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Genetic Diversity in Adults and Seedlings of *Celastrus orbiculatus* Thunb. (Asian Bittersweet) Near a Site of Initial Introduction in Asheville, North Carolina

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Abstract
Asian Bittersweet (*Celastrus orbiculatus*) is a woody non-native species introduced to the United States in the 1860s as an ornamental. *C. orbiculatus* threatens the environment and other plants, especially its native congener, American Bittersweet (*Celastrus scandens*). The two lianas hybridize easily on the landscape, and *C. orbiculatus* can outcompete the native plant to cause local extinctions. The objectives of this study were to test the efficacy of new microsatellite markers, then use those markers to determine the genetic diversity of seedlings and adults of *C. orbiculatus* from western North Carolina, near one site of *C. orbiculatus*' introduction, to gain insight into this non-native species' evolutionary forces and genetic pattern. DNA was extracted from the leaves of both adults and seedlings, and extracts were PCR amplified at seven variable microsatellite loci. All the loci tested were good enough to provide information about Asian Bittersweet. 4 or 5 of the microsatellite markers were sufficient to analyze the genetic diversity of Asian Bittersweet. Results showed that broad genetic similarities between adults and seedlings across all sites, and although allelic diversity was higher in adults, the difference was not statistically significant. This could indicate that natural selection, nonrandom mating, and/or genetic drift reduce the next generation's diversity. This study is one of the first to compare diversity across generations of a non-native species, and results could have implications for other systems.

Introduction
Invasive species are non-native organisms introduced intentionally or unintentionally to an ecosystem. Invasive species have been shown to compete with native species for nutrients and affect soil nutrient composition; because they tolerate harsh conditions they often, spread faster leading to the decline of native species[1,2]. Non-native species typically have a high growth rate and rapid dispersal that aids their spread and invasion. Introduced species with potential hybridization in isolated populations tend to show unidirectional gene flow which increases variation in a population[2,3]. Invasive species are ecologically flexible and have a high hybridization rate, and knowing their genetic patterns could allow researchers to characterize their status and potential for spread.

Asian Bittersweet (*Celastrus orbiculatus*) is an invasive, twining tetraploid liana native to China, Japan, and Korea that evolved in the Pliocene era[4]. *C. orbiculatus* was introduced from Asia to North America in the 1860s as an ornamental plant and it is now more common than American Bittersweet (*Celastrus scandens)*, a North American native species.[5,6] Mislabeling of *C. scandens* and *C. orbiculatus by* vendors and horticulturists due to their vegetative and fruit resemblance has led to the decline of the native species. The seedlings of both *C. scandens* and *C. orbiculatus* can be hard to differentiate but mature *C. scandens* fruits are heavier and their pollen is yellow.[7,8] American Bittersweet has an orange capsule with its fruits growing at the apex of the vine while Asian Bittersweet has a yellow capsule with fruits growing axially[7]. It was introduced as an ornamental plant and became popular because of its bright fruits. Multiple sources cultivated and distributed *C. orbiculatus* before it escaped from captivity, but the origin of its invasion is somewhat unclear. The Arnold Arboretum (Harvard University; Boston, MA) was an early cultivator of *C. orbiculatus* and is believed to be the source of ornamental *C. orbiculatus* for nearby estates[7]. The New York Botanical Garden was also an early cultivator of *C. orbiculatus*, and it was found to have escaped its viticetum as early as 1898[7]. Finally, the Biltmore Nursery in Asheville NC was a major known cultivator and distributor of the liana and advertised its sale in catalogs for decades[7]. Different control methods: including pesticide, burning, and cutting have proven futile in controlling *C. orbiculatus* and might have inadvertently caused the destruction of *C. scandens*.

Asian Bittersweet's invasiveness and spread were aided by bird dispersal and its ability to girdle trees. It outcompetes American Bittersweet because it has a broader niche and more prolific fruit production. *C. orbiculatus* has the ability to resist pesticides, photosynthesize in any environment due to its seasonal leaf plasticity traits, and sprout and regrow after being burned and cut[9,10]. The burning and cutting of *C. orbiculatus* have increased its spread and diversity in regions with sandy soil, leading to northeastern states declaring it an invasive species[2,9]. Currently, Asian Bittersweet occupies woodlands throughout eastern North America where its range overlaps significantly with American Bittersweet. It is therefore important to study the genetic diversity of *C. orbiculatus* to understand its current status and potential spread.

Genetic diversity is the proportion of traits and alleles within a species, a result of recombination, genetic drift, gene flow, natural selection, and mutation[11]. Genetic diversity can allow adaptation to different environmental conditions[11,12]. Patterns of diversity can show how fast a species is spreading and evolving. Changes in genetic diversity from generation to generation can occur in a population due to isolation, mutation, gene flow, and breeding patterns. Higher genetic diversity in the older generation could be caused by isolation, inbreeding, and natural

selection while in the younger generation the cause for higher genetic diversity could be explained by immigration. It is important to study genetic diversity because it provides past and present information about a species.

Hybridization of two closely related species has been going on for centuries to give rise to fit individuals and it has also led to outnumbering of native species and the evolution of invasive species. Most of the northeastern USA's cause of *C. scandens* decline is hybridization and the use of biological and chemical methods with the intention to manage and eradicate invasive species. Recent studies [3,14] have shown that *C. orbiculatus* have higher flower production than *C. scandens* which increases fertilization, seed germination, and hybridization. *C. orbiculatus* hybridizes easily with *C. scandens* and in doing so has both unidirectional gene flow and the ability to reject heterospecific pollen[3,15]. The studies also find that invasive species' spread is increasing because of their bidirectional pollen and when they hybridize with their native congener, thus, an introgression pattern towards the invasive species can be observed on the hybrids. Compared to Asian Bittersweet, *C. scandens* has bidirectional pollen that can cause a waste of pollen thus low fertility rate[15,16]. The genetic diversity of *C. orbiculatus* from different regions has been analyzed by previous research, but these studies have used less powerful markers and intergenerational genetic comparisons on invasive species have yet to be examined. A study of genetic diversity can be done by assessing molecular markers which allows the calculations of allelic frequency in populations and migration patterns of species[12,13]. This study will test recently developed molecular genetic markers for their ability to distinguish among Asian Bittersweet individuals and reveal patterns in populations. The study will also use the new molecular genetic markers to compare the genetic diversity of seedlings and adult generations of *C. orbiculatus*.

Methods

We conducted the study by collecting Asian Bittersweet (*Celastrus orbiculatus*) from 18 random sites within 100 km of Biltmore Estate in Asheville, NC. At each site, we obtained 6 leaf samples: 3 from adult plants and 3 from seedlings. DNA extraction was performed by grinding the leaves with a mortar and pestle in liquid N_2 to make a fine paste then using a modified CTAB method [17]. The DNA quality and quantity were measured using gel electrophoresis and a Nanodrop ND-1000 Spectrophotometer™. We performed polymerase chain reaction (PCR) by first making a mastermix that contained 0.5 µL of fluorescent forward primer, 0.5 µL reverse primer, 9 µL GoTaq Green Master Mix, and 8 µL PCR water. The 18 µL mastermix was mixed with 2 µL DNA extract and then taken to BIO-RAD T100TM thermal cycler for 2min at 94 ˚C followed by 35 cycles of the 40s at 94˚C,40s at appropriate annealing temperature, and 60s at 72 ˚C and final step for 10min at 72 ˚C. PCR products were multiplexed and then sent to the Genomic Sciences Laboratory at North Carolina State University for genotyping. Each PCR plate well contained 2 µL PCR products that worked, 0.5 µL LIZ genescan 500 ladder, and 7.5 µL formamide. Fragment analysis was performed in Geneious 2021.1 using the microsatellite plug-in, and population genetic indices were calculated in the *polysat* and *poppr* packages in RStudio[18,19].

Table 1. Characteristics of microsatellite loci used for genetic analysis

Locus	Nucleotide Repeat	Annealing T (^{0}C)	Forward Sequence, 5'to 3'	Reverse Sequence, 5'to 3'	Fluorophore
4	AG	59	CTTGGATTTGTGGACGCCTC	GTTTGGAGGTGAGGTGGTTATAGAG	6FAM
18	AG	59	AGAGAGAGATTCGAAGCCAGG	GTTTCAAACCAACCTTTCGCCTG	VIC
1353	AAG	58	CGCCTCCGTCTCAGTTATG	GTTTACCTTCTTACTCTCCGTTGTG	PET

1813	GTTT	58	GTTTCCCACATGTAAGCT TGGCC	ATACGCGTGAAACCA TCGAC	VIC
1845	GT	59	CTTCCGGTTGAGTAAAGG CC	GTTTCCTTTGCTTTAG AGGCTTCC	NED
4150	AG	56	GTTTTATGCCACCTTTGTT TGCC	CAGATGGCAAGTAAC AAACCAC	6FAM
4960	AAAC	59	GTTTCGTGCGTTCATTTCT TCCG	GAGACAAATTCCCTT GGGCG	NED

Results

Objective 1: Microsatellite Markers Resolution

After passing initial screening with a small subset of samples, one microsatellite locus (1813) had a low amplification success rate and was thus excluded from further analysis. A genotype multilocus curve (Figure 1) revealed that 4 or 5 out of the 6 loci tested were sufficient to analyze 40 multilocus genotypes (out of 40 samples). The loci were equally good enough to provide knowledge about Asian Bittersweet. All loci had high heterozygosity, evenness, and allele diversity. The total number of microsatellite repeat lengths found across 40 individuals per locus ranged from 9-17 and a mean Simpson Index of 0.86 (Table 2). Locus 4 was the most polymorphic, with 17 alleles, while loci 1353 and 4960 were less polymorphic, possessing 9 alleles (Table 2).

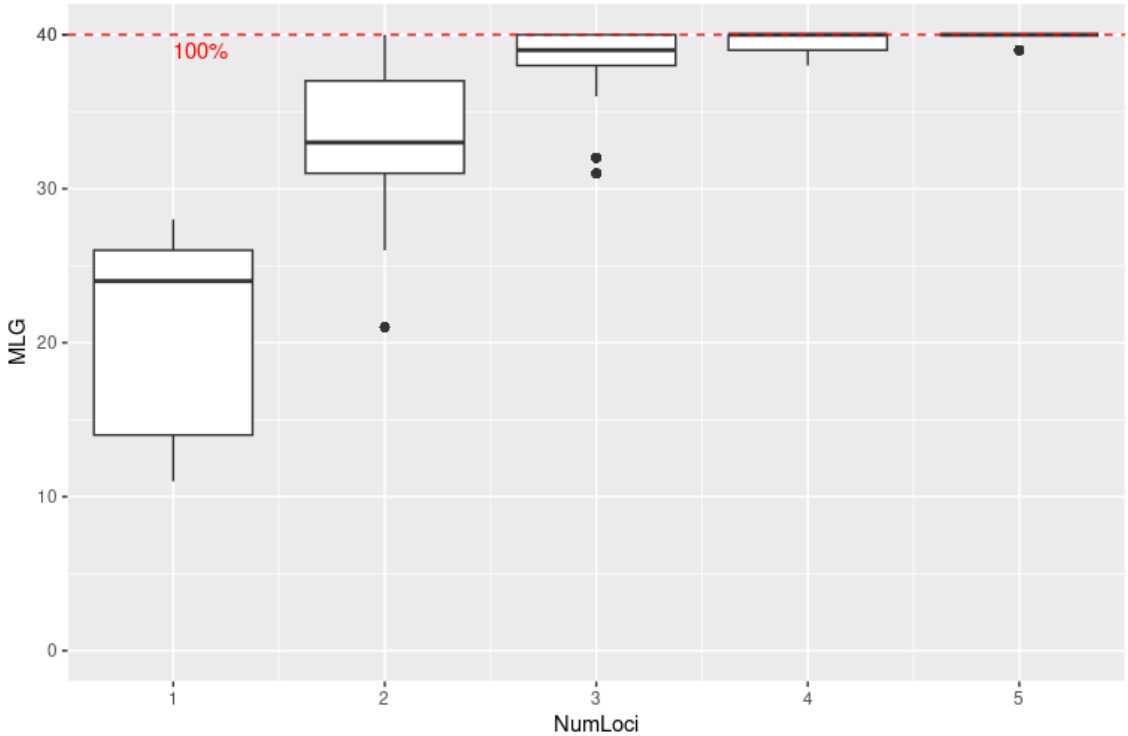

Figure 1. The number of Multilocus Genotypes (MLGs) detected per locus

Table 2. Repeat type, total alleles, Simpson Index, heterozygosity, and evenness of microsatellite loci

Locus	Repeat Type	Alleles	Simpson Index	H	Evenness
Loc4	dinucleotide	17	0.86	0.87	0.70
Loc18	dinucleotide	15	0.86	0.88	0.71
Loc1353	trinucleotide	9	0.86	0.87	0.91
Loc1845	dinucleotide	13	0.90	0.93	0.87
Loc4150	dinucleotide	16	0.90	0.91	0.81
Loc4960	tetranucleotide	9	0.77	0.79	0.67
mean		*13.17*	*0.86*	*0.87*	*0.78*

Objective 2: Genetic diversity of adults and seedlings of Asian Bittersweet

Adults had more alleles across all loci, ranging from 7 to 15 (Table 3); however, this difference was not statistically significant ($F_{1,10} = 2.98$, $P = 0.115$). Both adults and seedlings of *C. orbiculatus* showed a similar genetic pattern with high heterozygosity, and equal genotypic richness but a low index of association. Adults had a heterozygosity of 0.898 and an index of association (I_a) of 0.179, whereas seedlings had a heterozygosity of 0.824 and I_a of 0.083. Adults and seedlings were genetically similar when compared across and within sites ($F_{ST} = 0.01975$). Based on genetic distance, the samples were clustered into 4 categories; most of the samples were grouped with their geographical neighbors indicating being genetically similar (Figure 2).

Table 3. Allele diversity of adults and seedlings of *C. orbiculatus* across all loci

	Loc4	Loc18	Loc1353	Loc1845	Loc4150	Loc4960	*All Loci*
Adults	14	14	12	7	8	15	70
Seedlings	9	9	7	4	9	13	51
Overall	17	15	13	9	9	16	

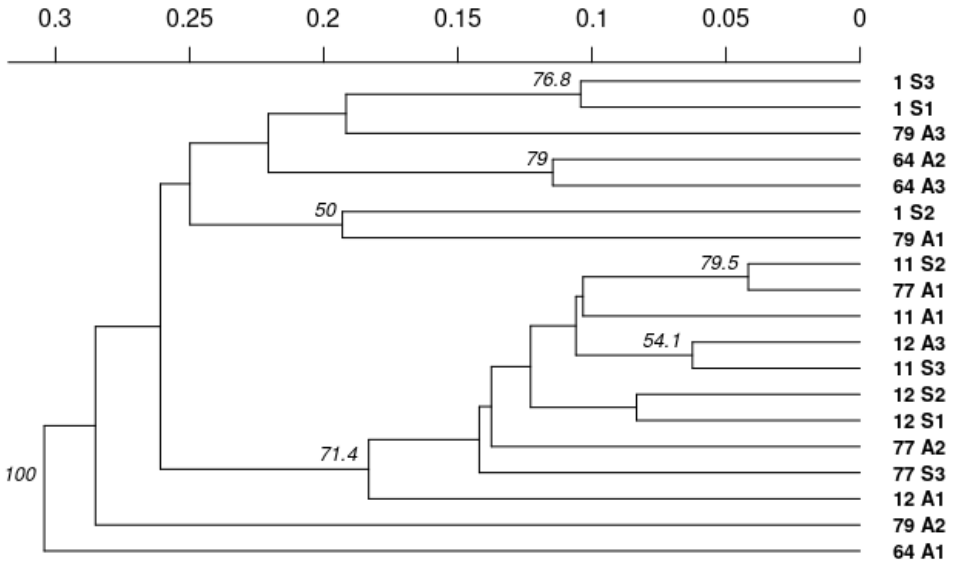

Figure 2. Rooted neighbor-joining tree of 6 Asian Bittersweet sites with 3-4 samples per site. The tree is based on Provesti's genetic distance with N = 1,000 bootstraps, and bootstrap values ≥ 50 are shown on branches. For each label, the first number is the site and the second is the adult (A) or seedling (S) number.

Discussion

The study examined new microsatellite markers and their ability to distinguish adults and seedlings of Asian Bittersweet. All loci tested had high allele diversity and expected heterozygosity therefore considered to be equally good at discriminating MLGs among individuals and capturing *C. orbiculatus* genetic diversity. The loci also had high allele distribution, therefore, being useful for studying the genetics of other plants.

Combining the markers allowed examining the allele distribution, heterozygosity, and genetic diversity of the samples. Results showed broad genetic similarities between adults and seedlings; adults had higher allelic diversity

but were not statistically significant. Overall, both showed high heterozygosity and genetic diversity across loci and within sites. High allele diversity in adults could be explained by founder effects, isolation, and natural selection[21,22] however the results indicated similar genetic forces acting upon the two populations. The founder effect gives rise to reduced genetic and allele diversity in the new population of an introduced species[20,21]. However, this study found no evidence of the founder effect because both generations had high genetic diversity. No founder effect found in Asian Bittersweet intergeneration could be due to multiple multilocus genotypes that were sold at Biltmore estate for decades[10] or mutation and/or gene flow occurring since the initial introduction of the species.

In a small population, low genetic diversity and increased inbreeding are expected as a consequence of isolation, genetic drift, and disrupted gene flow[23]. However, Asian Bittersweet samples showed limited clonality indicating that plants are undergoing sexual reproduction.[23,24] Recent studies have found that species/populations with increased genotypic richness and small sample distance have reduced clonal reproduction[24,] which was evident from the analysis. High genetic diversity is mostly seen in species from different geographical locations with fewer (re)introductions of nonnative species, and it should be noted that multiple introductions could weaken a species' local adaptation to its new environment[22,23].

The neighbor-joining tree showed that sometimes samples from the same location clustered, indicating their genetic similarity. In some other cases, samples within sites are dispersed on the joining tree which is a result of immigration or nonrandom mating in that population. The clustering pattern could have resulted from geographical isolation and low dispersal rates. Asian Bittersweet dispersal distance is expected to be long because its main dispersal agents are birds[25]. Asian Bittersweet can also be dispersed by humans, especially in densely populated regions that still use it for wreath making.

This study is one of the first to compare diversity across generations of a non-native species, and results could have implications for other systems. To provide more robust results, future studies should analyze more microsatellite markers since more/ number of loci yields more statistical power for distinguishing individuals. Analysis of more samples with a reduced/increased distance between samples to examine if intergenerational genetic diversity could change due to geographical distance.

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