Adaptive trait variation in the federally endangered Lindera melissifolia (Lauraceae), as it relates to genotype and genotype-environment interaction1

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Adaptive trait variation in the federally endangered *Lindera melissifolia* (Lauraceae), as it relates to genotype and genotype-environment interaction[1]

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Abstract. Molecular markers have shown a high level of genetic diversity in most extant populations of the federally endangered *Lindera melissifolia* (Walt.) Blume in the southeastern United States. However, it is unknown if diversity in adaptive traits exists. We quantified adaptive trait variation in 17 *L. melissifolia* genotypes by growing genets from two disjunct populations in the Lower Mississippi Alluvial Valley in a common-garden environment. We then examined genotype-environment interaction through introduction of a flooding treatment. Within a common-garden environment, variation in adaptive traits was affected by genotype. Stem height, stem diameter, leaf number, and stem architecture accounted for 56% of variation among genotypes, and an additional 23% of variation was attributed to differences in vegetative fecundity and foliar chlorophyll content. There was no divergence in plant morphology between disjunct populations. Plastic response to a winter flooding regime varied in magnitude and direction among male genotypes, and only in magnitude in female genotypes. A decrease in *L. melissifolia* population sizes would diminish genetic diversity, as well as adaptive trait variation, and may threaten long-term persistence of extant populations.

Key words: adaptive traits, endangered plant species, genetic diversity, Lauraceae, *Lindera*

Molecular markers have proven valuable in providing estimates of genetic diversity within and among plant populations. Researchers are able to identify processes such as gene flow and genetic divergence, as well as determine the presence of founder effects, genetic swamping, or outbreeding depression during restoration of populations of threatened or endangered species (Friar *et al*. 2000, Hufford and Mazer 2003, Oleas *et al*. 2018). Because molecular markers often represent neutral genetic variation (Holderegger, Kamm, and Gugerli 2006), their variability may be unrelated to plant performance and/or fitness. Consequently, marker variation may not be representative of adaptive trait variation (Storfer 1996; Holderegger, Kamm, and Gugerli 2006). Indeed, Reed and Frankham (2001) found that molecular measures explain only 4% of intraspecific variation in quantitative traits.

Variation in quantitative traits (*e.g*. plant height, biomass accumulation) is ecologically relevant because it is related to survival (Storfer 1996). Intraspecific variation in phenotype is influenced by genetics, environment, and the interaction of genotype and environment (Booy *et al*. 2000). One approach to estimating the genetic component that contributes to trait variation is to grow individuals of known genetic relationships under uniform conditions, that is, to conduct a common-garden experiment. Within this controlled environment, differences in quantitative traits among individuals must be attributed to genetic differences (Booy *et al*. 2000; Hufford and Mazer 2003; Holderegger, Kamm, and Gugerli 2006). Similarly, to quantify variation in genotypic response to environment, environmental manipulations may be introduced and treated as a fixed factor with genotypes treated as a random factor (Richards *et al*. 2006).

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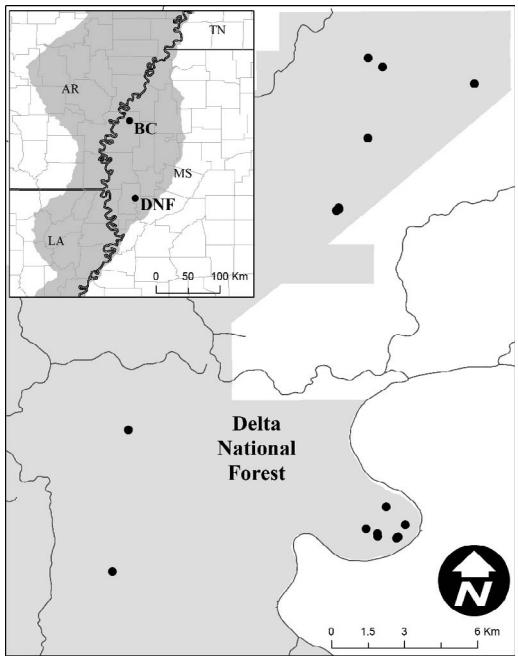

FIG. 1. Locations of *Lindera melissifolia* colonies within Delta National Forest (DNF, shaded), and locations of DNF and Bolivar County (BC), *L. melissifolia* populations (inset) sourced for stock plants to micropropagate genets used in a common-garden experiment.

Lindera melissifolia (Walt.) Blume is a federally endangered (US Fish and Wildlife Service 1986) dioecious shrub that is endemic to the southeastern United States (Steyermark 1949). Populations are composed of spatially segregated, unisexual colonies, and are male-colony biased (Wright 1994, Hawkins *et al*. 2007, Gustafson, Gunta, and Echt 2013). Sexual reproduction is characterized by heavy fruit set (Connor *et al*. 2007, Hawkins *et al*. 2010); however, ramet production via rhizomes is the predominant mode of successful reproduction (Gustafson, Gunta, and Echt 2013).

Extant *L. melissifolia* populations occur in isolated depression wetlands in the Southeastern Coastal Plain (SCP; Beckley and Gramling 2013), and in seasonally flooded bottomland hardwood forests in the Lower Mississippi Alluvial Valley (LMAV; Hawkins *et al*. 2009b). Genetic divergence between *L. melissifolia* populations in the SCP and LMAV is consistent with the Appalachian Mountain discontinuity (Echt, Demeer, and Gustafson 2011) found in other eastern North American plant species (Soltis *et al*. 2006). *Lindera melissifolia* populations in both physiographic regions are characterized by excess heterozygosity and absence of inbreeding depression, and within in each region, genetic divergence between populations is present, albeit less than between regions (Echt, Demeer, and Gustafson 2011).

The current genetic assessment of *L. melissifolia* populations in the LMAV and SCP indicates the potential for population persistence through maintenance of genetic diversity, primarily through clonal propagation (Echt, Demeer, and Gustafson 2011). Asexual propagation (*e.g*., ramet production) extends the life of an individual's genotype (Premoli and Steinke 2008) and may maintain population genetic diversity when there is little recruitment of sexually produced individuals (Bond and Midgley 2001; Clarke, Lawes, and Midgley 2010). However, long-term population persistence also relies on intraspecific variation in adaptive traits in order for plants to fully exploit niches in an existing heterogeneous environment, as well as in newly created niches caused by natural or anthropogenic disturbance. In light of this, the first objective of our study was to quantify intraspecific variation in plant traits related to plant performance and fitness among *L. melissifolia* genotypes sourced from two disjunct populations in the LMAV. An abiotic variable common among *L. melissifolia* habitats is seasonal flooding that may vary in timing and duration (Hawkins *et al*. 2010). Therefore, our second objective was to evaluate variation among genotypes in response to a winter flooding treatment.

Materials and Methods. SOURCE POPULATIONS AND SITE DESCRIPTIONS. Plants used in our study were micropropagated from stock plants (see Hawkins *et al*. 2007) from *L. melissifolia* colonies growing in Delta National Forest, Sharkey County, MS, and on privately owned land in Bolivar County (BC), MS (Fig. 1). Delta National Forest (DNF) is habitat for the southernmost *L. melissifolia* population in the LMAV. The soil series is characterized by poorly drained, fine-textured clayey surface soils and subsoils formed from Mississippi River alluvium (Scott and Carter 1962). Bottomland flooding at DNF most often occurs during late winter through early spring, but flood timing and duration may vary annually, and spatially throughout the forest (Hawkins *et al*. 2010). The *L. melissifolia* population at DNF is noncontiguous, with colonies concentrated in the southern (Fig. 1, below inset) and the northern

Table 1. Source populations and identification codes for 17 micropropagated genotypes of *Lindera melissifolia* used in a common-garden experiment.

Site	Site code	Location	Genet identification codes	
			Male	Female
Delta National Forest north	DNFN	Sharkey County, MS	24M, 27M, 37M, 41M, 43M	39F, 40F
Delta National Forest south	DNFS	Sharkey County, MS	2M, 4M, 15M, 16M	12F, 14F, 56F
Bolivar County	BC	Bolivar County, MS	5M	6F, 7F

(Fig. 1, above inset) areas of the approximately 24,000 ha of bottomland forest and associated wetlands that comprise DNF. Echt, Demeer, and Gustafson (2011) described the population as an admixture of two ancestral genetic clusters that are geographically dispersed among individuals within DNF. This population also has the highest genetic diversity of all *L. melissifolia* populations growing in the LMAV (Echt, Demeer, and Gustafson 2011). Micropropagated genets from DNF included in our study were five male genotypes and two female genotypes from north DNF (DNFN), and four male genotypes and three female genotypes from south DNF (DNFS; Table 1).

The BC *L. melissifolia* population grows in a 30-ha forest surrounded by agricultural fields and is approximately 133 km north of DNF (Fig. 1). Soil series is the same as that of DNF (Rogers 1958). Forest flooding is artificially controlled by the land owner and occurs annually beginning in winter and lasting until early spring (Hawkins *et al*. 2010). Although this site supports a relatively small *L. melissifolia* population, 13 distinct genotypes were identified by Echt, Demeer, and Gustafson (2011) at this site. One male and two female genotypes from BC were included in our study (Table 1).

COMMON-GARDEN ENVIRONMENT. Thirty-six micropropagated plants from each genotype, all of similar size (height, approximately 13 cm), were transplanted in January 2003 ($time_0$) into 23 cm (height) × 20 cm (diameter) pots (approximately 7.2 L) containing a 2 peat:1 sand by volume medium supplemented with superphosphate (467 g · m^{-3}), 10:10:10 (N:P:K, 1,130 g · m^{-3}), and Milorganite® (2,267 g · m^{-3}). Potted plants were placed randomly on tables in a climate-controlled greenhouse. Plants received ambient light, and diurnal temperatures were maintained at 23.3 ± 2.0 °C (day)/18.9 ± 2.0 °C (night) during the first 4 mo of growth to aid in plant acclimation. Thereafter, plants received ambient temperatures throughout the year, except during summer months when daytime temperatures in the greenhouse were prevented from exceeding 35 °C.

Stem height, stem diameter, and foliar chlorophyll content were measured, and number of leaves, branches (stem architecture), and ramets (vegetative fecundity) were counted in May 2004 (second growing season; $time_{16\ mo}$). Stem height (cm) was measured from the surface of the growing medium to the tip of the main stem. Stem diameter (mm) was measured at the surface of the growing medium. Leaf number represented all leaves on each plant (*i.e*., those on the main stem and branches). Foliar chlorophyll content was measured using a SPAD-502 chlorophyll meter, and meter indices were converted to mg * cm^{-2} (see Hawkins, Gardiner, and Comer 2009a).

In June 2004, ($time_{17\ mo}$), a malfunction of the greenhouse climate control system allowed summer daytime temperatures to reach a high of 45 °C for several hours for two consecutive days. This resulted in leaf and apical meristem necrosis on all plants. Once climate control was restored (*i.e*., high temperature not to exceed 35 °C), dead or damaged tissue was completely removed from all plants by clipping the stem to 20 cm above the growing medium surface. Plants responded rapidly by producing a single epicormic stem that replaced the one that had been removed. Leaf production and branching occurred sequentially with stem growth. The sequence and time of events for the common-garden experiment, as well as the genotype-environment interaction experiment (below) are summarized in Table 2.

GENOTYPE-ENVIRONMENT INTERACTION. In August 2004 ($time_{19\ mo}$), plants from each genotype were divided equally, placed in outdoor flooding wells, and assigned one of two flooding treatments: winter flooding (90 days) or winter nonflooding. For plants receiving the flooding treatment, water was maintained approximately 2.5 cm above the soil medium beginning on January 4, 2005 ($time_{24\ mo}$), and ending on March 31, 2005 ($time_{26\ mo}$).

Table 2. Summary of the sequence and timing of events for common-garden (genotype) and flooding (genotype-environment) experiments for *Lindera melissifolia* (Lauraceae). Time refers to the number of months since initiation of experiments in January 2003.

Date (month/year)	Time (months)	Environment[a]	Event
January 2003	0	Greenhouse	Initiation of common-garden experiment
May 2004	16	Greenhouse	Morphological measurements
June 2004	17	Greenhouse	Stems cut back following greenhouse high-temperature control malfunction
August 2004	19	Outdoors	Plants moved to empty flooding wells
January 2005	24	Outdoors	Initiation of flooding treatment experiment
March 2005	26	Outdoors	Flooding dropped down
June 2005	29	Outdoors	Plants harvested

[a] Greenhouse = climate controlled Jan 2003–May 2003. Thereafter, climate noncontrolled to 35 °C. Outdoors = ambient temperature, 100% light availability.

All plants were harvested in June 2005 ($time_{29\ mo}$). Roots were washed free of soil, and ramets were separated from parent plants. Ramets and parent plants of each genet were placed in labeled paper bags, and oven-dried at 70 °C until desiccated. Dried plant material was weighed to the nearest 0.001 g.

Statistical Analysis. Means and standard errors were calculated for all measured traits for each genotype. All ANOVAs were conducted using a binomial distribution generalized linear model with partitioned analysis of least significant means. In the common-garden experiment, a one-way ANOVA was used to test for effects of genotype on plant traits. Principal component analysis (PCA) was then used to further explore trait variation and similarities in multiple-trait space.

In the genotype-environment experiment, a two-way ANOVA was used to test for fixed effects and interaction of genotype and flooding on parent plant total biomass and ramet total biomass. A one-way ANOVA was used to compare mean total biomass accumulation of parent plants and ramets within and between treatments. Tukey's honestly significant difference test was used as the multiple comparison procedure. SigmaPlot Version 13.0 (Systat Software Inc., San Jose, CA) was used to perform PCA, and the SAS procedure GLIMMIX (SAS system for Windows, release V9.2. SAS Institute, Cary, NC) was used to perform all other analyses.

Results. Common-Garden Environment. All measured variables were significantly affected by genotype: stem height ($F_{16,35} = 33.51$, $P < 0.0001$), stem diameter ($F_{16,35} = 33.15$, $P < 0.0001$), number of leaves ($F_{16,35} = 12.45$, $P < 0.0001$), branching($F_{16,35} = 11.56$, $P < 0.0001$), foliar chlorophyll content ($F_{16,35} = 3.80$, $P < 0.0001$), and vegetative fecundity ($F_{16,35} = 2.36$, $P < 0.01$). The two leading axes of a standardized PCA explained approximately 78% of variation among genets (Fig. 2). The first principal component had high positive loadings for stem height,

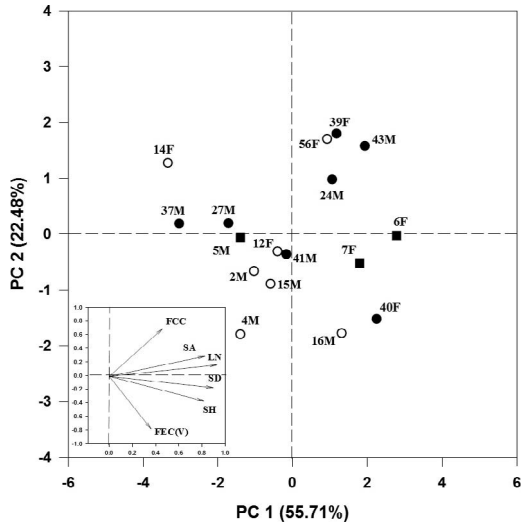

Fig. 2. Principal component analysis (PCA) and component loadings (inset) of adaptive traits for micropropagated *Lindera melissifolia* genets from: • north Delta National Forest (seven genotypes) and ○ south Delta National Forest (seven genotypes), Sharkey County, MS, and ■ Bolivar County, MS (3 genotypes) at 16 mo of growth in a common-garden environment. Genotypes are identified by a number followed by a gender identity signifier (M = male and F = female). FCC = foliar chlorophyll content (mg · cm^{-2}); SA = stem architecture (branching); LN = number of leaves; SD = stem diameter; SH = stem height; and FEC(V) = vegetative fecundity (number of ramets).

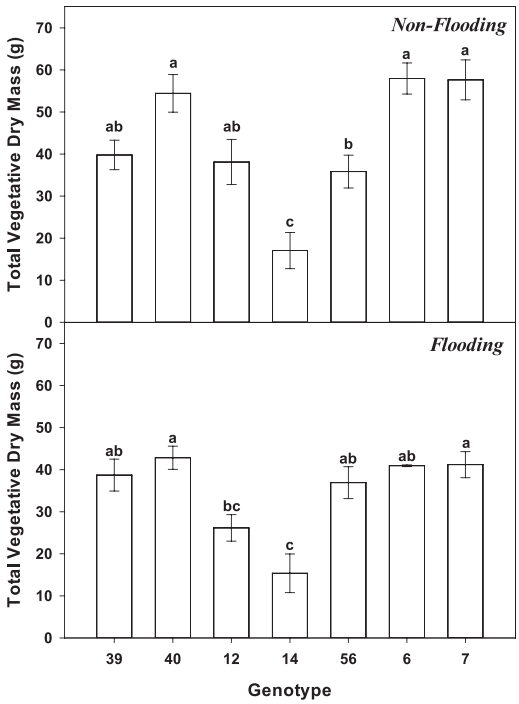

FIG. 3. Mean (± SE) total vegetative dry mass for seven genotypes ($N_{plants} = 16$/treatment) of female *Lindera melissifolia* plants receiving winter flooding (90 days) and winter nonflooding (0 days) treatments. Means with dissimilar lowercase letters within a given treatment are significantly different (ANOVA, $P < 0.05$).

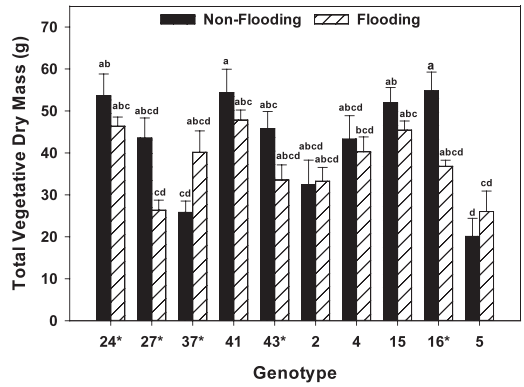

FIG. 4. Mean (± SE) total vegetative dry mass for 10 genotypes ($N_{plants} = 16$/treatment) of male *Lindera melissifolia* plants receiving winter flooding (90 days) and winter nonflooding (0 days) treatments. Means with dissimilar lower caseletters are significantly different; an asterisk indicates a significant difference in mean total biomass between flooding treatments for a given genotype (ANOVA, $P < 0.05$).

stem diameter, leaf number, and stem architecture, and the second component had a high positive loading for foliar chlorophyll content and high negative loading for vegetative fecundity (Fig. 2). Genets from DNFN and DNFS were distributed throughout all four quadrats in the ordination plane, and there was no clear grouping of genets based on locality within DNF, nor between DNF and BC. Although trait expression in BC genets was within the variation expressed by DNF genets, the first principal component separated female BC genets from the male BC genet (Fig. 2).

GENOTYPE-ENVIRONMENT INTERACTION. Partitioned analysis showed that interaction of variables was confined to male genets; therefore, results are presented by gender for clarity. Mean total biomass (parent plant) in female genets was affected independently by genotype ($F_{6,15} = 14.03$, $P = 0.0001$) and treatment ($F_{1,15} = 13.20$, $P < 0.01$). Four of seven female genotypes receiving the winter flooding treatment accumulated less total biomass than genets in the nonflooding treatment, and there was no effect on the remaining three genotypes (Fig. 3). Despite this pattern, there was no significant interaction of main effects ($F_{6,210} = 1.69$, $P = 0.13$).

Mean total biomass in male genets (parent plants) was affected independently by genotype ($F_{9,15} = 6.62$, $P < 0.0001$)) and treatment ($F_{1,15} = 6.72$, $P = 0.01$), and by the interaction of genotype and treatment ($F_{9,300} = 2.37$, $P = 0.01$). Genets in four of 10 male genotypes that received winter flooding accumulated significantly less total biomass than those receiving no flooding, and genets in one genotype accumulated significantly greater biomass following winter flooding (Fig. 4).

Notably, neither genotype nor treatment (or interaction) affected ramet mean total biomass in male (genotype: $F_{9,15} = 1.55$, $P = 0.13$; treatment: $F_{1,15} = 0.07$, $P = 0.79$) or female genets (genotype: $F_{6,15} = 1.76$, $P = 0.11$; treatment: $F_{1,15} = 1.48$, $P = 0.23$). Mean total ramet biomass for male and female genets was 0.38 ± 0.12 g and 0.28 ± 0.15 g, respectively.

Discussion. When grown in a common-garden environment, variation in plant traits among *L. melissifolia* genotypes indicated a genetic basis for this variation. Additionally, PCA (axis 1) revealed that four traits related to plant size—stem height, stem diameter, stem architecture, and leaf number—accounted for 55.7% of variation in plant

morphology. In several other plant species, these traits are directly related to competitive ability (Gaudet and Keddy 1995, Weiher *et al*. 1999), and thus we can infer that heterogeneity in competitive interactions may exert some influence on distribution of *L. melissifolia* genotypes throughout the habitat.

Spatial separation of disjunct populations is not necessarily accompanied by adaptive (or genetic) differentiation (Montalvo and Ellstrand 2000, 2001; Petit *et al*. 2001; Raabová, Münzbergova, and Fischer 2007), and we found this to be true for *L. melissifolia* genotypes in our study. Principal component analysis did not cluster or segregate genet morphologies by source location, or between colonies growing at DNFN and DNFS. Biotic and abiotic descriptors for these source sites (Hawkins *et al*. 2009b, 2010) suggest similar selective pressures, which in turn would yield similarity in local adaptive traits. On the other hand, female BC genotypes displayed greater mean values in growth traits than the male BC genotype. While this may have the appearance of gender effect, the sample size from BC included only one male genotype and two female genotypes. Consequently, it would be premature to conclude there was a more general influence of either genetics or gender on morphology.

At the population level, *L. melissifolia* plants exhibit phenotypic and/or physiological plasticity in response to light availability, flooding regime, plant density, and the interaction of these variables (Aleric and Kirkman 2005; Hawkins *et al*. 2009c, 2016; Lockhart *et al*. 2017). However, at the individual level, we found that a plastic response to winter flooding was dependent upon genotype when this response was measured as a function of parent plant total biomass. With the exception of one genotype (37M) that exhibited increased biomass in response to the flooding treatment, flooding response of genets in half (8 of 16) of all other genotypes resulted in decreased biomass. In *L. melissifolia*, extended soil inundation reduces leaf photosynthetic rate, and it may disrupt photosynthate translocation from leaves, particularly in high light availability (Aleric and Kirkman 2005, Lockhart *et al*. 2017). Therefore, we may hypothesize that decreased biomass in genotypes displaying plasticity may have resulted from a flood-induced lag time in physiological processes that contribute to biomass accumulation. Although not all genotypes exhibited a plastic response to the flooding treatment, this does not suggest that it would not occur in other light availabilities or flood durations. Indeed, several authors caution that a given genotype may not display plasticity for a specific trait in one set of environmental conditions, but will be plastic for that same trait in a different set of conditions (Bradshaw 1965, Sultan *et al*. 1998, Richards *et al*. 2006).

Fecundity-related biomass measurements such as reproductive allocation (RA) or reproductive output (RO) are often used to assess plant fitness and compare relative fitness among genotypes (Younginger *et al*. 2017). Within the common-garden environment, genotype had a significant effect on the number of ramets produced. Genets in the lower range of mean foliar chlorophyll content, and with fewer leaves and less branching produced more ramets. These data suggest a relationship between genotypically influenced growth variables and asexual reproduction at this stage of the *L. melissifolia* life cycle. However, genotype, genotype-environment, and gender had no significant influence on fecundity as quantified by RO (flooding experiment) at a later stage of the life cycle. Collectively, these data suggest that relative fitness among *L. melissifolia* genotypes may change over the course of a genet's life history. This dynamic indicates a need for future studies that more closely examine the relationship of genotype and growth variables with sexual and asexual reproductive output throughout the species' life history. Studies of this kind would not only provide a more accurate assessment of relative fitness among genotypes, but would also indicate potential for perpetuation of existing genotypes through asexual reproduction (ramets), as well as the introduction of novel genetic material through sexually generated seeds.

The US Fish and Wildlife Service (2007) defines an *L. melissifolia* population as one or more colonies separated by at least 1 mile (1.6 km) from other colonies. The colonies in DNFN and DNFS are separated by approximately 4 miles (6.0 km); however, Echt, Demeer, and Gustafson (2011) describe these colonies as an admixture of two ancestral genetic clusters geographically dispersed among individuals throughout DNF. By spatial definition, *L. melissifolia* colonies in DNFN and DNFS may be considered two populations, but an absence of differentiation in neutral molecular markers indicates a single population. Similarly, we did not identify divergence in quantitative

adaptive traits. We attribute the latter to similarity in selective pressures in north and south DNF. Although BC genets are not morphologically distinct from those at DNF, BC could be considered a distinct population from DNF by degree of genetic divergence (Echt, Demeer, and Gustafson 2011) and geographic separation.

Bottomland forests in the LMAV are characterized by heterogeneity in biotic and abiotic components that shape community structure (Collins and Battaglia 2002). Variation in adaptive traits and phenotypic plasticity among *L. melissifolia* genotypes indicates potential for continued persistence through co-occurrence of genotypes adapted to different microenvironments within these forests. This, in tandem with preservation of genetic diversity through clonal growth, confers *L. melissifolia* populations tolerance to slight changes in environment. However, effective population size is critical to maintaining genetic diversity and phenotypic variation. Natural or anthropogenic disturbances causing a demographic decrease in populations would diminish genetic and quantitative trait diversity that may further imperil the species.

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