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Genetic Diversity of Biofuel and Naturalized Napiergrass (*Pennisetum purpureum*)

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Biofuel crops such as napiergrass possess traits characteristic of invasive plant species, raising concern that biofuels might escape cultivation and invade surrounding agricultural and natural areas. Napiergrass biofuel types are being developed to have reduced invasion risk, but these might be cultivated in areas where naturalized populations of this species are already present. The successful management of napiergrass biofuel plantations will therefore require techniques to monitor for escaped biofuels as distinguished from existing naturalized populations. Here we used 20 microsatellite DNA markers developed for pearl millet to genotype 16 entries of napiergrass, including naturalized populations and accessions selected for biofuel traits. Use of the markers showed a clear genetic separation between the biofuel types and naturalized entries and revealed naturalized populations undergoing genetic isolation by distance. These findings demonstrated the utility of microsatellite marker transfer in the development of an important tool for managing the invasion risk of a potential biofuel crop.

Nomenclature: Napiergrass, *Pennisetum purpureum* Schumach.; pearl millet, *Pennisetum glaucum* (L.) R. Br.

Key words: Invasion risk, microsatellite marker transfer, naturalized population.

Cellulosic biofuels offer the potential to reduce greenhouse gas emissions and fossil fuel consumption, while enhancing food and energy security and economic opportunities (Demirbas 2009; Dornburg et al. 2010; Lemus and Lal 2005; Tilman et al. 2009). There is concern, however, that dedicated biofuel crop production will have unintended environmental consequences such as invasions into surrounding natural areas (Flory et al. 2012; Low et al. 2011; Raghu et al. 2011; Sheppard et al. 2011). Several traits desired in biofuel crops, such as rapid growth, vegetative propagation, resistance to pests and disease, and tolerance to abiotic stress, are commonly found in nonnative invasive plant species (Barney and DiTomaso 2011; Buddenhagen et al. 2009; Flory et al. 2012; Raghu et al. 2006). Furthermore, the cultivation and transportation of biofuels on a large scale creates numerous opportunities for cultivars to escape into natural areas or to hybridize with adjacent naturalized populations, potentially increasing invasion risk (Figure 1; Richardson and Blanchard 2011). Risk management strategies for biofuel production include the judicious selection of species and varieties, incremental tests to evaluate invasion potential of selected species and varieties, guidelines for plantation design and maintenance, and government regulation (Byrne and Stone 2011; Davis et al. 2012; Flory et al. 2012; Jørgensen 2011; Low et al. 2011; Richardson and Blanchard 2011).

Application of these biofuel risk management strategies requires a means to monitor the genetic purity and containment of biofuel plantings. DNA-based markers provide such a tool (Gaskin et al. 2011; Hufbauer 2004; Le Roux and Wieczorek 2009; Slotta 2008). Of these, microsatellite or simple sequence repeat (SSR) markers offer high levels of polymorphism, lab-to-lab transferability, and reproducibility (Powell et al. 1996; Tautz and Renz 1984; Weber and May 1989). Furthermore, the polymerase chain reaction (PCR) primer sets that are needed to amplify polymorphic SSR regions can often be transferred from a related species, saving time and expenses associated with marker development (Kalia et al. 2011). Ellis and Burke (2007) identified over 70 invasive plant species having a congeneric species expressed sequence tag (EST) database that has been or could be used in developing SSR markers to monitor the species of ecological concern.

Napiergrass (*Pennisetum purpureum* Schumach.), also known as elephantgrass, has significant potential as a

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Management Implications

Cellulosic biofuels offer opportunities for sustainable energy production, but many traits of biofuel crop species increase the potential for escape and invasion into surrounding natural areas. Biofuel crop accessions might be selected for high biomass and reduced invasion risk, but management of biofuel plantings will nevertheless require a means to monitor for the escape of cultivated varieties, especially in areas where naturalized populations of the same species are also present. DNA microsatellite markers offer technical and practical advantages for this purpose, including marker transferability. Markers that have already been developed for a crop or model species can be transferred to a related species, sparing the time and cost of marker development. Napiergrass (*Pennisetum purpureum*) is a crop that has tremendous potential for biofuel production in areas where naturalized populations are already present and creating weed problems. Exploiting napiergrass as a biofuel will require risk management strategies that include a means to genetically track both the cultivated and naturalized types. We found that microsatellite markers developed for pearl millet (*Pennisetum glaucum*) readily distinguished the naturalized populations of napiergrass collected across the state of Florida from the napiergrass selected for biomass traits. These findings demonstrate that naturalized, weedy populations of napiergrass have a different genetic origin from the biomass types and validate the transferred DNA markers as an effective management tool for distinguishing between the two.

cellulosic biofuel crop in the southern United States and in other areas of the world. This tetraploid C_4 grass species is broadly adapted to tropical and subtropical climates (Barney and DiTomaso 2011; Hanna et al. 2004) and generally out-performs other grasses in dry biomass yield (Anderson et al. 2008; Bouton et al. 2002; Hanna and Gupta 1999; Vicente-Chandler et al. 1974). *Pennisetum purpureum* was introduced to the United States from Africa in 1913 (Thompson 1919) as forage for cattle, but over time has become a weed in sugarcane plantings and invasive in natural areas (Arceneaux 1967; Gordon et al. 2011). As a cellulosic feedstock, *P. purpureum* could potentially contribute almost half of the U.S. biofuel production (USDA 2010) but monitoring of feedstock containment will be required (Gordon et al. 2011) to ensure cultivated varieties do not escape into surrounding agricultural and natural areas (Figure 1).

No SSR marker resource has been developed specifically for *P. purpureum*, but SSR markers developed for pearl millet [*Pennisetum glaucum* (L.) R. Br.] were evaluated in a study of the Embrapa active germplasm bank of napiergrass (Embrapa-BAGCE) (Azevedo et al. 2012). Our application of these markers revealed genetic diversity between *P. purpureum* entries selected for biofuel traits and naturalized *P. purpureum* populations collected throughout north, central, and south Florida. This approach validated a DNA marker set for invasion risk management of napiergrass biofuels and demonstrated the general utility of SSR

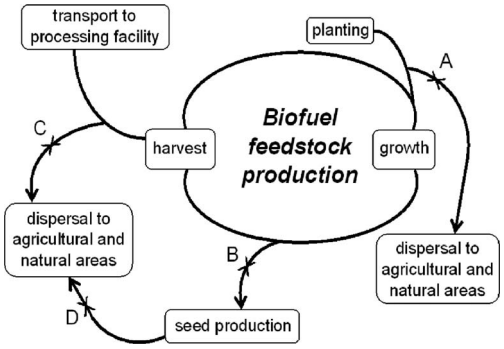

Figure 1. Conceptual diagram showing the process of biofuel feedstock production from planting through transport to the final processing facility. The risk of escape and invasion into agricultural and natural areas may occur during (A) planting of vegetative material, (B, D) through seed production and dispersal, or (C) during transport of biofuel to processing facilities. X indicates a point of invasion risk.

marker transfer in the development of tools for the management of invasion risk in biofuel crops.

Materials and Methods

Plant Material. We used six *P. purpureum* entries selected for biofuel traits, including biomass production, and 10 naturalized populations collected from fields, roadsides, and natural areas throughout Florida (Table 1). The naturalized populations were named according to the Florida counties where they were collected. Five of the six selected biofuel types—PI 300086 (USDA 1968), Merkeron (Burton 1989), N13, N43, and N51—were collected originally from the *P. purpureum* nursery at the U.S. Department of Agriculture–Agricultural Research Service Coastal Plain Station in Tifton, GA. The sixth, UF1, is an experimental line developed at the University of Florida. All of the naturalized entries were collected from October to December 2008 and planted, along with the selected biofuel types, in replicated common garden plots at the University of Florida Plant Science Research and Education Unit near Citra, FL. A hybrid *P. glaucum* cultivar, Tifleaf 3 (Hanna et al. 1997), was used as a positive PCR control for each of the primer sets.

DNA Extraction and Amplification. Leaf tissue (3 g [0.11 oz]) was individually collected from 10 plants of each study entry, freeze-dried, and stored at −80 C (−112 F). The procedure of Dellaporta et al. (1983) was used to extract genomic DNA from all 160 individuals and the *P. glaucum* entry, Tifleaf 3. Amplified fragment-length polymorphism (AFLP) markers previously revealed genetic variation within *P. purpureum* entries (Harris et al. 2009). DNA samples were therefore pooled to efficiently capture within-entry variation. Equivalent quantities of the 10

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Table 1. The *Pennisetum purpureum* entries and *Pennisetum glaucum* control investigated for pearl millet microsatellite marker transfer.

Entry no.	Entry identification no.	Type	Collection site[a]
1	PI 300086	Biofuel	N/A[b]
2	N13	Biofuel	N/A
3	N43	Biofuel	N/A
4	N51	Biofuel	N/A
5	Merkeron	Biofuel	N/A
6	UF1	Biofuel	N/A
7	Alachua	Naturalized	29.640°N, 082.362°W
8	Marion	Naturalized	29.473°N, 082.229°W
9	Lake	Naturalized	28.803°N, 081.911°W
10	Polk	Naturalized	27.853°N, 081.826°W
11	Hardee	Naturalized	27.594°N, 081.972°W
12	Manatee	Naturalized	27.594°N, 082.540°W
13	Lee	Naturalized	26.70941°N, 081.760°W
14	Palm Beach	Naturalized	26.89391°N, 080.612°W
15	Miami-Dade	Naturalized	25.445°N, 080.562°W
16	Broward	Naturalized	26.338°N, 080.541°W
17	Tifleaf 3 (*P. glaucum*)	Cultivated	N/A

[a] Global positioning system latitude and longitude coordinates of Florida collection sites for the naturalized population entries.

[b] N/A, not applicable.

individual DNA samples were combined, and the total volume of the sample was increased to achieve a final DNA concentration of 100 ng μl^{-1} (1×10^{-4} oz fl oz^{-1}) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Finally, an aliquot from each pool was diluted to 10 ng μl^{-1} in TE for use as PCR template.

Twenty microsatellite primer sets (Table 2), originally developed for *P. glaucum* and shown to amplify *P. purpureum* DNA templates (Azevedo et al. 2012), were used to genotype the 16 study entries. These primer sets were originally derived based upon *P. glaucum* genomic (Budak et al. 2003) and EST (Mariac et al. 2006) resources. The original primer designations were retained here and the primer sequences are reported in the respective references (Budak et al. 2003; Mariac et al. 2006). PCR reactions of 50 µl were prepared to contain 1.25 units of TaKaRa Ex Taq® DNA Polymerase Hot-Start Version (Clontech Laboratories, Inc., Mountain View, CA), 5 µl of 10X Ex Taq® buffer, 0.2 mM of each dNTP, 0.2 µM of each primer, and 20 ng of genomic DNA. Amplifications were performed in a PTC-100 PCR system (MJ Research, Inc., Waltham, MA, currently Bio-Rad Laboratories Inc., Hercules, CA). A step-down PCR program (Don et al. 1991) was used with the following conditions: 95 C for 5 min; 10 cycles of 1 min denaturation at 94 C, 1 min 30 sec annealing at 65 to 55 C, stepping down 1 C each cycle, and 1 min extension at 72 C; followed by 29 additional cycles using the final annealing temperature of 55 C. Two technical replications were performed for each PCR reaction. Reactions with pearl millet DNA were always included as positive PCR controls and reactions without template DNA were always included as negative PCR controls.

PCR reactions were screened for the presence of DNA amplification products (amplicons) by electrophoresis through 4% Ultra Pure™ Agarose-1000 gels (Invitrogen Corp., Carlsbad, CA). For enhanced resolution of PCR product length polymorphisms, successful PCR reactions were then fractionated on 5%, 10%, or gradient (4–20%) Criterion™ precast polyacrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA) in 1× TBE buffer (100 mM Trizma base, 100 mM boric acid, 2.5 mM EDTA; pH 8.3). Amplicon sizes were estimated by comparison to 50-bp and 100-bp DNA ladders (Promega Corp., Madison, WI). Electrophoresis was at 90 or 100 V for 2 or 3 h at 0 C. Gels were stained in ethidium bromide and photographed over a ultraviolet transilluminator in a Molecular Imager Gel Doc™ XR System (Bio-Rad).

Data Analysis. To identify the primer sets that were most informative for the study entries, diversity index (DI) values were computed for each primer set by the approach used in studies of polyploid potato (Milbourne et al. 1997). The entire pattern of amplicons produced in a PCR reaction was treated as a single genotype and the frequencies of the amplicon patterns were used for calculation of the DI value. The DI values were calculated as $1 - \sum fg^2$, where fg is the frequency of each genotype among the 16 napiergrass entries. A monomorphic marker has a DI value of 0, whereas a highly polymorphic marker has a DI value approaching 1.

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Table 2. *Pennisetum glaucum* microsatellite primers transferred to *Pennisetum purpureum*.

Primer[a]	DI[b] value	No. amplicons per 16 entries	No. of amplicons per entry	Size range (bp)
CTM-8	0.84	7	3.1	310–250
CTM-10	0.49	3	2.5	205–230
CTM-12	0.82	17	8.8	300–1500
CTM-25	0.91	24	8.0	200–920
CTM-26	0.80	17	6.8	220–850
CTM-27	0.77	24	9.6	290–700
CTM-59	0.94	20	7.1	170–900
PGIRD5	0.23	9	3.6	155–295
PGIRD12	0.00	5	5.0	120–295
PGIRD13	0.91	28	6.1	210–550
PGIRD19	0.89	17	4.2	285–605
PGIRD21	0.68	9	6.3	230–340
PGIRD25	0.80	11	4.7	150–330
PGIRD43	0.84	9	3.9	100–900
PGIRD44	0.86	17	6.8	105–250
PGIRD46	0.87	13	4.4	60–260
PGIRD49	0.90	15	7.9	180–520
PGIRD54	0.72	6	4.2	70–370
PGIRD56	0.70	11	6.4	150–680
PGIRD57	0.82	9	3.5	100–660
Average	0.74	14	5.6	180–570
Range	0.00–0.94	3–28	2.5–9.6	60–1,500

[a] CTM, genomic-derived primer designations of Budak et al. (2003) and PGIRD, expressed sequence tag–derived primer designations of Mariac et al. 2006.

[b] Abbreviation: DI, diversity index.

Phylogenetic analysis was performed based on DNA fragments that were reproducibly amplified from the pooled DNA templates in two technical replications. DNA bands were recorded as dominant genetic markers (present or absent) in a binomial data matrix. The FreeTree program (Pavlíček et al. 1999) was used to generate pairwise Dice genetic similarity (GS) values (Dice 1945) based upon shared amplicons (Nei and Li 1979). Higher values of the GS coefficient reflect closer relationships of the entries being compared. A phylogenetic tree was generated based upon the unweighted pair-group method with arithmetic averages (Sneath and Sokal 1973) and 500 bootstrap resampled data sets (Felsenstein 1985). The TreeView program (Page 1996) was used to generate the tree graphic, which reflects the genetic distances (calculated as 1-GS) among the study entries. *Pennisetum glaucum* Tifleaf 3 was not included as part of the phylogenetic analysis since, in contrast to the *P. purpureum* DNA templates, this PCR-positive control DNA template was not comprised of pooled DNAs extracted from 10 individual plants.

The correlation of genetic and geographic distances was tested for the naturalized *P. purpureum* populations. The GPS Visualizer web site (Schneider 2003) was used to create a location map for the naturalized populations and to calculate a matrix of geographic distances in miles between each pair of collection sites. The geographic and genetic distance matrices were tested for correlation by a Mantel test performed on the Isolation by Distance Web Server (Jensen et al. 2005) with the default parameters.

Results and Discussion

Microsatellite Marker Transfer. The *P. glaucum* primer sets used to genotype the Embrapa-BAGCE materials (Azevedo et al. 2012) effectively amplified *P. purpureum* DNA templates in the present study. The transferred primer sets therefore provided a robust resource for *P. purpureum* genotyping across genetic materials and experimental conditions. When used to genotype the biofuel and naturalized napiergrass accessions, the primers produced from 3 to 10 amplicons of varying intensity per entry (Table 2; Supplemental Figure 1), as expected for a heterozygous, tetraploid species such as *P. purpureum* (2n = 4x = 28) (Burton 1942). Amplification of a single locus is expected to generate one to four distinct PCR products

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Table 3. Genetic similarity matrix for 16 *Pennisetum purpureum* entries based upon amplicons from 20 *Pennisetum glaucum* microsatellite primer sets.

Identification		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	PI 300086	1.00															
2	N13	0.58	1.00														
3	N43	0.61	0.67	1.00													
4	N51	0.63	0.71	0.94	1.00												
5	Merkeron	0.63	0.68	0.95	0.95	1.00											
6	UF1	0.66	0.63	0.80	0.79	0.79	1.00										
7	Alachua	0.55	0.64	0.69	0.70	0.69	0.61	1.00									
8	Marion	0.54	0.59	0.66	0.65	0.66	0.62	0.87	1.00								
9	Lake	0.54	0.60	0.68	0.68	0.68	0.60	0.88	0.93	1.00							
10	Polk	0.56	0.65	0.68	0.67	0.68	0.65	0.77	0.80	0.77	1.00						
11	Hardee	0.53	0.58	0.69	0.67	0.65	0.59	0.85	0.89	0.89	0.76	1.00					
12	Manatee	0.56	0.62	0.71	0.70	0.70	0.67	0.79	0.78	0.78	0.83	0.81	1.00				
13	Lee	0.54	0.63	0.67	0.66	0.65	0.63	0.72	0.70	0.69	0.76	0.74	0.79	1.00			
14	Palm Beach	0.57	0.62	0.71	0.70	0.71	0.63	0.75	0.73	0.78	0.77	0.75	0.76	0.73	1.00		
15	Miami-Dade	0.61	0.61	0.63	0.64	0.64	0.66	0.57	0.56	0.54	0.55	0.55	0.59	0.57	0.61	1.00	
16	Broward	0.60	0.64	0.62	0.65	0.63	0.59	0.61	0.60	0.64	0.60	0.59	0.61	0.57	0.63	0.69	1.00

differing in band intensity depending upon the dosage of each allele present (Milbourne et al. 1997). The size variation of the amplicons produced by most of the primer sets was greater than expected for microsatellite length variation alone. *Pennisetum purpureum* microsatellite loci might vary not only from expansion and contraction of the microsatellite repeat but also from insertion–deletion polymorphisms in the microsatellite flanking regions, as seen in *Zea mays* L. (Matsuoka et al. 2002) and *Cynodon* spp. (Kamps et al. 2011). For the primers designed from an EST library, the gain or loss of intron sequences might create size variation among amplicons. The amplification of duplicate loci can also contribute to amplicon number and length variation, as seen for primer set CTM-12, which produced two amplicon clusters of distinctly different sizes on both the *P. glaucum* control and the *P. purpureum* entries (Supplemental Figure 1).

Genetic Diversity. The *P. purpureum* DNA amplicons provided informative genetic markers that distinguished each of the study entries. Nineteen of the 20 primer sets revealed genetic polymorphisms among the naturalized and biofuel *P. purpureum* entries. The DI values for the 20 markers on the 16 study entries ranged from 0.00 for the monomorphic, EST-derived PGIRD12 to 0.94 for the genomic-derived CTM-59. The average DI value for the set of 20 markers on the *P. purpureum* templates was 0.74 (Table 2). The Dice GS coefficients for all 16 *P. purpureum* entries ranged from 0.53 to 0.95, with a mean value of 0.68 (Table 3).

A genetic distance phylogram based upon 272 amplicons reproducibly amplified by the 20 markers separated the 16 *P. purpureum* entries into three major clusters, with the PI 300086 branching separately (Figure 2). A cluster of eight naturalized entries was separated from a cluster of five biofuel types, supported by a bootstrap value of 59, and from another group of two naturalized entries (Miami-Dade and Broward) by a bootstrap value of 50. The naturalized Florida populations descend from an unspecified African source introduced for forage in the early 20th century (Thompson 1919). The clustering of eight naturalized entries was consistent with a common genetic origin for these populations. The naturalized Miami-Dade and Broward entries might have originated from a different introduction, based upon their genetic distances from both the biofuel types (0.41 to 0.34) and the other eight naturalized entries (0.46 to 0.36) (Figure 2; Table 3; Supplemental Table 1). Prior randomly amplified polymorphic DNA (Lowe et al. 2003) and AFLP (Harris et al. 2009) marker studies grouped *P. purpureum* accessions largely corresponding to geographic origin. Lowe et al. (2003) found that accessions from southern Africa grouped separately from those originating in East Africa. All of the naturalized Florida entries were genetically distinct from the South African PI 300086 (USDA 1968) and the Zimbabwean N13 (USDA 1969). East Africa might therefore be the origin of the naturalized Florida entries.

Although the naturalized populations comprised two distinct groups, each population was genetically unique. Within the naturalized cluster of eight entries, there was a trend for the entries to branch according to their north, central, or south Florida collection sites (Figures 2 and 3a). All of the naturalized entries were therefore tested for correlation between genetic distance and geographic distance.

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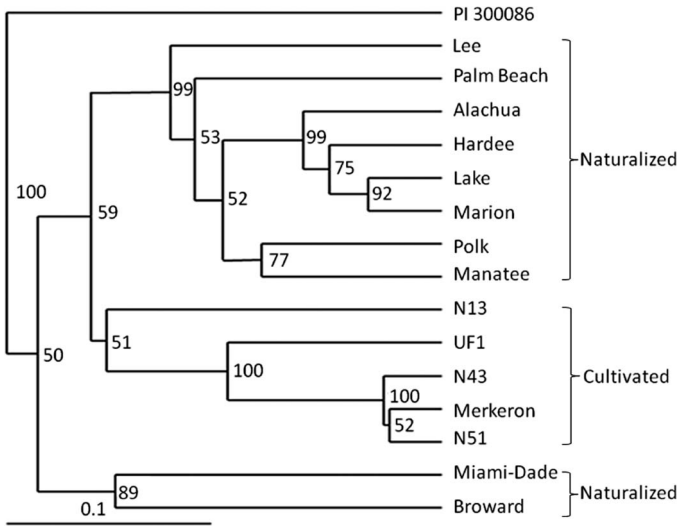

Figure 2. Unweighted pair-group method with arithmetic averages (UPGMA) phylogram of 16 *Pennisetum purpureum* entries based on 20 *Pennisetum glaucum* genomic and expressed sequence tag–derived simple sequence repeat markers. The genetic similarity (GS) matrix (Table 3) was converted to the UPGMA phylogram as described in Materials and Methods. Bootstrap resampling values of 50 or greater are indicated at their respective nodes. The scale bar represents 0.1 unit of genetic distance or dissimilarity (1-GS).

A Mantel test performed on the Isolation by Distance Web Server (Jensen et al. 2005) revealed a positive correlation ($r = 0.5$, $P < 0.0030$) for the genetic and geographic distances separating the naturalized types (Figure 3b; Supplemental Table 1). The naturalized populations might therefore be undergoing isolation by distance, acquiring genetic differences due to geographic limitations on genetic exchange. The transferred microsatellite markers will allow for detailed studies of the genetic diversity within the naturalized populations and for comparisons with more broadly collected materials for further investigation of their origin.

The *P. glaucum* markers were also informative with respect to the biofuel entries. With the exception of PI 300086, this group of entries had high GS values, ranging from 0.63 to 0.95 (Table 3), yet each was uniquely distinguished. There is almost no overlap of genetic materials investigated among the various *P. purpureum* marker studies (Azevedo et al. 2012; Harris et al. 2009; Lowe et al. 2003), but the biofuel types N51 and Merkeron were investigated in the present work and in the AFLP study of Harris et al. (2009). Both studies found these entries to be closely related. In the context of our study, unique combinations of amplicons can be used for the genetic fingerprinting of cultivars selected for use as biofuel crops. Genetic fingerprints provide an essential tool for biofuel crop risk management strategies (Gaskin et al. 2011; Hufbauer 2004; Le Roux and Wieczorek 2009; Slotta 2008), enabling both researchers and managers to monitor the escape of propagules at the multiple points of invasion risk (Figure 1) and pollen gene flow from cultivated to naturalized stands.

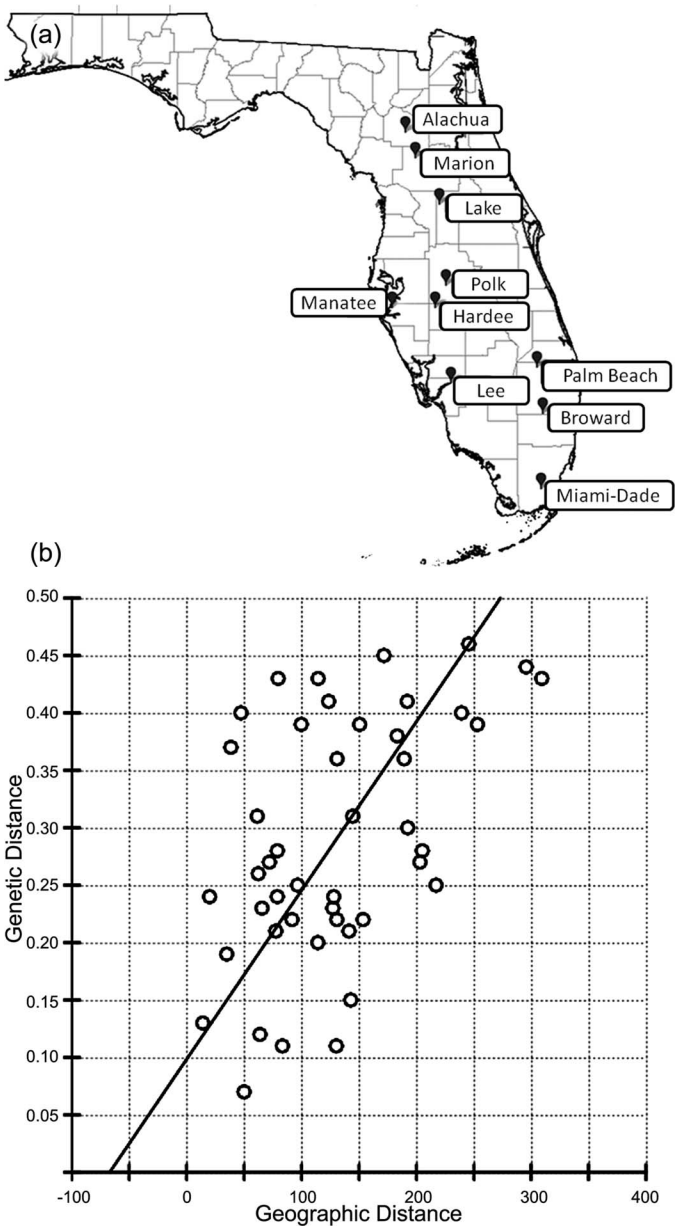

Figure 3. (a) Global positioning system map of the collection sites for naturalized Florida *Pennisetum purpureum* entries. (b) Positive correlation between genetic and geographic distance matrices for naturalized Florida *P. purpureum* entries.

Ellis and Burk (2007) suggested microsatellite marker transfer as an efficient, cost-effective tool for population genetic studies of rare, endangered, or invasive plant species. Our study demonstrated the efficacy of this approach with respect to *P. purpureum*, a promising biofuel crop with significant invasion risk. The microsatellite transfer

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approach provided an informative marker set revealing genetic relationships among selected and naturalized *P. purpureum* entries without costly investment in genomic information. This and other recent examples (Croxton et al. 2011; Kelager et al. 2013) actuate the strategy proposed by Ellis and Burke (2007) for the application of marker transfer to species of environmental importance. The *P. glaucum* genetic markers provided information on genetic diversity of *P. purpureum* and, based upon that diversity, a method to detect the escape of biofuel plantings or the hybridization between cultivated and naturalized populations.

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