithmic mean dendrogram (radial tree) representing 254 kikuyugrass accessions from Australia (90 accessions), California (86 accessions), the University of California, Riverside, collection (16 accessions), the Mealani Research Station, University of Hawaii collection (48 accessions),the island of Hawaii (12 accessions), and cultivars Whittet and AZ-1

curve. From the plots, the estimated optimal K is 2. Multiple runs produced similar results. These tests and analyses consistently differentiated two groups of accessions, based on the level of genetic differentiation.

Filtered 1,964 silicoDArT markers were used to generate a dendrogram of the accessions under study (Figure 3). The analyzed accessions did not aggregate (group) according to their geographic origin. Both major clusters include accessions from all three geographic origins, but in somewhat different proportions. Cluster 1 contains a group of 53 very closely related accessions (Figure 3; Group 1A), including most of the samples collected from a single field at the PBI Lansdowne Farm at Cobbitty. This group also contains two accessions collected in California, seven accessions from

the MRS collection, and two accessions collected around the Island of Hawaii. Cluster 2 did not contain such closely related accessions.

3.3 | California: University of California, Riverside, collection and in-state diversity

All of the 16 UCR collection accessions grouped in Cluster 2 and showed little genetic diversity (Figure 3, red lines). Accessions C1, C11, and C17 were originally collected in California to act as controls when rating UCR accessions. Ten UCR selections and C17 grouped together. C1, C11, and three remaining UCR selections grouped in a separate subcluster.

Out of the 86 samples collected in California, 80 grouped in Cluster 1 (Figure 3, orange lines). The remaining six samples present in Cluster 2 were all collected in southern California. Diversity within the same location in California was low. In case of seven locations (Seven Oaks Country Club [CC], Bakersfield; Belmont CC, Fresno; Rolling Hills Memorial Park, Richmond; Tilden Park Golf Course, Berkeley; Canyon Crest CC, Riverside; Los Angeles National Cemetery, Los Angeles; and Avalon, Catalina Island), all of the collected samples grouped closely together. Accessions from only five locations (Riviera CC, Pacific Palisades; Bel Air CC, Los Angeles; Black Hills CC, Yorba Linda; Mesa Verde CC, Costa Mesa; and Mission Viejo CC, Mission Viejo) were present in both clusters. Three samples from these locations grouped closely to Whittet and AZ-1, a cultivar selected from Whittet.

3.4 | Hawaii: The Mealani Research Station collection and in-state diversity

Accessions collected from the MRS in Kamuela (Figure 3, bright green lines) and from other locations on the Island of Hawaii (Figure 3, dark green lines) were present in both clusters, however only 16 out of 60 were present in Cluster 1. Nine of those were very closely related. Other Hawaiian accessions were present in two subclusters of Cluster 2. Thirty lines from the MRS collection grouped with the UCR collection lines, and another six formed their own subcluster.

3.5 | Australia: Plant Breeding Institute collection

Australian lines were scattered throughout both clusters (Figure 3, blue lines). Cluster 1 contained 58 accessions, and Cluster 2 contained 32 accessions, including 18 hybrids created at PBI. Most of the accessions collected from a single abandoned turf field adjoining PBI's Lansdowne Farm at Cobbitty formed a closely related group within Cluster 1 (Figure 3, Group 1A). With one exception, all samples collected through the Camden district were also present in Cluster 1, mostly clustering with PBI accessions. Within this group of closely related accessions, there were lines considered, on circumstantial evidence, to represent the original introduction material (1918 and 1920) or to be closely related to it. The collection also contains samples from other parts of the country: various regions of New South Wales, Queensland, Victoria, South and Western Australia, and Tasmania. Accessions originating from New South Wales were present in both clusters. Lines from Northern New South Wales were present in both clusters, two of them grouped not very closely in Cluster 2, and one was present in Cluster 1. Most of central New South Wales accessions were present in a closely related Group 1A of Cluster 1, and only two samples from this area were present in Cluster 2. All samples collected in southern New South Wales clustered in Cluster 1. Out of two samples from Queensland, one located in Cluster 1, and the other located in Cluster 2, close to Whittet. Samples from Victoria and Tasmania clustered with PBI and Camden district accessions in Cluster 1. A sample from Western Australia was found in Cluster 2. One of the two samples from South Australia grouped in Cluster 1, the other in Cluster 2. Among the three selections obtained via chemical mutagenesis of Whittet, two grouped together with Whittet in Cluster 2; however, only one of them was close to Whittet. The third mutagen of Whittet was present in Cluster 1. Selections of Whittet, Breakwell, and Noonan grouped together with Whittet in Cluster 2.

3.6 | Analysis of molecular variance

AMOVA of kikuyugrass populations of different geographical origin based both on silicoDArT and SNP data showed that 76% genetic variation was observed within populations, whereas 24% was observed among populations (PhiPT for silicoDArT = 0.237, PhiPT for SNP = 0.241 at $P < .001$). Pairwise PhiPT (analogue of fixation index used for assessing genetic differentiation) between populations of different origin for silicoDArT markers ranged from 0.038 between accessions from Australia and the island of Hawaii to 0.484 between accessions from California and the UCR collection (Table 1). Pairwise PhiPT values based on SNP markers ranged from 0.045 between accessions from Australia and the island of Hawaii to 0.437 between accessions from California and the UCR collection (Table 2).

TABLE 1 Pairwise PhiPT values (a measure of population genetic differentiation, bottom part) and their significance (upper part of the table) for silicoDArT markers from kikuyugrass populations

Population	Australia	California	Hawaii MRS[a] collection	Island of Hawaii	California UCR[b] collection
Australia		0.001	0.001	0.078	0.001
California	0.071		0.001	0.001	0.001
Hawaii MRS collection	0.274	0.379		0.001	0.017
Island of Hawaii	0.038	0.171	0.185		0.001
California UCR collection	0.346	0.484	0.060	0.242	

[a]MRS, Mealani Research Station. [b]UCR, University of California, Riverside.

TABLE 2 Pairwise PhiPT values (a measure of population genetic differentiation, bottom part) and their significance (upper part of the table) for single nucleotide polymorphism (SNP) markers from kikuyugrass populations

Population	Australia	California	Hawaii MRS[a] collection	Island of Hawaii	California UCR[b] collection
Australia		0.001	0.001	0.069	0.001
California	0.128		0.001	0.001	0.001
Hawaii MRS collection	0.268	0.353		0.002	0.004
Island of Hawaii	0.045	0.235	0.210		0.001
California UCR collection	0.317	0.437	0.071	0.218	

[a]MRS, Mealani Research Station. [b]UCR, University of California, Riverside.

3.7 | Principal coordinates analysis

The PCoA shows genetic divergence among the accessions. The first two axes together explain 43.47 and 43.48% of variance for silicoDArT and SNP data, respectively (Figures 4 and 5). The PCoA graphs generated using silicoDArT and SNP markers were similar and consistent with the dendrogram. Most of the samples from California were located in the left quadrants, whereas all UCR collection accessions were located in the right quadrants. Australian samples were scattered over the entire graph area. Similarly to the dendrogram grouping, PCoA shows a group of highly related accessions, consisting mostly of Australian samples collected from a single field at the PBI in Cobbity, but also several samples from Hawaii (MRS and the island of Hawaii) and California.

3.8 | Average genetic distance

Average genetic distances within populations ranged from 0.09 (UCR) to 0.16 (MRS). The most distant were populations of accessions collected in California and from the MRS collection (0.36), whereas populations of UCR and MRS collections turned out to be the closest (0.13, Table 3).

4 | DISCUSSION

The DArTseq array proved its robustness in generating large number of markers (102,294 of silicoDArT and of 19,159 SNPs). Both types of markers showed comparable quality to other species where DArT was applied. The PIC average and median of silicoDArT and SNP were .34 and .37, respectively. This was higher than for rye (silicoDArT mean = 0.22, SNP mean = 0.37) and macadamia (*Macadamia integrifolia* Maiden & Betche, *Macadamia tetraphylla* L. Johnson; silicoDArT average = 0.29, SNP average = 0.21), but similar to that of pigeonpea (silicoDArT average = 0.34) and strawberry (*Fragaria* × *ananassa* Duch.; silicoDArT average = 0.30) (Alam, Neal, O'Connor, Kilian, & Topp, 2018; Sanchez-Sevilla et al., 2015; Targonska-Karasek et al., 2017). SilicoDArT in this study produced only 1.78% of markers with PIC < .1, whereas SNP produced 11.29%. Within the PIC range of .4–.5 was 44.27% of silico DArT and 47.02% of SNP markers; however, this percentage of highly polymorphic silicoDArT markers was obtained with more stringent criteria of call rate filtering (95% for silicoDArT and 75% for SNP). Therefore, silicoDArT markers were selected as more reliable. Based on that, silicoDArT markers were selected to analyze genetic distances between individuals and create a dendrogram. SilicoDArT markers are dominant; therefore, this method was chosen for its suitability for this kind of markers. The RESTDIST program assumes nucleotide substitution model for restriction sites and because of that is considered appropriate for dominant markers (Grünwald, Everhart, Knaus, & Kamvar, 2017). GenAlEx, unlike RESTDIST, supports analysis of both dominant (silicoDArT) and codominant (SNP) markers and was used to perform PCoA analysis with both type of markers,

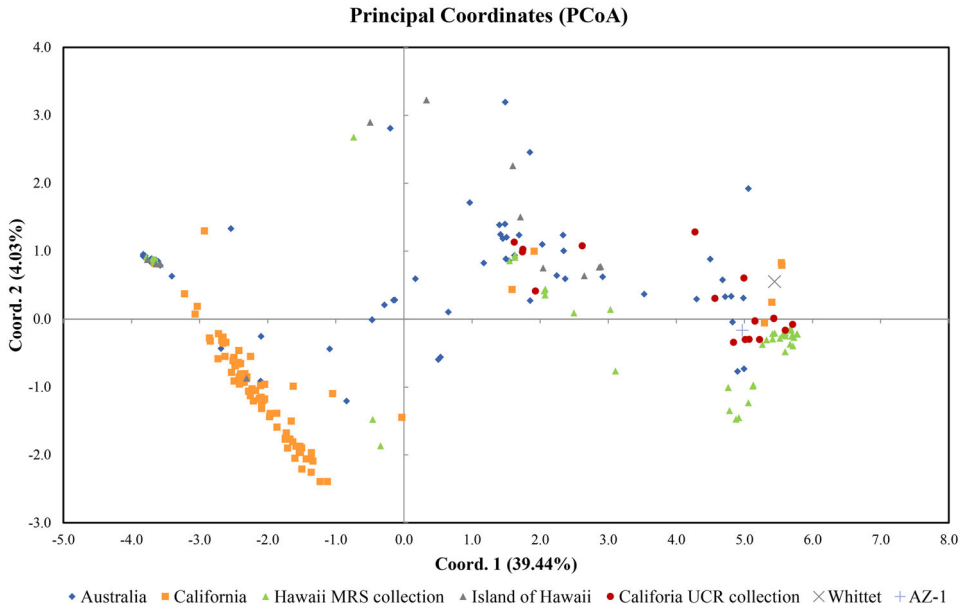

FIGURE 4 Principal coordinates of silicoDArT markers analysis of 254 kikuyugrass accessions from Australia (90 accessions), California (86 accessions), the University of California, Riverside, collection (16 accessions), the Mealani Research Station, University of Hawaii collection (48 accessions), the island of Hawaii (12 accessions), and cultivars Whittet and AZ-1. Axis 1 explains 39.44% of variance, axis 2 4.03%. These two axes contribute the most to variance explanation and together explain 43.47%

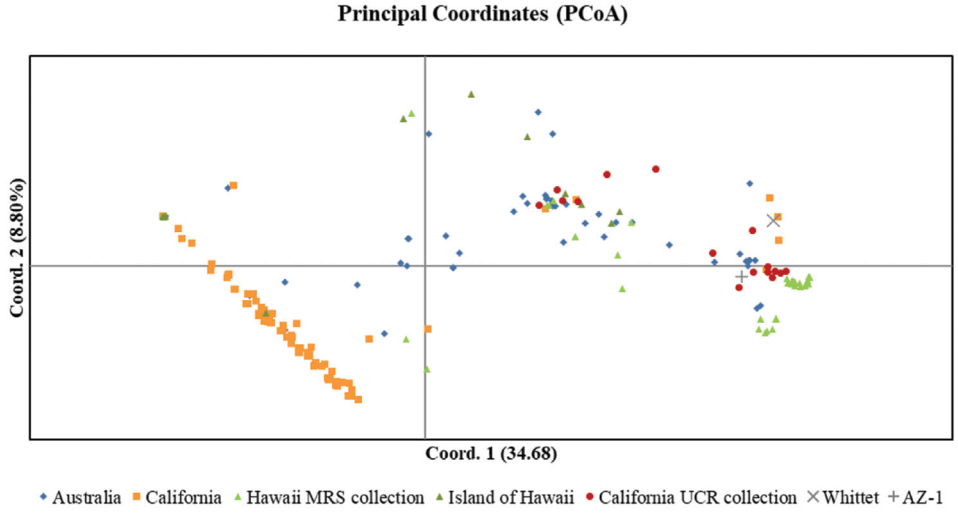

FIGURE 5 Principal coordinates of single nucleotide polymorphism (SNP) markers analysis of 254 kikuyugrass accessions from Australia (90 accessions), California (86 accessions), the University of California, Riverside, collection (16 accessions), the Mealani Research Station, University of Hawaii collection (48 accessions), the island of Hawaii (12 accessions), and cultivars Whittet and AZ-1. Axis 1 explains 34.68% of variance, axis 2 8.80%. These two axes contribute the most to variance explanation and together explain 43.48%

to investigate if SNP data confirm results obtained with silicoDArT. Both silicoDArT and SNP makers consistently formed two distinct groups on the dendrogram and PCoA graph.

A breeding system, along with the life form, is closely associated with the among-species variation. Self-pollinating and mixed-mating species tend to have lower genetic diversity and higher differentiation compared with outcrossing species (Hamrick & Godt, 1996). This may be a consequence of smaller effective population sizes of selfers, bottlenecks occurring during colonization, genetic hitchhiking associated with local adaptation, and clonal propagation (Ellstrand & Roose, 1987; Pannell & Dorken, 2006). Kikuyugrass appears to be predominantly

TABLE 3 Average genetic distances of kikuyugrass populations based on the distance matrix produced by RESTDIST

Population	Distances within population	Distance between populations				
		California	California UCR[a] collection	Hawaii MRS[b] collection	Island of Hawaii	Australia
California	0.12	x				
California UCR collection	0.09	0.34	x			
Hawaii MRS collection	0.16	0.36	0.13	x		
Island of Hawaii	0.12	0.14	0.20	0.20	x	
Australia	0.12	0.15	0.21	0.21	0.18	x

[a]UCR, University of California, Riverside. [b]MRS, Mealani Research Station.

self-pollinating. In tests of isolated flowering stems and tillers, we observed 72.7% seed set (data not shown). In addition, cross-pollination is believed to occur and may increase genetic diversity within this species. Overall, genetic diversity observed in this study was low, but this does not hinder the invasiveness of plant species (Bossdorf et al., 2005; Geng et al., 2007; Poulin, Weller, & Sakai, 2005); therefore, it does not appear to hinder adaptability. Fountaingrass [*Pennisetum setaceum* (Forssk.) Chiov.], a highly apomictic species, shows no or very low genetic diversity with high invasiveness (Poulin et al., 2005). Populations of kikuyugrass in this study showed low to moderate genetic differentiation. Moderate differentiation among populations was observed also in other grasses, such as *Stipa pennata* L. (PhiPT = 0.34), a species with a mixed mating system, incorporating self-pollination, similarly to kikuyugrass, or cleistogamy (Wagner et al., 2012). Japanese populations of rice (*Oryza sativa* L.), an autogamous species, showed PhiPT values between 0.117 and 0.356 (Yamasaki & Ideta, 2013). However, low genetic diversity would certainly hinder any breeding efforts, leading to new, better adapted, and perhaps less aggressive cultivars.

The genetic basis of separation into two distinct clusters is obscure. It may reflect different geographic origins of various introductions, to each of the three sampled programs and regions, with accessions from each sampled program represented in each cluster. Interestingly, the two Australian accessions believed to represent the original introductions are present in the same cluster.

Records of various transfers of the germplasm are far from clear. The initial introductions to California (Garner, 1925), Australia (Morris, 2009), and Hawaii (Hosaka, 1958) were from Africa using plant material originating most likely from Kenya. Some of these importations were facilitated by University of Pretoria in Southern Africa, but no clear records on the sources of the original samples, and the pattern of further distribution, are available. Research institutions breeding kikuyugrass were also exchanging germplasm with one another (e.g., in 1939, plant material from University of California was sent to Australia, and in 1991, a collection at UCR was started from seeds including material imported from Australia). In addition, cv. Whittet, which is a selection of kikuyugrass from Kenya and was released in Australia, was extensively used in breeding programs (e.g., cv. Hosaka from Hawaii is a Whittet selection, and cv. Noonan, another Australian release, is a hybrid between Whittet and Breakwell; Hanna et al., 2004; Morris, 2009).

Records indicate that there were two independent introductions of kikuyugrass into California: the first one in 1915, by the USDA from an unspecified location (but most likely Africa), and the second in 1920, from the University of Pretoria, Republic of South Africa. In early 1991, a third known introduction took place when a sample of seed was imported from Australia and, together with accessions collected within the state, gave rise to the current UCR collection.

In Hawaii, kikuyugrass was introduced in 1925. Since then, several cultivars have been released there, including cv. Hosaka developed by Dr. Ukio Urata (Fukumoto & Lee, 2003). Some of the unnamed hybrids from Dr. Urata's breeding program were included in this study, but their parentage and the relationship to Hosaka is unknown. There are indications that some cultivars of kikuyugrass were imported into Hawaii by individual growers, from unknown sources.

The first introduction of kikuyugrass into Australia was recorded in 1918, followed by several subsequent introductions: in 1920 from an unknown source, in 1925 from Africa (South African Department of Agriculture), and in 1939 from the University of California (Morris, 2009). A breeding effort there led to the release of cvs. Whittet, Breakwell, Croft, and Noonan. More introduction events, together with popularity as a forage and turf crop in Hawaii and Australia, are probably responsible for higher genetic diversity among accessions from these areas.

Across the experiment, more genetic variation was observed within than among populations (76 vs. 24%, respectively). This may be a consequence of homogenization of poorly maintained collection plots of old stocks, both at the UCR collection and at the MRS col-

lection in Hawaii. Multiple sampling of individual plots of the old UCR collection clearly showed the effects of either plot invasion or intercrossing across plots. In each case, at least one of the five samples taken from a single plot turned out to be genetically different from the remaining ones. Since only old collections at UCR and MRS had multiple samples taken, direct comparison of intra- with intervariation is impossible. Similar population structure was reported for unmanaged populations of *Pennisetum ciliare* L. Link. in northwestern Mexico, where only 22.6% of variation was due to variation among populations (Gutierrez-Ozuna, Eguiarte, & Molina-Freaner, 2009).

This study included kikuyugrass cultivars Whittet and AZ-1. In addition, among PBI lines were unregistered selections of cultivars ('Breakwell' and 'Noonan') and lines derived from chemical mutagenesis of Whittet. Except for one, they grouped together, showing low genetic diversity of commercial cultivars. This may create a bottleneck during the conversion of germplasm from forage to turf type, which was observed by Baird et al. (2012) in tall fescue turf-type cultivars, and negatively affects the breeding process. Low genetic diversity among cultivars and mutants of Whittet was also observed by Lowe, Bowdler, Sinclair, Holton, and Skabo (2010).

4.1 | California: University of California, Riverside, collection and in-state diversity

There were two distinct groups of Californian accessions: those collected throughout the state at the onset of this experiment in 2013, and the kikuyugrass collection at UCR. The latter is what remains from the work of S.T. Cockerham and his team in the early 1990s. Their effort was directed at selection for more desirable turf-type lines, with less aggressive growth and finer texture. The starting population for that selection included 17 entries selected from 21 accessions collected in the state of California, 400 seedlings from seeds donated by a private kikuyugrass breeder, and an unknown number of seedlings from a seed sample obtained from Australia. Some variation in phenotypic traits and heritability of the traits was originally present among the selections. However, with the lack of proper plot maintenance over the past ~20 yr, much of that original variation has disappeared, probably as a consequence of encroachments across plots and cross hybridization. Expecting this effect, plots were sampled carefully, with five samples taken from each accession. In most cases, at least one of the samples did not group with other replicates from the same plot, showing extensive cross-contamination (data not shown). Most of these UCR accessions grouped with cv. Whittet and other kikuyugrass cultivars, suggesting that the original seed samples obtained from external sources were probably some derivatives or selections from established cultivars.

In the UCR collection, accessions C1, C11, and C17 were meant as checks for the Cockerham selections. They were originally collected in California, but since then, they appear to have either hybridized or were invaded and taken over by other accessions, and they no longer cluster with the newly collected Californian accessions. Geographic origin of these three accessions (C1, C11, and C17) is unknown, but quite likely they were collected from golf courses. Two of the newly collected Californian accessions, those from the Mesa Verde and Mission Viejo CCs, did cluster with C1 and C11, which supports this assumption. The absence of UCR accessions in Cluster 1 containing most of the Californian accessions suggests that they have not mixed with other Californian germplasm since their outdoor planting 24 yr ago.

Fewer introduction events in California may be responsible for lower genetic diversity of kikuyugrass in the state. Most of the Californian accessions were related with each other (Figure 3), and also with accessions collected from PBI's farm at Cobbity, Australia (Figure 3, Group 1A). Low genetic diversity of kikuyugrass from golf courses in California was also observed by Wilen et al. (1995). There were several samples present in Cluster 2, more related to Whittet and other cultivars than to other Californian accessions. In several locations in California, kikuyugrass was planted from seed of released cultivars as the desired turf species. In some cases, that turf was invaded or intercrossed with "common" kikuyugrass. This course of events may explain why these accessions were somehow related to Whittet, rather than to other accessions collected through California for this study.

4.2 | Hawaii: Mealani Research Station collection and in-state diversity

Kikuyugrass is an important pasture grass in Hawaii and covers large areas of higher altitude rangelands (Hanna et al., 2004; Urata, 1982). Based on the records left by Dr. Urata, most of the accessions from MRS collection were originally collected in the state of Hawaii or were hybrids of Hawaiian accessions. The collection established by Dr. Urata was left without maintenance over a long time and appears to have homogenized to a considerable degree. This explains close grouping of many of these accessions in Cluster 2. There were some more distant accessions in this cluster, as well as in Cluster 1, which may indicate more genetic diversity within this collection in the past. From 48 samples collected from abandoned Dr. Urata's plots, 30

accessions group near cv. Whittet, as well as the Noonan and Breakwell selections. Since several kikuyugrass cultivars were successfully introduced to Hawaii (Fukumoto & Lee, 2003), the accessions collected from the islands by Dr. Urata and the hybrids he produced may well be related to those cultivars or originated from the same stocks as Australian introductions.

Samples of kikuyugrass collected from wild stands around the Island of Hawaii were genetically diverse. They were sampled from various locations around the island: roadsides, closed golf course, and the pasture belonging to MRS. These samples were scattered in both clusters. Such variation may result both from several introductions, original from Africa and following introductions of cultivars, as well as from mutations arising over the time, or both. Increased mutation rates are common in grasses, higher than in dicotyledonous plants (Wicker et al., 2016).

4.3 | Australia: Plant Breeding Institute collection diversity

Australian accessions were scattered among both clusters, but at the same time, many PBI accessions showed no, or very low, genetic distance (Figure 3, Group 1A). The Group 1A samples were collected in 2005 from a 1.2-ha field of kikuyugrass on an out-of-production turf (sod) farm adjoining the PBI's Lansdowne Farm at Cobbitty, near Camden. There was obvious morphological differentiation in this field, in the form of patches distinct from the "common" turf-type kikuyugrass originally planted in that field some 15 yr before. No fertilizer or irrigation had been applied since the end of 2002, and it was quite likely that reduced sward density under stress allowed numerous seeds from the seed bank to germinate and establish clonal patches of better adapted genotypes. Similar patching was observed by Wilen et al. (1995) at the golf course in Palo Alto, CA, with high salinity. Kikuyugrass is tolerant to moderate salinity (Fraser, Sharp, Ahmad, Morris, & Trethowan, 2017; Radhakrishnan, Waisel, & Sternberg, 2006), but high salinity at the Palo Alto golf course may have accelerated selection for better adapted genotypes. As the clustering of accessions from the turf farm at Cobbity illustrates, selection factors among these accessions were probably insufficient to create unique genotypes, so perhaps the observed morphological variation within the field is a demonstration of kikuyugrass plasticity (Morris, 2009). A similar adaptability to various environmental conditions was observed in closely related *Pennisteum setaceum* Forssk. (Chiov.). This apomictic species introduced in Hawaii was able to adapt to a wide range of altitudes, despite very low genetic diversity (Williams & Black, 1993).

A large proportion of closely related accessions affected the average genetic distance within Australian samples (0.12), but the presence of Australian accessions in different branches of both clusters indicates a moderately high genetic diversity of this group. Earlier, Holton et al. (2007) and Morris (2009) assessed genetic diversity of kikuyugrass accessions from various regions of eastern and southern Australia by DAF and RAPD markers and showed a significant level of genetic variation, forming three different clusters. On the other hand, a study by Lowe et al. (2010) on Australian "common" kikuyugrass and commercial cultivars showed two broad groups of accessions, with one of the clusters formed mostly by cultivars and their selections. Genotypic variation of Australian kikuyugrass lines examined using RAPD markers was higher than expected (Morris, 2009).

Although there is noticeable genetic diversity among kikuyugrass, accessions analyzed in this study did not group according to their geographic origin. This may reflect the role of the University of Pretoria as a clearinghouse for the distribution of germplasm out of Africa. Germplasm from collections developed at UCR, MRS, partially from PBI, and Whittet and other cultivars and their selections are related to each other, but not necessarily to accessions collected from other sources in the state (California or Hawaii) or country. Possible low diversity among introduced plant material, which was further exchanged in an attempt to extend genetic variation, combined with wide use of Whittet in the breeding process, could contribute homogenization of genetic resources of these institutions, instead of broadening their genetic diversity. In the case of California, it is visible in clear separation of the UCR collection accessions, which are related to Australian lines and cv. Whittet, and samples collected throughout the state, which are probably descendants of early importations from Africa, now known as "common" kikuyugrass. Samples collected from the island of Hawaii showed higher genetic diversity than accessions from the MRS collection. All samples from Australia were from the PBI collection, but this collection includes accessions from various, distant locations across Australia, which can explain more diversity within it. Even in this case, many hybrids developed at PBI are related to Whittet and other cultivars. Import restrictions to California are contributing to lower genetic diversity within the state, compared with Australia and Hawaii, which continued importing and distributing kikuyugrass as a valuable forage grass.

Despite a mating system promoting low genetic diversity and with limited introduction events, significant genetic diversity exists within and among kikuyugrass populations from different geographic regions. Australia and Hawaii appear to be valuable sources of genetically diverse germplasm, but prohibition on new introductions makes

those gene pools inaccessible to breeding efforts within the state. However, there is some genetic variation among accessions already present within the state. Whether this is sufficient for a sensible progress in breeding is not clear at this time. If import restrictions could be waived, germplasm from other areas would certainly be a major asset in new cultivar development within the state. Phenotyping can be helpful in germplasm selection for hybridization, but due to high adaptability and plasticity of kikuyugrass, selection of mating parents should be supported by tests of the genetic distance between them.

ACKNOWLEDGMENTS

This work was supported by the California Turfgrass & Landscape Foundation (CTLF), the U.S. Golf Association (USGA), the Western Municipal Water District (WMWD), and the Metropolitan Water District (MWD).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Pudzianowska M, Mock TJ, Martin PM, Lukaszewski AJ, Baird JH. Kikuyugrass germplasm collections in the United States and Australia show low levels of genetic diversity as revealed by DArTseq genotyping. *Crop Science*. 2020;1–14. https://doi.org/10.1002/csc2.20231