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Genetic variation in the endemic Hawaiian *Gardenia brighamii*: conservation and horticultural implications

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Introduction

Gardenia brighamii (Rubiaceae – *nanu* or *n'au*) is one of three endemic *Gardenia* species in the Hawaiian Islands. The species was federally listed as endangered in 1985 (U.S. Fish and Wildlife Service, 1985), and with three populations totaling 11 mature individuals remaining in the wild on leeward sides of Lāna'i, and O'ahu (U.S. Fish and Wildlife Service, 2008), is considered in immediate danger of extinction. On Maui, the species is considered extirpated, and the last trees on Molokai were confirmed dead in 2005 (Perlman, 2006). Once an important component of lowland dryland forests on all the main Hawaiian Islands, the species is threatened by the loss of dryland forest habitat, urbanization, invasive plant species, and grazing and browsing domestic and feral animals.

Concern has been raised recently by managers of living collections and horticulturalists as to the identity of a "robust" form of *Gardenia brighamii* in cultivation that has larger flowers and glossy leaves in comparison to herbarium specimens and wild collected individuals in cultivation. Plant propagators have indicated that the robust form of *G. brighamii* more readily takes from cuttings than typical *G. brighamii*. The introduced *Gardenia* species, *G. taitensis* has a superficial resemblance to *G. brighamii* (e.g.,U.S. Fish and Wildlife Service, 1993), and this may have resulted in some taxonomic confusion. *G. taitensis* is highly variable throughout its range (Smith, 1974; Wagner *et al*. 1999b). Indeed, H. St. John (1978, 1979) described a new species, *Gardenia weissichii*, collected from the Ko'olau Mountains, O'ahu, that was later determined to be *G. taitensis* (Wagner *et al*., 1999).

Given the status of *Gardenia brighamii* as endangered or extinct on most of the main Hawaiian Islands, it is essential that the genetic variation within extant individuals of *G. brighamii* be determined and the identity of the robust form be confirmed. *Gardenia brighamii* has significant ornamental and horticultural value, and the robust form is particularly appealing to horticulturalists and landscape architects. This form has been widely distributed and is located in several living collections and botanical gardens. This study uses molecular fingerprinting techniques to help resolve this issue.

Materials and Methods

An initial study of the genetic variation in wild and commonly cultivated *Gardenia* species found in Hawai'i was undertaken in 2002 using the fingerprinting technique known as Randomly Amplified Polymorphic DNAs (RAPDs) (Williams *et al*., 1990). This study, reported here, indicated that a specimen of the robust form was intermediate in genotype between *Gardenia taitensis* and *G. brighamii*. Specimens of the robust and typ-

Table 1. *Gardenia* specimens collected for this study, their collection location, and locality of source material, if known.

	PCMB No.	Collection Location	Original Source Locality	Form
Gardenia augusta				
A1	28	Propagated, O‘ahu		variegated
A2	29	Propagated, O‘ahu		
A3	30	Propagated, O‘ahu		‘White Gem Daisy’
A4	31	Propagated, O‘ahu		‘Winifred’
A5	32	Propagated, O‘ahu		
A6	33	Propagated, O‘ahu		‘Gallery-Augusta’
A7	39	Propagated, Wailalae Iki Ridge, O‘ahu		‘Radicans’
A8	183	Propagated, Maunawili, O‘ahu		‘Pinwheel’
Gardenia brighamii				
L1	36	Kehawai, Lāna‘i	Kehawai, Lāna‘i	typical
L2	43	Kānepu‘u Reserve, Lāna‘i	Kānepu‘u, Lāna‘i	typical
L3	112	Koko Crater Botanic Gardens, O‘ahu	Kānepu‘u, Lāna‘i	typical
L4	1596	Koko Crater Botanic Garden, O‘ahu	Kānepu‘u, Lāna‘i	typical
L5	1597	Koko Crater Botanic Garden, O‘ahu	Kānepu‘u, Lāna‘i	typical
NS1	179	Lili‘uokalani Botanical Garden, O‘ahu	Nānākuli -S, O‘ahu	typical
NS2	180	Lili‘uokalani Botanical Garden, O‘ahu	Nānākuli -S, O‘ahu	typical
NS3	1588	Lili‘uokalani Botanical Garden, O‘ahu	Nānākuli -S, O‘ahu	typical
NS4	1598	Koko Crater Botanic Garden, O‘ahu	Nānākuli -S, O‘ahu	typical
NS5	1603	The Nature Conservancy, Kunia, O‘ahu	Nānākuli -S, O‘ahu	typical
NN	2787	Nānākuli Valley, north branch, O‘ahu	Nānākuli -N, O‘ahu	typical
PK*	1601	The Nature Conservancy, Kunia, O‘ahu	Pu‘u Ku‘ua, O‘ahu	typical
PK*	1602	The Nature Conservancy, Kunia, O‘ahu	Pu‘u Ku‘ua, O‘ahu	typical
PK1	606	Leeward Community College, O‘ahu	Pu‘u Ku‘ua, O‘ahu	robust
PK2	607	Aiea, O‘ahu	Pu‘u Ku‘ua, O‘ahu	robust
PK3	1599	Koko Crater Botanic Garden, O‘ahu	Pu‘u Ku‘ua, O‘ahu	robust
PK4	1606	Propagated, O‘ahu	Pu‘u Ku‘ua, O‘ahu	robust
U1	91	Propagated, O‘ahu	unknown	robust
U2	1593	Big Island Nursery, Hawai‘i	unknown	robust
Gardenia mannii				
GM	1595	Wahiawā Botanic Garden, O‘ahu	O‘ahu	
Gardenia taitensis				
T1	14	O‘ahu		double flower
T2	24	O‘ahu		
T3	35	Wailupe Peninsula, O‘ahu		‘Tiare’
T4	178	Kamehameha Shopping Center, O‘ahu		
T5	181	University of Hawaii at Mānoa, O‘ahu		
T6	182	Maunawili, O‘ahu		

ical *Gardenia brighamii* forms were subsequently collected from the wild, living collections, and from propagators (Table 1), and a second genetic analysis was undertaken using the fingerprinting technique, Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995).

Specimen collection

Young leaves from cultivated *Gardenia augusta* and *G. taitensis* were collected from gardens and plant nurseries around O‘ahu (Table 1). *Gardenia brighamii* was collected from wild individuals at Kānepu‘u, Lāna‘i, and Nānākuli Valley, O‘ahu, and cultivated individuals growing in Queen Liliuokalani and Koko Crater Botanical Gardens, O‘ahu. The majority of the robust individuals of *Gardenia brighamii* were traced to the now extinct Pu‘u Ku‘ua *G. brighamii* mother plant. Cuttings from the mother plant were sampled for the AFLP analysis. A single specimen of *Gardenia mannii* was collected from Wahiawa Botanic Garden. The cultivated voucher of *Gardenia weissichii* historically accessioned at Wahiawa Botanic Gardens was found to have died and could not be included in the analysis. Sufficient genomic DNA for RAPDs analysis was also obtained from herbarium voucher specimens of *G. augusta*, *G. brighamii,* and *G. taitensis* housed in the *Herbarium Pacificum*.

Fresh collected specimens were immediately placed within activated silica. Dried samples were stored at -20 °C. Genomic DNA was extracted from either 6–10 mg dried or 20 mg fresh plant material, ground in 95% ethanol using DNeasy Plant Mini Kits (QIAGEN Inc.) following the recommended protocol. DNA vouchers for the specimens are held within the Pacific Center for Molecular Biodiversity, Bishop Museum, Honolulu, and voucher herbarium specimens are housed within the *Herbarium Pacificum*, Bishop Museum.

RAPDs analysis

Nine ten-mer primers (University of British Columbia) were used in PCR reactions (5´-3´): 153: GAG TCA CGA G; 184: CAA ACG GCA C; 212: GCT GCG TGA C; 218: CTC AGC CCA G; 234: TCC ACG GAC G; 250: CGA CAG TCC C; 265: CAG CTG TTC A; 346: TAG GCG AAC G; 347: TTG CTT GGC G; 391: GCG AAC CTC G; 431: CTG CGG GTC A; 478: CGA GCT GGT C. PCR was performed in a volume of 15 µL containing 2 mM $MgCl_2$, 0.24 µM of each dNTP, 15 ng BSA, 0.36 µM primer, 0.3 ng genomic DNA, and 0.6 unit of *Taq* DNA polymerase (Promega M1861), well mixed and the surface covered with sterile mineral oil (Sigma M3516). DNA amplification was performed in a programmable thermal cycler (MJ Research Inc.) using a thermal cycle of 94 °C (1.5 min) for initial strand separation, then 40 cycles of 38 °C (2 min), 72 °C (2 min), 91 °C (1 min). Two additional steps of 38 °C (2 min) and 72 °C (5 min) were used for final extension. Amplification products were analyzed by electrophoresis (72–75 V, 60 min) in 1.5% agarose gels and detected by staining with ethidium bromide. Bands were scored as present or absent. Similarity measures were computed as presence/absence matches, divided by the maximum observed value for that character over all specimens. The 124 polymorphic loci were analyzed using principal components analysis using NTSYSpc 2.1 (Rohlf, 2000). The scores of each component were statistically compared for each species using one-way analysis of variance. Similarity between each of the individuals within the species was used to compare the variability between and within the species.

AFLP analysis
AFLP band profiles were generated following the protocol of the AFLP Analysis System I (Invitrogen Corp.). Restriction enzymes *EcoR* I and *Mse* I were used to digest 250 ng genomic DNA, and the ligation of adapters and preamplification reactions were performed following the published protocol. Selective amplification was undertaken using with five primer pairs (*Eco*ACC or *Eco*AAG with *Mse*CTA, *Mse*CAT, or *Mse*CAC where *Eco* is 5´GAC TGC GTA CCA ATT C3´ and *Mse* is 5´GAT GAG TCC TGA GTA A3´). The *Eco* primers were fluorescently labeled with WellRED dyes D3-PA or D4-PA (Proligo LLC). The fluorescently labeled amplified fragments were accurately sized using the Beckman-Coulter CEQ8000 genetic analysis system with the inclusion of size standards (DNA Size Standard Kit 400, Beckman-Coulter, Inc.) for calibration. Fragments differing by <1bp were scored as present or absent, and the 156 polymorphic loci were analyzed using principal components analysis using NTSYSpc 2.1 (Rohlf, 2000).

Results
RAPDs analysis
Principal components analysis of 124 RAPDs bands separated the *Gardenia* species in ordinate space, with 36.7% and 26.5% of the variance being explained by the first and second axes, respectively (Figure 1). *G. augusta* was placed at the positive end of the first axis, *G. taitensis* to the negative end of the first axis, and *G. brighamii* at the negative end of the second axis. The average similarity between samples of *G. taitensis* was 88.8 ± 1.5%, and 81.6 ± 1.5% for *G. augusta*. Average similarity between samples of *G. brighamii* was 81.7 ± 2.9%, which increased to 88.6 ± 1.7% with specimen U1, an outlier, removed. The robust *G. brighamii* specimen, U1, grouped most closely with *G. taitensis* with 74.1 ± 0.9% similarity, and only 67.7 ± 1.9% similarity to *G. brighamii*. All specimens of *G. brighamii* were grouped closely, with no obvious distinction between the Lāna‘i and O‘ahu specimens.

AFLP analysis
Cuttings from the extinct Pu‘u Ku‘ua *G. brighamii* (PK*: 1601 & 1602) were different in their AFLP band profile from all the robust forms, with only 65% band similarity. As for the RAPDs analysis, the robust forms of *G. brighamii* fell between *G. brighamii* and *G. taitensis* in their AFLP profiles, having bands unique to both species. *Gardenia manii* and *G. taitensis* had a similarity of 67% and 35%, respectively, to wild collected *G. brighamii* specimens. Similarity within typical *G. brighamii* specimens ranged from 62 to 88% similarity, with specimens from living collections showing the greatest genetic variation (Figure 2).

Discussion
This study indicates that the native *Gardenia brighamii* is genetically discernable from the two most common propagated species in the Hawaiian Islands, *G. augusta* and *G. taitensis*, and the O‘ahu endemic *G. mannii*, using RAPDs and AFLP fingerprinting analyses. The Lāna‘i and O‘ahu populations of *G. brighamii* were very similar in RAPDs banding, with the ‘robust’ specimen, U1, falling between *G. brighamii* and *G. taitensis* in ordinate space. Similarly, all morphologically robust specimens of *G. brighamii* were intermediate between *G. taitensis* and *G. brighamii* in the AFLP analysis. The robust form of *G. brighamii* was traced to seed collected from the last remaining Pu‘u Ku‘ua indivdual formerly located in the Wai‘anae Mountains, O‘ahu. However, analysis of samples from cut-

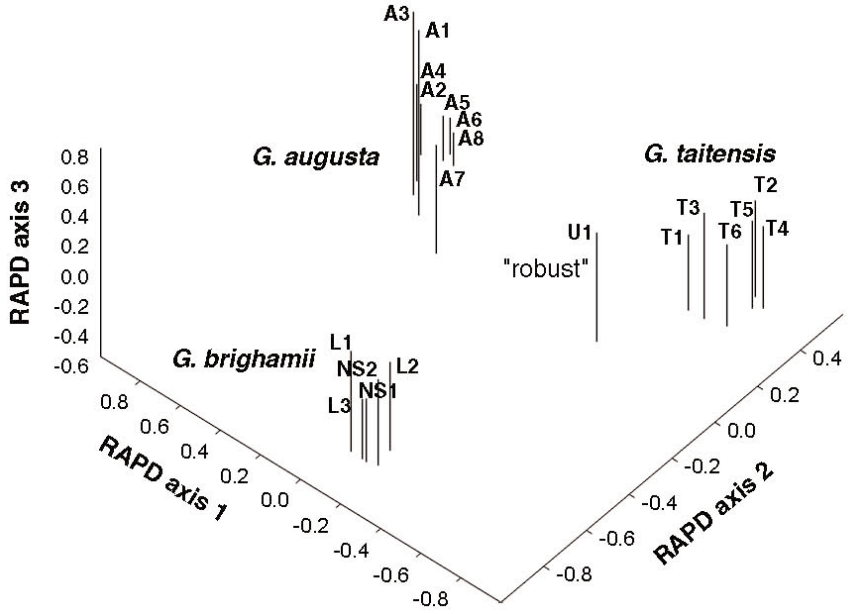

FIGURE 1. Principal Components Analysis of 124 RAPDs bands for specimens of *Gardenia augusta, G. brighamii,* and *G. taitensis*. Specimen details are given in Table 1.

tings directly from the last remaining Pu‘u Ku‘ua mother plant were significantly different in their genetic fingerprint than the robust specimens and had a greater similarity to wild collected specimens from Nānākuli Valley than to the robust individuals supposedly from the same mother plant. Given the data presented here, it appears unlikely that the robust form is true *Gardenia brighamii.* Care must be taken in restoration and augmentation projects to outplant true genotypes that are from the most appropriate population source in order to maintain the genetic integrity of the species. It is also important that the genetic variation within the endangered or extinct wild populations of *G. brighamii* from Moloka‘i, Hawai‘i, and Maui be determined, if specimens in cultivation can be located. This study has shown potential inconsistency between the recorded sources of propagation material and the genetic fingerprints of those individuals within living collections. Maintaining records in living collections as to the true origin and source (cutting vs. seed source) of specimens is critical for the maintenance of the genetic integrity of the species. No phylogenetic studies have, to date, been completed on the *Gardenia* species of the Pacific region, and including samples of *Gardenia taitensis* from throughout its range and other Fijian *Gardenia* species in future studies would help to clarify the ancestry of Hawaiian *Gardenia* species, and the genetic origin of the robust *G. brighamii*.

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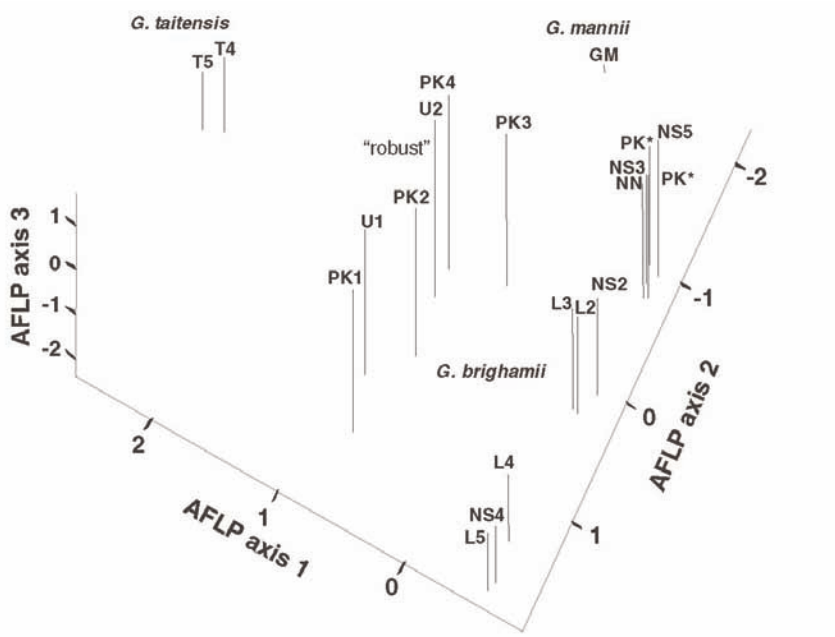

FIGURE 2: Principal Components Analysis of 17 *Gardenia brighamii* specimens from wild and cultivated sources, *G. taitensis* and *G. manii*. 156 polymorphic loci were used in the analysis. Specimen details are given in Table 1.

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New Hawaiian plant records for 2007

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Ongoing fieldwork, collections, and research continue to produce new, previously unpublished distributional records for the Hawaiian flora. In this paper, 4 state or new naturalized records, 48 new island records, 3 notable rediscoveries, and 3 range extensions are reported. Additionally, there are notes on 2 recently described species in the endemic Hawaiian genus *Cyanea*. A total of 59 taxa (11 indigenous) in 30 plant families are discussed. Seven are pteridophytes, 3 are gymnosperms, 27 are dicotyledonous angiosperms, and 22 are monocots. Information regarding the formerly known distribution of flowering plants is based on the *Manual of Flowering Plants of Hawai'i* (Wagner *et al*. 1999a) and information subsequently published in the *Records of the Hawaii Biological Survey* from 1995 through 2007. Distribution and taxonomy of ferns follows *Hawai'i's Ferns and Fern Allies* (Palmer 2003). Voucher specimens are deposited at B.P. Bishop Museum *Herbarium Pacificum* (BISH), Honolulu, with duplicates at the National Tropical Botanical Garden (PTBG), Lāwa'i, Kaua'i. A few specimens may be at only one facility; only in these cases will the herbarium acronym be cited.