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RAPID EVOLUTION OF REPRODUCTIVE ISOLATION BETWEEN INCIPIENT OUTCROSSING AND SELFING *CLARKIA* SPECIES

Ryan D. Briscoe Runquist,[1,2] Eric Chu,[1] Justin L. Iverson,[1] Jason C. Kopp,[1] and David A. Moeller[1]

[1] *Department of Plant Biology, University of Minnesota, St. Paul, Minnesota 55108*

[2] *E-mail: rbriscoe@umn.edu*

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A major goal of speciation research is to understand the processes involved in the earliest stages of the evolution of reproductive isolation (RI). One important challenge has been to identify systems where lineages have very recently diverged and opportunities for hybridization are present. We conducted a comprehensive examination of the components of RI across the life cycle of two subspecies of *Clarkia xantiana*, which diverged recently (ca. 65,000 bp). One subspecies is primarily outcrossing, but self-compatible, whereas the other is primarily selfing. The subspecies co-occur in a zone of sympatry but hybrids are rarely observed. Premating barriers resulted in nearly complete isolation in both subspecies with flowering time and pollinator preference (for the outcrosser over the selfer) as the strongest barriers. We found that the outcrosser had consistently more competitive pollen, facilitating hybridization in one direction, but no evidence for pollen–pistil interactions as an isolating barrier. Surprisingly, postzygotic isolation was detected at the stage of hybrid seed development, but in no subsequent life stages. This crossing barrier was asymmetric with crosses from the selfer to outcrosser most frequently failing. Collectively, the results provide evidence for rapid evolution of multiple premating and postzygotic barriers despite a very recent divergence time.

KEY WORDS: **Bateson–Dobzhansky–Müller incompatibility, hybridization, mating system, pollination, self-fertilization, speciation.**

Speciation requires the evolution of reproductive isolating mechanisms that provide barriers to hybridization between diverging lineages (Dobzhansky 1937a; Mayr 1942; Coyne and Orr 2004). Incipient species can be effectively reproductively isolated by one or multiple prezygotic or postzygotic mechanisms as long as they provide a strong barrier to gene flow (Dobzhansky 1937a; Coyne 1992; Schluter 2001; Coyne and Orr 2004; Nosil et al. 2005; Kay and Sargent 2009). Natural selection is often an important component of the speciation process and ecological factors have the ability to promote speciation through divergent selection between populations, which may contribute to both pre- or postzygotic mechanisms of isolation upon secondary contact (Orr and Smith 1998; Schluter 2001; Sobel et al. 2010; Nosil 2012). Determining the timing and relative strength of prezygotic and postzygotic barriers particularly in young, rapidly diverging lineages will provide valuable insight into the speciation process even in the face of ongoing gene flow.

During flowering plant diversification, one of the most frequent transitions is in mating system, especially from outcrossing to selfing (Stebbins 1950). This transition is often accompanied by changes in floral morphology and timing (e.g., smaller less-attractive flowers, reduced herkogamy, reduced protandry, more rapid development) generally termed the "selfing syndrome" (Ritland and Ritland 1989; Goodwillie et al. 2010; Sicard and Lenhard 2011; Kalisz et al. 2012; De Vos et al. 2014). These characteristic changes may reduce gene flow between taxa through a variety of premating isolating mechanisms, directly promoting reproductive isolation (RI) between the derived selfing lineage

EVOLUTION
INTERNATIONAL JOURNAL OF ORGANIC EVOLUTION

and the progenitor outcrossing lineage (Levin 1978; Coyne and Orr 2004; Hodges 2005; Martin and Willis 2007). Where RI has been studied between incipient species with divergent mating systems, floral divergence associated with mating system has proved important to RI (Martin and Willis 2007).

Even when RI is strong, gene flow is common between incipient species that are in secondary contact (Sambatti et al. 2012; Abbott et al. 2013). Patterns of introgression in hybrid zones are often observed to be asymmetrical and these asymmetries can arise from variations in the strength and direction of both premating and postmating reproductive isolating mechanisms (Petit et al. 2004; Buggs 2007; Scascitelli et al. 2010; Toews and Brelsford 2012). Under traditional genetic models of intrinsic postzygotic RI through Bateson–Dobzhansky–Müller (BDMs) incompatibilities or chromosomal rearrangements, crossing barriers are expected to be symmetrical (Bateson 1909; Dobzhansky 1937b; Müller 1942; Levin 1978). Yet, in animal, fungal and plant systems, crossing barriers between incipient species are frequently asymmetric (Oliver 1978; Harrison 1983; Orr and Coyne 1989; Gallant and Fairbairn 1997; Tiffin et al. 2001; Willett and Burton 2001; Presgraves 2002; Dettman et al. 2003; Bolnick and Near 2005; Palma-Silva et al. 2011; Ruhsam et al. 2011). In plants, crossing barrier asymmetry is particularly common in species that have diverged in mating system (Lewis and Crowe 1958; Brandvain and Haig 2005). Asymmetric crossing barriers can occur as a consequence of (a) pollen–pistil interactions (e.g., gametophyte–sporophyte interactions: Lewis and Crowe 1958; Kay 2006; Aagaard et al. 2013; Goodwillie and Ness 2013), (b) cytonuclear interactions (Burton et al. 2013), and (c) divergence in gene expression, including differential imprinting (Bushell et al. 2003). Phenomena occurring after a potential crossing barrier can also contribute to asymmetric patterns of introgression. For example, differential selection against reciprocal hybrids (Campbell et al. 2008) or asymmetric backcrossing from hybrids to one parent can influence the magnitude of introgression (Cruzan and Arnold 1994; Muranishi et al. 2013; Ruhsam et al. 2013).

Studies that thoroughly dissect RI between recently diverged lineages are necessary to identify and characterize the order and timing of mechanisms that contribute most to speciation, yet there are still relatively few comprehensive studies and even fewer that examine very recently diverged lineages. In studies that quantify the strength and actual contribution of multiple components of RI between plant species pairs, the predominant finding is that prezygotic barriers are stronger than postzygotic barriers (Ramsey et al. 2003; Lowry et al. 2008; Dell'Olivo et al. 2011; Palma-Silva et al. 2011), although there is variation in which prezygotic barriers (and whether they are pre- or postmating) are of greatest importance (Lowry et al. 2008). Alternately, in some systems multiple pre- and postzygotic mechanisms are important for RI to maintain species integrity (Sambatti et al. 2012; Scopece et al. 2013). Further, although mating system transitions are often associated with speciation events in plants, there is only one previous study of an outcrosser–selfer pair that systematically documented components of RI. Martin and Willis (2007) found several prezygotic barriers, including pollinator isolation (associated with mating system divergence) and flowering phenology that resulted in nearly complete RI between *Mimulus guttatus* and *M. nasutus*. More studies, particularly in recently diverged lineages that differ in mating system, are needed to better understand the timing and order of the origin of RI mechanisms.

Here, we examine the evolution of RI between two subspecies of *Clarkia xantiana* (Onagraceae): subsp. *xantiana* and subsp. *parviflora* (hereafter *xantiana* and *parviflora*, respectively). *Xantiana* is primarily outcrossing whereas *parviflora* is primarily self-fertilizing, with reduced herkogamy, protandry, and corolla size (Runions and Geber 2000; Moeller 2006; Moeller et al. 2012). Phylogenetic analyses revealed that *parviflora* populations constitute a monophyletic group, which is recently derived (ca. 65,000 years before present) from its outcrossing progenitor, *xantiana* (Pettengill and Moeller 2012a). The taxa diverged to some extent in allopatry and have since come into secondary contact following range expansion (Pettengill and Moeller 2012b). At sympatric sites, hybrids have been rarely observed. Population genetic studies have confirmed that there is some introgression, albeit limited to the narrow zone of sympatry. Introgression was detected primarily from *parviflora* to *xantiana* suggesting a possible asymmetry in the strength and direction of RI (Pettengill and Moeller 2012a).

Xantiana and *parviflora* have been subject to divergent natural selection in their respective ranges, and such ecological divergence has been suggested to be important in many instances of incipient speciation (Sobel et al. 2010). The combined range of x*antiana* and *parviflora* spans a roughly west-to-east gradient of declining precipitation and temperature in the Sierra Nevada Mountains of California (Eckhart and Geber 1999). The two taxa have differentiated most strongly in flowering phenology and floral traits, including those associated with mating system (Eckhart and Geber 1999; Runions and Geber 2000). The subspecies exhibit strong local adaptation to their respective ranges (Eckhart et al. 2004; Geber and Eckhart 2005) and there is evidence of adaptive differentiation across the same west–east gradient within *xantiana* (Gould et al. 2014). The subspecies also experience different pollination environments, and mating system differentiation within and between subspecies is correlated with the availability of effective pollinators (Moeller and Geber 2005; Moeller 2006). Solitary bees specialized on *Clarkia* (MacSwain et al. 1973), which are important pollinators (Moeller 2005; Eckhart et al. 2006), are only found in *xantiana*'s range and where the subspecies are sympatric (Fausto et al. 2001; Moeller 2006).

Our first objective was to examine individual components of RI throughout the life cycle of *xantiana* and *parvilfora* using a series of field studies and greenhouse experiments over four years. From these studies, we quantified the contribution of premating, postmating-prezygotic, and postzygotic isolation to the total RI. Given the very recent divergence time of the two taxa, we tested two main hypotheses: (1) RI is conferred primarily by premating isolation whereas intrinsic postzygotic isolation contributes minimally to RI (either individual or absolute); (2) premating RI arises primarily as a by-product of adaptation to contrasting environments and that the most strongly adaptively differentiated traits (flowering phenology and floral/mating system traits) contribute most significantly to premating isolation. Our second objective was to examine alternative hypotheses for observed asymmetric introgression to the outcrosser, *xantiana*, from its selfing derivative, *parviflora*. Here, we tested whether an asymmetric crossing barrier (caused by postmating-prezygotic or postzygotic barriers) limited introgression into one taxon, or whether processes occurring after hybrid formation influenced hybrid fitness and the potential for backcrossing.

Methods

STUDY SYSTEM

Clarkia xantiana A. Gray ssp. *xantiana* (hereafter *xantiana*) and *C. xantiana* A. Gray ssp. *parviflora* H. Lewis & P. R. Raven (hereafter *parviflora*) are winter annual plants endemic to the southern Sierra Nevada foothills, primarily in Kern and Tulare counties, CA (Lewis and Lewis 1955). The two subspecies have a parapatric distribution along a west-to-east environmental gradient: *xantiana* is found in western foothill oak woodlands whereas *parviflora* populations are found in eastern, more xeric habitats extending nearly to the Mojave Desert. There is a narrow area of sympatry (~5 km) at the eastern range edge of *xantiana*'s and the western range edge of *parviflora*'s range (Eckhart and Geber 1999; Eckhart et al. 2004) (Fig. 1). Within this zone of sympatry, subspecies can be found within meters to tens of meters of each other. No formal quantitative analysis of cross-compatibility has previously been reported.

TOTAL RI BETWEEN *C. XANTIANA* SUBSPECIES

We quantified the magnitude of RI for both subspecies across most of the life cycle including potential premating and postmating isolating barriers. RI mechanisms are sequential; those that promote RI later in the life cycle can only contribute to RI after accounting for earlier or simultaneously acting mechanisms. We calculated the contributions of individual RI mechanisms and the absolute contributions of RI mechanisms following the methods of Sobel and Chen (2014); this approach relates observations of gene flow to random expectations and allows for comparisons between pre- and postzygotic isolation. We computed the total RI by summing all of the absolute contributions of RI mechanisms. Summing across components of RI to obtain total RI requires that the quantification of prezygotic barriers account for the expected probability of mating to be comparable to postzygotic components (Martin and Willis 2007). A total RI of one indicates complete isolation and a total RI of zero indicates no isolation.

PREMATING BARRIERS

Ecogeography

We quantified RI due to ecogeography using data on the frequency of populations of each taxon found in sympatry versus allopatry. We gathered site record information on all known *C. xantiana* populations for both subspecies from the Jepson Herbarium (http://ucjeps.berkeley.edu/interchange.html) and populations found through extensive searches over ca. 20 years by our research group and colleagues (Fig. 1). We defined sympatry in two ways. We considered populations of each taxon to be sympatric if they occurred within 1 or 3 km of the nearest heterospecific population (Table S1). We quantified RI due to ecogeographic overlap for *xantiana* and *parviflora* using the methods from Sobel and Chen (2014) for barriers that affect co-occurrence (equation RI_{4C}), where $RI_{ecogeographic} = 1 -$ (number of sympatric populations for a subspecies / number of total populations for a subspecies).

Phenology

To document the flowering phenology of *xantiana* and *parviflora*, we conducted repeated censuses every three to four days (mean = 13.2 visits/site) at four sympatric sites (sites 7, 65, 106, 11) from 6 May to 21 June 2013. At all sites, we walked semipermanent transects that were stratified across each site (mean total transect length = 78.6 m/site). Two *parviflora* populations (sites 65, 111) were exhaustively sampled because they were small and patchy (for details see Table S2). During the first survey at each site, we counted all fruits, flowers, and buds for both subspecies to estimate total reproductive effort for the season. During each subsequent visit, we counted fruits, flowers, and buds on all plants that were still actively flowering. We quantified flowering overlap in two ways. First, we calculated the proportion of flowers for each subspecies that bloomed during the period of overlap. Second, we calculated the proportion of days that the blooming periods overlapped for the two subspecies. Because 2013 was an extremely dry year, we were not able to record the date of first flower for any *parviflora* population. During another extremely dry year (1999), the date of first flower was never before 30 April (Eckhart and Geber 1999). Therefore, we conservatively estimated the date of first flower for all *parviflora* populations as

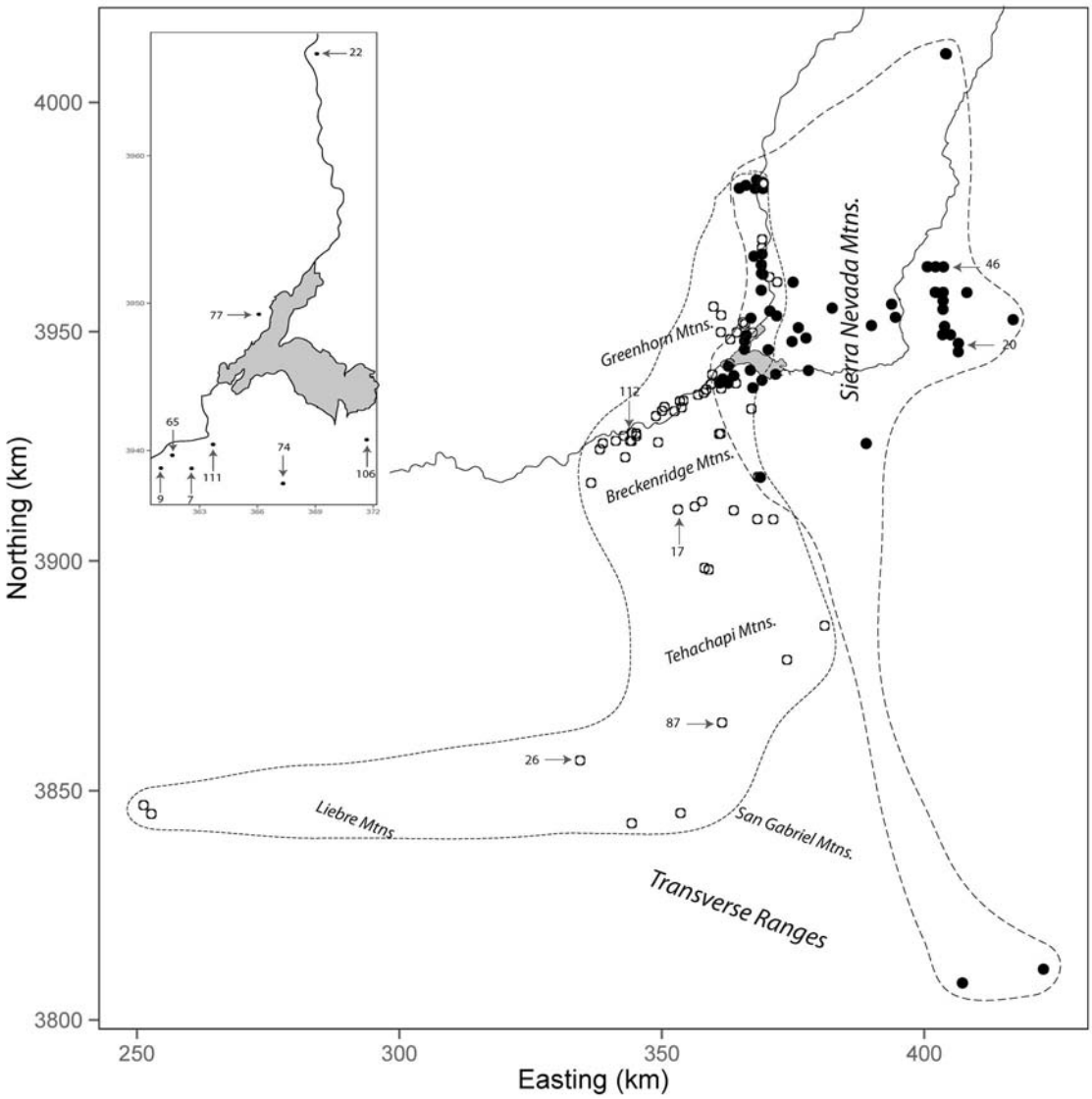

Figure 1. Map of geographic ranges and area of overlap for *xantiana* and *parviflora* in southern California, USA. All known *xantiana* sites are shown as open circles and all known *parviflora* sites are shown as closed circles. Axes are the Northing and Easting in kilometers for UTM zone 11S. The known geographic range of *xantiana* is outlined in a dotted line and the known range of *parviflora* is outlined in a dashed line. The area of sympatry is where the two ranges intersect in and around Lake Isabella. Populations used for experimental manipulations to assess RI are marked with arrows and their corresponding population identification number. The inset map shows the sympatric populations used for experimental manipulations in the Lake Isabella region.

25 April 2013. We calculated $RI_{phenology}$ using equation RI_{4S2} in Sobel and Chen (2014) for each site individually and averaged across sites to obtain an estimate of mean $RI_{phenology}$. This metric of RI accounts for differences in flowering time and relative abundance. It assesses deviations from random mating, where populations flower simultaneously and have equal abundances.

Differential pollen production

To quantify pollen production for each subspecies, we grew plants of both subspecies from two sympatric populations (74 and 77, Fig. 1) and collected all four long anthers from one flower per individual at anthesis ($N = 20$ plants/subspecies/population). We gently macerated anthers with a probe and vortexed with 75 μl of lactophenol aniline blue. We counted two 10 μl aliquots for each sample using a dissecting microscope. We analyzed differences in pollen production using a linear model with population, subspecies, and their interaction as fixed factors. We calculated $RI_{Differential\ pollen\ production}$ using equation RI_{4A} of Sobel and Chen (2014), which calculates deviations from expectations of random mating, where pollen production is equal between subspecies.

Pollinator preference and constancy

To measure pollinator preference and constancy, we placed arrays of potted plants in the field at three sympatric sites (site 9, 77, and 22). We performed two sequential rounds of the experimental arrays at each site. Each round was in the field for 10–11 days and composed of different individual plants. Plant genotypes in an array originated from the site in which the arrays were placed. We constructed circular arrays (diameter of ca. 5 m), composed of 64 individuals, half from each subspecies, and systematically rotated subspecies around the array (spaced ca. 25 cm apart). Arrays were placed into the field just prior to the flowering of natural *xantiana* populations to minimize any influence of local populations on pollinator behavior in arrays. Bee visitors, including specialists, were common in arrays after a two-day acclimation period. Three researchers simultaneously watched different positions (10–12 plants) in an array. During 10-min observation periods, we recorded individual pollinator movements, keeping track of the plant genotype and the sequence of flowers visited for each bee. In each round, we watched each array for a total of 12 h (72 observation hours in total).

We estimated pollinator preference for each observation period by calculating the proportion of *xantiana* flowers visited and the proportion of flowers that were *xantiana* in that section of the array. We then used a Wilcoxon signed rank test to determine if pollinators visited *xantiana* at a greater frequency than would be expected by chance. We calculated RI for preference by comparing the expected visitation per flower with the observed visitation per flower for the two subspecies using equation RI_{4A} from Sobel and Chen (2014), where the expected per flower visitation is equal. We estimated pollinator constancy by extracting all pollinator bouts that included a visit to at least one flower of each subspecies and recorded all of the transitions within and between subspecies. We pooled data across sites because only a small proportion of foraging bouts included intersubspecific transitions. We then used a chi-square analysis with a permutation test of significance to determine if the observed pattern of transitions differed from random expectation based on the proportion of *xantiana* and *parviflora* visited. For calculations of RI, we compared the observed number of transitions between subspecies in either direction to the expected number of transitions between subspecies in either direction using equation RI_{4C} from Sobel and Chen (2014) for reproductive barriers with unequal random mating expectations.

POSTMATING-PREZYGOTIC BARRIERS

Pollen tube growth

We quantified postmating-prezygotic isolation by measuring pollen tube growth rates following single-donor intra- and intersubspecific crosses in a greenhouse. Crosses were performed between subspecies within each of two sympatric sites (74 and 77, Fig. 1). One individual from 20 maternal families per subspecies per site was grown in conetainers and randomized across conetainer racks such that individuals from both subspecies from both sites were found in each rack. We performed 249 crosses within and between the two subspecies by gently swiping fresh pollen on all lobes of fully receptive stigmas. For *parviflora*, we bud-emasculated flowers to prevent autonomous self-fertilization. After 4–6 h, we excised the styles, fixed them in FAA (15% Formaldehyde, 2.5% Glacial Acetic Acid, and 50% Ethanol in water), and stained using lactophenol aniline blue. We measured the maximum length of the style and the length of the longest pollen tube in the style.

We analyzed pollen tube growth rate using a linear mixed model. Fixed factors included maternal subspecies and paternal subspecies. Random factors included population, date of cross, and rack. We also included a covariate of the number of minutes from pollination to style fixation to account for some variation among crosses in the total period of pollen tube growth. Analyses were carried out in the R statistical environment (R Core Development Team 2012) using the lmer function in the lme4 package (Bates et al. 2013) and the pamer.fnc function using the conservative degrees of freedom in the LMERConvenienceFunctions package (Tremblay 2012). We calculated RI using equation RI_{4A} (Sobel and Chen 2014), which compares pollen tube relative growth rates in heterospecific versus conspecific pistils to assess deviations from random mating expectations.

Gametophyte competition

We performed reciprocal crosses between subspecies using mixed pollen loads of the two subspecies to quantify the effect of male gametophyte competition on RI. As above, we used two sympatric sites, 74 and 77 (Fig. 1). All crosses ($n = 351$) were performed within populations and for all intraspecific crosses we avoided pairing individuals of the same genotype. In order to eliminate pollen load as a potential cause of variation in seed set, we applied standardized pollen loads for each cross that far exceeded the number of ovules. Based on estimates of the number of pollen grains per anther for both subspecies, we applied 0.25 of a *xantiana* anther (half of one locule) and 1.5 *parviflora* anthers to each stigma to approximately standardize pollen loads. We rotated between applying *parviflora* versus *xantiana* pollen first to the stigma to account for any effect of order and to examine the effect of pollen precedence on fertilization rates. Fruits were allowed to ripen on the plant and collected before dehiscence.

To quantify hybridization rates, we planted up to 24 seeds from each fruit ($n = 2124$). Plants were grown in a greenhouse until flowering, at which time we measured four floral traits (petal size, herkogamy, protandry, and petal color), which are diagnostic of subspecies. From these traits, we determined whether offspring

were hybrids or not. Microsatellite genotyping has confirmed that floral traits are highly informative at scoring unknown offspring as *xantiana*, *parviflora*, or hybrid (see Supporting Information for more detail on scoring procedures).

We determined if there were differences in the probability of hybrid formation between subspecies using a binomial mixed model with a logit link function. We treated maternal subspecies, first pollen donor and their interaction as fixed factors. Population and the crossing round were treated as random factors. Analyses were carried out using the glmer function in the lme4 package (Bates et al. 2013) in the R statistical environment (R Core Development Team 2012). We calculated RI using equation RI_{4A} from Sobel and Chen (2014) to determine how hybrid seed production differed from parity. We did not include this measure of RI in the estimation of absolute contributions to RI because it overlaps with RI generated from both pollen tube growth and single-donor crosses.

POSTZYGOTIC BARRIERS

Single-donor crosses

We performed intra- and intersubspecific crosses in the greenhouse to test for and quantify the extent of postzygotic isolation at the level of seed production. We used individuals from sympatric sites 74 and 77 and allopatric 17, 26, 87, and 112 for *xantiana* and 20 and 46 for *parviflora* (Fig. 1). For sympatric sites, we performed crosses within subspecies and between the two sympatric subspecies from the same site. For allopatric sites, we performed crosses within each population and between each pair of allopatric populations from the opposite subspecies. For *parviflora*, we performed intraspecific crosses by outcrossing one set of flowers and selfing a second set. When *parviflora* flowers were outcrossed, they were first bud emasculated to prevent self-pollination. For the sympatric crosses, 50 crosses were conducted for each cross-type for each of the two populations. For the allopatric crosses, between 50 and 70 crosses were conducted for the pure cross-types ($x \rightarrow x$ and $p \rightarrow p$, *p*self) in each population, whereas between 15 and 70 crosses were conducted for the heterospecific crosses between two specific populations ($n =$ 1375 total crosses). Pollen loads for crosses were standardized for the two subspecies using the same procedure as described above (see *gametophyte competition*). For each fruit, we determined the number of seeds, fertilized but aborted ovules, and unfertilized ovules. We verified our ability to visually distinguish between fertilized and unfertilized ovules using an experiment (see Supporting Information for details).

We analyzed seed set per fruit and aborted ovules per fruit separately using a binomial mixed model with a logit link function. We treated region (allopatric vs. sympatric), maternal subspecies, and paternal subspecies as fixed factors. Maternal and paternal populations were treated as random factors. Analyses were carried out using the glmer function in the lme4 package (Bates et al. 2013) in the R statistical environment (R Core Development Team 2012). We calculated RI for number seeds produced from intersubspecific versus intrasubspecific crosses using RI_{4A} (Sobel and Chen 2014) for sympatric crosses.

F1 hybrid seed viability

We tested hybrid seed viability by assessing the proportion of seeds from intersubspecific crosses that were viable compared to seeds from intrasubspecific crosses. First, we assessed germination for each cross-type using seeds from eight greenhouse-generated maternal families from site 22. For each family, we placed 20 fully formed seeds on filter paper moistened with 2 ml of ultrapure water in a Petri dish and sealed the dishes with parafilm. We kept Petri dishes in a cold room set to 12°C with full lights for 24 h/day. We counted the number of seeds that had germinated after three weeks and planted them to assess growth (see below). We determined the viability of the remaining seeds by cutting them in half and soaking them in a solution of 0.2% tetrazolium chloride. After 48 h, we determined whether seeds stained red and were viable or did not stain red and were inviable. We analyzed the number of viable seeds (germinated + ungerminated but viable) out of all seeds tested for each maternal family using a fully fixed general linear model with a quasibinomial error structure and logit link function to account for overdispersion in the data. We included maternal subspecies, paternal subspecies, and their interaction as factors in the model. Analyses were carried out using the glm function in the R statistical environment (R Core Development Team 2012). We calculated RI using RI_{4A} (Sobel and Chen 2014) to compare observed differences in parental and F1 seed viability with the expectation that seeds would be equally viable.

F1 hybrid growth

We grew plants from the seed viability study (above) to assess hybrid performance. Germinated seedlings were carefully transferred from the filter paper to conetainers filled with soil and grown in the greenhouse and fertilized. After plants were established and entering a rapid growth phase ($\sim$ 6 weeks after planting), we counted all leaves on each plant twice, two weeks apart. We determined performance using growth rates. To assess growth rates, we analyzed the number of leaves added over two weeks using a mixed model ANCOVA. Maternal subspecies, paternal subspecies, and their interaction were included as fixed factors and the number of leaves at first measurement was included as a covariate to account for differences in initial plant size. We also included block and maternal family as random factors. Analyses were carried out using the lmer function in the lme4 package of the R statistical environment (R Core Development Team 2012). The two subspecies differ inherently in growth rates, so we expected viable hybrids to exhibit intermediate growth rates (and final plant

size), whereas we expected less-viable hybrids to exhibit slower growth rates (and smaller final size) than either of the parental subspecies. Because the potential intermediacy of hybrid growth rates is not indicative of hybrid inviability, we did not include this measure in overall calculations of RI.

F1 hybrid pollen fertility

We tested for a reduction in hybrid fertility by assessing the proportion of viable pollen in F1 progeny compared to parental subspecies. We germinated seeds from parental families and F1 hybrids, both of which were generated from single-donor crosses for two populations, sites 74 and 77 (Fig. 1). Plants were reared in growth chambers and completed development in the greenhouse as described above. For one flower of each individual plant, we collected all four long anthers. We placed anthers in a microcentrifuge tube, macerated the tissue gently with a probe, and vortexed the tube with 75 μL of lactophenol aniline blue. Lactophenol aniline blue will stain viable pollen grains deep blue while inviable grains remain unstained or only lightly stained. Viable pollen grains are also larger than inviable grains. We counted viable and inviable pollen grains from two 25 μl aliquots of the solution under a dissecting microscope. For the analysis, we used a fully fixed binomial model with a logit link using the glm function. We included population and the maternal and paternal subspecies of the individual and all interactions as independent factors. We excluded individuals of all cross-types with pollen viability of less than 2% as outliers ($N = 4$ records), although this did not change the overall results. We calculated RI using RI_{4A} (Sobel and Chen 2014) to compare observed differences in parental and F1 pollen viability with the expectation that pollen would be equally viable.

Results

TOTAL RI BETWEEN *C. XANTIANA* SUBSPECIES

The subspecies of *C. xantiana* are highly reproductively isolated and the isolating barriers are asymmetric in their strength. We found some RI for at least one subspecies at every component we measured (Table 1; Fig. 2A). Including all components of RI, *parviflora* is 98.7% isolated and *xantiana* is 99.8% isolated (Table 1). Much of the isolation is due to premating barriers including ecogeography, phenology, and pollinator preference. When ignoring ecogeography and considering RI only in sympatry, total RI decreases only slightly for *parviflora* to 96.5% and for *xantiana* to 99.5% (Table 1; Fig. 2B). For postzygotic mechanisms alone, *xantiana* has much higher levels of RI (30.8%) compared to *parviflora* (8.2%; Table 1).

PREMATING BARRIERS

Ecogeographic

The latitudinal and longitudinal expanse of *xantiana* and *parviflora*'s range is approximately 125 and 80 km, respectively, with an overlap zone of approximately 5–10 km (Fig. 1). Despite this narrow overlap, 43% of all known *xantiana* sites are within 3 km of a *parviflora* site and 44% of all *parviflora* sites are within 3 km of *xantiana*. When the radius is decreased to 1 km, 29% of *xantiana* sites are sympatric and 31% of *parviflora* sites are sympatric (Table S1).

Phenology

At all sites, *parviflora* was considerably less abundant than *xantiana*. On average, *parviflora* population sizes, measured in number of individuals, were 27.5% of *xantiana* population sizes (range among four sites: 11–689 *parviflora* and 670–1343 *xantiana*). *Parviflora* populations also produced on average only 17.9% of the flowers produced by *xantiana* populations (range among four sites: 31–1713 *parviflora* and 1990–9417 *xantiana*). On average across sites, *parviflora* had 26% of all flowers and buds remaining when *xantiana* began to bloom (range among four sites: 19–44%) and 86% of *xantiana* flowers bloomed before the *parviflora* season ended (range among four sites: 70–100%; see Table S2 for specific sites). Seventy percent of the total flowering season included flowers of both *xantiana* and *parviflora* (see Table S2 for specific sites).

Differential pollen production

Xantiana anthers produced nearly five times the amount of pollen as *parviflora* anthers (sample means: *xantiana* = 321.78 ± 14.93; *parviflora* = 65.3 ± 15.23), which was a significant difference ($F_{1,78} = 145.77$; $P < 0.0001$). There was no difference in pollen production between populations ($F_{1,78} = 2.26$; $P = 0.1366$) or interaction between population and subspecies ($F_{1,78} = 0.52$; $P = 0.4733$).

Pollinator movement

Pollinators exhibited strong and consistent preference for *xantiana* over *parviflora* (Wilcoxon signed rank test: $V = 35.23$, $P < 0.0001$). Compared to random expectations, pollinators overvisit *xantiana* by approximately 26%. Of all recorded pollinator observation periods, 81% (223 out of 276) included only visits to *xantiana* flowers despite the presence of *parviflora* flowers.

Of 289 pollinator foraging bouts, 27 bouts included at least one transition between subspecies (90 transitions within and among subspecies across the 27 bouts). When considering only these foraging bouts, there was no evidence of constancy to either subspecies. Our observed transition probabilities did not differ significantly from random expectations ($\chi^2 = 0.93$, $P = 0.807$; Table S3).

Table 1. Absolute and actual contribution of reproductive isolating mechanisms to total reproductive isolation (RI) between *xantiana* and *parviflora*.

	(A) Individual contribution RI		(B Absolute contribution		(C) Absolute contribution in sympatry		(D) Absolute contribution of postzygotic mechanisms	
	parviflora	*xantiana*	*parviflora*	*xantiana*	*parviflora*	*xantiana*	*parviflora*	*xantiana*
Premating prezygotic								
Ecogeographic[1]	0.5645	0.5730	0.5645	0.5730	–	–	–	–
Phenology[2]	0.9591	0.7850	0.4177	0.3352	0.9591	0.7850	–	–
Differential pollen production[3]	−0.6626	0.6626	−0.0118	0.0608	−0.0271	0.1425	–	–
Pollinator preference[3]	0.7600	0.7600	0.0153	0.0260	0.0351	0.0610	–	–
Pollinator constancy[4]	0.0639	−0.0384	0.0011	−0.0004	0.0025	−0.0009	–	–
Postmating prezygotic								
Pollen tube growth rate[3]	−0.1174	0.1420	−0.0020	0.0013	−0.0046	0.0030	–	–
Postzygotic								
Competitive crosses[3]	0.5500	0.9092	–	–	–	–	–	–
Single-donor crosses[3]	0.0604	0.2200	0.0017	0.0015	0.0039	0.0034	0.0604	0.2200
F1 seed viability[3]	0.0982	−0.0081	0.0024	0.0000	0.0055	−0.0001	0.0973	−0.0077
F1 pollen viability[3]	−0.0770	0.1023	−0.0018	0.0005	−0.0042	0.0011	−0.0760	0.0956
Total reproductive isolation			0.9870	0.9979	0.9647	0.9950	0.0817	0.3079

(A) Individual contribution of components to reproductive isolation. (B) Absolute contribution of the focal barrier after accounting for previous RI mechanisms (Sobel and Chen 2014). (C) Absolute contribution of RI mechanism in areas of sympatry. (D) Absolute contribution of postzygotic mechanisms.

Equations used to calculate RI from Sobel and Chen (2014): $^{1}RI_{4C}$; $^{2}RI_{4S2}$; $^{3}RI4_{A}$; $^{4}RI4_{G}$.

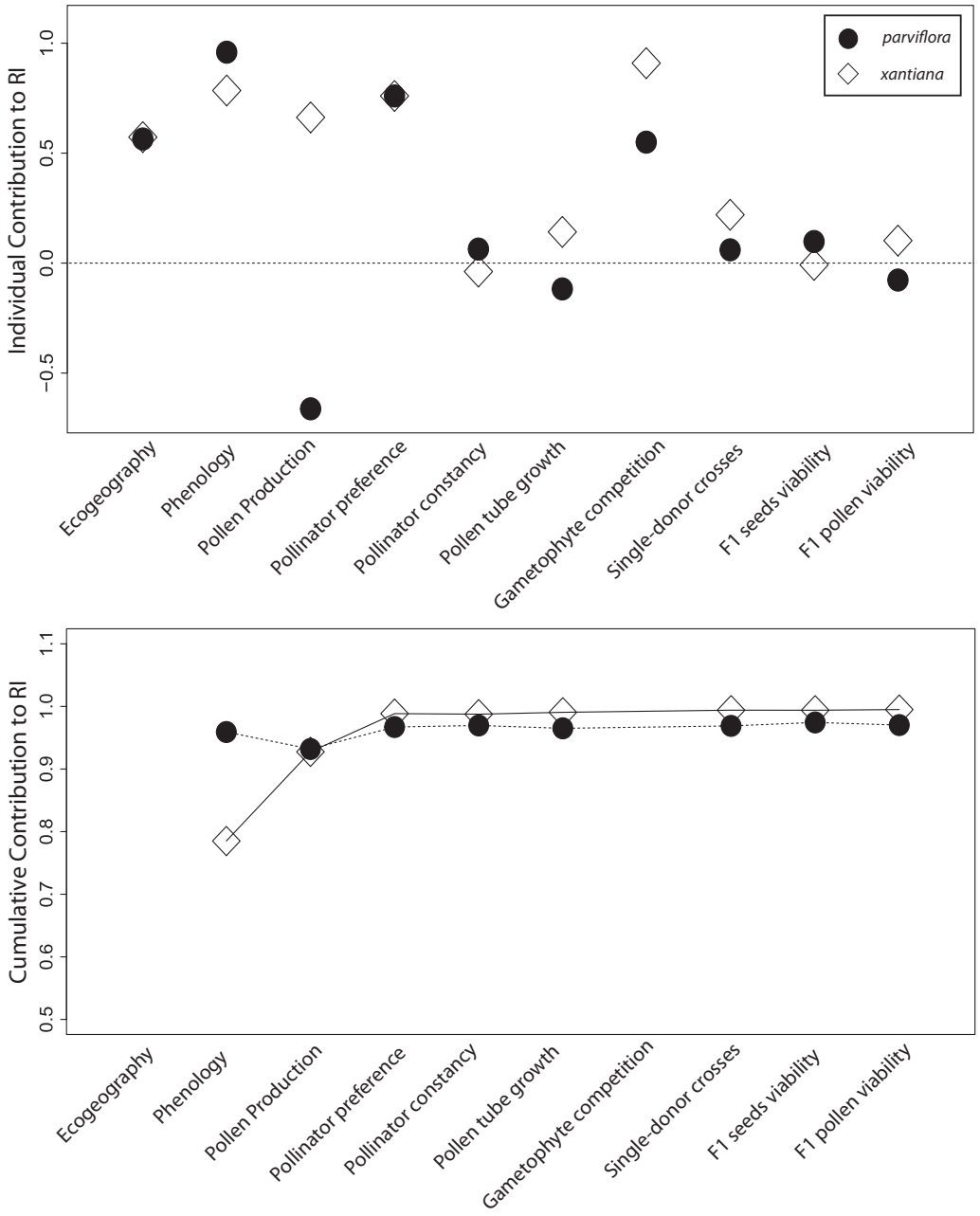

Figure 2. **Reproductive isolation (RI) between *parviflora* and *xantiana*. Panels show (A) the individual contribution of the isolating mechanisms to RI as calculated in the text and (B) the cumulative contribution to RI of a mechanism after accounting for previous mechanisms. The contribution of ecogeography is excluded from panel B to emphasize the relative importance of isolating mechanisms where the taxa are sympatric. *Parviflora* is shown in black circles and *xantiana* in open diamonds.**

POSTMATING-PREZYGOTIC BARRIERS

Pollen tube growth

Maternal and paternal subspecies both significantly influenced pollen tube growth rates, with maternal style environment having had the strongest effect. On average, pollen tubes grew 112% faster in *xantiana* than in *parviflora* styles ($F_{1,210} = 140.22$; $P < 0.0001$; Table S4). *Xantiana* pollen tubes grew significantly faster in both maternal style environments and on average 31% faster than *parviflora* pollen tubes ($F_{1,210} = 17.07$; $P < 0.0001$; Table S4).The maternal by paternal subspecies interaction had a marginally significant effect on growth rates ($F_{1,210} = 3.34$; $P = 0.069$; Table S4) due to a proportional increase in the growth rate of *xantiana* pollen tubes in *xantiana* styles (Fig. 3A). The covariate of growth period was not significant ($F_{1,210} = 0.04$; $P = 0.850$; Table S4).

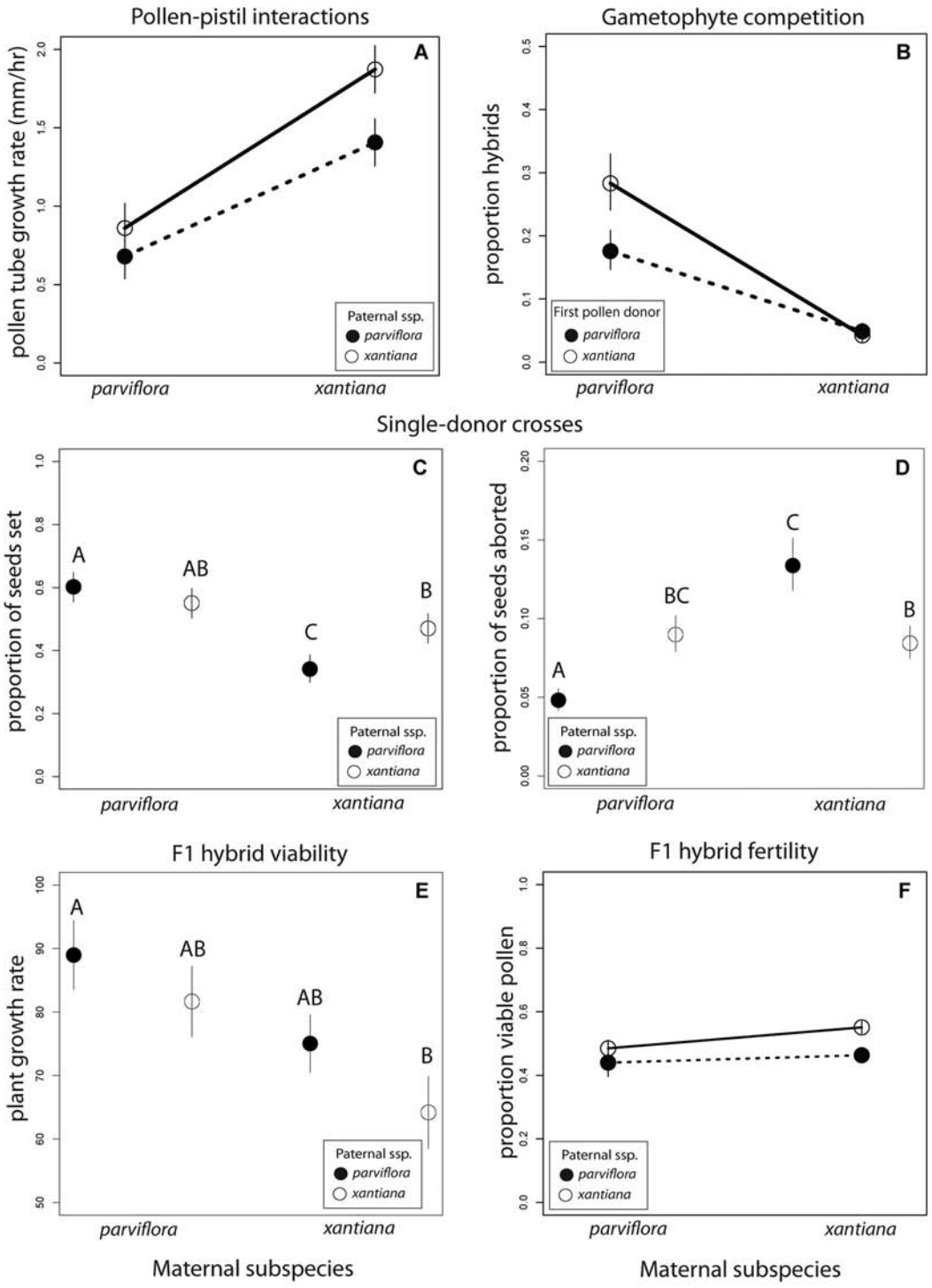

Figure 3. **Components of reproductive isolation between *parviflora* and *xantiana*. In all panels, the x-axis indicates the maternal subspecies in crosses within and between subspecies. Different symbols distinguish paternal subspecies contributions to the cross: open circles for *xantiana* and closed circles for *parviflora*. All estimates are least square means and error bars indicate standard errors. (A) Pollen tube growth rates (mm/h). (B) Pollen competition measured as the proportion of hybrids formed in fruits of a particular competitive cross. In these crosses, pollens from both subspecies were applied to each stigma. Different symbols indicate which subspecies was the first pollen donor of the cross. (C) Proportion of fully formed seeds set in fruits from single-donor crosses. (D) Proportion of aborted seeds from single-donor crosses. (E) Vegetative growth of reciprocal F1 hybrids and parentals (y-axis is the number of leaves added in a two-week period) in a greenhouse common garden study. (F) Proportion of viable pollen in reciprocal F1 hybrids and parentals in a greenhouse common garden study.**

POSTZYGOTIC BARRIERS

Gametophyte competition

The probability of hybrid formation in mixed crosses was significantly affected by the maternal parent, the subspecies of the first pollen donor, and their interaction (Fig. 3B; Table S5). *Parviflora* mothers were almost four times more likely to produce hybrids than *xantiana* mothers, resulting in a highly significant effect of maternal subspecies in the model ($\chi^2 = 118.39$; df = 1; $P < 0.0001$). There was a significant pollen precedence effect where stigmas pollinated first with *xantiana* were more likely to produce hybrids than those pollinated first with *parviflora* ($\chi^2 = 6.69$; df = 1; $P = 0.0097$). *Parviflora* that were first pollinated with *xantiana* pollen had a 61% higher probability of producing hybrids than those pollinated first with *parviflora* pollen (Fig. 3B). *Xantiana* was very unlikely to produce hybrids regardless of the subspecies of the first pollen donor (Fig. 3B).

Single-donor crosses

We detected a significant reduction in crossing success between subspecies compared to within. Notably, crosses from *parviflora* to *xantiana* resulted in lower seed set (proportion of ovules converted to seeds) than in the reciprocal direction; this asymmetry was apparent in a highly significant maternal by paternal subspecies interaction (Table S6; $\chi^2 = 746.95$; df = 1; $P < 0.0001$). The proportion of seeds set by *xantiana* when sired by *parviflora* was significantly reduced by 27.4% compared to when sired by *xantiana*. *Parviflora* seed set was reduced by 8.6% seeds when sired by *xantiana* compared to when sired by *parviflora* (Fig. 3C). We also detected significant regional interaction effects in the model (Table S6). The magnitude of the maternal by paternal subspecies interaction was somewhat stronger in allopatry than sympatry for the cross from *parviflora* to *xantiana* (the cross most likely to fail; Fig. S1).

The probability of seed abortion from single-donor crosses was significantly influenced by both the maternal and paternal subspecies identity. Maternal *xantiana* had a significantly higher probability of abortion overall compared to *parviflora* (61.5% greater). Intersubspecific crosses had higher abortion rates for both cross directions compared to intrasubspecific crosses (Fig. 3D; Table S6; $\chi^2 = 380.13$, df = 1, $P < 0.0001$). We did not detect significantly different abortion rates in sympatric and allopatric crosses.

F1 hybrid seed viability

The majority of seeds were viable for all cross-types (range: 61.25–94.38%). There was a significant main effect of paternal subspecies ($\chi^2 = 22.2$, df = 1, $P < 0.0001$), where fruits with seeds sired by *parviflora* pollen had 36.8% more viable seeds than those sired by *xantiana* pollen.

F1 hybrid growth

Hybrid growth rates were intermediate when compared to the growth rates of the two parental subspecies, suggesting that hybrid offspring exhibited similar viability to parental genotypes (Fig. 3E). Parental *parviflora* produced 38.6% more leaves than parental *xantiana*. There was a significant maternal effect ($F = 8.00$; df = 1, 98; $P = 0.0057$); plants with *parviflora* mothers produced 22.6% more leaves than plants with *xantiana* mothers. This maternal effect was evident but not significant in F1 hybrids.

F1 hybrid pollen viability

We did not find evidence for elevated pollen sterility in F1 hybrids. We detected maternal and paternal effects on pollen viability, where individuals with a *xantiana* mother and father had 9.7% and 14.7% higher pollen viability, respectively (maternal effect: $\chi^2 = 4.07$, df = 1, $P = 0.0438$; paternal effect: $\chi^2 = 10.04$, df = 1, $P = 0.0015$). However, we did not detect a significant two-way interaction indicating lower hybrid pollen viability (Fig. 3F; $\chi^2 = 0.14$, df = 1, $P = 0.7104$); there was only a subtle difference between parental *xantiana* and F1 hybrids with *xantiana* mothers in post hoc tests. We found a significant difference between populations ($\chi^2 = 44.98$, df = 1, $P < 0.0001$) and there was a significant three-way population by maternal by paternal subspecies interaction ($\chi^2 = 7.256$, df = 1, $P = 0.0071$; Table S7). Although we did not observe elevated hybrid sterility in either population, the pattern of the maternal by paternal subspecies interaction differed between them (Fig. S2).

Discussion

We found that RI between *xantiana* and *parviflora* was nearly complete (*xantiana*: 99.8%; *parviflora*: 98.7%) despite the relatively recent divergence time between the subspecies (ca. 65,000 years bp). Similar to findings in other species pairs, premating barriers contributed disproportionately to RI (Lowry et al. 2008). The absolute contributions of individual premating barriers were generally higher than for postmating mechanisms (Table 1), confirming our initial hypothesis. Further, when accounting for the sequential nature of isolating barriers, the reproductive barrier between the subspecies was nearly complete after accounting for all premating mechanisms (*xantiana*: 99.6%; *parviflora*: 98.5%) and the results were similar when considering barriers only in sympatric populations (*xantiana*: 99.1%; *parviflora*: 96.1%). Despite the limited overall contribution of postmating barriers to RI, we did discover a pronounced asymmetry in seed production for interspecific crosses. This postmating asymmetry is common between diverging plant species (Tiffin et al. 2001), but the mechanisms underlying most instances are unknown. In this system, the asymmetric barrier arose strictly during seed development and did not involve pollen–pistil interactions.

Xantiana and *parviflora* have experienced ecological divergence in traits due to environmental differences between their respective ranges, especially the pronounced west-to-east precipitation and temperature gradient (Eckhart et al. 2004; Geber and Eckhart 2005). Adaptation to their respective ranges has resulted in geographic differences that prevent mating opportunities in allopatric populations. For both *xantiana* and *parviflora*, approximately 57% of all known populations are considered allopatric, limiting the opportunity for the taxa to hybridize. Ecological divergence has also led to differences in flowering phenology between *xantiana* and *parviflora*. As part of adaptation to drier environments, *parviflora* has evolved a life-history strategy consistent with drought avoidance; plants do not exhibit elevated tolerance to drought conditions, but instead develop rapidly and flower prior to water stress (Mazer et al. 2010). In 2013, we found that 74% of *parviflora* flowers produced during the season did not overlap with *xantiana*, limiting mating opportunities. This was a very dry year where *parviflora*'s flowering season was earlier and compressed. Flowering phenology had one of the largest absolute contributions to RI for *parviflora*. Although this is undoubtedly an important mechanism and may greatly limit interspecific matings within a particular season, flowering phenology is also highly temporally variable. We have observed years in which sympatric populations have flowered nearly synchronously. Flowering time divergence may therefore be unreliable and of limited importance in promoting complete RI over long periods of time.

Aside from flowering time, *xantiana* and *parviflora* are most highly diverged in mating system (Eckhart and Geber 1999; Runions and Geber 2000). *Parviflora* evolved autonomous self-pollination and floral characteristics associated with the "selfing syndrome" (Goodwillie et al. 2010) in allopatry and subsequently experienced western range expansion to the area of sympatry (Pettengill and Moeller 2012b). *Parviflora* flower size is reduced with limited to no herkogamy or protandry (Runions and Geber 2000; Moeller 2006). This pattern of floral divergence is strongest in sympatric populations, especially for *parviflora* (Briscoe Runquist and Moeller 2014). Floral divergence had important consequences for pollinator behavior and the probability of interspecific movements. In experimental arrays, there was a pronounced preference for *xantiana* over *parviflora*. In individual contribution to RI, pollinator preference had a large effect (76%) and contributed 6.1% to total RI after accounting for differences in phenology and relative abundance of plants and pollen. Our results are similar to Martin and Willis (2007), the only other comprehensive study of RI between sister outcrossing and selfing taxa. In their study, flowering phenology and mating system were also the most important isolating mechanisms between *M. guttatus* and *M. nasutus*, which diverged approximately 200,000–500,000 years bp (Brandvain et al. 2013). Investigations of RI between young species pairs are particularly necessary for distinguishing mechanisms associated with the speciation process from subsequent evolution between diverging lineages. The very recent divergence of *xantiana* and *parviflora* (ca. 65,000 years bp; Pettengill and Moeller 2012a) highlights that isolating barriers that confer nearly complete RI can evolve quite rapidly.

In the absence of premating crossing barriers, *xantiana* and *parviflora* are partially isolated through postzygotic mechanisms; *xantiana* had a total postzygotic RI of 30.8% and *parviflora* had a total postzygotic RI of 8.2%. This degree of postzygotic RI between such recently diverged lineages is surprising and indicates the fixation of BDM incompatibilities in both lineages. Similar evidence of postzygotic isolation has been observed in other closely related outcrosser–selfer pairs including *Arenaria uniflora* (Fishman and Stratton 2004) and *M. guttatus* (Fishman and Willis 2001). Our experiments also compared postmating RI between allopatric populations to test the hypothesis that postmating RI is elevated in sympatry. The reinforcement hypothesis predicts that premating and postmating-prezygotic isolating barriers will be elevated in sympatry relative to allopatry (Coyne and Orr 1989). The experiment we present here only includes the contribution of postmating processes. We did not find support for this prediction as we find similar to slightly higher postmating RI between allopatric populations. Of the few previous studies examining sympatric and allopatric crosses, some find similar postmating barrier strength (Moyle et al. 2004) whereas others have found stronger pollen–pistil interactions in sympatry (Kay and Schemske 2008). Ongoing work on *C. xantiana* tests whether premating barriers are elevated in sympatry.

For *xantiana*/*parviflora*, some of the BDMs are unidirectional because there is asymmetry in the crossing barrier (Turelli and Moyle 2007). BDMs may have fixed either through drift during geographic separation or as a by-product of divergent selection in both lineages. Fixation of BDMs due to drift may be less likely, considering their very recent divergence time. Even when populations experience severe bottlenecks, such as in the evolution of selfing (Schoen et al. 1996), fixation of incompatibilities via drift is not often observed (Turelli et al. 2001). It may be more likely that genetic divergence due to selection and the accelerated fixation of advantageous alleles in both subspecies resulted in the crossing barrier. As previously discussed, selection in *xantiana* and *parviflora* has acted on a diverse array of traits affecting development, physiology, and mating system (Eckhart and Geber 1999; Runions and Geber 2000; Eckhart et al. 2004; Geber and Eckhart 2005; Moeller 2006; Mazer et al. 2010). Alleles fixed by positive selection during reciprocal adaptation to *xantiana* and *parviflora*'s respective ranges could contribute to allelic incompatibilities causing postzygotic RI.

The asymmetry that we observed is common in plants (Tiffin et al. 2001) and may be caused by pollen–pistil interactions

(Ruane 2009; Yost and Kay 2009), cytonuclear incompatibilities (Bomblies 2010), or expression-related maternal effects (Turelli and Moyle 2007). We found no evidence of pollen–pistil interactions, as *xantiana* pollen tubes consistently grew faster than *parviflora*. These results are consistent with theoretical predictions about outcrossers having higher male competitive ability than selfers (Kondoh and Higashi 2000; Brandvain and Haig 2005) and contradict a recent field study where *parviflora* was unexpectedly found to have higher pollen tube growth rates than *xantiana* (Hove and Mazer 2013). Beyond pollen tube growth rates, we have also observed successful fertilization of ovules in interspecific crosses, with no apparent inhibition of pollen tubes in styles of the other subspecies. Interestingly, our results run counter to those in the outcrosser–selfer pair, *M. guttatus* and *M. nasutus*, where conspecific pollen precedence is pronounced in *guttatus* styles but not *nasutus* styles (Kiang and Hamrick 1978; Fishman et al. 2008). Because postmating-prezygotic barriers can be shaped by selection, it has been predicted that they may evolve more rapidly than postzygotic barriers. Our findings do not support this prediction much like previous work by Moyle et al. (2004).

Asymmetry in postmating RI was more clearly observed during seed development. In crosses from *parviflora* to *xantiana*, we observed significantly reduced seed set and elevated seed abortion rates. In the opposite direction, we detected a less severe reduction in seed set and no strong evidence of elevated abortion rates. These results suggest that *xantiana* ovules are successfully fertilized by *parviflora* pollen tubes but fail to fully mature. Although this pattern could result from cytonuclear interactions, our evidence does not appear to support this mechanism. When cytonuclear interactions are important, hybrid inviability and sterility is common in F1s and/or segregating in F2s (e.g., chlorotic seedlings; Stubbe 1964; van der Meer 1974; Burton et al. 2013). In *C. xantiana*, however, after successful F1 seed development, we have not observed any reduction in seed viability, germination rate, growth, or fertility in F1s (reported here) or F2s (R. D. Briscoe Runquist and D. A. Moeller, unpubl. data). Expression-related maternal effects can also act as unidirectional BDMs. In plants, incompatibilities can occur in the developing zygote or in the development of the triploid endosperm (Cresti and Tiezzi 1997). Hybrid seed failure has frequently involved abnormal endosperm development due to interactions between the haploid male and double haploid female genetic components (Lin 1984; Haig and Westoby 1991; Katsiotis et al. 1995; Bushell et al. 2003; Gutierrez-Marcos et al. 2003). Differential imprinting may be one maternal effect that results in endosperm dysfunction (Haig and Westoby 1991), particularly when crosses occur between outcrossers and selfers (Brandvain and Haig 2005). The role of maternal effects in postzygotic RI between *xantiana* and *parviflora* is unknown and warrants further investigation.

Finally, population genetic analyses have indicated that there is introgression of *parviflora* alleles into *xantiana* genomic backgrounds, with introgressed alleles limited only to the narrow zone of sympatry (Pettengill and Moeller 2012a). Introgression from *xantiana* to *parviflora* is less apparent, but gene flow in this direction is harder to detect because segregating ancestral variation may persist in derived selfing lineages (Brandvain et al. 2013). Nevertheless, the direction of introgression is counter to that of the observed crossing barrier, which suggests that processes occurring after F1 formation may contribute more importantly to the pattern of introgression in the nuclear genome. For example, a higher probability of backcrossing from F1s to *xantiana* may more frequently introduce *parviflora* alleles into *xantiana* than the reverse. Because F1 hybrids' flowering time is intermediate between subspecies, the later flowering *xantiana* may disproportionately sire offspring on F1s with female phase flowers. In addition, because bee visitors discriminate against small *C. xantiana* flowers (D. A. Moeller, unpubl. data), backcrossing with *parviflora* may be particularly unlikely. Field experiments examining pollinator responses to hybrid phenotypes are needed to directly test this hypothesis.

Conclusions

Our examination of the components of RI between *xantiana* and *parviflora* indicate that both premating and postzygotic barriers have evolved rapidly because the recent divergence of the taxa. Premating barriers, especially flowering time and pollinator preference, result in limited opportunities for hybridization. These results are quite similar to those of Martin and Willis (2007), the one other study to comprehensively examine RI between sister outcrossing and selfing taxa. The genetics of postzygotic isolation, however, depart significantly from previous studies. Despite the very recent divergence time, we find substantial (and asymmetric) barriers to seed production that arise strictly during seed development. The absence of pollen–pistil interactions and hybrid inviability or sterility suggest that expression-related maternal effects on seed development may be a plausible mechanism. Taken together, the results explain the rarity of hybrids in sympatry and suggest that mating system divergence may have important consequences for the formation of new species.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.n4mm0.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Three-way interaction plot for regional, maternal subspecies, and paternal subspecies effects on seed set from single-donor crosses.
Figure S2. Proportion of viable pollen in reciprocal F1 hybrids and parentals in a greenhouse common garden study of two populations.
Figure S3. Correlations between pairs of traits measured from offspring that resulted from gametophyte competition crosses.
Figure S4. Proportion of seeds aborted from crosses involving different numbers of pollen grains.
Table S1. Proportion of sites within the sympatric zone.
Table S2. Phenological transect details.
Table S3. Test of pollinator constancy in sympatric arrays.
Table S4. Pollen tube growth rates.
Table S5. Gametophyte competition.
Table S6. Analysis of deviance tables for binomial mixed models of seed set and aborted seeds in single-donor crosses.