Mating System Biology of the Florida Native Plant: *Illicium parviflorum*

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

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August 2012

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DEDICATION

I dedicate this thesis to my mother and father,
who have taught me that nothing in life is stronger than
family.

ACKNOWLEDGEMENTS

First and foremost, I want to thank my adviser, Joe Williams, for his guidance on this project. I would also like to thank all of my committee members: Randall Small and Ben Fitzpatrick, as well as previous members Taylor Feild and Andreas Nebenführ for their feedback and patience. A special thanks to Ken McFarland for his help in the greenhouse (before and after it collapsed). Thanks to Joe Mays of the sequencing facility for his help on trouble shooting AFLPs.

I want to thank my wife, Michelle Connolly, for all of her help and encouragement through good times and bad. I also want to thank several of my friends and colleagues that have helped me with field work, lab work, and just plain keeping me sane: Andrew Moffat, Steve Furches, Matt Valente, Mackenzie Taylor, Todd Buchkovich, Jason Abercrombie, Annie Becker, Michelle Smith, Lisa Cantwell, and Patrick Hudson.

I appreciate the Florida Native Plant Society, the Department of Ecology and Evolutionary Biology at the University of Tennessee, the Hesler Fund, and the A.J. Sharp Fund for funding this work. I also would like to acknowledge Ashley Morris for help with choosing sites as well as the USDA Forest Service for land permission (FDAC permit #818).

ABSTRACT

Self-incompatibility is thought to have played a profound role in the evolution of the angiosperms. However, there is little evidence of self-incompatibility systems in early diverging lineages of flowering plants. *Illicium parviflorum*, one such early-divergent angiosperm, is an evergreen perennial species endemic to central Florida, particularly within the Ocala National Forest. Although locally abundant, *I. parviflorum* is currently listed as endangered at the state level due to being under constant threat of habitat disturbance and over-harvesting. Notably, this species had been described as self-incompatible due to its low seed-set. However, low seed set may also be a result of strong, early inbreeding depression. Using cross-pollinations, histology, and molecular analysis, I provide conclusive evidence that *I. parviflorum* possesses the ability to self-fertilize, while finding no evidence of a self-incompatibility system. Furthermore, cross-pollinations of individuals within and between populations revealed heterosis, while seeds collected from self-pollinations were smaller than those collected from out-crosses, suggesting that inbreeding may be reducing fitness within populations. An analysis used to estimate parental genotypes of individuals in a population using AFLP markers identified two out of 23 plants as having originated from natural self-pollination, while the mean ($\pm$ s.e.) pollen: ovule ratio of *I. parviflorum* was found to be 511 $\pm$ 86, a ratio consistent with a species that relies primarily, but not exclusively, on outcrossing. Pollen/ovule ratios of *I. parviflorum* and other small flowered *Illicium* are lower than their larger flowered, derived relatives, suggesting that the ancestral floral type to the *Illicium* lineage was self-compatible. These results support the hypothesis that early angiosperm species had the ability to self-fertilize and that self-incompatibility systems did not arise until after the origin of the bisexual flower.

Table of Contents

Introduction 8
Methods 11
  Illicium parviflorum 11
  Study sites 11
  Tests for self-incompatibility 11
  Presence of selfed pollen-tubes 12
  Testing for inbreeding depression 13
  Testing for apomixis or autogamy 14
  DNA isolation 15
  Genotyping 15
  AFLP analysis 17
  Pollen/ovule ratio 17
Results 19
  Diallel cross 19
  Inbreeding depression 19
  Apomixis or autogamy 21
  Pollen/ovule ratio 22
Discussion 22
Conclusion 26
References 36
Vita 40

Index of Figures

Figure 1. Flowers of Illicium parviflorum 28
Figure 2. Selfed pollen tubes growing on Illicium parviflorum 29
Figure 3. Diallel cross at Salt Springs.....30
Figure 4. Proportion of viable and aborted seeds according to treatment 31
Figure 5. Total seed measurements for each treatment.....32
Figure 6. Viable seed measurements for each treatment.....33
Figure 7. Pollen/Ovule ratios of Illicium species.....34
Figure 8. Illicium phylogeny.....35

Introduction

Self-incompatibility has been hypothesized to be a major factor in the evolution of the angiosperms (Allen and Hiscock, 2008). Self-incompatibility systems have been reported in nearly every major angiosperm lineage (de Nettancourt, 1977; Igic and Kohn, 2001) and are considered essential to promoting outcrossing in plants with hermaphroditic flowers (Allen and Hiscock, 2008). Self-fertilization can be beneficial in instances where population densities are reduced and the ability to mate with a neighboring individual is minimal (Baker, 1955). However, selfing limits gene flow and reduces heterozygosity within a population, consequently leading to a loss of population evolvability and eventually extinction (Stebbins, 1957). Thus, selfing is often categorized as an evolutionary dead-end since there are no known extant basal lineages that maintain an obligate selfing breeding system (Stebbins, 1957; Takebayashi and Morrell, 2001; Bartkowska and Johnston, 2009). If selfing is as detrimental to the proliferation of a species as Stebbins (1957) believed, then it can be presumed that self-incompatibility systems must have arisen early in the angiosperm lineage, coinciding with the origin of the bisexual flower (Whitehouse, 1950). Recent molecular phylogenetics have proposed that the ANITA grade angiosperms [Amborellales, Nymphaeales, and the Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae)] are successive sister lineages to all other angiosperms (Soltis et al. 1999; Chase et al. 2009; Soltis et al. 2011). Understanding breeding system evolution in these basal lineages is essential to elucidate evolutionary and developmental constraints present in more recently derived lineages. Furthermore, field studies examining

extant species from the ANITA group are vital to determining ancestral angiosperm reproductive traits that were crucial to the origin of the angiosperms.

Despite a number of cases of self-sterility being observed in putative early diverging lineages of angiosperms (Thien et al., 2000; Thien et al., 2009), there has been little to no evidence that such self-sterility is the result of a self-incompatibility system. For example, self-sterility had been noted in one species of Nymphaeaceae that produced deformed self-pollinated fruit (Schneider and Moore, 1977), yet this deformity may also have been a result of inbreeding depression. Meanwhile many other members of the water lilies exhibit autogamy (Kadono and Schneider, 1987; Wiersema, 1988) or geitonogamy (Osborn and Schneider, 1988; Wiersema, 1988). Studies on other taxa within the Austrobaileyales have reported self-sterility despite lacking evidence that indicates a genetic basis for self-incompatibility. For instance, Prakash and Alexander (1984) described *Austrobaileya scandens* as self-incompatible, though only one plant was observed over a single reproductive cycle. More recently, Williams and Kennard (2006) observed evidence of high genetic polymorphism within a population of *A. scandens* but also found a low, but significant, inbreeding coefficient. These data, along with evidence of selfing within a half-sib array, and the presence of self-pollen tube growth in selfed individuals, indicates that a strong, early acting self-incompatibility system is present in *A. scandens*.

Research on breeding systems of species within the genus *Illicium* (Illiciaceae; Austrobaileyales), also yield inconclusive evidence as to whether members of this genus are self-compatible. One recent study observed no clear differences in pollen germination and ovule fertilization between self-and outcrossed hand-pollinated flowers of *Illicium floridanum* (Koehl

et al., 2004). This result contradicts a prior study that observed no pollen tube growth or ovule penetration in self-pollinations (Thien and White, 1983). However, the lack of pollen tube growth observed by Thien and White (1983) may have been the result of hand pollinations occurring after anther dehiscence, as suggested in a later study by Koehl et al. (2004) that found complete protogyny (no overlap between stigma receptivity and male function). Interestingly, nearly all of the early diverging lineages of angiosperms that have bisexual flowers exhibit strong protogyny (Endress, 2010).

A study on *Illicium parviflorum* conducted by White and Thien (1985) concluded with the hypothesis that *I. parviflorum* is self-incompatible due to its naturally low fruit and seed set. Newell and Morris (2010) found high levels of homogeneity among populations of *I. parviflorum* using ISSR-markers, indicating that clonal reproduction or possibly apomixis may be important within these communities. Although *I. parviflorum* can reproduce asexually through rhizomes, high levels of homogeneity can also indicate a high degree of inbreeding within a population. Therefore, the naturally low fruit and seed set may be linked to self-sterility as a consequence of embryonic inbreeding depression rather then self-incompatibility. Consequently, Newell and Morris (2010) noted that a more accurate assessment of population homogeneity using additional molecular markers is needed to distinguish between inbreeding and clonality. The purpose of the current study is to determine whether self-sterility exists in *I. parviflorum* and, if so, whether the sterility is due to a genetically-based self-incompatibility system.

Methods

Illicium parviflorum – *Illicium parviflorum* is an evergreen woody perennial shrub, endemic to central Florida, predominantly within the St. John's River watershed. Though locally abundant, and planted widely as an ornamental (Newell and Morris, 2010), *I. parviflorum* is listed as endangered at the state level due to habitat disturbance and over-harvesting. Plants typically flower from May into July, while fruits ripen 2-3 months later. The light yellow flowers (Figure 1) are protogynous and are regularly visited by a variety of small insects, notably small Dipterans (White and Thien, 1985). Flowers do not open synchronously, therefor permitting geitonogamy (personal observation). As typical with all *Illicium*, seeds are distributed through ballistic dispersal (Roberts and Haynes, 1983). Aside from sexual reproduction, *I. parviflorum* may also reproduce through rhizome sprouting (White and Thien, 1985).

Study sites – Both field sites ('Salt Springs' and 'Alexander Springs') are within the Ocala National Forest, west of the St. John's River. Sites were chosen based on plant abundance and accessibility and are approximately 42 kilometers apart. Exact locations of plants are withheld to reduce environmental disturbance.

Tests for self-incompatibility – In May of 2009, I carried out a full diallel cross consisting of ten individuals (five from Salt Springs and five from Alexander Springs). Plants were chosen based on size (over 2.5 m tall) and flower availability. Reciprocal crosses (two replicates each)

were conducted for a total of 200 controlled pollinations. Prior to hand-pollination, buds from both pollen-donor and receiver were covered with pollination bags made with Spunbond Polypropylene non-woven fabric (maximum gap diameter of 70 μm) and allowed to mature until stigmas were receptive to pollen. Stigma receptivity was tested by staining flowers not involved in the pollination study with 3,5,3',5'-tetramethylbenzidine (TMB) for 5 min as per McInnis et al. (2006), where a deep blue coloration indicated stigma receptivity (Figure 1c). Pollen from a dehisced anther from the same or another plant was applied to the stigmatic tissue of a receptive flower using a toothpick. Pollinated flowers were rebagged to prevent fertilization from unknown pollen donors. Plants were revisited in July of 2009 and observed for the presence of swelling fruit, an indication of fertilization success.

Presence of selfed pollen-tubes – Multiple flowers were bagged on a single *I. parviflorum* plant in the University of Tennessee greenhouse and were hand self-pollinated as described previously. Flowers were collected up to three days later and fixed in FAA (40 % formaldehyde, glacial acetic acid, and 95 % ethanol). Carpels were then dissected out, dehydrated to 95% ethanol, and embedded in JB-4 polymer resin (Polysciences, Inc., Warrington, PA, USA) using standard protocols. Specimens were then serial-sectioned at 5 μM with a Sorvall Dupont JB-4 microtome (Newtown, Connecticut, USA), using glass knives. Sections were mounted on glass slides and stained with 0.1 % toluidine blue O for 45 seconds, rinsed with water for a minute and a half and then allowed to dry. Sections were viewed with a Zeiss Axioplan II compound microscope (Carl Zeiss, Oberkochen, Germany).

Additionally, carpels from fresh flowers were removed and hand-dissected to explore the pollen tube pathway as per Williams et al. (1993). Fresh carpels were splayed on a microscope slide, and stained in a drop of aniline blue (0.1mg/100mL aniline blue plus 1mg/mL of sodium azide in 0.33 M K_3PO_4 and 10 mL glycerol; Marshall and Diggle, 2001) for one hour. Carpels were then viewed under fluorescent light with a Zeiss Axioplan II compound microscope (Carl Zeiss, Oberkochen, Germany). Images were captured and analyzed using a Zeiss Axiocam camera and AxioVison 4.2 image analysis software.

Testing for inbreeding depression – In the summer of 2010, I conducted a controlled pollination experiment at the Salt Springs population to quantify early inbreeding depression. Using a random number generator, eight plants were selected to be crossed with pollen from one random individual in the population. Plants were not outcrossed with pollen from more than one individual. In addition to the random outcrosses, each plant was also hand self-pollinated. Each pollination was replicated twice for a total of 32 pollinations. Fruit from these crosses were collected that September and allowed to mature in paper envelopes in order to catch seeds as they dispersed. Fruit from 21 plants that underwent natural pollinations were also collected so as to determine natural variation in the population. After dispersal, seeds were weighed and measured for height, weight, and length in order to calculate seed density (weight / volume). Seed volume was calculated using the formula for determining the volume of an ellipsoid:

$$V = \frac{4\pi abc}{3}$$

where a, b, and *c* represent the radii of the height, length, and width, respectively. Seed density was calculated for each seed, averaged for each plant, and used as an estimate of fitness to calculate inbreeding depression as seed size has been noted to be correlated with seedling emergence and adult fitness (Stanton 1984; Bonfil 1998). Inbreeding depression (δ) was calculated using the formula:

$$\delta = 1 - (w_s / w_x)$$

where w_s and w_x represent the mean fitness (seed density) of self- and outcross progeny, respectively. Statistical analyses (ANOVA) and calculations (95% confidence intervals) were performed using JMP Pro v.9.0.2 statistical software (SAS Institute Cary, NC).

Testing for apomixis or autogamy– The presence of selfed fruit may often be mistaken for apomixis rather than autogamy. In order to discern the two reproductive tactics, I used amplified fragment length polymorphism (AFLP) primers to identify whether genetic differences were present between a maternal plant and its apparent selfed-seed offspring. In the summer of 2010, fruit were observed developing on a single, isolated *I. parviflorum* plant within the University of Tennessee greenhouse. Fruit were collected in August 2010 and allowed to mature in paper envelopes to catch seeds released by ballistic dispersal. Leaf tissue from the maternal plant and emrbyo/endosperm tissue from six seeds were fingerprinted using AFLP primers. Unlike more derived angiosperms that have triploid endosperm, endosperm from *Illicium* is diploid, having an identical genotype to the embryo (Williams and Friedman 2004). Consequently, endosperm was combined with embryonic tissue for DNA isolation.

To optimize AFLP primers for *I. parviflorum,* I screened 36 selective-AFLP primer pairs using 25 individuals from Salt Springs. These primer pairs were previously tested by Valente (2007) on *Schisandra glabra*, a related species also in the Austrobaileyales. Three primer combinations were selected and optimized for further analysis based on band size, number of bands, repeatability, and band variation.

DNA isolation – Primers were optimized by being tested on plants from the Salt Springs prior to being used on the parent-offspring combination. Leaves were collected, dried with silica gel, and stored at -80ºC until processing. To reduce the possibility of sampling multiple ramets of the same genet, I only sampled ramets greater than three meters from another sampled ramet. This distance is similar to that used by Newell and Morris (2010) who studied the same population using ISSR markers.

Extraction of DNA was completed using a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol. DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) and standardized to 100 ng/µl for PCR amplification. Extraction of DNA from seeds were identical to this procedure, though the seed coat was removed prior to grinding to prevent contamination from the maternal plant.

Genotyping– Amplified fragment length polymorphism (AFLP) reactions were conducted as in

Vos et al. (1995) with the following modifications. Restriction enzyme digestion and ligation occurred within an 11 µl PCR reaction consisting of 1.1 µl of 10x T4 DNA ligase buffer (Fisher Scientific), 1.1 µl 0.5M NaCl, 0.55 µl BSA (10ng/µl), 1 µl of the MseI adapter, 1 µl of EcoRI adapter, 0.1 µl MseI, 0.42 µl EcoRI, 0.33 µl T4 DNA ligase (Fisher Scientific) and 100 ng of DNA. The solution was incubated at 37ºC for two hours, followed by a deactivation step for 15 minutes at 72ºC, and then kept at 4ºC. The product of this reaction was diluted with 120 µl of Tris-HCl (pH 8.0). The pre-select PCR consisted of 2 µl PCR buffer (New England Biolabs), 0.5 µl dNTPs (10 µM), 1 µl EcoRI preselect primer (10 µM), 1 µl MseI preselect primer (10 µM), 0.3 µl Taq DNA polymerase (New England Biolabs), and 5 µl of the diluted RE/Ligation product into a 20 µl reaction. The pre-select reaction consisted of a single two minute cycle at 72ºC followed by 25 cycles of 20 seconds at 94ºC, 30 seconds at 56ºC, and two minutes at 72ºC. The reaction concluded with a two minute cycle at 72ºC, a 30 minute incubation at 60ºC, and a hold at 4ºC. The pre-select product was further diluted with 40 µl of Tris-HCl (pH 8.0). The select PCR consisted of 2 µl PCR buffer (New England Biolabs), 0.5 µl dNTPs (10 µM), 1 µl EcoRI preselect primer (10 µM), 1 µl MseI preselect primer (10 µM), 0.3 µl Taq DNA polymerase (New England Biolabs), and 3 µl of the diluted pre-select product into a 20 µl reaction. For the select reaction, the solution initially incubated at 94ºC for two minutes followed by ten cycles of 94ºC for 20 seconds, 60ºC for 30 seconds (diminishing by 1ºC each cycle), and 72ºC for two minutes. These ten cycles were followed by 24 cycles of 94ºC for 20 seconds, 56ºC for 30 seconds, and 72ºC for two minutes. To complete the reaction, a last cycle of 60ºC for 30 minutes was followed by a hold at 4ºC.

AFLP analysis – All fragments were sized on a 35 cm capillary, ABI 3100 automated sequencer. Each well was loaded with 0.5 µL of each selective, fluorescent-labeled primer combination (HEX, FAM, and NED), 0.4 µL of ROX-500 size standard (Applied Biosystems), and 13 µL of formamide (Applied Biosystems). Gels were first scored using the program GeneMarker V2.2.0 (Softgenetics 2012). Three of the 36 selective primer combinations that exhibited the greatest number of polymorphic alleles on 23 individuals from Salt Springs were chosen for further analysis. All individuals were run three times. Bands that were not present in all three runs were excluded as unrepeatable. Final profiles were generated with the three AFLP primer pairs: E-AAGT/M-CATG, E-ATAT/M-CATG, and E-AACA/M-CCAT which generated 160 bands, 93 of which were polymorphic (58.1%).

OptiFLP v1.41 (Molecular Ecology Group 2011) was used to develop pairwise matrices using the Jaccard Coefficient for determining genetic dissimilarity to estimate the relatedness of individuals. The program Colony v2.0.2.2 (Zoological Society of London 2008) was used for a parentage analysis of the 23 individuals at Salt Springs. Since individuals were mature plants, we used a conservative 0.5 probability that any of the Salt Springs plant genotypes could be a parent of any other Salt Springs individual. The analysis allowed for inbreeding, polygamy, and selfing and was run in ten replicates.

Pollen/ovule ratio – Pollen to ovule (P/O) ratio has previously been shown to be associated with breeding system (Cruden, 1977). High P/O ratios are necessary for species that are primarily

outcrossing due to the loss of pollen during transit between flowers, whereas low P/O ratios are associated with selfing as pollen typically is transferred efficiently within the same flower. Here, I tested whether *I. parviflorum* has a P/O ratio that corresponds to a primarily outbreeding or selfing breeding system as defined by Cruden (1977). Single flower buds were collected from 10 different plants just prior to anther dehiscence and fixed in FAA during the summer of 2009. Two anthers from each flower were dissected out and squashed in 200 µl of 1 % polyethylene glycol (PEG) in 95 % ethanol and mixed thoroughly. From this solution, 20 µl were pipetted onto a microscope slide and observed using light microscopy. The total number of pollen grains on the slide were counted and multiplied by ten then divided by two to estimate pollen grain number per anther. The estimated number of pollen grains per anther was then multiplied by the total number of anthers per flower and subsequently divided by the total number of carpels (*Illicium* carpels are uniovulate) to determine the P/O ratio for each flower.

To access whether P/O ratios vary among different *Illicium* species, I determined average P/O ratio for *I. floridanum, I. anisatum, I. henryi,* and *I. lanceolatum.* Single flower buds were collected from 10 different plants at Hurricane Creek, Tuscaloosa, AL for *I. floridanum* during the spring of 2012 and fixed in FAA and analyzed as per *I. parviflorum*. For *I. anisatum,* single flower buds were collected from 10 different plants located in the University of Tennessee greenhouse and analyzed as per *I. parviflorum.* In contrast, all 10 flowers used to determine the P/O ratio for *I. henryi,* and *I. lanceolatum* were collected from a single individual located in the basal angiosperm garden at the University of Tennessee, Knoxville. Analyses (ANOVA) were performed in JMP Pro v.9.0.2 statistical software (SAS Institute Cary, NC). Interestingly, pollen

for both *I. henryi,* and *I. lanceolatum* had to be collected earlier than other species as anthers dehisced while flowers were still in the bud stage of development.

Results

Testing for self-incompatibility – Among many hand-pollinated selfed flowers, several exhibited pollen-tube growth within the style and within the micropyle of the ovule (Figure 2). In the diallel cross, three of seven self-cross combinations (0.429) produced fruit, whereas eight of 21 outcross combinations (0.381) were successful (Figure 3). Three of the ten individuals set no fruit either as a pollen donor or receiver and were thus excluded. Notably, seven of the eight successful outcross combinations were from between-population outcrosses. These successes occurred despite a longer time between pollen collection and hand-pollination in the between-population crosses (~ 1hr or more). Not a single reciprocal cross was successful in both directions (0/12).

Inbreeding depression – Of the crosses testing for inbreeding depression, only one of eight families produced self seeds (1/8 = 0.125), whereas the success rate of the within-population outcrosses was greater than that of the previous year (4/7 = 0.571). Interestingly, some seeds were not obliquely ellipsoid as has been described previously in Olsen and Rutter (2001), but were instead thin (<1.2 mm), flattened and often indented. I categorized such flattened seeds as 'aborted' since dissected seeds exhibited a lack of endosperm, a trait observed in the aborted

seeds of other species (Sedgley et al., 1996; Lester and Kang, 1998). Seeds wider than 1.2 mm were subsequently characterized as 'viable' since these seeds exhibited a normal oblique shape. The proportion of viable seeds per ovule for the selfed family (0.538) fell within the 95% confidence intervals of both naturally-pollinated and outcross hand-pollinated treatments, however, the proportion of aborted seeds to total seeds produced (0.222) exceeded the 95% confidence intervals of the natural and outcross treatments (Figure 4). Notably, no aborted seeds were observed in any of the hand-outcrossed treatments. Furthermore, inbreeding depression using viable seed proportion as an estimate of fitness was calculated ($\delta = 0.222$).

Between hand-outcrossed and naturally pollinated fruits, there were no significant differences in overall seed length ($F = 0.1591$, d.f. = 23, $P = 0.6938$), height ($F = 0.9104$, d.f. = 23, $P = 0.3504$), width ($F = 1.8372$, d.f. = 23, $P = 0.1890$), volume ($F = 0.0365$, d.f. = 23, $P = 0.8502$), weight ($F = 0.3394$, d.f. = 23, $P = 0.5661$), or density ($F = 0.9274$, d.f. = 23, $P = 0.3460$; Figure 5). Notably, no significant differences were also found when excluding aborted seeds (seed length, $F = 0.6431$, d.f. = 23, $P = 0.4312$; height, $F = 2.3237$, d.f. = 23, $P = 0.1417$; width, $F = 0.4592$, d.f. = 23, $P = 0.5050$; volume, $F = 0.9068$, d.f. = 23, $P = 0.3513$; weight, $F = 1.9499$, d.f. = 23, $P = 0.1765$; density, $F = 1.7117$, d.f. = 23, $P = 0.2043$; Figure 6). Furthermore, natural and outcross treatments were not significantly difference with regards to the proportion of viable seeds to carpels ($F = 0.9905$, d.f. = 23, $P = 0.3304$) and proportion of total seeds aborted ($F = 1.3165$, d.f. = 23, $P = 0.2635$; Figure 4).

The selfed treatment differed from both the outcross and natural treatments, although data are limited to only one family. Overall mean values for selfed seeds with respect to width (1.84

mm), weight (0.0186 g), volume (24.80 mm^3), and density (0.000710 g/mm^3) were outside of and below the 95% confidence intervals of both outcross and natural treatments (Figure 5). This result was also found when excluding aborted seeds (width, 2.11 mm; weight, 0.0222 g; density, 0.000714 g/mm^3; Figure 6), however no difference was found with respect to mean viable seed volume (29.09 mm^3; Figure 6B). In light of the significant differences in both volume and weight, I used seed density as a composite estimate of fitness ($\delta = 0.2234$).

Apomixis or autogamy – The genetic dissimilarity between the greenhouse parent and the mean (SE) of the selfed seeds was 0.329 (0.031, $n = 6$) and not significantly different from the mean of the selfed seeds alone (0.369, s.e. = 0.033, $n = 6$; $F = 0.8195$, d.f. = 10, $P = 0.3866$). Notably, none of the offspring genotypes possessed a band that was not found in the maternal genotype, nor were any of the offspring genetically identical the parent plant, although both greenhouse genotypes shared bands with individuals from the Salt Springs population. However, mean genetic dissimilarity of the greenhouse selfed seeds (0.329, s.e. = 0.031, $n = 6$) was significantly lower ($F = 116.8$, d.f. = 28, $P < 0.0001$) than the mean genetic dissimilarity of the Salt Springs population (0.631, s.e. = 0.009, $n = 23$).

Colony reconstructed 19 possible parents to the individuals of the Salt Springs population, none of which were from the original 23 individuals analyzed. From this group there were estimated to be three biparental full-sibs and 31 half-sibs. Two of the 23 individuals were determined to be from selfing parents [probability of selfing was 1.0 for the two selfs while 0.02 $\pm$ 0.01 (mean $\pm$ s.e.) for outcrosses], and as such, outcrossing rate in this population is estimated

to be t = 0.913.

Pollen/ovule ratio – The mean (± standard error) P/O ratio of *Illcium parviflorum* was 511 ± 86 pollen grains per ovule (n = 10; Figure 7). *Illicium anisatum* had the highest ratio with 9253 ± 1277 pollen grains per ovule (n = 10). Both *I. floridanum* (6460 ± 888, n = 10) and *I. henryi* (5151 ± 883, n = 1) had similar P/O ratios to those reported by Luo et al. (2010) for *I. dunnianum* (5845 ± 374, n = 10) and *I. tsangii* (4190 ± 1158, n = 10). *Illicium lanceolatum* had the second lowest P/O ratio at 2821 ± 417 (n = 1).

Discussion

The purpose of this study was to determine whether *Illicium parviflorum* has the ability to self-fertilize, and if not, whether a self-incompatibility system is present. Here, I provide genetic, morphological, and reproductive evidence which indicates that *I. parviflorum* can and does self-fertilize in nature, thereby ruling out standard self-incompatibility systems.

The original study by White and Thien (1985) observed few fruit being produced in *I. parviflorum* populations. I also observed a very low fruit production rate in nature and in hand-pollinated crosses regardless of whether pollinations were outcrossed or selfed (Figure 1). However, I observed selfed fruit production in sets of crosses for two consecutive years at Salt Springs as well as the greenhouse. Histological evidence indicates that selfed pollen tubes can reach and enter ovules. Furthermore, apomixis is likely not a regular feature of reproduction,

since AFLP analysis indicated that seeds collected from a single greenhouse plant differed genetically from each other but were consistent with being biparentally derived from the seed parent alone. Notably, no unique paternal AFLP bands were expressed in the offspring, indicating that seeds were a result of selfing and not outcrossing.

Another result from the diallel cross between the two populations of *I. parviflorum* at Alexander Springs and Salt Springs showed that outcrosses between populations were more likely to succeed than outcrosses within populations. These results are indicative of heterosis (hybrid vigor), a factor commonly associated with populations diverging due to genetic drift (Busch, 2006). Therefore, gene flow may be constrained between the Alexander Springs and Salt Springs populations despite only being 19 km apart. Notably, a great deal of genetic over-lap exists between these two populations (Newell and Morris, 2010), suggesting that population fragmentation in Ocala National Forest is bottlenecking an already homogenous population into much smaller, distinct populations. Therefore, the success of *I. parviflorum* populations throughout the St. John's River watershed depends on how well these plants can survive inbreeding. Unfortunately, higher levels of aborted seeds within self-crosses (Figure 5) as well as a lower seed density in self- versus outcrosses (Figure 6-7) indicates inbreeding depression in Salt Springs population, suggesting that further conservation efforts are needed in order to preserve natural populations of *I. parviflorum*.

In contrast to a prior study by Newell and Morris (2010) that found evidence of clonality, I found no identical genotypes within the Salt Springs population. One possibility for this discrepancy may be an inherent problem with primer development. The current study specifically

targeted primers exhibiting variation within the Salt Springs population alone, whereas Newell and Morris (2010) targeted primers possessing variation among numerous *I. parviflorum* populations. Consequently, bands that were monomorphic within Salt Springs were excluded from the current analysis, but may have been included in Newell and Morris (2010) if polymorphisms existed in other populations. Another possibility may be a difference in the number of loci used in each study. While Newell and Morris (2010) used Inter Simple Sequence Repeat (ISSR) markers that produced 26 loci, only 10 were polymorphic, a factor that greatly reduced their ability to distinguish between closely related individuals. Conversely, my AFLP markers produced a total of 160 loci (93 of which were polymorphic), thereby allowing for greater resolution to detect the presence of clones within a population. Regardless, Newell and Morris (2010) provides a baseline study for estimating the genetic diversity between populations.

Although *I. parviflorum* has the ability to self-fertilize, the parentage analysis indicated that outcrossing is the primary means of reproduction in one population. *Illicium parviflorum* has a lower P/O ratio than each of its relatives (Figure 7), but still falls between facultative xenogamy and facultative autogamy (Cruden 1977), indicating a mixed mating system that is primarily outcrossing with some selfing. Additionally, the mean genetic dissimilarity between individuals of the Salt Springs population is much greater than the mean dissimilarity between the greenhouse plant and its selfed offspring, suggesting that the individuals at Salt Springs are the result of outcrossing and not selfing. However, individuals sampled in this study were all mature, full grown plants and not representative of their replacement seedlings. Notably, parentage analysis estimated that the 23 individuals in this study stemmed from only 19 parents,

suggesting an unequal contribution of genomic investment in future offspring, thereby indicating a smaller effective population size than is actually observed. A multigenerational genetic analysis on naturally pollinated individuals and their seeds would be needed to determine pollen flow, natural paternal origin, and how populations are responding to ecosystem disturbance.

Despite *I. parviflorum* being primarily outcrossing, this study is the first to identify successful selfing within a woody, perennial angiosperm of the ANITA group. Other *Illicium* species may also maintain the ability to self-fertilize. With the exception of *Illicium anisatum* and *I. floridanum* which exhibit large flowers with a ligulate tepal morphology, *Illicium dunnianum, I. tsangii, I. henryi,* and *I. lanceolatum* have small flowers with an orbicular tepal phenotype similar to *I. parviflorum* (Smith 1947). Indeed, the larger flowered species have higher P/O ratios than their smaller flowered relatives (Figure 7), suggesting that larger flowered species may have a greater dependence on outcrossing. The correlation between large flowered species and outcrossing has been described in numerous instances (Stebbins 1970; Cruden 1977; Li and Johnston 2010), therefore finding a correlation between P/O ratio and flower size is not unexpected. Yet, according to Cruden (1977), *I. dunnianum, I. tsangii, I. henryi,* and *I. lanceolatum* should also be xenogamous due to their high P/O ratios. However, higher than average P/O ratios are necessary for protogynous species that have some degree of selfing in their mating system (i.e. geitonogamy) despite a temporal disassociation between stigma receptivity and anther dihiscence within the same flower. Indeed, the protogynous morph of *Plantago ovata* is self-compatible despite having a mean P/O ratio of approximately 6500 (Sharma et al., 1992). Furthermore, *Scrophularia peregrina* has a P/O ratio of 1909.2 $\pm$ 414.2

though maintains incomplete protogyny (Olivencia and Alcaraz, 1993). *Lactoris fernandanzi*, a protogynous member of the Piperales, also has a high P/O ratio, despite being self-compatible. Notably, bisexual flowers of *L. fernandanzi* have a mean P/O ratio of, yet the presence of female flowers lowers the P/O ratio per plant to 1339 (Bernardello et al., 1999). *Caboma aquatica* (Cabombaceae), another self-compatible basal angiosperm with strong protogyny, has a mean P/O ratio of 1055 (Silva and Leite, 2011). Therefore, because *Illicium* species exhibit protogyny, P/O classification on species within this genus may be misleading, with many species being assigned to categories that have a greater reliance on outcrossing than is actually occurring.

Interestingly, a maximum parsimony analysis conducted by Morris et al. (2007) suggests that the small-flowered, orbicular tepal phenotype is plesiomorphic to the ligulate tepal phenotype and that ligulate tepals have arisen multiple times within the genus *Illicium* (Figure 8). Further supporting this hypothesis is that orbicular tepal morphology is also observed in other Austrobaileyales, including *Austrobaileya scandens, Schisandra sp.,* and *Kadsura sp.* Notably, the P/O ratio of *A. scandens* (Joe Williams, personal communication; Figure 7) is similar to numbers observed among small flowered *Illicium*, indicating possible self-compatibility. The presence of self-compatibility in *A. scandens* would explain the evidence of selfing observed previously by Kennard and Williams (2008). Furthermore, these results support the hypothesis that self-compatibility is the plesiomorphic state of the Austrobaileyales.

Conclusion – The presence of selfed pollen tubes, fruit and seeds resulting from self-crosses, and a low P/O ratio in *Illicium parviflorum* indicates the species is self-compatible. Indeed,

results from the AFLP analysis suggest that seeds were the result of selfing and not apomixis. However, seed abortion and seed density data indicate there is a moderate degree of early inbreeding depression in the Salt Springs population, consistent with the relatively high outcrossing rate of 92% calculated for the population. Introduction of individuals from other populations may increase fitness through heterosis, though gene flow between populations will still be reduced. *Illicium parviflorum*'s ability to self-fertilize is important to the evolution of the angiosperms because its presence within the Austrobaileyales, an ancient clade of woody perennials near the base of the angiosperm tree. The presence of protogyny, conserved tepal morphology, and similar P/O ratios within *Illicium* implies that self-compatibility may be more widespread throughout the Austrobaileyales, suggesting that the earliest flowering plants may also have had the ability to self.

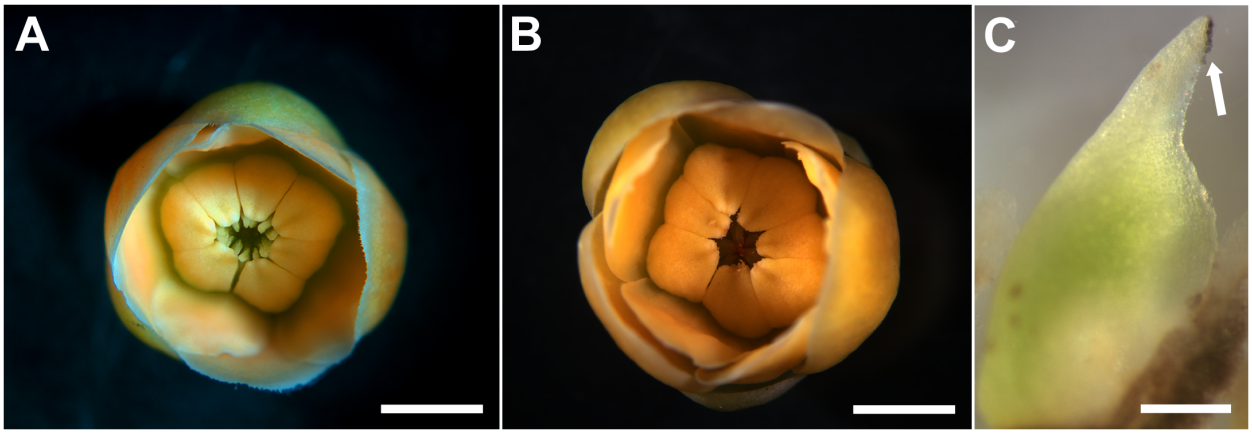
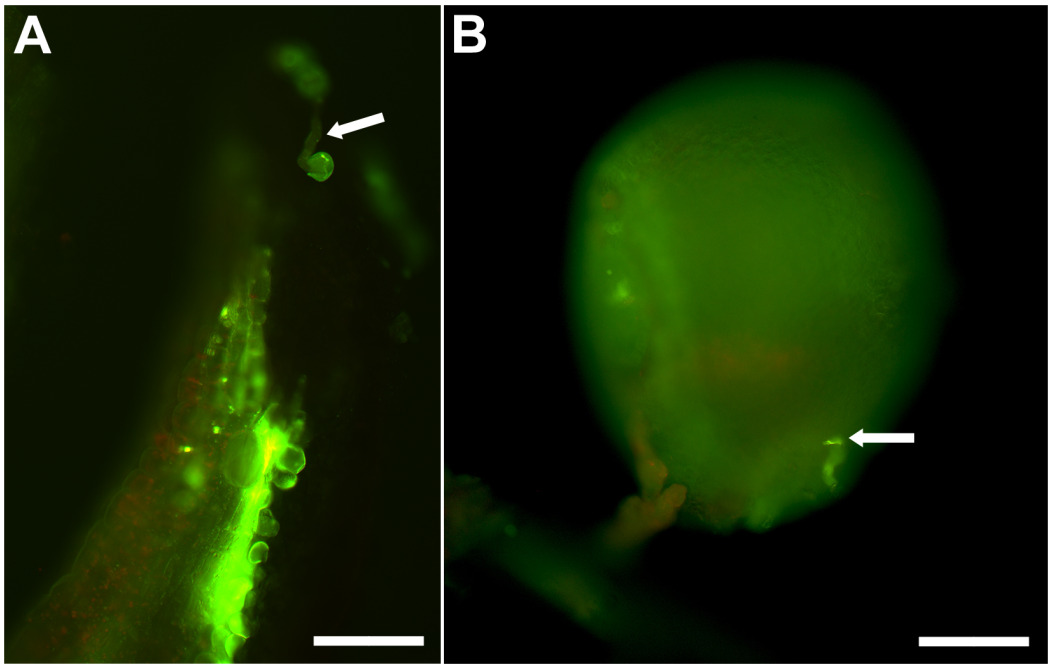


Figure 1. Flowers of *Illicium parviflorum*. Flowers are represented by both the female (A) and male (B) phase. C. Female phase carpel stained with 3,5,3',5'-tetramethylbenzidine (TMB). Dark blue coloration of the stigmatic surface (arrow) indicates oxidation and pollen receptivity. Scale bars = 2.5 mm for A and B, 0.5 mm for C.

Figure



2. Aniline blue fluorescence of self-pollen tube (arrow) growing on the stigmatic surface (A) and penetrating the ovule (B). Scale bars = 100 μm.

FEMALE PLANT		MALE PLANT: SS 1	SS 2	SS 3	SS 4	AS 5	AS 6	AS 7
SS	1	0.002	0.502	0.002	0.002	0.502	0.502	0.001
SS	2	0.002	0.502	0.002	0.002	0.002	0.502	0.002
SS	3	0.002	0.002	0.002	0.002	0.002	0.502	0.002
SS	4	0.002	0.002	0.002	0.002	0.502	0.002	0.002
AS	5	0.002	0.002	0.002	0.002	1.002	0.001	0.001
AS	6	0.002	0.002	0.002	0.002	0.002	0.002	0.001
AS	7	0.502	0.002	1.002	0.002	0.001		0.502

Figure 3. Fruit success from a diallel cross of *Illicium parviflorum* in May of 2009. Top numbers of each cell indicates the proportion of pollinations which produced fruit, whereas the lower number represents the number of crosses attempted. Green cells indicate a successful cross. Salt Springs and Alexander Springs are denoted as SS and AS, respectively.

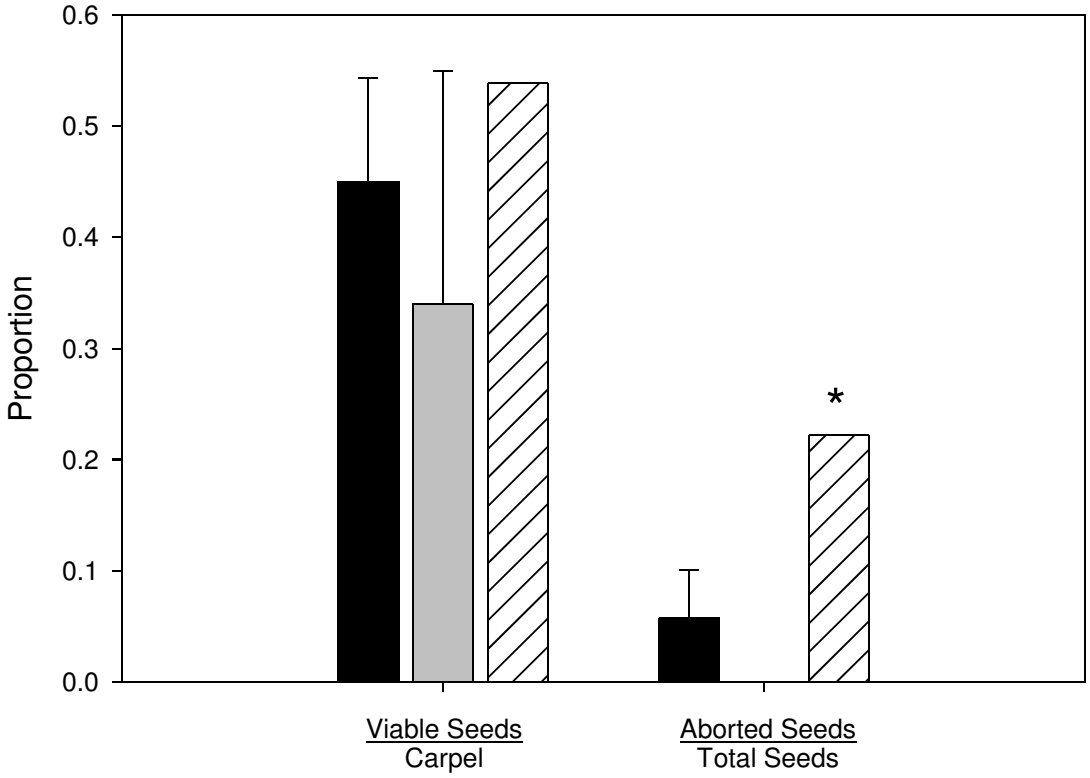


Figure 4. Viable seeds per carpel and proportion of total seeds that were aborted for natural (black), hand-outcrossed (grey), and hand-selfed (striped) crosses. Asterix indicates selfed seed abortion rate falls outside the 95% confidence interval for outcrossed seeds.

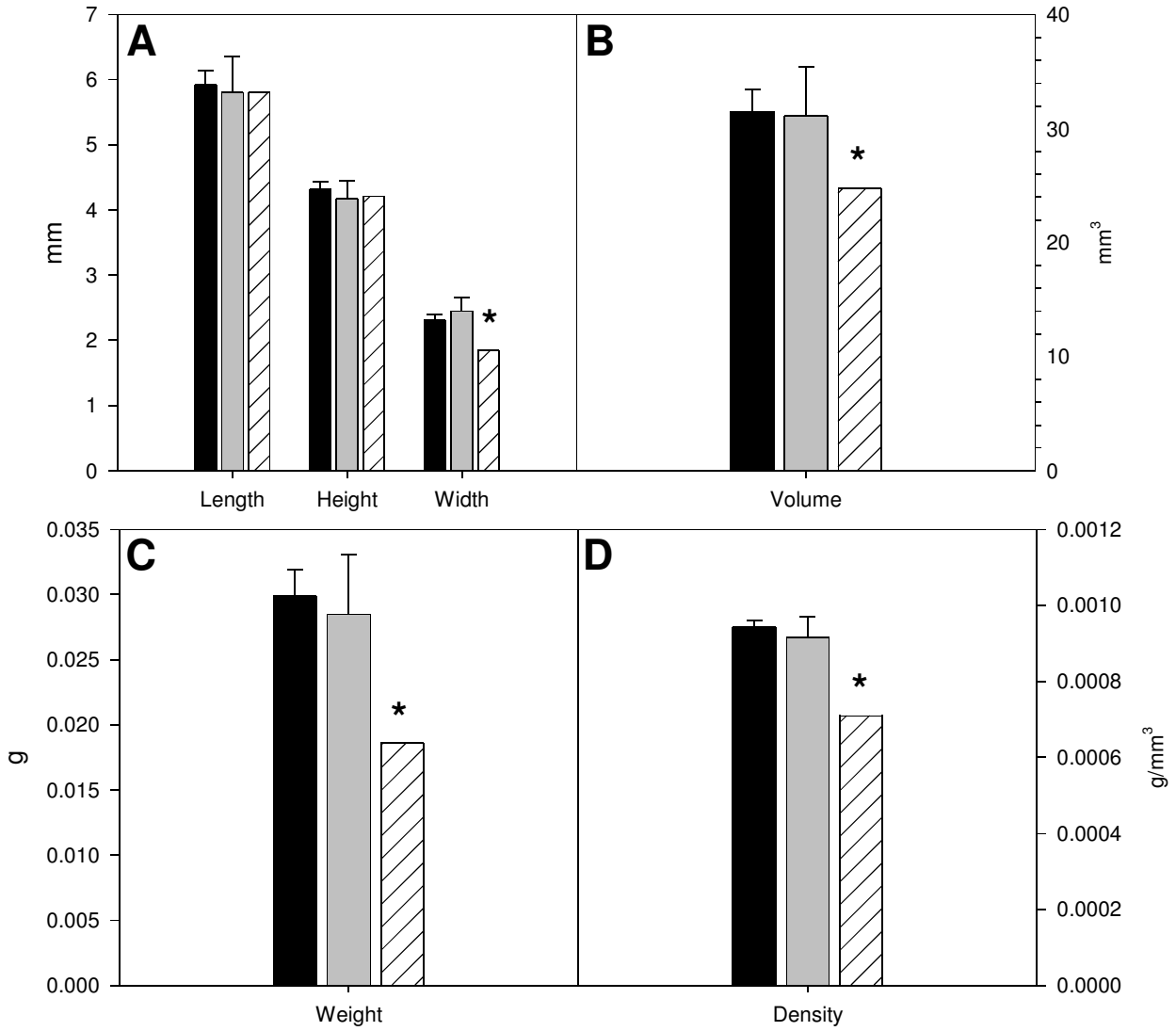


Figure 5. Mean and 95% confidence intervals for total seed (A) length, height, and width, (B) volume, (C) weight, and (D) density for natural (black), hand-outcrossed (grey), and hand-selfed (striped) crosses. Asterix indicates hand-selfed seeds falling outside the 95% confidence interval for hand-outcrossed seeds.

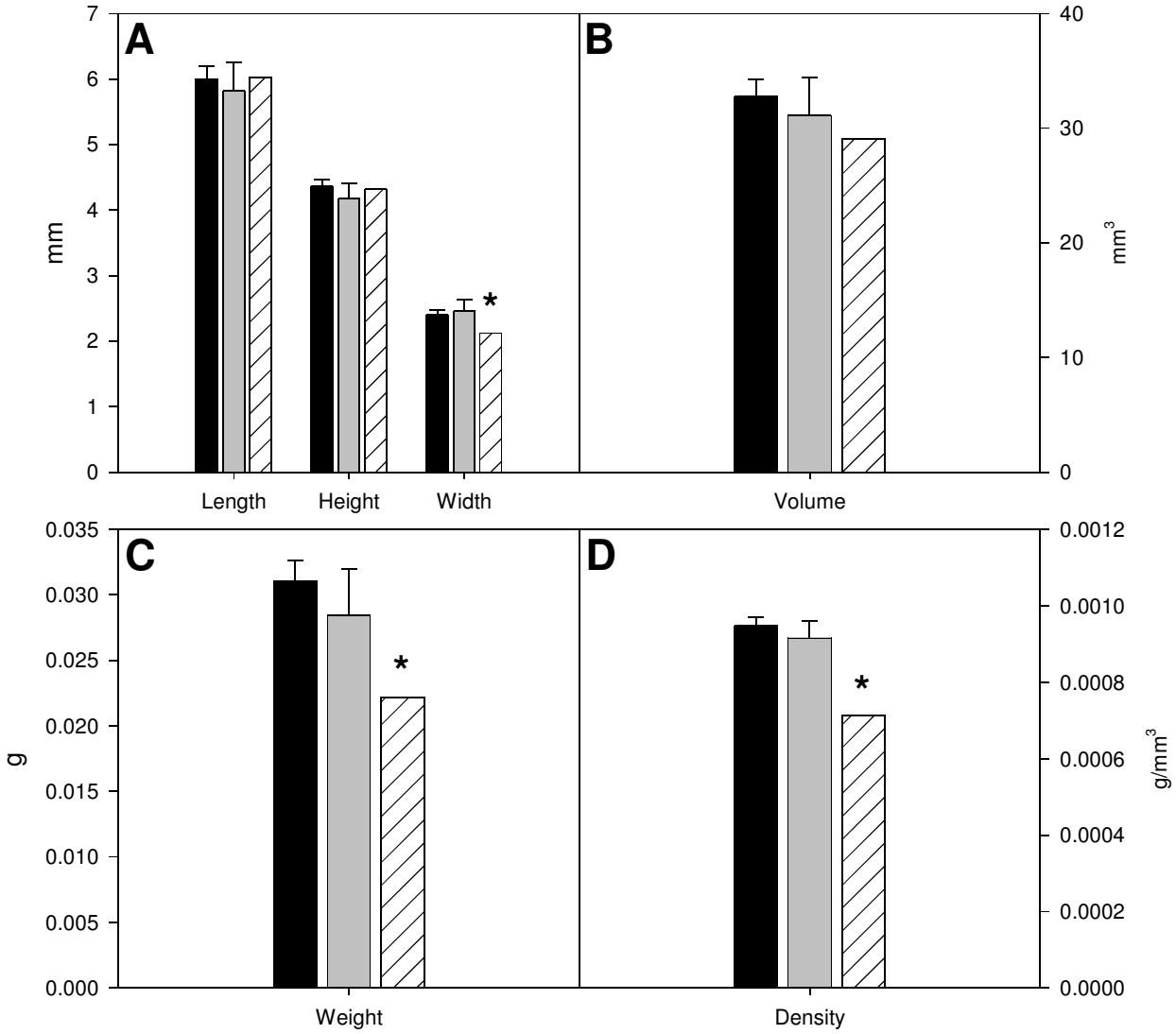


Figure 6. Mean and 95% confidence intervals for viable seed (A) length, height, and width, (B) volume, (C) weight, and (D) density for natural (black), outcrossed (grey), and selfed (striped) crosses. Asterix indicates hand-selfed seeds falling outside the 95% confidence interval for hand-outcrossed seeds.

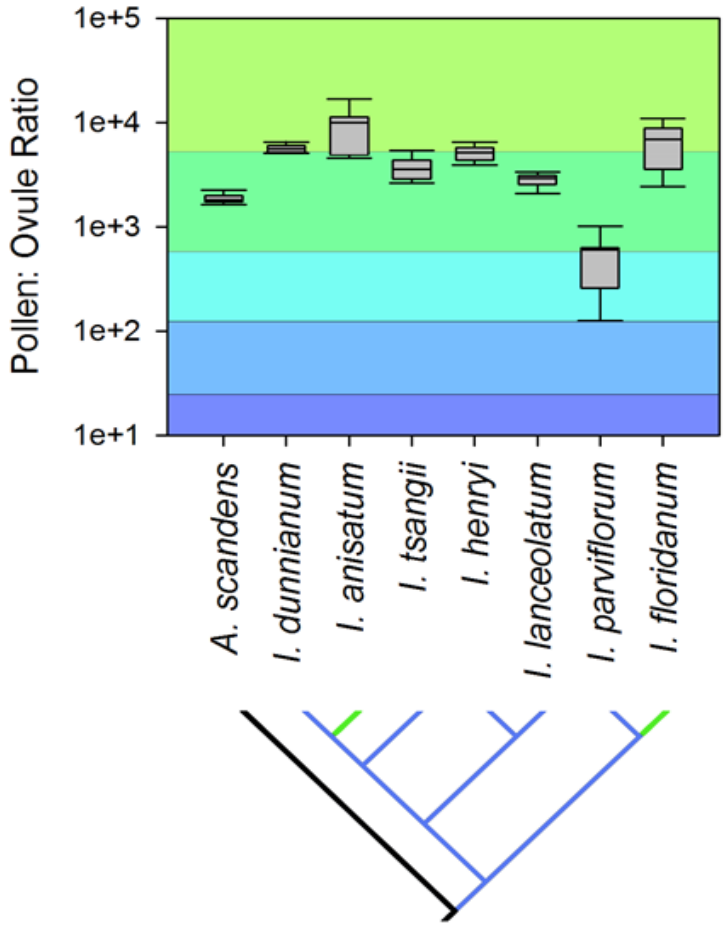


Figure 7. Log scale of Pollen/Ovule ratios and phylogeny for *Austrobaileya scandens* and numerous *Illicium* species. Color bars indicate P/O ranks according to Cruden (1977). Lower ratios suggests increased selfing. Modified phylogeny is from Morris et al. (2007) with blue lineages having orbicular and green lineages having ligulate tepal morphology.

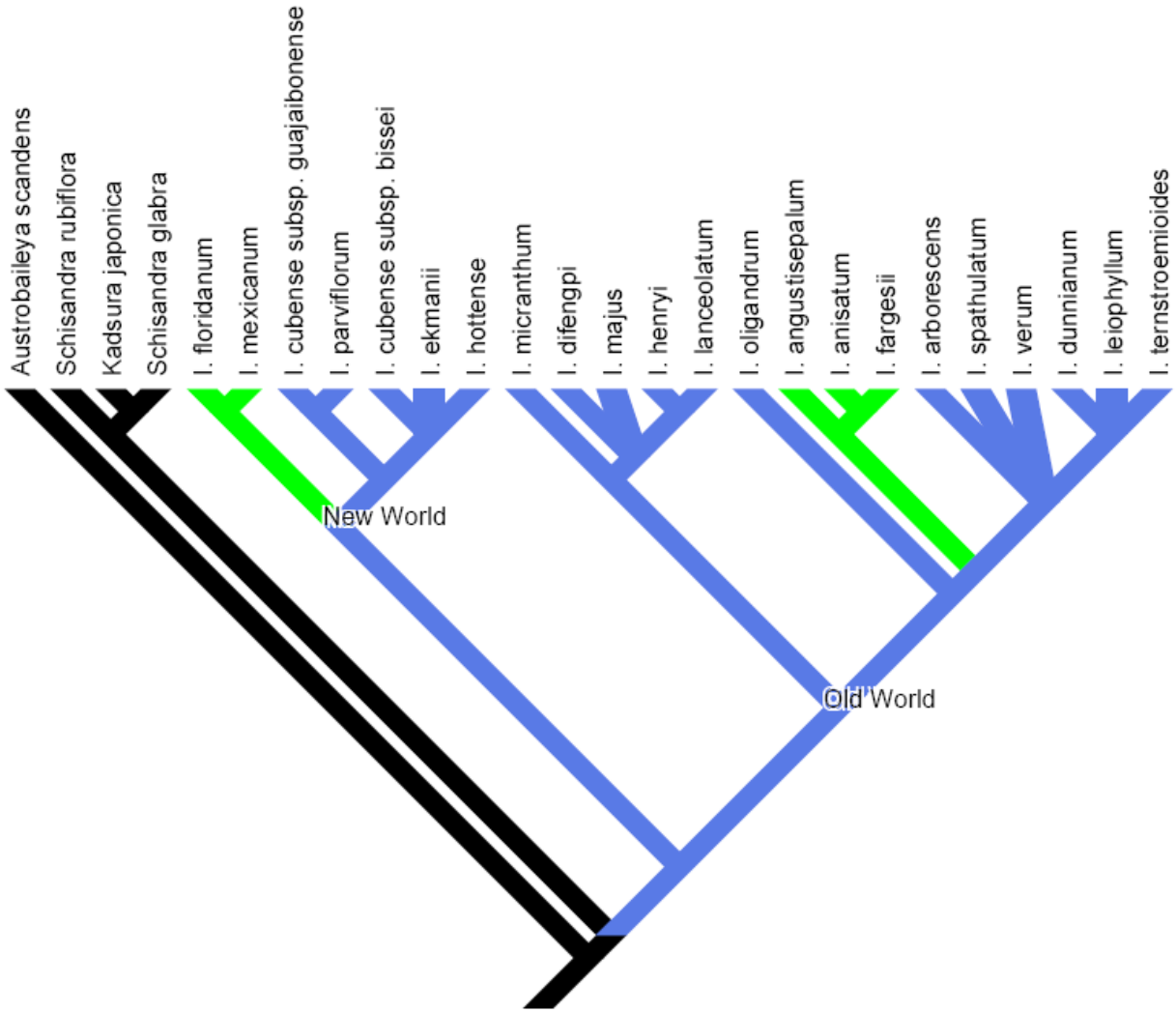


Figure 8. Phylogeny indicating multiple origins of a large flowered, ligulate tepals phenotype within the *Illicium* genus. Modified phylogeny is from Morris et al. (2007) with blue lineages having orbicular and green lineages having ligulate tepal morphology.

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Vita

Nicholas Buckley was born January 16, 1981 in the ocean side city of Newburyport, MA. After attending several elementary schools throughout New England, he graduated from Pentucket High School (West Newbury, MA) in 1999. Nicholas then attended Dalhousie University in Halifax, NS, Canada, where he earned a Bachelor's of Science degree in Biology in 2004. During his last year of his B.Sc., Nicholas worked with Dr. Mark O. Johnston on several projects including the mating system biology of *Amsinckia spectabilis* as well as a developmental study on mutants in *Arabidopsis thaliana*. Following a brief stint of work as a lab technician, Nicholas continued into higher education at Acadia University (Wolfville, NS, Canada) where he earned a Master's of Science in Applied Biology in 2008. While at Acadia, Nicholas worked with Dr. Germán Avila-Sakar on a project that investigated tradeoffs in tolerance and resistance to herbivory in the dioecious shrub *Ilex glabra*. Following his graduation, Nicholas accepted a graduate teaching assistantship at the University of Tennessee, Knoxville, working in a lab under the supervision of Dr. Joe Williams. Upon completion of his Master's of Ecology and Evolutionary Biology degree in August 2012, Nicholas plans on pursuing his love of teaching and botany.