# Genetic Diversity in Kentucky Spicebush Populations using Simple Sequence Repeat Markers

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ABSTRACT

Spicebush (*Lindera benzoin* L.) is a native small native shrub that grows in the moist, understory areas of Appalachia and has potential as a new niche crop for small farmers. Native Americans and early settlers used this plant traditionally as a tea, and used the berries for jam, to spice food, and possibly for health benefits. Native spicebush patches may serve an important role in forest ecosystems in terms of fruit production for animals, soil erosion control, and enhancing insect biodiversity. Spicebush may also serve to hold ecological niches by outcompeting invasive plants compared to those in unchallenged areas. Genetic diversity of native spicebush populations in Kentucky has not been examined. The objective of this study is to determine the genetic diversity in spicebush populations in Kentucky using simple sequence repeat (SSR) DNA marker systems. Leaf samples were collected from 120 spicebush plants in the forests at the Kentucky State University Environmental Education Center (EEC), at a location near the Kentucky River, and at Cove Spring Park. DNA was extracted using the DNAmite Plant Kit. Primers A7, A115, B105, B122, C4, and C10 were used to amplify SSR products that were separated with a 3130 Applied Biosystems capillary electrophoresis system. The software program Power Marker was used to examine genetic relationships among genotypes. The SSR markers generated showed genetic variation among the spicebush genotypes. A number of selections with unique genotypes will be sampled and propagated for study in the KSU germplasm collection for potential cultivar development.

KEYWORDS: *Lindera benzoin* (L.), Microsatellites, Simple Sequence Repeat (SSR), Perrenial shrub, Spicebush, Genetic Diversity.

# INTRODUCTION

Spicebush (*Lindera benzoin* L.) is a large, highly branched shrub or small tree. It is able to grow to over 12 feet tall and 15 feet wide. This plant is usually found as an understory plant in the forests understory of North America, especially in the eastern United States (Mooney, et al., 2010). Spicebush can be found near wetlands, woodlands, and in other shaded moist areas and in soils rich in limestone (Niesenbaum 1992). The leaves of the spicebush are one of the first to bloom in the spring or late winter compared to other plants. The leaves are obovate and alternate, and contribute to its name because of the aromatic spicy scent that comes from them as well as the berries and stems of the plant. Historically, the leaves of this shrub have been used for tea and medicine by Native Americans and early settlers (Anderson et al., 1992; Meuninck, 2007). The tea made from the leaves has been used to relieve pain, fever, arthritis, and breathing difficulties (Anderson et al., 1992; Meuninck, 2007). The oil from the berries was sometimes used as a first aid treatment. Further research is needed to determine the effectiveness of these uses (Allison 2003). The berries have been used in jams, and are currently sold in combination with fruit such as blueberry and pawpaw to create flavors such as pawpaw spiceberry jam or blueberry spicebush jam. The berries have also been used as food flavoring by grinding the dried berries and using it as a seasoning on meat. The seeds, also known as drupes, are usually about 8mm long, a bright cherry red color when mature, and contain a seed. The fruit matures in late summer or early fall (Tucker and Maciarello, 1994; Meuninch, 2007). Interestingly, spicebush has no significant disease or pest problems which allows it to have the potential to be a new niche crop for small farmers.

Spicebush serves an important role in the ecosystems of forests. The fruit enhances biodiversity since it can be eaten by animals and insects, the root system in soils helps prevent

soil erosion around streams and rivers, and biodiversity is enhanced from species that thrive specifically on spicebush such as the Spicebush Swallowtail (*Papilio Troilus*) (Matlack, 1994). With the unique food and medicinal qualities, as well as the importance of this plant in the Kentucky ecosystem, spicebush is attractive to small farmers as a potential alternative crop, and as a plant for improving biodiversity in woodlands.

Microsatellites, or simple sequence repeats (SSRs), are a marker of choice for genetic diversity estimates, genetic mapping, and DNA fingerprinting (Wünsch and Hormaza, 2002). SSRs are short (1-6 bp) tandem repeat DNA sequences flanked by unique, conserved DNA sequences. The relative random distribution of microsatellites in the genome, co-dominant inheritance, high level of reproducibility and transportability across laboratories make these markers useful for assessing genetic diversity as well as fingerprinting (Kijas et al., 1995; Wünsch and Hormaza, 2002). SSR marker systems have been developed for a number of fruit species including pawpaw [*Asimina triloba* (L.) (Pomper et al., 2010)], hazelnut [*Corylus avellana* L. (Bassil et al., 2005)], blueberry [*Vaccinium corymbosum* L.(Boches et al., 2006)], peach [*Prunus persica* (L.) Batsch; (Aranzana et al., 2002)], cherry [*Prunus cerasus* L. (Cantini et al., 2001)], pear [*Pyrus communis* L. (Yamamoto et al., 2001)], and apple [*Malus sylvestris* (L.) Mill. (Hokanson et al., 1998, 2001)].

Spicebush can be found in a range of different light environments. There have been several previous limited studies assessing genetic diversity of spicebush in eastern Pennsylvania (Edwards and Niesenbaum, 2007; and Moony et al., 2010). Edwards and Niesenbaum (2007) developed 12 polymorphic SSR primer sets for examining genetic diversity in spicebush and Mooney et al. (2010) used these markers to examine genetic differences between spicebush in sun and shade environments. Genetic diversity of spicebush populations in Kentucky has not

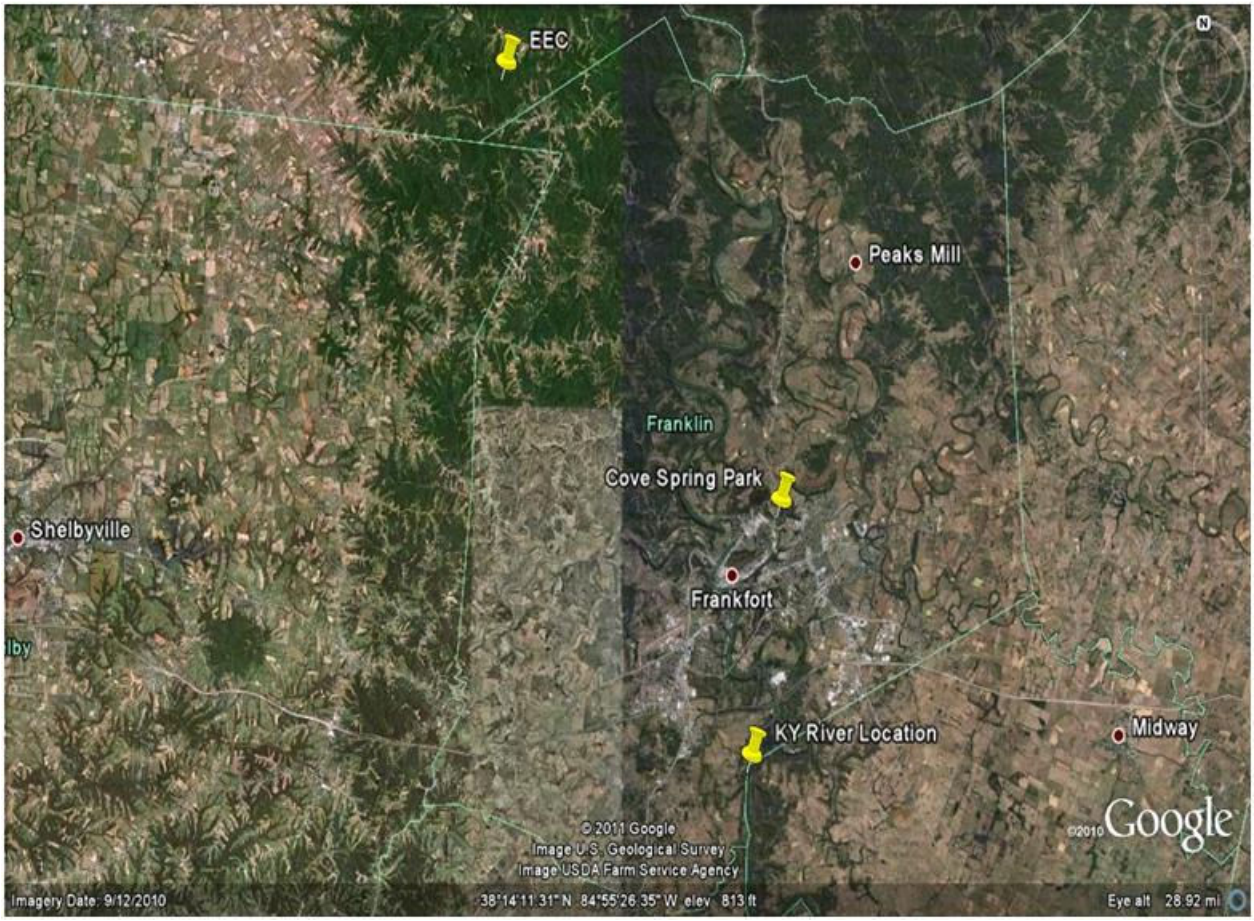
been examined. The long term goals of this research are to understand the cycling and spread of native spicebush patches as a part of Kentucky State University's mission and germplasm collection efforts in order to select genetically different varieties that can be grown by small farmers. Our objective was to determine the genetic diversity in spicebush populations in Kentucky using polymerase chain reaction (PCR) and SSR-DNA marker systems.

## MATERIALS AND METHODS

### Plant Material

Leaf samples were collected from 120 spicebush plants. At least 20 leaf samples were collected from plants from six different patches located in Central Kentucky (Figure 1). Each location yielded two patches with 20 leaf samples coming from each patch. The first location samples were collected from the forests at the Kentucky State University Environmental Education Center (EEC) in Henry County. The second location was at a location near the Kentucky River in Franklin County. The third location was at Cove Spring Park in Franklin County.

Figure 1. A map of the three locations that were sampled in the genetic study.




Patches from each location were far apart to ensure there was no overlap, and individual leaf samples were carefully collected to ensure the samples didn't come from the same plant with intertwining limbs. To ensure the best DNA, young leaf samples were collected. The leaf samples were placed in separately numbered ziplock plastic bags and stored at 4°C until needed for DNA extraction.

DNA Extraction

About 1-2cm$^2$ of young leaf was used. The DNA was extracted from the pieces of spicebush leaves using the DNAMITE Plant Kit (The Gel Company, San Francisco, CA). The DNA concentration and a 260/280 nm absorbance ratio were determined with GeneQuant™ pro

RNA/DNA calculator (GE Healthcare, Piscataway, NJ). Based on the concentrations, the samples were diluted to 1ng/µl. All samples were stored at 4°C until needed.

SSR-PCR Amplification

The SSR-PCR amplification was performed with GoTaq Flexi DNA polymerase (Promega Co., Madison, WI). The reactions were set up as follows: 4 µL of 5X colorless GoTaq Flexi buffer, 0.4 µL of 10 mM dNTPs solution, 2 µL of 25 mM $MgCl_2$, 0.3 µL of 30 µM forward primer (fluorescence labeled with FAM) solution and 0.3 µL of 30 µM reverse primer (unlabeled) solution, 0.2 µL of 5 units/µL GoTaq DNA polymerase, 2 µL of diluted 1 $ng \cdot \mu L^{-1}$ spicebush DNA, and 10.8 µL of $ddH_2O$ to bring the total volume to 20 µL. Six dinucleotide and trinucleotide primers created by Edwards and Niesenbaum, 2007 were selected and labeled with the fluorescent tag FAM: Spice-A115, Spice-A7, Spice-B105, Spice-B122, Spice-C4, and Spice-C10 for use in this study (Table 1). The PCR amplifications were performed using the Techne TC-5000 Thermal Cycler (Techne Inc., Burlington, NJ). The PCR program consisted of an initial period of 94 ºC for 3 min, followed with 30 cycles of 40 s denaturation at 94 ºC, 40 s annealing at 53 ºC, and 30 s extension at 72 ºC, and a final extension period of 5 min at 72 ºC. The PCR results were then stored at 4 ºC until analysis. Products were separated with ABI 3130 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) Capillary electrophoresis system with GeneScan™ 500 LIZ™ as an internal size standard. Individuals were analyzed and genotyped with GeneMapper version 4.0 software (Applied Biosystems Inc., Foster City, CA).

Table 1. The six loci tested, their forward and reverse sequences, and the repeat motif used in the SSR-PCR.

| Locus (GenBank) | Primer sequence (5′–3′) | Repeat motif |
|---|---|---|
| A7 | F: AAAACGGATCAGATACTCCC | $(AC)_{13}$ |
| EF193199 | R: GCAGCATTATTGGGTTAGTG | |
| A115 | F: AGGAGCTACCTCTGATTCTTGG | $(CT)_{19} + (AC)_{13}$ |
| EF193200 | R: TCACCCACATCTCAATATCATG | |
| B105 | F: ACAGGTCTTGACTTTGGGATAT | $(GA)_{11}$ |
| EF193201 | R: GGATGGCTTATGGAGTGG | |
| B122 | F: TGCTCAAGGAGAGATTCAAC | $(AG)_{17}$ |
| EF193202 | R: CTCAGCCGAGTCTACTATCG | |
| C4 | F: TTGGGTGGAGTTGATGAG | $(GAA)_{13}$ |
| EF193203 | R: ACGCATTATTGACAGCCTT | |
| C10 | F: TTCCTAAACCCTGTTGTAAAAC | $(AAG)_{15}$ |
| EF193204 | R: GCCAATCATGTGACTATTGTC | |

DATA ANALYSIS

Genetic relationships among patches were examined using PowerMarker version 3.25 to calculate the number of genotypes, number of alleles, allele size, expected heterozygocity ($H_e$), observed heterozygocity ($H_o$), and polymorphism information content (PIC) for all accessions. The major allele frequency is the frequency of the most common allele. The $H_o$ was calculated as the number of heterozygous genotypes at a given locus divided by the number of genotypes present at the locus. $H_e$ was defined as the probability that two randomly chosen alleles from the population are different. PIC was an estimate that the parental origin of an allele can be determined from the marker locus genotype in any given offspring. The equation for $H_o$, $H_e$, and PIC are given in the PowerMarker software manual. Genetic distance (D) between genotypes

was computed as (1 - proportion of shared alleles) (Bowcock 1994). The distance data were used to generate an unweighted pair group mean analysis method with an arithmetic mean (UPGMA) dendrogram.

## RESULTS AND DISCUSSION

All six of the SSR primer sets tested amplified SSR-PCR products in all three populations examined. On average, each locus produced over 18 different alleles and 42 different genotypes. Allele size ranged from 133 to 292 base pairs and heterozygosity was high ($H_e$ = 0.89; Table 2). When comparing populations, there was significant genetic diversity. The high average number of alleles per locus (21.0) is comparable to that in other woody perennial fruit and nut crop species such as 13.3 alleles per locus reported for hazelnut (Bassil et al., 2005) 12.1 for apple (Hokanson et al., 1998, 2001) and 9.1 for pear (Sisko 2009.). The $H_e$ (0.89) in our study indicated high levels of genetic diversity among the spicebush populations examined. Edwards and Niesenbaum (2007) examined genetic diversity of 29 eastern Pennsylvania spicebush plants using 11 SSR primer pairs and found $H_e$ ranging from 0.10 to 0.82, and alleles ranged from two to 16 per locus. Mooney et al. (2010) examined genetic diversity of 127 eastern Pennsylvania spicebush plants using 12 SSR primer pairs and found $H_e$ ranging from 0.61 to 0.65, and alleles ranged from 3.7 to 4.3 per locus. The higher $H_e$ determined for spicebush populations in our study likely reflects the wider sampling of populations separated by greater distance and therefore a more precise estimate of genetic diversity of spicebush in the wild.

UPGMA cluster analysis was used to generate a dendrogram (Figures 2, 3, 4, and 5). UPGMA analysis indicated the EEC and KYR populations were easily separated. Figure 2 shows there was a significant amount of diversity among the three locations. Figure 3 shows the genetic relationships between the six patches. The two Cove Spring patches were genetically

similar. Conversely, the EEC2 population was closer genetically to the KYR 2 population than to EEC1; even though the two EEC populations were closer in proximity. Figure 4 shows the genetic relationships in the EEC populations. This population showed genetic diversity, but samples EEC31 and EEC48 and EEC29 and EEC34 were more similar. Figure 5 shows the genetic relationships in the Cove Spring populations. This population showed more similarities between samples than the EEC population. With the Cove Spring samples, CS22 and CS47, CS46 and CS21, CS38 and CS39, and CS10 and CS12 were similar. Figure 6 shows the genetic relationships in the Kentucky River populations. Interestingly, with this population, three samples were similar, KYR13, KYR22, and KYR9. Each of the three populations showed genetic similarities among samples.

Table 2. The six loci tested, the number of genotypes, the number of alleles, the allele size, the expected heterozygocity, the observed heterozygocity, and the PIC for all loci tested.

| Marker | GenotypeNo | AlleleNo | Allele size (bp) | He | Ho | PIC |
|---|---|---|---|---|---|---|
| **A115** | 75 | 30 | 160-248 | 0.92 | 0.85 | 0.91 |
| **A7** | 39 | 13 | 233-250 | 0.86 | 0.73 | 0.85 |
| **B105** | 87 | 27 | 238-292 | 0.93 | 0.88 | 0.93 |
| **B122** | 69 | 23 | 191-267 | 0.89 | 0.78 | 0.89 |
| **C4** | 38 | 13 | 222-247 | 0.87 | 0.60 | 0.86 |
| **C10** | 42 | 17 | 133-230 | 0.87 | 0.82 | 0.85 |
| **Mean** | **58** | **21** | **133-292** | **0.89** | **0.78** | **0.88** |

Figure 2. A dendrogram comparing genetic relationships of all the samples at all six patches at the three locations.

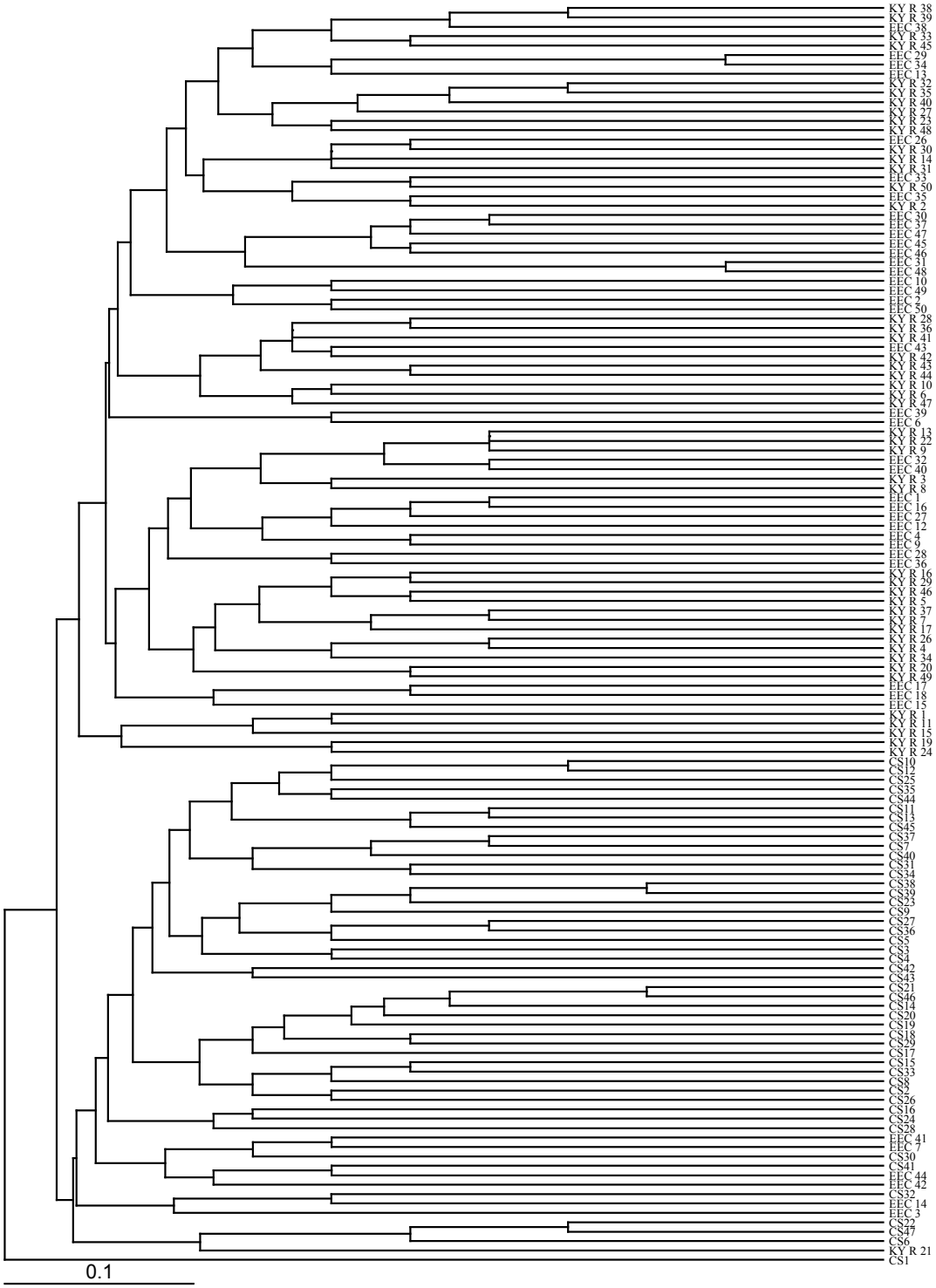


Figure 3. A dendrogram comparing the genetic relationships between the six patches.

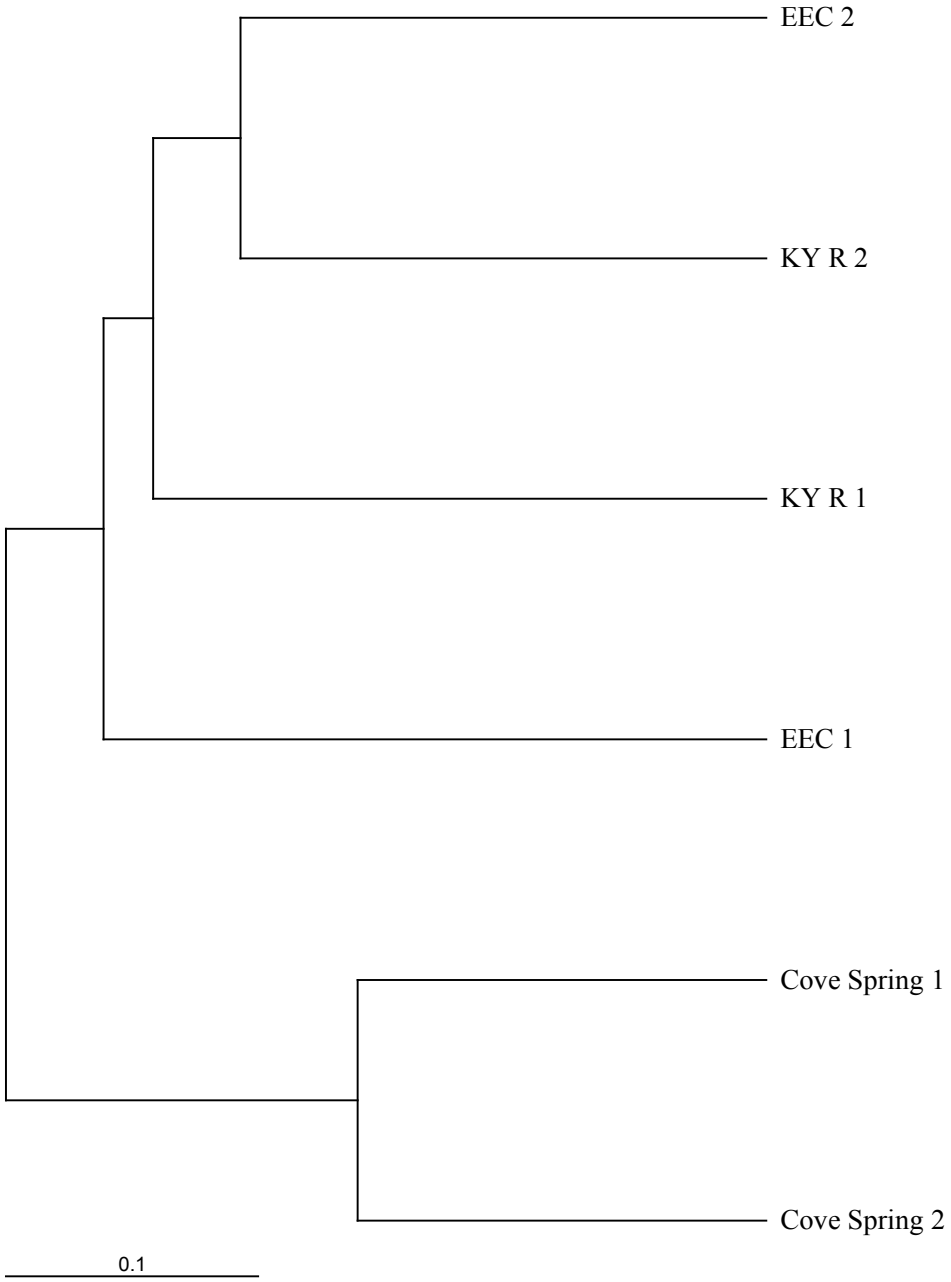


Figure 4. A dendrogram comparing the EEC location.

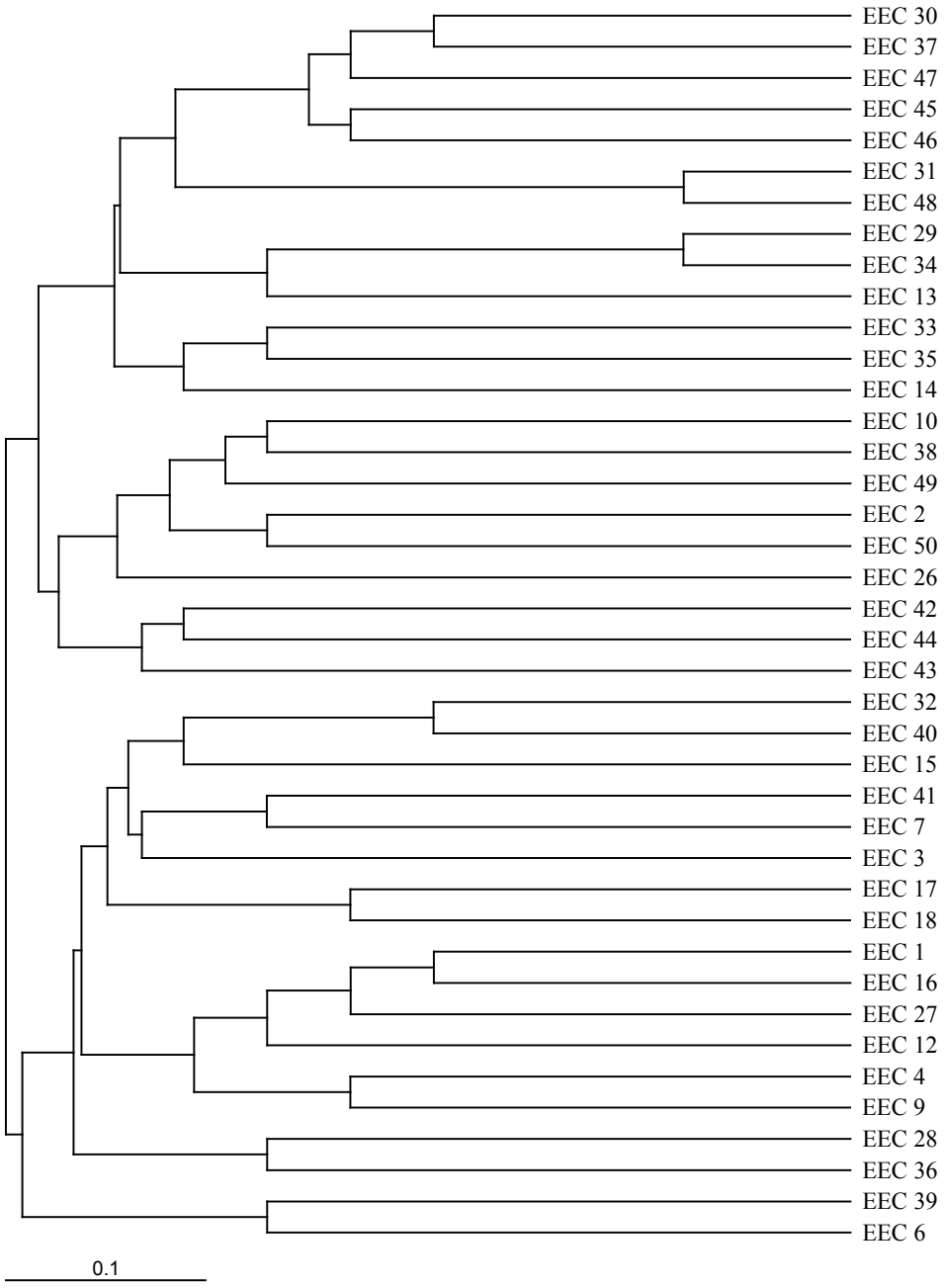


Figure 5. A dendrogram comparing the Cove Spring location.

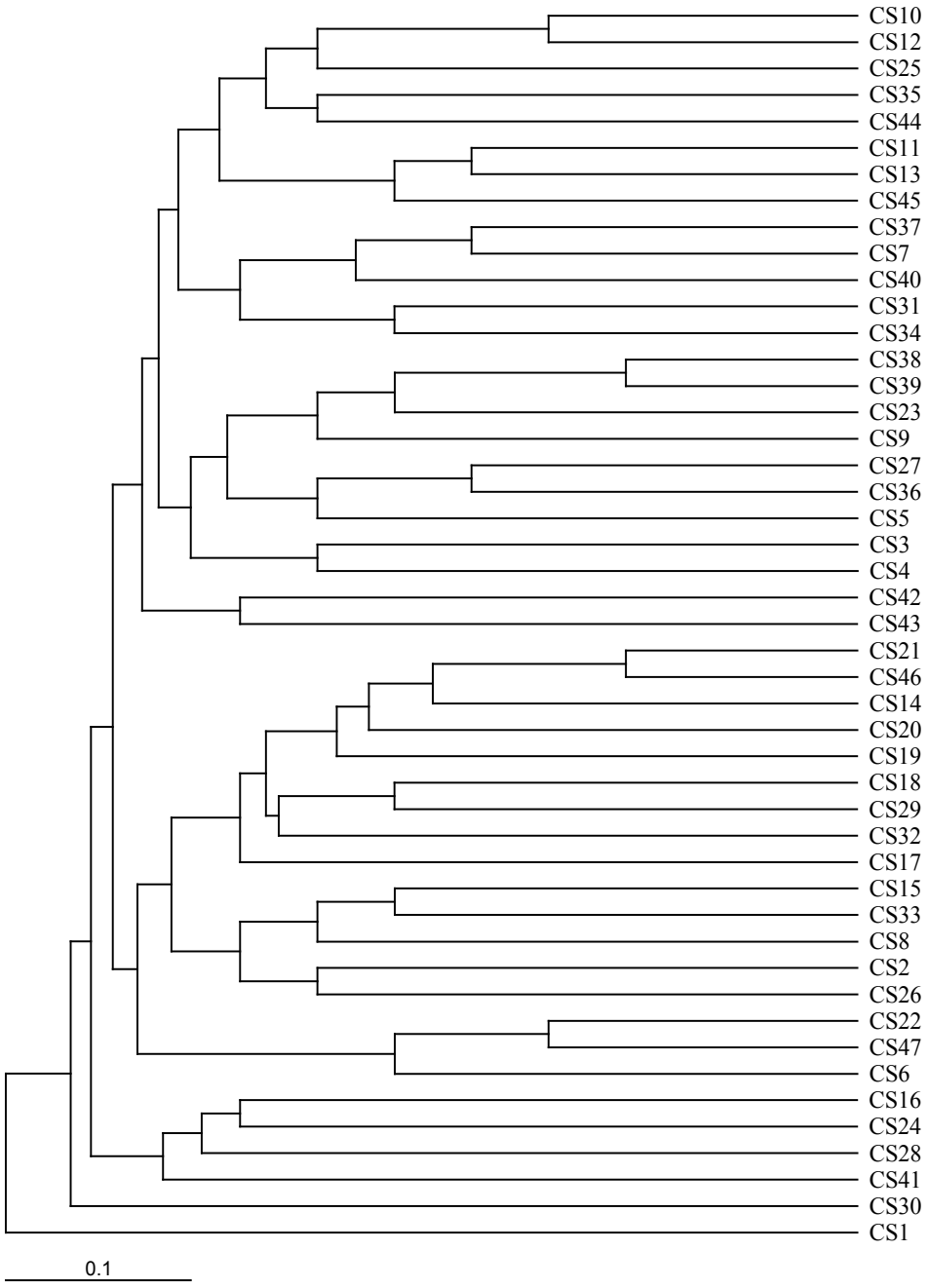
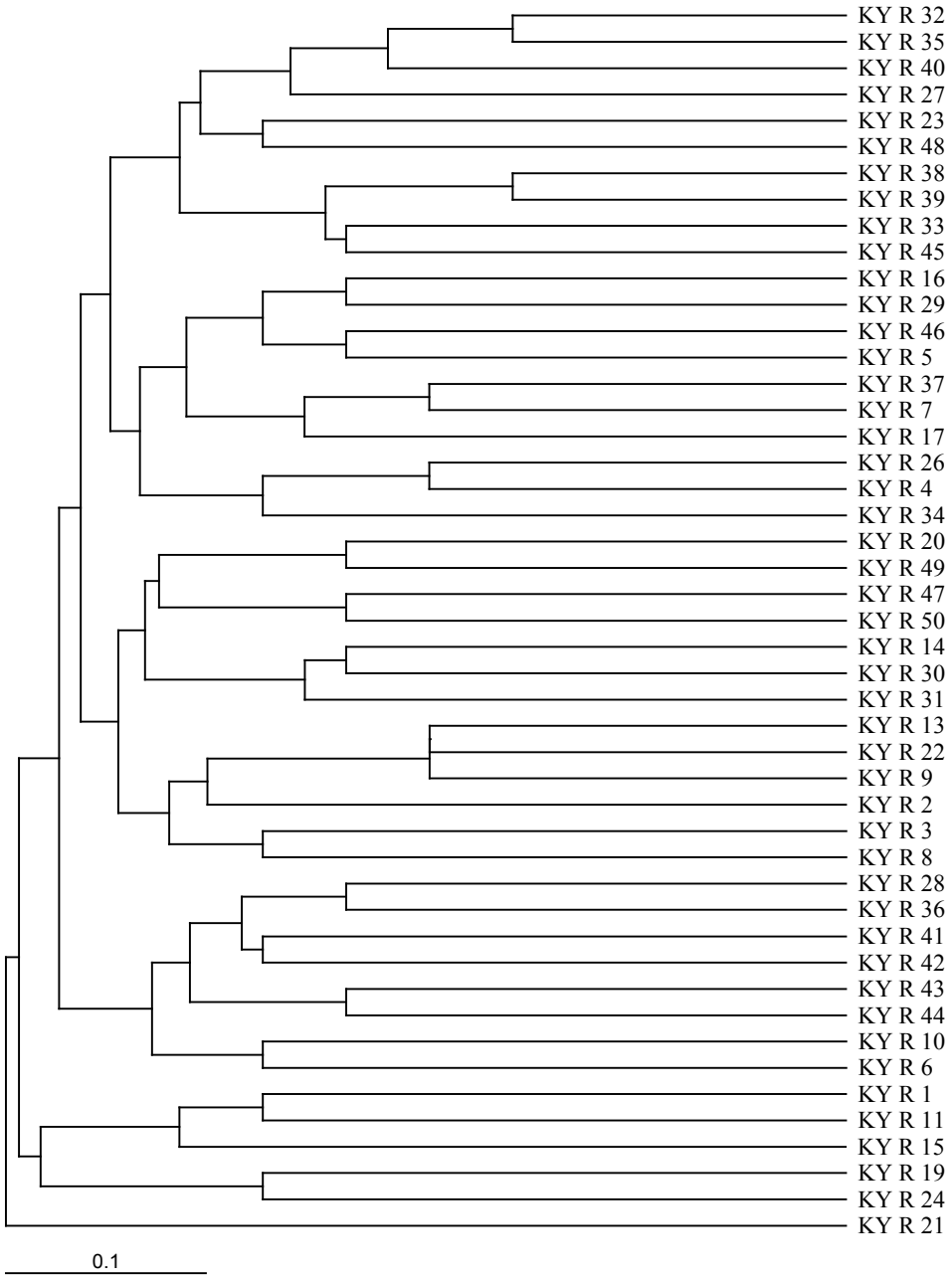


Figure 6. A dendrogram comparing the Kentucky River location.



In conclusion, there was significant genetic diversity observed in the spicebush populations examined. This is the first report of spicebush genetic diversity and relationships in Kentucky. The results of this study indicate the six primers used should be useful in examining additional spicebush populations. For the establishment of spicebush varieties for small farmers in Kentucky, varieties with broad genetic diversity will be important for future insect and disease resistance by plants when planted by farmers in larger monocultures that increase disease and pest pressure. Large fruit size will also be important to growers for spicebush to become a successful crop for small farmers. Future studies will include looking at fruit size among different populations. A number of selections with unique genotypes will be sampled and propagated for study in the KSU germplasm collection for potential cultivar development.

## ACKNOWLEDGMENT

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