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SHORT COMMUNICATION

Inbreeding, low genetic diversity, and spatial genetic structure in the endemic Hawaiian lobeliads *Clermontia fauriei* and *Cyanea pilosa* ssp. *longipedunculata*

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Abstract The endemic lobeliad genera *Cyanea* and *Clermontia* (Campanulaceae) are among the largest in the native Hawaiian flora, and contain large numbers of endangered and threatened taxa. As a baseline for future studies of rare species in this group, we used RAD markers to estimate genetic variation and spatial genetic structure in single populations of two common species, *Cyanea pilosa* ssp. *longipedunculata* on Hawai'i and *Clermontia fauriei* on Kaua'i. We found low to moderate levels of genetic diversity ($H_e \approx 0.13$ and $\pi_{all} \approx 0.0013$ for both species), consistent with studies of several other island plant species, and substantial inbreeding ($F_{is} \approx 0.17$). There was no evidence for spatial genetic structure in *Cyanea*, and in *Clermontia* SGS was weak and restricted to small spatial scales (<10 m). The relative dearth of genetic diversity and high levels of inbreeding in these common lobeliads may reflect pre-existing conditions in this group or selfing in response to a decline in native avian pollinators, raising concerns that inbreeding and loss of genetic variation may be even more severe in rare species of this highly diverse but endangered group.

Keywords *Cyanea* · *Clermontia* · Lobelioids · Spatial genetic structure · Hawaii

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Introduction

Oceanic islands are largely isolated from mainland sources of plant species, but adaptive radiation, repeated founder events, and geographic isolation within and among islands can lead to genetically divergent populations and new species (Carlquist 1970; Price and Wagner 2004; Givnish 2010; Pillon et al. 2014). The same processes of ecological specialization, narrow endemism, and repeated bottlenecks may also, however, create ecological conditions that are favorable for extinction and loss of genetic variation within and among populations (Givnish et al. 1995; Barrett 1996; Givnish 1998, 2010; Kapralov et al. 2013). Limited dispersal can foster genetic differentiation within species at small spatial scales, favoring speciation and repeated, parallel bouts of adaptive radiation, but also result in narrow ranges that make extinction more likely (Givnish et al. 1995; Givnish 1998, 2010). Limited dispersal may thus reduce genetic variation within and among populations of island plants in lineages undergoing extensive diversification, even in species that are moderately abundant.

In the Hawaiian Islands, roughly 1000 native plant species have evolved from 263 successful colonists; the 20 most diverse lineages account for 51 % of the native flora, 61 % of that derived in situ (Wagner et al. 2005; Givnish 2010). The largest clade in the native flora are the endemic Hawaiian lobeliads (Asterales: Campanulaceae), including six genera and ca. 130 species, which have undergone spectacular radiations in growth form, habitat, floral morphology, and leaf shape since colonizing the Hawaiian chain 13 million years ago (Givnish et al. 2009). The two largest genera of lobeliads, *Cyanea* (ca. 80 spp.) and *Clermontia* (22 spp.) are sister to each other, share fleshy fruits, and are native to the interiors and edges/canopies of Hawaiian wet forests, respectively. *Cyanea* is

the largest genus in the native flora and contains more endangered and extinct species than any plant genus native to the US (Center for Plant Conservation 2015). Givnish et al. (1995) hypothesized that understory groups with fleshy fruits, like *Cyanea*, may have limited dispersal due to reliance on understory birds, which are relatively sedentary (Diamond et al. 1976; Moore et al. 2008; Burney and Brumfield 2009). Such plant groups often do speciate extensively (Givnish 1998, 2010; Smith 2001), and in one such group, *Psychotria* (Rubiaceae), genetic differentiation within species does occur at small spatial scales, from roughly 10–100 m (Theim et al. 2014). By comparison, forest-edge birds are often more mobile, so that dependence on them for seed dispersal may lead to less spatial genetic structure.

In this study, we use restriction-associated DNA (RAD) sequencing to assess genetic diversity and spatial genetic structure (SGS) in two common Hawaiian lobeliads: *C. pilosa* ssp. *longipedunculata*, native to wet forest understories on windward Mauna Loa and Kilauea on Hawai'i, and *Clermontia fauriei*, native to wet forest edges and gaps in the Alaka'i Swamp atop Kaua'i. Our underlying hypothesis is that understory *C. pilosa* would exhibit greater SGS than the edge-dwelling *C. fauriei*. These data are among the first generated for Hawaiian plants using next-generation sequencing, and add to a small but growing number of studies of the population genetics of native Hawaiian plants (e.g., Gemmill et al. 1998; Marr and Bohm 1999; Friar et al. 2000; Morden and Gregoritza 2005).

Methods

Study species and sites

Cyanea pilosa ssp. *longipendiculata* (Rock) Lammers is a 1-m tall treelet endemic to the Big Island of Hawai'i. *C. fauriei* H. Lév. is a 2- to 3-m tall shrub, endemic to Kaua'i. Both lobeliads have fleshy fruits and small flowers visited by honeycreepers and various introduced birds. *Cyanea* is typically found in densely shaded understories, while *Clermontia* is found in open woodlands and forest edges and gaps. Neither is considered endangered or threatened, and indeed are among the most common species of Hawaiian lobeliads remaining. We studied *C. pilosa* in the 'Ola'a Small Tract of Hawaii Volcanoes National Park, in a wet forest (ca. 2900 mm rainfall/year) over young (ca. 300 year) volcanic ash at 1400 m elevation, densely shaded by native tree ferns (*Cibotium glaucum*) with scattered individuals of *Metrosideros polymorpha* persisting from a bout of displacement canopy dieback several decades previously (Stratton 1996). We studied *C. fauriei* in the Na Pali Kona Reserve, mainly to the west of the Pihea Trail near its intersection with the Alaka'i Swamp Trail, in wet forest (ca. 3500 mm rainfall/year) over old (4.1 My) shield volcanoes at 1400 m elevation, shaded by a low, open canopy of *Metrosideros* likely to have been disturbed by Hurricanes Iwa (1982) and Iniki (1992) (Fig. 1). Four parallel transects were run at regular intervals in a relatively flat, spatially uniform portion of the 'Ola'a Tract, while two long transects were run along a narrow ridge supporting *C. fauriei*, flanked by a bog and a steep drop-off, at Na Pali Kona. In both instances, all plants of the focal species within ca. 10 m of the study transects were sampled, although not all plants were successfully genotyped (see Results).

Tissue sampling

Leaf samples of both species were collected in late May and early June 2014. *Cyanea* was sampled along six parallel transects ca. 125 m long and 25 to 50 m apart. *Clermontia* was sampled along two transects paralleling the Pihea and Alaka'i Swamp trails near their intersection. From each individual sampled, we collected a 2.5 cm^2 tissue sample from one fully expanded, undamaged leaf. Samples were wiped with alcohol prep pads to remove surface contaminants, and dried and stored on silica gel at room temperature until use. Locations of sampled plants were measured using a handheld GPS unit (Garmin) or calculated based on distance and compass bearings from central GPS-located points using a precision compass and laser rangefinder (Laser Technologies, Inc.). All locations were converted to universal transverse Mercator (UTM) coordinates.

Library preparation and sequencing

Genomic DNA was isolated from tissue samples using the DNeasy 96 Plant Kit (Qiagen) following the manufacturer's instructions. DNA was prepared for sequencing using the double-digest RAD (ddRAD) protocol described by Peterson et al. (2012). Briefly, individual samples were digested with *HindIII* and *HpaII*, labeled with unique barcodes, pooled, and size-selected for fragments between 350 and 700 bp. The resulting library was sequenced on a single flowcell of the Illumina HiSEq 2500 platform in rapid run mode to generate single-end, 100 bp reads. Following removal of adapter-contaminated sequences using Cutadapt (Martin 2011), samples were demultiplexed using the *process_radtags* program in Stacks v1.09 (Catchen et al. 2013), which also excluded low-quality reads and reads with uncalled bases. SNPs were called separately for each species, using the *denovo_map* program in Stacks v1.27 with a minimum stack depth parameter $m = 3$, maximum distance between stacks $M = 3$, and a maximum distance between catalog loci $n = 2$. Only one SNP per

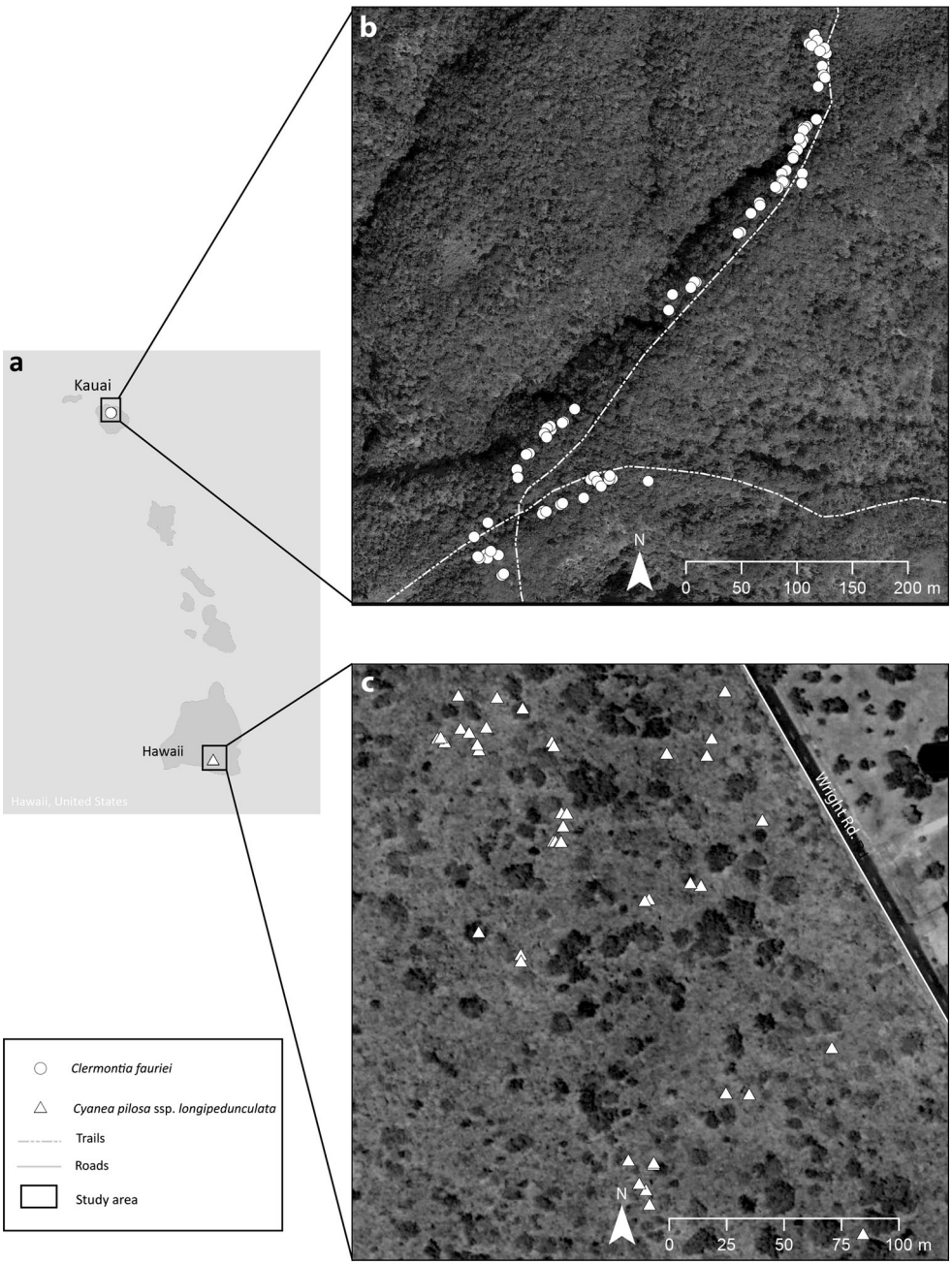

Fig. 1 Maps of the study populations of *Cyanea pilosa* ssp. *longipedunculata* at the ʻOlaʻa Tract on Hawaiʻi (*above*) and of *Clermontia fauriei* along the Alakaʻi and Pihea Trails on Kauaʻi (*below*). Each dot represents a sampled individual. Individuals were sampled along four transects perpendicular to Wright Road in the ʻOlaʻa Tract, and within 10 m of two boardwalks in the Alakaʻi Swamp

RAD locus was exported for analysis (–write_single_snp flag in the *populations* program).

Population genetic analysis

Expected heterozygosity (H_e), observed heterozygosity (H_o), and inbreeding coefficient (F_{is}) were calculated for each species in Stacks v1.27. Nucleotide diversities were calculated for the bases containing the SNPs themselves (π_{snp}) and for all RAD tag positions surveyed (π_{all}). To assess SGS, spatial autocorrelation analysis was performed in SPAGeDi v1.4 (Hardy and Vekemans 2002) by calculating the average kinship coefficient F (Loiselle et al., 1995) for all pairs of individuals within 5 m (*Clermontia* only) or 10 m (*Clermontia* and *Cyanea*) distance classes, with tests of significance based on 20,000 permutations. *Sp*, a measure of SGS intensity (Vekemans and Hardy 2004), was calculated as $Sp = -b_F/(1 - F_{(1)})$, where b_F is the slope of the regression of kinship on ln(distance) and $F_{(1)}$ is the average kinship of the first distance class.

Results

Sequencing produced 186,939,767 reads, 139,555,292 of which remained after cleaning and demultiplexing. Low-coverage samples were dropped, and loci were filtered to retain only biallelic loci with 25 % or less missing data and a minimum minor allele count of 2 to ensure that sequencing error was not misidentified as a SNP. The final dataset contained 40 individuals genotyped at 406 SNP loci for *Cyanea* and 80 individuals genotyped at 655 loci for *Clermontia*.

Population genetic statistics were similar for both species (Table 1). Diversity was moderately low (e.g. $H_e = 0.127$ for *Clermontia* and 0.138 for *Cyanea*; $\pi_{all} = 0.0014$ for *Clermontia* and 0.0012 for *Cyanea*). There was evidence for substantial inbreeding ($F_{is} = 0.167$ for *Clermontia* and 0.182 for *Cyanea*). SGS intensity measured as *Sp* was nearly zero for both species, indicating negligible spatial genetic structure (Table 1); b_F and thus *Sp* for *Cyanea* did not differ significantly from zero based on a t-test ($b_F = 0.00165 \pm 0.00139$ SE). Autocorrelation analysis showed weak but significant SGS in *Clermontia* for interplant distances less than 5 and 10 m, and no significant SGS in *Cyanea* (Fig. 2).

Discussion

Givnish et al. (1995, 2009) proposed that speciation should proceed more rapidly in species dependent on understory birds for seed dispersal, which are more sedentary and less likely to cross habitat barriers than birds native to forest edges and gaps. Support for this hypothesis comes from Smith (2001), who found that genera with fleshy fruits have more species than sister genera with capsular fruits among Neotropical understory plants, and from Theim et al. (2014), who found that four fleshy-fruited species of understory *Psychotria* from Panama exhibited fine-scale genetic structure and short dispersal distances. We therefore expected to observe greater small-scale genetic structure in *C. pilosa* ssp. *longipenduculata* than in *C. fauriei*. Although *Clermontia* showed significant SGS over

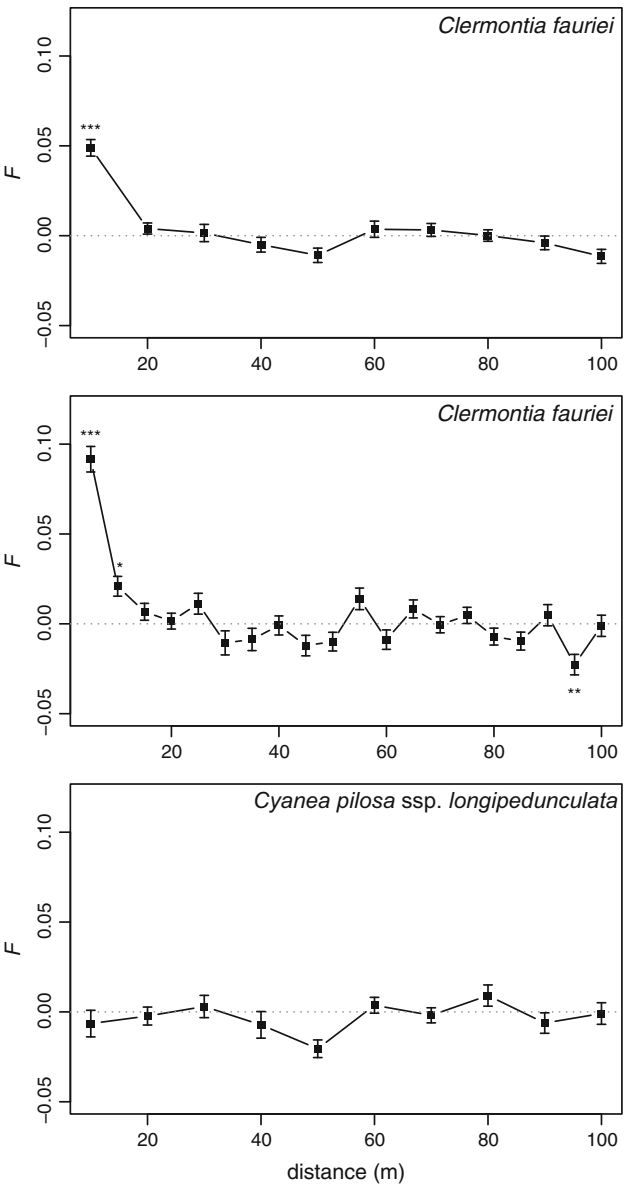

Fig. 2 Spatial autocorrelation of kinship (*F*) in study populations, binned by 5- and 10-m classes in *Clermontia fauriei* (above) and by 10-m classes in *Cyanea pilosa* ssp. *longipedunculata* (below). Points show average ± 1 SE of *F* for pairs within each range of distances. Significant deviations for expectations are indicated by stars: *P < 0.05, **P < 0.01, ***P < 0.001

Table 1 Summary statistics for single populations of *Clermontia fauriei* and *Cyanea pilosa* ssp. *longipedunculata*. Sample size (n), number of SNP loci, observed (H_o) and expected (H_e) heterozygosity, average nucleotide diversity for SNPs used (π_{snp}), nucleotide diversity for all RADtag positions (π_{all}), inbreeding coefficient (F_{is}), and SGS intensity (*Sp*) are shown

	n	loci	H_o	H_e	π_{snp}	π_{all}	F_{is}	*Sp*
Clermontia	80	655	0.106	0.127	0.128	0.0014	0.167	0.006
Cyanea	40	406	0.114	0.138	0.140	0.0012	0.182	−0.002

distances less than 10 m, *Cyanea* did not show any significant SGS, contrary to expectations (Fig. 2). Additionally, both showed very low values of *Sp* relative to other published studies (Vekemans and Hardy 2004; Theim et al. 2014), which for *Cyanea* did not differ significantly from zero. Very low values of *Sp* and a lack of SGS in *Cyanea* might reflect (1) reduced statistical power particularly at short distance classes due to small, low-density populations and sample dropout during genotyping (this especially would have affected the *Cyanea* dataset, which was reduced to $n = 40$ for SGS analyses) (2) limited genetic variation due to possible population crashes associated with pre-existing conditions in the group, or due to canopy dieback or hurricanes a few decades ago (see above); or (3) large-scale movement of fruits by open- or edge-habitat birds after such disturbances. Given that inter-plant distances in the samples for both species have a similar distribution over distances <150 m, it seems unlikely that differences between species in the distributions of sampled plants accounts for the different SGS patterns observed.

Gemmill et al. (1998) quantified isozyme variation in populations of *Brighamia insignis* and *B. rockii* in the only previous study of population genetics in Hawaiian lobeliads. The extent of genetic variation (H_e) within those species was typical of that seen in several other Hawaiian lineages (*Bidens,* silversword alliance, and *Tetramelopium* in Asteraceae, *Pritchardia* in Arecaceae) but low comparable to flowering plants in mainland settings. More extensive comparisons indicate that mainland and insular populations generally do not differ significantly in genetic diversity, except that narrowly distributed island endemics do indeed exhibit low genetic diversity (García-Verdugo et al. 2015). Our findings of moderately low SNP diversity in two endemic Hawaiian lobeliads appear consistent with these trends.

The Hawaiian lobeliads represent the most diverse plant radiation on any single oceanic island or archipelago (Givnish et al. 2009). Many species in this group are at risk or have already gone extinct due to a variety of factors, including loss of habitat and native pollinators (Givnish et al. 1995) including both honeycreepers and endemic ‘o‘o’s (Lammers and Freeman 1986; Fleischer et al. 2008; Pender et al. 2014). Evidence of inbreeding and low genetic diversity in populations of relatively common species of *Clermontia* and *Cyanea* may reflect some pre-existing characteristic of Hawaiian lobeliads, but might well be a result of the known loss of native pollinators. Other species of *Clermontia* and *Cyanea* appear to be mixed-mating and use self-pollination for reproductive assurance when pollinators are excluded (Cory et al. 2015). Given the relatively high inbreeding coefficients found in this study, it seems likely that *C. fauriei* and *C. pilosa* ssp. *longipedunculata* are also mixed mating. Low levels of genetic variation in *C. pilosa* ssp. *longipedunculata* in the ‘Ola‘a Tract may also reflect large fluctuations in population size there associated with massive dieback of canopy *Metrosideros* a few decades previously.

Because we only sampled one population per species, we are unable to determine if the low genetic diversity found is representative of the two species studied. Both *Clermontia* and *Cyanea*, however, were sampled in areas that are actively managed to reduce the impacts of feral pigs and other invasive species, so it is unlikely that they have been affected by the extreme disturbances that would be reducing population sizes in unprotected areas. Expanding this analysis to include other, potentially disturbed populations of *C. pilosa* ssp. *longipeduculata* and *C. fauriei* would increase our confidence in a loss of genetic diversity, possibly due to loss of pollinators or impacts on the canopy. Conservation efforts to maintain these two species may, in response to our findings, place special emphasis on the maintenance of genetic diversity across populations and require intentional cross-pollination to prevent excessive inbreeding and/or self-pollination.

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