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Developing Genetic Tools for Oriental Bittersweet (*Celastrus orbiculatus* Thunb.) Populations

Andrew A. Flynn
Biology
The University of North Carolina Asheville
One University Heights
Asheville, North Carolina 28804 USA

Faculty Advisor: Dr. Jennifer Rhode Ward

Abstract

Celastrus orbiculatus Thunb. (Oriental bittersweet) is an invasive liana from eastern Asia that now shares a large portion of its range with its native North American congener, *Celastrus scandens* (American bittersweet). *Celastrus orbiculatus* was originally introduced as an ornamental but is now a widespread pest species that harms native plants, is difficult to eradicate, and can both grow and spread quickly. The origin of invasion is difficult to pinpoint, but the Biltmore Nursery (Asheville, NC) could have been a primary source. To help determine the veracity of this claim, potential microsatellite loci were identified, then screened for consistent amplification and polymorphisms. DNA was extracted from adult and seedling *C. orbiculatus* leaves collected at various distances from the Biltmore Estate, then PCR amplified at 24 potential microsatellite loci. Fourteen loci amplified successfully, and 8 were polymorphic. Allelic diversity varied among populations, but genotypes did not cluster by distance from the putative source or by population. Greater genetic diversity was found in adult individuals than seedlings, suggesting some individuals are reproducing more than others. The successful development of these markers could help elucidate the origin of *C. orbiculatus*' invasion in the southern Appalachian Mountains or discover invasion routes and gene flow patterns.

1. Introduction

Celastrus orbiculatus Thunb. (Oriental bittersweet or Asiatic bittersweet; Celastraceae) is an invasive liana native to eastern Asia. The species grows rapidly, and its seeds are dispersed by birds, making it highly invasive [1,2,3]. *Celastrus orbiculatus* is capable of rapid growth in shaded habitats such as forest understories, in which it quickly ascends to the canopy level after disturbance; it can exclude or hybridize with its native congener[1,4,5]. When growing towards an opening in the canopy layer, *C. orbiculatus* girdles trees by wrapping tightly around the trunk, shades the plants below it by densely occupying the upper canopy, and can even weigh down the host tree enough to topple it[5,6,7]. Additionally, *C. orbiculatus* is very difficult to remove from an area once established. Roots left intact after cutting or pulling are capable of generating sucker shoots that lead to rampant growth[5,6].

C. orbiculatus' bright yellow leaves in the fall, yellow capsule, and bright red fruit made it a desirable ornamental in estates of affluent North Americans[6]. Reports of naturalization by *C. orbiculatus* had already begun in the early twentieth century[1], and its potential as an invasive species and pest began to be recognized[6]. Since its introduction, *C. orbiculatus* has spread over a great deal of the range of its native congener, *Celastrus scandens* L.[1]. *Celastrus scandens* is now considered a preferable horticultural alternative to *C. orbiculatus* to avoid further invasive spread. However, the species look very similar when not fruiting, and *C. orbiculatus* is often mislabeled and sold as *C. scandens*[2]. Its hardy attributes and effective dispersal by birds ensure *C. orbiculatus* is able to invade most eastern U.S. habitats regardless of forest layer or disturbances, even fire[8].

Multiple sources cultivated and distributed *C. orbiculatus* before its escape from captivity, but the origin of invasion is somewhat unclear. The Arnold Arboretum (Harvard University; Boston, MA) was an early cultivator of *C. orbiculatus* and believed to be the source of ornamental *C. orbiculatus* for nearby estates[6]. 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[6]. Finally, the Biltmore Nursery in Asheville NC was a major known cultivator and distributor of the liana and advertised its sale in catalogues for decades[6].

Microsatellite loci may prove to be useful in determining the origin of *C. orbiculatus* in western North Carolina. Microsatellite markers consist of tandem repeats of DNA motifs in semiconserved, intergenic regions of the genome[9,10]. Microsatellite loci have been found in many eukaryotic genomes, and markers have great utility for population genetics since repeat patterns can vary even among individuals within a population[9,11]. The number of repeats or mutations in microsatellites can be used to differentiate among populations. PCR can be used to amplify microsatellite loci, and the exact number of tandem repeats can be identified with fragment analysis. Differences in repeat number can be used to calculate population genetic parameters.

The goal of this research was to develop genetic tools for amplifying microsatellite loci in *Celastrus orbiculatus*, then use these primers to measure genetic diversity patterns of *C. orbiculatus* populations in the Asheville area around the Biltmore Estate. Before this, only two microsatellite loci had been discovered for the species, which is insufficient for population-level analyses[1]. It was expected that individuals would show less genetic diversity as distance from the Biltmore Estate increased if it is the origin of invasion for *C. orbiculatus* in western North Carolina, since founder populations tend to lose a great deal of genetic diversity followed by strong selection while adapting to the new environment[12].

2. Methods

Celastrus orbiculatus leaves were harvested in 2011-2012 by A. Maser and J. Rhode Ward. DNA was extracted from leaves by freezing them with liquid nitrogen and mechanically grinding tissue with a mortar and pestle. A Qiagen DNEasy Plant Mini Kit™ was then used to extract DNA from the tissue. DNA concentration and quality were assessed using a Nanodrop ND-1000™ spectrophotometer. This was done for twenty plant samples taken from various distances from the Biltmore Estate in North Carolina, and DNA samples were stored at 4 ℃ until use. Plant samples were harvested within a 70 km radius of the Biltmore Estate and included both seedling and adult individuals.

Potential microsatellites were identified using msatcommander[13], and primers suitable for PCR amplification were purchased from Eurofins Genomics©. Annealing temperatures for primers were selected from msatcommander data to ensure the left T_m and right T_m were within 1 °C of each other. PCR tubes each contained 6.5 μL of 2x GoTaq© green master mix (DNAP, dNTPs buffer), 0.15 mM forward primer, 0.15 mM 6-FAM fluorescent dye, 0.30 mM reverse primer, 0.03 mM of PCR H_2O, and 0.30 mM of DNA sample a total reaction volume of 13 μL in each container. PCR was performed in a Bio Rad T100™ thermal cycler. The thermocycler program consisted of 35 total cycles and began by heating to 94 °C for 5 min, 94 °C for 30 s, 30 s at the primer T_A (58 -60 °C), 30s at 72 °C, and ended with 5 min at 72 °C. After 35 cycles, PCR products were held at 12 °C until removed. DNA amplification success was assessed by 1% agarose gel electrophoresis using 1X TAE buffer. Fluorescently tagged PCR samples were mailed out to North Carolina State University's Genomic Science Laboratory for fragment analysis. NCSU fragment analysis data was examined in Geneious[14] to determine fragment sizes and more accurately identify polymorphisms. Data from Geneious were analyzed in RStudio™ with polysat package[15] to assess genetic diversity among populations.

Table 1. *Celastrus orbiculatus* harvest sites and distance from the Biltmore Estate

Site number	Distance (km)
10	10
21	20
23	20
56	50
57	50

58	50
67	60
69	60
70	70

3. Results

Twenty-two loci were screened for PCR amplification, and 14 amplified consistently. Loci included 6 dinucleotide repeats, 4 trinucleotide repeats, 2 tetranucleotide repeats, 1 pentanucleotide repeat, and 1 hexanucleotide repeat (Table 2, Table 3). Linear regression revealed no significant relationship between allelic diversity and distance from the Biltmore Estate (Figure 1; $F= 1.905$, $df = 7$, adjusted $R^2 = 0.1016$, $p = 0.2100$). Allelic diversity varied among populations (Table 4), but genotypes did not appear to cluster in populations sampled (Figure 2). Greater genetic diversity was found in adult individuals than seedlings ($t = 3.8093$, $df = 18$, $p = 0.0013$).

Table 2. Primer sequences, percent success, polymorphisms, and annealing temperatures for loci screened

Locus ID	% Success	Polymorphism (Y/N)	Annealing Temperature (°C)	Allelic Diversity	Repeats
9597	87%	N	58	N/A	N/A
501	50%	N	58	N/A	N/A
4150	100%	Y	58	6	2
405	63.20%	Y	59	6	2
688	60.90%	N	59	N/A	N/A
1941	78.90%	Y	59	12	2
191615	95.70%	N	58	N/A	N/A
3826	78.90%	Y	59	14	3
5351	89.50%	Y	58	4	3
1586	100%	Y	59	8	3
950680	100%	N	59	N/A	N/A
183	68.40%	Y	59	2	4
4044	78.90%	Y	59	15	5
116	55%	N	59	N/A	N/A

Table 3. Loci screened and repeat patterns

Repeat pattern	Locus ID	Microsatellite	Total number of loci amplified
Dinucleotide	9597, 501, 4150, 405, 688, 1941	AT, AT, AG, AC, AG, AT	6
Trinucleotide	191615, 3826, 5351, 1586	AAT, ATC AAG, AAC	4
Tetranucleotide	950680, 183	AAAG, ACAT	2
Pentanucleotide	4044	AAATG	1
Hexanucleotide	116	ACTGCC	1

Table 4. Total number of alleles by population and their distance from the Biltmore Estate

Site	Total Alleles	Distance (km)
10	10	10
21	8	20
23	4	20
56	13	50
57	10	50
58	12	50
67	11	60
69	14	60
70	6	70

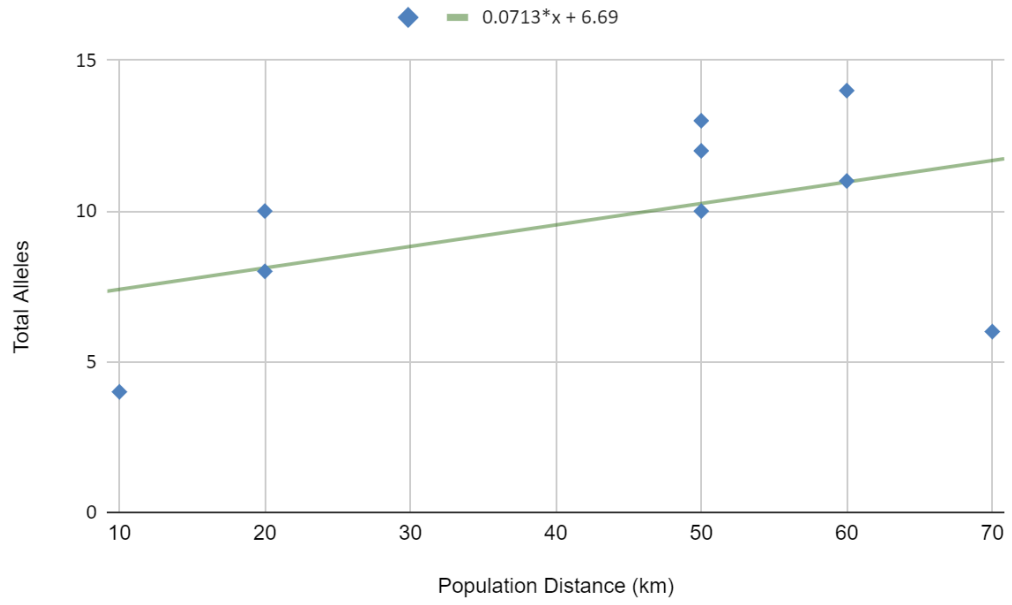

Figure 1. Allelic diversity of *Celastrus orbiculatus* populations as distance from the Biltmore Estate increases, with line of best fit. Adjusted $R^2 = 0.1016$

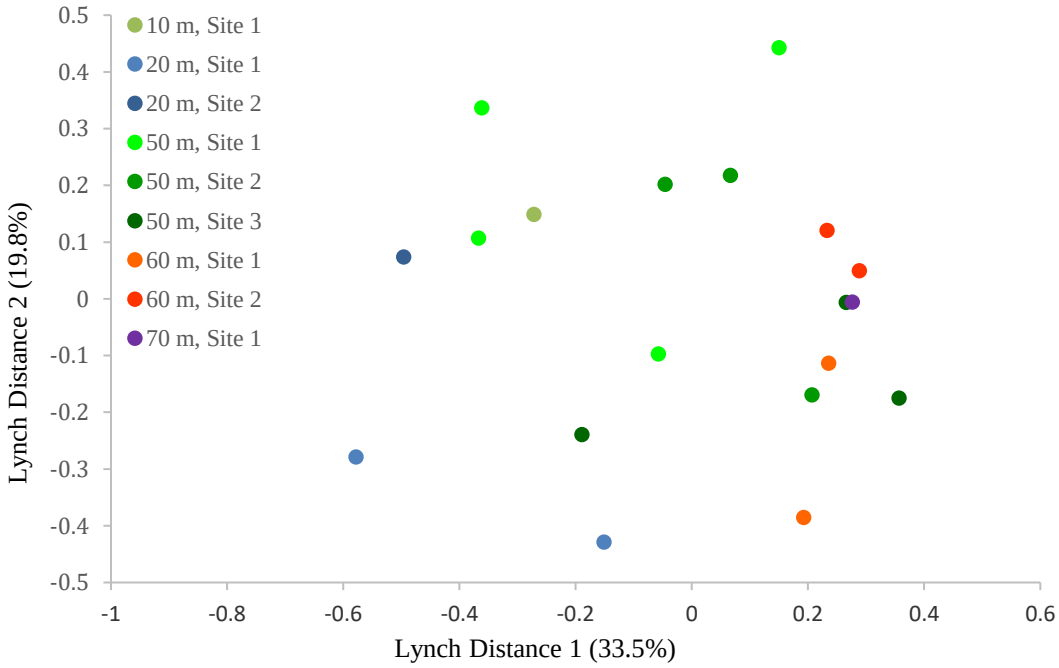

Figure 2. Principal component analysis of *C. orbiculatus* genotypes by population. Each color represents a different population, and each dot represents an individual. Multiple genetic variables have been collapsed into two that account for 53.3% of genotypic variability in individuals sampled. No clustering of genotype by population (color of dot) was observed.

4. Discussion

Fourteen primer pairs were developed for *Celastrus orbiculatus*. Until now, only two primer pairs have been previously developed, but having more available primers makes proper population studies more informative[1,9,10]. These genetic markers can be used to quickly and inexpensively acquire a large amount of genetic information about *C. orbiculatus* populations and potentially help manage its spread[9.11.13]. Lower genetic diversity in seedlings compared to adults could have multiple causes but may be due to a lower seedling sample size than adult, which could be remedied by examining more seedlings from target sites. However, the difference in genetic diversity could also be due to which individuals are sexually reproducing, which may be a smaller subset of the overall *C. orbiculatus* population. Collection of more samples in the same populations over time may reveal if loss of genetic diversity continues, possibly due to selective pressure for a particular genotype[12].

Genotypes were not clustered by population in sites sampled, and there was no significant relationship between allelic diversity and distance of a population from the Biltmore Estate. Genotypic clustering would likely indicate the population originating from a common source or geographic location[12]. A lack of genotype clustering by sample site could be due to individuals originating from multiple locations after avian dispersal. An alternative explanation could be fitness benefits of having a genetically diverse population rather than selection for a particular genotype. No relationship between allelic diversity and distance from the Biltmore Estate could indicate the Biltmore Nursery was not the origin of invasion for the surrounding area, because we would expect diversity to decrease as distance increased due to selective pressure on the founder population. However, sampling sites at increasingly farther distances may be required to confirm or deny this lack of a relationship.

Future work could include using the developed markers to amplify samples from more individuals, especially seedlings, from the harvest sites, sampling individuals from populations further away from the Biltmore Estate, and comparing *C. orbiculatus* populations in near Asheville, NC to those near the Arnold Arboretum. Utilizing more samples in the Asheville area would counteract potential deficiencies in seedling sample size and site distance, while sampling from the Boston MA area could search for signs of the origin of invasion there. Development of more genetic markers utilizing tetranucleotide, pentanucleotide, and hexanucleotide repeats might also be useful, as relatively few of those were developed in this research compared to dinucleotide and trinucleotide markers.

5. Acknowledgements

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