MICROPROPAGATION AND EVALUATION OF THE GENETIC POPULATION STRUCTURE OF RIVER CANE *ARUNDINARIA GIGANTEA*, A SPECIES SUITABLE FOR RIPARIAN RESTORATION

by

ALEX CHARLES RAJEWSKI

(Under the Direction of Donglin Zhang)

ABSTRACT

River cane [*Arunindaria gigantea* (Walter) Muhl.] is an endangered species of endemic North American bamboo. Because of its ability to effectively control erosion along waterways and function as a riparian buffer, restoration of river cane is of great importance. However, there have been few investigations into propagation methods for river cane. Here a method of micropropagation on nodal segments was initiated and showed that 7 mg/L of benzyladenine produced superior shoot proliferation *in vitro*. To further inform restoration, the genetic population structure of three river cane population was investigated. Six simple sequence repeat loci and sequencing of three chloroplast regions showed that each population contains a dominant genotype accounting for 66%-93% of individuals, and genotypes are shared across populations, indicating low differentiation among populations

INDEX WORDS: River cane, Arundinaria, micropropagation, tissue culture, meta-topolin, microsatellites, simple sequence repeats, SSR, population genetics, chloroplast sequencing, endangered species, restoration

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DEDICATION

To my mother and sister, whose advice and guidance have shaped me immeasurably.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 PLANT DESCRIPTION AND IMPORTANCE

River cane [*Arundinaria gigantea* (Walter) Muhl.] is a bamboo species occurring natively in 22 states of the southeastern United States. River cane typically grows along rivers and streams in dense monotypic stands referred to as canebrakes. These canebrakes are ecologically important and form a habitat for at least 70 vertebrate species as well as many insect species (Platt et al., 2013). Additionally, canebrakes are riparian buffers whose dense rhizome and root networks mitigate the negative effects of nitrite runoff and erosion caused by agricultural land use. River cane conservation and restoration are also important issues to the Cherokee and many other Native American tribes, who use river cane as an artistic medium.

Starting as early as De Soto in the 16th century, early European scouts and settlers of North America described vast canebrakes in the southeastern United States (Ranjel et al., 1904). Although canebrakes were seldom measured exactly, various accounts mention canebrake sizes of up to one half mile wide and two miles long. The size of such canebrakes differs drastically from what is observed in the present day. As an explanation of this disparity, it has been suggested by Platt and Brantley (1997) that the unusually large canebrakes witnessed by the Europeans were indirectly the product of the arrival of the Europeans and not the status quo of the North American landscape. Old World diseases brought to America by the explorers decimated the Native American population. This decline in Native American population meant a corresponding decrease in agricultural land use. Since river cane canebrakes thrive in areas of

moderate ecological disturbance and Native American fields were often located on alluvial plains in which river cane grows naturally, the invasion of the abandoned fields by river cane was rapid. In the 200 years between the Native American population decline and the more extensive European exploration of the continent, rhizomes left over in the fields quickly regenerated and created the enormous canebrakes observed (Denevan, 1992; Platt and Brantley, 1997)

Since widespread European settlement, however, the overall size of these canebrakes has decreased by 98% (Noss et al., 1995). As evidenced by the Native Americans' use of the alluvial plains for farmland, areas suitable for the growth of river cane are often highly fertile; this correlation led to the destruction of canebrakes for the re-creation of farmland. An altered, more frequent regime of prescribed burning prevented the canebrakes from establishing themselves as they had previously done under the system of more infrequent fires employed by the Native Americans. Additionally, overgrazing of the leaves and trampling of the culms by cattle reduced the vitality and spread of river cane. Together, these two alterations caused the supplantation of river cane by other woody species (Platt and Brantley, 1997). As a result, canebrakes are now considered to be a critically endangered ecosystem (Noss et al., 1995).

1.2 PROPAGATION STRATEGIES

Based on their historic and cultural significance, but also more significantly on their potential environmental and ecological impacts, there is great interest in the reestablishment and management of canebrakes across their native range. However, the reestablishment of river cane canebrakes presents a set of unique challenges.

River cane is a temperate woody bamboo (Poaceae: Bambusoideae: Bambuseae; (Triplett and Clark, 2010)), and like many other bamboo species in its tribe, river cane is a gregariously flowering, semelparous species, meaning that all the members of a cohort tend to flower *en*

masse, produce massive amounts of seed, and then die shortly thereafter (Janzen, 1976; Mathews et al., 2009). Bamboos in general are also known to have very long intervals between flowering events in a population (intermast periods) and a very long period until sexual maturity (Janzen, 1976). For river cane within a canebrake, the reported intervals between mass flowering events range from three to 25 years (Triplett et al., 2010) although yearly flowering has occasionally been observed (Thomas Peters, personal communication). Though numerous seeds are produced, reports by Baldwin (Baldwin et al., 2009) suggest that river cane seed has a variable and low germination rate of 6% to 58% depending on temperature and the specific population sampled. Other research by Gagnon and Platt (2008) reported similarly variable seed production, but high germination rates, up to 95%. This research suggests that a system of seed-based mass propagation of river cane would be subject to high uncertainty in seed availability and viability.

As commercial propagation of other bamboo species is typically vegetative, it might seem appropriate to explore vegetative propagation as a restoration method. In fact, recent reports by several researchers have sought to ascertain the ideal conditions for vegetative propagation, establishment, and maintenance of river cane (Dalzotto, 2013; Schoonover et al., 2011; Zaczek et al., 2010). While this method does appear to be a viable option for restoration of river cane, vegetative propagation has several drawbacks. This production is limited by the amount of source material that can be collected from the field or that is available in nursery fields, making large-scale restoration efforts difficult. Additionally, vegetative propagation is hampered by the possibility of cavitation and death of the source material during transplant and by the logistic difficulties of harvesting, cleaning, transporting, and replanting the material.

Aside from sexual propagation and vegetative macropropagation, a third method for restoration is the use of micropropagation. Micropropagation or tissue culture is *in vitro* clonal

multiplication and is capable of producing large numbers of transplantable individuals from a very small amount of source material. Thus, micropropagation, unlike seed-based or vegetative macropropagation, offers the possibility to regenerate larger numbers of transplantable plants without uncertainties in source material acquisition. This technique has been successfully developed for a number of bamboo species (Agnihotri et al., 2009; Jain et al., 2007; Jiménez et al., 2006; Lin et al., 2007; Lin and Chang, 1998; Malay and Amita, 2005; Mudoi and Mina, 2009; Niladri et al., 2000; Priyanka and Rao, 2006; Ramanayake et al., 2006; Ramanayake et al., 2001; Ramanayake and Yakandawala, 1997; Shirin and Rana, 2007; Yogeshwar et al., 2008). Micropropagation research with river cane is limited to a single report attempting regeneration via shoot proliferation (Baldwin et al., 2009); however, multiplication rates were not calculated. Additionally root induction was not successful, and the method failed to produce transplantable plants. A refined method for river cane micropropagation with an emphasis on rooting is critical for effective and efficient restoration efforts to begin.

Initiation of micropropagation cultures is dependent upon generating disinfested explant material. Disinfestation protocols using mercuric chloride are extremely effective and very common with bamboo, which is notoriously difficult to disinfest (Jain et al., 2007). However, mercuric chloride is an extremely hazardous compound, and other combinations of less hazardous compounds can achieve similar results (Jain et al., 2007). A further consideration with culture initiation is the balance between disinfesting the explant and killing it. Extremely harsh methods will yield ascetic cultures but can also result in death of the plant material. Thakur (2006) developed a method for reduced contamination of bamboo explants using a combination of fungicide and antibiotic rinses of dissected tissue, and this method has yet to be evaluated in river cane.

After the establishment of disinfested material, the explants are typically grown in a media supplemented with a cytokinin in order to induce shoot proliferation. Previous research with river cane has shown that Murashige and Skoog (MS) medium performs comparably to Woody Plant Medium (WPM) and superior to Lepoivre (LP) and Driver & Kuniyuki Walnut (DKW) media (Baldwin et al., 2009). This same study used the synthetic cytokinin thidiazuron (TDZ) together with indole-3-butyric acid to promote shoot proliferation, however concerns regarding abnormal growth drive most micropropagation studies to use benzyladenine (BA) over TDZ. BA is the most widely used cytokinin for shoot proliferation in plant tissue culture. However, it has recently been shown that 9-β-glucopryanosyl-benzyladenine, a metabolic derivative of BA, can accumulate in basal tissues of some plants. This persistent metabolite can inhibit rooting up to nine weeks after the plants have been transferred to new media (Werbrouck et al., 1995). Meta-topolin (mT) is a natural analog of BA that has been shown to metabolize into derivatives that break down more readily and thus do not inhibit root formation *in vitro* or *post vitro* (Werbrouck et al., 1996). Micropropagation protocols for many difficult-to-root species now suggest mT as the preferred cytokinin. Additionally, mT been reported to produce shoots with increased vigor and fewer incidences of hyperhydricity (Bairu et al., 2007; Valero-Aracama et al., 2010). Given the previously observed difficulty in rooting river cane explants, an investigation into the ability of meta-topolin as an alternative cytokinin would be extremely useful to develop a refined micropropagation technique.

1.3 POPULATION GENETICS OF RIVER CANE

Implicit in all of these propagation methods is the need for a deeper understanding of the population structure of the canebrakes themselves. Growth of river cane is primarily vegetative (clonal) with new culms emerging from rhizomes, which extend leptomorphically from existing

culms (Judziewicz et al., 1999). River cane flowers protandrously and is assumed to be a primarily outcrossing species, although no thorough floral development studies or progeny analyses have been published, likely due to the rarity of flowering events. As mentioned previously, river cane is semelparous and gregariously flowering (Gagnon and Platt, 2008). Given this combination of factors, without adequate knowledge of the population structure of a canebrake, restored canebrakes could display vigorous vegetative growth for approximately 25 years, die back after a gregarious flowering event, and potentially produce no viable seed due to unrealized self-incompatibility. This scenario would require constant and costly observations and management of restored canebrakes to prevent their loss and would be unsustainable. It is therefore important to know the necessary genetic diversity, vis-à-vis number of distinct genotypes, to form a sustainable canebrake across generations. Although this cannot feasibly and empirically be proven, studying the diversity currently present in several natural canebrakes can give a reasonable approximation of the typical genetic diversity present.

Surveys of population genetic diversity across a variety of clonal species have shown a recurring trend; populations tend to have intermediate diversity spread evenly across populations, and extremely widespread clones are the exception not the rule (Ellstrand and Roose, 1987). While this study included 20 species of vegetatively propagating plants, no member of the bambusoideae subfamily was included. Given the unique life history of the bamboos, it is not unreasonable to assume that their population genetic structure could differ. In a survey of mass flowering behavior in *Sasa cernua*, a close relative of *Arundinaria*, Kitamura and Kawahara (2009) examined over 1200 culms in a single population using five simple sequence repeats (SSRs) and found only six multilocus genotypes (putative clones). They furthermore showed that a single clone accounted for 93% of the culms surveyed. Mathews et al. (2009) characterized the

flowering habits of two stands of river cane based on amplified fragment length polymorphism (AFLP) analysis of 22 and 37 individuals, respectively, sampled on transects. This study by found low polymorphism at the AFLP loci used but still was able to distinguish six unique clones for each of their two sample sites. No genotype was shared across stands, and each site was shown to have a dominant genotype that accounted for approximately 75% of the individuals surveyed. However, this AFLP study in particular set a very high threshold of genetic dissimilarity for distinguishing clones, 9% versus the typical 2%-5% (Meudt and Clarke, 2007), which was based on the reproducibility of replicate samples. Despite the similarity of results obtained between Mathews et al. and Kitamura and Kawahara, a more thorough survey of within- and among-population diversity for river cane with more thorough sampling would better inform efforts to restore canebrakes.

Several genetic techniques can be used to determine population level genetic diversity. Single nucleotide polymorphisms (SNPs) are the most informative technique currently used (Hudson, 2008). On a per locus basis, SNPs provide very little information or discriminatory power, but typical SNP analyses survey thousands of SNP loci across the genome. This large number of loci gives SNPs an advantage over other techniques. SNPs are not widely used in population genetic studies because there are many barriers to their use in non-model species (Helyar et al., 2011). Most non-model species lack reference genomes, making proper assembly of SNP data difficult, and the bioinformatics considerations for SNP studies can also be prohibitive. Currently, the closest relative to river cane with a sequence genome is *Phyllostachys edulis* (Peng et al., 2013). Therefore a technique requiring less genomic information is necessary. Many techniques do not require prior knowledge of the species' genome. Several PCR-based techniques such as AFLP, ISSR, SSR, and RAPD have been used extensively in population

genetic studies of a variety of species including many bamboos (Belaj et al., 2003; Mueller and Wolfenbarger, 1999; Yeasmin et al., 2014). These techniques are uniquely suited for many endangered species, which rarely have sequenced genomes. Lack of reproducibility of RAPDs has led to their disfavor, and they are no longer widely used (Jones et al., 1997). AFLP, ISSR, and RAPD are also dominant markers and cannot give information about the heterozygosity of the individuals assayed (Lynch, 1988). SSR by contrast are repeats of 2-5 nucleotides with an extremely variable repeat number and codominant inheritance, making them suitable markers for a variety of population genetic studies (Morgante and Olivieri, 1993). Importantly, SSR has also been shown to give higher estimates of within-population diversity than AFLP due to its increased variability (Nybom, 2004). Although there are no currently published SSR primers in the genus *Arundinaria*, it has been shown the SSRs developed in one species can often be applied for genetic studies in closely related species (Peakall et al., 1998). The *Arundinaria* species complex has low genetic diversity, akin to other bamboos (Triplett et al., 2010; Zeng et al., 2010). This is likely due to their extensive clonal growth and extremely infrequent flowering events. Therefore, the hypervariability of SSR markers and their transferability makes them suitable for examining the population structure of river cane.

While no SSR markers have been developed for *Arundinaria,* SSR primer sequences are available for several commercially important bamboos and other important bamboos. Primers have been developed in several bamboos in the *Sasa* genus (Kitamura et al., 2009; Miyazaki et al., 2008; Nayak and Rout, 2005), *Phyllostachys edulis (Jiang et al., 2013; Tang et al., 2010),* and several more distantly related bamboos (Dong et al., 2011; Kaneko et al., 2007; Sharma et al., 2009). Additionally, the rice (*Oryza sativa*) genome shows high homology with other sequenced bamboos (Peng et al., 2013), suggesting that the enormous number of rice SSR

markers might be applicable in *Arundinaria.* In populations of *Arundinaria*, a large amount of clonal growth is expected, and, due to low population density, the individual genotypes are expected to be closely related. Thus, the level of diversity at each locus within each population is expected to be extremely low, and a larger number of SSR loci will be needed to identify unique genotypes within stands.

Within the genus *Arundinaria,* most taxonomists currently recognize only the closely related North American species (Triplett and Clark, 2010). In addition to the type species, *Arundinaria gigantea*, the literature notes at least two other species, *A. tecta* and the newly discovered *A. appalachiana* (Hughes, 1951; McClure, 1973; Triplett, 2006). The taxonomic history of river cane and closely related species has seen them classified as one to three species, with varying numbers of subspecies and varieties (Triplett, 2006; Triplett and Clark, 2009; Ward, 2009). Additionally, the cane species have been subject to several taxonomic regroupings and renamings. In addition to the three currently recognized species and the putative hybrids that have been anecdotally mentioned, the scientific names *Arundinaria macrosperma, Arundinaria gigantea ssp. gigantea, Arundinaria gigantea ssp. tecta, Arundinaria gigantea ssp. macrosperma, Arundo gigantea*, and finally *Arundo tecta* designate a slew of historically overlapping names for potentially the same plants (Triplett and Clark, 2009; Ward, 2009). Common names for the species show an even higher level of overlap and inconsistency. The taxonomic confusion owes to the morphological similarity of the three putative species. Characteristics for distinguishing the species are often idiosyncratic and include presence of aerenchyma in the roots, number of top-knot leaves, leaf length and width, leaf texture, and leaf vestiture. For the three species, most of the diagnostic characters overlap, making a determination based on morphology nearly impossible.

A study of the three putative species by Triplett (Triplett et al., 2010) using AFLP markers showed that the genetic distance between *A. tecta* and *A. appalachiana* (Nei's D=0.081) was much smaller than the distance between these two species and *A. gigantea* (Nei's D=0.55). The authors found low overall diversity within and among the species as well as weak indications of isolation-by-distance, consistent with other bamboo genera. Their results generally support the three-species theory, but a lack of consistent clustering of the species in their study as well as no study of the fertility of the hybrids mentioned means that conclusive evidence for the species is lacking. The authors also included only one sample per population, assuming low diversity. Other studies, which used chloroplast genome sequences or nuclear gene sequences to investigate the phylogeny of bamboos in general, have reiterated the low diversity and the general relationship between the three species of *Arundinaria*. (Burke et al., 2012; Triplett and Clark, 2010; Yeasmin et al., 2014; Zeng et al., 2010; Zhang et al., 2012).

Any successful plan for sustainable restoration of river cane will require an investigation into the within- and among-population diversity of the species, which is so far lacking. Though previous studies have been conducted, their sampling strategies have not allowed a meaningful comparison of diversity across populations.

1.4 RESEARCH OBJECTIVES AND APPROACHES

The primary goal of this study was to determine a shoot proliferation protocol for river cane micropropagation to begin to create a stable source of material for restoration efforts. This was accomplished using nodal explants grown on either meta-topolin or 6-benzylaminopurine supplemented media, and their effects on plant growth were compared. The secondary objective of this study was to determine the genetic structure within and between populations of river cane so that reintroduction strategies can be developed that address the number of genotypes required

to maintain healthy populations. This was ascertained using SSR polymorphism data from loci developed in closely related species and was supplemented using chloroplast sequence data. The number and size of clones per population is reported along with measures of population structure, including F_{ST} and Nei's D, which will inform restoration efforts of new canebrakes.

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CHAPTER 2

BENZYLADENINE OUTPERFORMED META-TOPOLIN IN SHOOT PROLIFERATION OF RIVER CANE

2.1 INTRODUCTION

River cane [*Arundinaria gigantea* (Walter) Muhl.] is one of only three congeneric bamboo species that are native to North America (Judziewicz et al., 1999). This fast growing species can reach heights of more than six meters and naturally occurs along the lowland banks of streams and rivers, often forming monotypic stands called canebrakes. Within the United States, river cane is endemic to the southeastern states but grows as far north as Pennsylvania and extends west to Louisiana. Although river cane once covered vast areas of these states, conversion to farmland and altered burning regimes have shrunken river cane populations by an estimated 98%, making it a critically endangered species (Noss et al., 1995; Platt and Brantley, 1997).

The restoration of river cane populations is therefore critical from a conservation standpoint, but the species also has a profound ecological significance. Canebrakes possess a dense system of roots and rhizomes, which can control erosion along the waterways where it grows, and river cane's rapid growth allows it to absorb nitrogen fertilizer runoff that could potentially pollute waterways. Additionally, canebrakes form a habitat for many species including over 70 species of vertebrates (Platt et al., 2013). Direct human use of river cane is limited, but the plant has cultural and artistic significance for many Native American tribes who have used river cane in basketry, tapestries, and as weapons for over 500 years (Ranjel et al.,

1904). Because of its fast growth and ecological significance, river cane has recently been suggested as a species suitable for wetlands restoration efforts.

Although vegetative growth in river cane is rapid, natural establishment of new canebrakes is rare. Propagation of river cane in the wild is predominantly vegetative, with new culms arising from rhizomes, which extend leptomorphically from existing culms (Judziewicz et al., 1999). Similar to most other woody bamboos, river cane flowering events are extremely rare (every 30-40 years) and gregarious, with the flowering culms typically dying off in the following year (Brown, 1929; Gilly, 1943; Hughes, 1951; Janzen, 1976). Current propagation techniques for river cane employ rhizome divisions, but are labor intensive, slow, and potentially destructive to an existing canebrake (Dalzotto, 2013; Schoonover et al., 2011; Zaczek et al., 2004).

Development of an *in vitro* micropropagation technique for river cane has the potential to alleviate these problems and supply consistent, transplantable plants for wetlands restoration. Although many other bamboo species have well established micropropagation protocols (Jain et al., 2007), studies on river cane *in vitro* micropropagation are limited to a single published study, but no rooting of explants was achieved (Baldwin et al., 2009).

Benzyladenine (BA) is the most commonly used cytokinin for shoot proliferation *in vitro*, and indeed forms the basis of most micropropagation protocols due to its affordability. However, in some species 9-β-glucopyranosyl-benzyladenine, a metabolic derivative of BA, has been shown to accumulate in basal tissues of the plant and persist for up to nine weeks, inhibiting the rooting and acclimation phases of micropropagation (Werbrouck et al., 1995). *meta*-Topolin (mT), a natural analog of BA first isolated from poplar trees, has been shown to metabolize into derivatives that break down more readily and thus do not inhibit root formation *in vitro* or *post vitro* (Werbrouck et al., 1996). This compound has recently been shown to be the preferred

cytokinin for several difficult-to-root species *in vitro* and has also been reported to show increased vigor in the shoots and fewer incidences of hyperhydricity (Bairu et al., 2007; Valero-Aracama et al., 2010).

To date there are no published reports of micropropagation protocol employing mT in bamboo or studies of its effectiveness in bamboo. It is the objective of this study to compare the effects of differing concentrations of the widely used cytokinin BA and the relatively newer cytokinin mT on *in vitro* shoot proliferation of river cane. Additionally, the effects of these two cytokinins on rooting percentage will be compared.

2.2 MATERIALS AND METHODS

Culture Initiation and Proliferation

Multiple rhizomes, each with 6-7 nodes, were collected from a dense stand of river cane in January 2014 in Athens-Clarke County, Georgia. Each rhizome was planted horizontally in a 3-gallon pot and grown in a heated greenhouse at the University of Georgia. In early February 2015, the approximately 1-1.5 m plants were cut back to 10cm and treated with the systemic fungicide Procure 480 SC (Triflumizole, Chemtura Corp., 8 oz./gal). Culms with multiple nodes and a diameter of approximately 5 mm were harvested in late March for culture initiation (Figure 2.1). For each culm, the culm leaf sheathes were manually dissected away down to the nodal lines, exposing the leaf buds. The culms were then divided into 2-3 cm sections each containing a single node and bud (Figure 2.2).

Disinfestation followed a procedure adapted from Thakur (2006). The nodes were washed for 20 minutes in soapy water and rinsed three times with deionized water. Under aseptic conditions, the nodes were rinsed in 70% ethanol for 30 seconds, followed by sterile water for 3

minutes. The nodes were then transferred to a sealed 1 L Erlenmeyer flask containing a combined solution of antibiotic and fungicide (Rifampicin 30 mg/L [Sigma] and Procure 480 SC 0.625 mL/L) with Tween-20 and agitated on an orbital shaker at 100 rpm for 1 hour. After this, the explants were rinsed twice with sterile water for 5 minutes, and then subsequently in a solution containing 4% (v/v) sodium hypochlorite with Tween-20 for 5 minutes. Finally, the explants were rinsed three times with sterile water. Disinfested explants were placed vertically in test tubes. All cultures were maintained under 16-hour daylight (125 μmol m^{-2} s^{-1}) at 25 °C.

Cultures were initiated in test tubes containing 20 mL of media with Murashige and Skoog (MS) salts, 3% (w/v) sucrose, 1.5 mg/L nicotinic acid, 10.1 mg/L thiamine-HCl, 1.5 mg/L pyridoxine-HCl, 2 mg/L glycine, and 100 mg/L myo-inositol. The medium was gelled with 0.4% (w/v) Gelzan (PlantMedia, Dublin, OH, USA), and adjusted to pH 5.8 before autoclaving. Because the effects of cytokinins were to be evaluated, the media was left either free of plant growth regulators as a control or supplemented after autoclaving with filter-sterilized BA at 3-10 mg/L and naphthalene acetic acid (NAA) at 0.2mg/L or mT at 0.1-5mg/L (Table 2.1). After four weeks, the growing explants were aseptically transferred to new test tubes containing fresh media, and shoot height and number were recorded.

Root Initiation

Six weeks after culture initiation, the newly initiated shoots were excised together (groups of 1-6 shoots) from their original nodes, cut to approximately 3 cm in height, and transferred as a group to Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL, USA) containing 60 mL of media prepared as above but supplemented with 5 mg/L filter-sterilized indole-3-butyric acid (IBA).

Statistical Analysis

All statistical analyses were conducted with SAS™ software version 9.4 (SAS Institute, Inc, Cary, NC, USA). Data on heights of shoots were analyzed for mean differences and separations using ANOVA and Tukey's HSD, respectively. Count data for shoot number were analyzed using a Poisson regression of shoot number on media type. Before analysis, shoot number was transformed by subtracting one from the total for each individual in order to fit a Poisson distribution.

2.3 RESULTS AND DISCUSSION

Disinfestation

Previous attempts at micropropagation of *Arundinaria gigantea* resulted in very high rates of latent contamination, often in excess of 90% (data not shown). While many protocols for bamboo micropropagation employ mercuric chloride to disinfest explants (Negi and Sanxena, 2011; Yogeshwar et al., 2008), mercuric chloride is extremely hazardous, and often other methods can produce similar results (Jain et al., 2007). In the current study, an enhanced protocol for explant disinfestation without mercuric chloride was adapted from Thakur (2006). Additionally, all explants were cultured in individual vessels to minimize potential cross contamination, and the greenhouse source material was treated with a systemic fungicide. Despite this pretreatment and the intense disinfestation protocol, moderate contamination of cultures was observed but was restricted to mostly bacterial contamination (Table 2.1). Overall contamination rates were 41/146=28% and 5/146=3% for bacterial and fungal contamination, respectively. Because the media were supplemented (if necessary) with filter-sterilized growth regulators after autoclaving, equality of contamination rates between supplemented and unsupplemented (basal) media was tested and subsequently rejected (chi-squared, $p<<0.01$). The

increased efficacy of post-autoclave addition of growth regulators is well known (Nissen and Sutter, 1990). Unfortunately it is likely that this step introduced the majority of the bacterial contamination into the cultures.

Shoot Proliferation

Previous experiments with BA had shown a positive correlation between BA concentration and shoot proliferation (data not shown), and the levels of BA supplementation selected here were designed to determine the extent of this correlation. In contrast, mT is not widely used in micropropagation, and this is at present the first report of its use in a bamboo species. Therefore, a wide range of mT levels were selected here in order to roughly determine an appropriate concentration for future refinements.

Because counts of proliferated shoots were discrete and low, a Poisson regression was used to determine the effect of BA or mT treatments against the control. Although ANOVA is the more common analysis used for differences in shoot number, the underlying non-normal distribution, inconsistent variance structure, and typically low counts of shoot number violated the assumptions of the ANOVA model. Mize et al. (1999) have suggested that, in cases such as this, a Poisson regression is the most appropriate and most powerful statistical tool to detect differences in treatments. The outputs of the Poisson regression are shown in Table 2.2 and include an intercept as well as parameter estimates for the various treatment conditions. More readily interpretable fold multiplications calculated from the regression are also shown at the right of Table 2.2. The intercept corresponds to the basal media treatment and is output as the natural logarithm of the transformed (mean-1) fold multiplication. The parameter estimate for each of the treatments is also output as a natural logarithm; however; in this case, it is the natural logarithm of fold change caused by that treatment. For example, from Table 2.2, the intercept of

the regression is -1.79, indicating that the basal treatment had a mean shoot multiplication rate of: $e^{-1.79} = 0.167 + 1 = 1.167$. The parameter estimate for the 5 mg/L meta-topolin treatment is 1.6094, which indicates that this treatment is $e^{1.6094} = 5$ fold better than the basal treatment's transformed multiplication rate. This yields a fold multiplication of $5 \times 0.167 = 0.835 + 1 = 1.835$ fold for the 5 mg/L mT treatment.

Except for the 0.1 mg/L mT treatment, which was not significantly different, all treatments resulted in statistically higher fold multiplication than the basal media treatment (Wald Test, $p<0.05$, Table 2.2). The highest fold multiplication was observed in the 7 mg/L BA treatment (2.4 fold); however, multiple pairwise comparisons of the mean fold multiplication results showed that all BA-supplemented treatments and the mT treatments at 1 and 5 mg/L were statistically equal (Wald Test, $p\text{-}value>0.18$).

In order to further characterize the effects of BA and mT, the height of each shoot produced was recorded. The maximum shoot height and summed shoot height for each explant within a given treatment were analyzed in order to demonstrate the differing growth patterns. In the absence of exogenous BA and mT, the explants on basal media had the greatest maximum shoot height, averaging 11.9 cm after six weeks (Figure 2.3). Consistent with the application of high cytokinin, the explants grown on 10 mg/L BA media showed the smallest maximum shoot heights, averaging 8.6 cm. Explants from lower-concentration BA media as well as all mT media were not statistically different from the BA10 or basal explants (Figure 2.3, Tukey's HSD, $p<0.05$). Analyses of summed shoot height failed to show any differences among media types (Figure 2.3, ANOVA, $p>0.1$). Additionally, no differences in leaf morphology, hyperhyricidity, or albinism were noted among the treatments. Taken together, these results suggest that the application of exogenous BA or mT promotes shoot multiplication but is not necessary for

growth *in vitro* and does not noticeably increase explant vigor. However, established protocols for root initiation in bamboos commonly use explants with multiple shoots trimmed to a uniform height; therefore, application of exogenous cytokinin to promote shoot proliferation and reduce shoot height is likely to be necessary.

Rooting

Attrition of explant numbers due to contamination prevented robust statistical analysis of multiple rooting treatments. Instead, explants from all seven shoot proliferation treatments were transferred to a common media containing 5 mg/L IBA (Shirin and Rana, 2007). Although the explants displayed only weak shoot growth, after four weeks, no explant had developed roots.

2.4 CONCLUSIONS

This study presents a protocol for successful disinfestation and multiplication of *Arundinaria gigantea* shoot nodes using MS media supplemented with 7 mg/L BA and 0.2 mg/L NAA. These results also show that shoot multiplication of *A. gigantea* is statistically equal using 1 mg/L of mT, representing the first report of this growth regulator's use in a bamboo species. Because rooting was not achieved here, future studies comparing the rooting capability of BA and mT-grown explants with multiple rooting treatments are needed. Further refinement of mT concentration as well the effects of mT and NAA combinations could lead to enhanced shoot proliferation.

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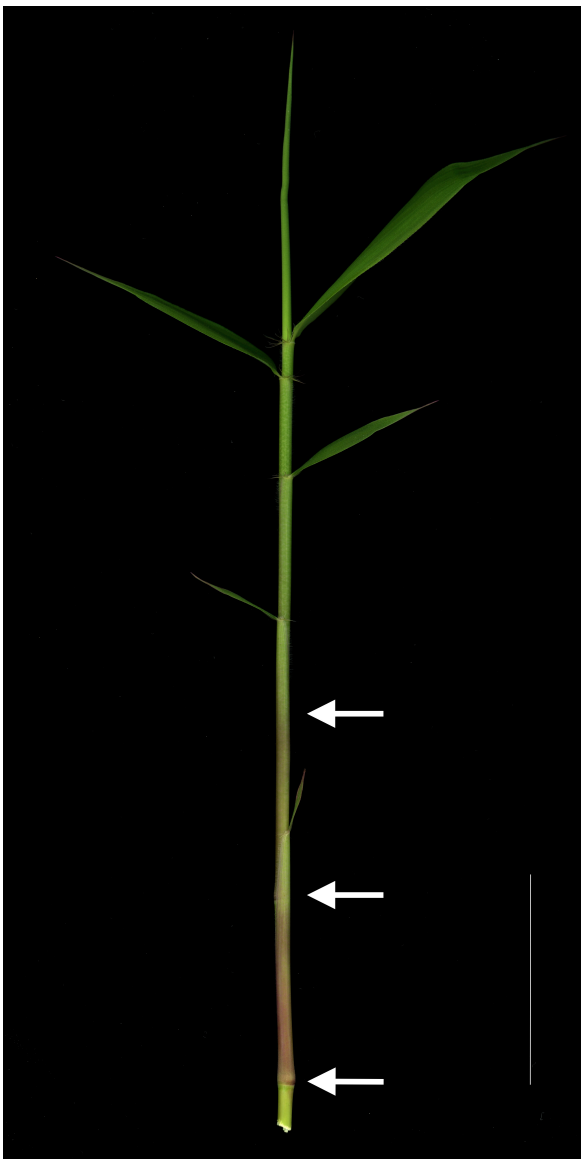

Figure 2.1. Typical shoot for node harvesting. Arrows indicate the position of nodes large enough for use. Scale bar indicates 4 cm.

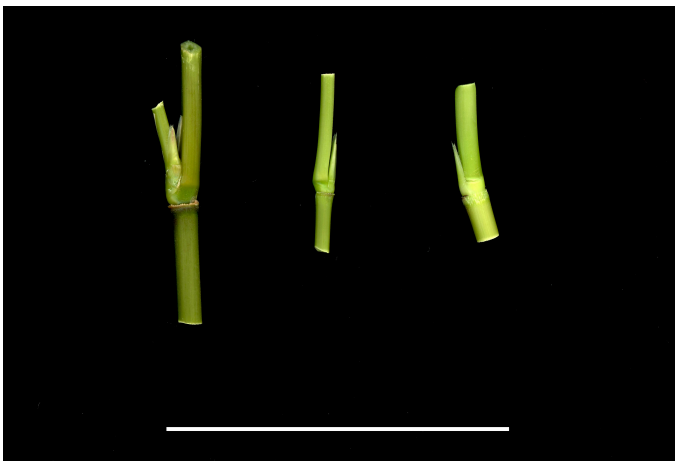

Figure 2.2. Examples of dissected shoot nodes with bud scales used for culture initiation. Scale bar indicates 4 cm.

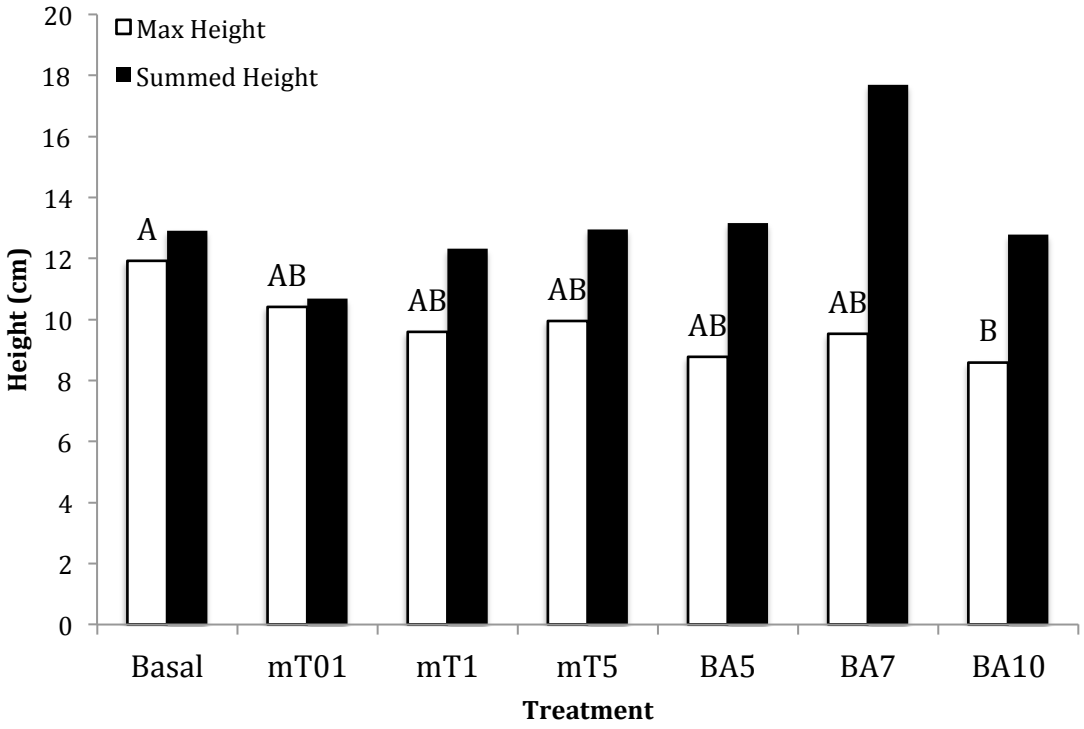

Figure 2.3. Comparison of maximum shoot height and summed shoot height by treatment. Mean separation letters by Tukey's HSD show the treatment with 10mg/L BA to have significantly shorter maximum heights than the basal treatment. No significant differences were observed for summed height.

Table 2.1. Summary of treatments including number of experimental units (n), concentrations of BA, NAA, and mT in mg/L, and the number of experimental units lost to bacterial or fungal contamination.

		BA			Contamination	
	n	(mg/L)	NAA	mT	Bacterial	Fungal
Basal	20	—	—	—	2	1
mT01	21	—	—	0.1	6	1
mT1	21	—	—	1	5	0
mT5	21	—	—	5	5	0
BA5	21	5	0.2	—	6	0
BA7	21	7	0.2	—	10	1
BA10	21	10	0.2	—	7	2

Table 2.2. Poisson regression parameter estimates, significance of treatment effects, and calculated fold multiplication values. Fold multiplication includes mean separation by pairwise Wald's tests (α=0.05).

	Regression Estimate	p-value	Fold Multiplication
Basal	-1.7918	0.011	1.17^{A}
mT01	-0.7732	0.528	1.08^{A}
mT1	1.7918	0.020	2.00^{B}
mT5	1.6094	0.034	1.83^{B}
BA5	1.9169	0.010	2.13^{B}
BA7	2.1282	0.004	2.40^{B}
BA10	1.9636	0.008	2.19^{B}

CHAPTER 3

SIMPLE SEQUENCE REPEATS AND PLASTID SEQUENCING ANALYSIS OF MULTIPLE RIVER CANE POPULATIONS

3.1 INTRODUCTION

The genus *Arundinaria* is a taxonomically complex group of temperate woody bamboos that includes the only species of bamboo native to North America. River cane [*Arundinaria gigantea* (Walter) Muhl.] is the type species for the genus and, along with Switch cane (*A. tecta*) and Hill cane (*A. appalachiana*), has a native range that encompasses the southeastern United States from Pennsylvania to Louisiana. Historically, these species often formed vast monotypic stands called canebrakes that covered large tracts of the landscape. During his explorations of the continent in the 16th century, De Soto described these canebrakes and their extensive use by the Native Americans (Ranjel et al., 1904). Although river cane still enjoys contemporary, though largely ceremonial use in Native American artwork, the size of these canebrakes has shrunken by an estimated 98% since they were first recorded, making river cane a critically endangered species (Noss et al., 1995).

In its native habitat, river cane fulfills a unique ecological niche, supporting many species of insects and birds, but it also functions as a noninvasive riparian buffer, preventing erosion and mitigating fertilizer runoff. Restoration of canebrakes in the southeastern United States has consequently received much attention in the last decade, and many studies have been conducted into developing and improving river cane propagation techniques (Peters, 2013; Schoonover et al., 2011; Zaczek et al., 2004).

However, meaningful and sustainable restoration of canebrakes requires knowledge of their genetic population structure in order to ensure maintenance of adequate diversity. A study by Triplett et al. (2010) of species-level diversity in the genus *Arundinaria* using AFLP and chloroplast sequencing suggested low genetic diversity within *A. gigantea*, *A. tecta*, and *A. appalachiana*, though structure and diversity within individual populations could not be examined. River cane and most other bamboos exhibit widespread clonal growth with rhizomes extending several hundred meters underground sending up genetically identical culms along their length (Suyama et al., 2000). Flowering in bamboos is also extremely infrequent, so studies of outcrossing rates and self-compatibility are rare, although observations from the Asian bamboo species *Sasa cernua* reported high rates of selfing with isolated mixed mating and a high inbreeding coefficient of 0.248 (Kitamura and Kawahara, 2011). Based on their extensive clonal growth and the evidence of high inbreeding from other bamboo species, within population genetic diversity for river cane is expected to be low. Mathews et al. (2009) used AFLP to examine clonal diversity in two river cane canebrakes. The two canebrakes were 65 m^2 and 5905 m^2, yielding 18 and 37 samples respectively; however in each canebrake only six distinct genotypes were detected. Differences in sampling strategies preventing direct comparison of genetic diversity between stands, and lowered reproducibility of AFLP fingerprints in the study limited discrimination of genotypes. Although this study was able to estimate clonal diversity, molecular genetic techniques with potentially greater resolution exist that could give a more precise estimate. Additionally, a direct comparison of clonal diversity between canebrakes could more thoroughly inform decisions on the number and diversity of genotypes needed to reestablish healthy canebrakes.

Currently, the most powerful molecular genetic technique for examining population structure and diversity is SNPs (Hudson, 2008). Although individual SNPs lack the discriminatory power of other techniques, the vastly increased number of loci examined in typical SNP analyses compensates. However, several obstacles prevent the widespread use of SNPs in non-model species (Helyar et al., 2011). Next generation sequencing (NGS) is routinely used in SNP assays, and while the cost of NGS is dropping, many population-level studies assay hundreds of individuals, making the use of NGS prohibitive. Furthermore, many non-model species lack the reference genomes that are necessary for proper assembly of SNP data, and to date *Phyllostachys edulis* is the only bamboo species with a draft genome (Peng et al., 2013). PCR-based techniques such as SSR, AFLP, ISSR, and RAPD have been used extensively in population genetic studies of a variety of species including many bamboos (Belaj et al., 2003; Mueller and Wolfenbarger, 1999; Yeasmin et al., 2014). AFLP, ISSR, and RAPD generate fingerprints of PCR bands and do not require prior knowledge of the sequence to be amplified. They are therefore uniquely suited for many endangered species, which rarely have sequenced genomes. Unfortunately AFLP, ISSR, and RAPD are dominant markers and cannot give information about the homozygosity or heterozygosity of the individuals assayed (Lynch, 1988). In contrast, SSR are codominant tandem repeats of 2-5 nucleotides that exhibit an extremely variable repeat number making them suitable markers for a variety of population genetic studies (Morgante and Olivieri, 1993). Importantly for within-population studies, SSR has been shown to give higher estimates of within-population diversity than AFLP due to increased variability on the part of SSR (Nybom, 2004). Although there are no published SSR primers for the genus *Arundinaria*, primers developed in one species can often be applied in congeners and occasionally across genera or (rarely) families, though typically with reduced polymorphism

(Peakall et al., 1998; Wang et al., 2005). The bamboo clade (Poaceae: Bambusoideae) is known to have notoriously low genetic diversity between species (Zeng et al., 2010), and primers have already been developed in a number of other bamboo species and used for clonal identification (Kitamura and Kawahara, 2009). The primary goal of this study was to determine the within and among population diversity for river cane, including number of individual genotypes, their geographic extent, and the genetic differentiation between populations, in order to inform restoration activities. SSR were chosen for this objective because of their codominant nature, extreme variability, and potential transferability between species.

In order to augment the information gained from SSR analysis, several genomic regions were selected for sequencing in a subset of individuals sampled. The Internal Transcribed Spacer (ITS) region is a nuclear ribosomal gene that has been used for phylogenetic studies of bamboos and other species (Guo et al., 2002; Li et al., 2011). This region is extremely variable and could be used within populations to increase resolution of genotypes.

The Bambusoideaea subfamily is known to contain several polyploid and diploid taxa with variable chromosome number, and *A. gigantea* has been classified as tetraploid with n=24 (Gould, 1960). Recent evidence suggests that the temperate woody bamboos, including *Arundinaria spp.,* (Tribe: Arundinarieae) are allotetraploids between two ancient temperate bamboo lineages (Triplett et al., 2014). Their polyploid origin makes the differentiation of homeologous nuclear gene sequences difficult; however, chloroplasts are typically uniparentally inherited, they have been widely used in studies of bamboos to avoid the problems of polyploidy (Leducq et al., 2012; Triplett and Clark, 2010; Triplett et al., 2010; Zeng et al., 2010). Several variable chloroplast regions have also been proposed for use in species identification of land plants, including *rbcL, trnH-psbA, atpF-atpH,* and *matK*, but the low level of nucleotide

divergence shown in previous studies of bamboos has reduced the genetic signal these commonly used regions can provide (Fazekas et al., 2008; CBOL Plant Working Group, 2009; Zeng et al., 2010). Recently, other chloroplast regions have been implemented among the bamboos with greater success. Zeng et al. (2010) used eight plastid regions to construct a phylogeny of temperate bamboos and reported several regions that showed elevated mutation rates among this clade. Two of those chloroplast regions (*rpl*32-*trn*L and *psa*A-ORF170), along with the more commonly accepted *matK* chloroplast region and the nuclear ITS regions were selected for analysis in the present study. A previous study of chloroplast haplotypes in the genus *Arundinaria* detected few haplotypes within each species, but this study was restricted to single samples per population (Triplett et al., 2010). To gain an understanding of haplotype diversity within populations, here multiple individuals from each sampling population were selected for analysis.

3.2 MATERIALS AND METHODS

Site Selection

Three sampling sites were selected for this study (Figure 3.1). The largest site was located in the Tallassee Forest near Athens-Clarke County, Georgia (33°58'13"N, 83°29'7"W) and consisted of several hundred culms growing sparsely over approximately 5,000 m^2 of land along the northern bank of the Middle Oconee River. River oats (*Chasmanthium latifolium*) and Japanese Stiltgrass (*Microstegium vimenium*) are the dominant understory species, with river cane intermingled among them. Despite the presence of the invasive Japanese Stiltgrass, the 219-hectare Tallassee Forest is a mature bottomland forest that is largely ecologically undisturbed. A second sampling site in Athens-Clarke County, Georgia was selected near Old Hull Road, along a tributary of the North Oconee River (33°58'30"N, 83°21'10"W). The Hull

Road site was the smallest and densest of the sampling sites, also consisting of several hundred culms but only spread over approximately 200 m^2. This site was monotypic except for peripheral invasion by Smilax (*Smilax spp.*). The two sites are approximately 13 km apart, and their respective rivers unite downstream of both sampling sites, so colonization of one site via seeds or rhizome nodes from the other is unlikely. The final site covered approximately 5,000 m^2 located near Tellico Plains, Tennessee along the Coker Creek (35°13'10"N, 84°20'13"W) and consisted of a few hundred culms growing tightly along the riverbank for approximately 500 m. This site included a diverse mixture of shrub and tree species interspersed with river cane. Though the culms occasionally invade a neighboring field, those are regularly mowed over or cut back by the landowner.

All sites were sampled on a 10 m grid delineated by GPS; however, dense canopy cover in the Tallassee Forest site reduced accuracy of the GPS signal. Due to its compact size, the Hull Road site was also subsampled on a 2 m grid. This sampling density yielded 114, 26, and 37 samples for the Tallassee, Hull Road, and Tennessee sites, respectively. In addition to these three populations, known samples of *A. gigantea* and *A. tecta* were grown in the greenhouse from rhizomes kindly provided by Brian Baldwin of Mississippi State University.

Sample Collection and DNA Extraction

For each sampled culm, six circular leaf punches approximately 6 mm in diameter were collected in 2 mL tubes, placed on dry ice, and then stored at -80 ºC until DNA extraction. One round BB was placed in each collection tube, and the samples were frozen in liquid nitrogen and ground for 60 seconds at 30 Hz using a TissueLyser (QIAGEN, Valencia, CA, USA) to a fine powder.

DNA for samples collected from the Tennessee site was extracted following the procedure from King et al. (2014) except that all centrifugation times were doubled to better pellet the materials and the final resuspension was performed in 200 µL of TE buffer. Total DNA from the Hull Road and Tallassee site samples was extracted using the E.Z.N.A Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA). All DNA concentrations were measured with a TECAN Infinite M200 and NanoQuant Plate (TECAN, Morrisville, NC, USA) and diluted to 20 ng/µL in TE for downstream use.

SSR Analyses

A total of 35 primer pairs (Table 3.1) were selected and synthesized (Sigma-Aldrich, St. Louis, MO), representing six species of woody bamboos as well as rice (*Oryza sativa*), the most closely related crop plant to bamboos (Jiang et al., 2013; Kaneko et al., 2007; Kitamura et al., 2009; Miyazaki et al., 2008; Nayak and Rout, 2005; Tang et al., 2010; Wang et al., 2005). Initially, a tag (5'-TGT AAA ACG ACG GCC AGT-3') was appended to the 5' end of each forward primer for use with a universal fluorescently labeled primer (Schuelke, 2000). Various PCR conditions were tested for the initial primer pairs and trios; however, this technique was abandoned due to extremely poor incorporation of the fluorescent primer (data not shown). The best performing 12 primer pairs under this system were selected and synthesized with 5' fluorescent labels (Integrated DNA Technologies, Coralville, IA). Published protocols for each primer pair were followed except for alterations to annealing temperatures (Table 3.1); however, the primer Phe23 required a modified touchdown PCR protocol to achieve consistent amplification. This protocol consisted of: a 95 ºC initial denaturation for 90 s; 10 cycles of 95 ºC for 15 s, a 62 ºC annealing step for 20 s decreasing by 1 ºC every cycle, and a 72 ºC extension step for 30 s; 35 cycles of 95 ºC for 15 s, 52 ºC for 20 s, and 72 ºC for 30 s; and a final extension

step at 72 ºC for 15 minutes. All PCR amplifications were performed in an Eppendorf Mastercycler Nexus Gradient thermal cycler in 20 µL reaction volumes comprising 40 ng of template DNA, 1x PCR Buffer (NEB; New England Biolabs, Ipswich, MA, USA), 100 µM dNTPs, 0.4 µM of the fluorescently labeled forward and unlabeled reverse primer, and 0.6 units of *Taq* DNA polymerase (NEB). Primer pairs showing consistent, single locus amplification via ethidium bromide-stained agarose gel electrophoresis were used for subsequent capillary electrophoretic analysis.

PCR amplification was conducted individually for each primer, and products were diluted in formamide with GeneScan-500 ROX as the internal size standard. Fragment sizes were analyzed using the Applied Biosystems 3730xl DNA Analyzer at the Georgia Genomics Facility (Athens, GA). Peak calling and allele sizing was conducted using Geneious version 8.1.3 (www.geneious.com, Kearse et al., 2012), and GenAlEx 6.5 (Peakall and Smouse, 2006; Peakall and Smouse, 2012) was used to identify multilocus genotypes and conduct AMOVA. PowerMarker version 3.25 (Liu and Muse, 2005) was used to generate the pairwise genetic distance matrix based on Nei's D_A (1983), the UPGMA tree of individuals, and summary statistics for the loci and populations in the study.

DNA Sequencing Analyses

Primers for amplification and sequencing of *matK* were selected from Fazekas et al. (2008), for *psa*A-ORF170 from Saltonstall (2001), and for *rpl*32-*trn*L from Shaw et al. (2007). Several ITS primers were used, including ITS1, ITS4, and ITS5 from White et al. (1990) as well as ITSL and 18S KRC from Hsiao et al. (1995). All PCR amplifications were performed in an Eppendorf Mastercycler Nexus Gradient thermal cycler in 20 µL reaction volumes comprising 40 ng of template DNA, 3% DMSO, 1x Phusion HF Buffer (NEB), 200 µM dNTPs, 0.5 µM each

of forward and reverse primers, and 0.4 units of Phusion polymerase (NEB). Annealing temperatures for the Phusion PCR were calculated with the NEB Tm calculator (tmcalculator.neb.com/) and were 68 ºC for ITS reactions, 59 ºC for both *psa*A-ORF170 and *rpl*32-*trn*L, and 55 ºC for *matK*. Amplification was performed according to the Phusion manufacturer's protocol and consisted of an initial denaturation step at 98 ºC for 30 s; 35 cycles of 5 s at 98 ºC, 20 s annealing step, and a 30 s extension at 72 ºC; and a final extension step for 10 minutes at 72 ºC. PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide. Bands were excised from the gels and purified with the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek). Ten approximately evenly spaced samples from each of the sampling sites were selected for analysis. Irregular distribution of samples from the Hull Road site was due to inadvertent loss of samples, which were not resampled for this analysis. The complete chloroplast genomes of *A. gigantea*, *A. tecta*, and *A. appalachiana* have already been sequenced and were used as references for the comparative three-region analysis (Burke et al., 2014; Burke et al., 2012). *P. edulis*, *S. cernua*, and *S. senanensis* were used as outgroups for the chloroplast analysis.

Pairs of reads from bi-directional Sanger sequencing for each region were aligned to one another using MUSCLE version 3.8.524 (Edgar, 2004). Consensus sequences for each individual were extracted, trimmed to a uniform length, and aligned to each other with MUSCLE. Trimmed alignments for the three regions were concatenated and analyzed using Maximum Likelihood (PHYML version 3.0, Guindon et al. (2010)) and Bayesian Inference (MrBayes version 3.2.2, Ronquist and Huelsenbeck (2003)). Haplotypes were identified by GenAlEx 6.5.

3.3 RESULTS AND DISCUSSION

SSR Analysis

During the initial screening of primers for this study, 35 primer pairs were selected and tested (Table 3.1). These primers were intended for use with a universal fluorescently labeled primer, which required the addition of an 18 base-pair 5' tag to each forward primer (Schuelke, 2000). A variety of PCR conditions were tested to obtain consistent and specific amplification, which proved difficult to achieve. A few primer pairs (e.g. BWSS5 and Sasa718) failed to show any amplification in *Arundinaria spp.* despite testing across a range of annealing temperatures. Other primers that proved unsuccessful showed specific amplification with only the forward and reverse primers but failed to show any amplification when combined with the third fluorescently labeled primer. In a few cases, such as with BWSS3, specific amplification with two primers was achieved, but the addition of the third primer caused non-specific amplification, which could not be removed with increasing annealing temperature. This three-primer system depends on incorporation of the 5' tag sequence into the PCR products to act as a binding site for the third primer and to allow incorporation of the fluorescent dye. Without the 5' tag incorporation, the forward and reverse primer can still produce specific products, but these products will lack the fluorescent dye necessary for analysis of product size. This appeared to be the case with a number of primers tested, as the specific products observed on agarose gels resulted in fluorescent peaks that were too weak to be interpreted. Because of problems encountered in determining appropriate reaction conditions and ensuring efficient incorporation of the third primer, this three-primer system was ultimately abandoned in favor of conventionally labeled forward primers. A subset of 12 primers that had consistently produced strong, specific products was selected for conventional analysis.

Of the 12 fluorescently labeled SSR primer pairs selected for final analysis (Table 3.1), Ba58 and BWSS1 failed to show amplification in *Arundinaria spp.* with their published PCR protocols. Two additional primer pairs, R44 and Sasa03E, showed non-specific amplification products across a range of annealing temperatures. R44 and Ba58 were developed in rice (*Oryza* sativa) and *Bambusa arundinacea,* respectively, and represent the most phylogenetically distant taxa used for primer sourcing in this study. The failure of these primers to produce single-locus amplification could be due to mutations in primer binding regions.

The remaining eight primer pairs showed single-locus amplification and were used for SSR analysis on the field-collected samples. PBM027 and PBM028 were monomorphic and were excluded from further analysis. Two primers, Phe23 and Sasa540, showed distinct peaks in samples collected from the Hull Road site as well as in the known sample of *A. tecta*; however, no bands were present in the known *A. gigantea* sample or individuals from either the Tennessee or Tallassee sites. Because the absence of peaks followed distinct species or population boundaries, the absence of peaks for these primers was assumed null alleles, which could be caused by mutations in primer binding sites or large insertions/deletions at the SSR locus preventing amplification. Five individuals had two or more loci that did not amplify at all and were excluded from further analysis

The number of alleles per primer pair was low, ranging from 2-4 alleles per locus, as was mean observed heterozygosity (0.15, Table 3.1). Despite low polymorphism, 15 distinct multilocus genotypes (MLG) were detected among the three populations (Figure 3.2 and Figure 3.3). In the Tallassee Forest site a total of 114 samples were collected and assayed. The vast majority of culms assayed, 90 of 114, belonged to a single MLG, which extended 431 m along the length of the population (MLG1; red squares). Several other MLGs were similarly

widespread, extending 346 m (MLG5, blue squares) and 289 m (MLG3, purple squares). In contrast, the remaining seven MLGs at this site were represented by two or fewer individuals each (Singletons: TL22, TL46, TL47, TL51, TL86, TL105; and doubles TL62 and TL95). The Tennessee site showed a similar pattern to that observed at Tallassee. Only three distinct MLGs were detected, and all of these were also present at the Tallassee Forest Site. The population was dominated by a single MLG, which accounted for 30 of 32 assayed culms and extended for 396 m (MLG1, red squares). At the Hull Road site, five distinct MLGs were observed. One of these MLGs accounted for 18 of 26 samples assayed at the site and extended for 13.4 m across the site (MLG11, red triangles). The second largest MLG comprised only four assayed culms, but extended for 10.2 m (MLG12, yellow triangles). Because two SSR loci amplified a private allele in every sample of this population, no MLGs were shared between this site and the other two. Since some MLGs were observed in both the Tallassee and Tennessee sites, which are separated by over 200 km and are not present on the same river system, it is unlikely that these MLGs represent true clones (individual ramets of the same genet).

A UPGMA dendrogram constructed from a matrix of pairwise genetic distances among the unique MLGs shows that the 10 MLGs from the Tallassee and Tennessee sites group together with the known sample of *A. gigantea* (Figure 3.3; Gig), which originates in Mississippi. Nei's genetic distance between the Tallassee and Tennessee populations was only 0.001, and the pairwise F_{ST} was 0.024, indicating very little differentiation between these two populations (Table 3.2). Culms assayed from the Hull Road population grouped with the known sample from *A. tecta* (Figure 3.3; Tec) in the UPGMA dendrogram and showed significantly larger pairwise F_{ST} and Nei's genetic distance values when compared with the Tallassee and Tennessee sites (Table 3.2). Partitioning of the diversity via AMOVA showed that a great deal of

the genetic diversity (73%) was apportioned among populations (Table 3.3); however, this very high result is likely due to the divergence of the Hull Road population from the other two. Partitioning via AMOVA across only the Tallassee and Tennessee populations shows that there is no between population diversity (data not shown). The clustering of the Hull Road samples with the *A. tecta* sample suggests that this site could have been Switch cane (*A. tecta*) misidentified as river cane; however, these samples share morphological similarities with other *A. gigantea* samples collected, suggesting the possibility of hybridity.

DNA Sequence Analysis

Amplification of the ITS region produced inconsistent, non-specific fragments that were not suitable for sequencing; however, all 32 samples selected for sequencing produced high quality sequence data for the three chloroplast regions assayed. After trimming, alignment, and concatenation, a matrix of 2,104 base pairs was used for haplotype determination and phylogenetic inference. A total of four chloroplast haplotypes were detected from among the samples (Table 3.4), and an additional four haplotypes were observed in the control samples. All samples from the Tallassee Forest site and the majority of the samples from the Tennessee site exhibited a common haplotype (Figure 3.4, "Haplo1"), which was also shared with both the known *A. gigantea* sample and the publically available *A. gigantea* chloroplast sequence. Three samples from the Tennessee site (samples TN24, TN25, and TN26) possessed a distinct haplotype ("Haplo2") from the other samples surveyed. The haplotype only differed from the *A gigantea* haplotype (Gig) with a single base-pair deletion in the *psa*A-ORF170 region and 5 tightly clustered substitutions and a two base-pair insertion in the *rpl*32-*trn*L region. Except for the final insertion, which is unique among the haplotypes, all polymorphisms that differentiate this haplotype from Haplo1 are shared with the haplotype detected in the known sample of

A. tecta, termed "Tec". In the Hull Road population, nine of the ten samples shared a common haplotype ("Haplo3"). This haplotype differed from Haplo1 with a single base pair substitution in *matK*, a single base pair deletion in *psa*A-ORF170, and three single base pair substitutions in *rpl*32-*trn*L. These polymorphisms were all also shared with the *A. tecta* haplotype. A minor haplotype ("Haplo4") detected in the Hull Road population was substantially similar to the Haplo3 haplotype but lacked one of the deletions in *psa*A-ORF170 that characterized Haplo3. Except for the final insertion in the Haplo2 haplotype, all polymorphisms in Haplo2, Haplo3, and Haplo4 were shared with the Gig, Tec, or *A. tecta* haplotypes.

The phylogenetic trees constructed from the concatenated three-region alignment using both Bayesian inference and maximum likelihood were extremely similar (Figure 3.5) and only differed in that the maximum likelihood tree did not group Haplo3 and Haplo4 together, making them both instead sister taxa to the Tec/*A. tecta*/*A. appalachiana* clade. All samples from the Tallassee Forest site, the majority of the Tennessee samples, and the Gig samples resolve as a polytomy in the middle of the tree with a shared identical haplotype (Haplo1 and Gig). The minor haplotype samples from the Tennessee site cluster between the Gig haplotype and the out group species. The Hull Road samples group tightly together and are intermediate between the Gig and Tec samples. This finding corroborates the UPGMA tree from SSR the analysis, which grouped the Hull Road samples with *A. tecta*. The intermediate grouping of the Hull Road chloroplast samples here and the hybrid appearance of the chloroplast sequences suggest recombination of the chloroplast genomes between congeneric species. A hybrid origin of the population would challenge the assumption of uniparental chloroplast inheritance in this species or at least within interspecific hybrids. Recombination of chloroplast genomes has been observed in algae and in hybrids of *Nicotiana*, but not in bamboo (Lemieux et al., 1984; Medgyesy et al.,

1985) Although there is substantial sequence similarity between the Haplo2 samples and the Haplo3, Haplo4 and Tec samples, the anomalous placement of Haplo2 could reflect the low support for the placement of the Haplo3 and Haplo4 clades, which in some trees was resolved as intermediate between Gig and the outgroups.

3.4 CONCLUSIONS

In an effort to inform conservation and restoration decisions in river cane, amplification of SSR loci from a variety of related species were attempted. Results of the SSR analysis showed very few distinguishable genotypes within the populations assayed (Figure 3.2), and several of these genotypes were present in multiple populations. Because of the large geographic separation between the sites, it is highly unlikely that these identical genotypes represent true clonal growth, but rather a lack of resolution by this technique with six loci. Despite their hypervariability, monomorphism or low allele number in SSR has been reported in other endangered species. In a study of genetic diversity in Wollemi pine (*Wollemia nobilis*), Peakall et al. (2003) were unable to detect any variation in 20 SSR loci developed *de novo* in the species. They extended this work to other species in the same family with similar results. The SSR results presented here almost certainly underestimate the true amount of genetic diversity present in river cane; however, the low number of alleles and low observed heterozygosity from the SSR loci suggest that genetic diversity in river cane is also very low. However, with the decreasing costs of next-generation sequencing, SNPs will become a useful and applicable technique for the elucidation of populations structure in non-model plants, and could detect previously overlooked diversity in river cane.

Low genetic diversity and heterozygosity are consistent with previous studies of bamboo populations. Kitamura and Kawahara (2011) used six SSR loci to show that selfing is the

predominant mating system in dwarf bamboo *Sasa cernua*, though outcrossing does occur in isolation. In a related study, Kitamura and Kawahara (2009) applied eight SSR loci to distinguish clones of the same dwarf bamboo. Only six distinct genotypes were detected among the 1200 samples culms, and 93% of culms sampled belonged to the same genotype. Echoing these studies, all populations of river cane surveyed here were dominated by a single genotype with low heterozygosity. At each site, the majority genotype accounted for 66%-93% of the culms sampled. This genotype often extended the entire length of the population, which, in the case of the Tallassee site, was over 400m. Such widespread MLGs suggest that either the individuals are genetically distinct but extremely closely related or that clonal growth across several hundred meters is common. These two hypothesis are not mutually exclusive, given the previous results of population diversity in bamboo as well as previous reports of extremely large clone size in other bamboos (Suyama et al., 2000).

The results of the SSR analysis were corroborated by the chloroplast sequence analysis. Across the three sampling sites, only four haplotypes were discovered. Each sampling site was dominated by a single haplotype, and in the case of the Tallassee and Tennessee sites this haplotype was shared. Interestingly, the minor chloroplast haplotypes in the populations did not correspond to individuals with distinct SSR genotypes. The Tallassee population displayed 10 SSR genotypes and only one chloroplast haplotype, and the Tennessee population contained a small cluster of individuals (Figure 3.4; TN24, TN25, and TN26) with a distinct chloroplast haplotype, but these individuals possessed the dominant SSR genotype. Chloroplasts are typically maternally inherited, although this has not been proven for *Arundinaria,* and the presence of a distinct clustered haplotype among this subset of individuals suggests that they share a common seed parent. This contrast highlights the underestimation of diversity provided

by the current SSR analysis, however the low number of haplotypes detected serves to underscore the overall low diversity in the populations.

Although the Tennessee and Tallassee populations are extremely similar, the Hull Road population diverged from the other two in both the SSR and chloroplast analyses (Figure 3.2 and Figure 3.4). Although the Hull Road samples consistently clustered with *A. tecta* in these analyses, given the variety of haplotypes observed within the populations, there is not enough information to unambiguously determine the species or potential hybrid status of the Hull Road samples. In a previous study of *Arundinaria* population structure and hybridization, Triplett et al. (2010) showed evidence of hybridization between congeneric *Arundinaria* species based on AFLP data. The authors also examined chloroplast haplotypes for a single intergenic region and showed that putative hybrids contained either *A. tecta* or *A. gigantea* haplotypes; however, in the present study, haplotypes from the populations share polymorphism with both *A. gigantea* and *A. tecta* samples. This apparent mixture of haplotypes could be the result of biparental chloroplast inheritance, but to date this has not been documented in bamboos (Hansen et al., 2007). Another potential explanation for the intermediate haplotype in the Hull Road population is incomplete lineage sorting, that is, ancestral chloroplast polymorphism has been retained in both species. Given the recent origin of *Arundinaria* (Burke et al., 2014) and the long generation times of bamboos (Janzen, 1976), it is possible that ancestral chloroplast polymorphism has simply not become fixed in the two species. This hypothesis is not supported by the fact that the intermediate haplotypes were not detected in the Tallassee population, and this larger population there should be a lesser chance for a particular haplotype to go to fixation.

Overall, the conclusions of this study build upon those of previous studies in *Arundinaria,* demonstrating that there is little polymorphism present in natural populations of

river cane and that natural canebrakes are dominated by a single widespread genotype; however, canebrakes are not exclusively clonal. In order to mimic typical levels of genetic diversity for a restored canebrake, the use of a single genotype is likely to be insufficient. Additionally, the results from one population surveyed here further suggest the potential for hybridization between species within this genus and underscore the need for future study of reproductive biology in bamboos.

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Figure 3.1 The locations of the three sample sites indicated by yellow pins. The Tallassee Forest and Hull Road sites were located in Athens-Clarke County, GA, and the Tennessee Site was located near Tellico Plains, TN. The Tennessee site is separated from the other two by approximately 200km.

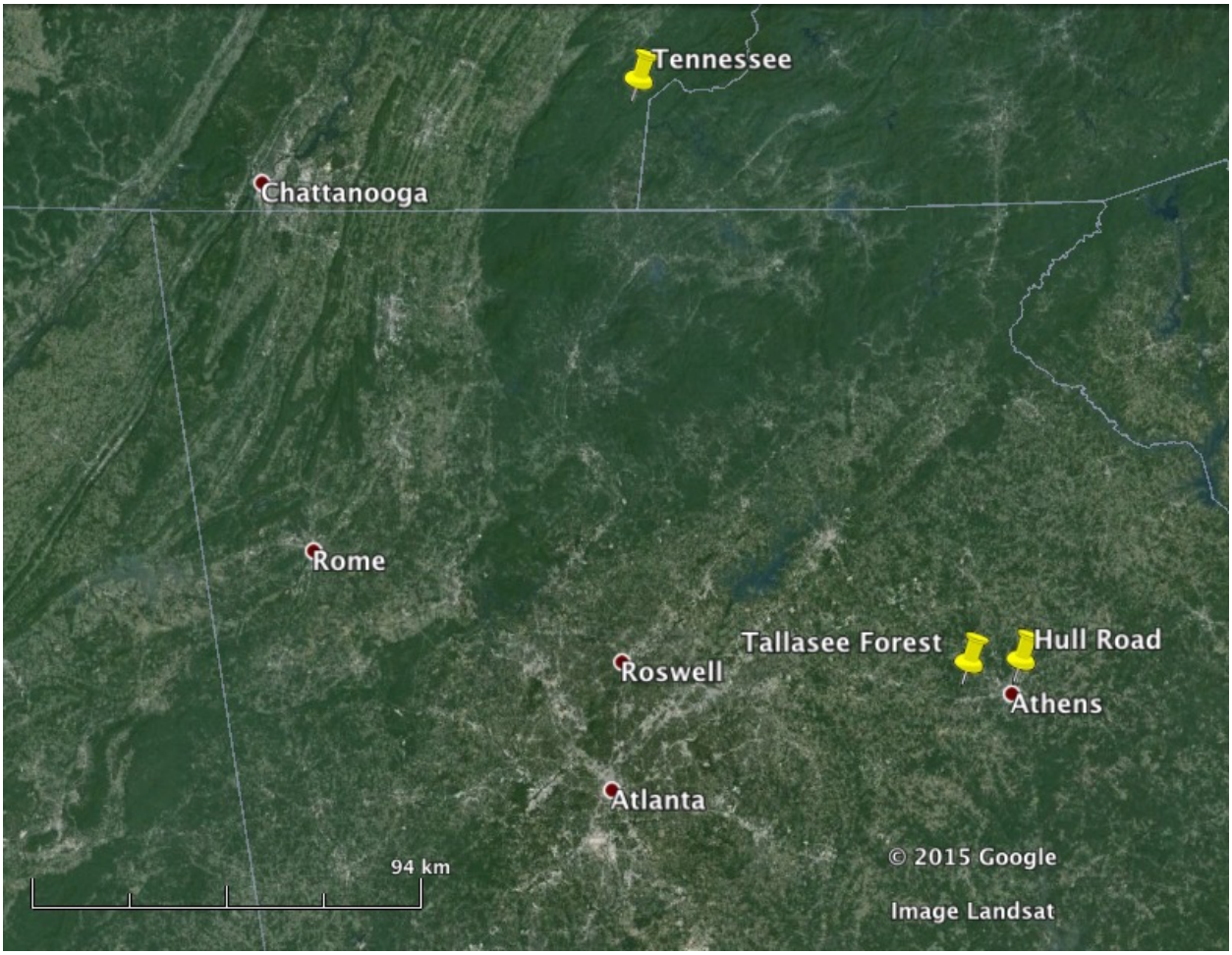
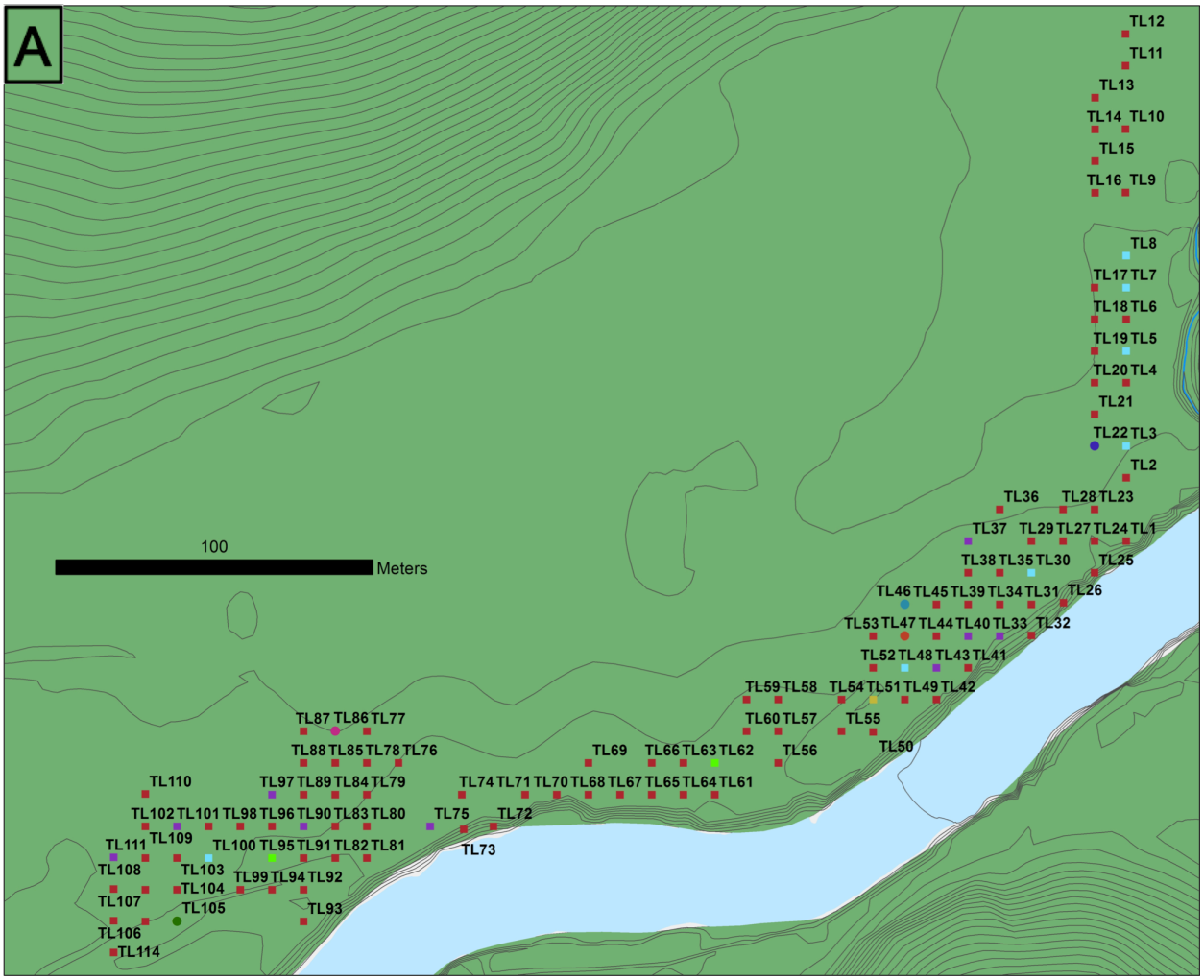
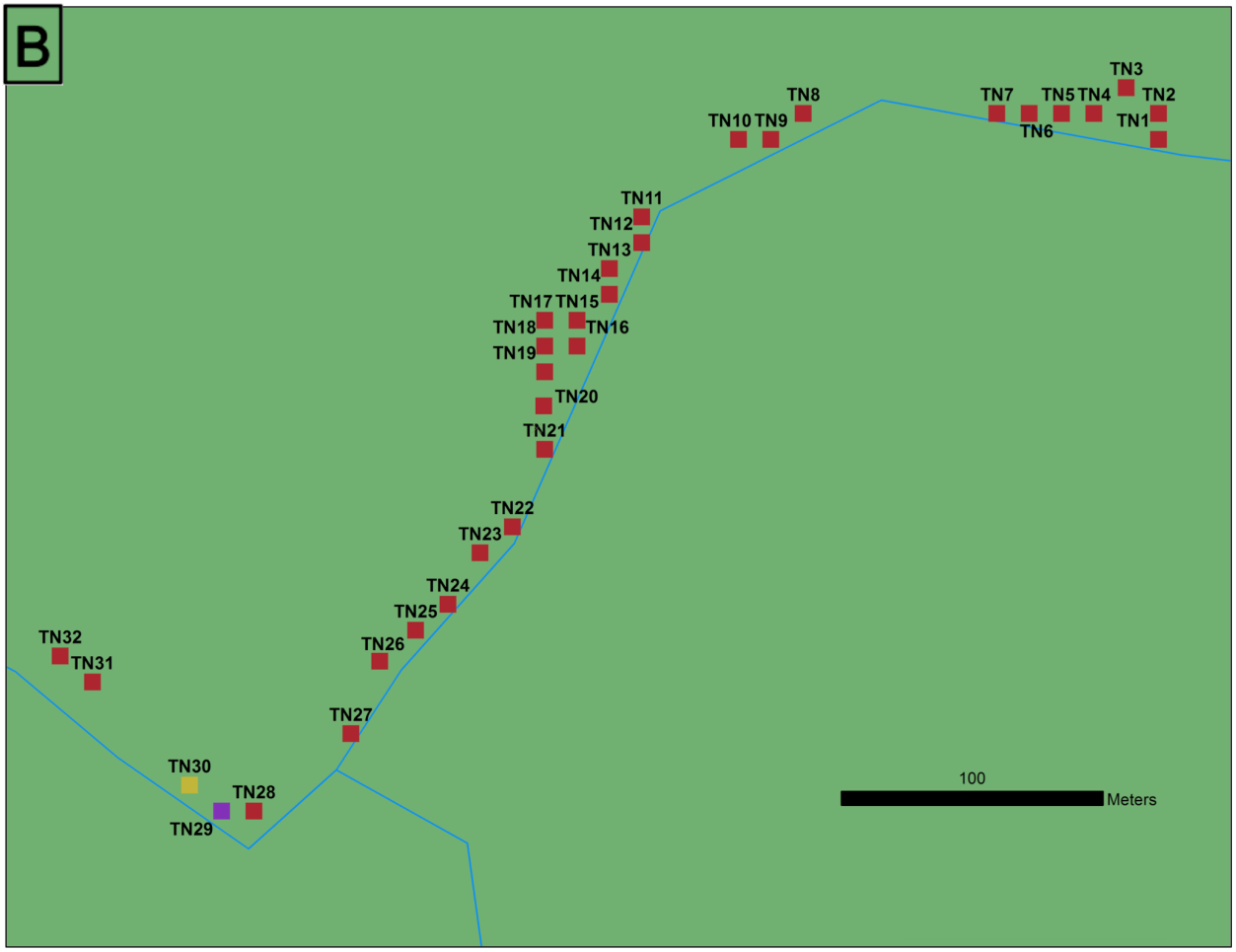


Figure 3.2 Multilocus genotypes (MLG) from six-locus SSR analysis in the (a) Tallassee Forest site (116 samples), (b) Tennessee site (32 samples), and (c) Hull Road site (26 samples). Distinct MLGs are indicated by color and marker shape.




B
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TN8
TN7
TN5
TN4
TN2
TN10
TN9
TN6
TN1
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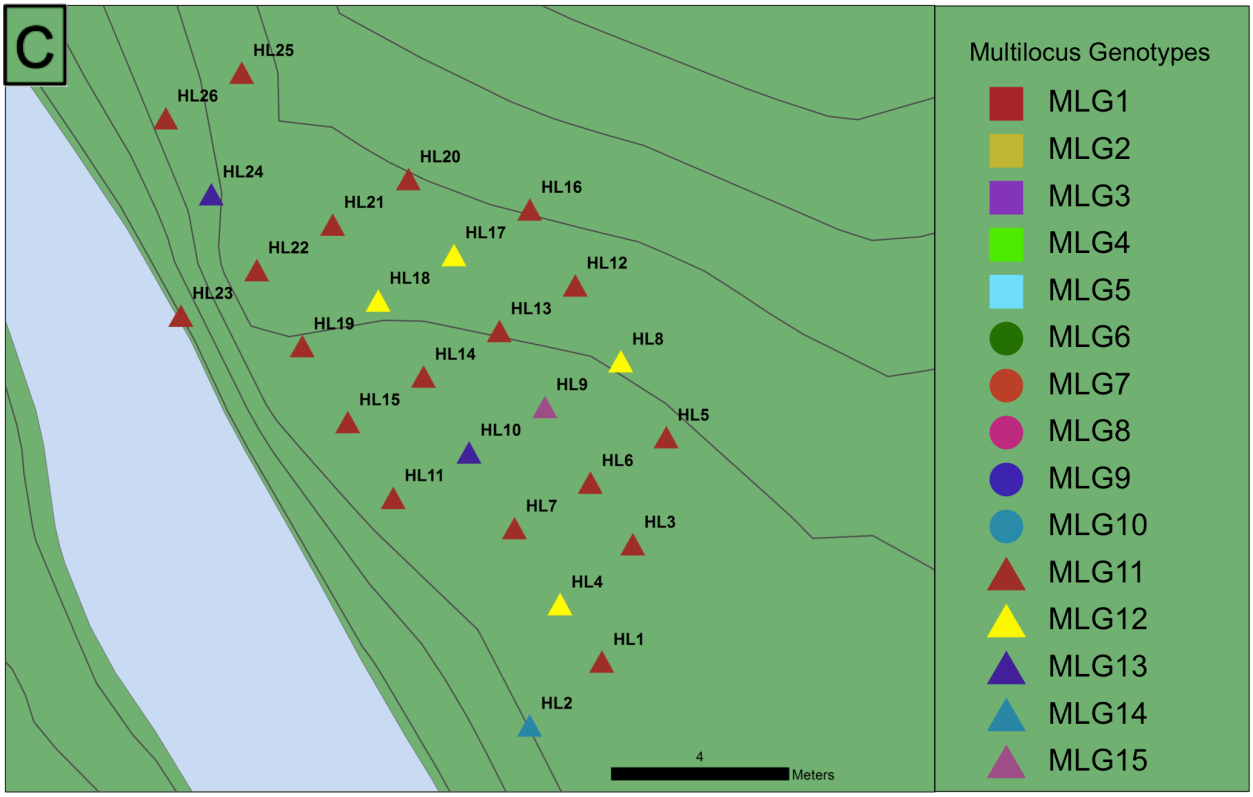
100
Meters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Multilocus Genotypes
MLG1
MLG2
MLG3
MLG4
MLG5
MLG6
MLG7
MLG8
MLG9
MLG10
MLG11
MLG12
MLG13
MLG14
MLG15

Figure 3.3 UPGMA dendrogram based on a matrix of Nei's genetic distance (D_A) for the 15 distinct multilocus genotypes (MLGs) observed in the SSR analysis. Number of individuals in each MLG is indicated in parenthesis. Known samples of *A. tecta* and *A. gigantea* are also present in the dendrogram as Tec and Gig, respectively.

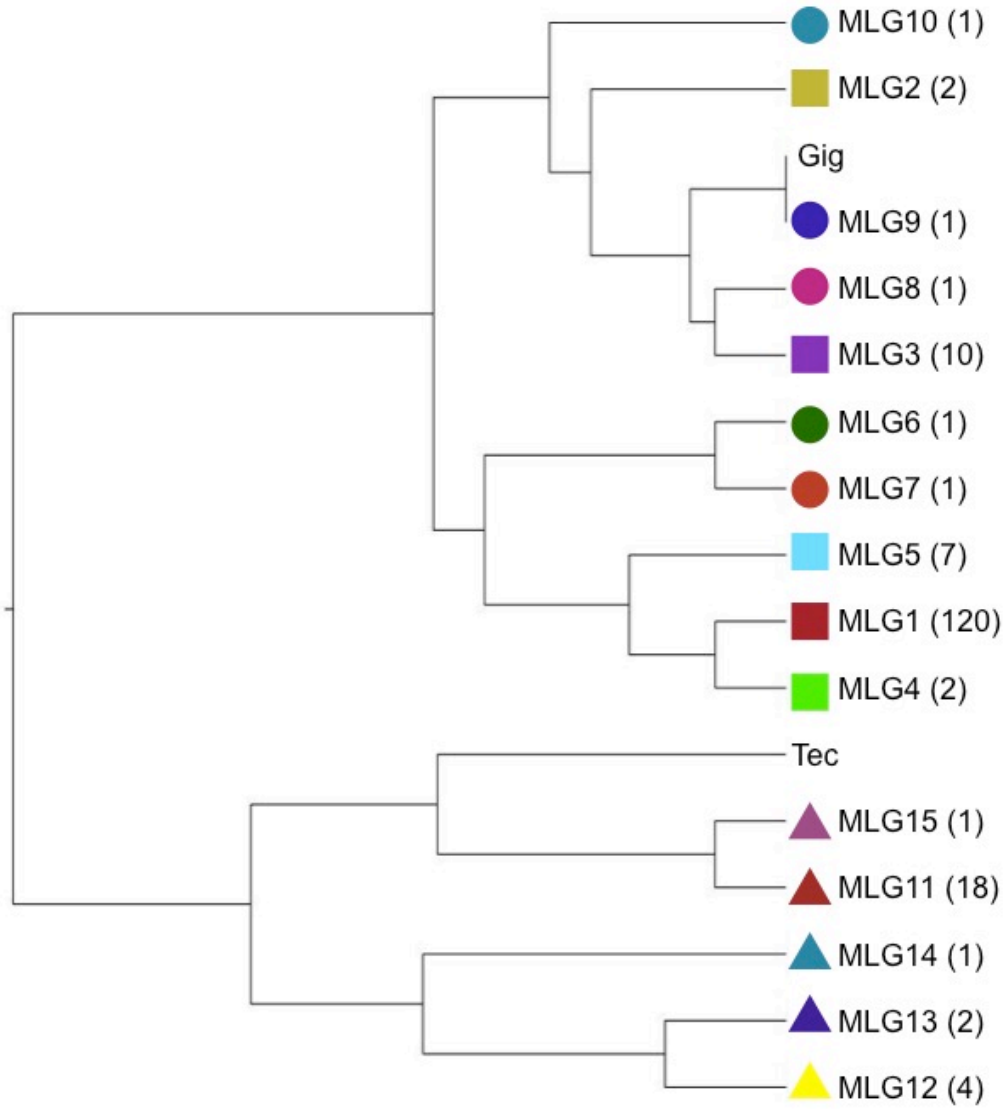
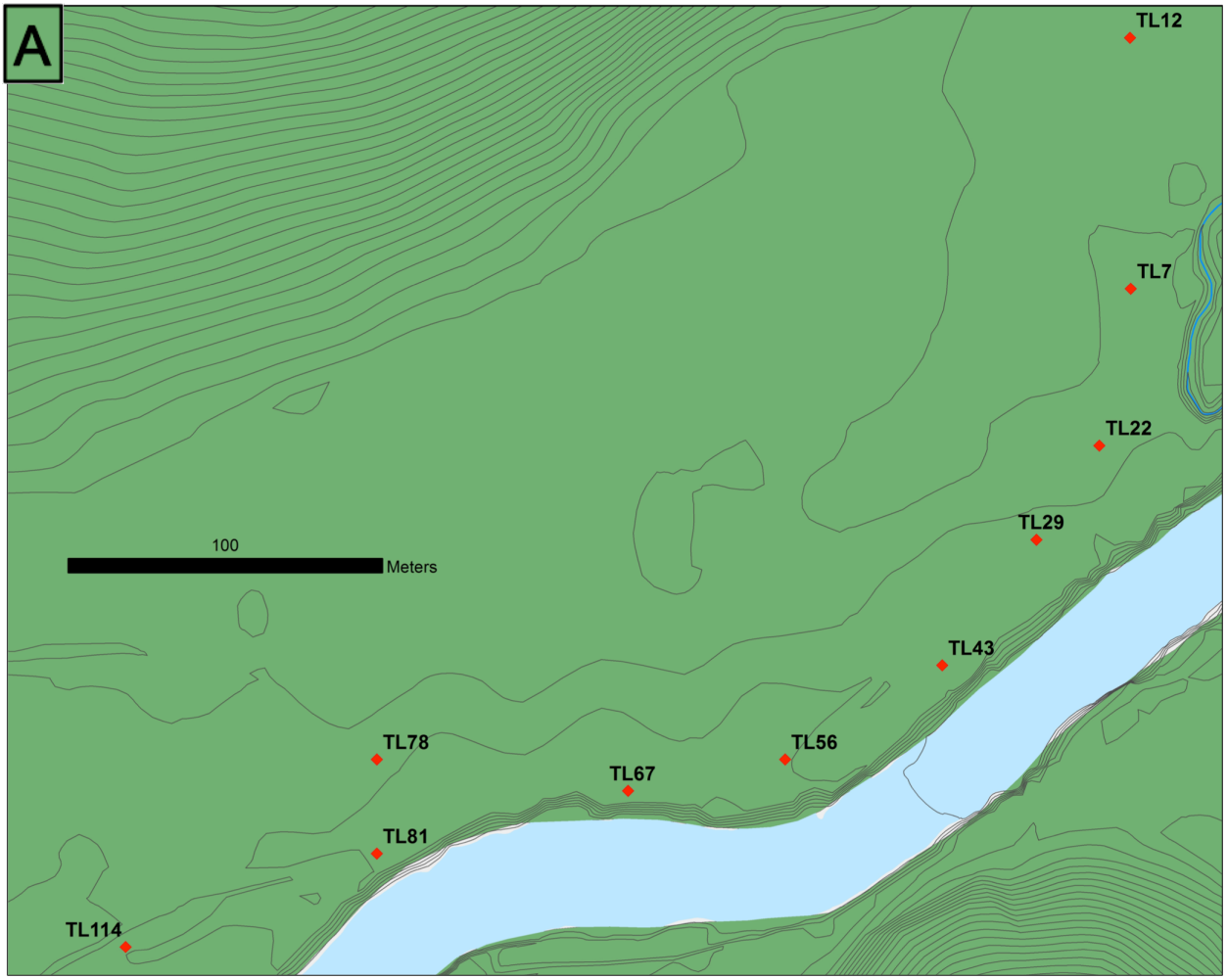
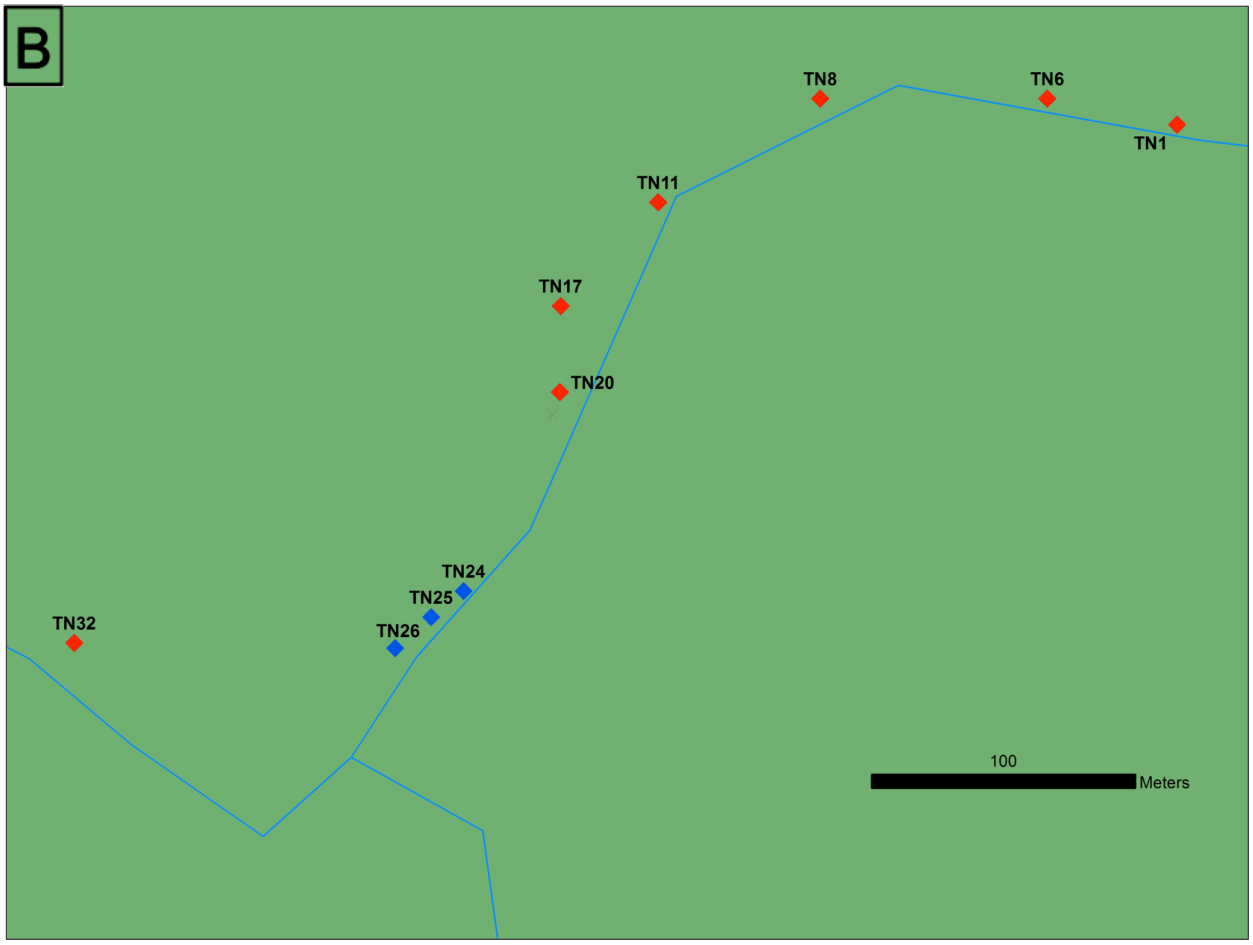


Figure 3.4 Haplotypes for the three chloroplast region sequences in the (a) Tallassee, (b) Tennessee, and (c) Hull Road sites. A subset of ten samples was chosen at each site and three chloroplast regions were sequences for each sample. Distinct haplotypes are identified by color.

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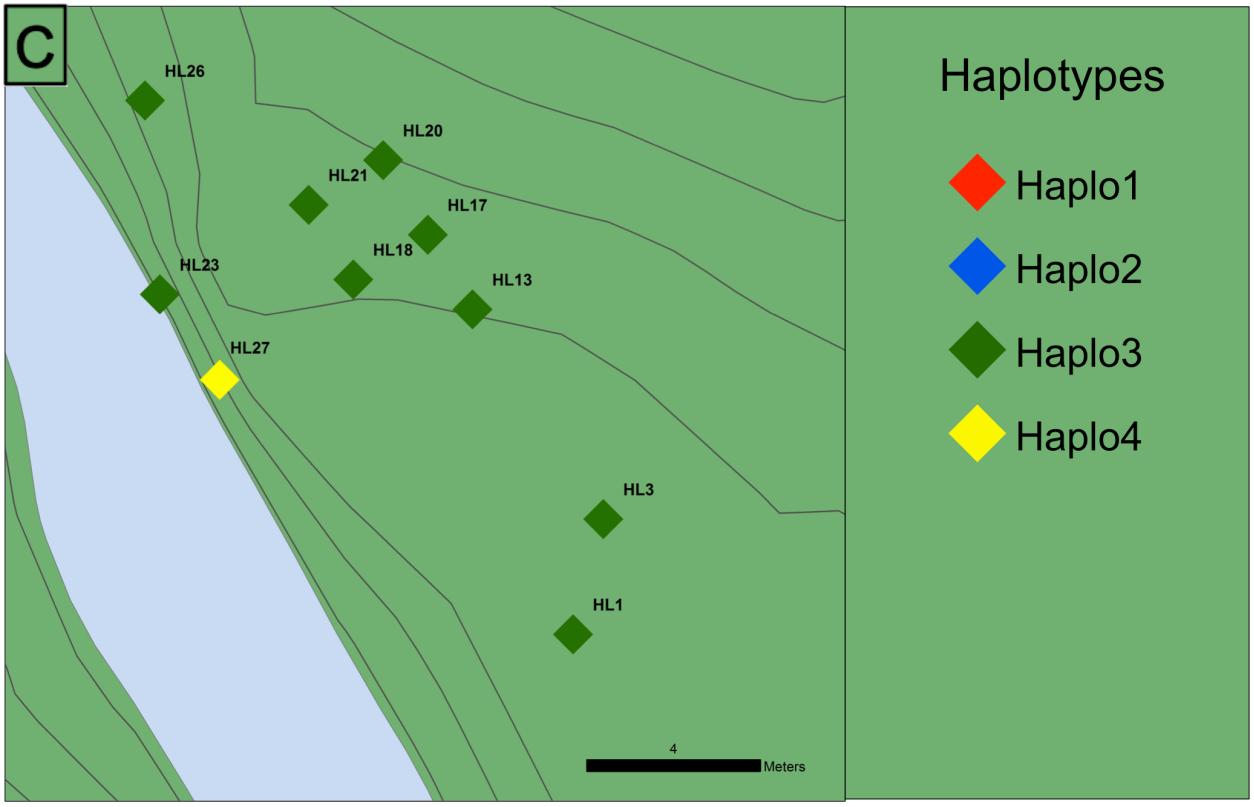
Meters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HL27
HL3
HL1
4
Meters
Haplotypes
Haplo1
Haplo2
Haplo3
Haplo4

Figure 3.5 Maximum likelihood and Bayesian tree of the three-region chloroplast alignment from 30 samples, outgroups, and controls. Branches are labeled with posterior probability (above) and bootstrap support (below, 100 bootstrap replicates). Italicized names represent publically available sequences, and Gig and Tec represent known samples for *A. gigantea* and *A. tecta*. Samples sizes are indicated in parenthesis.

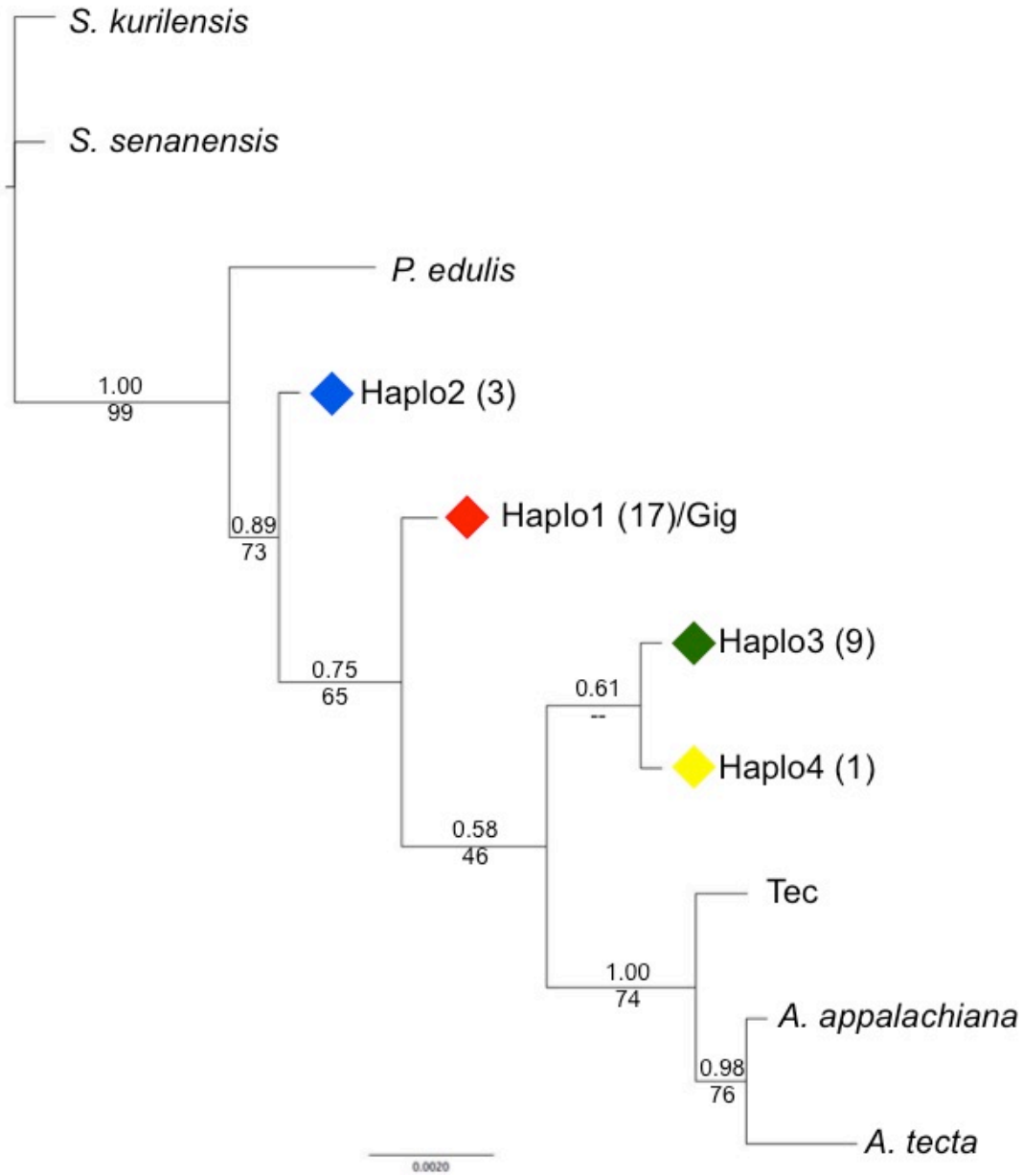

Table 3.1 Simple Sequence Repeat primers screened in *Arunindaria spp.* Species column refers to the species the SSR primers were developed in. Asterisk (*) indicates a touchdown PCR protocol was used. Number of alleles (Na), expected (H_E) and observed heterozygosity (H_O), and Polymorphism Information Content (PIC) were calculated using PowerMarker 3.25.

Primer	Species	Reference	Ta (ºC)	Amplification	Allele Size Range	Na	H_E	H_O	PIC
Ba02	*B. arnhemica*	Kaneko (2007)	50-64	Non-specific					
Ba18	*B. arnhemica*	Kaneko (2007)	50-64	Non-specific					
Ba45	*B. arnhemica*	Kaneko (2007)	50-64	Non-specific					
Ba14	*B. arundinacea*	Nayak (2005)	50-64	Non-specific					
Ba58	*B. arundinacea*	Nayak (2005)	43-55	No					
R16	*O. sativa*	Wang (2005)	53-66	Non-specific					
R39	*O. sativa*	Wang (2005)	53-66	Non-specific					
R4	*O. sativa*	Wang (2005)	53-66	Non-specific					
R44	*O. sativa*	Wang (2005)	53-66	Non-specific					
PBM014	*P. edulis*	Tang (2010)	52	Yes	285-298	4	0.602	0.118	0.222
Phe23	*P. edulis*	Jiang (2013)	52*	Yes	370, Null	2	0.415	0	0.216
PBM004	*P. edulis*	Tang (2010)	55-66	Non-specific					
PBM016	*P. edulis*	Tang (2010)	47-61	Non-specific					
PBM019	*P. edulis*	Tang (2010)	47-61	Non-specific					
PBM022	*P. edulis*	Tang (2010)	55-66	No					
PBM027	*P. edulis*	Tang (2010)	49	Non-specific					
PBM028	*P. edulis*	Tang (2010)	53	Non-specific					
Phe01	*P. edulis*	Jiang (2013)	50-61	No					
Phe10	*P. edulis*	Jiang (2013)	50-64	No					
Phe100	*P. edulis*	Jiang (2013)	51-63	Non-specific					
Phe28	*P. edulis*	Jiang (2013)	51-5	Non-specific					
Sasa500	*S. cernua*	Kitamura (2009)	57	Yes	125-131	3	0.604	0.647	0.409
Sasa540	*S. cernua*	Kitamura (2009)	57	Yes	230, Null	2	0.443	0	0.209
Sasa223	*S. cernua*	Kitamura (2009)	58-65	Non-specific					
Sasa718	*S. cernua*	Kitamura (2009)	51-63	No					

Sasa946	*S. cernua*	Kitamura (2009)	43-46	No					
Sasa03E	*S. kurilensis*	Kitamura (2009)	50-61	Non-specific					
Sasa06B	*S. kurilensis*	Kitamura (2009)	43-69	Non-specific					
BWSS4	*S. senanensis*	Miyazaki (2008)	52*	Yes	80-84	2	0.057	0.059	0.012
BWSS8	*S. senanensis*	Miyazaki (2008)	52*	Yes	89-96	2	0.057	0.059	0.006
BWSS1	*S. senanensis*	Miyazaki (2008)	43-58	No					
BWSS3	*S. senanensis*	Miyazaki (2008)	51-58	Non-specific					
BWSS5	*S. senanensis*	Miyazaki (2008)	50-64	No					
BWSS6	*S. senanensis*	Miyazaki (2008)	50-64	Non-specific					
BWSS7	*S. senanensis*	Miyazaki (2008)	43-58	Non-specific					
					Mean	2.6	0.363	0.147	0.311

Table 3.2 Population comparison matrix based on SSR date with pairwise F_{ST} above the diagonal and Nei's genetic distance below the diagonal.

	Hull	Tallassee	Tennessee
Hull		0.696	0.619
Tallassee	0.495		0.024
Tennessee	0.497	0.001	

Table 3.3 AMOVA table of SSR data calculated from three sampling populations. P value calculated based on 999 bootstraps.

AMOVA Table

Source	df	SS	MS	Est. Var.	%	p
Among Pops	2	177.565	88.783	2.034	73%	0.010
Within Pops	169	129.539	0.767	0.767	27%	
Total	171	307.105		2.800	100%	

Table 3.4 Four haplotypes were observed in the three populations from the 3-region chloroplast sequence alignment (Haplo1-4), and four control sequences were determined from known samples, publically available sequences, or both (italicized control samples). Sample sizes for each haplotype are indicated (n). Polymorphic sites are numbered individually for each region.

			matK	*psa*A-ORF170							
		n	1045	59-65	79-83	90	98	103-104	114	120	253-255
Control	*A. gigantea*	2	G	TCTACCG	TACT	T	-	GG	-	G	--T
	A. appalachiana	1	A	TCTACCG	TACT	G	-	GG	-	G	TTT
	A. tecta 1	1	G	-------	----	G	C	AT	G	T	TTT
	A. tecta 2	1	A	TCTACCG	TAC-	T	-	GG	-	G	-TT
Tallassee	Haplo1	10	G	TCTACCG	TACT	T	-	GG	-	G	--T
Tennessee	Haplo1	7	G	TCTACCG	TACT	T	-	GG	-	G	--T
	Haplo2	3	G	TCTACCG	TAC-	T	-	GG	-	G	--T
Hull Rd	Haplo3	9	A	TCTACCG	TAC-	T	-	GG	-	G	--T
	Haplo4	1	A	TCTACCG	TACT	T	-	GG	-	G	--T

		rpl32						
		41-59	169	184	237-245	331-332	434	490-491
Control	*A. gigantea*	----------	A	C	ATAATAAAA	TG	A	--
	A. appalachiana	----------	G	T	ATATTTTAT	AG	G	--
	A. tecta 1	ATTCCAATTATTTAATATG	G	T	ATATTTTAT	AG	G	-
	A. tecta 2	ATTCCAATTATTTAATATG	G	T	CTATTTTAT	AG	G	--
Tallassee	Haplo1	----------	A	C	ATAATAAAA	TG	A	--
Tennessee	Haplo1	----------	A	C	ATAATAAAA	TG	A	--
	Haplo2	----------	A	C	ATATTTTAT	TG	A	AA
Hull Rd	Haplo3	----------	G	C	ATAATAAAA	AG	G	--
	Haplo4	----------	G	C	ATAATAAAA	AG	G	--

CHAPTER 4

SUMMARY

River cane [*Arundinaria gigantea* (Walter) Muhl.] is an endangered, native species of North American bamboo. Ecologically river cane is important because it grows preferentially along waterways, where it effectively controls erosion, acts as a vegetative buffer, and serves as a habitat for diverse wildlife species. Efficient large-scale propagation of river cane is difficult. The *in vitro* micropropagation method developed here using nodal segments showed superior shoot proliferation compared to previous studies; however, the lack of rooting with *in vitro* methods remains a barrier to the use of micropropagation for restoration.

Genetic analysis of river cane populations using six simple sequence repeat loci and sequencing of three chloroplast regions showed that populations contained a dominant genotype and haplotype that accounted for the vast majority of samples collected. In two of the three populations surveyed, several genotypes and one haplotype were shared, resulting in very little genetic distance between these geographically distant populations. In the third population, there was extensive, though inconclusive, evidence of interspecific hybridization between river cane and Switch cane (*A. tecta*). This evidence of hybridization complicates restoration plans, because it is unknown how extensive this is in areas where the species overlap, and if these putative hybrids can produce viable offspring.

In river cane canebrakes (as opposed to those containing putative hybrids or mixtures of species), there seems to be little differentiation among populations. Ultimately, the use of totally

clonal material does not accurately represent the true population structure of natural canebrakes, and multiple genotypes will be necessary for effective and sustainable restoration efforts.