Postzygotic barriers isolate sympatric species of *Cyrtandra* (Gesneriaceae) in Hawaiian montane forest understories[1]

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PREMISE OF THE STUDY: Recent reviews of reproductive isolation (RI) in plants propose that boundaries between closely related species are maintained predominantly through prezygotic mechanisms. However, few experimental studies have explored how boundaries are maintained in long-lived species. Hawaiian *Cyrtandra* presents an intriguing challenge to our understanding of RI, as it comprises 60 shrub or small tree species that are almost exclusively restricted to wet forests, where sympatry of multiple species is common.

METHODS: We assessed the relative strengths of pre- and postzygotic barriers among four species of *Cyrtandra* occurring at the extremes of the main Hawaiian Island's natural island-age gradient, Kaua'i (4.7 Myr) and Hawai'i Island (0.6 Myr), to contrast the strengths and stages of reproductive isolation among species at different stages of divergence.

KEY RESULTS: A combination of F_1 seed germination, F_1 seedling survival, and F_1 seedling growth isolated (61–91%) three of the species from sympatric relatives. In contrast, the fourth species was isolated (59%) from its sympatric relative through phenological differences alone. Significant postzygotic barriers in between-island crosses were also observed in one species.

CONCLUSIONS: Results suggest that boundaries between sympatric *Cyrtandra* species in Hawaii are maintained predominantly through postzygotic barriers. Observations from between-island crosses indicate that postzygotic barriers can arise in allopatry, which may be important in the initial divergence of populations. Future studies of RI in *Cyrtandra* should include a broader range of species to determine if postzygotic isolating barriers are foremost in the maintenance of species boundaries in this large genus.

KEY WORDS angiosperms; cross-fertility; hand pollinations; Hawaii; hybridization; islands; reproductive isolation; species boundaries

Experimental studies to date propose that boundaries between closely related plant species are maintained predominantly through prezygotic barriers (reviewed in Rieseberg and Willis, 2007; reviewed in Widmer et al., 2009). These studies suggest that prezygotic barriers arise early during speciation and more effectively

reduce gene flow between plant species than postzygotic isolation (Chari and Wilson, 2001; Ramsey et al., 2003; Kay, 2006; Martin and Willis, 2007; Rieseberg and Willis, 2007; Lowry et al., 2008). Among the range of prezygotic barriers found to separate closely related plant species, ecogeographic isolation (habitat differences that reduce gene flow; sensu Ramsey et al., 2003) appears to be most important, followed by pollinator discrimination, breeding system incompatibility (e.g., absence of hybridization between sympatric outcrossing and selfing species), and divergence in flowering times (Rieseberg and Willis, 2007; Lowry et al., 2008). However, the majority of studies on isolating barriers in plants have focused on short-lived annuals or herbaceous perennials (Rieseberg and Willis, 2007; Lowry et al., 2008; Widmer et al., 2009), with few studies exploring patterns of RI in long-lived species. Long-lived species are typically defined by tremendous fecundity that lasts for many

years, high dispersal capacity, and predominantly outcrossed mating systems (Drake, 1992; Hamrick et al., 1992; Karrenberg et al., 2002; Petit and Hampe, 2006). Given that this suite of life history characters differs from that of most short-lived species, it may be that long-lived species also differ in patterns of RI, with postzygotic barriers playing an important role. While studies to date of RI in long-lived species report a range of pre- and postzygotic barriers (Mosseler, 1989, 1990; Potts and Dungey, 2004; Curtu et al., 2009; Lepais et al., 2013), further work is needed to discern which barriers are most important in species isolation, especially at the early stages of divergence.

Hawaiian *Cyrtandra* J. R. & G. Forster (Gesneriaceae) poses an intriguing challenge to our understanding of the maintenance of boundaries between closely related plant species. *Cyrtandra* in the Hawaiian Islands are remarkably similar in growth form and reproductive morphology, being long-lived perennial shrubs or small trees with white flowers and white fleshy fruits. The mating system in Hawaiian *Cyrtandra* also appears widely conserved. Although species are self-compatible they require a pollinator to set fruit, as autogamy (in which a plant is able to self-fertilize without biotic pollination) has not been observed in *Cyrtandra* (M. Johnson, unpub. data). This, coupled with the protandrous flowers, tendency to gynodioecy in a few species, and the occurrence of interspecific hybrids (see below), all suggest that Hawaiian *Cyrtandra* are strongly outcrossing (Gillett, 1967; Roelofs, 1979; Wagner, 1990; M. Johnson, pers. obs.).

Of the 60 *Cyrtandra* species currently recognized in Hawaii (see Wagner et al., 2005), the vast majority are confined to the understory of wet-to-occasionally mesic upland forests, where up to eight species coexist within a single community (Wagner, 1990), allowing ample opportunity for interspecies hybridization. *Cyrtandra* has the highest occurrence of interspecific hybridization in the Hawaiian flora, with 82 putative hybrid combinations reported to date (Wagner, 1990; K. Wood, personal communication), suggesting that pollinators of this group (likely nocturnal moths; see Gardener and Daehler, 2006) have low species fidelity. Hybrid identification is largely based on morphological intermediacy between the parents, sympatry of the putative parents and hybrid, and occasionally decreased fertility of the hybrid (Wagner, 1990; Luegmayr, 1993); 17 putative interspecies hybrids have been further supported by molecular markers (RAPDs; Smith et al., 1996). Estimates of putative hybrids based on morphological intermediacy in two natural populations suggest that hybridization may be less frequent between sympatric species on the older island of Kaua'i (3% putative hybrids), whereas hybridization between sympatric species on young Hawai'i Island appears to be rampant (52% putative hybrids; M. Johnson, unpub. data). In spite of the evidence of hybridization, 60 species of Hawaiian *Cyrtandra* persist as distinct taxa based on morphological (Wagner, 1990) and genetic characters (Clark et al., 2008, 2009; Pillon et al., 2013a, 2013b), suggesting that at least partial barriers to reproduction exist between species. Furthermore, observed variation among islands in the degree of interspecies hybridization suggests that the strength of reproductive barriers between sympatric species may increase with time since island colonization.

Several lines of evidence support the oldest main island of Kaua'i as the initial colonization point for *Cyrtandra* in Hawaii. Pillon et al. (2013a) found species-diagnostic plastid and nuclear haplotypes in each of the three *Cyrtandra* species examined from Kaua'i (including two species in the current study, *C. kauaiensis* and *C. longifolia*),

while unique haplotypes were not found within species from the younger islands of O'ahu (3.0 Myr) and Hawai'i (0.6 Myr). Greater haplotype diversity on Kaua'i, relative to that on the younger islands, has also been invoked as evidence in support of a "Kaua'i first" colonization hypothesis for Hawai'i Island's dominant native tree genus *Metrosideros* (Percy et al., 2008). A phylogenetic analysis using the internal and external transcribed spacer (*ITS* and *ETS*, respectively) nuclear regions and the *psbA-trnH* chloroplast region also suggest that Kaua'i *Cyrtandra* species are more diverged from each other than are species that co-occur on younger islands (Clark et al., 2009). Lastly, examination of genetic distance at nuclear *ITS* and *ETS* gene regions in two pairs of sympatric species suggests that the Kaua'i species are more diverged from each other than are the Hawai'i Island species (0.02% vs. 0.008%; M. Johnson, unpub. data).

Initial divergence of Hawaiian *Cyrtandra* species may have occurred through isolation of populations following infrequent dispersal events. Long-distance dispersal of *Cyrtandra* seeds by birds is likely rare between islands (see Price and Wagner, 2004). The rarity of dispersal events would lead to isolation of small populations of siblings for long periods until subsequent dispersal events or range shifts due to climate cycles (see Hotchkiss and Juvik, 1999) cause sympatry of morphologically diverged populations (i.e., species). Under this model, geographic isolation of populations would lead to genetic and phenotypic divergence through the accumulation of novel mutations independently in each population, and by natural selection and/or genetic drift (Mayr, 1942, 1963; Turelli et al., 2001; Gavrilets, 2004; Schluter, 2009). Reproductive barriers between geographically isolated populations would then arise as a byproduct of divergence (Dobzhansky, 1936; Rice and Hostert, 1993; Schluter, 2001; Turelli et al., 2001). In this model of speciation, the degree of divergence and strength of reproductive barriers between isolated populations are expected to increase with time since isolation (Mayr, 1947). Upon secondary contact through range shifts (Liou and Price, 1994; Servedio, 2000), RI may be reinforced through the evolution of prezygotic barriers to prevent the production of less-fit hybrids (Dobzhansky, 1937; Coyne and Orr, 1998).

The high rate of endemism within Hawaiian *Cyrtandra* appears consistent with infrequent dispersal events followed by divergence of founder populations, with 57 out of 60 species being single-island endemics. Furthermore, the majority of species have very narrow distributions within islands, such that many species are described as microendemics. If Hawaiian *Cyrtandra* fits a model of species emergence in allopatry, then prezygotic isolation via geographic separation would be the initial barrier to reproduction between diverging populations. The barriers that maintain species boundaries in sympatry, however, remain unknown.

To determine how species boundaries are maintained in this island radiation, we examined the strengths of prezygotic and early stage postzygotic RI among *Cyrtandra* species at the extremes of the main Hawaiian Islands' natural island-age gradient: Kaua'i (4.7 Myr) and Hawai'i Island (0.6 Myr; Clague, 1996). Examination of sympatric species on older Kaua'i and young Hawai'i Island allowed us to contrast RI between species that are at different stages of divergence, have likely coexisted in sympatry for different periods of time, and exhibit varying degrees of interspecies hybridization. Specifically, we estimated the relative and absolute contributions of premating prezygotic barriers (geographic isolation and flowering phenology), postmating prezygotic barriers (pollen tube growth), and postzygotic

barriers (fruit set, number of germinants per fruit, and F_1 seedling survival and growth to six months) to total RI. In addition, heterospecific crosses between plants on Kaua'i and Hawai'i Island were done to reveal if postzygotic barriers to gene flow have formed between species in allopatry, which may play an important role in the initial divergence of species in this group.

MATERIALS AND METHODS

Study System—*Cyrtandra* is the largest genus in the Gesneriaceae family, with over 800 species distributed across Southeast Asia and the Pacific (Atkins et al., 2013). The 60 *Cyrtandra* species currently recognized in Hawaii form a monophyletic group that derives from a single introduction (Cronk et al., 2005), likely from the Fijian Region (Clark et al., 2009). We used the 4-million-year age gradient of the main Hawaiian Islands to investigate reproductive barriers among four species of Hawaiian *Cyrtandra* with study sites located on Kaua'i (4.7 Myr) and Hawai'i Island (0.6 Myr; Clague, 1996). Study sites were selected based on the following criteria: (1) occurrence on the

oldest or youngest main Hawaiian Island, (2) accessibility, and (3) relative similarity of ecosystem types (i.e., wet-to-mesic montane forests dominated by native species). The Kaua'i study site was located on the northwest side of the island in Kōke'e State Park at 1250 m elevation in montane mesic forest habitat (Fig. 1). The Hawai'i Island study site was located on the east side of the island in the Upper Waiākea Forest Reserve (UWFR) at 975 m elevation, which is at the lower limits of montane wet forest habitat (Fig. 1).

Species examined in this study were chosen based on their conservation status (apparently secure; see Wagner et al., 2005), occurrence at one of the two study sites, a population size of at least 25 reproductive individuals, and morphological distinctness (see Fig. 2 for characters used to distinguish species). *Cyrtandra longifolia* (Wawra) Hillebr. ex. C. B. Clarke, and *C. kauaiensis* Wawra are both endemic to Kaua'i, while *C. platyphylla* A. Gray occurs on Maui and Hawai'i Island, and *C. paludosa* Gaud. var. *paludosa* is found on Kaua'i, O'ahu, Maui, and Hawai'i Island (Wagner, 1990; Price et al., 2012). While *C. paludosa* var. *paludosa* occurs in Kōke'e State Park on Kaua'i, we did not observe individuals of this species to be sympatric with the population of *C. longifolia* and *C. kauaiensis* studied
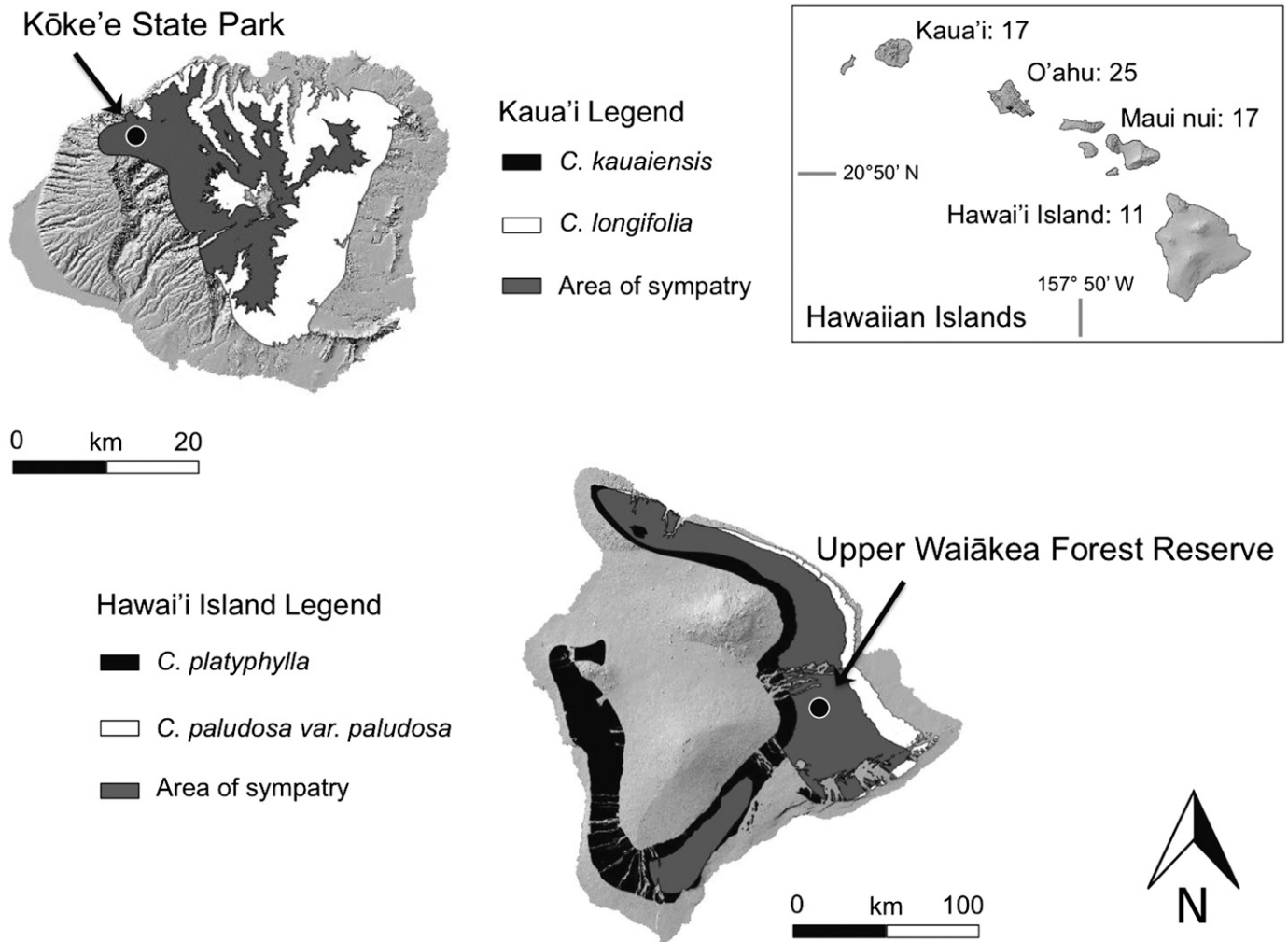

FIGURE 1 Location of study sites on Kaua'i (Kōke'e State Park) and Hawai'i Island (Upper Waiākea Forest Reserve). *Cyrtandra longifolia* is sympatric with *C. kauaiensis* across 44% of its range on Kaua'i, while *C. platyphylla* is sympatric with *C. paludosa* var. *paludosa* across 59% of its range on Hawai'i Island. Inset map of the main Hawaiian Islands shows the number of species occurring on each island.
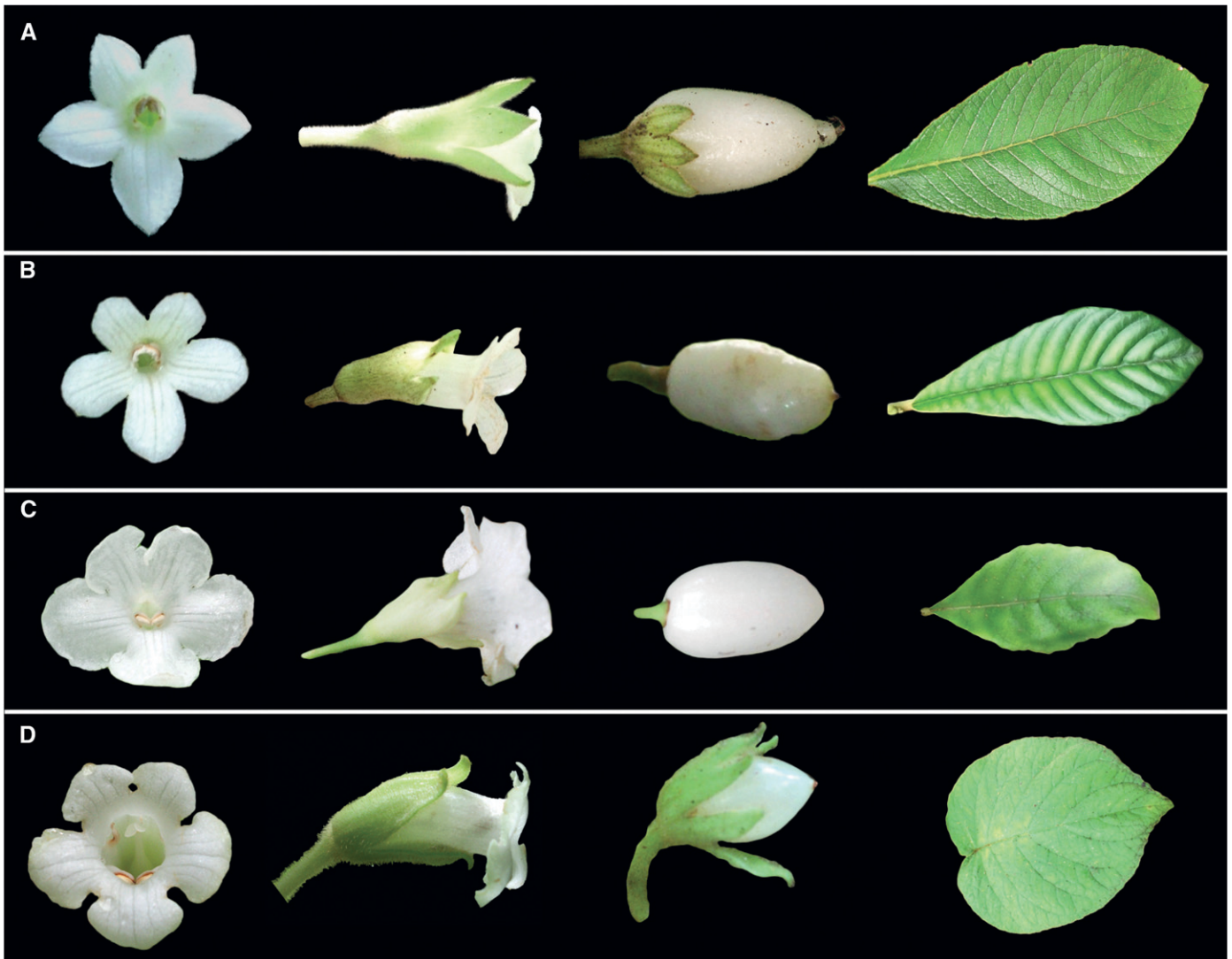
FIGURE 2 Floral and vegetative characters used to identify the four Hawaiian *Cyrtandra* study species in the field: (A**)** *C. kauaiensis*, (B) *C. longifolia*, (C) *C. paludosa* var. *paludosa*, and (D) *C. platyphylla*. From left to right: corolla shape, calyx symmetry, calyx persistence on fruits, and leaf shape and pubescence.

here (see Appendix 1 for voucher information), although we acknowledge that these populations may have been sympatric in the past. The following putative hybrid combinations (direction of cross unknown) have been reported among these four species: *C. longifolia* × *C. kauaiensis*, *C. kauaiensis* × *C. paludosa* var. *paludosa*, and *C. platyphylla* × *C. paludosa* var. *paludosa*. Ideally, only single-island endemic species would have been included in our study, because a broad distribution may influence the strength and stages of RI of a species in unpredictable ways. However, *C. platyphylla* and *C. paludosa* var. *paludosa* were chosen because all other endemic species on Hawai'i Island are rare, making it difficult to acquire the desired population size of 25 reproductive individuals. Cytologically, all species examined have a diploid chromosome number of 34 (Kiehn, 2005), and there is no evidence to date of polyploidy in the group.

Premating prezygotic isolation: geography—Geographic isolation between species occurring on the same island was quantified using range maps produced by Price et al. (2012), which are derived from data on the distribution of species by geographic region, major habitat type, and elevation range (Price, 2004) in the context of corresponding geographic information system (GIS) layers. Quantum GIS v. 1.8.0 (QGIS Development Team, 2012) was used to calculate the maximum potential range size from each species map. Although *C. platyphylla* and *C. paludosa* var. *paludosa* have multi-island distributions, only the area occupied by each species on Hawai'i Island was considered for the purposes of this study. We chose to exclude *C. paludosa* var. *paludosa* from Kaua'i because of the following: (1) this species was not observed in sympatry with the population studied here, and (2) range maps are not available for this particular variety (i.e., range maps are only available for *C. paludosa* as a species, yet *C. paludosa* var. *microcarpa* and *C. paludosa* var. *paludosa* are known to have very different distributions on Kaua'i). Geographic isolation between sympatric species was calculated using the methods described in Sobel and Chen (2014) with the equation:

$$RI = 1 - \left(\frac{S}{S+U} \right). \tag{1}$$

The proportion of space shared (S) by the respective pairs of species on Kaua'i and Hawai'i Island was equal to the area of overlap between the two species ranges. The proportion of unshared space (U) was equal to the area occupied by a single species, and this calculation was performed separately for each of the four respective species. Geographic isolation was assumed to be complete for species on different islands.

Premating prezygotic isolation: phenology—To assess the possibility of prezygotic isolation among sympatric species via divergence in flowering phenology, 25 haphazardly selected adults of each species were censused monthly over a 16-mo period. The tall stature and small flower size of some species precluded quantification of the number of flowers per individual; therefore, only presence/absence of open flowers on each individual was noted. The significance of flowering time overlap was assessed using a Student's paired t test, in which the percentage of sympatric individuals flowering each month was compared between species. We used the general method for calculating reproductive isolation outlined in Sobel and Chen (2014) to estimate prezygotic isolation via flowering time differences between sympatric species:

$$RI = 1 - 2 \times \left(\frac{H}{H+C} \right). \tag{2}$$

Here, both heterospecific (H) and conspecific (C) matings are taken into account, with H = % heterospecific flowering individuals, and C = % conspecific flowering individuals. For this particular barrier, we calculated RI for each month individually and averaged across months to obtain an estimate of mean RI.

Postmating prezygotic isolation: pollen tube growth—We quantified postmating prezygotic isolation between sympatric species by measuring in the field pollen tube growth following single-donor conspecific and heterospecific crosses. For each of the four species, 10 haphazardly selected flowering adults served as maternal plants, and 10 served as pollen donors for sympatric conspecific (control) and sympatric heterospecific crosses. The lifespan of a *Cyrtandra* flower is generally seven days, with the stigma being receptive upon exposure of the two stigma lobes on the fourth day until senescence (Roelofs, 1979; M. Johnson, pers. obs.). Fine-mesh pollinator exclusion bags were placed over flowers prior to stigma receptivity, and were replaced after hand pollinations to prevent pollinator visitation. Anther emasculation was not necessary because of the temporal separation of stigma and anther maturation (species are strongly protandrous, and do not set fruit when flowers are bagged to exclude pollinators; Gillett, 1967; Roelofs, 1979; M. Johnson, unpub. data).

Pollen was applied to receptive stigmas using flat wooden toothpicks; pollen loads were standardized by saturating the receptive stigma surface with all the pollen grains from both anthers of the male donor flower. For each maternal plant, one flower was hand-pollinated with sympatric conspecific pollen (N = 10), and one flower with sympatric heterospecific pollen (N = 10). Styles were excised four days postpollination and preserved in 70% ethanol.

Styles were then stained with decolorized aniline blue (0.03% in 0.1 M K_2HPO_4) for 24 h following the method of Kearns and Inouye (1993), and viewed with a fluorescent microscope. Pollen tube growth was estimated as the density of pollen tubes nearest the ovary (low, medium, high), as well as the length of the pollen tube bundle for each sample. Pollen tube lengths were compared between sympatric conspecific and heterospecific crosses within each maternal species using nonparametric Mann-Whitney U-tests. We calculated RI using Eq. (2) above (see Sobel and Chen, 2014), where H = mean length of the longest heterospecific pollen tube, and C = mean length of the longest conspecific pollen tube.

Postzygotic isolation: fruit set—To assess the importance of early acting postzygotic barriers, a second set of hand-pollinations was conducted on the four species both within and between sites on Kaua'i (K) and Hawai'i Island (H) from March 2010 to October 2011. Sympatric species crosses were performed between *C. longifolia* (K) and *C. kauaiensis* (K), and between *C. platyphylla* (H) and *C. paludosa* var. *paludosa* (H). Interisland allopatric species crosses involved all four species. Hand-pollinations were also performed between sympatric conspecific individuals of each of the four species to serve as controls. Conspecific crosses involved cross-pollinating individuals of the same species occurring in the same population (no self-pollinations were used for this cross type). Because of the associated difficulties of performing a large cross-pollination experiment within and between islands (e.g., availability of flowers, transporting pollen between sites, timing of anthesis and stigma receptivity, insect predation, etc.) our target sample size was N = 10 per cross type.

Individuals of each species were selected for the crossing study based on the timing and number of flowers produced, with plants added to the study as flowers became available. The limited number of reproductive individuals at each site (27 *C. kauaiensis*, 40 *C. longifolia*, 35 *C. platyphylla*, and 50 *C. paludosa* var. *paludosa*) prevented a fully independent crossing design such that we were unable to use different maternal and paternal plants for each of the three cross types (this would have required 80 reproductive individuals per species for bidirectional crosses). Given this limitation, our initial aim was to use the same 20 maternal plants of each species in the three cross types, with each maternal plant receiving pollen from one sympatric conspecific donor, one sympatric heterospecific donor, and two allopatric heterospecific donors. However, because of the low number of flowers produced at any given time per plant (typically 1–2), not all maternal plants received pollen from all four donors, or were involved in all possible cross types. The high incidence of insect predation on flowers and fruits (see results) also prompted an attempt to maximize hand-pollination opportunities, which sometimes resulted in using the same maternal and/or paternal plant multiple times for a given cross type. However, for all statistical analyses, only independent crosses were included, such that a given plant was used only once as a pollen donor or pollen recipient for each cross type. Lastly, because *C. longifolia* is gynodioecious (female and hermaphroditic individuals coexist in the same population), and female plants may have higher fertility than hermaphroditic plants, we selected 50% female individuals and 50% hermaphroditic individuals as maternal plants.

All cross types were performed in both directions such that each species provided and received pollen. Each hand-pollination involved pollen from a single donor flower and one receptive flower per maternal plant; to standardize pollen loads and ensure maximum

fertilization, the stigma surface was completely saturated with all of the pollen grains from both anthers of the male donor flower. Flowers from pollen donors were collected on the first day of anthesis and stored at 4°C until maternal flowers were receptive. Storage at 4°C delayed floral senescence, keeping pollen viable until it could be transported between study sites. Viability tests were performed on transported pollen for all four species as follows. Five donor flowers were collected from each species on the first day of anthesis and stored at 4°C. Pollen from donor flowers was applied to five receptive stigmas per species at 1, 2, 3, 4, and 5 d after collection. Styles from pollinated flowers were collected four days postpollination, stained with a decolorized aniline blue solution, and examined under a fluorescent microscope. Pollen tube length and density were compared among the five treatments for each species, and the results (not presented) showed vigorous pollen tube growth in all treatments, indicating that pollen was viable for a *minimum* of five days after collection. To ensure maximum pollen viability, all hand-pollinations were done within five days of donor flower collection.

All hand-pollinated flowers from this second set of pollinations were monitored for fruit set. Fruits were collected when mature (white in color), and time to maturation was recorded in days. Binary logistic regression was used to test for differences in fruit set (yes/no) among conspecific, sympatric heterospecific, and allopatric heterospecific crosses, and a Kruskal-Wallis test was used to test for differences in fruit maturation time among cross types. To calculate RI, we again used Eq. (2) above (see Sobel and Chen, 2014). For this particular barrier, H = % heterospecific fruit set, and C = % conspecific fruit set.

Postzygotic isolation: number of germinants per fruit—Given the extremely small size of the seeds (0.2–0.6 mm in length) and high number of seeds per fruit (potentially thousands in some crosses), we were unable to assess percent seed viability or percent seed germination. However, preliminary germination trials using the four study species showed that seeds assumed to be inviable based on appearance (brown, shriveled) failed to germinate in all cases, while seeds that were assumed to be viable (tan, round) exhibited consistently high germination, ranging from 87–100% across all species (M. Johnson, unpub. data). Given these preliminary data on seed viability, we chose to compare the total number of germinants per fruit between crosses as estimates of postzygotic RI.

For each of the three cross types, one mature fruit per maternal plant was scored for the number of germinants/fruit at the University of Hawai'i at Hilo Agricultural Farm located in Pana'ewa, East Hawai'i Island. Seeds from experimental fruits were isolated from the fleshy tissue and surface sown into pots filled with moist sphagnum moss. For each fruit, all of the seeds were randomly distributed between two 3 × 4-cm pots. Pots were arranged haphazardly in trays and placed in a mist house set to water every 10 min for 5 s during daylight hours. After eight weeks, trays were moved to greenhouse benches set to water three times per day for 5 min. Trays were rotated among benches every two weeks in both the mist house and greenhouse to avoid area-specific effects on seedling survival and growth. Trays were kept under 80% shade cloth to approximate forest understory light levels, and all seedlings were fertilized with a 15:30:15 foliar feed once per month. The total number of germinants per fruit was recorded weekly for 12 wk.

A Kruskal-Wallis test was used to compare the mean number of germinants per fruit at 12 wk for conspecific, sympatric heterospecific, and allopatric heterospecific crosses, followed by Mann-Whitney

U-tests to assess differences between cross types. Given the difficulty of acquiring a sample size of 10 mature fruits for each cross type (see comments on insect predation under results for fruit set), and the fact that we attempted two distinct allopatric cross types per maternal species, we later chose to pool results from the two allopatric heterospecific crosses for each maternal species. We felt this was justified given that the results of the two allopatric heterospecific crosses were highly similar for each maternal plant. To calculate RI we used Eq. (2) above (see Sobel and Chen, 2014). Here, H = mean number of heterospecific germinants per fruit, while C = mean number of conspecific germinants per fruit.

Postzygotic isolation: survival and growth—The proportion of surviving seedlings in each family was recorded six months after the seeds were sown. Also at six months, 25 seedlings from each family were haphazardly selected from the pool of larger seedlings for height measurements. Separate Kruskal-Wallis tests were used to assess variation in survival and height among cross types, followed by post hoc comparisons of means with Mann-Whitney *U*-tests. All statistical analyses were performed in the R statistical environment (R Core Team, 2014). To calculate postzygotic RI via differences in survival and growth between sympatric species, we used Eq. (2) above (see Sobel and Chen, 2014). For survival, H = % of surviving heterospecific seedlings at six months, while C = % of surviving conspecific seedlings at six months. For growth, H = mean heterospecific seedling height at six months, and C = mean conspecific seedling height at six months.

Total reproductive isolation—The individual strength of each reproductive isolating barrier was estimated independently for each of the four maternal species; this standardized measure of RI can vary from 1 (complete isolation), to 0 (random mating), to −1 (heterospecific gene flow is facilitated). The absolute contribution of each individual barrier to total isolation was calculated such that each isolating barrier reduces gene flow that has not already been inhibited by earlier mechanisms of reproductive isolation (Coyne and Orr, 1989; Kay, 2006). Total reproductive isolation was then calculated as the sum of all absolute contributions. Absolute contributions of each barrier and total isolation were calculated with (1) geographic isolation removed to represent the potential for gene flow in sympatry, and (2) all barriers in the analysis. In addition, total RI was estimated only for sympatric species, because RI between allopatric species was assumed to be complete (i.e., such species are geographically isolated).

RESULTS

Geography—Analysis of the maximum potential range of each species revealed that on Kaua'i, *C. longifolia* (615.63 km^2) is sympatric with *C. kauaiensis* (268.78 km^2) across 44% of its range. In contrast, the entire area occupied by *C. kauaiensis* is within the range of *C. longifolia*, such that *C. kauaiensis* exhibits a complete lack of geographic isolation. *Cyrtandra longifolia* has a broad distribution that extends across the majority of upland Kaua'i, while *C. kauaiensis* appears to be restricted to eastern/central mountainous regions (Fig. 1).

On Hawai'i Island, *C. platyphylla* has a broad distribution covering 3667.67 km^2 across the east and west sides, and reaches slightly higher elevations relative to *C. paludosa* var. *paludosa*. In contrast,

C. paludosa var. *paludosa* has a smaller range (2559.55 km^2) and is restricted predominantly to the east side of Hawai'i Island, although it extends its range slightly below that of *C. platyphylla* into lower elevation wet forests (Fig. 1). *Cyrtandra platyphylla* is sympatric with *C. paludosa* var. *paludosa* across 59% of its range, while *C. paludosa* var. *paludosa* is sympatric with *C. platyphylla* across 84% of its range.

***Phenology*—**On Kaua'i, *C. kauaiensis* and *C. longifolia* exhibited considerable overlap in flowering phenology throughout the 16-mo observation period (Fig. 3A). The percentage of individuals flowering was not significantly different between these two species (two-tailed; $t = -0.82$, df = 12, $P = 0.43$). In contrast, the percentage of flowering individuals of *C. platyphylla* and *C. paludosa* var. *paludosa* on Hawai'i Island was significantly different throughout the observation period (two-tailed; $t = 4.62$, df = 15, $P < 0.001$), with *C. platyphylla* only flowering for 4–6 mo out of the year (Fig. 3B). However, both species exhibited a peak in flowering during the drier months (June–August) over two consecutive years (Fig. 3B).

***Pollen tube growth*—**Pollen tube growth was examined in conspecific and sympatric heterospecific crosses in each maternal species, except *C. paludosa* var. *paludosa,* which showed no difference in the number of germinants per fruit among cross types (high in all crosses) and therefore was not expected to show reduced pollen tube growth in any cross. Reduced sample sizes for *C. longifolia* and *C. platyphylla* were due to damaged experimental flowers caused by unidentified insect larvae, which were observed immediately prior to flowers falling from the maternal plant. However, for those flowers that remained intact, there were no differences in pollen tube growth among cross types; pollen tube bundles reached the base of the style at day two and grew into the ovary by day four. Pollen tube density was uniformly high for all crosses (Appendix S1; see Supplemental Data with the online version of this article). The length of pollen tube bundles did not differ between sympatric heterospecific and sympatric conspecific crosses for any of the three maternal species (*C. kauaiensis*: 11.47 vs. 12.53 mm, $N = 10$ sympatric heterospecific and 10 conspecific pollinations, $W = 126.0$, $P = 0.12$; *C. longifolia*: 10.61 vs. 10.62 mm, $N = 7$ sympatric heterospecific and 6 conspecific pollinations, $W = 42.5$, $P = 1.00$; *C. platyphylla*: 15.33 vs. 14.65 mm, $N = 4$ sympatric heterospecific and 4 conspecific pollinations, $W = 17.0$, $P = 0.89$).

***Fruit set*—**Of the 368 hand-pollinations that were done, only 116 resulted in fruit set. Thirteen of these mature fruits were randomly selected for removal from the study because of nonindependence, leaving a total of 103 mature fruits produced from 103 unique crosses. Fruits that had fallen from the maternal plant before reaching maturity were recorded as aborted. In all cases of fruit abortion on both Kaua'i and Hawai'i Island, unidentified insect larvae and/or holes made by insect larvae were seen in the fruits and/or pedicels immediately prior to abortion from the maternal plant. In contrast, mature fruits showed no signs of insect predation. Thus, insect predation was implicated as the cause of fruit abortion in all cases (although we did not test this experimentally). Additionally, 3 of the 20 *C. longifolia* maternal plants were removed from the study because of damage from feral ungulates, resulting in lower sample sizes for cross types involving this species. The proportion of unique hand-pollinations yielding mature fruits was examined to determine if there was any difference in fruit set among cross types. Fruit set ranged from 9–61% across species, but did not differ among cross types for any of the four species examined (*C. kauaiensis*: $G_2 = 3.37$, $P = 0.19$; *C. longifolia*: $G_2 = 5.18$, $P = 0.08$; *C. paludosa* var. *paludosa*: $G_2 = 1.16$, $P = 0.56$; *C. platyphylla*: $G_2 = 0.67$, $P = 0.72$; Fig. 4). Mean fruit maturation time also did not differ among cross types, although the range across species was 125–282 d (Appendix S2).

***Number of germinants per fruit*—**For both Kaua'i species, the mean number of germinants per fruit at 12 wk was significantly lower for sympatric heterospecific crosses than for conspecific crosses (*C. kauaiensis*: 374 vs. 1126 germinants/fruit, $N = 11$ sympatric
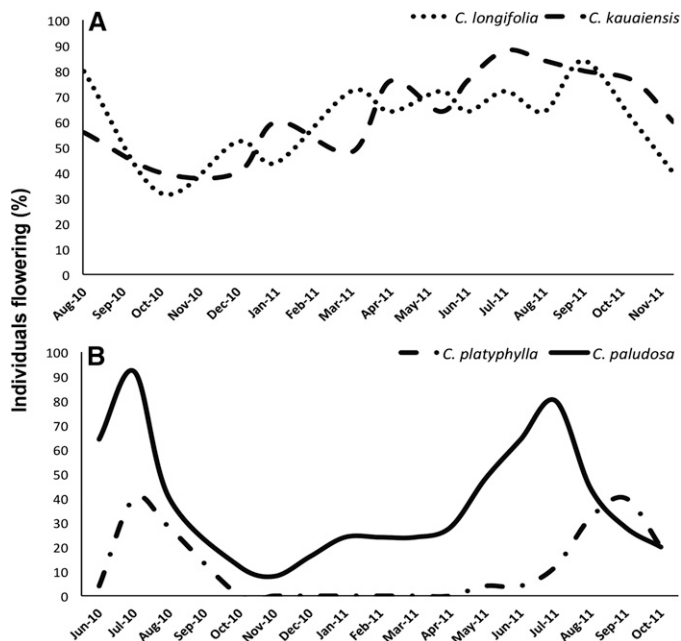

FIGURE 3 Flowering phenology for Kaua'i (A) and Hawai'i Island (B) *Cyrtandra* species over a 16- to 17-mo period from 2010–2011.
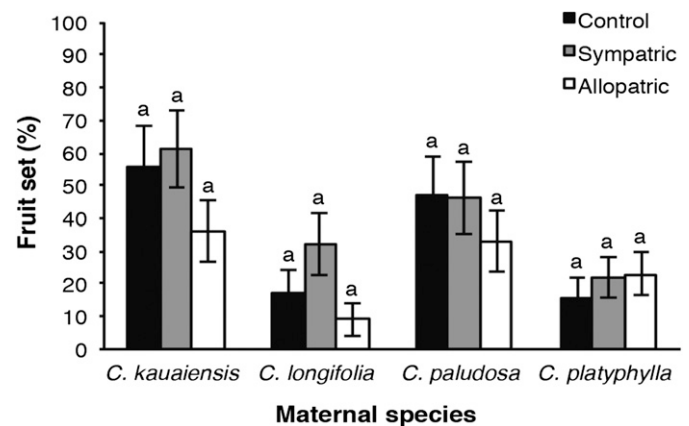

FIGURE 4 Percentage (mean ±1 SE) of hand-pollinated flowers resulting in mature fruits for sympatric conspecific (control), sympatric heterospecific, and allopatric heterospecific crosses involving *Cyrtandra* species on Kaua'i (*C. kauaiensis* and *C. longifolia*) and Hawai'i Island (*C. paludosa* and *C. platyphylla*). Within each graph, lowercase letters represent significant Mann-Whitney differences in means.

heterospecific and 10 conspecific pollinations, $W = 91$, $P = 0.01$; *C. longifolia*: 38 vs. 141 germinants/fruit, $N = 8$ sympatric heterospecific and 5 conspecific pollinations, $W = 34$, $P = 0.04$; Fig. 5). For both species, the majority of fruits resulting from sympatric heterospecific crosses was observed to have a high number of inviable seeds (brown, shriveled), while fruits from all conspecific crosses contained only viable seeds (tan, round). In contrast, mean germination at 12 wk in allopatric heterospecific crosses was at least as high as that for conspecific crosses (*C. kauaiensis*: 1484 vs. 1126 germinants/fruit, $N = 10$ allopatric heterospecific and 10 conspecific pollinations, $W = 64$, $P = 0.32$; *C. longifolia*: 156 vs. 141 germinants/fruit, $N = 3$ allopatric heterospecific and 5 conspecific pollinations, $W = 6$, $P = 0.79$; Fig. 5).

For *C. paludosa* var. *paludosa* the mean number of germinants per fruit did not vary among crosses ($N = 10$ sympatric heterospecific, 10 allopatric heterospecific, and 9 conspecific pollinations, $H_2 = 0.55$, $P = 0.76$; Fig. 5). At 12 wk, the mean number of germinants/fruit ranged from 388–477 for all cross types. For *C. platyphylla*, the mean numbers of germinants/fruit at 12 wk were significantly lower for sympatric heterospecific crosses (382, $N = 10$; $W = 61$, $P = 0.01$) and allopatric heterospecific crosses (472, $N = 10$; $W = 7$, $P = 0.007$) relative to conspecific crosses (1261, $N = 7$; Fig. 5). Nine out of 20 heterospecific crosses (six sympatric and three allopatric) to *C. platyphylla* produced fruits that contained mostly inviable seeds (brown, shriveled).

Survival and growth—Seedling survival at six months did not vary among sympatric heterospecific, allopatric heterospecific, and conspecific cross types for either Kaua'i species (*C. kauaiensis*: $H_2 = 3.28$, $P = 0.19$; *C. longifolia*: $H_2 = 1.30$, $P = 0.52$; Fig. 6). Seedlings from allopatric heterospecific crosses (mean ± SE; 4.33 ± 0.75 cm) were significantly taller than sympatric heterospecific (1.26 ± 0.54 cm; $W = 6$, $P = 0.008$) and conspecific-cross seedlings (1.91 ± 0.37 cm; $W = 13$, $P = 0.02$) when *C. kauaiensis* was the maternal plant (Fig. 7). Six-month seedling heights for crosses involving *C. longifolia* as a maternal plant revealed no difference among cross types ($H_2 = 0.75$, $P = 0.69$; Fig. 7).
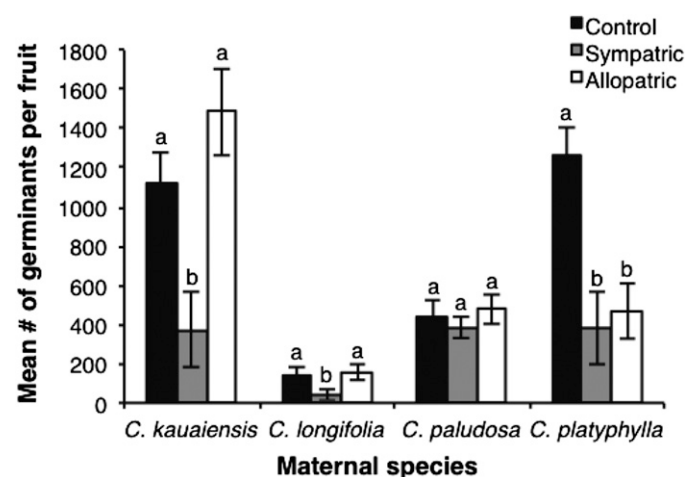

FIGURE 6 Percent survival (mean ±1 SE) of Hawaiian *Cyrtandra* seedlings over a six-month period from sympatric conspecific (control), sympatric heterospecific, and allopatric heterospecific crosses. Within each graph, lowercase letters represent significant Mann-Whitney differences in means.
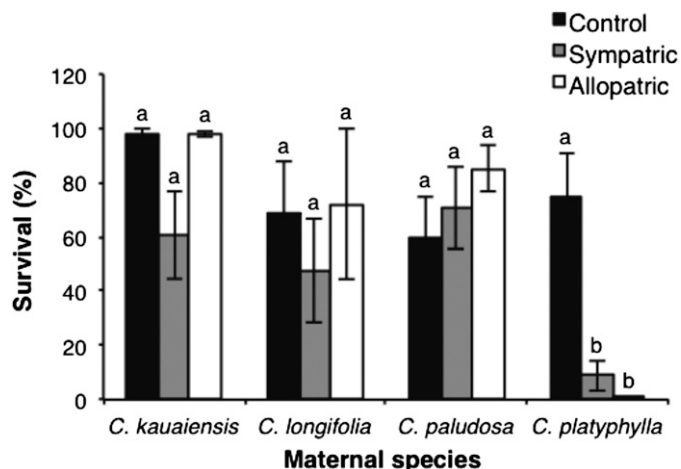
Seedling survival and growth at six months did not differ among sympatric heterospecific, allopatric heterospecific, and conspecific crosses involving *C. paludosa* var. *paludosa* as a maternal plant (Survival: $H_2 = 0.87$, $P = 0.65$, Fig. 6; Growth: $H_2 = 2.75$, $P = 0.25$, Fig. 7). In stark contrast, most of the hybrid seedlings produced from heterospecific crosses (both sympatric and allopatric) with *C. platyphylla* as the maternal plant failed to produce any true leaves (i.e., only the two cotyledons were evident; Fig. 8). Relative to conspecific crosses, seedlings from sympatric heterospecific crosses ($W = 42$, $P = 0.02$) and allopatric heterospecific crosses experienced significantly greater mortality by six months ($W = 1$, $P < 0.001$; Fig. 6). Furthermore, the hybrid seedlings from sympatric and allopatric heterospecific crosses that produced true leaves exhibited a nonsignificant trend of
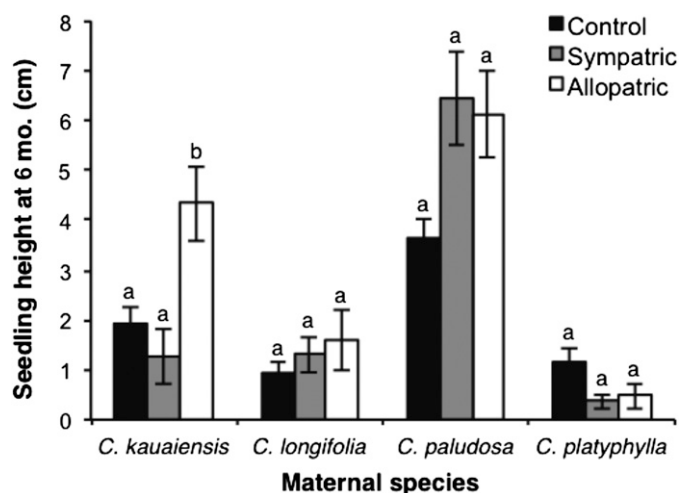

FIGURE 5 Mean (±1 SE) number of germinants per fruit at 12 wk for Kaua'i (*C. kauaiensis* and *C. longifolia*) and Hawai'i Island (*C. paludosa* and *C. platyphylla*) *Cyrtandra* species following sympatric conspecific (control), sympatric heterospecific, and allopatric heterospecific crosses. Within each graph, lowercase letters represent significant Mann-Whitney differences in means.

FIGURE 7 Heights (in centimeters) of six-month old seedlings from sympatric conspecific (control), sympatric heterospecific, and allopatric heterospecific crosses between Hawaiian *Cyrtandra* species on Kaua'i (*C. kauaiensis* and *C. longifolia*) and Hawai'i Island (*C. paludosa* and *C. platyphylla*). Within each graph, lowercase letters represent significant Mann-Whitney differences in means.
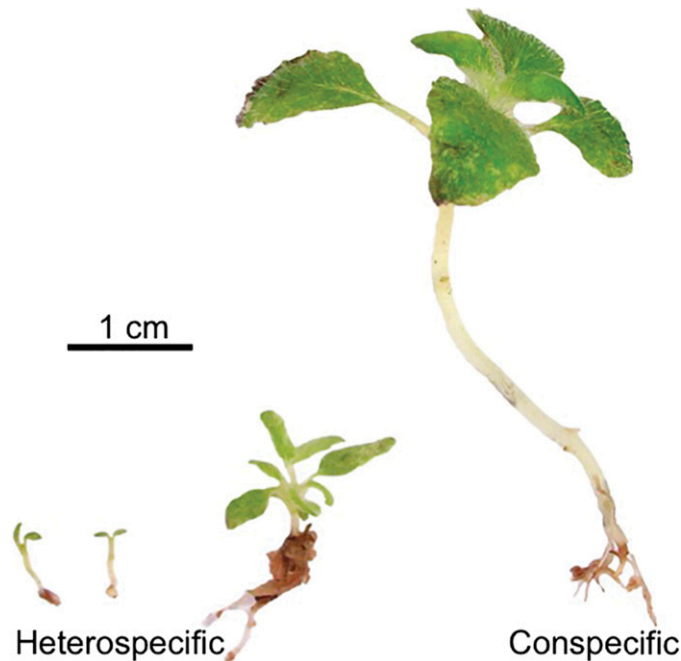
FIGURE 8 Six-month old seedlings from growth trials involving *Cyrtandra platyphylla* as a maternal plant. Seedlings from the majority of sympatric and allopatric heterospecific crosses did not produce true leaves (left), while a few sympatric heterospecific crosses produced seedlings with stunted growth and deformed true leaves (center); in contrast, all conspecific crosses produced large healthy seedlings (right).

stunted growth relative to conspecific seedlings at six months of age ($H_2 = 5.07$, $P = 0.08$; Fig. 7); the true leaves of these seedlings were also deformed (Fig. 8).

Total reproductive isolation—Total estimated RI was moderate for sympatric species on Kaua'i, with a value of 0.611 for *C. longifolia* and 0.707 for *C. kauaiensis* (Table 1). A reduction in the number of germinants/fruit made the largest relative contribution to RI for both *C. kauaiensis* (0.501) and *C. longifolia* (0.575; Table 1). On Hawai'i Island, total estimated RI for sympatric species varied from moderate to essentially complete, with a value of 0.587 for *C. paludosa* var. *paludosa* and 0.907 for *C. platyphylla*. A reduction in F_1 seedling survival (0.788) made the largest relative contribution to RI in *C. platyphylla* (Table 1). In contrast, flowering phenology (0.682) was the sole reproductive barrier in *C. paludosa* var. *paludosa*

(Table 1). When including geography, total RI increased somewhat for three of the species: *C. longifolia* (0.830), *C. paludosa* var. *paludosa* (0.654), and *C. platyphylla* (0.946), but not for *C. kauaiensis* (Appendix S3).

DISCUSSION

Summary—The present work describes the strength and stages of reproductive isolation among species of Hawaiian *Cyrtandra*, a lineage that presents an intriguing challenge to our understanding of reproductive barriers in angiosperms. *Cyrtandra* is an important component of the Hawaiian flora, being distributed across six of the main Hawaiian Islands in predominantly wet montane forests, where multiple species often co-occur. *Cyrtandra* has been described as having the highest incidence of hybridization in the Hawaiian flora, yet 60 species are distinguished based on genetic and morphological characters. This suggests that at least partial barriers to hybridization must exist between species. While recent reviews indicate that prezygotic isolation more strongly reduces gene flow between plant species than postzygotic isolation, our observations of two pairs of sympatric Hawaiian *Cyrtandra* species suggest that three of the four species are maintained predominantly through postzygotic barriers. The fourth species, in contrast, appears to be maintained solely through the single prezygotic mechanism of flowering time differences, due to the limited flowering period of its sympatric relative. We also report variation in the strength of postzygotic barriers in allopatric crosses, with one of four species exhibiting strong postzygotic RI in crosses with allopatric pollen donors.

Prezygotic isolation—Our estimates of range overlap within islands suggest that the two species pairs examined are sympatric across a large portion of their ranges. As such, geographic RI within islands does not appear to be a major barrier to reproduction between these species. However, our calculations are based on the estimated natural range of species prior to human disturbance. In addition, these are estimates of current species distributions and do not include any information about historical ranges. Given that range sizes have undoubtedly changed over time because of climate cycles and habitat disturbance, the estimates of range overlap presented here must be interpreted with caution.

The overall pattern of phenology on Kaua'i is that of sympatric species exhibiting overlapping flowering times, with an average of 62% (*C. longifolia*) to 65% (*C. kauaiensis*) of the individuals flowering throughout the observation period. In contrast, *C. platyphylla*

TABLE 1. Relative and absolute contributions to total reproductive isolation (RI). The strength of each barrier varied from 1 (complete isolation), to 0 (random mating), to −1 (heterospecific gene flow is facilitated). Contributions were computed for the two sympatric species pairs examined in this study (Kaua'i: *Cyrtandra longifolia* and *C. kauaiensis*, and Hawai'i Island: *C. platyphylla* and *C. paludosa*).

RI barriers	Relative contribution to reproductive isolation				Absolute contribution to RI in sympatry			
	C. kauaiensis	*C. longifolia*	*C. paludosa*	*C. platyphylla*	*C. kauaiensis*	*C. longifolia*	*C. paludosa*	*C. platyphylla*
Geography	0	0.563	0.159	0.413	—	—	—	—
Phenology	0.031	−0.031	0.682	−0.682	0.031	−0.031	0.682	−0.682
Pollen tube growth	0.044	0	0	−0.023	0.043	0	0	−0.039
Fruit set	−0.043	−0.306	0.011	−0.158	−0.040	−0.315	0.003	−0.272
F_1 seed germination	0.501	0.575	0.056	0.535	0.484	0.774	0.018	1.066
F_1 survival	0.205	0.166	−0.277	0.529	0.099	0.095	−0.082	0.490
F_1 growth	0.236	0.184	−0.089	0.788	0.090	0.088	−0.034	0.344
Total RI					**0.707**	**0.611**	**0.587**	**0.907**

exhibited a limited flowering time from May–October, with an average of only 11% of the observed individuals producing flowers. All other species in this study produced flowers year round, including the widely distributed *C. paludosa* var. *paludosa*, with an average of 39% of the individuals flowering throughout the observation period. Although 42% of the total flowering season included both *C. platyphylla* and *C. paludosa* var. *paludosa*, these sympatric species appear to be isolated from one another because of the restricted flowering of *C. platyphylla*. While this may be a significant prezygotic barrier between these two species, it is important to recognize that flowering phenology is highly variable over time. Over two consecutive years, *C. platyphylla* was observed to vary by as much as 20% in terms of the number of flowering individuals, and also showed variation in the length of flowering season (4–6 mo). Thus, flowering time divergence may be unreliable and of limited importance to total RI over long time periods.

Prezygotic barriers in the form of reduced pollen tube growth do not appear to contribute to RI between sympatric species. On both Kaua'i and Hawai'i Island, pollen tube growth was vigorous in all of the conspecific and sympatric heterospecific species crosses. The individual contribution of this barrier to total RI was low, ranging from –0.023 to 0.044 across all species.

While there are other prezygotic barriers that were not assessed in this study, the likelihood of these contributing significantly to species isolation seems low. The high degree of similarity in floral morphology among species, and the fact that interspecific hybrids have been reported across the main Hawaiian Islands makes pollinator discrimination seem unlikely, although this possibility cannot be ruled out. The only pollinator documented for Hawaiian *Cyrtandra* to date is a nocturnal moth seen visiting an individual of *C. hawaiensis* over several consecutive nights, with the plant later setting fruit (Gardener and Daehler, 2006). We did attempt to film nocturnal pollinators over several evenings at both study sites, but these attempts recorded no floral visitors. Data on pollinators should be pursued in future studies. Finally, there is also a possibility of a late-stage prezygotic barrier, wherein the pollen tube fails to penetrate and fertilize the ovule. While visualization of pollen tubes entering the ovules was not possible in this study, the full development and maturation of all fruits from sympatric and allopatric heterospecific crosses (aside from those impacted by insect predation) suggests that fertilization did occur, and that the presence of late-stage prezygotic barriers is unlikely (Nitsch et al., 1960; Gillaspy et al., 1993).

Postzygotic RI between sympatric species—On the older island of Kaua'i, early postzygotic isolation was observed between sympatric species in the form of significantly lower mean numbers of germinants/fruit. *Cyrtandra kauaiensis* as a maternal plant produced few-to-no germinants in 8 of 11 sympatric heterospecific crosses with *C. longifolia*, while *C. longifolia* as a maternal plant produced few to no germinants in six of eight sympatric heterospecific crosses with *C. kauaiensis*. Recent work has shown that postzygotic barriers can evolve rapidly (Widmer et al., 2009), resulting in barriers being polymorphic among individuals within a species (Scopece et al., 2010). Fruits from sympatric heterospecific crosses resulting in few to no germinants were observed to contain large numbers of inviable seeds (although we did not attempt to quantify this), while inviable seeds were not observed in conspecific crosses. Significant differences between cross types in the number of germinants per fruit may be due to some combination of variation in embryo

abortion/seed development and germination rate of fully formed seeds. Further studies will be needed to discern the contributions of these two barriers to postzygotic isolation in Hawaiian *Cyrtandra*.

Our RI estimates suggest that sympatric species on the oldest island of Kaua'i are strongly, though incompletely, isolated from one another (Total RI = 0.611 and 0.707). The relatively few seedlings produced through sympatric heterospecific crosses on Kaua'i that yielded viable seeds had high survival and growth. These measures were recorded in a greenhouse, however, which may lead to overestimation of seedling fitness in the parental environment (Hatfield and Schluter, 1999). If survival of these individuals to adulthood in nature is high, it may be that these viable F_1 hybrids have reduced fertility (reviewed in Burke and Arnold, 2001), or that further reductions in fitness may appear in later hybrid generations (reviewed in Coyne and Orr, 1998, 2004). Long-term studies involving experiments in the natural environment of the parental species are required to fully quantify postzygotic barriers and total RI in *Cyrtandra*.

While sympatric species on Kaua'i showed similar patterns of postzygotic RI, crosses between sympatric species on young Hawai'i Island spanned the full spectrum from absent (*C. paludosa* var. *paludosa*) to strong (*C. platyphylla*) postzygotic RI through to the six-month growth and survival of F_1 seedlings. The mean number of germinants/fruit was significantly lower for sympatric and allopatric heterospecific crosses involving *C. platyphylla* as the maternal plant, with 9 of 20 crosses producing few to zero germinants/fruit. Seedlings from sympatric and allopatric heterospecific crosses also experienced significant mortality at six months, with the few surviving seedlings exhibiting stunted growth and deformed leaves. The severe reduction in germination, survival, and vegetative growth of hybrid seedlings from *C. platyphylla* may be a rare example of unidirectional hybrid necrosis (Bomblies and Weigel, 2007). This type of necrosis is where imbalances between the mitochondrial and nuclear genomes in hybrids results in misregulation of programmed cell death in pollen-forming cells; this in turn causes the more commonly seen cytoplasmic male sterility (CMS) in hybrids (Frank and Barr, 2003). Observation of male-sterile individuals in *C. longifolia* and *C. paludosa* var. *microcarpa* on Kaua'i (M. Johnson, pers. obs.) implies that CMS alleles exist in *Cyrtandra*. Spill-over of the programmed cell death response caused by cytonuclear incompatibility into tissues other than anthers has been invoked as an explanation for deleterious hybrid phenotypes that are characteristic of hybrid necrosis, including dwarfism and leaf deformation (Frank and Barr, 2003). Fixation of similar alleles in *C. platyphylla* that cause hybrid necrosis could account for the strong RI observed in this species.

Variation in postzygotic RI between allopatric species—Interisland crosses to three of the four maternal species (*C. kauaiensis*, *C. longifolia*, and *C. paludosa* var. *paludosa*) resulted in a mean number of germinants/fruit that was at least as high as that in conspecific crosses. In addition, the resulting seedlings from allopatric heterospecific crosses were taller at six months than seedlings from conspecific crosses when *C. kauaiensis*, *C. longifolia*, and *C. paludosa* var. *paludosa* were the maternal plants. High cross-fertility between species of Hawaiian *Cyrtandra* may result from the following criteria: (1) recency of colonization (< 5 Mya), such that an insufficient amount of time has passed to allow the formation of reproductive barriers between species, (2) high genetic similarity between species (≥98%; M. Johnson, unpub. data) across the archipelago

such that interfertility is also high, or (3) heterosis, such that the cumulative effects of deleterious alleles in small parental populations (approximately 30–50 reproductive individuals; see comments in Methods) are overcome by increases in heterozygosity of F_1 hybrids (Burke and Arnold, 2001). All three of these scenarios seem plausible given what is known about *Cyrtandra* in Hawaii.

In sharp contrast with these results, we also observed significant reductions in the number of germinants/fruit, F_1 seedling survival, and F_1 seedling growth to six months in both heterospecific crosses involving maternal plants of Hawai'i Island *C. platyphylla*. This finding demonstrates that postzygotic barriers can arise between *Cyrtandra* species in allopatry, and is consistent with the idea that geographic isolation of populations may be important in the early stages of species divergence. Further crosses involving other allopatric species will be necessary, however, to determine if postzygotic barrier formation between geographically isolated species is a widespread phenomenon in this group. Lastly, our finding of strong postzygotic RI in *C. platyphylla*—a species that is endemic to the younger islands of Maui and Hawai'i Island—indicates that reproductive barrier formation may be relatively rapid (< 1.5 Myr) in at least some species.

Hybridization and introgression in Hawaiian Cyrtandra—While natural hybridization has been well documented in Hawaiian *Cyrtandra*, the evolutionary significance of this phenomenon is unclear. It is common for populations that have developed incomplete reproductive barriers to be in contact at some stage of divergence, possibly due to changes in range size, allowing the opportunity for gene flow between them. If these populations come into secondary contact, initial divergence may either break down or barriers to gene flow may strengthen and promote speciation. For incompletely isolated populations to move toward speciation, associations among the loci that influence RI (i.e., those loci that are under divergent selection or contribute to reduced hybrid fitness) must build up (Smadja and Butlin, 2011). However, those loci that do not contribute to RI are likely to introgress between hybridizing populations (Barton and Bengtsson, 1986).

While a large fraction of introgressed variation is likely to be deleterious or have no long-term impact (Abbott et al., 2013), introgressed genetic variation can also enhance the ability to coexist and help expand the range of a hybrid population (Prentis et al., 2008). Previous phylogenetic work suggests that introgression between Hawaiian *Cyrtandra* species (where some genes cross a species barrier via hybridization and backcrossing; Rieseberg, 1997), may contribute to a lack of resolution at the species level (Clark et al., 2008), as well as incongruence among nuclear and chloroplast loci (Pillon et al., 2013b). In contrast to the other species examined in the current study, postzygotic barriers were absent in *C. paludosa* var. *paludosa* on Hawai'i Island, with heterospecific gene flow being facilitated at the F_1 seedling growth and survival stages when *C. paludosa* var. *paludosa* was the maternal plant. Pillon et al. (2013b) cited introgression into *C. paludosa* as a plausible cause for gene discordance in this species. At the *Cyrt17* locus, *C. paludosa* var. *microcarpa* from Kaua'i were more closely related to other Kaua'i species than to conspecifics from O'ahu and Hawai'i Island (Pillon et al., unpub. data). In addition, phylogenetic analysis at the *ITS* and *ETS* gene regions suggests that *C. paludosa* var. *paludosa* from the UWFR on Hawai'i Island are more closely related to species from Kaua'i than to all other taxa, including conspecifics on O'ahu (M. Johnson, unpub. data). These findings suggest that introgression occurred into *C. paludosa* sometime after its origin on

Kaua'i, but prior to its colonization of Hawai'i Island (Pillon et al., 2013b). It is therefore possible that introgressed genetic variation in *C. paludosa* var. *paludosa* has facilitated its successful colonization of four of the main Hawaiian Islands, a feat that is rare in the genus. To date, 16 putative hybrid combinations (20% of the total number of putative *Cyrtandra* hybrid combinations in Hawaii) have been reported to involve this taxon—at least twice the number of any other species in this group (Wagner, 1990; K. Wood, pers. comm). Nonetheless, that this taxon can be distinguished based on morphological and genetic characters suggests that it is isolated from other species at a few loci at least (Schaal et al., 1998). It is possible that the partial isolation between *C. paludosa* var. *paludosa* and other species is due to incomplete barriers acting at stages unexamined in this study. Future studies of RI in this group should examine prezygotic barriers via pollinator preference and postzygotic barriers through to the fitness of second-generation hybrids.

Time and the evolution of RI—While we observed strong postzygotic barriers for both pairs of sympatric species examined, these barriers were incomplete. The pattern of incomplete isolation observed suggests that the evolution of isolating mechanisms may lag behind the origin of new lineages in this group. If our model of species emergence in allopatry is true, then the evolution of intrinsic barriers to reproduction are not necessary for the origin of these lineages, as species initially become reproductively isolated through geographic barriers. Intrinsic isolating mechanisms are often incomplete or absent between young species (Carr and Kyhos, 1986; Coyne and Orr, 2004; Wiens et al., 2006). Indeed, the origin of the Hawaiian radiation of *Cyrtandra* occurred sometime within the last five million years (Clark et al., 2009), making this an evolutionarily young lineage. Interestingly, a paucity of putative hybrids in both herbarium collections (Gillett, 1967) and field observations (M. Johnson, pers. obs.) of *Cyrtandra* species in Fiji suggests that reproductive barriers between species are stronger in this earlier diverging lineage (~11.4–8.9 MYBP; Clark et al., 2009), despite a high degree of sympatry (i.e., 2–11 sympatric species in any given area). Combined with the results from Hawaii, these observations support the idea that the strength of reproductive barriers among sympatric species of *Cyrtandra* increases gradually over time.

CONCLUSIONS

The findings presented here indicate that postzygotic mechanisms are the predominant barriers to mating between closely related sympatric species of *Cyrtandra* in Hawaii. A combination of several strong postzygotic barriers were found to isolate three of the four species from sympatric taxa, while a single prezygotic barrier in the form of flowering time differences appears to isolate the fourth species from its sympatric relative. Crosses between species on distant islands revealed that *C. platyphylla* on young Hawai'i Island has evolved strong postzygotic barriers to reproduction with both Kaua'i species, consistent with the idea that geographic isolation may be important in the early stages of diversification within this group. Lastly, the range of RI patterns observed among the four species examined suggest that the maintenance of species boundaries in this group is complex. Future studies of RI should include a broader range of species as well as additional stages of RI, including pollinator preference and F_1/backcross viability and fertility. The large number of species, high degree of sympatry, and young

evolutionary age of Hawaiian *Cyrtandra* makes this an ideal model for further studies of the maintenance of species boundaries in flowering plants.

LITERATURE CITED

Abbott, R., et al. 2013. Hybridization and speciation. *Journal of Evolutionary Biology* 26: 229–246.

Atkins, H. J., G. L. C. Bramley, and J. R. Clark. 2013. Current knowledge and future directions in the taxonomy of *Cyrtandra* (Gesneriaceae), with a new estimate of species numbers. *Selbyana* 31: 157–165.

Barton, N. H., and B. O. Bengtsson. 1986. The barrier to genetic exchange between hybridizing populations. *Heredity* 57: 357–376.

Bomblies, K., and D. Weigel. 2007. Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. *Nature Reviews. Genetics* 8: 382–393.

Burke, J. M., and M. L. Arnold. 2001. Genetics and the fitness of hybrids. *Annual Review of Genetics* 35: 31–52.

Carr, G. D., and D. W. Kyhos. 1986. Adaptive radiation in the Hawaiian silversword alliance (Compositae-Madiinae). II. Cytogenetics of artificial and natural hybrids. *Evolution* 40: 959–976.

Chari, J., and P. Wilson. 2001. Factors limiting hybridization between *Penstemon spectabilis* and *Penstemon centranthifolius*. *Canadian Journal of Botany* 79: 1439–1448.

Clague, D. A. 1996. The growth and subsidence of the Hawaiian-Emperor volcanic chain. *In* A. Keast, and S.E. Miller [eds.], The origin and evolution of Pacific island biotas, New Guinea to Eastern Polynesia: patterns and processes, 35–50. SPB Academic, Amsterdam, Holland.

Clark, J. R., R. H. Ree, M. E. Alfaro, M. G. King, W. L. Wagner, and E. H. Roalson. 2008. A comparative study in ancestral range reconstruction methods: retracing the uncertain histories of insular lineages. *Systematic Biology* 57: 693–707.

Clark, J. R., W. L. Wagner, and E. H. Roalson. 2009. Patterns of diversification and ancestral range reconstruction in the southeast Asian-Pacific angiosperm lineage *Cyrtandra* (Gesneriaceae). *Molecular Phylogenetics and Evolution* 53: 982–994.

Coyne, J. A., and H. A. Orr. 1989. Patterns of speciation in *Drosophila*. *Evolution* 43: 362–381.

Coyne, J. A., and H. A. Orr. 1998. The evolutionary genetics of speciation. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 353: 287–305.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer, Inc., Sunderland, Massachusetts, USA.

Cronk, Q. C. B., M. Kiehn, W. L. Wagner, and J. F. Smith. 2005. Evolution of *Cyrtandra* (Gesneriaceae) in the Pacific Ocean: the origin of a supertramp clade. *American Journal of Botany* 92: 1017–1024.

Curtu, A. L., O. Gailing, and R. Finkeldey. 2009. Pattens of contemporary hybridization inferred from paternity analysis in a four-oak-species forest. *BMC Evolutionary Biology* 9: 284–292.

Dobzhansky, T. 1936. Studies on hybrid sterility II. Localization of sterility factors in *Drosophila pseudoobscura* hybrids. *Genetics* 21: 113–135.

Dobzhansky, T. 1937. Genetics and the origin of species. Columbia University Press, New York, New York, USA.

Drake, D. R. 1992. Seed dispersal of *Metrosideros polymorpha* (Myrtaceae): a pioneer tree of Hawaiian lava flows. *American Journal of Botany* 79: 1224–1228.

Frank, S. A., and C. M. Barr. 2003. Programmed cell death and hybrid incompatibility. *The Journal of Heredity* 94: 181–183.

Gardener, M. C., and C. C. Daehler. 2006. Documenting floral visitors to rare Hawaiian plants using automated video recordings. *Pacific Conservation Biology* 12: 189–194.

Gavrilets, S. 2004. Fitness landscapes and the origin of species (Monographs in Population Biology 41). Princeton University Press, Princeton, New Jersey, USA.

Gillaspy, G., H. Ben-David, and W. Gruissem. 1993. Fruits: a developmental perspective. *The Plant Cell* 5: 1439–1451.

Gillett, G. W. 1967. The genus *Cyrtandra* in Fiji. *Contributions from the United States National Herbarium* 37: 107–159.

Hamrick, J. L., M. J. W. Godt, and S. L. Sherman-Broyles. 1992. Factors influencing levels of genetic diversity in woody plant species. *New Forests* 6: 95–124.

Hatfield, T., and D. Schluter. 1999. Ecological speciation in sticklebacks: environment-dependent hybrid fitness. *Evolution* 53: 866–873.

Hotchkiss, S., and J. O. Juvik. 1999. A late-Quaternary pollen record from Ka'au Crater, O'ahu, Hawai'i. *Quaternary Research* 52: 115–128.

Karrenberg, S., P. J. Edwards, and J. Kollmann. 2002. The life history of Salicaceae living in the active zone of floodplains. *Freshwater Biology* 47: 733–748.

Kay, K. M. 2006. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. *Evolution* 60: 538–552.

Kearns, C. A., and D. W. Inouye. 1993. Techniques for Pollination Biologists. University Press of Colorado, Niwot, Colorado, USA.

Kiehn, M. 2005. Chromosome numbers of Hawaiian angiosperms: new records and comments. *Pacific Science* 59: 363–377.

Lepais, O., G. Roussel, F. Hubert, A. Kremer, and S. Gerber. 2013. Strength and variability of postmating reproductive isolating barriers between four European white oak species. *Tree Genetics & Genomes* 9: 841–853.

Liou, L. W., and T. D. Price. 1994. Speciation by reinforcement of premating isolation. *Evolution* 48: 1451–1459.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 363: 3009–3021.

Luegmayr, E. 1993. Pollen of Hawaiian *Cyrtandra* (Gesneriaceae) including notes on southeast Asian taxa. *Blumea* 38: 25–38.

Martin, N. H., and J. H. Willis. 2007. Ecological divergence associated with mating system causes nearly complete reproductive isolation between sympatric *Mimulus* species. *Evolution* 61: 68–82.

Mayr, E. 1942. Systematics and the Origin of Species. Columbia University Press, New York, New York, USA.

Mayr, E. 1947. Ecological factors in speciation. *Evolution* 1: 263–288.

Mayr, E. 1963. Animal species and evolution. Harvard University Press, Cambridge, Michigan, USA.

Mosseler, A. 1989. Interspecific pollen-pistil incongruity in *Salix*. *Canadian Journal of Forest Research* 19: 1161–1168.

Mosseler, A. 1990. Hybrid performance and species crossability relationships in willows (*Salix*). *Canadian Journal of Botany* 68: 2329–2338.

Nitsch, J., C. Pratt, C. Nitsch, and N. Shaulis. 1960. Natural growth substances in Concord and Concord seedless grapes in relation to berry development. *American Journal of Botany* 47: 566–576.

Percy, D. M., A. M. Garver, W. L. Wagner, H. F. James, C. W. Cunningham, S. E. Miller, and R. C. Fleischer. 2008. Progressive island colonization and ancient origin of Hawaiian *Metrosideros* (Myrtaceae*). Proceedings. Biological Sciences* 275: 1479–1490.

Petit, R. J., and A. Hampe. 2006. Some evolutionary consequences of being a tree. *Annual Review of Ecology Evolution and Systematics* 37: 187–214.

Pillon, Y., J. Johansen, T. Sakashima, S. Chamala, W. B. Barbazuk, E. H. Roalson, D. K. Price, and E. A. Stacy. 2013a. Potential use of low-copy nuclear genes in DNA barcoding: a comparison with plastid genes in two Hawaiian plant radiations. *BMC Evolutionary Biology* 13: 35–45.

Pillon, Y., J. B. Johansen, T. Sakashima, E. H. Roalson, D. K. Price, and E. A. Stacy. 2013b. Gene discordance in phylogenomics of recent plant radiations, an example from Hawaiian *Cyrtandra* (Gesneriaceae). *Molecular Phylogenetics and Evolution* 69: 293–298.

Potts, B. M., and H. S. Dungey. 2004. Interspecific hybridization of *Eucalyptus*: key issues for breeders and geneticists. *New Forests* 27: 115–138.

Prentis, P. J., J. R. U. Wilson, E. E. Dormontt, D. M. Richardson, and A. J. Lowe. 2008. Adaptive evolution in invasive species. *Trends in Plant Science* 13: 288–294.

Price, J. P. 2004. Floristic biogeography of the Hawaiian Islands: influences of area, environment and paleogeography. *Journal of Biogeography* 31: 487–500.

Price, J. P., and W. L. Wagner. 2004. Speciation in Hawaiian angiosperm lineages: cause, consequence, and mode. *Evolution* 58: 2185–2200.

Price, J. P., J. D. Jacobi, S. M. Gon III, D. Matsuwaki, L. Mehrhoff, W. Wagner, M. Lucas, and B. Rowe. 2012. Mapping plant species ranges in the Hawaiian Islands—Developing a methodology and associated GIS layers: U.S. Geological Survey Open-File Report 2012– 1192, 34 p., 1 appendix (species table), 1,158 maps. Available at http://pubs.usgs.gov/of/2012/1192/.

QGIS Development Team. 2012. QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org

Ramsey, J., H. D. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* 57: 1520–1534.

R Core Team. 2014. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.Rproject.org/

Rice, W. R., and E. E. Hostert. 1993. Laboratory experiments on speciation: what have we learned in 40 years? *Evolution* 47: 1637–1653.

Rieseberg, L. H. 1997. Hybrid origins of plant species. *Annual Review of Ecology and Systematics* 28: 359–389.

Rieseberg, L. H., and J. H. Willis. 2007. Plant speciation. *Science* 317: 910–914.

Roelofs, F. M. 1979. The reproductive biology of *Cyrtandra grandiflora* (Gesneriaceae) on Oahu. *Pacific Science* 33: 223–231.

Schaal, B. A., D. A. Hayworth, K. M. Olsen, J. T. Rauscher, and W. A. Smith. 1998. Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* 7: 465–474.

Schluter, D. 2001. Ecology and the origin of species. *Trends in Ecology & Evolution* 16: 372–380.

Schluter, D. 2009. Evidence for ecological speciation and its alternative. *Science* 323: 737–741.

Scopece, G., C. Lexer, A. Widmer, and S. Cozzolino. 2010. Polymorphism of postmating reproductive isolation within plant species. *Taxon* 59: 1367–1374.

Servedio, M. R. 2000. Reinforcement and the genetics of nonrandom mating. *Evolution* 54: 21–29.

Smadja, C. M., and R. K. Butlin. 2011. A framework for comparing processes of speciation in the presence of gene flow. *Molecular Ecology* 20: 5123–5140.

Smith, J. F., C. C. Burke, and W. L. Wagner. 1996. Interspecific hybridization in natural populations of *Cyrtandra* (Gesneriaceae) on the Hawaiian Islands: evidence from RAPD markers. *Plant Systematics and Evolution* 200: 61–77.

Sobel, J. M., and G. F. Chen. 2014. Unification of methods for estimating the strength of reproductive isolation. *Evolution* 68: 1511–1522.

Turelli, M., N. H. Barton, and J. A. Coyne. 2001. Theory and speciation. *Trends in Ecology & Evolution* 16: 330–343.

Wagner, W. L. 1990. *Cyrtandra. In* W.L. Wagner, D.R. Herbst, and S.H. Sohmer, [eds.], Manual of the flowering plants of Hawai'i (vol. 1), 735–781. University of Hawaii Press and Bishop Museum Press, Honolulu, Hawaii, USA.

Wagner, W. L., D. R. Herbst, and D. H. Lorence. 2005. Flora of the Hawaiian Islands [online]. Website http://botany.si.edu/pacificislandbiodiversity/hawaiianflora/index.htm [Accessed 12 March 2014].

Widmer, A., C. Lexer, and S. Cozzolino. 2009. Evolution of reproductive isolation in plants. *Heredity* 102: 31–38.

Wiens, J. J., T. N. Engstrom, and P. T. Chippindale. 2006. Rapid diversification, incomplete isolation, and the "speciation clock" in North American salamanders (Genus Plethodon): Testing the hybrid swarm hypothesis of rapid radiation. *Evolution* 60: 2585–2603.

APPENDIX 1   Voucher specimen information.

Taxon; Voucher specimen, Collection locale; Herbarium.

Cyrtandra kauaiensis Wawra; Johnson 03, Kōke'e State Park, Kaua'i; Rancho Santa Ana Botanic Garden (RSA).

Cyrtandra longifolia (Wawra) Hillebr. ex. C.B. Clarke; Johnson 04, Kōke'e State Park, Kaua'i; RSA.

Cyrtandra paludosa **var. paludosa** Gaud.; Johnson 01, Upper Waiākea Forest Reserve, Hawai'i Island; RSA.

Cyrtandra platyphylla A. Gray; Johnson 02, Upper Waiākea Forest Reserve, Hawai'i Island; RSA.