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Assessing Genetic Diversity and Population Structure of *Kalmia latifolia* L. in the Eastern United States: An Essential Step towards Breeding for Adaptability to Southeastern Environmental Conditions

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Abstract: *Kalmia latifolia* L. (mountain laurel), an attractive flowering shrub, is considered to be a high-value ornamental plant for the eastern United States. Limited information on the genetic diversity and structure of *K. latifolia* is available, which obstructs efficient germplasm utilization and breeding for adaptability to southeastern environmental conditions. In this study, the genetic diversity of 48 wild *K. latifolia* plants sampled from eight populations in the eastern U.S. was assessed using eight inter simple sequence repeat (ISSR) markers. A total of 116 bands were amplified, 90.52% of which (105) were polymorphic. A high level of genetic diversity at the species level was determined by Nei's gene diversity (0.3089) and Shannon's information index (0.4654), indicating that *K. latifolia* was able to adapt to environmental changes and thus was able to distribute over a wide latitudinal range. In terms of the distribution of genetic diversity, Nei's genetic differentiation and analysis of molecular variance (AMOVA) showed 38.09% and 29.54% of diversity existed among populations, respectively, elucidating a low-to-moderate level of among-population genetic differentiation. Although a relatively large proportion of diversity was attributed to within-population variation, low diversity within populations (mean genetic diversity within populations (H_S) = 0.19) was observed. Both STRUCTURE and unweighted pair group method with arithmetic mean (UPGMA) dendrograms exhibited the clustering of populations that inhabit the same geographic region, and four clusters correlated with four geographic regions, which might be attributed to insect pollination, small population size, and environmental conditions in different habitats. These results function as an essential step towards better conserving and utilizing wild *K. latifolia* resources, and hence promoting its genetic improvement and breeding for adaptability to southeastern environmental conditions.

Keywords: genetic diversity; genetic differentiation; population structure; germplasm resources; breeding for adaptability; ornamental

1. Introduction

The genus *Kalmia* is a member of *Ericaceae* along with genera *Rhododendron* and *Vaccinium*. It consists of seven species that primarily distribute in temperate zones. Most of them are shrubs and subshrubs, some are herbs, and a few are trailing vines [1]. *Kalmia latifolia* L. (mountain laurel, 2n = 2x = 48) is the most famous species in genus *Kalmia* [1]. It is an outstanding spring–summer blooming shrub and a promising ornamental in the eastern United States [2].

Since the early 1960s, the breeding of *K. latifolia* has led to the release of various new cultivars. More than 140 cultivars have been documented by The European Kalmia Society (http://www.kalmia-society.org/cultivars.php?&lang=gb), of which about 30 obtained popularity because of their attractive characteristics and superior landscape performance. Efforts from breeders (Dr. Richard Jaynes at Broken Arrow Nursery (Hamden, CT) who devoted himself to genus *Kalmia* for more than 40 years, for instance) have notably increased the availability of cultivars and market shares of *K. latifolia* in the northeastern U.S. It hence has become a popular ornamental plant in the northeastern region and was elected as the state flower of Connecticut [1]. Conversely, the low tolerance of these *K. latifolia* cultivars to heat and intense light has limited their acceptance in areas of USDA zone 7, in the southeastern U.S. in particular [3]. In the southeastern U.S., few cultivars are available on the market, and those cultivars are principally produced by niche Ericaceous nurseries. Although being produced in a few southeastern nurseries, *K. latifolia* is rarely utilized in southeastern landscapes. In order to enhance production and utilization of *K. latifolia* in the southeastern U.S., the breeding of improved plants that have adaptability to southeastern environmental conditions is urgently needed.

Historically, wild plant resources have been involved in breeding programs worldwide to introduce desirable traits and increase genetic diversity, and therefore aid plant improvement [4]. In the United States, *K. latifolia* is naturally distributed in the east, specifically from southern Maine (USDA Zone 4a) west through southern New York (4a) to central Ohio (6a), south to eastern Louisiana (8b), southern Mississippi (8b), Alabama (8b), Georgia (8b), and northwestern Florida (8b) [1]. *K. latifolia* plants in wild populations, particularly southeastern populations, are valuable resources for developing improved cultivars. Thus, collecting and conserving wild *K. latifolia* plants has been considered as the foundation of breeding for adaptability to southeastern environmental conditions [5]. Due to the consideration of lowering facility and labor cost, it is always desired to minimize the quantity of plants collected and conserved and meanwhile maximize the genetic diversity that collection may provide [6]. Genetic diversity and population structure studies are therefore urgently needed for efficient *K. latifolia* germplasm collection and conservation. Additionally, yet as a cross-pollinating species, *K. latifolia* was found to be able to self-pollinate, while the significant reduction in survival and vigor of seedlings from self-pollinated plants indicated inbreeding depression in *K. latifolia* [7]. From a breeders' standpoint, in order to prevent inbreeding depression as well as maximize genetic variations among progenies, individuals that are genetically distinct should be used as parents in cross hybridization. Genetic relationship information will therefore tremendously benefit breeding programs by providing a guidance for effectively utilizing wild *K. latifolia* plants as parental materials.

Many studies have been carried out to assess the genetic diversity of wild populations of genus *Rhododendron* that are closely related to *K. latifolia* (genus *Rhododendron* and *Kalmia* are members of *Ericoideae*, a subfamily of *Ericaceae*) in recent years. The genetic diversity of *Rhododendron* was principally attributed to within-population variation (62–83%), while a low level of genetic diversity was observed among populations [8–10]. These results illuminated the importance of individual plants within a population and revealed the levels and patterns of genetic variation for *Rhododendron*, which facilitate cultivar development by efficiently maximizing genetic diversity in both conservation and cross hybridization. Similar studies on *K. latifolia* are needed as an essential step toward better collecting, conserving, and utilizing wild resources.

Molecular markers have been widely used to elucidate genetic diversity in woody species due to the reliability and efficiency [11–14]. The inter simple sequence repeat (ISSR) markers detect polymorphisms in inter-microsatellite loci and can produce a much greater number of fragments per primer, with the advantages of high reproducibility and relatively low cost. Moreover, ISSR exists regardless of the availability of information about the genome sequence [15]. Therefore, ISSR has been employed in *Cunninghamia lanceolata* [16], *Prunus* spp. [17], *Quercus susber* [18], *Haloxylon salicornicum* [19], and *Rhododendron* spp. [20,21] to reveal genetic variation and population structure. Such information played fundamental roles in establishing an effective strategy for conservation and breeding purposes. In the present study, ISSR markers were employed to assess (1) the levels of the genetic diversity within

and among eight investigated *K. latifolia* populations in the eastern U.S., (2) the degree of genetic differentiation among populations, and (3) the genetic relationship and population structure of 48 wild *K. latifolia* individuals sampled from eight populations. The results provide guidance for efficient germplasm conservation and utilization, and thus promote the breeding of *K. latifolia* plants that have adaptability to southeastern environmental conditions.

2. Materials and Methods

2.1. Plant Materials and Collection

K. latifolia naturally distributes in the eastern U.S. (Figure 1). Wild populations were identified in the native range, and eight populations with a minimum of 15 individuals were selected for sampling (Figure 1, Table 1). In each population, a random sample of six individual plants was obtained. Individuals were evenly distributed throughout the population and were at least 10 m away from each other. The youngest leaves were individually collected from six sampled plants and placed in six envelopes. Envelops were carefully labeled and placed in a heavy-duty zip-lock bag filled with 100 g silica gel. Leaf tissues were maintained in the bag until being transported back to the laboratory and stored at −80 °C. Genomic DNA was extracted from 100 mg leaf tissue using Plant DNAzol® Reagent (Invitrogen, Carlsbad, CA) following manufacturer's protocols. The DNA samples were diluted to 10 ng·μL^{-1} and then stored at −20 °C until further analysis.

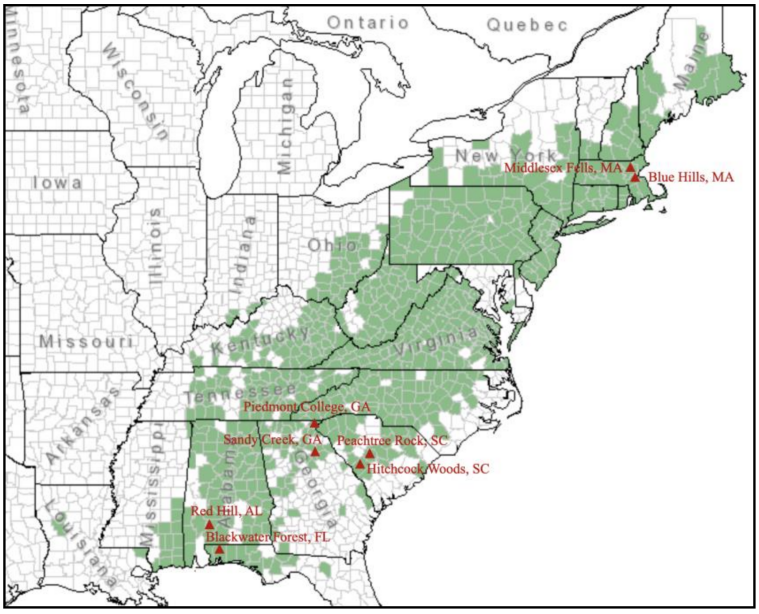

Figure 1. Natural distribution of *K. latifolia* and geographic location of eight investigated populations in the eastern United States.

Table 1. Geographic information on eight investigated populations of *K. latifolia*.

Population	Location	Latitude	Longitude	Sample nos.
Middlesex Fells (MF)	Middlesex, Massachusetts	42°26′51″ N	71°5′26″ W	MA1-6
Blue Hills (BH)	Norfolk, Massachusetts	42°12′55″ N	71°3′3″ W	MA7-12
Peachtree Rock (PR)	Lexington, South Carolina	33°49′47″ N	81°11′57″ W	SC1-6
Hitchcock Woods (HW)	Aiken, South Carolina	33°33′9″ N	81°44′33″ W	SC7-12
Red Hill (RH)	Monroe, Alabama	31°44′17″ N	87°21′30″ W	AL1-6
Blackwater Forest (BF)	Santa Rosa, Florida	30°47′23″ N	86°47′33″ W	FL1-6
Piedmont College (PC)	Rabun, Georgia	34°51′50″ N	83°23′16″ W	GA1-6
Sandy Creek (SC)	Clarke, Georgia	34°0′58″ N	83°22′32″ W	GA7-12

2.2. ISSR Procedure

Cultivars "Olympic Fire", "Ostbo Red" (maternal parent of "Olympic Fire"), and "Starburst" were used to screen 96 ISSR primers designed by the University of British Columbia (UBC 801-896). Eight primers were selected based on the strong, clear, reproducible, and polymorphic banding patterns they produced. ISSR-PCR amplifications were performed in a total volume of 20 µL consisting of 2 µL (20 ng) template DNA, 2 µL primer, 10 µL master mix (Applied Biosystems, Foster city, CA, USA), and 6 µL double-distilled water. The amplifications were programmed in a Mastercycler nexus gradient (Eppendorf, Hamburg, Germany) under following conditions: an initial denaturation step at 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 52 °C for 50 s, and 72 °C for 120 s; followed by an extension for 7 min at 72 °C. Samples were then cooled to 4 °C and placed in a refrigerator until being loaded into gels. The PCR products were electrophoresed on 1.2% (*w*/*v*) agarose gels that, in 0.5 × TBE buffer solution (Fisher Scientific, Fair Lawn, NJ, USA) (80 V for 3.5 h) and stained with 0.5 $mg{\cdot}L^{-1}$ ethidium bromide solution (Sigma-Aldrich, St. Louis, MO, USA). 100-bp DNA ladder (Invitrogen, Carlsbad, CA, USA), were loaded onto the outside two lanes of each gel to estimate the size of amplified fragments. Gels were visualized and photographed using a BioDoc-It^{TM} Imaging System (UVP, Upland, CA, USA).

2.3. Data Analysis

ISSR amplified fragments were scored based on the presence (1) or absence (0) for each sample, and values recorded in Excel. POPGENE version 1.32 [22] were used to calculate the following parameters: total number of observed alleles (Na), mean observed number of alleles (Noa), mean effective number of alleles (Nea), Nei's gene diversity (h), Shannon's information index (I), percentage of polymorphic bands (PPB) for each population, Nei's unbiased genetic identity and distance [23], the total genetic diversity (H_T), mean genetic diversity within populations (H_S), coefficient of genetic differentiation among populations [$G_{ST} = (H_T\text{-}H_S)/H_T$], and pairwise G_{st} between populations [24]. Analysis of molecular variance (AMOVA) was employed to determine the partitioning of genetic variance within and among populations using Arlequin version 3.5.2.2 [25]. The significance of this F-statistic was tested with 1000 random permutations. The average genetic distance (AD) of each individual was firstly calculated. A group of genetically distinct individuals was subsequently identified by a means of iteration through the stepwise selection (proportional increment of 0.1 with 9 steps) of individuals with the highest AD values and AMOVA for the assessment of the change of genetic differentiation in the selected individuals using AveDissR [26]. Additionally, PCA was performed on a pairwise genetic distance matrix using AveDissR. Eigenvectors were obtained using "vegan" cmdscale, and the first two principal components were selected for a scatter plot. The generated PCA plot of the genetic associations between the identified group and original samples was used to assess the representativeness of the selected individuals [26].

The genetic structure of the investigated populations was analyzed using STRUCTURE version 2.3.4 [27]. The number of discontinuous K was estimated from two to ten with 20 replicates; both the value of Markov chain Monte Carlo (MCMC) and the length of burn-in period were set to 100,000 times. Subsequently, structure harvester (http://taylor0.biology.ucla.edu/structureHarvester) [28] was used to harvest the optimum number of cluster (K) according to the greatest delta K value. CLUMPAK was then used to perform repeated sampling analysis and generate genetic structural plot [29]. In this approach, each sample was assigned to populations (named RPs and MIX) according to its maximum membership coefficient (Q value) using a threshold value of 0.65 based on the optimum K value. Each sample was assigned to RPs if its Q value was above 0.65; otherwise, it was grouped into MIX [30]. An unweighted pair group method with arithmetic mean (UPGMA) dendrogram, based on Nei's unbiased genetic distance, was generated to determine the relationships among all individuals using NTSYS-pc version 2.10e [31]. Additionally, Mantel tests were conducted between the matrices of geographic distance and pairwise G_{st}, geographic distance and Nei's unbiased genetic distance, and Nei's unbiased genetic distance and pairwise G_{st} using R, respectively.

3. Results

3.1. ISSR Polymorphism

Ninety-six ISSR primers were screened in this study, and eight primers were employed to assess the genetic diversity and structure of *K. latifolia* in the eastern U.S. based on the strong, clear, reproducible, and polymorphic banding patterns they produced. A total of 116 bands were generated in 48 *K. latifolia* individuals by eight ISSR markers, of which 105 (90.52%) were polymorphic. The number of bands amplified per primer ranged from 10 to 19, with an average of 14.5 (Table 2). The molecular weight of bands was found between 150 and 2000 base pairs (bps).

Table 2. Total number of bands, number of polymorphic bands, and percentage of polymorphic bands (PPB) generated by eight inter simple sequence repeat (ISSR) primers across 48 sampled *K. latifolia* individuals.

ISSR Primers	Sequence (5′ – 3′)	Total Number of Bands	Number of Polymorphic Bands	PPB
UBC808	(AG)8C	14	13	92.86%
UBC814	(CT)8A	10	8	80%
UBC835	(AG)8YC	13	12	92.31%
UBC836	(AG)8YA	16	13	81.25%
UBC841	(GA)8YC	13	11	84.62%
UBC856	(AC)8YA	15	14	93.33%
UBC864	(ATG)6	19	19	100%
UBC873	(GACA)4	16	15	93.75%
Total		116	105	90.52%

3.2. Genetic Diversity

Among eight investigated populations, the percentage of polymorphic band (PPB) ranged from 40.52% to 56.03% (Table 3). With the largest PPB, Piedmont College population also had the largest number (97) of observed alleles (Na), Nei's gene diversity (h) of 0.2213, and Shannon's information index (I) of 0.3249, while Blue Hills, with the smallest PPB, showed the smallest Na (78), h of 0.1485, and I of 0.2224. The overall within-species genetic diversity results were PPB = 90.52%, h = 0.3089, and I = 0.4654, assessed by ISSR analysis.

Table 3. Genetic diversity of *K. latifolia* populations analyzed using ISSR primers.

Population	Na	Noa	Nea	h	I	PPB
MF	81	1.4569	1.3130	0.1801	0.2648	45.69
BH	78	1.4052	1.2485	0.1485	0.2224	40.52
PR	89	1.5517	1.3812	0.2174	0.3194	55.17
HW	90	1.5086	1.3362	0.1949	0.2884	50.86
RH	87	1.5086	1.3239	0.1906	0.2837	50.86
BF	94	1.5431	1.3357	0.2007	0.3000	54.31
PC	97	1.5603	1.3881	0.2213	0.3249	56.03
SC	90	1.4741	1.2981	0.1762	0.2629	47.41
Total	116	1.9052	1.5191	0.3089	0.4654	90.52

Na = total number of observed alleles, Noa = mean observed number of alleles, Nea = mean effective number of alleles, h = Nei's gene diversity, I = Shannon's information index, and PPB = percentage of polymorphic bands.

Both Nei's genetic differentiation and AMOVA analysis were performed to investigate the distribution of genetic diversity within and among populations. The total genetic diversity at the species level (H_T) and average genetic diversity within populations (H_S) assessed by ISSR were 0.3089 and 0.1912, respectively. The coefficient of genetic differentiation (G_{ST}) for eight populations was estimated as 0.3809, which indicated that 38.09% of the total genetic diversity was distributed among

populations (Table 4). Pairwise G_{st} between populations varied from 0.1818 to 0.3586, with smaller values between populations in a geographic group (0.1818–0.2023), while larger values between populations inhabited different geographic regions (0.2049–0.3586) (Tables 4 and 5). The AMOVA analysis revealed that 29.54% ($P < 0.001$) of genetic variation occurred among populations, while 70.46% ($P < 0.001$) of variation was observed within populations (Table 6), which was consistent with the results of Nei's genetic differentiation analysis.

Table 4. Analysis of Nei's genetic differentiation among eight *K. latifolia* populations.

Population	H_T	H_S	G_{ST}
Massachusetts group (MF and BH)	0.2059	0.1643	0.2023
South Carolina group (PR and HW)	0.2523	0.2062	0.1827
Southernmost group (RH and BF)	0.2391	0.1956	0.1818
Georgia group (PC and SC)	0.2474	0.1988	0.1965
Total	0.3089	0.1912	0.3809

H_T = total genetic diversity, H_S = mean genetic diversity within populations, and G_{ST} = coefficient of genetic differentiation.

Table 5. Pairwise G_{st} for eight *K. latifolia* populations.

Population	MF	BH	PR	HW	RH	BF	PC	SC
MF								
BH	0.2023							
PR	0.2095	0.2554						
HW	0.2699	0.3252	0.1827					
RH	0.2925	0.3426	0.2420	0.2452				
BF	0.2656	0.3177	0.2392	0.2761	0.1818			
PC	0.2049	0.2431	0.2083	0.2347	0.2675	0.2356		
SC	0.3155	0.3586	0.2541	0.2733	0.3142	0.3039	0.1965	

Table 6. Analysis of molecular variation (AMOVA) for eight *K. latifolia* populations.

Source of Variation	d.f.	Sum of Squares	Variance Component	Percentage of Variance	*P*
Among populations	7	327.542	5.58	29.54	<0.001
Within populations	40	532.333	13.31	70.46	<0.001
Total	47	859.875	18.89		

3.3. Population Structure

An admixture model-based approach was performed to evaluate the population structure of 48 *K. latifolia* samples. The optimal cluster value (K) of sampled individuals was four, with the highest values of both LnP(K) (log probability of data, - 3417.37) and delta K (4.24) obtained from the structure harvester (Figure 2a,b). Based on K of four, a bar plot of estimated membership coefficients of each sample was displayed in Figure 2c. Each sample was represented by a vertical line, and each color displayed the proportion of membership of each sample to the four clusters. The individual with the maximum membership coefficient (Q value) higher than 0.65 was recognized as a pure one and lower than 0.65 was an admixture one. In the analysis, 45 individuals were relatively pure and the other three were more complex. The blue cluster consisted of 12 Massachusetts pure individuals, and the green cluster included 12 pure individuals sampled from the southernmost range. The orange and purple group comprised 12 South Carolina individuals with 11 pure and one admixture one and 12 Georgia individuals with 10 pure and two admixture ones, respectively.

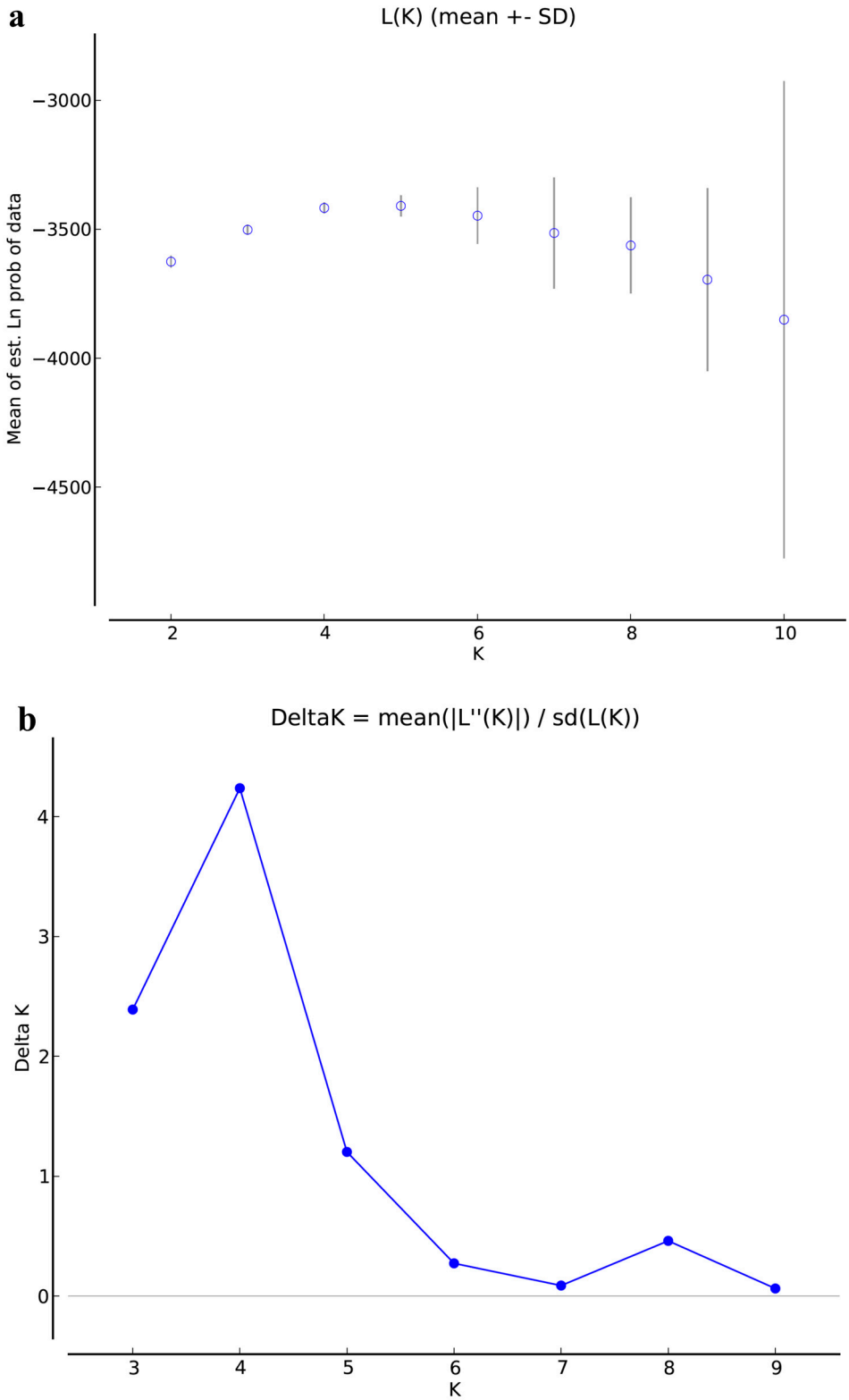

Figure 2. *Cont.*

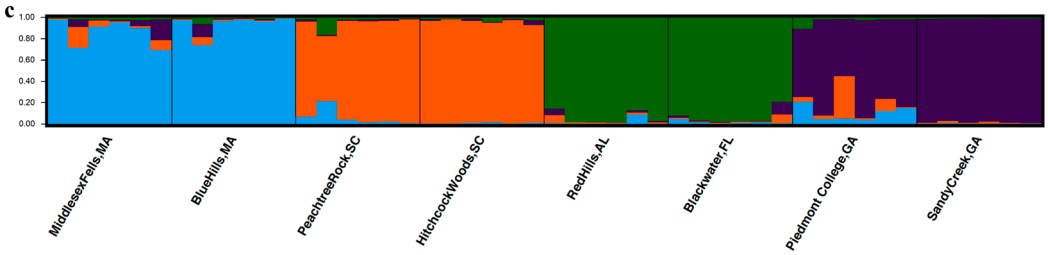

Figure 2. Population structure of 48 *K. latifolia* samples based on ISSR data. (**a**) Estimation of population structure using mean of estimated log probability of data (LnP(K)) with cluster value (K) ranging from 2–10. (**b**) Estimation of population using delta K with cluster value (K) ranging from 2–10. (**c**) Four estimated clusters (K = 4) of 48 *K. latifolia* samples are presented in different colors inferred by STRUCTURE analysis. Four clusters are presented by blue, orange, green, and purple, respectively. Each sample (represented by a vertical bar) is partitioned into colored segments representing the estimated membership coefficients (Q values).

Nei's unbiased genetic distance between populations ranged from 0.0868 to 0.2201, with smaller distance (0.0868–0.1061) between populations in the same geographic group than that between populations inhabiting different geographic regions (0.1151–0.2201) (Table 7). The UPMGA dendrogram was then generated based on Nei's unbiased genetic distance between *K. latifolia* individuals, which showed similar results to the STRUCTURE analysis (Figure 3). Six individuals within the population were clustered together. Meanwhile, populations located in a geographic region were grouped into a cluster and four clusters correlated with four geographic regions were observed. Average genetic distance (AD) of 48 sampled individuals varied from 0.2777 to 0.3650 (Figure 4a). Nine groups were then generated through stepwise selection, which included 5, 9, 14, 18, 25, 30, 35, 40, and 45 individuals with the highest AD values, respectively. By assessing the changes in genetic differentiation with the increased selection of individuals with the highest AD values using AMOVA, 40 individuals (in the eighth step) were identified as the genetically distinct ones because they largely maintained the extent of genetic differentiation (percentage of variance among populations of 29.33%) in 48 original samples (29.54%). The PCA plot of the genetic associations between 40 selected and 48 original individuals showed the representativeness and genetic distinctness of selected individuals (Figure 4b).

Table 7. Nei's unbiased genetic distance (below diagonal) and geographic distance (km) (above diagonal) between *K. latifolia* populations.

Population	MF	BH	PR	HW	RH	BF	PC	SC
MF		29.45	1533.70	1594.86	2158.12	2203.19	1593.25	1667.28
BH	0.0868		1528.87	1590.03	2153.30	2198.36	1588.42	1662.45
PR	0.1178	0.1451		64.86	708.11	711.33	271.98	238.18
HW	0.1659	0.2033	0.0994		645.35	646.95	251.06	191.51
RH	0.1875	0.2201	0.1550	0.1466		147.74	597.07	534.30
BF	0.1647	0.1993	0.1574	0.1857	0.0920		626.03	550.39
PC	0.1151	0.1354	0.1341	0.1514	0.1859	0.1554		114.91
SC	0.2031	0.2263	0.1597	0.1670	0.2098	0.2117	0.1061	

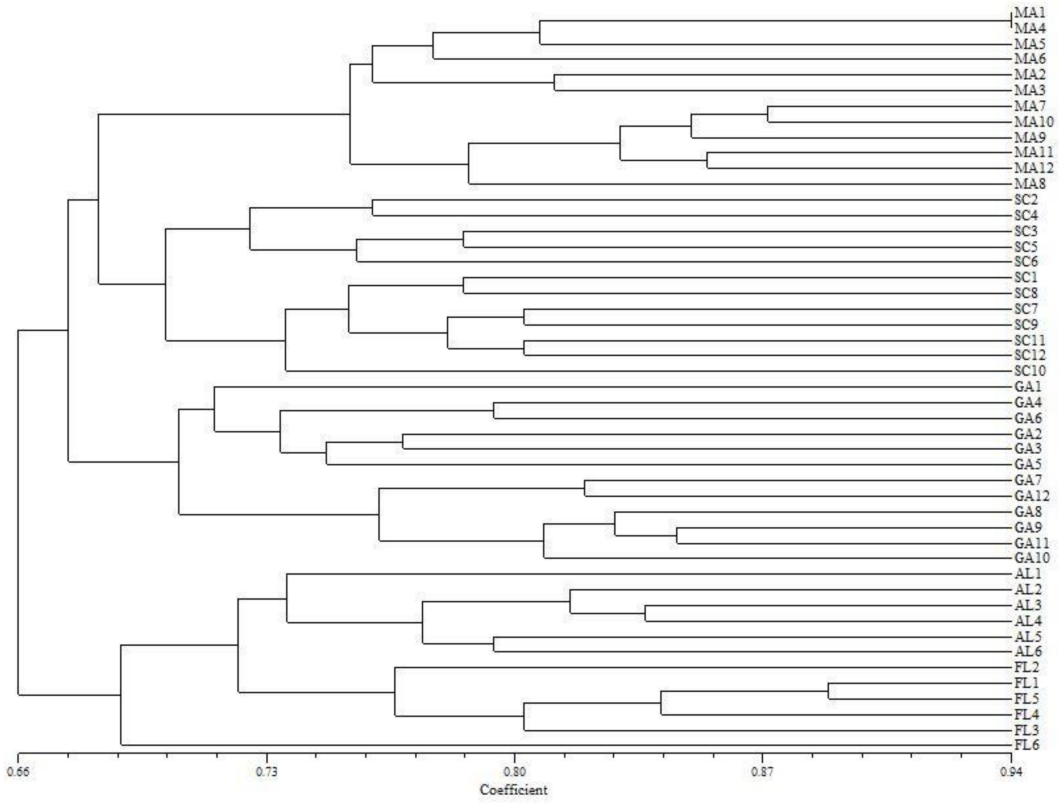

Figure 3. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram of 48 *K. latifolia* individuals (six individuals sampled from each of eight populations) constructed based on Nei's unbiased genetic distance matrix. Six individuals within the population were clustered together. Meanwhile, populations located in a geographic region were grouped into a cluster and four clusters correlated with four geographic regions were observed.

Mantel tests between the matrices of geographic distance and pairwise G_{st} (Figure 5a), geographic distance and Nei's unbiased genetic distance (Figure 5b), and Nei's unbiased genetic distance and pairwise G_{st} (Figure 5c) were subsequently performed, respectively. The results indicated that the correlation between geographic distance and pairwise G_{st} ($r^2 = 0.5646$, $P = 0.0004$) as well as between geographic distance and genetic distance ($r^2 = 0.4715$, $P = 0.0166$) were not as significant as that between genetic distance and pairwise G_{st} ($r^2 = 0.9629$, $P = 0.0001$).

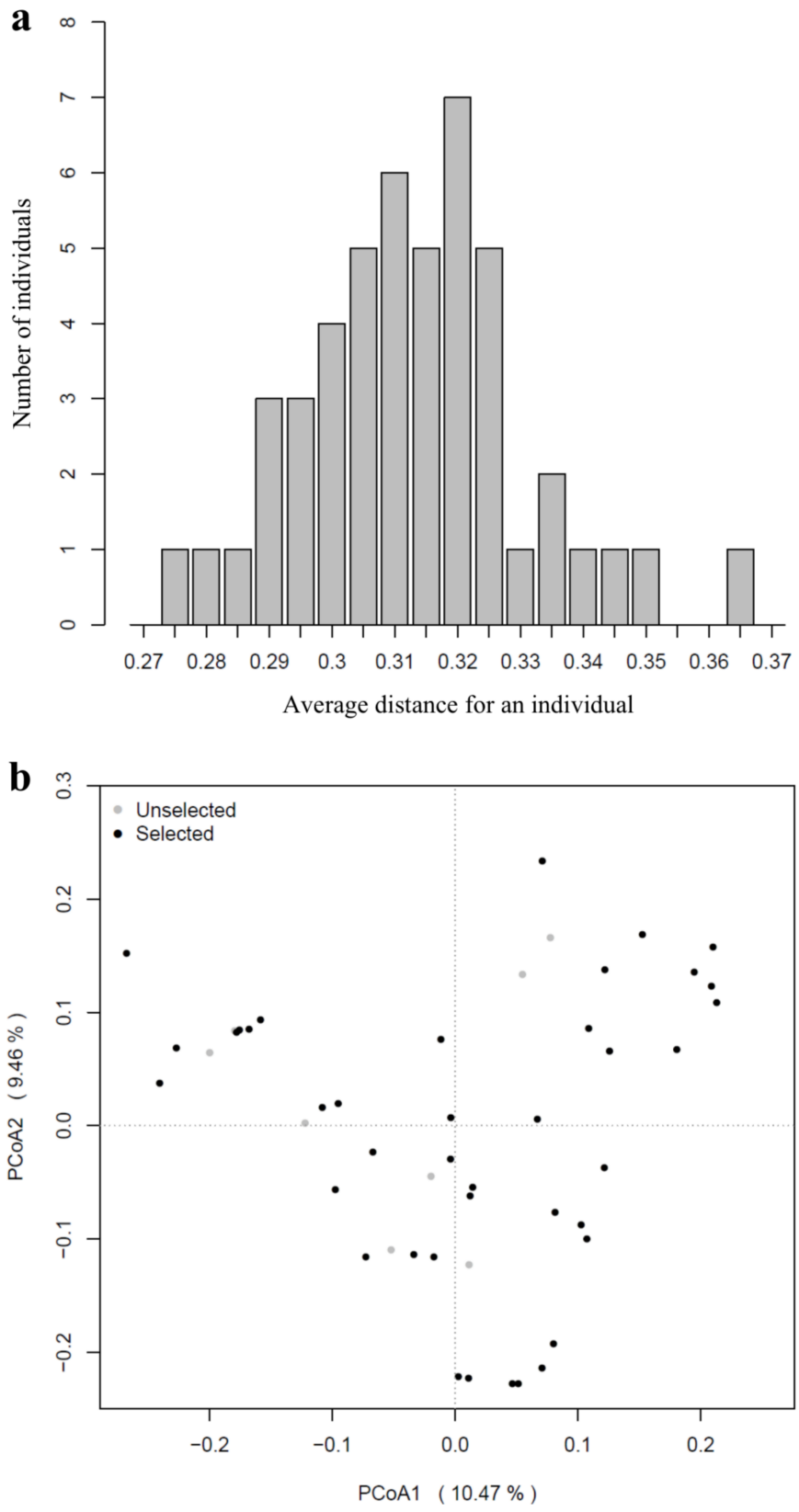

Figure 4. Genetic distinctness of 48 *K. latifolia* individuals assessed using AveDissR. (**a**) Average distance (AD) frequency distribution in 48 individuals. (**b**) PCA plot for the genetic association of 40 individuals selected based on the highest AD values and original 48 individuals.

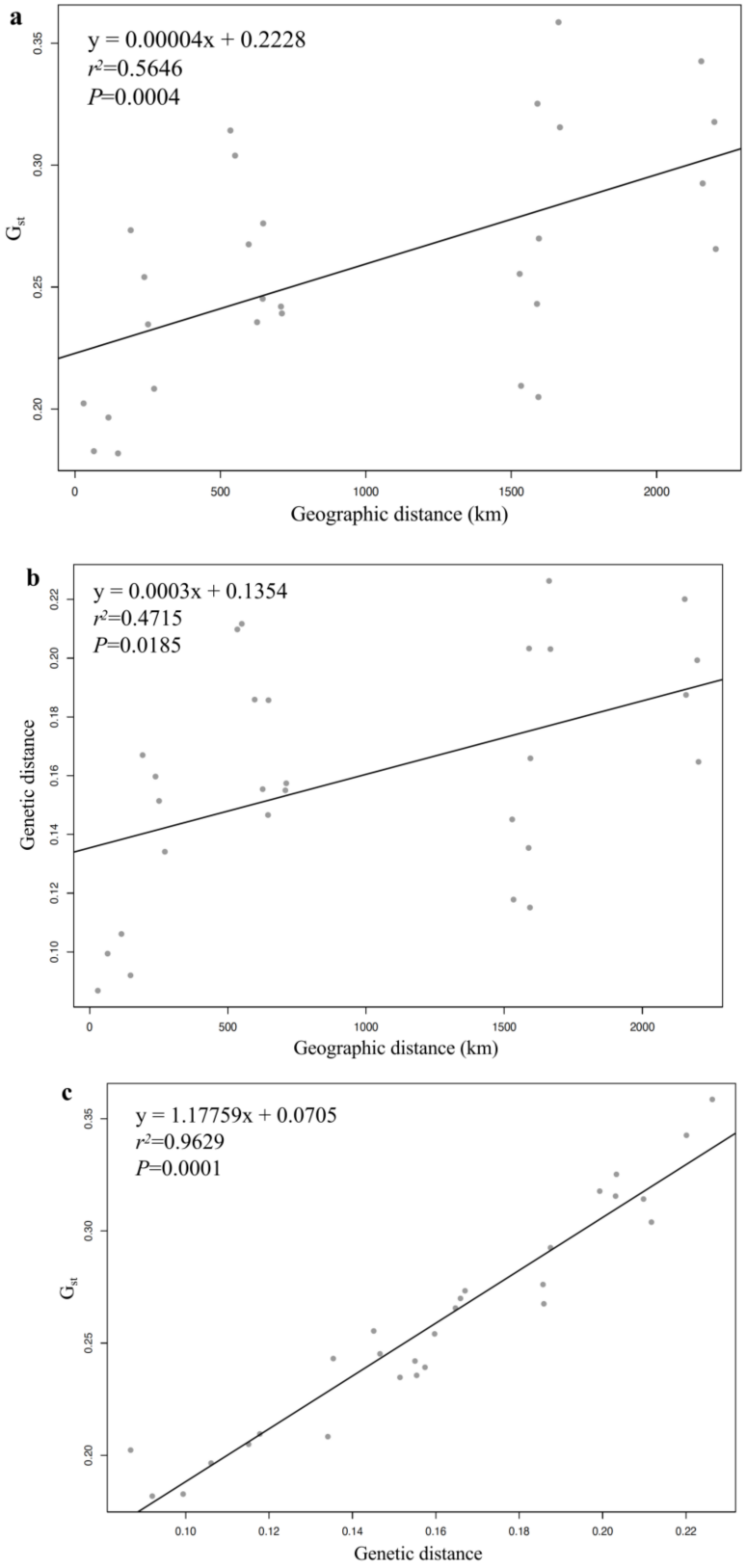

Figure 5. Mantel tests between the matrices of geographic distance and pairwise G_{st} (**a**), geographic distance and Nei's unbiased genetic distance (**b**), and Nei's unbiased genetic distance and pairwise G_{st} (**c**) for eight *K. latifolia* populations.

4. Discussion

4.1. Effectiveness of ISSR in K. latifolia

Although the reduced accuracy of estimation of genetic diversity using dominant markers was reported, it has been well documented that ISSR is a reliable technique for detecting DNA polymorphisms and assessing genetic diversity of many woody plant species [16–18]. Even though no ISSR analysis study has been carried out on *K. latifolia*, numerous similar researches have been conducted on other two genera, *Rhododendron* and *Vaccinium*, in Ericaceae. Zheng et al. found that 13 ISSR primers amplified 106 fragments in *Rhododendron hybrdum* in total, of which 85.48% were polymorphic, and each primer produced 9.5 bands in average [21]. In nine cold-hardy *Rhododendron* spp., thirteen ISSR primers amplified fragments between 300 and 2000 bps, and 90.63% of them were polymorphic [20]. In blueberries, six ISSR primers generated 87 bands, of which 80.4% were polymorphic [32]. Similar to the results reported by these studies, ISSR produced reproducible and highly polymorphic fragments in present study, which indicated that ISSR was a reliable and effective technique to amplify the loci between microsatellites in *K. latifolia*.

4.2. Genetic Diversity and Differentiation of K. latifolia

Genetic diversity in wild plant species is generally related to life form, mating/breeding system, seed dispersal, geographic range, and population size. In this study, ISSR analysis of eight investigated *K. latifolia* populations revealed a high level of genetic diversity at the species level (h = 0.3089, I = 0.4654, PPB = 90.52%) (Table 3). The considerable existing genetic diversity in *K. latifolia* could be predominantly explained by life form, breeding system, and geographic range. Generally, long-lived perennial species having a mixed breeding system and regional distribution have relatively high genetic diversity [33]. *K. latifolia* is a long-lived, perennial, evergreen shrub that is regionally distributed in the eastern U.S. It is a cross-pollinating species that relies on insects, bumble bees in particular, for pollination [1]. Anthers eventually release without a visitor at the end of floral life, and this autonomous selfing assures reproduction in the absence of pollinators. Hence, *K. latifolia* has been considered as a species with predominant out-crossing and low selfing ability [34]. This association has resulted in a high level of within-species genetic diversity of *K. latifolia*. The observed high genetic diversity indicated that *K. latifolia* should be able to adapt to environmental changes, and thus being able to distribute along different latitudes.

For dominant markers, the proportion of genetic diversity attributed to among-population variation (G_{ST} = (H_T-H_S)/H_T) has been widely used to estimate genetic differentiation among populations [35,36]. G_{ST} of eight *K. latifolia* populations was 0.38, which indicated that a low-to-moderate proportion of total genetic diversity was observed among populations (38.09%) whereas a relatively high level of diversity was found within populations (61.91%) (Table 4). The low G_{ST} values have been found in *Rhododendron* spp. (0.37 in *R. aureum*, 0.26 in *R. prunifolium*) that closely related to *K. latifolia* and other out-crossing species (mean G_{ST} of 0.22) [8,10,36]. Because of the high potential for pollen dispersal, out-crossing species tend to have higher level of gene flow among populations, which leads to a similar complement of alleles in similar frequencies and thus a low level of genetic diversity observed at the population level [35], whereas compared with other out-crossers, the level of genetic differentiation among *K. latifolia* populations was relatively higher [36], which might have been due to geographic distribution pattern of *K. latifolia*. Wild *K. latifolia* plants have been restricted in the eastern U.S. while distributed over a wide latitudinal range. Different phenological phases caused by temperature, light, and other environmental factors between different latitudinal populations have led to reduced pollination across them. For instance, *K. latifolia* populations at the southernmost range usually have a flowering time one month earlier than populations in Massachusetts according to our investigation. This gap in blooming period, coupled with the large geographic distance, could prevent insect pollination [37]. Therefore, a relatively higher level of among-population differentiation was observed on *K. latifolia* than other out-crossing species. Geographic distribution pattern could also

account for the difference in pairwise G_{st} between populations. Due to limited barriers and phenotypical differences between populations within in a geographic group, insect pollination could take place easily between them, resulting in more frequent gene flow and hence smaller G_{st} values (0.1818–0.2023) than that between populations located at different geographic regions (0.2049–0.3586) (Tables 4 and 5) [38]. AMOVA results (Table 6) indicated a small proportion of variation observed among populations (29.54%; $P < 0.001$) and a high level of variation within populations (70.46%; $P < 0.001$), which was consistent with observed G_{ST} value (0.38). The relatively low G_{ST} value corresponding with the low level of genetic variation among populations revealed by AMOVA elucidated that individuals within populations tended to be genetically different, but each population comprised a similar complement of alleles in similar frequencies [38].

One of the most commonly employed methods to estimate within-population diversity is H_S, Nei's average genetic diversity within populations. In contrast to the proportion of genetic diversity partitioned among and within populations estimated by G_{ST} and AMOVA, H_S is a direct measure of average diversity within populations [36]. H_S of eight *K. latifolia* wild populations was 0.1912 (Table 4), which was lower than that of other out-crossing species (mean $H_S = 0.27$) [36] yet reasonable. One likely cause for overall lower diversity within populations was small population size. A trend toward lower genetic diversity for smaller populations was illustrated by previous studies [8,10,39]. The populations surveyed in present study inhabit a relatively small geographic area. Differing from larger populations, insects could cover the entire population and easily transport pollen among all individuals of the population, leading to gene flow among individuals and thus significant reductions in heterozygosity. In addition to population size, the capability of selfing of *K. latifolia* might be a factor causing the lower diversity within populations, compared with other out-crossers, supported by the H_S approaching that of species having mixed breeding system (mean $H_S = 0.18$) [36]. Some extent of inbreeding due to small population size and selfing ability could increase the expression of recessive deleterious alleles and result in inbreeding depression in individuals that were likely to be eliminated, which could reduce the genetic diversity within populations [10]. The difference in within-population genetic diversity was observed among populations, which might be predominantly accounted by population size. The lowest genetic diversity was observed in Blue Hills population ($h = 0.1485$, $I = 0.2224$), which was the smallest of eight investigated populations (<20 individuals), followed by Sandy Creek population, where approximately 20 individuals formed a small patch (Table 3). In contrast, Piedmont College population, which inhabits the largest geographic area, exhibited the highest genetic diversity ($h = 0.2213$, $I = 0.3249$).

4.3. Population Structure of K. latifolia

The population structure of 48 *K. latifolia* individuals assessed by STRUCTURE indicated four clusters corresponding with four geographic regions (Figure 2c). Among 48 individuals, 45 were relatively pure according to $Q > 0.65$ as the pure standard. The other three, including two individuals sampled from Piedmont College and one from Peachtree Rock population, were more complex, which suggested that they were genetically admixed. Individuals within populations tended to be genetically diverged, resulting in higher genetic diversity in Piedmont College ($h = 0.2213$, $I = 0.3249$) and Peachtree Rock population ($h = 0.2174$, $I = 0.3194$), compared with other six populations (Table 3). Nei's unbiased genetic diversity and UPGMA dendrogram revealed the population structure that was consistent with STRUCTURE result. Smaller Nei's unbiased genetic distance was observed between populations in a geographic group (0.0868-0.1061) and compared with that between populations inhabiting different geographic regions (0.1151–0.2201) (Table 7). For instance, two populations in South Carolina, Peachtree Rock, and Hitchcock Woods had the genetic distance of 0.0994, while Hitchcock Woods population was 20.33% genetically different to Blue Hills population in Massachusetts. Correspondingly, UPGMA dendrogram revealed four clusters, which were correlated with four geographic regions (Figure 3).

The population structure of *K. latifolia* was found to be correlated with its geographic distribution, which however might not be directly attributed to geographic distance between these populations. Mantel tests revealed positive relationship between pairwise G_{st} and geographic distance ($r^2 = 0.5646$, $P = 0.0004$), as well as between genetic distance and geographic distance ($r^2 = 0.4715$, $P = 0.0166$), indicating that genetic differentiation and genetic distance were not significantly correlated with geographic distance between populations. It has been concluded in previous studies that there was no direct correlation between the geographic distance between populations and the distribution of genetic diversity [30,35,40]. As the geographic region correlated with the genetic structure found on *Rhododendron* spp. in the eastern U.S. [8], several factors related to breeding system, population size, and environmental conditions most likely led to this population structure of *K. latifolia*. Cross pollination of *K. latifolia* relies on insects. Small size of investigated populations in the study allowed insects to cover all individuals throughout the entire population, so relatively smaller genetic distances were observed among individuals within the population and they were clustered together. In general, environmental conditions, including temperature, precipitation, light, and soil, gradually vary as the geographic distance between populations increases. Due to insect pollination and similar environmental conditions, out-crossing can easily occur between populations that distribute in a geographic region. Frequent gene flow led to low level of genetic differentiation and small genetic distance between them [38]. By contrast, gene flow between populations inhabiting different geographic regions was reduced because of different phenological phases (e.g., blooming time) caused by different environmental conditions [37]. In addition to limited gene flow, the result of plants' adaptations to different growing conditions might also account for the higher level of genetic differentiation and larger genetic distance between populations that distribute in different geographic regions. Hence, both STRUCTURE and UPMGA analysis suggested that populations in the same geographic region were less genetically different and fell into a cluster, and eight investigated *K. latifolia* populations were classified into four clusters correlated with four geographic regions (Figures 2c and 3).

4.4. Conservation and Breeding Strategy

Four points have been illustrated by present study in relation to the conservation and breeding of *K. latifolia*. (1) High genetic diversity was observed at the species level, which indicated that *K. latifolia* could adapt to environmental changes and would thus function as a valuable resource for breeding for adaptability to different environmental conditions. (2) In general, decreased levels of genetic diversity within populations might affect population viability in short term by reducing individual fitness, and could also influence long-term persistence by reducing adaptability to changing environmental conditions [39]. The low genetic diversity within populations ($H_S = 0.1912$) observed on *K. latifolia* in the eastern U.S. indicated the potential risk of population declines, which illuminated the importance of collecting and conserving its wild genetic resources. (3) A low-to-moderate level of among-population differentiation of *K. latifolia* revealed by Nei's genetic differentiation (38.09%) and AMOVA analysis (29.54%) (Tables 4 and 6) elucidated that collecting individuals from populations across wide geographic ranges might not further increase genetic diversity in its conservation and breeding program, while the individuals that tended to genetically diverge, which could be identified via STRUCTURE analysis (Figure 2c), need to be conserved and utilized for maximizing genetic diversity [17]. (4) The most genetically redundant individuals were revealed according to their lowest average genetic distance values, and removing them from sampled individuals did not show any large changes from the original genetic differentiation (Figure 4). This information could aid in identification and exclusion of redundant resources, and thus improve the efficiency of collecting, conserving, and utilizing *K. latifolia* genetic resources.

5. Conclusions

Genetic diversity and population structure information is essential for both conservation and breeding program of *K. latifolia*. ISSR markers exhibited a high level of efficiency in assessing genetic

diversity and structure of *K. latifolia* populations in the eastern U.S. The observed high genetic diversity at the species level indicated that *K. latifolia* could adapt to environmental changes; wild *K. latifolia* would thus function as a valuable genetic resource for breeding for adaptability to various environmental conditions. In terms of genetic differentiation, a low-to-moderate proportion of diversity was observed among populations due to factors related to breeding system and geographic distribution pattern, which elucidated that collecting individuals from populations across wide geographic ranges might not further increase genetic diversity in *K. latifolia* conservation and breeding programs. The low average diversity within *K. latifolia* populations indicated the potential risk of losing genetic variation due to the small size of most populations, illuminating the significance of collecting and conserving their wild genetic resources. *K. latifolia* in the eastern U.S. exhibited a population structure that was correlated with its geographic distribution, which might be attributed to insect pollination, small population size, and environmental conditions in different habitats. These results would function as an essential step toward better collecting, conserving, and utilizing wild *K. latifolia* resources, thus promoting the breeding of *K. latifolia* plants that have adaptability to southeastern environmental conditions.

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