

FLORAL BIOLOGY, BREEDING SYSTEM, POLLINATION ECOLOGY, AND *EX SITU*  
GENETIC DIVERSITY OF THE ENDANGERED HAWAIIAN SPECIES, *BRIGHAMIA*  
*INSIGNIS* A. GRAY (CAMPANULACEAE)

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CHAPTER 1  
BREEDING SYSTEM AND FLORAL BIOLOGY OF *BRIGHAMIA INSIGNIS* A. GRAY  
(CAMPANULACEAE)

**Abstract**

*Brighamia insignis* A. Gray is a functionally extinct Hawaiian lobeliad endemic to Kauaʻi. Its putative pollinator is believed to have gone extinct and this is thought to be one of the major reasons for its decline. Floral characters were examined to provide a best guess of the type of animal pollinator(s) to which *B. insignis* is adapted. The breeding system was examined to assess whether the species is able to set fruit and produce seeds on its own without a pollinator. The flowers have a long corolla tube, are yellow to white, contain copious amounts of sucrose-rich nectar, and emit a strong floral scent, which was found to contain benzyl alcohol, linalool, and methyl salicylate. This combination of traits points to a moth pollination syndrome. Knowledge of a plant's breeding system is crucial in deciding how to best conserve or restore a rare plant species, particularly for those with specialist pollinator(s) that are rare or extinct. Flower phenology and pollen manipulation treatments conducted in 2011 suggested that the plant is primarily outcrossing. Flowers remained open between five and seven days, with a temporal and physical separation of male and female reproductive phases, and fruit formation occurred only in the two outcross treatments. However, some breeding system traits examined, and results of the pollen manipulations in two of the three years of treatments, do not point to a strict xenogamous mating system. A pollen to ovule ratio of 161 ( $\pm$  12) pointed to an autogamous mating system. No fruit formed in either of the outcross treatments in 2012 or 2013, whereas only a single fruit formed in each of the control and self treatments in those two years combined. Our findings indicate that in the absence of pollinators, active management (such as human assisted cross pollination) would be required in most cases for *B. insignis* to set fruit and seed.

**Introduction**

Understanding plant breeding systems is a fundamental component of rare plant conservation (Gargano et al., 2009). The need for information is even more pressing for rare

plants that have co-evolved with specialist pollinators that may also be rare or even extinct (Campbell, 2008). If a plant species is incapable of full or partial autogamy (selfing), a total loss of reproductive output may be experienced with loss of a pollinator. Conversely, selection towards autogamy may occur when such offers reproductive assurance (Knight et al., 2005). Such shifts may also lead to inbreeding depression (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987), further compounding the effect of the loss of pollinator(s). Although the risk of inbreeding depression is likely to be high for rare plants that shift to complete selfing, it may, at least for some time, provide a means for the species to persist when there are few to no pollinators left (Hill et al., 2008).

*Brighamia* is an endemic genus of two morphologically similar species (*B. insignis* A. Gray and *B. rockii* H. St. John, variously called ālula, ‘ōlulu, or pua‘ala in Hawaiian) distinct from other Hawaiian lobeliad genera most obviously in its caudiciform habit. Both species are listed as federally endangered. Both species have yellow to white salverform-shaped, fragrant flowers (Figure 1.1). In addition, *B. insignis* has been said to contain sucrose-rich nectar (Lammers, 1989). These are all characteristics consistent with a moth pollination syndrome (Herrera and Pellmyr, 2002). A plant's pollination syndrome can be defined as a specific suite of combined floral traits that together, attract and utilize a specific group of animals as pollinators (Fenster et al., 2004). It has been speculated that the natural pollinator of both *Brighamia* species was a native hawk moth that is now either extremely rare or extinct (Lammers and Freeman, 1986; Lammers, 1989; Hannon and Perlman, 2002). Whether or not there was a single, separate or shared pollinator species for each *Brighamia* species, and/or multiple, shared or separate pollinator species for both or either *Brighamia* species, has not been discussed. In contrast, the other Hawaiian lobeliad genera (*Clermontia*, *Cyanea*, *Delissea*, *Lobelia*, and *Trematolobelia*) are believed to have evolved a bird pollination syndrome, the traits of which include hexose-rich nectar, deep, wide, tubular flowers, and no odor (Lammers and Freeman, 1986).



Figure 1.1. *Brighamia insignis* (left) and *B. rockii* (right), variously called ālula, ‘ōlulu, or pua‘ala in Hawaiian. Both species have yellow to white salverform-shaped, fragrant flowers. Photo courtesy of Joel Lau.

*Brighamia insignis* is endemic to the island of Kaua‘i and historically to Ni‘ihau (Wagner et al., 1999; see Figure 1.2 for photos of *B. insignis* habit and flowers). Despite the species being listed as federally endangered since 1994, much remains unknown about its biology and ecology. Moth pollination for *B. insignis* has been proposed based solely on a few floral trait observations and quantifications indicative of a moth pollination syndrome (Lammers and Freeman, 1986; Lammers, 1989). Although no moth pollinator has ever been observed visiting *Brighamia* flowers, there are three native endemic species belonging to the Sphingidae family (commonly known as hawk moths, sphinx moths, and hornworms) reported to occur on Kaua‘i. These three species are *Hyles calida*, *Manduca blackburni*, and *Tinostoma smaragditis* (Nishida, 2002). Other species may have gone extinct before being described. A cohesive study to formally

quantify floral traits in detail has not been completed. It was stated in the previous paragraph that *B. insignis* contains sucrose-rich nectar, but this was only mentioned in Lammers (1989) with a ‘personal communication’ citation. Published data on the actual ratios of different sugars was not found. Therefore, a nectar sugar composition analysis was conducted here to examine sugar ratios. Furthermore, although a floral scent analysis was done for both *Brighamia* species by Kaiser (2010), sampling for *B. insignis* was only done in the morning, between 8:00 and 11:15.



Figure 1.2. *Brighamia insignis* habit (left) and flowers (right).

Here, floral scent of *B. insignis* was sampled during both the day and at night, to examine whether there were any differences in compounds emitted and emission rates of compounds diurnally versus nocturnally. Day and night differences in floral scent emissions have been observed for other moth-pollinated plant species, with higher emission rates in the evening

(Hoballah et al., 2005; Martinell et al., 2010). In addition to examining nectar sugar composition and floral scent as described above, cultivated plants were used to document average floral organ measurements and colors, nectar standing crop, and position of nectar as it relates to reproductive organs, in order to more confidently support or refute the hypothesis that *B. insignis* evolved a moth pollination syndrome. Since the species' breeding system has never been studied experimentally, flower phenology and pollen-ovule ratios were examined and pollen manipulations on cultivated plants undertaken to assess the hypothesis that *B. insignis* is self-incompatible. The viability of pollen from a subset of the plants used for pollination treatments in the third flowering season (2013) was also examined.

## **Methods**

### *Floral measurements*

Fourteen male-phase flowers (1–4 flowers per plant from 7 different plants) and fifteen female-phase flowers (1–3 flowers per plant from 10 different plants) were collected from the National Tropical Botanical Garden (NTBG) Conservation and Horticulture Center, McBryde Garden in Kalaheo, Kaua'i on May 17 and 18, 2011 and at Queen Kapiolani Garden (QKG) in Honolulu, O'ahu in June 2011 and February 2012. Flowers of *Brighamia insignis*, like all of the Hawaiian Campanulaceae, are protandrous, which means they first pass through a male-phase before going into a female-phase (Lammers, 2004; Drake and Morden, 2006; personal observation). In the male-phase, pollen is shed as the style elongates up through the column and pushes the pollen out (see Figure 1.3 for flower morphology). Once the style exerts past the staminal column and apex of anthers, the stigmatic lobes separate and expand, transitioning into a female-phase with an exposed, receptive stigma. Fourteen measurements were made on each flower. Two and four additional measurements were made on flowers during male (anther length and width) and female (stigma lobe length and width and stigma exertion above corolla lobes and above anthers) phases, respectively. Table 1.4 lists all floral characteristics and organs that were measured. A flexible plastic ruler was used to record all measurements to the nearest half mm.

### *Floral organ color*

The color of 10 floral organs and organ parts of eight female-phase (1–2 flowers per plant from 6 different plants) and eight male-phase (1–2 flowers per plant from 5 different plants) flowers were determined by comparison to the Royal Horticultural Society (RHS) color charts. Flowers used were collected from the NTBG Conservation and Horticulture Center, McBryde Garden in May 2011 and at QKG in June 2011 and February 2012. RHS color codes were then translated into colors named by the Universal Color Language (UCL) using a comparison table on the Azalea Society of America's website (<http://azaleas.org/index.pl/rhsmacfan1.html>).

### *Nectar standing crop*

Seven male-phase (1–2 flowers per plant from 4 different plants) and 12 female-phase (1–2 flowers per plants from 6 different plants) unvisited flowers were harvested and maintained in an upright position (so that nectar was not lost). Within two hours after collecting, the corollas of all flowers were partially dissected by hand and the nectar they contained was immediately collected and the volume calculated to obtain an average measurement of nectar standing crop using a calibrated micropipette (0–200  $\mu$ L). Nectar samples were stored in 1.5 mL microcentrifuge tubes for immediate percent mass sucrose measurements and then subsequently stored at  $-20^{\circ}\text{C}$  for future high-performance liquid chromatography (HPLC) analysis.

### *Position of nectar in flowers*

The same flowers from which nectar was sampled to measure the nectar standing crop were used to measure the distances between the upper reaches of nectar in the corolla tube and the 1) apex of the anther, 2) stigma apex in female-phase flowers, 3) corolla opening, and 4) base of the corolla (Figure 1.3). The corollas were partially dissected by hand and distances measured to the nearest half mm using a flexible plastic ruler. These measurements were used to estimate the proboscis length required for a flower visitor to access nectar while simultaneously coming into contact with reproductive organs.

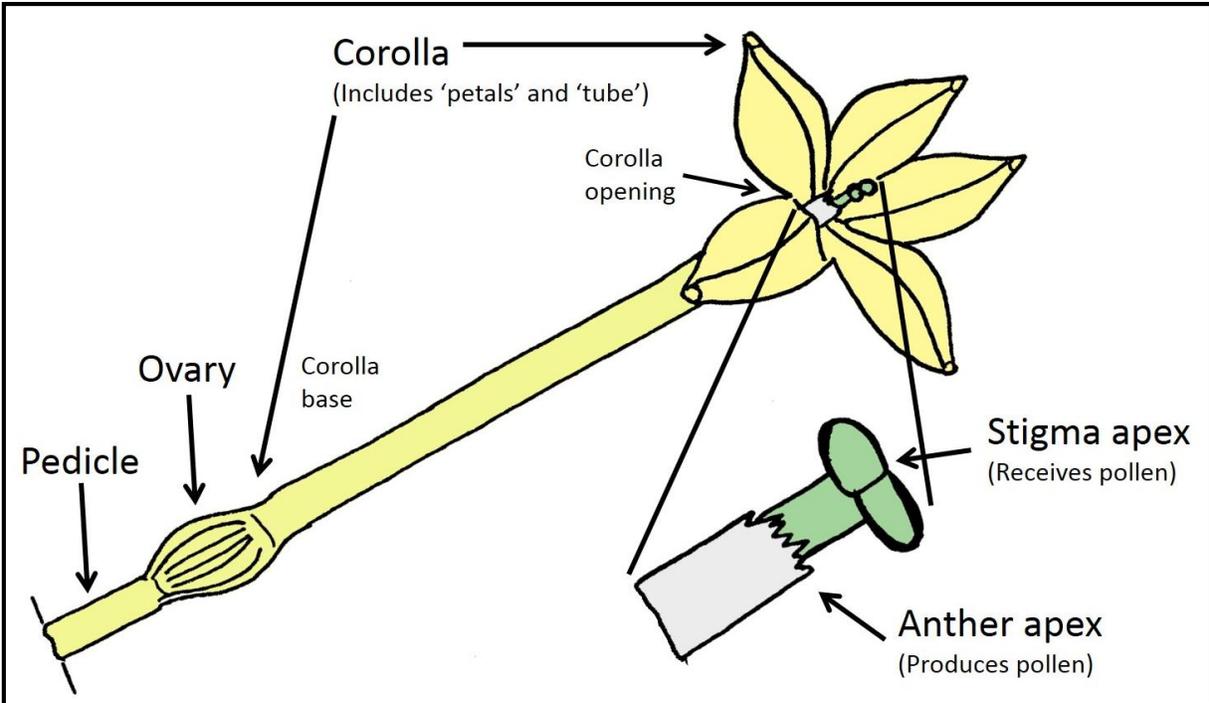


Figure 1.3. Hand drawing of *B. insignis* flower displaying select organs and parts, including the following which were used to measure distances between the upper reaches of nectar in the corolla tube: 1) apex of the anther, 2) stigma apex, 3) corolla opening, and 4) base of the corolla.

### *Nectar sugar composition*

Using the same flowers from which nectar standing crop was measured, and at the same time (within two hours after collecting), a handheld refractometer (model: Eclipse 0–50% w/v; Bellingham and Stanley Ltd, Basingstoke, UK) was used to quantify the percent mass sucrose in nectar of each flower. HPLC analysis was later conducted by Dr. Robert Paull (Department of Tropical Plant and Soil Sciences, University of Hawai‘i at Mānoa (UHM)) using a Shimadzu Model 20 HPLC with a CBM-20A controller, LC-20AT pump, SIL-20A automatic injector, CTO-20A column oven and a ELSD-LT-II Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto, Japan) with a Fast Carbohydrate Analysis Column (100 mm × 7.8 mm) with a precolumn (Bio-Rad Laboratories, Hercules, California, USA). The methods followed those of Pender et al. (2014) to identify and quantify the percent of different sugars (sucrose, glucose, and fructose) in the nectar.

### *Floral scent*

On December 30, 2013, floral scent samples from a flowering *B. insignis* individual behind the Botanical Research Center (BRC) at NTBG on Kaua‘i were collected by Dr. Robert R. Junker (University of Salzburg, Department of Organismic Biology). The scent of two open flowers on the same plant, one in an early male-phase and one that was in a later female-phase, was collected using the dynamic headspace sampling method. Scent was sampled once during the day (16:00) and once at night (19:30) by enclosing the flowers in scentless oven bags. Headspace (air surrounding the flowers) was enriched between 40 and 53 minutes (time varied slightly for each sample) and the scented air was sucked through volatile traps for 2 min by a membrane pump with a flow rate of 200 mL/min. Volatile traps contained a mixture of 1.5 mg Tenax-TA (mesh 60/80; Supelco, Germany) and 1.5 mg Carbotrap B (mesh 20/40; Supelco, Germany). Volatiles were desorbed from traps using an automatic TD (thermal desorption) system (model TD-20; Shimadzu, Japan).

Samples were analyzed at the University of Salzburg by coupled gas chromatography (GC) and mass spectrometry (MS) (model QP2010 Ultra EI; Shimadzu, Japan). The GC was equipped with a 60 m long column (Zebron ZB-5; Newport Beach, USA) with an inner diameter of 0.25 mm and a film thickness of 0.25  $\mu$ m. The column flow of a helium carrier gas had a rate of 1.5 mL/min. GC oven temperature was kept constant at 40°C for 1 min, then increased by 6°C/min until a maximum temperature of 250°C was reached. The MS interface and ion source were set at 260°C and 200°C, respectively.

For identification of the scent compounds, GCMSsolutions Software Version 2.72 (Shimadzu Corporation) was used by comparing mass spectra with authentic standards, computer libraries (ADAMS, ESSENTIALOILS-23P, FFNSC 2, W9N11) and Kovats indices generated using n-alkanes.

### *Phenology of individual flowers*

Eight flowers in bud were selected at random among four different plants, tagged and monitored over the course of their lifespan at the NTBG Conservation and Horticulture Center, McBryde Garden between May 17 and May 25, 2011. Occurrence of male (pollen presentation)

and female (stigmatic) phases were monitored and noted each morning (between 7:30 and 10:30) and each evening (between 15:30 and 18:30) on tagged flowers. Flowers were considered transitioned from a male-phase to female-phase once the two rounded stigmatic lobes were fully expanded and reflexed partially. Flowers were considered no longer female when the stigma became flaccid and the corolla browned (senescence).

#### *Pollen/ovule ratio*

Fourteen flowers from six different plants (2–3 flowers per plant) at QKG in Honolulu were harvested while in bud, labelled with an assigned plant and flower number, and placed in air-tight preserving jars containing 70% isopropyl alcohol. These were later dissected to count the ovules. The ovaries of *B. insignis* flowers have two distinct locules. To expedite counting, ovaries were cut in half using a razor blade and ovules from just one of the two locules were removed using a dental pick, counted using a dissecting microscope, and then multiplied by two for a total count of ovules in the whole ovary.

Anthers from 12 of those same flowers were used to make pollen counts. Using a petri dish containing a thin layer of distilled water, anthers were held with tweezers and divided in two along their suture lines with a scalpel. Using a dental pick, pollen was removed from the anthers into the petri dish. Using a squeezable wash bottle filled with distilled water, the anthers and equipment were repeatedly rinsed into a pre-weighed beaker to collect the suspended pollen/liquid solution. The petri dish was rinsed with distilled water until all the pollen from an individual anther was collected in the beaker. Additional distilled water was added to the beaker until there was 50 mL of suspended pollen in distilled water. Total anther pollen was further diluted by adding 150 mL of 25% glycerol and 75% 0.2  $\mu\text{m}$  filtered seawater (electrolyte) solution and then weighed. Pollen from each flower was counted separately on the Beckman-Coulter Multisizer III Coulter Counter (Model 2000) located in the Department of Oceanography at UHM. A 400  $\mu\text{m}$  aperture on the machine was used and the standard operating method time was set at 30 seconds. A magnetic stirrer maintained the pollen in suspension.

This diluted pollen solution was then run on the instrument and checked to make sure the particle concentration was less than 10%. If it was not, the sample was diluted further with the

electrolyte solution and the volume used to dilute noted for a known total dilution volume. The sample was then weighed again, the solution analyzed with stirring for 30 sec, weighed again, and the difference between the first weight and second weight was the volume run. Weighing and running (analyzing) was repeated three times to give three replicates per sample.

The relevant size range of the three counts was used to obtain an average of the number of pollen grains counted in 30 seconds. The difference in weight between runs was used to estimate the volume run for each of those counts. For example, if an average of 100 mL were run in 30 seconds and an average of 1,000 pollen grains were counted in those 100 mL, that's 10 pollen grains per 1 mL. If total initial volume of solution with diluted pollen grains was 300 mL, then that is 3,000 pollen grains per anther.

### *Pollination treatments*

To assess if pollinators are necessary for fruit and seed production in *B. insignis*, manipulative pollination treatments were conducted on cultivated plants over the course of three years. In the summer of 2011, 15 cultivated *B. insignis* plants were purchased from the Maui Native Plant Nursery based in Kula, Maui and shipped to O'ahu in a refrigerated container. Pollination treatments were conducted with these plants that flowered in fall 2011 and fall 2012. In 2012, four additional plants were purchased from horticulturist Frani Okamoto (Leeward Community College (LCC), O'ahu) and three plants were borrowed from Dr. Dan Rubinoff (Department of Plant and Environmental Protection Sciences, UHM). All plants were kept on the fifth floor balcony of the St. John Plant Science building on the UHM campus for the duration of the study. In 2011, pollen donors for the outcross treatments were from plants growing at the QKG in Honolulu. These plants produced copious amounts of pollen. In 2012, the plants at QKG had died, so pollen for outcross treatments was from different sources (e.g., plants from Maui Native Nursery, LCC, and Dr. Rubinoff). Unfortunately, it is unknown whether the plants used for outcrossing were close relatives (siblings). Provenance data of specific populations from which source material of the cultivated plants came, with the exception of NTBG's accessions, is unavailable. In the summer and fall of 2013, 32 cultivated plants representing 10 different NTBG accessions were utilized for pollen manipulations at the Conservation and Horticulture Center at

the NTBGMcBryde Garden in Kalaheo, Kaua‘i. Outcross treatments with these plants were between different accessions.

Manipulative pollinations consisted of five treatments (Table 1.1). Bridal-veil bags (15 cm x 4 cm) held in place with zip ties were used to exclude potential pollinators in all treatments except the control treatment. Bags were left in place until flowers senesced. Flowers to which any treatments were applied were labeled with jewelry tags. Table 1.2 provides a breakdown of the number of flowers and plants used in each manipulative pollination treatment each season (2011, 2012, and 2013) and in all seasons combined.

Table 1.1. Name, purpose, and description of each manipulative pollination treatment.

<b>Treatment</b>	<b>Purpose</b>	<b>Description</b>
Control	To assess how plants reproduce naturally without any manipulation or bagging	Flowers randomly tagged and left unmanipulated
Self	To test if the plants are autogamous (autonomous self-pollination; self-compatible within a single flower without assistance)	Flowers randomly tagged, bagged while in bud, and left unmanipulated
Plus self pollen	To test if the plants are geitonogamous (self-compatible between different flowers of the same individual plant)	Flowers randomly tagged, bagged while in bud, and monitored until stigma became receptive. When stigma was receptive, anthers from a male-phase flower of same plant were removed, opened and gently dabbed on stigma. Flowers were rebagged
Plus outcross pollen	To assess pollen limitation	Flowers randomly tagged, bagged while in bud, and monitored until stigma became receptive. When stigma was receptive, anthers from male-phase flower of different plant were removed, opened and gently dabbed on stigma. Flowers were rebagged
Emasculation plus outcross pollen	To assess pollen limitation while reducing the risk of unwanted self-pollen transfer and fertilization	Flowers randomly tagged and bagged while in bud. When bud was just about to open or in early male-phase, stamens were gently removed with a razor blade and flowers were rebagged. When stigma was receptive, anthers from male-phase flower of different plant were removed, opened and gently dabbed on stigma. Flowers were rebagged

Table 1.2. Number of flowers and plants used in each manipulative pollination treatment each season (2011, 2012, and 2013) and in all seasons combined. To note, two plants that were used in the control and two used in the self treatments in 2012 were also used in 2011. One of the plants used in each of the other three treatments was used in both 2012 and 2011. All other plants used among all seasons were different individuals.

<b>Treatment</b>	<b># of flowers (plants) used in 2011</b>	<b># of flowers (plants) used in 2012</b>	<b># of flowers (plants) used in 2013</b>	<b>Total # of flowers (plants) used</b>
Control	23 (8)	21 (6)	51 (20)	95 (34)
Self	21 (7)	37 (9)	57 (23)	115 (39)
Plus self pollen	8 (4)	9 (3)	3 (2)	20 (9)
Plus outcross pollen	18 (6)	3 (3)	18 (13)	39 (22)
Emasculation plus outcross pollen	8 (5)	3 (2)	13 (10)	24 (17)

Any fruits that matured did so about one month after the pollination treatment was conducted. All mature fruits were collected, cut open using a razor blade and the seeds extracted using a dental pick. Seeds were counted, and all of them were weighed together to obtain an average weight per seed.

*Pollen viability*

The viability of pollen from a subset of plants used in the manipulative pollination treatments (outcross treatments and self-pollen supplementation treatments; see Table 1.3 for a breakdown of all accessions and plants used to test pollen viability) during the third flowering season (September 7 through December 2, 2013) was assessed using a stain method developed by Peterson et al. (2010), which is similar to Alexander’s stain (Alexander, 1969) but simplified and less toxic. However, samples were not fixed prior to staining and the stained pollen for this study was not heated, as suggested by Peterson et al. (2010). Trial stains were conducted both with and without heating and no distinguishable differences in the way the pollen stained were observed. Pollen was collected from flowers in late bud to male-phase, stored in small plastic vials, and stained within an hour of collecting. Anthers were cut in half and the pollen removed using a small scalpel. Pollen was placed on the center of a glass microscope slide, followed by two small drops of the stain. A coverslip was placed directly on top of the stained pollen and

sealed with clear nail polish. Pollen grains that stained magenta-red were considered viable (non-aborted) while those that stained blue-green were considered non-viable (aborted). Three-hundred pollen grains per slide, in three random views at 100x or 400x magnification, were scored as viable or non-viable (with one exception of a sample that contained 356 pollen grains total, in which case all pollen grains were counted) by viewing under a compound light microscope. The total number of viable pollen grains was divided by 300 (the total number of pollen grains counted), giving a percentage of pollen viability for each sample.

Table 1.3. Breakdown of all accessions and plants used to test pollen viability. Eighteen different individual plants representing nine NTBG accessions were used and 24 replicates total done to examine pollen viability of plants used in manipulative pollination treatments in 2013.

Accession # (NTBG accession #)	Plant # (replicates)
8 (100652)	1 (2), 65 (1)
3 (9900842)	3 (1), 4 (1)
9 (990836)	4 (2), 2 (1)
1 (120043)	11 (1), 3 (1), 12 (1)
4 (050389)	8 (5)
10 (990840)	3 (1), 2 (1), 1 (2)
5 (100651)	93 (1), 164 (1)
11 (no accession # on tag)	1 (1)
2 (990833)	6 (1), 4 (1)

#### *Data analysis*

Statistical analyses were undertaken in Minitab version 14. All means are presented  $\pm$  1SE. Two-sample t-tests were used when means were compared.

## **Results**

### *Floral measurements*

The average corolla tube length of *B. insignis* flowers was  $102.8 \pm 2.5$  mm excluding the corolla lobes and  $113.8 \pm 2.1$  mm including the corolla lobes. The average corolla tube width was  $5.3 \pm 0.1$  mm halfway down the length of the corolla tube from the outside,  $6.6 \pm 0.1$  mm at the top of the corolla tube just below the lobes from the outside and  $4.2 \pm 0.1$  mm at the top of the corolla tube looking down into it. Mean measurements of all floral characteristics and organs are listed in Table 1.4.

Table 1.4. Mean measurements (mm) of all floral characteristics and organs measured.

<b>Floral organ measurements</b>	<b><i>N</i></b>	<b>Mean (<math>\pm</math> 1SE)</b>
Ovary length	30	16 ( $\pm$ 0.5)
Ovary width (widest point)	30	8.3 ( $\pm$ 0.2)
Sepal length	30	1.1 ( $\pm$ 0.1)
Sepal width	30	1.7 ( $\pm$ 0.04)
Corolla tube length (excluding lobes)	30	102.8 ( $\pm$ 2.5)
Corolla tube length (including lobes)	30	113.8 ( $\pm$ 2.1)
Corolla tube width (halfway point outside)	30	5.3 ( $\pm$ 0.1)
Corolla tube width (apex below lobes outside)	30	6.6 ( $\pm$ 0.1)
Corolla tube width (apex inside)	30	4.2 ( $\pm$ 0.1)
Corolla face width	30	33.2 ( $\pm$ 1.8)
Corolla lobe length	148	21.2 ( $\pm$ 0.3)
Corolla lobe width	148	9.6 ( $\pm$ 0.1)
Pedicel length	30	32.4 ( $\pm$ 1.5)
Pedicel width	30	4.1 ( $\pm$ 0.1)
Anther length	17	13.2 ( $\pm$ 0.4)
Anther width	17	3.2 ( $\pm$ 0.1)
Stigma lobe length (fully expanded)	15	2.5 ( $\pm$ 0.1)
Stigma lobe width (fully expanded)	15	2.3 ( $\pm$ 0.1)
Stigma exertion above corolla lobes (where they attach)	15	4.6 ( $\pm$ 0.5)
Stigma exertion above anthers	15	9.7 ( $\pm$ 0.9)

#### *Floral organ color*

During the female phase, upper corolla color ranged from brilliant greenish yellow ( $n=6$ ) to light greenish yellow ( $n=2$ ). Flowers in the male phase possessed an upper corolla color ranging from brilliant greenish yellow ( $n=6$ ) to brilliant yellow ( $n=1$ ) and vivid greenish yellow ( $n=1$ ; see Figure 1.4). Table 1.5 indicates the most common color recorded for 10 floral organs and parts of organs in female-phase and male-phase flowers examined.

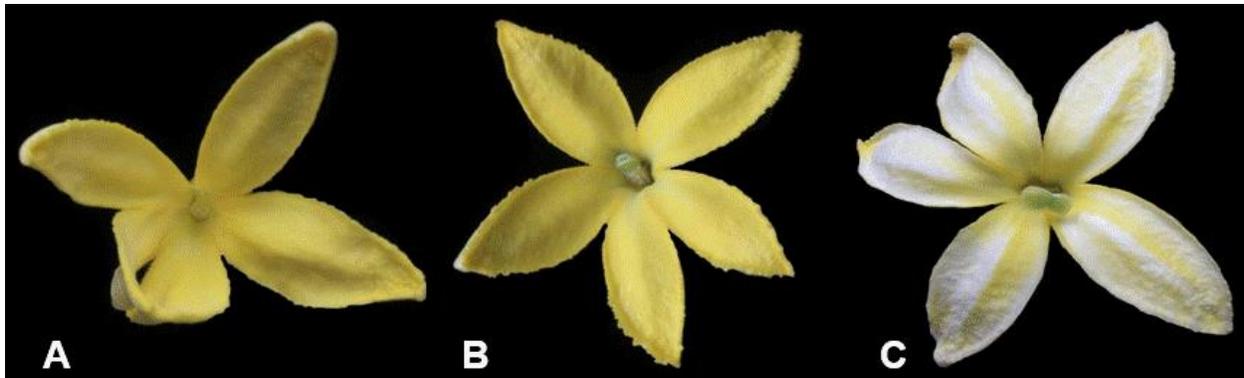


Figure 1.4. Flower coloration sequence in early male (A), inter-phase between male/female (B), and late female (C) phase flowers (photos follow flowering sequence). Flowers are brilliant greenish yellow to brilliant yellow when in the male-phase and early female-phase, fading to brilliant greenish yellow to light greenish yellow to eventually white in the late female-phase and as they senesce.

Table 1.5. Most common color (UCL) of selected floral organs and parts of organs in female-phase and male-phase flowers.

Floral organ	Female phase ( $n=8$ )	Male phase ( $n=8$ )
Pedicle	Light Yellowish Green	Light Yellowish Green
Ovary (outside)	Brilliant Yellowish Green	Brilliant Yellowish Green
Sepals (outer)	Strong Yellowish Green	Strong Yellowish Green
Corolla tube (outside)	Brilliant Yellowish Green	Strong Yellowish Green
Corolla lobes (upper)	Brilliant Greenish Yellow	Brilliant Greenish Yellow
Corolla lobes (lower)	Brilliant Yellowish Green	Light Greenish Yellow
Stigma	Brilliant Yellowish Green	N/A
Style	Light Yellowish Green	N/A
Anther	N/A	Pale Greenish Yellow to Pale Yellow
Anther hairs	N/A	Yellowish White

#### *Nectar standing crop*

The mean nectar standing crop of all flowers sampled, both female-phase and male-phase flowers combined, was  $94 \mu\text{L} \pm 15$ . Female-phase flowers contained significantly more nectar than male-phase flowers (female =  $137 \mu\text{L} \pm 12$ ; male =  $22 \mu\text{L} \pm 3$ ;  $t = 8.9$ ,  $df = 12$ ,  $P < 0.001$ ).

#### *Position of nectar in flowers*

Table 1.6 summarizes the distances between parts of select floral organs and the uppermost position of nectar within the corolla tube. The distance between the upper distribution

of nectar and anther apex in male-phase flowers was 54.5 mm while the distance between the uppermost position of nectar and stigma apex in female-phase flowers was 35 mm. There was more nectar in female-phase flowers (personal observation).

Table 1.6. Mean distance (mm) between anther apex, stigma apex, corolla opening, base of corolla and the upper distribution of nectar within the corolla tube.

	<b>Female phase (n=8)</b>	<b>Male phase (n=8)</b>
<b>Distance between nectar and anther apex</b>	26 ( $\pm$ 5)	54.5 ( $\pm$ 6)
<b>Distance between nectar and stigma apex</b>	35 ( $\pm$ 5)	N/A
<b>Distance between nectar and corolla opening</b>	32.5 ( $\pm$ 4.5)	63 ( $\pm$ 6)
<b>Distance between base of corolla and upper limit of nectar</b>	57.5 ( $\pm$ 4)	32.5 ( $\pm$ 1.5)

#### *Nectar sugar composition*

The mean percentage sucrose (refractometer measurements in % w/v) was 8.0% ( $\pm$  0.4). There was no significant difference ( $P = 0.188$ ) in sucrose percentage between female-phase and male-phase flowers. HPLC results showed a mean sucrose to hexose ratio (% sucrose)/(% fructose + % glucose) of 0.9. Nectar samples were rich in sucrose (46%  $\pm$  4) and fructose (44%  $\pm$  3), but contained only small amounts of glucose (10%  $\pm$  2).

#### *Floral scent*

Sixteen volatile organic compounds (VOCs) in total were found in the floral scent bouquet of *B. insignis*, 12 of which were identified by comparing their mass spectra and retention times with those of standard substances. Proportional emission rates during the day and at night of all volatiles detected are listed in Table 1.7. The two dominant volatiles were benzyl alcohol and linalool. Benzyl alcohol made up an average of 46% and 52% of the total volatile compounds identified in the floral scent samples during the day and at night, respectively. Linalool made up an average of 41% during the day and 33% at night of total emissions.

Table 1.7. Day and night proportional emission rates of all volatile organic compounds (VOCs) revealed in *B. insignis* floral scent analysis.

Family or Class of Compounds	Volatile organic compound	Proportion during day	Proportion at night
Aliphatics			
	Decanal	0.002	0
	1-Hexanol	0.020	0.015
	(Z)-3-Hexen-1-ol	0.015	0.009
	Z-3-Nonenol	0.003	0.006
Benzenoids			
	Benzyl alcohol	0.459	0.522
	Methyl salicylate	0.021	0.010
Monoterpenes			
	1,8-Cineole	0	0.005
	delta-3-Carene	0.003	0.001
	Heptyl butyrate	0	0.008
	Linalool	0.406	0.334
	(Z)-Linalool oxide furanoid	0.001	0.001
Sesquiterpenes			
	(E)-Nerolidol	0.029	0.019
Unknown			
	unknown1	0.003	0.004
	unknown3	0	0.002
	unkown2	0.026	0.025
	unkown4	0.011	0.037

#### *Phenology of individual flowers*

Flowers remained open between five and seven days (mean = 6 days  $\pm$  0.2). Flowers remained in the male-phase, on average, for 39 hours ( $\pm$  3.7) (ca. 1.6 days) and in the female-phase for 110 hours ( $\pm$  70) (ca. 4.6 days).

#### *Pollen/ovule ratio*

Per flower, the mean number of ovules was 963 ( $\pm$  37) and the mean number of pollen grains was 150,655 ( $\pm$  10,627). The mean pollen-ovule ratio was 161 ( $\pm$  12).

*Pollination treatments*

Combining results of the three flowering seasons during which the breeding system study was conducted (2011, 2012, 2013), most fruit and seed formation occurred in the two pollination treatments that utilized outcross pollen ('plus outcross pollen' and 'emasculatation plus outcross pollen'). All but one fruit in each of the other pollination treatments ('control', 'self', and 'plus self pollen') aborted shortly after the flowers senesced in all three years of treatments (Table 1.8). However, in 2012 and 2013, no mature fruits formed in either of the outcross treatments. Formation of the single mature fruit for the 'plus self pollen' treatment occurred in 2012 and one each for the 'control' and 'self' treatments occurred in 2013 (Table 1.8).

Table 1.8. Percent fruit formation in each pollination treatment by flowering season (year) and in all seasons combined.

<b>Treatment</b>	<b>Season 1 (2011)</b>	<b>Season 2 (2012)</b>	<b>Season 3 (2013)</b>	<b>All seasons combined</b>
Control	0/23 = 0%	0/21 = 0%	1/51 = 2%	1/95 = 1%
Self	0/21 = 0%	0/37 = 0%	1/57 = 2%	1/115 = 1%
Plus self pollen	0/8 = 0%	1/9 = 11%	0/3 = 0%	1/20 = 5%
Plus outcross pollen	13/18 = 72%	0/3 = 0%	0/18 = 0%	13/39 = 33%
Emasculatation plus outcross pollen	6/8 = 75%	0/3 = 0%	0/13 = 0%	6/24 = 25%

There were also differences observed in the mean number of seeds formed per fruit among the pollen manipulation treatments (Table 1.9). However, statistical analyses to examine the significance of these differences were not done because only a single fruit formed in three of the treatments.

Table 1.9. Mean seed formation in fruits of each pollen manipulation treatment.

<b>Treatment</b>	<b><i>n</i></b>	<b>Seed count (mean ± 1SE)</b>
Control	1	142
Self	1	41
Plus self pollen	1	53
Plus outcross pollen	13	326 (± 32)
Emasculatation plus outcross pollen	6	454 (± 73)

*Pollen viability*

Average *B. insignis* pollen viability in 2013 was 4.7%. Table 1.10 shows the percent viability for each flower examined. The non-viable pollen grains not only stained blue-green, an indication of non-viability, but also appeared shriveled and smaller compared to those that stained pink and hence, were considered viable. The amount of pollen within the anthers was noted to be substantially higher ('lots of pollen') in the *B. insignis* individual with the highest percent viability (74%) compared to all others, which were noted to have 'barely any', 'very little', 'a little' or 'some' pollen (in order of increasing amount of pollen). Pollen from a single flower of a *B. rockii* individual was also examined. The anthers contained copious amounts of pollen and the viability was 86.3%.

Table 1.10. Percent pollen viability of all NTBG *B. insignis* accessions and plants examined.

Accession # (NTBG #)	Plant #	# viable pollen grains	# non-viable pollen grains	Percent viable
1 (120043)	3	9	347	3.0%
1 (120043)	11	0	300	0.0%
1 (120043)	12	0	300	0.0%
2 (990833)	4	0	300	0.0%
2 (990833)	6	0	300	0.0%
3 (9900842)	3	0	300	0.0%
3 (9900842)	4	1	299	0.3%
4 (050389)	8	0	300	0.0%
4 (050389)	8	0	300	0.0%
4 (050389)	8	22	278	7.3%
4 (050389)	8	78	222	26.0%
4 (050389)	8	19	281	6.3%
5 (100651)	93	0	300	0.0%
5 (100651)	164	1	299	0.3%
8 (100652)	1	0	300	0.0%
8 (100652)	1	0	300	0.0%
8 (100652)	65	0	300	0.0%
9 (990836)	2	2	298	0.7%
9 (990836)	4	0	300	0.0%
9 (990836)	4	0	300	0.0%
10 (990840)	1	0	300	0.0%

10 (990840)	1	0	300	0.0%
10 (990840)	2	223	77	74.3%
10 (990840)	3	0	300	0.0%
11 (no info on tag)	1	0	300	0.0%

## Discussion

Traditional views of differences in flower shape are that they reflect adaptations to different groups of pollinators (Faegri and van der Pijl, 1979). Tube-shaped flowers, like those of *B. insignis*, are considered to be adapted to hovering or perching (if adjacent structures are present) behaviors because a floral visitor would not be able to enter the tube to reach nectar. Rather, the visitor would need to possess a long proboscis (e.g., Lepidoptera) or long, narrow beak (some birds) (Faegri and van der Pijl, 1979).

The likely adaptation of *Brighamia* to moth pollination is exemplified in one way by the drastic differences in flower morphology (e.g., flower shape and corolla tube length) compared to the other lobeliad genera, which have shorter, curved corollas adapted for bird pollination. The time required for pollinator-mediated selection of floral traits, such as spur length, is thought to be relatively short (Whittall and Hodges, 2007). The single, initial colonist of the Hawaiian lobeliads is thought to have arrived in the Hawaiian island chain around  $13.6 \pm 3.11$  million years ago (mean  $\pm$  SD), with all current genera having diverged within  $3.39 \pm 0.48$  million years of initial colonization (Givnish et al., 2009).

The upper corolla (inner surface) color of *B. insignis* flowers, when initially opening, is bright greenish yellow to brilliant yellow, eventually fading to white during senescence (Figure 1.4). Upper corolla color is one of several floral traits that, in combination with other traits, is used to infer the pollination syndrome of a plant species. Plant species having a nocturnal moth pollination syndrome typically possess corollas that are white because it's easier to perceive, compared to darker colors, under low light conditions (Herrera and Pellmyr, 2002). Interestingly, the closely related *B. rockii* possess corollas that are white and subsequently fade to pale yellow with age, the opposite sequence to *B. insignis* (Richard Pender, personal communication). The separate species may have evolved due to selection pressures from specialized pollinators, each of whom favored the different flower colors we see in *B. insignis* and *B. rockii* corollas.

Alternatively, differences in the order of changes in flower color between these two closely related sister taxa could be due to genetic drift or pleiotropy (the influence of one gene on multiple phenotypic traits) (Rausher, 2008; Brothers and Atwell, 2014).

Nectar volumes have been found to differ significantly among pollination syndromes (Wolff, 2006 with the exception of ornithophilous versus sphingophilous pollinated species examined). The mean nectar standing crop of *B. insignis* flowers examined in this study (both male-phase and female-phase combined) was 94  $\mu\text{L}$  ( $\pm 15$ ), higher than the ranges found in studies of other moth-pollinated plant species (Perret et al., 2001; Wolff, 2006). The average single day nectar standing crop in covered flowers of the eight different Sphingidae-pollinated plant species examined by Wolff (2006) was 48  $\mu\text{L}$  ( $\pm 1\text{SE}$ : 12; range  $5.4 \pm 1.7$  to  $102.9 \pm 42.1$ ). Nectar standing crop of the single moth-pollinated species Perret et al. (2001) examined was 20  $\mu\text{L}$ . Interestingly, the nectar standing crop of species belonging to another Hawaiian lobeliad genus (*Clermontia*) was high (Pender et al., 2014) as well in comparison to species examined in Wolff's study (2006) with the same pollination syndrome (bird). The mean volume of nectar per flower among species of *Clermontia* was 177.8  $\mu\text{L}$  ( $\pm 1\text{SD}$ : 112) whereas the average nectar standing crop of the 13 different ornithophilous plant species examined by Wolff (2006) was 21  $\mu\text{L}$  ( $\pm 1\text{SE}$ : 5; range  $1.6 \pm 0.6$  to  $51.9 \pm 7.1$ ).

Female-phase *B. insignis* flowers contained significantly more nectar than male-phase flowers. This result may be interpreted in two ways: 1) either female-phase flowers produce greater quantities of nectar when compared to male-phase flowers or, 2) nectar simply accumulated with increased time, as flowers begin as males and mature to females. To conclusively show if nectar production varies during the life of the flower, sampling of nectar would need to be conducted consecutively during the course of the flower's life. However, because the corolla tube is fused to the stamen filaments for much of its length, nectar could not be accessed without damaging the flower, making repeated nectar sampling over the life of the flower very difficult. Because there is a larger volume of nectar in female-phase flowers, nectar in the corolla tube is closer to the anther and stigma in female-phase flowers than in male-phase flowers (Table 1.6). If nectar is produced in larger quantities during the female phase, such a trait may have evolved to enable the pollinator (or a wider range of pollinators) to forage with greater

ease, increasing the chances that pollen may be deposited on a receptive stigma. However, without further investigation of the rates of nectar secretion in *B. insignis* flowers, the underlying mechanisms cannot be fully identified.

The position of nectar within the corolla tube has important implications for the ability of an organism to successfully forage for nectar from *B. insignis* flowers and serve as an effective pollinator. Our results indicate that a hawk moth, for instance, would need a proboscis at least 54.5 mm long to access nectar in male-phase flowers and simultaneously come into contact with the stamens, and at least 35 mm long to access nectar in female-phase flowers and come into contact with the stigma (Table 1.6). Information on the proboscis lengths of hawk moth species present on Kaua'i is unavailable and unfortunately, measuring proboscis length is not something that can be easily and precisely measured from pinned specimens since they are dry and can no longer be uncoiled (Will Haines, personal communication, February 2015). Such information would be one indication as to whether any of the hawk moth species on the island would be able to forage for nectar from *B. insignis* flowers while simultaneously making contact with the reproductive organs. It is not uncommon elsewhere for hawk moths to have a proboscis as long as 35 or 54.5 mm. Proboscis lengths of hawk moth species that have been observed pollinating various North American plant species ranged from 23 to 138 mm (averaging 60 mm) and in Costa Rican communities, proboscis lengths ranged from 10 to 200 mm (averaging 49 mm) (Miller, 1997).

It could also be possible that a lower volume of nectar in male-phase flowers, therefore, a greater distance between the upper reaches of nectar (the reward) and anthers (pollen) was selected for. This could be resulting in the “tricking” of a putative moth pollinator with a shorter proboscis than what is required to reach nectar during the male-phase, to make unsuccessful attempts at reaching it (Cliff Morden, personal communication, March 2015). In trying to access nectar during the male-phase and failing, a moth would have picked-up pollen during the attempt but then likely, subsequently visit another flower still in search of nectar. The next flower visited would more likely be female, since flowers remain in a female-phase (110 hours  $\pm$  70) ca. 2.8 times longer than in the male-phase (39 hours  $\pm$  3.7). Since there is a greater volume of nectar during the female-phase (therefore, a shorter distance between the uppermost position of nectar

and the stigma), the putative moth pollinator would be rewarded with nectar while simultaneously coming into contact with the receptive stigma and depositing the pollen that had been picked-up from the previously visited male-phase flower. In addition, although a long corolla tube does not exclude the potential for insects to visit *B. insignis* flowers to collect pollen as a foraging resource, inadvertently pollinating stigmas in the process, such behaviors were not observed during 29.5 and 21.5 hours of diurnal and nocturnal floral visitor observations, respectively (Chapter 2).

The mean percentage sucrose (refractometer measurements in % w/v) was  $8\% \pm 0.4$ . HPLC results showed a mean sucrose to hexose ratio ( $\% \text{ sucrose} / (\% \text{ fructose} + \% \text{ glucose})$ ) of 0.9 ( $46\% \pm 4$  sucrose,  $44\% \pm 3$  fructose, and  $10\% \pm 2$  glucose). With a sucrose to hexose ratio between 0.5 and 0.999, the nectar sugar of composition of *B. insignis* is considered sucrose-rich (Baker and Baker, 1983). Higher proportions of hawk moth (Sphingidae) pollinated plant species have been found to be sucrose-dominated or sucrose-rich in contrast to hexose-rich or hexose-dominated species that are adapted to other groups of animal pollinators (Baker and Baker, 1983; Chalcoff et al., 2006). Floral nectar studies examining the sucrose to hexose ratio of other Hawaiian lobeliad genera (*Clermontia*, *Cyanea* and *Lobelia*) found them to be hexose-rich and hexose-dominated, further supporting that the other Hawaiian lobeliads are, in contrast, adapted to bird pollination (Lammers and Freeman, 1986; Pender et al., 2014).

Benzyl alcohol and linalool made up a large proportion of the volatile emissions of *B. insignis* flowers. Both of these VOCs have been described as emitted by flowers of plant species that are visited and pollinated by moths (Dobson, 2006; Jurgens et al., 2002; Riffell et al., 2012). However, both volatiles are also commonly emitted by a high diversity of plants, many of which are not visited by moths (Knudsen et al., 2006). Interestingly, a study that examined the floral scent of moth versus bee pollinated *Petunia* species found that the predominant VOCs of the moth-pollinated species included benzyl alcohol and methyl benzoate and that these two volatile compounds elicited the highest antennal responses of the moth, *Manduca sexta* (Hoballah et al., 2005).

Benzyl alcohol was present in the highest amounts at 46% and 52% of the total VOCs identified in the floral scent samples during the day and at night, respectively (Table 1.7).

Hoballah et al. (2005) found that VOC emission rates of the moth pollinated *Petunia* species were higher at night compared to the day and that the night-collected odors stimulated the antenna of *Manduca sexta* significantly more. In contrast, the bee-pollinated species they examined showed no difference in day and night VOC emission rates. The fact that benzyl alcohol was the most abundant VOC emitted by *B. insignis* flowers, and that the emission rate was higher at night compared to during the day, offers additional support to the hypothesis that *B. insignis* evolved with a moth as its primary pollinator. However, these day and night differences must be treated with caution as the sample size is small.

The presence of methyl salicylate is also noteworthy. A recent study examined the floral scent of four Hawaiian *Schiedea* species (Caryophyllaceae) which contained both linalool and methyl salicylate among other VOCs (Jurgens et al., 2012). *Schiedea kaalae* contained the highest proportions of linalool and methyl salicylate among the four species examined. Subsequent floral observations revealed that *S. kaalae* was being visited by moths (an unidentified species of microlepidoptera) at dusk in a common garden on O‘ahu (Weisenberger et al., 2014). Moths were not observed visiting during the day and no other visitors were observed landing on open flowers. In addition, a pollination biology study of another closely related Hawaiian *Schiedea* species found that the most frequent floral visitor was a Pyralid moth (Norman et al., 1997).

Our floral scent bouquet of *B. insignis* differs from that of Kaiser’s (2010) analysis in that he found the VOC with the highest emission rate to be (E)-nerolidol (41%). In Kaiser’s analysis, linalool was the VOC with the fourth highest emission rate at 3% whereas our analysis showed it to have the second highest emission rate (Table 1.7). Our analyses are similar in that three of our four VOCs with the highest emission rates were the same ((E)-nerolidol, benzyl alcohol, and linalool).

Formally quantifying flower phenology of *B. insignis* and examining the breeding system by conducting pollen manipulation treatments suggested that the plant is primarily outcrossing. *Brighamia insignis* flowers remain open for just over 6 days, spending a shorter period of time in the male phase (ca. 1.6 days) than in the female phase (ca. 4.6 days). Besides exhibiting dichogamy, or a temporal separation of stamen and stigma maturation, there was also a mean

physical separation of stamens and receptive stigma (i.e., herkogamy) of 9.7 mm ( $\pm$  0.9). Both of these strategies (dichogamy and herkogamy) are thought to be evolutionary adaptations to promote outcrossing and limit selfing (Affre and Thompson, 1998; Dart et al., 2012). One or both of these strategies are present in nearly all outcrossing angiosperm species (Webb and Lloyd, 1986). Although pollen is shed primarily during the male-phase, it is possible that some pollen could cling to anther hairs below the receptive stigma during the female-phase when the stigma lobes recurve. Hence, the spatial separation of anther and receptive stigma, although not drastic, may further reduce autonomous self-pollination in *B. insignis* flowers.

A longer period of time of *B. insignis* flowers in the female-phase could have evolved to increase the chances of successful pollination of the stigma and fertilization of the ovules, which relates to what was described above about the potential “tricking” of putative moth pollinators during the male-phase. It is also possible that the long period of time in the female-phase is an adaptation for the pollen tube to reach and fertilize the ovules of these flowers that have a long corolla tube. Of flowering plant species examined, pollen tube growth rates are quite variable, ranging (*in vitro*) from 0.06 to 20 mm per hour (Tangmitcharoen and Owens, 1997). However, the pollen tube growth rate of *B. insignis* is unknown.

Pollen to ovule ratio (P:O) results, based on Cruden’s (1977) predictions (cleistogamy,  $4.7 \pm 0.7$ ; obligate autogamy,  $27.7 \pm 3.1$ ; facultative autogamy,  $168.5 \pm 22.1$ ; facultative xenogamy,  $796.6 \pm 87.7$ ; xenogamy,  $5859.2 \pm 936.5$ ), suggest that *B. insignis* should be facultatively autogamous (mean ratio = 161). However, expectations of plant breeding systems based on P:O values given by Cruden (1977) have been shown to be frequently inconsistent (Michalski and Durka, 2009). Michalski and Durka (2009) suggest that other factors (e.g., pollination mode, life form), not just breeding system, as Cruden (1977) suggests, may play important roles in the P:O of a plant species. Furthermore, breeding system designations based off P:O ratios made by Cruden (1977) result from only a relatively small number of diverse species (cleistogamy, N = 6; obligate autogamy, N = 7; facultative autogamy, N = 20; facultative xenogamy, N = 38; xenogamy, N = 25). Species with different evolutionary histories might have very different P:O ratios while possessing the same type of breeding system (Preston, 1986). To note, the *B. insignis* flowers used to measure P:O ratios ended up containing markedly more

pollen than most of the other plants with which I worked during this synthesis of different studies, including the pollen manipulations. If those flowers that were producing only very little pollen were used for the P:O measurements, expected results based on Cruden's (1977) predictions may have suggested an even stricter autogamous breeding system; however this would directly contradict findings from the pollen manipulation studies, where fruit only rarely formed from self-pollination and those fruits contained fewer seeds than cross-pollinated fruits. It is also possible that ovule numbers of flowers producing low amounts of pollen are lower in relation, but pollen and ovule counts with those flowers were not done here.

Lloyd and Schoen's (1992) literature review of self-compatible species revealed that self-incompatible species, or those with "low Autofertility Indices", were concentrated in a few families, which included the Campanulaceae. However, these did not include any Hawaiian Campanulaceae. They also found that all species exhibiting dichogamy, which *B. insignis* does, had low auto fertility; this included species of *Lobelia* (Lloyd and Schoen, 1992), an unknown species of which was likely the founder for the Hawaiian lobeliad group (Givnish et al., 2009).

Results of the pollination treatments are difficult to interpret. Fruit and seed formation only occurred in the two outcross treatments in 2011 (72% in the 'plus outcross pollen and 75% in the emasculation plus outcross pollen). Then in 2012 and 2013, no fruits formed in either of the outcross treatments. Formation of the single mature fruit for the 'plus self pollen' treatment occurred in 2012 and one each for the 'control' and 'self' treatments occurred in 2013 (Tables 1.8 on fruit formation and Table 1.9 on seed formation). Home and commercial growers with cultivated plants have indicated that fruit and viable seed have formed when they have applied self pollen, and that plants have been grown up from such seed (Hank Oppenheimer, personal communication, August 2012; Robin Schaap, personal communication, February 2013). In addition, I was able to confirm viability of seeds from the single fruit that formed in the 'plus self pollen' treatment. Seeds were germinated on an agar media in growth chambers at the Seed Conservation Laboratory, part of the Hawaiian Rare Plant Program at Lyon Arboretum. However, there was 0% germination of seeds from the single fruit that formed in the 'self' treatment. In any case, propagation from self-pollination is possible, although fruits from outcrossing in the pollination treatments that were conducted here produced more than double

the number of seeds (Table 1.9). Lack of fruit set after applied outcross treatments in 2012 and 2013 may be the result of low production of predominantly non-viable pollen in the cultivated plants that were used those years.

Most of the cultivated plants worked with at UHM in 2011 and 2012, and the vast majority of the 32 cultivated plants used at NTBG on Kaua‘i in 2013, produced very little pollen. Interestingly, I observed individual flowers on the same plant produce more pollen than other flowers on occasion. Others have noted the same on plants they have worked with (Lesley Randall, personal communication, January 2014; Lynn Janik, personal communication, March 2015). In addition, pollen viability of the plants used in 2013 for pollen manipulations was low (average 4.7%). These factors could have affected the ability of the plants to form fruits and seeds in all of the treatments. Interestingly, the single fruit formations that each occurred in the ‘self’ and ‘control’ treatments in 2013 occurred on the individual plant that was found to be producing some viable pollen (NTBG accession #050389, plant #4 behind the Botanical Research Center). Three out of the five flowers that were examined for pollen viability from this particular plant contained viable pollen grains. Average pollen viability for the plant (from all five flowers combined) was 7.9% (range: 0% to 26%).

Fruits were produced abundantly from outcrossing in 2011, suggesting that pollen viability was likely high in those plants that were used as pollen donors that year. Since the plants used as pollen donors were outplanted in a public, high traffic area (QKG), pollen manipulation treatments had not been applied to those plants; they were used only as pollen donors. If treatments had been applied, it is possible that fruits would have formed for the self treatments, since those plants were producing copious amounts of seemingly viable pollen.

There are at least three possible reasons why the vast majority of all other plants utilized in this study were producing low amounts of pollen (Curt Daehler, personal communication, November 2013). First, low pollen production could have been due to an environmental response (e.g. changes in lighting, temperature, rainfall, relative humidity, nutrient availability) (Lau and Stephenson, 1993; Astiz and Hernandez, 2013; Mercuri et al., 2013; Donders et al., 2014). Second, some plants have been observed to tend toward female function (Gemmill et al., 1998). Third, inbreeding might reduce pollen production (Hayes et al., 2005). The plants have been

severely inbred, with most of those currently in cultivation originating from the Waiahuakua population. The reason for low pollen production observed in cultivated *B. insignis* plants used for pollination treatments remains unknown, but could be related to a combination of the three factors mentioned above.

Steve Perlman worked with natural populations of *B. insignis* in the 70's, 80's and 90's, and he estimated that only about one-third of the plants produced good (copious and viable) pollen (Steve Perlman, personal communication, September 2012). Observations by Gemmill et al. (1998) indicated that ca. 5% of flowers in natural populations and garden collections of the genus *Brighamia* produced pollen. This and other observations of reproductive biology led them to suggest that *Brighamia* may be either gynomonocious or monoecious (Gemmill et al., 1998).

This study utilized cultivated *ex situ* *Brighamia insignis* plants to provide observational and experimental data on reproductive biology in order to contribute to the management of this extremely rare taxon. The combination of floral traits examined here, namely corolla tube length and width, corolla color, nectar standing crop, distance between reproductive organs (stamens and stigma) and the upper reaches of nectar in the corolla tube, nectar sugar composition, and floral scent, provide support for the hypothesis that *B. insignis* is adapted to a hawk moth pollinator. Whether there would have been a single or multiple native hawk moth pollinator(s) is unknown. It would be interesting to experimentally assess whether any of the three native or seven non-native Sphingidae reported to occur on Kaua'i would visit and serve as effective pollinators of *B. insignis*. However, no moth pollinator has ever been observed visiting *B. insignis* flowers (Chapter 2).

Flower phenology, the fact that *B. insignis* is dichogamous and herkogamous (spatial separation of anthers and receptive stigma), and overall results of pollen manipulations, suggest a primarily xenogamous breeding system. However, results of the pollen manipulations are difficult to confidently interpret. All of this, coupled with the fact that even human-assisted hand pollination has often been unsuccessful at producing fruit and viable seed in recent years (Ashly Trask, personal communication, January 2015 and this study), demonstrates that *B. insignis* faces severe challenges in capacity to reproduce even with intense human intervention. Chapter 4 synthesizes the results of this chapter and others with detailed step-by-step conservation

management recommendations that may be necessary if it is decided by the plant conservation community to work towards restoring the species *in situ*.

## CHAPTER 2

### *EX-SITU* POLLINATION ECOLOGY OF *BRIGHAMIA INSIGNIS* A. GRAY (CAMPANULACEAE)

#### **Abstract**

Research on the pollination biology of species of conservation importance is needed to facilitate conservation and restoration efforts. The Kaua‘i endemic lobeliad, *Brighamia insignis* A. Gray, is effectively extinct in the wild. One possible reason for its decline is the speculated loss of its native pollinator, possibly a moth. To determine whether restoration outplantings of *B. insignis* are being pollinated, diurnal and nocturnal floral visitor observations were conducted in the fall of 2013 and 2014 at the National Tropical Botanical Garden Limahuli Garden and Preserve on Kaua‘i. Visitation to *B. insignis* flowers was rare. Across days, an average of  $2.5 \pm 1.7$  SE % of the plants were visited per hour during the day and  $5.0 \pm 3.7$  SE % per hour at night. The average percent of available flowers that were visited and resulted in floral reproductive organ contact was  $1.1 \pm 0.7$  SE % per hour during the day and  $1.2 \pm 0.7$  SE % per hour at night. No moth visitors were observed. It appears unlikely that anything is effectively pollinating *B. insignis* outplantings at Limahuli Garden, and no fruits were observed, raising the question of whether populations can be self-sustaining. The loss of pollinators for *B. insignis* and other rare Hawaiian plant species could hinder restoration efforts and warrants further research in order to formulate the best management decisions on a species-by-species basis.

#### **Introduction**

In recent years, a suspected decline in pollinators worldwide has been referred to as a “pollination crisis”. This has prompted increased interest in understanding the roles played by—and the importance—of pollinators (Pauw, 2007). Since many agricultural crops and natural plant populations depend on insect pollinators for reproduction, loss of pollinating insects is a matter of public concern (Biesmeijer et al., 2006). From the plant's perspective, the decline of a pollinator could lead to pollen limitation, leading to lowered reproductive output and lowered recruitment that can be exacerbated by Allee effects (Wilcock and Neiland, 2002; Knight, 2003;

Knight et al., 2005). The Allee effect refers to the strong positive relationship between mean individual fitness and population density. In other words, if a population is small to begin with, reproductive output and hence population growth, could be either slowed down (but not becoming negative) if there is a “weak” Allee effect or halted completely and enter a negative growth rate if there is a “strong” Allee effect (Deredec and Curchamp, 2007). In Hawai‘i, insects make up the vast majority of the native fauna, with close to 5,500 described species at last count and nearly all of them being endemic (Nishida, 2002; Medeiros et al., 2013). They provide important ecological services, including pollination services, to an estimated 67% of endemic Hawaiian flowering plant species (Sakai et al., 2002; Medeiros et al., 2013).

Floras of isolated islands are particularly vulnerable to pollinator mutualism decline and disruption. Such ecosystems typically have high levels of endemism, limited population distributions, lower animal to plant species ratios (Olsen and Jordano, 2002), and are particularly vulnerable to invasion by alien species (Denslow et al., 2009) which may displace, compete with, or cause the extinction of plant-pollinator mutualists (Kaiser-Bunbury et al., 2010). The Hawaiian archipelago exemplifies these scenarios. The native angiosperm flora, composed of over 1,000 species, has lost many species from key pollinator guilds, particularly birds. Fifty-two native Hawaiian bird species that were present at the time of European contact are now extinct (Cox and Elmqvist, 2000). Many Hawaiian endemic insects have also gone extinct and many of those that are known and described are listed as endangered (Cox and Elmqvist, 2000). In actuality, only a small fraction of at-risk insect species are even recognized as at-risk. Funding for insect research and conservation is disproportionately lower compared to vertebrates and plants, and insects are largely ignored altogether in current conservation practices (Medeiros et al., 2013).

Problems faced by most of the hundreds of endangered plant species in Hawai‘i include a lack of knowledge of their pollination biology (Duffy and Kraus, 2008). Floral biology and field-based pollination ecology studies of Hawaiian endangered plants have only occasionally been undertaken (Norman et al., 1997; Drake and Morden, 2006; Pender et al., 2014; Krushelnycky, 2014; Weisenberger et al., 2014). Impacts of introduced pollinators, such as honeybees (*Apis*

*mellifera*) and Japanese white-eyes (*Zosterops japonicus*), are only beginning to be addressed (Aslan et al., 2014).

*Brighamia insignis* A. Gray is an endangered lobeliad (Campanulaceae) endemic to the island of Kauaʻi and historically to Niʻihau (Wagner et al., 1999). This unique species has fragrant, yellow to white flowers with salverform corollas that are about 114 mm long and produces sucrose-rich nectar (Chapter 1). This combination of floral characters is associated with a moth pollination syndrome (Lammers, 1989; Herrera and Pellmyr, 2002). A pollination syndrome can be defined as a specific suite of combined floral traits that together, attract and utilize a specific group of animals as pollinators (Fenster et al., 2004). The remaining Hawaiian lobeliad genera (*Clermontia*, *Cyanea*, *Delissea*, *Lobelia*, and *Trematolobelia*), in contrast, are believed to have evolved an ornithophilous (bird) pollination syndrome (Lammers and Freeman, 1986). The Hawaiian endemic nectarivorous passerines in the Drepanidinae and Mohoidae have been suggested as the native pollinators of these five lobeliad genera; the two groups (passerines and lobeliads) likely co-evolved (Lammers and Freeman, 1986). According to the U.S. Fish and Wildlife Service's Endangered Species Database (USFWS, 2015), 69 Hawaiian lobeliad species in the genera *Clermontia*, *Cyanea*, *Delissea*, *Lobelia*, and *Trematolobelia* are also endangered, threatened or listed as candidate species and 36 species have already gone extinct (Wagner et al., 2005), and one of the major causes is thought to be the loss of their pollinators (Drake and Morden, 2006).

Only a single naturally-occurring individual of *B. insignis* remains, on the Nā Pali Coast of Kauaʻi (Steve Perlman, personal communication, June 2013). Hence, the species is effectively extinct in the wild. Hurricanes, invasive plant and animal species (particularly goats that eat the plants and increase landslides in the natural cliff side habitat), and the suspected loss of its pollinator(s) (hawk moths based on floral biology; Lammers, 1989) are thought to be some of the main reasons for its decline. The National Tropical Botanical Garden (NTBG), in an effort to ameliorate this situation, has outplanted over a hundred *B. insignis* individuals at the Limahuli Garden and Preserve on the north shore of Kauaʻi. These plantings started in the living collection or lower garden portion in 1992 and in the Limahuli Lower Preserve in 2006 (Kava Vale, personal communication, January 2015). Outplantings were also done at the Kīlauea Point

National Wildlife Refuge on the northeast coast of Kaua‘i by the Department of Land and Natural Resources (DLNR) and volunteers in 1995 (Figure 2.1; Jennifer Waipa, personal communication, January 2015). The Kīlauea Point outplanting, however, was not self-sustaining and all of those planted have since died (Kim Uyehara, personal communication, April 2013). Furthermore, NTBG and other institutions locally, nationally, and even internationally, maintain cultivated accessions. At NTBG, staff have been hand pollinating plants to obtain more seed since fruit set has been rare and, when it has occurred, seed set was low. As these efforts have increased seed set, it appears likely that *B. insignis* may be pollen limited. Currently, however, even human-assisted pollination has been unsuccessful at producing viable seed (Ashly Trask, personal communication, January 2015).

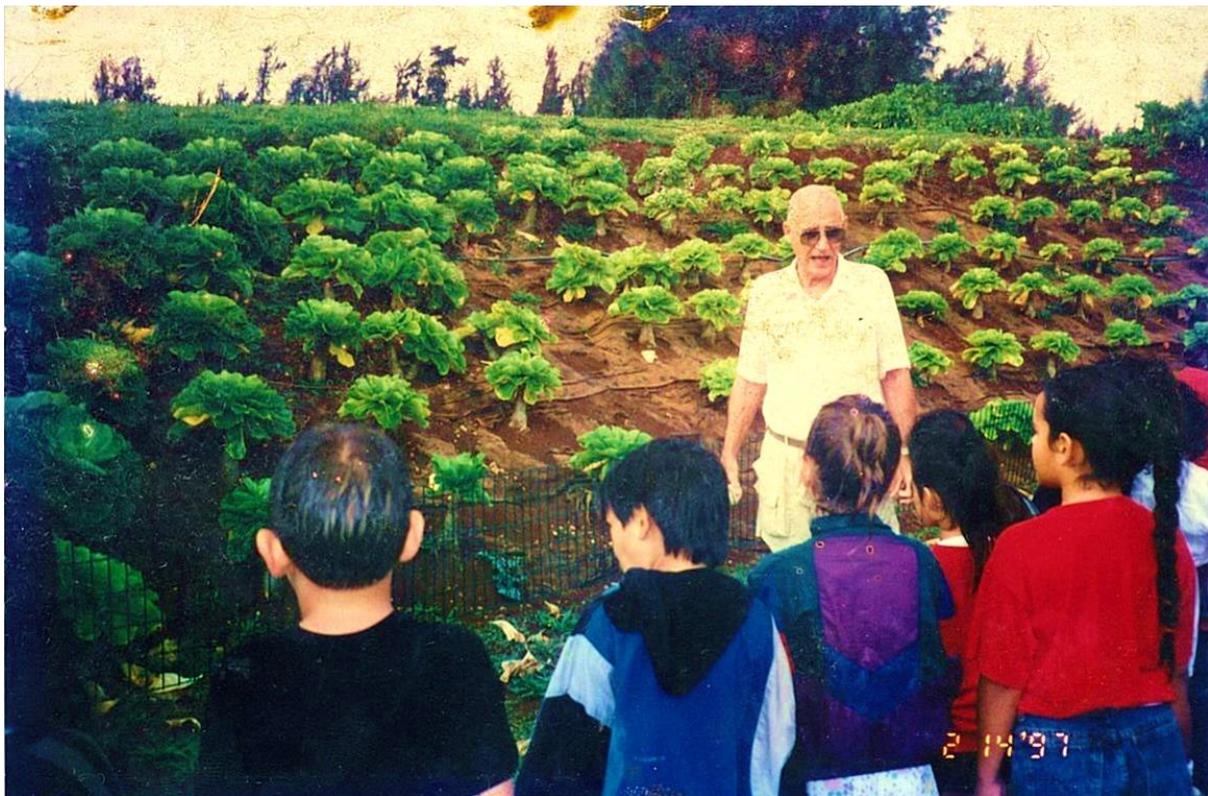


Figure 2.1. *Brighamia insignis* plants at Kīlauea Point National Wildlife Refuge in 1997, two years after outplanting. The last plant died around 2011 (Jennifer Waipa, personal communication, January 2015). Photo courtesy of U.S. Fish and Wildlife Service.

Despite the species being listed as federally endangered since 1994, no formal pollination study has been undertaken. The purpose of this study was to investigate whether outplanted individuals at the NTBG Limahuli Garden and Preserve are being visited and potentially pollinated by non-native and/or native animals. My prediction, based on no published records or even anecdotal observations of anything ever visiting *B. insignis* flowers, no signs of recruitment, and that the outplanting site is in a modified habitat (landscaped garden), was that there are no effective pollinators visiting outplanted individuals at Limahuli Garden today.

## **Methods**

### *Study site*

All potential pollinator visitation observations were undertaken with outplanted individuals at the NTBG Limahuli Garden and Preserve in Hā'ena, Kaua'i, Hawai'i. This area is situated very near to the species' natural historic range. Population GIS data show that the closest distance from Limahuli Valley to known former natural populations is approximately 2 kilometers (Ken Wood, personal communication, January 2015). Known historic distributions of *B. insignis*, through survey work done by Steve Perlman and Ken Wood at NTBG, and the outplanting site at Limahuli, are shown in Figure 2.2. They are also listed in Table 2.1, along with the elevation and annual rainfall data of those localities obtained from the "Hawaii statewide gis program rainfall Hawaii 1990" ArcGIS layer (Ben Nyberg and Ken Wood, personal communication, February 2015). According to this information, the outplanting site at Limahuli Valley, at an elevation between 26 and 84 m with an annual rainfall of about 1,980 mm, is situated in an area that does not fall into known elevation and rainfall ranges of the species' natural habitats. The outplanting site is slightly lower in elevation and receives slightly more rainfall in comparison.

Table 2.1. Locations of known historic natural populations of *B. insignis*, and the outplanted site at Limahuli, and their elevation and annual rainfall ranges.

<b>Locality</b>	<b>Elevation (m)</b>	<b>Rainfall (mm)</b>
Nā Pali (Ho‘olulu and Waiahuakua)	183 – 213	1,500 – 1,775 mm
Niumalu	122	1,400 – 1,500
Hā‘upu	480	1,400 – 1,500
Limahuli Valley	26 – 84	1,980

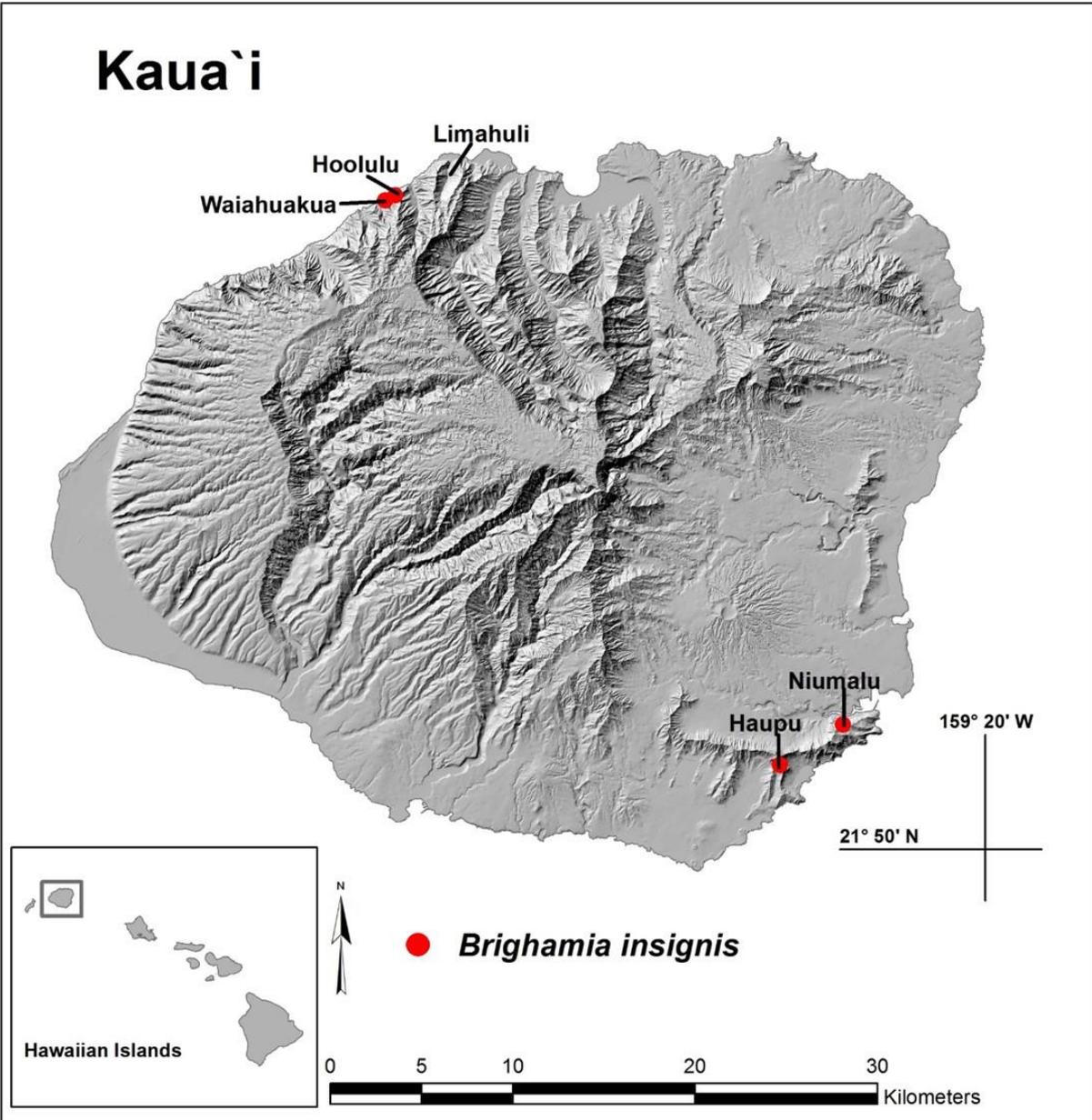


Figure 2.2. Known historic distributions of *B. insignis*. Outplanting site at the NTBG Limahuli Garden and Preserve is on the north side of the island, approximately 2 kilometers from the nearest known former natural population. Map courtesy of Ken Wood.

*Pollination study*

All observations of floral visitors were conducted in the fall of 2013 (18 plants) and 2014 (13 plants). Floral visitor observations took place over six days in 2013 (September 4 through 6

and October 4 through 6) and three days in 2014 (September 19 through 21). A Scientific Permit for Native Invertebrate Research and Collection effective May 15 through December 1 2013 was obtained from the Department of Land and Natural Resources, Division of Forestry and Wildlife (DLNR-DOFAW). In 2013, no endangered invertebrates were observed so the permit was not renewed in 2014 (I opted to photograph any visitors rather than collecting them).

Diurnal and nocturnal observations totaled 29.5 and 21.5 hours, respectively. Diurnal floral visitor observations took place between 6:50 and 19:20. Nocturnal observation hours started at civil dusk which, depending on the date, started as early as 18:45, and were conducted until as late as 23:55. At night, a headlamp with red light was used to monitor potential pollinators, following methods of recent studies that conducted nocturnal pollinator observations using either a light covered with red cellophane (Danieli-Silva and Varassin, 2013; Manning et al., 2012) or a red light (Miller et al., 2014). All flowers on an individual plant were monitored for visitors by standing or sitting within two meters of the focal plant during 15 minute long observation periods. Before the 15 minute observation period began, notes on the weather (e.g., cloud cover and wind) and the total number of male-phase and female-phase flowers on a plant, was recorded (Table 2.2). Flowers of *B. insignis*, like all of the Hawaiian Campanulaceae, are protandrous, which means they first pass through a male phase before going into a female phase. During the observation period, the type, frequency and fate of visit (landed on reproductive organ(s) versus other flower parts (e.g., corolla lobes or tube) versus other parts of plant (e.g., leaves)) of any organisms visiting *B. insignis* was recorded. The total duration of contact with a floral reproductive organ (i.e., stigma and/or tip of the stamens where the pollen is discharged) was timed using a stopwatch. Floral visitors were photographed with an Olympus Tough TG-1iHS 12 megapixel waterproof digital camera with optical zoom and later identified by professional entomologists through email communication and the Hawaiian Entomological Society Facebook page.

## **Results**

Visitation to *B. insignis* flowers was rare. During any single date of diurnal or nocturnal observations, between 0 and 10 percent of available flowers were visited by an organism that

came into contact with the stigma or stamens. Across days, the average percent of available flowers that were visited and resulted in floral reproductive organ contact was  $1.1 \pm 0.7$  SE % per hour during the day and  $1.2 \pm 0.7$  SE % per hour at night. An average of  $2.5 \pm 1.7$  SE % of the plants were visited per hour during the day and  $5.0 \pm 3.7$  SE % per hour at night (Table 2.2).

Table 2.2. Breakdown of the number of plants and flowers observed (male and female) and visited during all diurnal and nocturnal pollination observation days. Only visits that resulted in contact with floral reproductive organs (stigma and/or stamens) were counted here. Notes on the weather during all observation periods are also included.

Date	Hours of observations	# plants observed	# plants visited	# female (& male) flowers observed	# flowers visited	Weather
<b>Diurnal</b>						
Sept. 4 2013	1.75	4	1	7 (3)	1	17:00–19:00: 0% clouds; gentle breeze.
Sept. 5 2013	4.75	6	0	9 (2)	0	7:00–10:00: 20% clouds; no wind. 10am–13:30: 20% clouds; light breeze. 13:30–19:00: 80% clouds; light breeze.
Sept. 6 2013	3	6	0	9 (2)	0	8:30–11:00: 50% clouds; no wind. 11:00–12:00: 80% clouds; light breeze.
Oct. 4 2013	3.5	9	1	10 (8)	1	14:00–17:00: 30% clouds; moderate winds. 17:00–18:45: 30% clouds; no wind.
Oct. 5 2013	6.25	12	1	16 (18)	1	9:30–17:00: 30% clouds; light breeze. 17:00–18:15: 30% clouds; windy. 18:15–18:45: 30% clouds; no wind.
Oct. 6 2013	4.25	12	0	23 (16)	0	10:15–18:45: 100% clouds; light breeze; scattered drizzle.
Sept. 19 2014	0.5	2	0	1 (2)	0	18:30–19:00: 20% clouds; no wind.
Sept. 20 2014	5.5	13	2	38 (11)	2	6:45–9:30: 0% clouds; light breeze. 9:45–11:30: 50% clouds; light breeze. 17:00–19:00: 25–75% clouds; no wind to light breeze.
<b>Nocturnal</b>						
Sept. 4 2013	2.25	5	1	8 (2)	1	20:15–22:45: 0% clouds; light breeze.
Sept. 5 2013	2.5	6	0	9 (2)	0	19:30–22:45: 0% clouds; no wind.
Sept. 6 2013	2.5	6	0	10 (3)	0	19:15–22:00: 0% clouds; no wind.
Oct. 4 2013	2.5	6	0	6 (6)	0	18:45–22:15: 50% clouds; light breeze.
Oct. 5 2013	1	3	1	19 (11)	1	18:45–20:15: 30% clouds; light breeze.

Oct. 6 2013	2.25	6	0	9 (4)	0	18:45–21:15: 70% clouds; scattered showers.
Sept. 19 2014	4.25	10	1	38 (5)	2	19:00–24:00: 0% clouds; light breeze.
Sept. 20 2014	2.25	9	0	32 (4)	0	19:10–21:30: 0% clouds: no wind.
Sept. 21 2014	2	8	0	25 (7)	0	19:00–21:20: 0% clouds; no wind.

A total of six different invertebrate species came into contact with floral reproductive organs while visiting *B. insignis* flowers. Three of these visitors were non-native ants. One of the ant species was a diurnal visitor tentatively identified as *Ochetellus glaber* (Steven Montgomery, Bernice P. Bishop Museum, personal communication, January 2014). Two separate individuals of this species visited on two separate dates during the day. Another ant species was a nocturnal visitor tentatively identified as *Brachymyrmex obscurior* (William Haines, University of Hawai‘i Entomology, personal communication, October 2014). Two separate individuals of this species visited two different flowers on the same plant during the same observation period at night. The third ant species, a small black ant (Family Formicidae), visited a flower once during the day and once at night. Two of the other visitors were identified to the level of order. One was a fly (Order Diptera) and one was a thrips (Order Thysanoptera) (Mach Fukada, Hawai‘i Department of Agriculture, personal communication, January 2015). I was unable to obtain a photograph or capture one of the organisms, possibly a beetle (Order Coleoptera), which came into contact with floral reproductive organs. It was ca. 7 mm in length. All nine individual visitors were observed visiting just a single flower on a single occasion.

Five of the visits occurred during the day and four occurred at night. Four visits resulted in contact with stamens for an average of 6.3 seconds (range: 1–15 sec). However, the organisms did not appear to be purposefully seeking out pollen on the stamens, but rather inadvertently coming into contact with them. The other five visits resulted in contact with the receptive portion of the stigma for an average of 31.6 seconds (range: 1–120 sec). During the day, three out of the five visits resulted in contact with the stamens while three out of the four visits at night resulted in contact with the stigma.

All visitors to *B. insignis* plants that were photographed are displayed in Figure 2.3. All visitors to *B. insignis* plants, including those that did not contact reproductive organs nor were photographed, are listed in Table 2.3. All visitors to *B. insignis* plants were non-native based on identifications at the species level and best approximation of higher taxonomic level by Hawai'i-based professional entomologists.



Figure 2.3. All visitors to *B. insignis* plants that were photographed during an observation period: A) Spider (Family Theridiidae), B) *Brachymyrmex obscurior*, C) Click beetle (Family Elateridae), D) *Neoscona theisi*, E) *Bactrocera dorsalis*, F) Jumping spider (Family Salticidae), G) *Entomobrya sauteri*, H) *Musca sorbens*, I) *Gyponana germari*, J) Thrips (Order Thysanoptera), K) *Ochetellus glaber*, L) *Drosophila suzukii*, M) *Anolis carolinensis*, N) *Lepidodactylus lugubris*, O) *Aedes albopictus*, P) Spider (Order Araneae), Q) Fly (Order Diptera), R) *Apis mellifera*, S) *Melormenis basalis*, T) Small black ant (Family Formicidae).

Table 2.3. All visitors to *B. insignis* plants, separated by diurnal and nocturnal observations. Visits that resulted in contact with reproductive organs are listed first in each category.

Insect	Identifier	# of different dates observed visiting	Part(s) of plant landed upon	# of flowers observed visiting	Contacted reproductive organs?	Photo
<b>Diurnal</b>						
Large black ant ( <i>Ochetellus glaber</i> )	S. Montgomery, Bishop Museum, pers. comm., Jan. 2014	6	stamen, corolla	ca. 28	Yes	Yes
Small black ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	5	stamens, corolla	11	Yes	Yes
Fly (Order Diptera)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	3	stigma, bud	3	Yes	Yes
Thrips (Order Thysanoptera)	M. Fukada, Hawai'i Department of Agriculture, pers. comm., Jan. 2015	2	stamens, corolla	2	Yes	Yes
Medium brown ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Oct. 2013	1	corolla	2	No	No
Small brown ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2014	1	corolla	5	No	No
Small red ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	3	corolla	8	No	No
Carolina anole ( <i>Anolis carolinensis</i> )	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	1	leaves	0	No	Yes
European honey bee ( <i>Apis mellifera</i> )	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	2	corolla, bud	2	No	Yes
Jumping spider ca. 4 mm (Family Salticidae)	A. Ciccù, Hawaiian Entomological Society Facebook page, pers. comm., Oct. 2014	1	corolla	1	No	Yes
Lady beetle (Family Coccinellidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	1	bud	1	No	No

Leafhopper ( <i>Gyponana germari</i> )	K. Magnacca, University of Hawai'i Entomology, pers. comm., Oct. 2014	1	bud	1	No	Yes
Muscid fly ( <i>Musca sorbens</i> )	K. Magnacca, University of Hawai'i Entomology, pers. comm., Nov. 2014	1	bud	1	No	Yes
Spotted-wing drosophila ( <i>Drosophila suzukii</i> )	S. Montgomery, Bishop Museum, pers. comm., Jan. 2014	4	corolla, leaf	5	No	Yes
Springtail ( <i>Entomobrya sauteri</i> )	K. Magnacca, University of Hawai'i Entomology, pers. comm., Nov. 2014	1	corolla	1	No	Yes
Tephritid fruit fly ( <i>Bactrocera dorsalis</i> )	K. Magnacca, University of Hawai'i Entomology, pers. comm., Oct. 2014	2	bud, leaf	1	No	Yes
<b>Nocturnal</b>						
Large brown ant ( <i>Brachymyrmex obscurior</i> )	W. Haines, University of Hawai'i Entomology, pers. comm., Oct. 2014	1	stigma, corolla	2	Yes	Yes
Small black ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	1	stamen, corolla	1	Yes	Yes
Possibly beetle ca. 7 mm (Order Coleoptera)	S. Walsh, University of Hawai'i Botany, pers. obs., Oct. 2013	1	stigma	1	Yes	No
Large black ant ( <i>Ochetellus glaber</i> )	S. Montgomery, affiliation, pers. comm., Jan. 2014	5	corolla	8-10	No	Yes
Medium black ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2013	2	corolla	3	No	No
Small brown ant (Family Formicidae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2014	2	corolla	10	No	No
Click beetle (Family Elateridae)	K. Magnacca, University of Hawai'i Entomology, pers. comm., Oct. 2014	1	corolla	1	No	Yes
Common web- spider	W. Haines, University of Hawai'i	1	leaves	0	No	Yes

( <i>Neoscona theisi</i> )	Entomology, pers. comm., Oct. 2014					
Mourning gecko ( <i>Lepidodactylus lugubris</i> )	A. Miyamoto, Hawaiian Entomological Society Facebook page, pers. comm., Nov. 2014	2	leaves	0	No	Yes
Plant hopper ( <i>Melormenis basalis</i> )	K. Magnacca, University of Hawai'i Entomology, pers. comm., Jan. 2015	1	bud	1	No	Yes
Spider (Order Araneae)	S. Walsh, University of Hawai'i Botany, pers. obs., Sept. 2014	1	corolla	1	No	Yes
Spider ca. 4 mm (Family Theridiidae)	W. Haines, University of Hawai'i Entomology, pers. comm., Oct. 2014	1	corolla	1	No	Yes
Spotted-wing drosophila ( <i>Drosophila suzukii</i> )	S. Montgomery, Bishop Museum, pers. comm., Jan. 2014	2	corolla	3	No	Yes
Tiger mosquito ( <i>Aedes albopictus</i> )	S. Montgomery, Bishop Museum, pers. comm., Jan. 2014	1	corolla	1	No	Yes

## Discussion

Based on these observations, it appears unlikely that anything is effectively pollinating *B. insignis* outplantings at NTBG's Limahuli Garden and Preserve. Very minimal floral visitations were observed, all of which appeared inadvertent since none of the visitors were exhibiting any sort of foraging behavior (with the exception of ants crawling deep into the corolla tube to potentially reach nectar). At least eight out of the nine visits that resulted in floral reproductive organ contact also involved crawling over or landing on the corolla lobe(s) and/or tube and/or bud. I also did not observe mature fruit on any plants during visits. However, I did not formally examine and quantify this nor did I return to see if any fruits matured approximately a month after flowering ended, which is about how long it takes for a fruit to mature if it had been fertilized (personal observation).

Most of the plants were infested with introduced carmine spider mites (*Tetranychus cinnabarinus*), which could possibly deter potential floral visitors indirectly as they negatively affect the overall health of the plant. The mites were seen all over the plant, but predominantly the undersides of leaves and on the corollas. The leaves and flowers of plants most heavily infested were discolored, limp and seemingly had more rapid leaf and flower senescence compared to lightly infested or uninfected plants (personal observation; Figure 2.4). Carmine spider mites were listed as a major threat of *B. insignis* in the U.S. Fish and Wildlife Services Federal Register report (USWFS, 2003) and have been observed to cause leaf loss in both cultivated and wild individuals (CWCS 2015).

Although many honey bees were flying above and next to *B. insignis* flowers, there were no observations where they came into contact with floral reproductive organs. During one visit, a honey bee landed on the corolla and during another, a flower bud, but no attempts toward reproductive organs were made. The bees were frequently visiting ‘ilima (*Sida fallax*) flowers growing in close proximity and appeared to be foraging for pollen during these visits.



Figure 2.4. *Brighamia insignis* plants infested with carmine spider mites (*Tetranychus cinnabarinus*) had yellow leaves (left). Individual flowers heavily infested were limp and seemingly senescing early (right).

Lack of pollination (personal observation) coupled with the fact that human-assisted hand pollination has drastically increased fruit and seed set in the recent past (Ashly Trask, personal communication, January 2015; personal observation), suggests that the native pollinator for *B. insignis* may indeed be extremely rare or extinct and this may be one of the major reasons *B. insignis* is functionally extinct in the wild. This combination of factors has previously been suggested by professionals familiar with *B. insignis* (e.g., Steve Perlman and Ken Wood) and in formal reports (e.g., USFWS, 2007) as some of the major reasons for decline of the species.

Some others who work with *B. insignis* have observed fruit formation without human-assisted pollination. In one case, fruit formed when an abundance of ants had visited the flowers (Frani Okamoto, personal communication, September 2012). This case involved potted plants in a greenhouse at Leeward Community College (Pearl City, O‘ahu). Whether ants could serve as viable pollinators in nature remains to be seen and seems unlikely based on field observations here. On occasion, mature capsules containing viable seeds have been observed on outplanted *B. insignis* at Limahuli Garden without human-assisted pollination, but the circumstances that allowed this are unknown (Kawika Winter, personal communication, January 2015).

Successful reintroductions of rare plant species into, or near, their natural habitat is a common goal for endangered species management. Definitions and criteria of plant reintroduction success are variable since different practitioners have different definitions and criteria to evaluate success (Godefroid et al., 2011). Reintroductions can sometimes be a best first step to restore plant populations (Keir and Weisenberger, 2014). However, it is often difficult to even get to that “first step” because all threats at an outplanting site must be dealt with and likely, managed indefinitely. In the case of plant species that are functionally extinct in the wild, like *B. insignis*, reintroductions are the only *in situ* conservation management option. If all threats are managed in a potential, appropriate outplanting site, an important primary question to answer before reintroductions take place is whether or not the plant species will likely be effectively pollinated if it needs to be, in order to set fruit and viable seed. Furthermore, to define success here, not only should reintroduced populations be fruiting and setting viable seed, but new seedlings should be sprouting on their own and surviving to complete the cycle for future

generations, demonstrating that the population will spread and persist. There must be evidence of recruitment from seed to deem a population self-sustaining (Wilcock and Neiland, 2002).

Traditionally, biodiversity monitoring focuses on species numbers and population sizes, perhaps because such measurements are easier to quantify compared to mutualistic interactions (Kaiser-Bunbury et al., 2010). For plant species with breeding systems that make them more susceptible to Allee effects (e.g., such as a predominantly outcrossing species with reduced or lack of pollination services; Forsyth, 2003), successful management strategies may require the simultaneous management of their animal pollinator(s) (Knight, 2003) in addition to monitoring population size and structure of the plant. For example, one thing to consider with a plant's pollinator in mind is nearest flowering neighbor distances since this could affect reproductive success and hence, population stability of an outcrossing plant species (Carr et al., 1986; Forsyth, 2003; Krushelnycky, 2014). At Haleakalā National Park on Maui, lower seed set was found in individuals of *Argyroxiphium sandwicense* subsp. *macrocephalum* that were more isolated and very closely-spaced (Krushelnycky, 2014). One hypothesis for less seed set exhibited in the more isolated plants was a failure of the floral visitors (which were predominately *Hylaeus* bees) to transfer non-self pollen across such distances (Krushelnycky, 2014). Carr et al. (1986) also found that closely spaced *A. sandwicense* subsp. *macrocephalum* individuals produced a lower number of viable seeds, attributing it to cross-incompatibility since they were likely too closely related as siblings. Additional, general recommended actions for conserving threatened species *in situ* include (1) introducing cross-compatible genotypes to populations where only a single or very few genotypes exist and/or (2) establishing new populations consisting of several different genotypes (Spira, 2001; Wilcock and Neiland, 2002).

Another method for managers to consider in restoration planning for rare plant species that are pollinator limited is “rewilding”, which is the introduction of a species to replace the functional role (e.g., pollinator) of a species that has gone extinct (Kaiser-Bunbury et al., 2010). Currently on Kaua‘i, the Sulcata Tortoise (*Centrochelys sulcata*) from sub-Saharan Africa is being evaluated as a potential functional replacement for the once abundant native *moa nalo*, or giant flightless ducks and geese that were herbivores and seed dispersers driven to extinction soon after the first humans arrived to the Hawaiian Islands. In this case currently, however, the

Sulcata Tortoises are primarily being evaluated for their ability to control non-native invasive plant species at the Makauwahi Cave Reserve (Burney et al., 2012).

There are many factors to take into account when considering the reintroduction of a rare plant species *in situ*. This study was done with the intention that results would provide conservation managers with information that could help in deciding how to best manage *B. insignis* both *ex situ* and *in situ*. This is in line with one of the targets for Objective 1 of the Hawai'i Strategy for Plant Conservation, which includes increasing research on the pollination biology and limiting factors of species of conservation importance (SCI) to facilitate restoration efforts and inform conservation practice (Keir and Weisenberger, 2014). Chapter 4 synthesizes the results of this chapter and others with detailed step-by-step conservation management recommendations that may be necessary if it is decided by the plant conservation community to work towards restoring the species *in situ*.

CHAPTER 3  
*EX SITU* GENETIC DIVERSITY OF *BRIGHAMIA INSIGNIS* A. GRAY  
(CAMPANULACEAE)

**Abstract**

*Brighamia insignis* A. Gray, a lobeliad endemic to the island of Kaua‘i and historically to Ni‘ihau, is one of 358 federally listed endangered plant species in Hawai‘i. Only a single individual remains in the wild. For this and other extinct or nearly-extinct species, the only hope for survival comes from *ex situ* collections, which may be able to support eventual *in situ* conservation efforts. The National Tropical Botanical Garden (NTBG), and other institutions around the world, maintain cultivated accessions as living collections. The purpose of this study was to examine genetic diversity maintained within and among as many *B. insignis ex situ* collections as possible, to see if some collections maintain diversity underrepresented in the NTBG collection that could then be used in a potential future breeding program. Ninety individuals representing 48 accessions from 24 total *ex situ* collections in seven countries were included in this study. ISSR markers were used to assess genetic diversity. Our results indicate 1) there is a high level of variation among the collections, and 2) variation among NTBG accessions does not reflect the extent of this total variation. Specific collections were identified that could be best incorporated into a breeding program to potentially increase levels of genetic diversity. Since NTBG maintains the largest *ex situ* collection of *B. insignis* and it is on the island upon which a restoration project would take place, it is recommended that NTBG obtain seeds from plants held in three collections (Botanischer Garten Copenhagen, Chicago Botanic Garden, and Missouri Botanical Garden) to then grow and use as pollen donors in crosses with NTBG accessions. Plants in these collections grouped predominantly into an inferred genetic cluster least represented in NTBG’s collection and showed statistically significant levels of genetic differentiation compared to NTBG. By pooling resources, collaboration among institutions can lead to more efficient and effective *ex situ* conservation action.

## **Introduction**

### *The importance of genetic diversity*

Theory holds that higher genetic diversity within a species is advantageous because there is more variation upon which evolutionary forces can act (e.g., if a species needs to adapt to a changing environment). This is more important now than ever with rapid environmental changes due to continued habitat destruction and the global movement of non-native species, all of which are being, and will continue to be, compounded by global climate change (Jump et al., 2009). Engelhardt et al. (2014) found that population performance of *Vallisneria americana* was affected by three different, yet non-mutually exclusive, scales of genetic diversity (heterozygosity, at the individual scale; genotypic diversity, at the population scale; and local adaptation, at the regional scale) and that effect size of the different scales will depend on the population source and environmental conditions. Based on results of their study, some of their recommended guidelines to protect, maintain or restore managed populations include 1) avoiding low levels of heterozygosity (inbreeding) to maximize vigor of individuals, and 2) maximizing genotypic diversity to enhance reproductive potential (Engelhardt et al., 2014).

It has been recommended for restoration projects using rare taxa to first evaluate genetic variation using molecular methods and then to use the maximum possible number of sources of material for restoration (Gorbunov et al., 2008). By knowing the genetic diversity remaining in a threatened or endangered species, conservation managers can hopefully conserve or even augment that diversity through their management plans and practices, and, through the use of pedigrees, minimize inbreeding within collections. Understanding diversity is vital for establishing populations with higher chances of long-term success (Maschinski and Haskins, 2012). It is therefore important to have an understanding of the genetic variability of restoration propagules before, not after, restoration outplantings take place (Fant et al., 2008; Maschinski and Haskins, 2012). Results of a multivariate analysis found that using material originating from multiple populations increased success of plant reintroductions (Godefroid et al., 2011). They also found higher survival rates of target species in restoration efforts that incorporated genetic diversity into the project design (Godefroid et al., 2011).

The importance of incorporating genetic diversity into rare plant reintroductions is likely the consequence of efforts to avoid two processes, inbreeding depression and genetic drift, which reduce fitness and future adaptability of smaller plant populations (Reed and Frankham, 2003). Genetic drift is of special concern in restoration because of the potential loss of genetic diversity and changes in allele frequency through the establishment of a new population with only a small number of individuals (“founder effect”; Lesica and Allendorf, 1999). *Ex situ* collections are sometimes founded from a small number of individuals that do not adequately represent the genetic variation of source populations, resulting in low diversity within the *ex situ* populations (Rucińska and Puchalski, 2011). Inbreeding depression happens when there is a reduction in fitness due to mating among close relatives (Keller and Waller, 2002). In small populations or populations with few founders, the likelihood of mating between close relatives increases. This in turn increases the likelihood of inbreeding, and therefore inbreeding depression, in these populations. A study that compared success of transplanted outbred versus inbred individuals of a rare *Silene* species found higher survivorship in the outcrossed progeny versus the open- and self-pollinated progeny (Kephart, 2004). The greater success of the outbred individuals (mortality was 17% in the outbred versus 26% in the inbred transplanted progeny) was likely due to overcoming negative impacts of inbreeding. Results of studies such as this have direct implications for planning outplantings of rare species with a greater likelihood of success.

However, genetic diversity in a reintroduction is not a panacea. This is because the reintroduction may experience outbreeding depression if plants included are too genetically different. Outbreeding depression occurs when there is a reduction in fitness due to mating among individuals that are too distantly related, caused by one of two mechanisms: 1) extrinsic (environmental), where locally adapted alleles are diluted, or 2) intrinsic (genetic), due to the loss of coadapted gene combinations (Hufford et al., 2012). Montalvo and Ellstrand (2001) found increasing levels of outbreeding depression in fitness components (such as seed production and seedling emergence) following controlled crosses between more genetically distant (as measured by allozymes) populations of *Lotus scoparius*. Outbreeding depression was highest in crosses between the most genetically distinct groups of the species (two different varieties) that have been used indiscriminately in restoration projects in California (Montalvo and Ellstrand, 2001).

Frankham et al. (2011) point out that probability of outbreeding depression has been shown to increase with an increase in any or all of the following four factors: 1) selection differential (this increases as the difference between the environment to which the population was adapted and the new environment increases), 2) heritability of reproductive fitness, 3) effective population size (this is the number of individuals in a population that could potentially contribute offspring to the next generation; smaller effective populations tend to show stronger genetic differentiation among populations; Huff et al., 2011) and 4) generations since isolation of the population. They also provide a decision tree to determine the probability of outbreeding depression following the crossing of individuals between two populations, and advocate to increase gene flow between fragmented populations when there is a low probability of outbreeding depression (Frankham et al., 2011). In addition, studies that can define optimal outcrossing seed provenance zones (e.g., Hufford et al., 2012) can prevent outbreeding depression, as well as inbreeding depression, from happening in species and/or population level management plans.

#### *Ex situ conservation approaches*

Botanical gardens and other institutions play a critical role in maintaining *ex situ* collections of rare species. Their importance is apparent at the international level, in Target 8 of the Global Strategy for Plant Conservation (GSPC) developed by the United Nations' Convention on Biological Diversity (CBD), and at the local level, in Targets 8 and 9 of the Hawai'i Strategy for Plant Conservation (HSPC). The goal of Target 8 of the GSPC calls for "at least 75% of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20% available for recovery and restoration programmes." There has been some progress towards the target but it has not been achieved. On a global level, only 29% of IUCN Red List species are known in *ex situ* collections, and "more effort is needed to ensure that *ex situ* collections are genetically representative of species populations" (Sharrock et al., 2014). The goals of Targets 8 and 9 of the HSPC are that "at least 75% of SCI [Species of Conservation Importance] are secured with adequate *ex situ* collections by 2020" and "collections of at least 20% of SCI are available for rare species recovery, habitat restoration and watershed protection projects", respectively (Keir and Weisenberger, 2014). Hawai'i is close to reaching its goal of *ex*

*situ* representation of SCI, with 73% (528 of 724 taxa) secured at some level of representation. However, of those 528 taxa, 64% are represented by collections from only 10% or less of the known wild individuals (Weisenberger and Keir, 2014).

There are many potential ways to build *ex situ* collections and each of these will impact the genetic diversity of material that is available for reintroduction projects. Different approaches include: cryopreservation (seeds, pollen, or tissue frozen in liquid nitrogen), seed banking, tissue culture storage and propagation, and cultivation of living collections in dedicated conservation facilities, controlled environments, or mixed display or reference collections (Maunder et al., 2004). Ultimately, factors such as reproductive biology, seed characteristics (e.g., orthodox versus recalcitrant), and adaptability to *ex situ* conditions of the target plant species will determine the type of plant material maintained. Species with orthodox seeds, seeds that can be dried and stored at low temperatures for many years, provide the greatest conservation value and potential to maintain the most diversity at the lowest cost (Kramer et al., 2011).

All approaches of maintaining a species *ex situ* have different potential to capture genetic diversity and ensure that diversity is not lost over time. There are drawbacks to living collections, such as relatively high costs per individual to maintain, short longevity, and maintenance of low levels of genetic diversity compared to seed banking, cryopreservation, and tissue culture. However, living collections are valuable in replenishing seed bank and tissue culture collections and serve important, indirect conservation purposes primarily through research and outreach (Kramer et al., 2011). Another important concern with the maintenance of *ex situ* living collections and subsequent seed bank and tissue culture collections if they are replenished from living collections, is adaptive responses to the *ex situ* environment, which is inevitable to some degree through differential mortality (Husband and Campbell, 2004). Regardless, source material from living *ex situ* collections can be extremely valuable for conserving diversity (Guerrant and Fiedler, 2004), and is sometimes the only option. The ultimate goal in sampling of material for *ex situ* collections is to capture variation representative of the species (Husband and Campbell, 2004). A well-documented *ex situ* collection, that captures the highest levels of genetic diversity of the species possible, will have the greatest conservation value (Kramer et al., 2011). Unfortunately, for many collections worldwide,

provenance data is unknown or the plants were cultivated from only a single founder (Weisenberger and Keir, 2014).

### *Study species*

Although Hawai‘i is considered one of the world’s biodiversity hotspots, it is also unfortunately known as the ‘endangered species capital of the world’. The U.S. Fish and Wildlife Service’s online endangered species database lists 420 Hawaiian plant and animal species that are currently listed as federally endangered. Three-hundred fifty-eight of those (or 85%) are plants (USFWS 2015). *Brighamia insignis* A. Gray, a lobeliad (Campanulaceae) endemic to the island of Kaua‘i and historically to Ni‘ihau (Wagner et al., 1999), is one of those 358 species. Only a single individual remains in the wild, on the Nā Pali Coast of Kaua‘i (Steve Perlman, personal communication, June 2013). The other *Brighamia* species, *B. rockii* H. St. John, is endemic to Moloka‘i and also listed as federally endangered (USFWS 2015). These two species are morphologically similar and are found in similar habitats of the different islands. Although *B. insignis* and *B. rockii* were officially listed as federally endangered in 1994 and 1992, respectively, it was noted by St. John as early as 1969 that populations of the whole *Brighamia* genus were declining, which he attributed primarily to feral goats. In 1977, the National Tropical Botanical Garden (NTBG) started assessing populations of both *Brighamia* species. In 1977 and 1978, then NTBG field botanist Steve Perlman collected seeds from the Waiahuakua population of *B. insignis* on the Nā Pali coast of Kaua‘i and distributed them to various botanical gardens around the world (Hannon and Perlman, 2002). It was through this foresight and these efforts that *B. insignis* is still extant *ex situ* today.

In a continued effort to manage the species *ex situ*, NTBG started outplanting individuals at the Limahuli Garden living collection (lower garden portion) in 1992 and in the Lower Preserve in 2006 (Kava Vale, personal communication, January 2015). Limahuli Valley is approximately 2 kilometers from the known and documented natural historic range of the species (Ken Wood, personal communication, January 2015). In 1995, the Department of Land and Natural Resources (DLNR) and volunteers conducted outplantings at the Kīlauea Point National Wildlife Refuge. Unfortunately, all of those planted have since died (Kim Uyehara, personal

communication, April 2013). In addition, NTBG and other institutions locally, nationally, and internationally, continued maintaining cultivated accessions, many of which very likely derived from those seed distribution efforts of Steve Perlman in 1977 and 1978. In the Netherlands, Plant Planet cultivates *B. insignis* by the hundreds of thousands and sells them by the botanically inaccurate name, “The Hawaiian Palm” (Robin Schaap, Plant Planet Manager, personal communication, February 2013). Maunder et al. (2004) in *Ex situ plant conservation: supporting species survival in the wild*, opens chapter one by stating “Botanic gardens and other *ex situ* facilities such as seed banks are among the most extensive yet underused plant conservation resource in the world.” In the case of *B. insignis* and other extinct or nearly-extinct species, cultivated individuals kept in *ex situ* facilities, primarily botanic gardens, are all that is left. For these species, the only hope for species survival comes from *ex situ* collections, which may be able to support eventual *in situ* conservation efforts. Such *in situ* reintroductions would not be an option without appropriate *ex situ* collections from which to obtain material.

The purpose of this study was to investigate genetic diversity of *ex situ* accessions of *B. insignis*. Many collections locally, nationally, and internationally maintain *B. insignis* in their living collections. Of those collections included in this study, only 25% of them also maintain the species via other methods, such as seed storage. In addition to maintaining by far the largest living collection, NTBG also maintains the largest seed collection of *B. insignis*. They have a total of 11,900 seeds stored representing 36 different accessions. However, it is essential that they keep replacing these stored seeds because viability drastically declines after only ca. five years in storage (Margaret Clark, personal communication, March 2015). The Seed Conservation Lab at Lyon Arboretum stored *B. insignis* seeds in the past but no longer does so, since there is a seed bank at NTBG on Kaua‘i, and any restoration would be done there (Marian Chau, personal communication, March 2015).

It is likely that the species has continued to be maintained in as many living collections as it has been because it is unique in form, has beautiful, fragrant flowers, and has been an icon for plant conservation. All of the institutions with *ex situ* collections are most certainly putting considerable resources into maintaining them, especially since pests, such as spider mites, are reported to require constant management (Lynn Janik, personal communication, January 2014;

Margaret Yeakel-Twum, personal communication, January 2014; Dylan Hannon, personal communication, March 2014). With the recent, functional extinction of *B. insignis* in the wild, and low numbers of collections maintaining the species *ex situ* using other approaches such as seed banking, these living collections are the only way aging seed bank germplasm can be replaced. By knowing the genetic characteristics of these different collections, we can design a pedigree breeding program in order to obtain more diverse progeny. In summary, this study was done with the intention of providing conservation managers with information to help make decisions to best manage *ex situ* collections, and perhaps eventual *in situ* populations, of the species.

In 1998, Gemmill et al. conducted a population genetic study of both *Brighamia* species (*B. insignis* and *B. rockii*). Using isozyme analyses, they investigated levels of genetic diversity at the population and species level and whether genetic variation of *ex situ* collections at NTBG were representative of wild populations. To compare *ex situ* collections to wild populations, they compared an *ex situ* collection representative of a single population of both species to its corresponding natural population. For *B. insignis*, this equated to 48 *ex situ* individuals that had been propagated from seed collected at a Mt. Hā'upu population (NTBG accession #940074). For *B. rockii*, this equated to 13 individuals that had been propagated from seed collected at a Ka'aloa population (NTBG accession #950422). The intention of their study was that results would help form better conservation management programs. At the time, it was apparent that both species, especially *B. insignis*, were at high risk of extinction if drastic conservation management actions were not taken. The authors found little genetic variation within species, similar levels of genetic variation between *ex situ* collections and wild populations, higher levels of inbreeding within *ex situ* collections, and distinct genetic differences between the two species (Gemmill et al., 1998).

Since Gemmill et al. (1998), no additional formal genetic research has been done on *Brighamia* despite the fact that neutral genetic markers are now available that are often more polymorphic than allozymes and which therefore may provide additional resolution when assessing genetic diversity of *ex situ* collections. Additionally, *B. insignis*: 1) is an iconic species for plant conservation in Hawai'i, 2) has been a federally listed endangered species since 1994

and now is functionally extinct in the wild, and 3) is a species for which the U.S. Fish and Wildlife Service, in their 5-Year Review from 2007, recommended conducting “a more detailed genetic study of the individuals in the wild and in cultivation to determine micro-level distinction between populations and individuals, to assist in determining suitable material to use of hand pollinated crosses” (USFWS, 2007).

For this study, the following questions were asked: (1) Do the different *ex situ* collections differ in the amount of genetic diversity they harbor? (2) How does the amount of genetic diversity of collections at NTBG compare to collections throughout the rest of the world? (3) Which *ex situ* collection(s) hold accessions or individuals that are the most genetically distinct from those at NTBG? Results of this work are discussed from the perspective of being able to use this information for a breeding program that could potentially introduce genetic diversity into restoration propagules at NTBG on Kaua‘i.

## **Methods**

### *Collection procedures*

Before collecting any fresh leaf samples, I verified whether or not permits were required because *B. insignis* is a federally listed endangered species. A state permit was not required since the plants I was working with are in cultivation (Charmian Dang, Rare Plant Permit Coordinator, personal communication, January 2013). The majority of individuals and accessions included in this study were collected from NTBG on Kaua‘i (18 different NTBG accessions total), which contains the largest *ex situ* collection world wide of the species. Permission to collect from NTBG was obtained by signing a Plant Distribution Agreement.

When the idea to conduct a genetic diversity study of *B. insignis* in cultivation first came to mind, a colleague mentioned a similar project had been done at Chicago Botanic Garden (CBG; Tim Gallaher, personal communication). Through a Google search of ‘cultivated *Brighamia* genetic diversity’, I came across a poster showcasing preliminary results of the author’s project titled ‘Using Botanic Garden Collections to Augment Genetic Diversity For Conservation of *Brighamia insignis*’ that was done in 2002. I was able to get in touch with one of the author’s, Dr. Jeremie Fant, who is the Molecular Ecology Lab Manager at CBG’s

Conservation Science Laboratories. Dr. Fant and I had mutual interest in collaborating to expand and complete this project he and Kayri Havens started 10 years prior.

Ninety individuals representing 48 accessions from 24 total *ex situ* collections in seven countries were included in this study (Table 3.1). Five collections were from Hawai‘i, 12 from the continental United States, and seven from the rest of the world. An accession is believed to come from one source, and may contain many individuals (likely related). In cases where collection records lacked accession data, all individuals within that collection were assumed to represent a single accession. In this study, I am referring to a ‘collection’ as any entity containing at least one living *ex situ* individual. These ‘collections’ were represented by universities, community colleges, botanic gardens, nurseries, and other public and private gardens.

All representative collections obtained in 2013 and 2014, that are outside of Hawai‘i, were found through a search of ‘*Brighamia insignis*’ on the Botanic Gardens Conservation International (BGCI) PlantSearch database. An email request for silica-dried leaf samples was sent to all collections indicating they had *B. insignis* in their living collection. Additionally, I signed a Data Supply Agreement to obtain a spreadsheet from BGCI listing all institutions that reported *B. insignis* in their living collections. The list provided the institution name, type, address, phone, email and contact person. From this, I was able to contact each person directly to request leaf material. I was able to obtain leaf samples only from those who responded and were willing to collect and send them. CBG obtained samples from eight different collections in the early 2000’s; four of these collections I did not obtain samples from again in 2013 and 2014 because either 1) I did not get a response through the BGCI PlantSearch database email request or through direct contact (Botanic Garden Meise), 2) the collection was not listed in the spreadsheet of direct contact information (Botanischer Garten Copenhagen), or 3) collections dwindled to a smaller number of individuals compared to what was initially included through CBG’s efforts in the early 2000’s (CBG and Waimea Arboretum Foundation). The original, natural source population for all these cultivated plants, with the exception of NTBG collections, is unknown or unclear. Collected leaves were immediately placed in seed envelopes and then into ziplock bags containing silica sand or beads. Silica dried leaves that were mailed were done so within one week after collecting.

Table 3.1. List of different collections (any entity containing at least one living *ex situ* individual) organized by regional groupings assigned. These collections included universities, community colleges, botanic gardens, nurseries, and other public and private collections. The location and putative number of accessions and individuals from each collection that were included in this study and total in the collection, and totals within the regional grouping, are displayed. Collections with an asterisk (\*) are those from which CBG obtained leaf material in the early 2000's and were not duplicated.

Collection	Location	Source of plants in collection	Seed stored?	# of accessions (individuals) in collection	# of accessions (individuals) included in study
<b>NTBG</b>					
National Tropical Botanical Garden	Kaua'i, Hawai'i	Wild populations	Yes	<b>22 (900)</b>	<b>18 (27)</b>
<b>Hawai'i minus NTBG</b>					
Leeward Community College	O'ahu, Hawai'i	NTBG and Lyon Arboretum	No	1 (10+)	1 (3)
Maui Native Nursery (Native Nursery LLC)	Maui, Hawai'i	Ho'olawa Farms	No	1 (3+)	1 (3)
Queen Kapiolani Garden	O'ahu, Hawai'i	Hui Kū Maoli Ola	No	0 (0)	1 (4)
Waimea Arboretum Foundation*	O'ahu, Hawai'i	?	?	1 (1)	1 (1)
<b>REGION TOTAL</b>				<b>3 (13+)</b>	<b>4 (11)</b>
<b>USA</b>					
Atlanta Botanical Garden	Atlanta, Georgia, USA	NTBG	No	1 (2)	1 (2)
C & J Cactus Nursery	Vista, California, USA	Gary James who got seeds from Honolulu Botanical Gardens	Yes	1 (20+)	1 (9)
Chicago Botanic Garden*	Glencoe, Illinois, USA	Missouri Botanical Garden	No	1 (3)	1 (6)
Denver Botanic Gardens	Denver, Colorado, USA	Grün Stadt Zürich	No	2 (5)	1 (1)
Ganna Walska Lotusland	Santa Barbara, California, USA	San Diego Botanic Garden and Adrianus Cornelis Dekker	No	2 (4)	2 (2)

Humboldt State University	Arcata, California, USA	Unknown	No	1 (22)	1 (2)
Longwood Gardens	Kennett Square, Pennsylvania, USA	Meadowbrook Farm	No	1 (2)	1 (1)
Marjorie McNeely Conservatory (Como Park Zoo)	St Paul, Minnesota, USA	A gardening friend 15+ years ago	Yes, but likely not viable	1 (9)	1 (2)
Missouri Botanical Garden	St Louis, Missouri, USA	University of California – Davis and Santa Barbara	Yes	1 (1)	1 (6)
San Diego Zoo	San Diego, California, USA	Ho‘omaluhia Botanical Garden	No	8 (36)	4 (6)
University of Connecticut	Storrs, Connecticut, USA	Connecticut Cactus and Succulent Society	Yes	1 (3)	1 (1)
University of Wisconsin - Eau Claire	Eau Claire, Wisconsin, USA	Olbrich Botanical Gardens and Marjorie McNeely Conservatory	No	1 (6)	1 (2)
<b>REGION TOTAL</b>				<b>21 (113+)</b>	<b>16 (40)</b>
<b>Rest of the world</b>					
Botanic Garden Meise*	Meise, Belgium	Longwood Gardens (1979); Agora in Aartselaar (2014)	No	1 (5)	1 (1)
Botanischer Garten Copenhagen*	Copenhagen, Denmark	Unknown nursery in Germany	Yes	1 (?)	1 (3)
Botanischer Garten der Universität Osnabrück	Osnabrück, Germany	Botanischer Garten Heidelberg	?	1 (1)	1 (1)
Grün Stadt Zürich	Zürich, Switzerland	Botanischer Garten Heidelberg	No	6 (21)	4 (4)
Jardin Exotique de Monaco	Monaco	Italy	No	1 (1)	1 (1)
Mairie de Lyon Jardin Botanique	Lyon, France	Unknown	No	? (?)	1 (1)

Trompenburg Tuinen and Arboretum	Rotterdam, Netherlands	Hawai'i (Ampie) Giuseppe Oslanoto	No	1 (1)	1 (1)
<b>REGION TOTAL</b>				<b>11 (29)</b>	<b>10 (12)</b>
<b>GLOBAL TOTAL</b>				<b>57 (1,055+)</b>	<b>48 (90)</b>

#### *DNA extraction and PCR reactions*

All DNA extractions of samples obtained in 2013 and 2014 were done in Dr. Cliff Morden's molecular lab at the University of Hawai'i at Mānoa. Total DNA was extracted from 0.05-2 g of silica-dried leaves using Doyle and Doyle's (1987) CTAB method with minor adjustments following Morden et al. (1996). All samples were accessioned into the Hawaiian Plant DNA Library (HPDL) (Morden et al., 1996). Extractions done over a decade ago at CBG were also done using a modified CTAB protocol as described by Doyle and Doyle (1990). CBG DNA samples have been stored and preserved in a  $-80^{\circ}\text{C}$  freezer. The quality and quantity of DNA in all samples used were measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

An initial search on GenBank in December 2013 indicated that no microsatellite primers had been developed for this species, or for close relatives within Campanulaceae. Microsatellite markers would have been preferred because 1) they are co-dominant, which means inbreeding can be examined, 2) they are highly polymorphic, which means they can detect high levels of differentiation, and 3) they are highly reproducible (Arif and Khan, 2009). A good molecular marker alternative, a PCR-based method, is inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994). These hypervariable nuclear markers, the primers of which are designed from common sequences of simple sequence repeats, have been used quite extensively in genetic diversity studies of plant species in both cultivation (Farsani et al., 2012; Choudhary et al., 2013; Hu et al., 2014; Zhang et al., 2014) and at the population level in the wild (Esselman et al., 1999; Fant et al., 2008; Hadian et al., 2014; Qian et al., 2014; Zhou et al., 2014). A comparison of ISSRs to other PCR-based methods (restriction fragment length polymorphism, or RFLP, and random amplified polymorphic DNA, or RAPD) found that ISSRs are superior in terms of cost and speed and have higher reproducibility compared to RAPDs (Yang et al., 1996). We therefore used ISSR markers to assess genetic diversity within and among *ex situ* collections of *B. insignis*.

Polymorphic bands are scored as diallelic (presence/absence of band) and, like RAPD data, analyzed as dominant markers. (Fant et al., 2008; Wolfe et al., 1998).

All samples were screened for reproducibility and polymorphic bands using the University of British Columbia (UBC) microsatellite set #9 ISSR primers. Fant and colleagues had already identified a short list of six primers (UBC807, UBC814, UBC841, UBC846, UBC848, and UBC835; see Table 3.2 for anchoring sequence of primers) to test. From these, four (UBC807, UBC814, UBC841, and UBC835) were selected for analysis of all individuals because they yielded distinct, multiple, and reproducible polymorphic bands. Optimization reactions included varying annealing temperatures (45.1, 45.9, 47, 48, 49, 50, 51.5°C), and a matrix of different DNA, primer, and gel concentrations to produce clear bands for easy scoring. Smear bands were initially a problem, which would have made scoring them extremely difficult. After optimization, standard 15  $\mu$ l polymerase chain reaction (PCR) conditions were 5  $\mu$ L primer, 1  $\mu$ L of ca. 15 ng/ $\mu$ L genomic DNA, and 7.5  $\mu$ L Promega PCR Mastermix (Madison, Wisconsin, USA). PCR was performed in Eppendorf thermocyclers using the following conditions: one cycle of 94°C for 90 seconds, followed by 35 cycles of 94°C for 40 seconds, 49°C for 45 seconds, 72°C for 90 seconds, and then finishing with 20 minutes at 72°C. PCR reactions were run on a gel-electrophoresis kit at 3% agarose gels in 1x TE buffer for 2–3 hours at 100 mA and visualized using SYBR Green (UVP, Inc., Upland, CA, U.S.A.).

Table 3.2. University of British Columbia (UBC) microsatellite set #9 ISSR primers screened. From these, four (UBC807, UBC814, UBC841, and UBC835) were selected for analysis of all individuals.

<b>UBC Primer Set #9 (Microsatellite)</b>	<b>Anchoring Sequence</b>
807	AGA GAG AGA GAG AGA GT
814	CTC TCT CTC TCT CTC TA
835	AGA GAG AGA GAG AGA GYC
841	GAG AGA GAG AGA GAG AYC
846	CAC ACA CAC ACA CAC ART
848	CAC ACA CAC ACA CAC ARG

### *Data analysis*

Two replicate PCR reactions and gel runs were performed per primer for all DNA accessions to confirm reproducibility; any two replicates that did not match were repeated for a third time or discarded. ISSR bands were visualized and photographed under ultraviolet light using the Kodak Photo ID program. Only variable loci were included in the analysis. Scoring by eye, presence of a band was recorded as "1" and absence of a band was recorded as "0". All data were entered into an Excel spreadsheet and then organized for input into Arlequin ver 3.5.1.3 software (Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA) was used to measure the percentage of genetic variation among collections and different regional groupings, to determine how much neutral genetic variation was captured in any single collection. Due to the small size of most collections, samples were also pooled into the following four regional groups: NTBG, other Hawaiian collections, other US collections and collections outside the USA. The first AMOVA (model 1) treated all collections individually and then pooled into the four regional groupings (described in Table 3.3). To determine if NTBG was successful in collecting most of the diversity of this species, in model 2, NTBG was compared to the three regional groups combined ('all other collections'; Table 3.3). Finally, to determine how NTBG influenced the results, model 3 for the AMOVA excluded the NTBG collection from the analysis. Arlequin software was also used to measure the pairwise genetic distances (fixation index;  $F_{ST}$ ) among all collections.  $F_{ST}$  values range from 0 to 1 with values closer to 0 indicating less genetic differences.

Genetic structure within and among collections was investigated and analyzed using the program Structure 2.3.4 (Pritchard et al., 2000), which infers population structure through a Bayesian model clustering method using a Markov chain Monte Carlo (MCMC) algorithm. The program estimates the number of genetic clusters ( $K$ ) and assigns individuals to each of the inferred clusters. To enter the dominant data into Structure, we coded bands as 1 and -9, where 1 designates the dominant allele and -9 designates the unknown second allele. Absence of a band was scored as 0 and 0. Assuming an admixture model, 20 independent models were run for maximum  $K$  of 26 with a burn-in of 1,000,000 and 1,000,000 MCMC repetitions. Structure Harvester (Earl and vonHoldt, 2012) was used to determine the most appropriate value of  $K$

following techniques described by Evanno et al. (2005). In addition, a principal coordinate analysis (PCoA) was conducted using the Gower general similarity coefficient in MVSP 3.1 (Multi-Variate Statistical Package; Kovach Computing Services, 1987-2009). Results of the PCoA are presented in the regional groupings of collections, as grouped for model 1 in the AMOVA described above and in Table 3.3.

Table 3.3. Descriptions of different groupings compared (models) for analyses.

<b>Model</b>	<b>Description of grouping</b>	<b># of ‘populations’ (collections) and groups compared</b>
1	Separated into the following regional groups: ‘Hawai‘i minus NTBG’, ‘NTBG’, ‘USA minus Hawai‘i’, and ‘rest of the world’	24 collections, 4 groups
2	Separated into two groups: ‘NTBG’ and all the other regional groups combined (‘all other collections’)	4 regional groups, 2 groups
3	Excluding ‘NTBG’ and separated ‘all other collections’ into the following groups: ‘Hawai‘i minus NTBG’, ‘USA minus Hawai‘i’, and ‘rest of the world’	23 collections, 3 groups

## Results

According to banding patterns using 24 polymorphic bands from four different primers (range of 4 to 10 polymorphic bands scored per primer with an average of 6), there were 72 different haplotypes identified among all *B. insignis* individuals included in this study. Levels of polymorphism detected among *B. insignis* individuals, within NTBG’s collection, and within the regional groupings of ‘USA minus Hawai‘i’ and ‘rest of the world’, were comparatively high (range from 70.8 to 95.8%), while percent polymorphism within the smallest regional grouping, ‘Hawai‘i minus NTBG’, was low (54.2%) (Table 3.4). NTBG and the three groupings were compared for genetic similarities based on the Nei and Li (1979) genetic identity (*I*), where a value of 0.0 indicates no markers in common and 1.0 indicates complete genetic identity. Within a collection or group, the genetic similarity among individuals was highest for the ‘Hawai‘i minus NTBG’ regional group (0.821), followed by NTBG’s collection (0.789), the ‘USA minus Hawai‘i’ (0.723) group, and the ‘rest of the world’ (0.711) group. Similarity between groups of collections showed that NTBG’s collection and the ‘rest of the world’ regional group was the

least similar (0.694). NTBG was the most similar to the ‘Hawai‘i minus NTBG’ group (0.801) among all group comparisons (Table 3.5).

Table 3.4. Number of identifiable ISSR markers and percent polymorphism within all *B. insignis* individuals included and within the NTBG collection and three regional groups (‘Hawai‘i minus NTBG’, ‘USA minus Hawai‘i’, and ‘rest of the world’).

Group	N <sup>a</sup>	Markers <sup>b</sup>	P <sup>c</sup>
<i>B. insignis</i>	90 (24)	23	95.8%
<b>Hawai‘i minus NTBG</b>	11 (4)	13	54.2%
<b>NTBG</b>	26 (1)	18	75.0%
<b>USA minus Hawai‘i</b>	41 (12)	20	83.3%
<b>Rest of the world</b>	12 (7)	17	70.8%

<sup>a</sup>Number of individuals (and collections) sampled

<sup>b</sup>Number of ISSR markers identified

<sup>c</sup>Percent polymorphism

Table 3.5. Levels of genetic similarity within and among NTBG’s collection and the following three regional groupings of collections: ‘Hawai‘i minus NTBG’, ‘rest of the world’, and ‘USA minus Hawai‘i’.

	NTBG	Hawai‘i minus NTBG	USA minus Hawai‘i	Rest of the world
<b>NTBG</b>	0.789			
<b>Hawai‘i minus NTBG</b>	0.801	0.821		
<b>USA minus Hawai‘i</b>	0.728	0.752	0.723	
<b>Rest of the world</b>	0.694	0.712	0.704	0.711

The AMOVA (as a weighted average over loci), model 1 showed that 73% of the genetic variation is accounted for within an average collection (e.g., NTBG, San Diego Zoo), while 26% is accounted for between collections within their regional grouping, and 1% is accounted for among the four different groupings (Table 3.6). When NTBG was compared to all other collections (model 2), we see that average genetic variation accounted for within a regional group increases 15% from model 1 (88% total). Interestingly, we still see that an average of 10% of diversity varies between regional groups within the larger collective group (‘all other collections’) of all those regional groups combined. Only 1% of genetic diversity is accounted for between groups ‘NTBG’ and ‘all other collections’ (Table 3.6). When plants maintained within the NTBG collection were excluded from the analysis (model 3), we see that the genetic

variation accounted for within an average collection drops to 64%. Hence, more than one-third of diversity is not accounted for within a collection. In addition, diversity accounted for between collections within their regional group jumps to 28%, suggesting that almost a quarter of the variation is being maintained by multiple collections within a geographical region. Furthermore, there is greater percentage of diversity found among the three large geographic groupings (8% compared to 1%) (Table 3.6). All models showed that the large majority of genetic variation is distributed at the collection level. However, there was a substantial amount of variation also detected between regional groups or the higher hierarchical collective grouping of ‘NTBG’ and ‘all other collections’.

Table 3.6. Hierarchical analyses of molecular variance (AMOVA) results as a weighted average over loci of *B. insignis* collections for three different models; see Table 3.3 for description of different groupings for each model and Table 3.1 for a breakdown of collections (populations) within each of those groupings. SS = sum of squares; VC = variance components.

Source of variation	SS	VC	Variation	P
<b>Model 1 (4 regional groups and 24 collections)</b>				
Among regional groups	12.92	0.01	0.91 %	0.286
Among collections within groups	37.22	0.35	26.41 %	0.000
Within collections	63.14	0.96	72.68 %	0.000
Total	113.28	1.32		
<b>Model 2 (2 groups and 4 regional groups)</b>				
Among global group and ‘NTBG’ group	7.03	0.02	1.34 %	0.328
Among regional groups within global group and ‘NTBG’	8.54	0.17	10.41 %	0.001
Within regional groups and ‘NTBG’ group	123.18	1.44	88.25 %	0.000
Total	138.75	1.63		
<b>Model 3 (3 regional groups and 23 collections)</b>				
Among regional groups	7.99	0.11	8.22 %	0.037
Among collections within groups	37.22	0.38	27.83 %	0.000
Within collections	34.92	0.87	63.95 %	0.000
Total	80.13	1.36		

The NTBG collection was compared to each of the other 23 collections by examining population pairwise  $F_{ST}$  values. Over half of the collections only had 1-2 individual plants so the power of the analyses in those instances was weak and differences not statistically significant. Of those collections with differences that were statistically significant ( $P$ -value  $\leq 0.050$ ), the following five collections were the most different from plants held at NTBG, with  $F_{ST}$  values

above 0.25 indicating high genetic differentiation (Hartl and Clark, 1997): CBG ( $F_{ST} = 0.402$ ), the University of Wisconsin - Eau Claire ( $F_{ST} = 0.382$ ), Missouri Botanical Garden ( $F_{ST} = 0.372$ ), Botanischer Garten Copenhagen ( $F_{ST} = 0.336$ ), and Atlanta Botanical Garden ( $F_{ST} = 0.311$ ) (Table 3.7).

Table 3.7. Population pairwise  $F_{ST}$  values of the NTBG collection compared to all other 23 collections. Those that are statistically significant with a  $P$ -value  $\leq 0.050$  are in bold.

Collection	$F_{ST}$	$P$
<b>Atlanta Botanical Garden</b>	<b>0.311</b>	<b>0.045</b>
Botanic Garden Meise	0.388	0.991
<b>Botanischer Garten Copenhagen</b>	<b>0.336</b>	<b>0.000</b>
Botanischer Garten der Universität Osnabrück	0.480	0.991
C & J Cactus Nursery	0.038	0.117
<b>Chicago Botanic Garden</b>	<b>0.402</b>	<b>0.000</b>
Denver Botanic Gardens	0.316	0.991
Ganna Walska Lotusland	0.294	0.099
Grün Stadt Zürich	0.112	0.081
<b>Humboldt State University</b>	<b>0.221</b>	<b>0.000</b>
Jardin Exotique de Monaco	0.371	0.991
Leeward Community College	0.000	0.685
Longwood Gardens	0.000	0.991
Mairie de Lyon Jardin Botanique	0.209	0.991
Marjorie McNeely Conservatory (Como Park Zoo)	0.135	0.216
Maui Native Nursery (Native Nursery LLC)	0.000	0.432
<b>Missouri Botanical Garden</b>	<b>0.372</b>	<b>0.000</b>
Queen Kapiolani Garden	0.000	0.676
San Diego Zoo	0.000	0.640
Trompenburg Tuinen and Arboretum	0.320	0.991
University of Connecticut	0.514	0.991
<b>University of Wisconsin - Eau Claire</b>	<b>0.382</b>	<b>0.018</b>
Waimea Arboretum Foundation	0.209	0.991

To further investigate genetic variability among and within collections, data were analyzed with the program Structure (Pritchard et al., 2000). Structure Harvester (Earl and vonHoldt, 2012) identified the smallest number of genetic clusters ( $K$  value) that best explained the data to be  $K = 3$ . Most individuals (72 of 90) showed  $> 75\%$  assignment to one of the three genetic groups (Table 3.8; Figure 3.1). Most collections were represented predominantly by one or two of the genetic clusters, with only five collections out of the 24 (21%) showing  $\geq 10\%$

representation of each of the three clusters. These five collections are Grün Stadt Zürich, Longwood Gardens and Marjorie McNeely Conservatory (Como Park Zoo) with majority of assignment to inferred cluster 3 (green), and NTBG and San Diego Zoo with majority of assignment to inferred cluster 2 (red) (Table 3.8; Figure 3.1). Most collections (13/24 or 54%) were predominantly assigned to cluster 3 (green), with  $\geq 66\%$  assignment to that cluster. Twenty-nine percent of collections (7/24) were predominately assigned to cluster 2 (red). However, the percent assignment to that cluster ranged from 55% to 78%. Only 17% (4/24) of collections were predominantly assigned to cluster 1 (blue), with 75% to 96% assignment to that cluster. These four collections were Botanischer Garten Copenhagen, Botanischer Garten der Universität Osnabrück, CBG, and Missouri Botanical Garden (Table 3.8; Figure 3.1).

NTBG was predominantly assigned to inferred clusters 2 (red) (62%) and 3 (green) (28%), with only 10% assignment to cluster 1 (blue). Interestingly, a majority of the other Hawaiian collections (Leeward Community College, Maui Native Nursery and Queen Kapiolani Garden) also predominantly grouped into inferred cluster 2 (red), with 55% to 74% assignment to that cluster. The single individual from Waimea Arboretum Foundation grouped into inferred cluster 3 (green) with 91% assignment. The percent assignment to cluster 1 (blue) of the other Hawaiian collections was also low, as it was for NTBG, ranging among collections from only 2% to 3% assignment (Table 3.8; Figure 3.1).

Table 3.8. Average percentage of each genetic cluster (*K*) within each collection. The highest percentage assignment of a cluster within a collection is in bold.

Collection	<i>K</i> = 1 (blue cluster)	<i>K</i> = 2 (red cluster)	<i>K</i> = 3 (green cluster)
Atlanta Botanical Garden	10%	3%	<b>87%</b>
Botanic Garden Meise	3%	2%	<b>96%</b>
Botanischer Garten Copenhagen	<b>80%</b>	16%	4%
Botanischer Garten der Universität Osnabrück	<b>91%</b>	1%	9%
C & J Cactus Nursery	4%	<b>78%</b>	18%
Chicago Botanic Garden	<b>96%</b>	3%	1%
Denver Botanic Gardens	2%	3%	<b>96%</b>
Ganna Walska Lotusland	3%	3%	<b>95%</b>
Grün Stadt Zürich	21%	11%	<b>68%</b>
Humboldt State University	2%	18%	<b>80%</b>
Jardin Exotique de Monaco	5%	3%	<b>92%</b>
Leeward Community College	2%	<b>66%</b>	32%
Longwood Gardens	21%	<b>56%</b>	23%
Mairie de Lyon Jardin Botanique	8%	11%	<b>81%</b>
Marjorie McNeely Conservatory (Como Park Zoo)	15%	20%	<b>66%</b>
Maui Native Nursery (Native Nursery LLC)	3%	<b>74%</b>	23%
Missouri Botanical Garden	<b>75%</b>	5%	20%
National Tropical Botanical Garden	10%	<b>62%</b>	28%
Queen Kapiolani Garden	2%	<b>55%</b>	44%
San Diego Zoo	21%	<b>63%</b>	16%
Trompenburg Tuinen and Arboretum	5%	4%	<b>91%</b>
University of Connecticut	2%	1%	<b>97%</b>
University of Wisconsin - Eau Claire	3%	2%	<b>94%</b>
Waimea Arboretum Foundation	3%	6%	<b>91%</b>

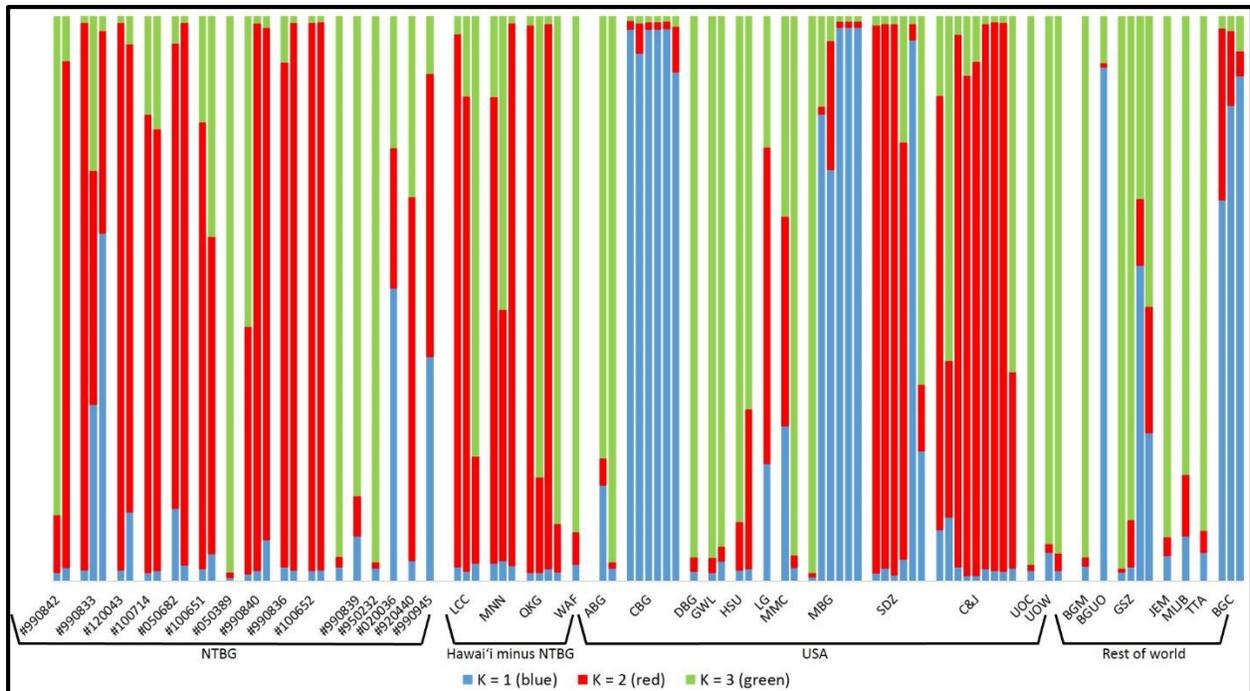


Figure 3.1. Estimated genetic structure of all *B. insignis* individuals from all collections included in this study. Individual samples are represented by vertical bars, which are divided into colored segments that represent estimated membership fractions in each of  $K=3$  clusters. Accessions at NTBG collection are grouped together. Individuals within collections are grouped together. NTBG and regional groups ('Hawai'i minus NTBG', 'USA', and 'rest of the world') are grouped together and identified by labels below the graph. LCC=Leeward Community College; MNN=Maui Native Nursery; QKG=Queen Kapiolani Garden; WAF=Waimea Arboretum Foundation; ABG=Atlanta Botanical Garden; CBG=Chicago Botanic Garden; DBG=Denver Botanic Gardens; GWL=Ganna Walska Lotusland; HSU=Humboldt State University; LG=Longwood Gardens; MMC=Marjorie McNeely Conservatory; MBG=Missouri Botanical Garden; SDZ=San Diego Zoo; C&J=C & J Cactus Nursery; UOC=University of Connecticut; UOW=University of Wisconsin-Eau Claire; BGM=Botanic Garden Meise; BGUO= Botanischer Garten der Universität Osnabrück; GSZ=Grün Stadt Zürich; JEM=Jardin Exotique de Monaco; MLJB= Mairie de Lyon Jardin Botanique; TTA=Trompenburg Tuinen and Arboretum; BGC= Botanischer Garten Copenhagen.

Results from the PCoA reinforced some of the same patterns that were revealed in the AMOVA and Structure analyses (Figure 3.2). There is overlap in the distribution of individuals from within the NTBG collection and all three regional groupings, across both axes. This pattern was reflected in the AMOVA model 1, which showed that there is low genetic variation among NTBG and the regional groups. However, we see that the second axis slightly separates NTBG from the ‘rest of the world’ regional group. This pattern was reflected in Structure with the inferred genetic cluster 1 (blue) being more prevalent in the ‘rest of the world’ group in comparison to NTBG, in which assignment of individuals to this cluster was underrepresented, and underrepresentation of cluster 2 (red) in the ‘rest of the world’ group.

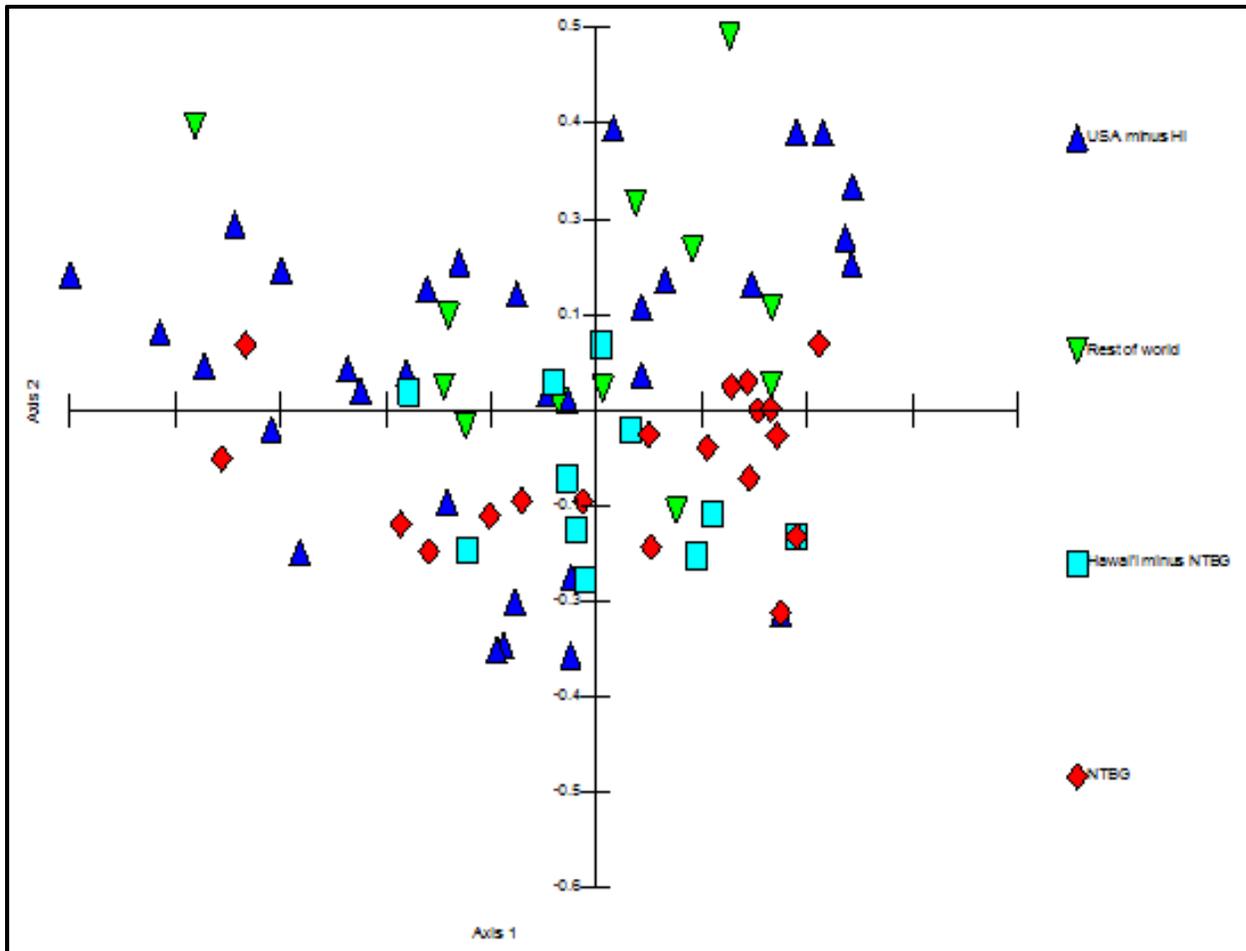


Figure 3.2. Principal coordinate analysis (PCoA) of ISSR data for *B. insignis* individuals from all collections included in this study. PCoA axes 1 and 2 accounted for 36.1% of the total variation.

## Discussion

AMOVAs found that most diversity is maintained at the collection level. In model 1, when all collections included in this study were pooled into NTBG and the following regional groups: ‘Hawai‘i minus NTBG’, ‘USA’, and ‘rest of the world’, an average of 73% of the genetic variation was accounted for within an average collection. However, roughly one-quarter (26%) of the neutral diversity not explained by a collection is quite high; this could reflect important adaptive diversity missing from these collections. When plants maintained within the NTBG collection were excluded from the analysis (model 3), the amount of genetic variation accounted for within an average collection dropped to 64%. However, when the NTBG collection was compared to the three regional groups combined (model 2), only 1% of the genetic variation, on average, was accounted for between those two groupings (‘NTBG’ and ‘all other collections’), an indication that cohesively, all other collections included in this study maintain levels of genetic diversity comparable to what NTBG maintains.

Examining differences in genetic diversity between NTBG’s collection and all other 23 collections individually is especially useful because it tells us which specific collections are the most genetically distinct from NTBG. This suggests that although NTBG was shown to hold a large proportion of the known neutral genetic diversity, there was some diversity in other collections that was underrepresented in NTBG’s collection. Population pairwise  $F_{ST}$  values indicated that the CBG ( $F_{ST}=0.402$ ), the University of Wisconsin - Eau Claire ( $F_{ST}=0.382$ ), Missouri Botanical Garden ( $F_{ST}=0.372$ ), Botanischer Garten Copenhagen ( $F_{ST}=0.336$ ), and Atlanta Botanical Garden ( $F_{ST}=0.311$ ) collections were the most genetically different compared to NTBG’s collection. None of the other collections maintained in Hawai‘i were significantly genetically distinct from NTBG’s collection. This suggests that crossing plants between NTBG and other Hawaiian collections (at least those collections included in this study) would be unlikely to increase or enhance levels of diversity already present in NTBG’s collection. Results of Structure further support this. All Hawaiian collections, including NTBG, were predominantly represented by genetic clusters 2 (red) and 3 (green). There was a very low percent assignment of the Hawaiian collections, including NTBG, to genetic cluster 3 (blue). Only the following four collections, out of all 24 included in this study, were predominately assigned to cluster 1 (blue):

Botanischer Garten Copenhagen, Botanischer Garten der Universität Osnabrück, CBG, and Missouri Botanical Garden. Interestingly, three of these collections (Botanischer Garten Copenhagen, CBG, and Missouri Botanical Garden) were also ones that showed strong, significant pairwise  $F_{ST}$  values compared to NTBG.

Insight into the genetic diversity maintained in *ex situ* collections of *B. insignis* all over the world, and the differences in those collections compared to NTBG, points out specific collections that would be best to incorporate into a breeding program to potentially increase levels of genetic diversity within NTBG's collection. Since NTBG maintains the largest *ex situ* collection of *B. insignis* and it is on the island upon which a restoration project of *B. insignis* would take place, I recommend that NTBG obtain seed from the following three collections, to then grow and use those plants as pollen donors in crosses with their different accessions: Botanischer Garten Copenhagen, CBG, and Missouri Botanical Garden. These collections predominantly grouped into an inferred genetic cluster least represented in NTBG's collection (and all other Hawai'i collections included in this study) and showed statistically significant, high genetic differentiation ( $F_{ST}$  values) compared to NTBG. Of those three, I recommend starting off with plants maintained at CBG because it had the highest  $F_{ST}$  value compared to NTBG and logistically, it would be the easiest. Although none officially remain in the collections there, Dr. Andrea Kramer (who was a major collaborator for this study) maintains three individual plants in her office and is willing to help NTBG with a breeding program by providing seeds (or pollen, if feasible, to cross with NTBG's plants). In addition, the plants continue to flower every fall, during the same time plants at NTBG have been observed flowering the past several years (Andrea Kramer, personal communication, February 2015; personal observation). If seeds were unable to be obtained through selfing or crossing between the plants available, and hence pollen was the only other option, it would be necessary that the amount and viability of the pollen produced be examined prior to making arrangements to collect and ship pollen to NTBG. However, very little is known about pollen storage for Hawaiian plants (Tim Kroessig, personal communication, April 2015) and hence, what it would require to maintain viability of pollen being shipped to Hawai'i.

Unfortunately, Missouri Botanical Garden currently maintains only a single live specimen of *B. insignis* that originated from the University of California – Davis. Results of Structure showed that this single individual grouped into the inferred cluster 3 (green) with 99% assignment. Other individuals from Missouri Botanical Garden, from which DNA samples were included in this study, grouped into inferred genetic cluster 1 (blue). However, these plants are no longer living. It turns out that *B. insignis* plants historically maintained at Chicago Botanic and Missouri Botanical Gardens were the same after learning of the historical trading of *B. insignis* material between the two institutions. CBG originally acquired their plants from Missouri. As would be expected, based on this history, these two collections predominantly grouped into the same inferred cluster 1 (blue) in the Structure analysis. Furthermore, when we compared genetic diversity between CBG and all other 23 collections individually, the lowest genetic differentiation was between CBG and Missouri Botanical Garden ( $F_{ST} = 0.207$ ;  $P = 0.009$ ).  $F_{ST}$  values between CBG and all the other collections ranged between 0.250 and 0.876 (average = 0.671) (data not shown). Missouri Botanical Garden has stored seeds from one of those plants that had been traded with CBG, of which originated from the University of California - Santa Barbara. I expect these were seeds acquired from those plants that had grouped into inferred genetic cluster 1 (blue). Missouri Botanical Garden is willing and able to send these seeds if NTBG is interested in utilizing this germplasm for conservation efforts (Caroline Bernard, personal communication, January 2014).

The third recommended collection from which to obtain seed and/or pollen to use in a breeding program at NTBG is Botanischer Garten Copenhagen. Staff there have expressed interest in a planned breeding program and are likely willing to help however they can. They maintain just a single accession but have multiple plants scattered around the garden (Ole Seberg, personal communication, March 2015). They also maintain seeds in storage (Martin Aarseth-Hansen, personal communication, April 2015).

There were additional collections with high  $F_{ST}$  values compared to NTBG. Although not statistically significant, they could be considered for use in a breeding program if utilizing the three aforementioned collections was unsuccessful. The five additional collections with the highest  $F_{ST}$  values, ranging from highest value to lowest, were: University of Connecticut ( $F_{ST} =$

0.514), Botanischer Garten der Universität Osnabrück ( $F_{ST}= 0.480$ ), Botanic Garden Meise ( $F_{ST}= 0.388$ ), Jardin Exotique de Monaco ( $F_{ST}= 0.371$ ), and Trompenburg Tuinen and Arboretum ( $F_{ST}= 0.320$ ).

The only *ex situ* collection with a substantial collection of stored seeds of *B. insignis* is NTBG, with a total of 11,900 seeds representing 36 different accessions. It is possible that some of the NTBG accessions not included in this study are representative of the inferred cluster 1 (blue) from the Structure analysis; 20 accessions are currently represented only in seed storage (Kava Vale, personal communication, March 2015) and not all 30 unique accessions currently represented in living collections were included in this study. However, something to keep in mind is that seed viability drastically declines after about five years in storage (Margaret Clark, personal communication, March 2015). The fact that NTBG is the only collection with a substantial seed storage collection highlights the need and urgency for backup seed collections of all living collections maintained around the world before more genetic diversity is lost.

The Seed Conservation Lab at Lyon Arboretum stored *B. insignis* seeds in the past and results from their 1999 research collection showed 96% initial viability with no significant decline in viability after about five years in dry/refrigerated storage. After ca. 10 years, however, viability dropped to 26%. In dry/frozen storage, viability dropped to 79% after only ca. five years and then frozen stored seeds were depleted (Marian Chau, personal communication, March 2015). Although this was from just one collection, it suggests that seeds are freeze sensitive and can only be stored dry/refrigerated for less than 10 years. It has been extremely difficult in recent years for NTBG staff to obtain viable seed from *B. insignis* plants (Ashly Trask, personal communication, January 2015), which is a current major concern and set-back since the NTBG Seed Lab needs to replace aging seed germplasm and has been trying to provide fresh seed to the National Center for Genetic Resources Preservation (NCGRP) to test optimal seed storage methods (Margaret Clark, personal communication, March 2015; Marian Chau, personal communication, March 2015). New seeds could be produced from crosses between NTBG and the three previously recommended collections. Not only would this likely result in the production of viable seeds for storage and for research at NCGRP, it would decrease the likelihood of

producing inbred offspring, since we would know that the mother and father are from different genetic clusters.

With 72 different haplotypes identified among the 90 individual plants included in this study, surprisingly high levels of genetic variation were found within and among *ex situ* collections of *B. insignis*. This differs from the 1998 study (Gemmill et al.) that used allozymes that found little genetic variation within both *Brighamia* species. However, allozymes, which analyze genetic variation in proteins, have lower levels of polymorphism compared to ISSRs, which analyze genetic variation in DNA (Cavalli-Sforza, 1998) because not all variation in DNA translates into variable protein products. For example, some DNA base changes may result in the same amino acids after translation. In studies that are testing whether or not populations are genetically distinct from one another, a lack of neutrality in the marker being used can be a disadvantage (Freeland et al., 2011). However, there are also limitations to ISSRs. Due to their dominant nature, only one allele at each locus can be identified so heterozygotes cannot be differentiated from homozygotes, making it difficult to calculate allele frequencies (Freeland et al., 2011).

Referring back to the Gemmill et al. (1998) study, it is reassuring that similar levels of genetic variation were found between NTBG's *ex situ* collections and wild populations. If today's *ex situ* collection is representative of the *ex situ* collection from years previous, there is hope that we can, not only continue to maintain levels of genetic diversity in NTBG's *ex situ* collection, but enhance it through a breeding program previously described. In potential future genetic work with *B. insignis*, I recommend including *B. rockii* given the potential for hybridization and/or misidentification since they are very similar morphologically and plant identifications from collections have been reported to often contain mistakes (Gorbunov et al., 2008).

Management plans for breeding programs of animals and other plant species have been informed by genetic studies. In these cases, molecular tools to examine genetic diversity were used to guide crossing designs that would produce more diverse progeny. A 2013 study (Marsden et al.) that examined the genetic diversity of African wild dogs within the European zoo network was used to create a management plan to inform a captive breeding program among

zoos. Based on their results, recommendations in the plan included 1) selecting breeding groups that provide equal representation of each of the founder families of the captive population to maximize mixing of different founder lines, and 2) continue separate management of captive populations within different countries to help maintain variation across the small captive populations, with occasional exchanges between regional programs to introduce new genetic diversity and reduce inbreeding (Marsden et al., 2013). As found with *B. insignis*, Marsden et al. (2013) found a lack of provenance data (studbook information); information was often incomplete and not informative about genetic diversity. The neutral genetic marker data was hence informative in understanding genetic structuring between captive populations, indicating that integrating incomplete pedigree data with genetic assessments can likely provide the strongest basis for genetic management plans.

A recent study by Da Silva et al. (2012) examined genetic structure of the critically endangered cycad, *Encephalartos latifrons*, with fewer than 60 individuals left in the wild. Their study included all known wild individuals and individuals from collections in Kirstenbosch National Botanical Garden which lack provenance data. Their study provided an opportunity to test a recommendation that augmentation should be based only on plants of known provenance, whereas there were 18 mature plants *ex situ* with potential value for increasing genetic diversity of natural populations. The authors found that the *ex situ* collection had similar levels of genetic diversity to wild stock, and included one genotype group extinct in nature. Therefore, the authors recommended these individuals be used to supplement the wild population through artificial fertilization or translocation. The authors urged potential breeding programs to focus on combinations that would maintain genetic diversity in the F<sub>1</sub> generation and highlighted the importance of *ex situ* collections, including those of unknown provenance (Da Silva et al., 2012). Points brought up in Da Silva et al. (2012) are interesting and relevant for future, potential restoration efforts of *B. insignis* since the current policy across agencies for outplanting of threatened and endangered species is that there must be provenance information with outplanting material. However, it seems that provenance data would have been a stand-in for genetic diversity in the past, whereas now we have the ability to examine actual genetic diversity,

making this an interesting policy question for the future (Margaret Clark, personal communication, March 2015).

Restoration outplantings of *B. insignis* on Kaua‘i have so far been unsuccessful. The outplanting conducted in 1995 at Kīlauea Point failed and plants at Limahuli have not shown signs of recruitment. Although one of the reasons for failure and lack of recruitment in previous and current outplantings could be unsuitable habitats, another confounding factor could be inbreeding depression. Since *B. insignis* is functionally extinct in the wild, restoration propagules would come from *ex situ* collections maintained at NTBG on Kaua‘i. Results of this study could help in the design of a breeding program to potentially introduce genetic diversity from other collections into NTBG’s collection, which may enhance the vigor and adaptability of plants used for restoration. By pooling resources, collaboration among institutions can lead to more efficient and effective *ex situ* conservation action (Kramer et al., 2011). In Chapter 4, specific step-by-step conservation recommendations for this rare species are outlined, which also take into account results of other studies that were conducted simultaneously on the biology and ecology of *B. insignis*.

## CHAPTER 4

### CONSERVATION AND RESTORATION MANAGEMENT IMPLICATIONS AND RECOMMENDATIONS

Motivation behind this thesis research was to help inform best management practices of the extremely rare, *B. insignis* on Kaua‘i. Botanical gardens and other institutions all over the world have maintained the species in their living collections for decades and have used it as a symbol and talking point for plant conservation (Oldfield, 2010). People across Europe care for the species as a potted plant in their homes as the Netherlands based, Plant Planet, cultivates *B. insignis* by the hundreds of thousands and sells them by the botanically inaccurate name, “The Hawaiian Palm” (Robin Schaap, Plant Planet Manager, personal communication, February 2013). *Brighamia insignis* has been an icon for plant conservation not only in Hawai‘i, but worldwide (Oldfield, 2010). Unfortunately, however, the species continues to battle an exhausting list of threats; even after being listed as federally endangered in 1994, populations continued to drastically decline (Ken Wood, personal communication, April 2015). In 1994, there were 60 to 70 plants remaining in the wild (USFWS, 1995). Then just four year later, the total number of naturally-occurring individuals went down to ca. 29 (Gemmill et al., 1998). When this project first started with preliminary data collection in 2011, two naturally-occurring individuals remained on the Nā Pali coast. Today, the species is functionally extinct in the wild with the last known remaining individual likely deceased (Steve Perlman, personal communication, June 2013). The urgency with which we need to work together to save this iconic species from extinction is apparent.

Findings within each chapter are discussed in the context of how they inform conservation management of the species, with specific management recommendations written out. While some findings confirmed hypotheses and long held predictions/speculations, some results were surprising yet still important to the conservation of *B. insignis*, confirming the importance of applied scientific research to inform management. This research also contributes broadly to our overall understanding of breeding systems, floral biology, pollination ecology, and *ex situ* genetic diversity of rare and endangered Hawaiian plant species.

### *Summary of findings*

Floral traits examined provided support for the hypothesis that *B. insignis* is adapted to moth pollination. Flower phenology and overall results of pollination treatments suggested a primarily xenogamous breeding system. However, results of the manipulative pollinations were difficult to confidently interpret since pollen quantity and quality produced were extremely low when examined in 2013. Based on 29.5 and 21.5 diurnal and nocturnal floral observation hours, respectively, it appears unlikely that anything is effectively pollinating *B. insignis* outplantings at NTBG's Limahuli Garden and Preserve. No moths were observed and all six of the different floral visiting species were non-native and did not appear to be serving as pollinators. Overall, these findings demonstrate that *B. insignis* faces severe challenges to reproduce on its own. Finally, results of a collaborative study that examined genetic diversity of *ex situ* collections of *B. insignis* found that most genetic diversity is maintained at the collection level. It also pointed out several collections that are genetically distinct from NTBG accessions and other Hawaiian collections included in the study.

### *Implications and recommendations*

Considering the extremely low fruit and seed set after pollination treatments conducted in 2012 and 2013, and low pollen quantity and quality (viability) produced by cultivated *B. insignis* plants being maintained *ex situ* at NTBG on Kaua'i examined in 2013 (Chapter 1), I initially recommend incorporating additional genetic diversity into plants at NTBG. Although the reason for low pollen quantity and quality observed remains unknown, inbreeding could be one of the reasons (Hayes et al., 2005). A breeding program, using plants at NTBG as mothers and individuals from specific, recommended collections that were included in the *ex situ* genetic diversity study (Chapter 3) as pollen donors, could introduce diversity underrepresented in NTBG's *ex situ* collection. This could result in more vigorous progeny that later on, may produce higher quantities of viable pollen, as other studies have clearly demonstrated that inbreeding adversely affects traits related to male function, such as pollen production (Carr and Dudash, 1996; Willis, 1999; Good-Avila et al., 2003; Hayes et al., 2005) and viability (Carr and Dudash, 1997; Willis, 1999). Such a project may be able to either verify or rule out inbreeding as

the reason for low pollen production and/or viability and hopefully alleviate it, which could also potentially increase the likelihood of fruit and viable seed production. It has been extremely difficult in recent years for NTBG staff to obtain viable seed from *B. insignis* plants (Ashly Trask, personal communication, January 2015), which is a concern and setback since the NTBG Seed Lab needs to replace aging seed germplasm and provide fresh seeds to the National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, Colorado to test optimal seed storage methods as part of a recent grant (Margaret Clark, personal communication, March 2015; Marian Chau, personal communication, March 2015). It was noted as early as 1995 that traditional seed storage methods (e.g., drying and freezing) had generally not been successful for long-term seed storage of this species and that research into long-term seed storage is needed (USFWS, 1995).

The three collections (in the following order) from which I initially recommend that NTBG obtain seeds to then grow up plants to use as pollen donors in crosses with their different accessions are: Chicago Botanic Garden, Missouri Botanical Garden, and Botanischer Garten Copenhagen. Detailed reasons for recommending these three collections, and specific recommendations on who to contact and how to conduct it, are outlined in Chapter 3. Since 20 NTBG accessions are currently represented only in seed storage (Kava Vale, personal communication, March 2015) and not all 30 unique accessions currently represented in living collections were included in this study, it is possible that some of those NTBG accessions are representative of the inferred cluster 1 (blue) from the Structure analysis. Therefore, I also recommend that NTBG strive to maintain all accessions as living collections, in addition to maintaining them in seed storage, since seed viability drastically declines after about five years in storage (Margaret Clark, personal communication, March 2015). In potential future genetic work with *B. insignis*, I recommend incorporating all NTBG accessions, which would reveal whether those accessions not included in the genetic study here are part of that blue cluster.

In conjunction with the above recommended project, or afterwards if the project is unsuccessful in its goal to increase fruit and seed set in plants at NTBG and increased fitness in progeny thereafter, I suggest NTBG consider conducting a controlled, experimental hybridization project between the two *Brighamia* species. Since *B. insignis* is already

functionally extinct in the wild and *B. rockii* remains endangered with most threats continuing to be largely unmanaged (USFWS 2014), examining whether F<sub>2</sub> or later progeny of hybrids express signs of hybrid vigor (increase in vigor of progeny compared to parents following hybridization), as opposed to hybrid breakdown (decrease in vigor of progeny compared to parents following hybridization) (Fridman, 2015), would be not only interesting in itself, but worthwhile in an effort to potentially reintroduce *B. insignis in situ* and augment existing populations of *B. rockii*. If hybrid progeny are vigorous, backcrossing those progeny with individuals of either and/or both *Brighamia* species, in order to incorporate more genetic diversity into the species' gene pool, could potentially greatly increase the fitness for either and/or both species. Similar work done on other species has demonstrated that this is possible (Burke and Arnold, 2001). Currently, further inbreeding (and likely inbreeding depression) of *B. insignis* is inevitable, and hence, it is unlikely that any future reintroductions would be successful long-term.

If either or both of the above recommendations are successful in increasing fitness of *B. insignis* progeny (e.g., increase in pollen production and viability), I recommend examining (in a controlled, experimental setting) whether any of the native and/or non-native moths present on Kaua'i would likely visit *B. insignis* and serve as effective pollinators. There are seven non-native and three native endemic species belonging to the Sphingidae family reported to occur on Kaua'i. The seven non-native species are *Agrius cingulata*, *Daphnis nerii*, *Hyles lineata*, *Macroglossum pyrrhoticum*, *Psilogramma menephron*, *Theretra nessus* (Nishida, 2002) and *Hippotion rosetta* (Kumashiro et al., 2001). The three native endemic species are *Hyles calida calida*, *Manduca blackburni*, and *Tinostoma smaragditis* (Nishida, 2002). Moths could be released into an enclosure with blooming *B. insignis* plants to observe whether they will visit and function as effective pollinators. Something to keep in mind for such a project, however, is that it may be difficult to get moths (especially large species that are strong fliers) to behave naturally in a cage (Will Haines, personal communication, April 2015).

In January 2015, I visited the J. L. Gressitt Center for Research in Entomology at Bishop Museum to see if specific collection localities of the aforementioned moth species were described in detail on voucher specimen labels. Specifically, I wanted to know if any collections were made within the natural historic range of *B. insignis* or at Limahuli Valley where the

species is currently outplanted. Unfortunately, locality descriptions were vague, indicated as either “Kipu”, “Koke‘e”, “Koloa”, “Hā‘ena”, or “Kaholuamano (near Waialae Falls)”. The more detailed locality descriptions included “Rice Residence”, “NASA station”, or “Kalalau lookout”. In a 2000 publication, *Tinostoma smaragditis* or the Fabulous Green Sphinx of Kaua‘i, was reported to have been collected within the natural historic range of *B. insignis*, at Ho‘olulu Valley in 1992 (Heddle et al., 2000). This was one of only 18 individuals known to have ever been collected on the island, or in the world for that matter since it is considered endemic to Kaua‘i. Consequently, much remains unknown about this moth species (including the host plant(s)) and no further studies are currently being funded to find out more, despite its rarity and the fact that it is the only species of an endemic genus (Heddle et al., 2000; Medeiros et al., 2013). Thus, I would also recommend additional research be conducted on aspects of the biology and ecology of *T. smaragditis*. It is not unreasonable to speculate that the Fabulous Green Sphinx of Kaua‘i could be the single or one of several, native pollinator(s) of *B. insignis*.

Other information that would be helpful to narrow down moth species to potentially include in a controlled, pollination experiment is knowledge of their proboscis lengths. By examining the mean distances between the uppermost position of nectar in the corolla tube and floral reproductive organs in female-phase and male-phase flowers, a hawk moth, for instance, would need a proboscis at least 54.5 mm long to access nectar in male-phase flowers and simultaneously come into contact with the stamens, and at least 35 mm long to access nectar in female-phase flowers and come into contact with the stigma (Chapter 1). However, the Discussion section in Chapter 1 also speculates on the possibility of a greater distance between the upper reaches of nectar (the reward) and anthers (pollen) during the male-phase having been selected for by a putative moth pollinator with a shorter proboscis than what is required to reach nectar during the male-phase. Unfortunately, information on proboscis lengths for the moth species on Kaua‘i is unavailable. Proboscis lengths are typically not reported and measuring the length from dried pinned specimens is not something that can be easily and precisely measured (Will Haines, personal communication, February 2015). In 2000, Heddle et al. conducted extensive searches of *T. smaragditis* on Kaua‘i. In the beginning of the Results and Discussion section of their publication (Heddle et al., 2000), it states that “the second specimen was

photographed, and its proboscis was unrolled and measured.” Unfortunately, however, the proboscis length is not listed therein and I have been unable to track down that missing information.

To find out the proboscis lengths of hawk moth species present on Kaua‘i, I recommend starting with a combination of more in-depth literature-based research and personal communication with professional entomologists that made the collections and/or studied the moth species. If these options are unsuccessful, I recommend conducting a survey/collection of the moth species to measure their proboscises. Of course, all necessary permits for such research would need to be obtained. By knowing the proboscis lengths of each moth species, one could narrow down the species to use in a controlled pollination experiment that would test whether any of them could potentially serve as effective pollinators of *B. insignis*. Only those species with proboscis lengths at least 35 mm long need be included. If one or multiple hawk moth species are found to visit and effectively pollinate *B. insignis* flowers, research should assess their host plant species. If a particular hawk moth already present on the island is found to pollinate *B. insignis*, it is possible that the moth is not located within the natural habitat and outplanting areas of *B. insignis* because the host plant of the moth is not in those areas. By knowing the host plant of a potential moth pollinator, a translocation of a moth with the moth’s host plant (if the plant species is native or non-invasive), could help establish a resident population of the moth, which may increase pollination services for *B. insignis*. If a non-native moth is already established on the island, they are likely already occupying their potential niche. In other words, if they do not occur in a particular area, it is likely because the required conditions and/or host plant(s) are not there, as opposed to the moth simply not having yet reached a particular area (Will Haines, personal communication, April 2015). Therefore, it would likely be unnecessary to assess the risk of herbivory the caterpillars of moth species already present on Kaua‘i would pose to *B. insignis* and other nearby native Hawaiian plant species.

The natural habitat of *B. insignis*, which is also home to many other rare, threatened and endangered species, must be protected if there is any chance for natural reestablishment or successful, future *in situ* reintroductions of the species. These habitats encompass Niumalu and Hā‘upu on the southeast coast and Ho‘olulu and Waiahuakua on the Nā Pali coast of Kaua‘i. As

described in the last USFWS 5-Year Review (2007), goats “eliminated much of the native vegetation and destroyed individual plants” (USFWS 2007). They are one of the greatest threats to the species directly and the habitat overall. Feral pigs are also degrading the habitat of *B. insignis* and native vegetation is being outcompeted by invasive plant species such as *Melinis minutiflora*, *Setaria parviflora*, *Sporobolus africanus*, *Lantana camara*, *Psidium cattleianum*, *Psidium guajava*, *Bryophyllum pinnatum*, *Ageratum conyzoides* and *Stachytarpheta* sp. (USFWS 1995). Other direct threats to the plants include rats, slugs and carmine spider mites (USFWS 2007). Recommended management for natural habitat of *B. insignis* includes fencing and subsequent removal and control of invasive animals and weed species within those fenced units. Eradicating goats within future fenced units should be a primary management goal for the recovery of the species. It will also protect many other endangered plant species in those same habitats, and decrease landslides caused by goats. Other federally listed threatened and endangered plants that occur within the natural habitat of *B. insignis* include *Polyscias racemosa*, *Canavalia napaliensis*, *Charpentiera densiflora*, *Peucedanum sandwicense*, *Pittosporum napaliense* and *Pteralyxia kauaiensis* (Michelle Clark, personal communication, March 2015). Goat eradication within fenced units in Hawai‘i has resulted in increased establishment and recovery of native plant species (Baker and Reeser, 1972; Scowcroft and Hobdy, 1987). In the Hono O Nā Pali Natural Area Reserve (NAR) Management Plan, there are plans for replacing strategic fencing and conducting feral ungulate control for Lower Pōhākea, which could provide a safe place for future reintroductions of *B. insignis* (DLNR-DOFAW, 2011; Michelle Clark, personal communication, March 2015). Landslides and falling rocks obviously pose a high risk to hikers on the Kalalau Trail as well, which has increased in usage over the past decade with over double the number of annual trail users in 2009 (209,510 annual trail users) compared to 1998 (104,025 annual trail users) (Martha.E.Yent, Division of State Parks, Department of Land and Natural Resources, personal communication, April 2015).

Finally, if all aforementioned recommendations are successful (e.g., more genetically diverse progeny are produced through a breeding program, appropriate moth pollinators and their host plant(s) on Kaua‘i are found, and natural habitat is fenced with all goats eradicated and other invasive species controlled) and a well thought out reintroduction plan is set-up, plants producing

high quantities of predominantly viable pollen could be outplanted and appropriate moth species and their host plant(s) introduced to the outplanting site(s). Managers should also consider including in potential future reintroduction plans, introducing native and/or non-invasive plant species that may help attract pollinators. For example, although non-native, *Bougainvillea* sp. has been observed to attract moth pollinators in Hawai‘i (Curt Daehler, personal communication, April 2015). It is possible that moths are not being attracted to *B. insignis* because there is not a critical enough mass of plants and flowers to attract them. It will require the careful analysis by numerous conservation managers working together to decide whether the costs of following these recommendations are worth trying, with no guarantee that *B. insignis in situ* restoration will be successful.

Another major consideration when deciding on appropriate habitat for reintroductions of endangered plant species is the future effects of climate change in Hawai‘i. Climate change may affect rare ecosystems and species, and distribution and abundance of invasive species within Hono O Nā Pali NAR by altering rainfall patterns and amounts (DLNR-DOFAW, 2011). Fortini et al. (2013) recently published results of a model-based approach to determine overall climate change vulnerability for Hawaiian plant species, defining vulnerability as “the inability of species to exhibit any of the response necessary for persistence under climate change.” Their assessment found that *B. insignis* is highly vulnerable (vulnerability rank of 0.867, where a rank of 1 indicates the most vulnerable species and a rank of 0 the least) to the impacts of climate change (Fortini et al., 2013). It is difficult to incorporate these projections into future conservation management plans when something needs to be done now and the future microclimates of specific areas cannot be predicted with certainty.

Obviously, all of this would require a considerable amount of time and resources. If it is decided that the costs of following these recommendations are much too high to try to restore *B. insignis in situ* with no guarantee of success, another option is to simply increase and improve *ex situ* conservation. This should be done simultaneously regardless. Although many institutions all over the world have maintained *B. insignis ex situ* for decades, there is much room for improvement. For example, in most cases, provenance data are unavailable and seeds are not stored. There has been recent progress towards increased and improved global collaborations for

*ex situ* plant conservation with the creation of the Global Strategy for Plant Conservation (Sharrock et al., 2014). Since many institutions maintain *B. insignis* in their collections, improving *ex situ* conservation for this species will require a collaboration among many people within many institutions all over the world. Working together towards this will strengthen overall collaborations among institutions, building networks and working relationships that in the future, could increase capacity for improved *ex situ* conservation of other rare, threatened, and endangered species.

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