

POLLINATOR SPECIFICITY DRIVES STRONG PREPOLLINATION REPRODUCTIVE ISOLATION IN SYMPATRIC SEXUALLY DECEPTIVE ORCHIDS

Michael R. Whitehead^{1,2} and Rod Peakall¹

¹Evolution, Ecology and Genetics, Research School of Biology, The Australian National University, Canberra, Acton 0200, Australia

²E-mail: michael.r.whitehead@gmail.com

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Few studies have quantified the full range of pre- and postzygotic barriers that limit introgression between closely related plant species. Here, we assess the strength of four isolating mechanisms operating between two morphologically similar and very closely related sympatric orchid taxa, *Chiloglottis valida* and *C. aff. jeanesii*. Each taxon sexually attracts its specific wasp pollinator via distinct floral volatile chemistry. Behavioral experiments with flowers and synthetic versions of their floral volatiles confirmed that very strong pollinator isolation is mediated by floral odor chemistry. However, artificially placing flowers of the two taxa in contact proximity revealed the potential for rare interspecific pollination. Although we found hybrid vigor in F1 hybrids produced by hand-crossing, genetic analysis at both nuclear and chloroplast loci showed significant and moderate-to-strong genetic differentiation between taxa. A Bayesian clustering method for the detection of introgression at nuclear loci failed to find any evidence for hybridization across 571 unique genotypes at one site of sympatry. Rather than inhibiting gene flow, postpollination barriers surveyed here show no contribution to overall reproductive isolation. This demonstrates the primacy of pollinators in maintaining species boundaries in these orchids, which display one of the strongest known examples of prepollination floral isolation.

KEY WORDS: *Chiloglottis*, floral isolation, floral odor, hybridization, pollinator isolation, population genetics, prezygotic isolation, reproductive isolation, sexual deception.

Reproductive isolation inhibits the free exchange of genetic variation between lineages and is central to the generation and maintenance of species (Coyne and Orr 2004). Isolating barriers act as sequential obstacles to gene flow, and are typically classified by their order of appearance in the life cycle, with early-acting barriers exerting a proportionately larger effect on overall reproductive isolation (Coyne and Orr 2004). For plants, these include the pre-pollination barriers of geographic isolation, temporal isolation, and floral isolation by mechanical and ethological means (Grant 1949; Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2008; Schiestl and Schluter 2009). Postpollination barriers then include pollen competition, pollen–pistil interactions, and gametic incompatibilities, which form the final barriers in prezy-

gotic isolation. Postzygotic barriers act last and include embryo abortion, hybrid sterility, and depressed hybrid fitness (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004; Rieseberg and Willis 2007).

Floral isolation is the inhibition of gene flow due to divergent use of pollinators and has long been hypothesized to be a key driver in the radiation of flowering plants. This is because pollinator shifts or divergent specialization for different pollinators can in theory lead to rapid and effective reproductive isolation (Grant 1949; Stebbins 1970; Johnson 2006; van der Niet and Johnson 2012). The trend emerging from published studies however suggests that pollinator isolation seldom acts alone, with reproductive isolation more often arising as a consequence



of synergistic interactions between early- and late-acting barriers (Hodges and Arnold 1994; Morrison et al. 1994; Chari and Wilson 2001; Husband and Sabara 2004; Kay 2006; Scopece et al. 2013; Van der Niet et al. 2014). For example, although *Mimulus lewisii* and *M. cardinalis* attract distinct pollinators with high fidelity (bumblebee and hummingbird pollination, respectively), they occur sympatrically in only part of their range and are otherwise ecogeographically separated by altitude. Further, interspecific crosses result in lower seed set and F1 hybrids exhibit depressed germination (Ramsey et al. 2003). In another example, partially sympatric *Petunia* species that are morphologically adapted to different pollinators are actually strongly isolated by the combination of minimal range overlap, divergent pollinators, as well as gametic incompatibilities (Dell'Olivo et al. 2011).

Despite the progress, our understanding of reproductive isolation in plants is still built on just a few case studies of closely related taxa [reviewed in Lowry et al. (2008) and Scopece et al. (2013)] and a few wide comparative studies (Moyle et al. 2004; Scopece et al. 2007; Jewell et al. 2012). More studies quantifying the range of pre- and postpollination isolating barriers are therefore required to build generalities and identify informative exceptions (Ramsey et al. 2003; Moyle et al. 2004; Martin and Willis 2007; Lowry et al. 2008).

The orchids should be a major focal point for examining the role of pollinators in reproductive isolation because they often exhibit highly specific pollinator relationships (Tremblay et al. 2005; Schiestl and Schluter 2009). Yet, despite their central place in the emergence of the field of pollination biology (Darwin 1877), the orchids remain surprisingly underrepresented in evolutionary studies (Peakall 2007). Perhaps one of the more extreme forms of pollinator specificity is found in sexually deceptive orchids that attract their pollinators via the mimicry of sexual signals (Schiestl et al. 1999, 2003; Schiestl 2005; Ayasse et al. 2011). After attraction, male insect pollinators are duped into receiving or transferring pollen when mating routines are stimulated by chemical and tactile cues of the flower. This intriguing pollination strategy has evolved independently on at least four continents (Africa, Australia, Europe, and South America), and is known to be employed by several hundred orchid species (Gaskett 2011). Furthermore, new discoveries of sexual deception continue to be made both within (e.g., Phillips et al. 2014) and beyond the Orchidaceae (e.g., within a daisy [Ellis and Johnson 2010] and an iris [Vereecken et al. 2012]).

There are presently very few plant species for which the strength of multiple potential reproductive barriers have been comprehensively investigated (Rieseberg and Willis 2007; Scopece et al. 2013). Among sexually deceptive orchids, only one case in the European genus *Ophrys* (Xu et al. 2011) has been investigated in detail. Thus, there is a need for more studies of reproductive isolation between closely related species to build our

understanding in both sexually deceptive systems and flowering plants more generally.

Here, we report a detailed study on the nature and strength of pre- and postpollination reproductive barriers in two sympatric species of Australian *Chiloglottis* orchids. First, we described and quantified prepollination reproductive isolation through floral volatile chemistry analysis and behavioral experiments with flowers and synthetic versions of the floral volatiles they emit. Second, we investigated postpollination barriers by germination of seed from experimental crosses. Lastly, we quantified genetic divergence between the taxa at both nuclear and chloroplast microsatellite loci and used this population genetic data in a thorough hybrid analysis designed to detect rare hybridization events.

Methods

STUDY SPECIES

The subjects of this study were the terrestrial orchids *Chiloglottis valida* and an undescribed taxon. Analysis indicates that the undescribed taxon shares genetic affinity with *C. jeanesii*, a taxon considered to be locally restricted to Victoria, southeastern Australia. Hence, we refer to this taxon as *C. aff. jeanesii*. These taxa are typical of their genus in that they are self-compatible and form clonal colonies in the understory of moist forest habitats in southeastern Australia (Peakall et al. 1997). The specific interaction between *Chiloglottis* orchids and their wasp pollinators is known to involve one, two, or three compounds from a pool of six related natural products, all 2,5-dialkylcyclohexane-1,3-diones or “chiloglottes” (Schiestl et al. 2003; Franke et al. 2009; Peakall et al. 2010).

Chiloglottis valida is known to attract its male thynnine pollinator *Neozeleboria monticola* with the single volatile compound, 2-ethyl-5-propylcyclohexane-1,3-dione or chiloglottone 1 (Schiestl and Peakall 2005). Similarly, evidence including gas chromatography with electroantennographic detection (GC-EAD), gas chromatography with mass selective detection (GC-MS), and bioassays with synthetic compound has revealed that *C. aff. jeanesii* attracts its undescribed pollinator (a *Neozeleboria* species in the *impatiens* species complex, *N. sp.* (*impatiens2*), see Griffiths et al. 2011) by a structural isomer of chiloglottone 1; 2-butyl-5-methylcyclohexane-1,3-dione or chiloglottone 3 (Whitehead and Peakall 2009; Peakall et al. 2010).

These two study taxa are each representative of sister species complexes within the “valida” clade, one of the three main clades of *Chiloglottis* sensu lato (Peakall et al. 2010). Within each of these two species complexes, there is very limited phylogenetic resolution (based on nuclear internal transcribed spacer [ITS] and 10,000 bp of noncoding cpDNA intron/spacer region DNA sequence in phylogenetic analysis including seven taxa allied to

C. valida and three taxa allied to *C. aff. jeanesii*). Furthermore, genetic differentiation among pairs of taxa spanning the two complexes falls well within the range of that typical for population-level, rather than species-level differentiation in orchids (e.g., F_{ST} ranges from 0.23 to 0.36 between *C. aff. jeanesii* and three species allied to *C. valida*, as estimated across 13 nuclear microsatellite loci, see Peakall and Whitehead (2014) for details, and Phillips et al. (2012) for a meta-analysis of orchid differentiation). Thus, we knew a priori that our two study taxa exhibited some divergence at cpDNA loci, while at the same time being very closely related. This enabled us to simultaneously investigate introgression at nuclear loci and assess whether incompatibilities had accumulated subsequent to the initiation of isolating barriers among the species complexes.

These two taxa further offer a particularly compelling reason for the study of floral isolation, in that the only known populations of *C. aff. jeanesii* are sympatric with *C. valida*, the range of the former embedded within the latter, making them truly ecogeographically sympatric (Peakall and Whitehead 2014). At sites of sympatry, the two orchids can commonly be found flowering in patches separated by 2–10 m and sometimes within the same patch (<1 m). In addition, the known occurrence of flowering hybrids between *C. valida* (in the *valida* clade) and more distantly related *C. trapeziformis* (in the *formicifera* clade; Peakall et al. 1997; Schiestl and Peakall 2005) suggests that F1 hybrids (if they exist) could be viable and detectable in the field. These species therefore fulfill all criteria as ideal systems for investigating the influence of floral odor and pollinator behavior on population genetics and speciation (Whitehead and Peakall 2009). Namely, they should be closely related, sympatric, coflowering, morphologically similar, and interfertile.

GENETIC AND CHEMICAL SAMPLING

Two levels of sampling across the taxa were undertaken. First, a “taxon-level” sample of individuals across two known sites of sympatry (approximately 180 km apart) was obtained to delineate taxon boundaries with respect to floral odor, validate diagnostic chloroplast markers, and assess nuclear population genetic differentiation. Each of these taxon-level samples was collected in flower and analyzed for floral scent chemistry before completing the genetic analysis. Second, we obtained an extensive fine-scale “population-level” sample at a single site of sympatry in Kanangra-Boyd National Park (KBNP), NSW, designed to detect rare hybrids. Here, in 2007, we mapped a 2 m × 2 m grid onto a 100 m × 24 m transect and collected one leaf sample per grid square. For the population sample, we collected a flowering specimen where possible, however because only a fraction of plants flower in any one season (Peakall et al. 1997), many individuals were only collected as a leaf. This made taxonomic diagnosis by morphology or floral chemistry impossible and for these samples

we used chloroplast DNA as a diagnostic marker (see below). For each grid square without a flowering specimen, we preferentially collected a random leaf in the southeastern quarter so as to space collections from adjacent grid squares. If a leaf was not available from this quarter, we rotated our preference clockwise until we had collected a sample. Over subsequent flowering seasons (2008–2011), tissue collections, floral volatile samples, and pollination data were collected from flowering individuals over several days each season (see Table S1 for breakdown of sampling effort).

For floral volatile analysis, single labella were extracted for 5 min in 200 µL of high-performance liquid chromatography (HPLC) grade dichloromethane (DCM), within 48 h of collection (following Peakall et al. 2010). Labella of volatile analysis samples were preserved in ethanol for later morphological analysis and a single leaf was taken as a DNA sample for genetic analysis.

FLORAL CHEMISTRY

To confirm that floral volatile composition corresponds with species boundaries, we applied GC-MS to identify the active compound in flowers from the taxon-level sample. GC-MS was performed following Peakall et al. (2010) and employed selective ion monitoring designed to specifically target chiloglottones from single-labellum extracts (Whitehead and Peakall 2009).

OBTAINING GENETIC DATA

Leaf samples were lyophilized and then DNA was extracted using a Qiagen (Valencia, CA) Plant-Mini kit as per the manufacturer’s instructions. For our taxon-level sample, we employed both nuclear and chloroplast microsatellites as markers for genetic analysis. Our set of 13 nuclear loci was drawn from the 16 loci developed for *Chiloglottis* by Flanagan (2006), who also describe the DNA extractions, PCR, and genotyping methods followed in this study. For chloroplast DNA analysis, we employed the set of 64 cpDNA genetic markers designed by Ebert et al. (2009) to target both intra- and interspecific polymorphic cpSSRs and cpIndels in *Chiloglottis*. These genetic makers were discovered within *Chiloglottis* by first employing the universal set of cpDNA sequencing primers of Ebert and Peakall (2009) with the final set of markers spread across more than 19 kb of noncoding sequence within the large single copy region of the chloroplast. The cpDNA laboratory methods followed Ebert et al. (2009).

To increase efficiency in analyzing our fine-scale population sample, we reduced the number of markers used to the six nuclear loci that gave the highest resolution from the 13 described above (Whitehead 2012). As many of the population samples were collected as a leaf only, we also used an agarose-based PCR assay to identify the taxon based on two diagnostic cpIndels (see Supporting Information: Additional Methods).

Table 1. Summary of genetic statistics based on nuclear and chloroplast DNA analysis of two sympatric orchid populations; Kanangra Boyd National Park (KBNP) and Tallaganda State Forest (TA).

	<i>Chiloglottis valida</i>		<i>C. aff. jeanesii</i>	
	KBNP	TA	KBNP	TA
<i>N</i> samples	22	10	27	27
Nuclear SSR summary statistics (13 loci)				
H_o	0.505 (0.068)	0.600 (0.063)	0.325 (0.083)	0.407 (0.084)
H_e	0.584 (0.059)	0.633 (0.052)	0.340 (0.079)	0.440 (0.091)
F	0.124 (0.079)	0.068 (0.048)	0.115 (0.07)	0.058 (0.033)
Haplotypes based on cpIndels (23 loci) + cpSSRs (41 loci) = (64 loci)				
Haplotypes	8	6	8	12
Private haplotypes	6	4	4	8
Shared haplotypes	0	0	7	7
Number of effective haplotypes	4.321	5	5.481	7.078
Haplotype diversity (h)	0.769	0.800	0.818	0.859

See Ebert et al. (2009) for formulae used in these calculations.

GENETIC DATA ANALYSIS

Unless otherwise stated, GenAlEx 6.5 (Peakall and Smouse 2006, 2012) was used for population genetic analysis of both the nuclear and chloroplast genetic markers. For the taxon and population sample, we discarded samples amplifying at fewer than 12 and five loci, respectively. We identified and discarded clones with matching multilocus genotypes and retained only one sample in any such match. The numbers of samples in the taxon-level analysis are reported in Table 1 and we included 571 unique genotypes in the population-level hybrid analysis (see below). For both marker types, we calculated descriptive statistics such as heterozygosity, allele and haplotype frequencies, as well as performing analysis of molecular variance (AMOVA; Excoffier et al. 1992; Peakall et al. 1995) to investigate the partitioning of genetic variation within and among a priori groupings of the samples and taxa. AMOVA was carried out separately for chloroplast and nuclear genetic data, and tests for departure from the null hypothesis of no genetic differentiation among groups were performed by random permutation (999 permutations per run). In addition to the standard estimators of genetic differentiation (F_{ST} and Φ_{ST}), we also employed AMOVA to estimate standardized F'_{ST} and Φ'_{ST} and their regional analysis equivalents following the formulae of Meirmans (2006) for standardizing F_{ST} -analogs.

GenAlEx was also employed to prepare files for export of the chloroplast haploid data to the software package Network 4.6 (Fluxus Technology Ltd. 2010). This package was used to reconstruct median-joining networks of the nonrecombining haploid chloroplast data, following the manufacturer guidelines. For background to the statistical procedures that underpin this analysis, see Bandelt et al. (1999) and Forster et al. (2000).

POLLINATOR FIDELITY AND POLLINATOR ISOLATION

To experimentally test the influence of floral volatiles on specific pollinator attraction and the potential for interspecific pollination, we conducted pollinator choice tests with mixed arrays of flowers from both taxa. Pollinator choice tests with synthetic pheromone baits were also conducted, following Peakall et al. (2010). At two sites (Tallaganda State Forest (TA) and KBNP) where both taxa of orchid and pollinator were known to occur, we simultaneously presented four baits: chiloglottone 1, chiloglottone 3, a 1:1 blend of both chiloglottones, and a DCM control. Synthetic chiloglottones dissolved in HPLC-grade DCM were checked for purity and standardized for concentration (1 mg/mL) at the beginning of each field season (Poldy et al. 2008, 2009). A total of 20 μ L per compound was applied to the 4-mm diameter head of a dress-maker's pin. Thus, for the 1:1 blend, a total volume of 40 μ L was applied, whereas 20 μ L of pure DCM was added to the single-compound tests to standardize the solvent applied. The solvent was then allowed to evaporate before use, leaving the chiloglottones behind. Pins were stuck to bamboo skewers 5 cm from the point, which were then pushed into the ground in a row perpendicular to any prevailing wind, each one separated by approximately 20 cm and their order randomized between trials. We scored the relative attractiveness of volatile compounds by recording the behavior of visiting wasps in 4-min trials. Behavioral responses scored were *approaches* if a wasp flew toward or lingered in proximity to the bait, *landings* if the wasp landed on the bait or the ground below the bait, and *copulations* if the copulatory routine including abdominal probing was observed. The two different species were identified by different coloration on the wing and abdominal spotting patterns (see Griffiths et al. 2011 for details).

In the field, it is not uncommon to find mixed colonies of both species of orchid. We investigated the plausibility of heterospecific pollination and limits to pollinator fidelity by manipulating the distance between flowers of different species. We simulated the extreme scenario of both species' flowers contacted at the stem as well as interspecies separation of 20–40 cm (approximating the closest distance between the two species we have observed in nature). We did this by translocating whole adult plants into three vials with water: one *C. valida*, one *C. aff. jeanesii*, and a combination vial containing one plant of each taxon. As in trials with synthetic chiloglottones, these three vials were presented in randomized arrays in choice trials of 4 min in which we scored wasp responses to each individual flower in the array. If no wasp responses were scored for the first minute of the trial, the trial was abandoned and a new one restarted in a different location. Flowers used in the trials were subsequently extracted for floral volatile analysis, and DNA collections were made for genotyping.

POSTPOLLINATION ISOLATION: HAND CROSSES AND SYMBIOTIC GERMINATION

To assess postzygotic compatibility within and among taxa, we conducted hand crosses with plants collected from the field. After dehiscence of pods, seed viability was quantified by staining seeds in acetocarmine glycerol jelly (Marks 1954) and 600 per pod were counted for presence or absence of an embryo. Orchid seed do not contain maternal provisions for germination and require specific fungal interactions to initiate germination. We therefore germinated hand-crossed seed following the symbiotic methods of Roche et al. (2010) and Linde et al. (2014), with additional details provided online in Supporting Information: Additional Methods.

CALCULATION OF THE STRENGTH OF REPRODUCTIVE ISOLATION

We calculated the strength of individual pre- and postzygotic reproductive barriers after the general form:

$$RI = 1 - w,$$

where w is the relative fitness of F1 hybrids, obtained by dividing mean fitness of F1 hybrids by that of pure crosses (Coyne and Orr 1989, 2004; Ramsey et al. 2003). To detect asymmetry in the operation of barriers between the taxa, we calculated each level of RI separately for each taxon as seed mother (Martin and Willis 2007).

To quantify the contribution of pollinator fidelity to prepollination reproductive isolation, $RI_{pollinator}$, we estimated the observed proportions of conspecific interactions (pollinator fidelity) and heterospecific interactions (breakdown in fidelity) from the pollinator experiments. Because the behavioral experiment simulated an extreme and unnatural form of sympatry by presenting

a mixed-species pair of flowers at 1 cm separation, we calculated two $RI_{pollinator}$ estimates: one for the whole experiment including the mixed pair and two single flowers, the other a more realistic scenario based solely on data from the heterospecific flower pair presented at 20–40 cm apart. For each of these two spatial scenarios, we calculated $RI_{pollinator}$ based on combined total visits (rolling all data for different behaviors into one overall visitation value) and for attempted copulations only (the critical behavior for pollination). We therefore present four values for $RI_{pollinator}$ calculated from the observed visitation proportions for each taxon following Martin and Willis (2007):

$$RI_{pollinator} = 1 - \frac{\text{observed/expected heterospecific interaction}}{\text{observed/expected conspecific interaction}}.$$

The expected term is calculated as the probability of hybrid or pure crosses in the absence of pollinator fidelity. Because our pollinator visitation data were obtained under equal frequency of the two plant taxa, the expected probabilities are both 0.5, which simplifies the equation as:

$$RI_{pollinator} = 1 - \frac{\text{observed heterospecific interaction}}{\text{observed conspecific interaction}}.$$

Asymmetrical pollinator isolation requires systemic temporal precedence of either the hetero- or conspecific interaction, which due to observed coflowering and coflight times of pollinators is both unlikely to be occurring and unmeasurable in this study. We therefore calculate our final value of $RI_{pollinator}$ as an average for both taxa.

Pollination with incompatible interspecific pollen may result in failure to set fruit, therefore we estimated this postpollination, prezygotic component to reproductive isolation using data on successful fruit set in hybrid and pure hand-crosses. Following Scopece et al. (2007), the formula used was:

$$RI_{postpoll-prezygotic} = 1 - \left(\frac{\text{proportion fruit set heterospecific}}{\text{proportion fruit set conspecific}} \right).$$

Both seed viability and seedling growth were measured to estimate separate components of postpollination postzygotic isolation. Postzygotic isolation was quantified separately by the formula:

$$RI_{postzygotic} = 1 - \left(\frac{P_{heterospecific}}{P_{conspecific}} \right),$$

where P is the mean proportion of seeds bearing embryos in viability counts or mean growth index across plates in germination trials.

NUCLEAR DNA ANALYSIS FOR HYBRID DETECTION

To identify hybrids in our population-level sample, we implemented the Bayesian clustering analysis for admixture detection offered in the software NEWHYBRIDS (Anderson and

Thompson 2002). NEWHYBRIDS avoids any a priori designation of allele frequencies for groups of interest, but it does assume an admixed population of two parental species and that pure parent species, F1, F2, and respective backcrosses can all be represented in the sample. Through a Markov chain Monte Carlo simulation, the program then divides each sample's posterior probability between six genotype classes (two purebreds, F1, F2, and two backcrosses to the respective parent species). The threshold probability value (TQ) to assign a sample to any one class is arbitrary, with higher thresholds being more conservative. We chose to focus on detecting F1 hybrids as the detection of successive backcross classes requires increasingly more loci and is better achieved in populations with greater divergence in allele frequencies. We used TQ values of 0.9, 0.75, and 0.5, and in detecting F1 hybrids, these were used as cutoffs for both the posterior probability value given strictly for the F1 genotype class as well as a combined value summed from probabilities in all nonpure genotype classes.

To assess performance of the method on our data, we simulated hybrid genotypes. We based our simulations on observed parental allele frequencies in the subset of samples from KBNP that were taxonomically defined by both floral volatile and chloroplast haplotype data. In our simulation, 10 population pairs were created, each composed of one pure parental population for each taxon ($n = 1000$). These were created by randomly drawing each simulated individual's genotype from the observed allele frequency distribution for that taxon. These pure parental population pairs then underwent one round of simulated random mating before producing three offspring populations of $n = 1000$: one pure population for each taxon and one F1 hybrid population. These simulations were performed in a customized version of GenAIEx 6.5, using the simulation framework outlined in Banks and Peakall (2012). Each NEWHYBRIDS analysis was then run on the three offspring populations together with no prior information offered. We measured power to detect hybrids and pure parental taxon status for simulated genotypes by the proportion of correctly assigned individuals to the actual number of individuals in that class (Vähä and Primmer 2006; Burgarella et al. 2009). This was calculated under both hybrid detection criteria described above for varying levels of TQ. Finally, we assessed type I error by the number of pure individuals wrongly assigned hybrid status over the total number of individuals of pure parental stock.

In preliminary runs of the simulated data, varying our choice of priors and the number of sweeps during and after burn-in made no difference to the results. We chose to run all final analyses with "Jeffreys-like" priors for both the mixing proportions and allele frequencies with a burn-in of 50,000 sweeps preceding 100,000 sweeps. Lastly, we ran two analyses on real genotype data from the population-level sample combined with taxon-level samples from KBNP. One analysis was run without any prior information,

whereas the other specified pure taxon status a priori in only those samples for which we had chemistry and haplotype data to define taxon.

Results

FLORAL CHEMISTRY AND TAXON GENETIC DIVERGENCE

Our taxon-level sample found broadly similar cpDNA genetic diversity within each of the two taxa, however nuclear genetic diversity at 13 loci appeared lower within *C. aff. jeanesii* (Table 1). We found strict correspondence between cpDNA-defined groups and the floral volatile chemotypes. This is illustrated by the median-joining network (Fig. 1), which shows the haplotype network is composed of two clusters, separated by a minimum of 44 mutations, with each cluster exclusively associated with their respective chemotypes. Not surprisingly, an AMOVA analysis of the cpDNA/chemotype revealed very strong differentiation ($\Phi_{RT} = 0.792$, $\Phi'_{RT} = 0.885$, $P < 0.001$, Table 2) among the chemically defined taxa, whereas at the nuclear loci, only moderate differentiation was found among the taxa ($F_{RT} = 0.188$, $F'_{RT} = 0.414$, $P < 0.001$, Table 2). This finding is consistent with the expectation that the uniparentally inherited cpDNA will show more extreme differentiation than nuclear loci (Ebert and Peakall 2009).

All samples in both the taxon-level ($n = 86$) and population-level analysis ($n = 571$) showed internal consistency between the two diagnostic cpDNA indel markers. Furthermore, the inferred species diagnosis based on cpDNA was invariably correlated to floral chemistry with *C. valida* volatile samples matching to chiloglottone 1 ($n = 46$) and *C. aff. jeanesii* matching chiloglottone 3 ($n = 83$).

POLLINATOR SPECIFICITY AND FLORAL ISOLATION

In synthetic chemical choice trials, we scored 77 total visits for *N. impatiens* over 25 trials and 245 visits for *N. monticola* in 31 trials (Fig. 2). Consistent with previous studies (Peakall et al. 2010), we demonstrated that *N. impatiens* was attracted to chiloglottone 3 (proportion of total visits = 0.987) and not to chiloglottone 1, or a 1:1 blend of the two semiochemicals. *N. monticola* was attracted to chiloglottone 1 (proportion of total visits = 0.876), did not respond to chiloglottone 3, but showed some tendency to also approach and land on the 1:1 blend (proportion of total visits = 0.118). As expected, we scored no responses to the DCM control.

Bioassays with live plants generated a total of 86 *N. impatiens* visits in 20 trials and 135 *N. monticola* visits in 17 trials (Fig. 3). Both pollinators displayed strong pollinator constancy despite the presentation of a close heterospecific pair of flowers. Although the overwhelming majority *N. monticola*'s landings were on *C. valida*

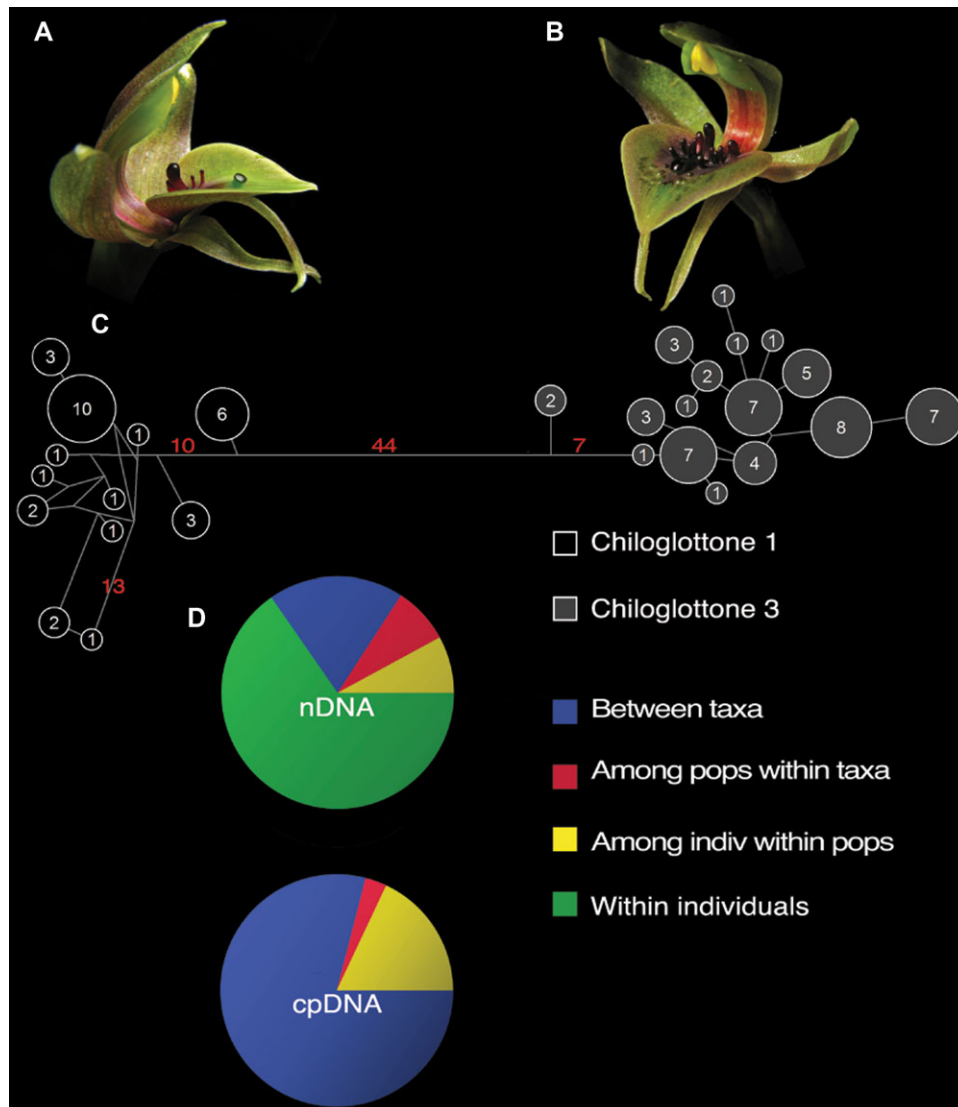


Figure 1. Population genetic summary for sympatric orchids (A) *Chiloglottis valida* and (B) *C. aff. jeanesii*. The chloroplast haplotype network (C) shows clear separation between chemically defined taxa and this is supported by the substantial proportion of between-taxon population genetic variance (D) in both nuclear and chloroplast DNA. The numbers inside the circles in the haplotype network (C) depict the number of samples bearing each 64-locus chloroplast haplotype and the number of mutation steps between some of the large divergences are depicted in red.

(94/107 cases), some breakdown in pollinator specificity (11/88 lands and 2/19 attempted copulations with the wrong species) occurred when the two taxa were presented together (joined at their stems). Given the heterospecific pairing represented an extreme and artificial scenario, we calculated pollinator isolation (Table 3) for the entire dataset including the 1 cm heterospecific separation ($RI_{\text{pollinator}} = 0.91$) and separately for the single flowers presented at more natural 20–40 cm separation ($RI_{\text{pollinator}} = 0.97$).

It is important to note that successful pollen pickup and deposition requires the wasp to align with the column. This positioning is usually achieved during attempted copulation with the

labellum, in which such movements bring the wasp in contact with the column. It is therefore worth examining data on attempted copulation in isolation. Attempted copulation behavior in the flower choice tests showed very strong pollinator constancy: *N. impatiens* did not display copulatory behavior on *C. valida* at all whereas *N. monticola* was only recorded twice attempting to copulate with *C. aff. jeanesii* in the heterospecific pair out of a total of 19 instances of pseudocopulation across all trials. The resulting estimates for pollinator isolation when considering only attempted copulations were $RI_{\text{pollinator}} = 0.83$ and $RI_{\text{pollinator}} = 1.00$ for 0 and 20–40 cm heterospecific distances, respectively (Table 3).

Table 2. AMOVA results for genetic differentiation within and between sympatric orchid taxa. Probability values were estimated by random permutation ($n = 999$).

Source	nSSR (13 loci)			cpDNA (64 loci)		
	df	Variance estimate	Percentage	df	Variance estimate	Percentage
Among taxa	1	0.797	19	1	12.725	79
Among populations within taxa	2	0.322	8	2	0.489	3
Among individuals within populations	82	0.338	8	82	2.851	18
Within individuals	86	2.785	66	—	—	—
Total	171	4.242	100	171	16.066	100

Level	nSSR (13 loci)			cpDNA (64 loci)		
	Statistic	Value	<i>P</i>	Statistic	Value	<i>P</i>
Among taxa	F_{RT}	0.188	0.001	Φ_{RT}	0.792	0.001
	F'_{RT}	0.414	—	Φ'_{RT}	0.885	—
Among populations within taxa	F_{SR}	0.093	0.001	Φ_{PR}	0.146	0.001
	F'_{SR}	0.179	—	Φ'_{PR}	0.161	—
Among all populations	F_{ST}	0.211	0.001	Φ_{PT}	0.823	0.001
	F'_{ST}	0.410	—	Φ'_{PT}	0.827	—
Within individuals	F_{IS}	0.108	0.001			

POSTPOLLINATION REPRODUCTIVE ISOLATION

To estimate postpollination–prezygotic isolation, we recorded the ratio of fruit set for 2010 hand crosses. We found no substantial difference in proportion of successful fruit set between conspecific (*C. valida*: 0.778, $n = 9$; *C. aff. jeanesii*: 0.700, $n = 10$ as pollen recipient) and heterospecific artificial crosses (*C. valida*: 0.923, $n = 13$; *C. aff. jeanesii*: 0.786, $n = 14$ as pollen recipient). Using these ratios of fruit set we determined $RI_{postpoll-prezygotic} = -0.19$ and -0.12 for *C. valida* and *C. aff. jeanesii*, respectively (Table 3).

Seed viability, as measured by embryo counts (Fig. 4), showed that the mean proportion of embryo-bearing seeds for conspecific crosses (0.818, $SE = 0.022$, $n = 23$) was similar to viability of heterospecific crosses (0.850, $SE = 0.026$, $n = 28$). The resulting component of postzygotic reproductive isolation was calculated $RI_{viability} = -0.15$ and -0.05 for *C. valida* and *C. aff. jeanesii*, respectively (Table 3).

Growth of symbiotically cultured orchid seed was measured as an estimate of fitness for postzygotic reproductive isolation (Fig. 4). Consistent with other orchid symbiotic germination studies, we found wide variation and nonnormal distributions in our seed germination data (Batty et al. 2001; Swarts et al. 2010), necessitating nonparametric tests. Kruskal–Wallis tests for interactions between isolate, taxon, and type of cross revealed that the isolate had no significant effect on the growth index, whereas taxon and type of cross showed significant effects across treatments. We found significantly higher fitness in F1 hybrids than controls (hybrids: mean growth index = 127.97, $SE = 23.99$, $n = 66$; within-taxon outcrosses: mean growth index = 44.94,

$SE = 12.50$, $n = 47$; Kruskal–Wallis test $H = 5.52$, $df = 1$, $P = 0.02$), which translated to negative reproductive isolation for F1 seedling fitness (Table 3). The strength of hybrid vigor depended greatly on species with a much stronger difference in hybrid versus pure progeny found in *C. aff. jeanesii* as mother ($RI_{germination} = -4.92$) compared to *C. valida* mothers ($RI_{germination} = -1.00$). When data were combined regardless of taxon, we found $RI_{germination} = -1.27$.

We have avoided presenting total reproductive isolation (RI_{total}) by summing RI values across barriers for the same reasons outlined in Martin and Willis (2007). This is because total RI values based on additive accumulations of individual RI values obscure important information about the operation of asymmetrical barriers and the interaction between barriers. Here, total RI ignores the higher likelihood for orchid–wasp mismatching in *N. monticola* (Fig. 3) and the more prominent hybrid vigor found in hybrids mothered by *C. aff. jeanesii* relative to pure crosses (Fig. 4). Additionally, the summed calculation of RI fails to represent interactions between barriers dependent on their sequence. Each isolating mechanism can only act on gene flow funneled from preceding barriers, giving later acting barriers the power to increase but not decrease isolation. Nevertheless, in systems with very strong ecogeographic or pollinator-driven $RI_{prezygotic}$, the common potential for strong hybrid vigor at fruit set, embryo viability, germination, biomass, and fertility can quickly reduce RI_{total} to levels equivalent to those with weak $RI_{prezygotic}$. An indication of the prezygotic propensity for hybrids to form is then quickly lost from the RI_{total} statistic by the high levels of hybrid

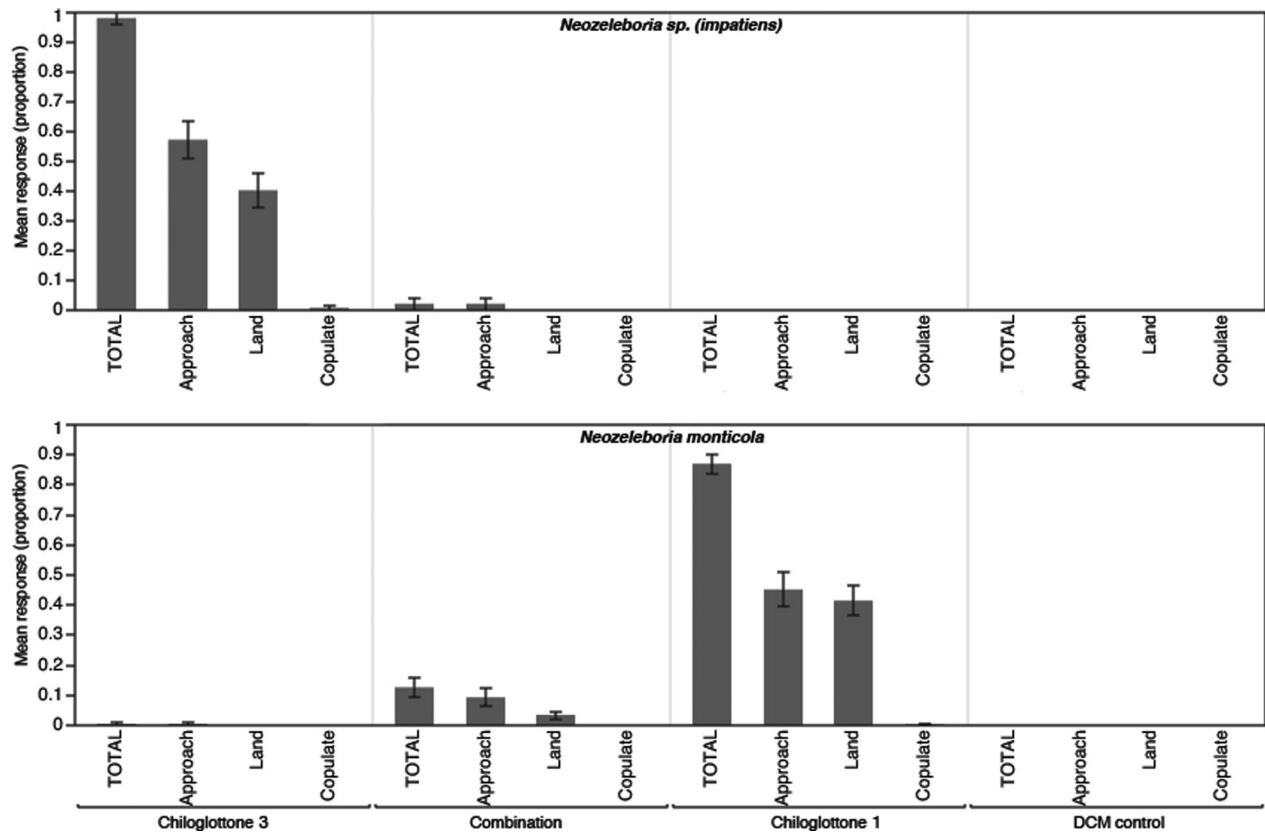


Figure 2. Behavioral responses of two wasp pollinators to a choice of synthetic pheromone baits: Chiloglottone 3, Chiloglottone 1, and a 1:1 blend of the two semiochemicals. Error bars give SE.

vigor observed in this study (e.g., —four to five times hybrid advantage in measures of seedling growth). We therefore argue that there is no straightforward interpretation of RI_{total} .

HYBRID DETECTION

An average of 95% of the simulated F1 hybrids were correctly assigned under a relaxed threshold ($TQ \geq 0.5$) and this dropped to 82% under the strict threshold ($TQ \geq 0.9$; Fig. 5). Basing assignments on strict F1 probabilities versus rolling all nonpure probabilities into one value did not substantially affect the power to detect hybrids. The range in power of correctly assigning simulated *C. valida* and *C. aff. jeanesii* under varying TQ was 88–95% and 96–99%, respectively. If NEWHYBRIDS misassigned one of our simulated pure genotypes, they were (with a single exception assigned to the other taxon) misassigned to the F1 hybrid genotype class. Type I error was very low with a range 0.027–0.006 across relaxed-to-strict TQ . The conservative threshold ($TQ \geq 0.9$) therefore performed very well at assigning pure parents to the correct class, but detected significantly fewer hybrids than under more relaxed thresholds.

Our population-level sample included 571 unique multilocus genotypes for which we typed at least six nuclear loci and the

taxon diagnostic cpIndels. Of these, 279 multilocus genotypes lacked a provisional taxon field identification because they were sampled as leaf material. The simulation results demonstrated strong power to detect existing hybridization, yet within our population sample hybrid detection analysis did not assign a single individual to the F1 genotype class. The maximum posterior probability for a sample in the F1 genotype class was just 0.046. There were however a small number of samples that assigned to one of the other nonparental genotype categories (F2, backcrosses). The proportion of these varied depending on whether chemistry and haplotype data were used as prior information in the analysis (Table 4). When chemistry was included as a prior, the number of inferred nonpure genotypes was below the number expected under $\alpha = 0.05$ as determined by simulation across all levels of TQ . When we included no such prior, the proportion of nonpure samples was just above that expected under $\alpha = 0.05$. Thus, we have been unable to find a single genotype that appears to be an F1 hybrid or a later generation hybrid, suggesting that hybridization, if it occurs, is indeed very rare. The absence of hybrids is further supported by the taxon-level chloroplast analysis that showed no haplotype sharing and wide phylogenetic separation between haplotype clusters belonging to the two chemically defined taxa.

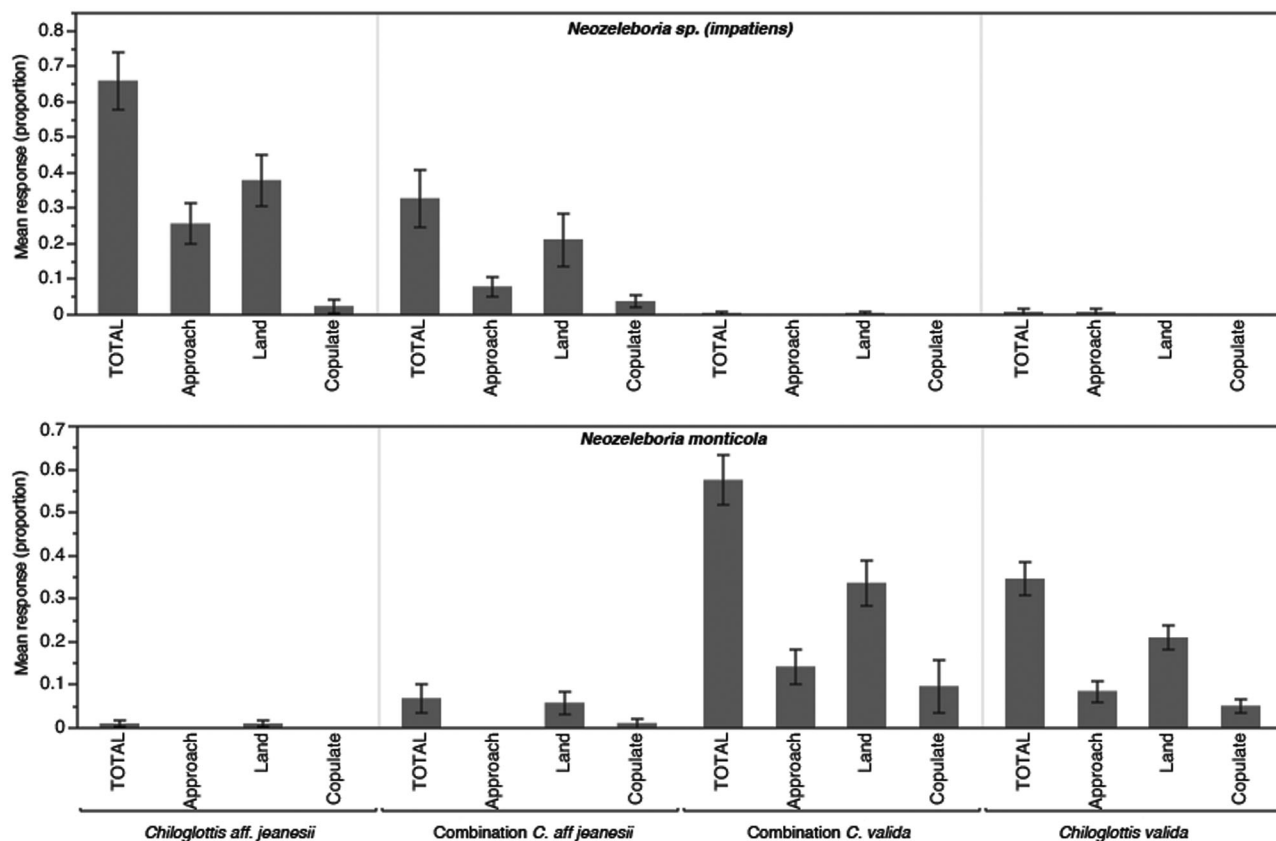


Figure 3. Behavioral responses of two wasp pollinators to a choice of flowers: *Chiloglottis* aff. *jeanesii* and *C. valida* presented in isolation or in combination. The “combination” treatment is separated here to split data into interactions with the two respective orchid taxa while in the combination. Error bars represent SE.

Table 3. *RI* estimates for the strength of pre- and postzygotic reproductive isolating barriers under scenarios of spatial separation of >20 or 1 cm between heterospecific flower pairs.

Isolating barrier	>20 cm		1 cm	
	CVA	CAJ	CVA	CAJ
F1 seed parent				
Pollinator (all visits)	0.97		0.91	
Pollinator (copulations)	1.00		0.83	
Fruit set	−0.19	−0.12	−0.19	−0.12
Seed viability	−0.15	−0.05	−0.15	−0.05
Germination	−1.00	−4.92	−1.00	−4.92

CVA = *Chiloglottis valida*; CAJ = *C. aff. jeanesii*.

Discussion

Our study has provided strong evidence that floral volatile chemistry, by controlling pollinator specificity, provides the majority contribution to reproductive isolation in this pair of sympatric orchid taxa. Furthermore, the floral volatile composition delineates taxonomic boundaries that are strongly supported by the chloroplast and nuclear genetic analysis. Interestingly, pollinator exper-

iments showed that artificially positioning flowers in very close proximity can result in rare breakdowns in pollinator specificity. Furthermore, the potential for introgression is likely not impeded by postpollination reproductive barriers, which we found to be effectively nonexistent in life stages up to and including the protocorm. Despite these findings, and the finding of hybrid vigor in the germination rates of artificially produced F1 offspring, a comprehensive and rigorous genetic screen of nuclear DNA variation failed to detect any F1 hybrids among a mixed population of adult plants. Consequently, we conclude that this is a compelling case for strong reproductive isolation mediated by pollinator isolation.

DOES FLORAL ODOR DELINEATE TAXONOMIC BOUNDARIES?

The strict correlation we found between floral volatile composition and chloroplast haplotype (based on $n = 129$) shows that floral odor chemotype reflects taxonomic boundaries in these sympatric taxa. This finding gives us confidence that cpDNA diagnosis of taxon is an accurate proxy for morphology and floral volatile profile in samples collected without floral material. Our

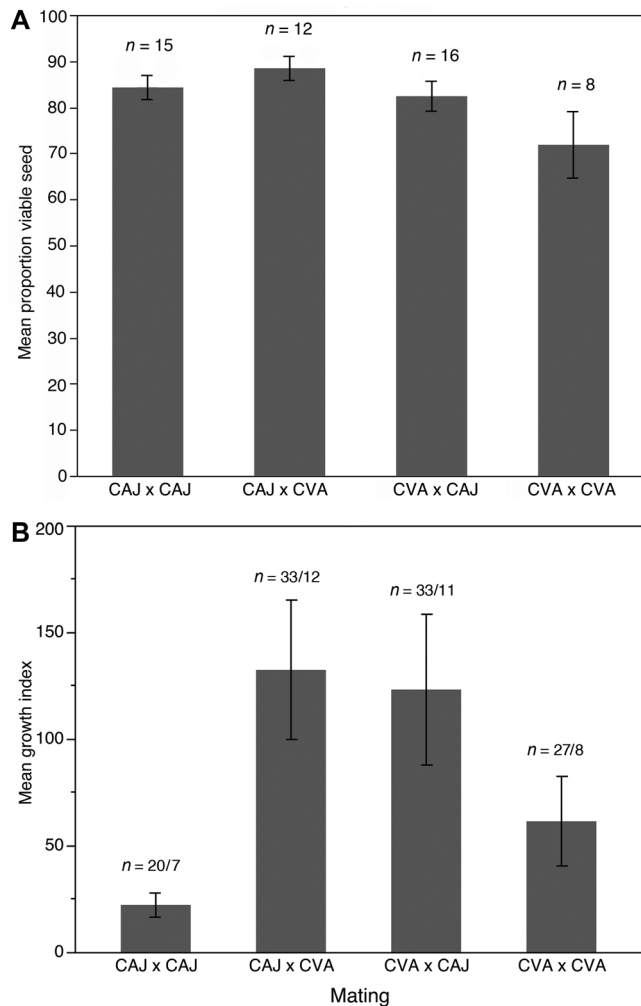


Figure 4. (A) Grand mean percentage of viable seeds by cross type, (B) symbiotic germination and growth of seedlings (growth index averaged among plates) by cross type. (CAJ, *Chiloglottis aff. jeanesii*; CVA, *C. valida*, n = number of total plates/number of unique crosses). Intraspecific versus hybrid crosses were significantly different (Kruskal–Wallis test $H = 5.52$, $df = 1$, $P = 0.02$). Error bars represent SE.

taxon-level sample divided into chemistry/cpDNA-defined taxa showed a considerable level of genetic differentiation between taxa (Table 2) especially for sympatric interfertile closely related congeners. Barriers to gene flow must be both substantial and long-standing to have given rise to this level of genetic differentiation in sympatric coflowering populations.

DOES FLORAL ODOR CONTROL POLLINATOR SPECIFICITY?

Behavioral assays with synthetic chemicals are an effective way of controlling for the influence of other cues (such as morphology or color) in pollinator attraction. Our results for synthetic chemical choice trials mirror previous results that showed pollinator

specificity to be controlled by a limited suite of floral volatiles (Peakall et al. 2010). However, the highly specific attraction of *N. monticola* to chiloglottone 1 and *N. sp. (impatiens)* to chiloglottone 3 can be disrupted to some degree by artificial blends (Fig. 2). By carrying out equivalent behavioral assays with live plants, we were able to test for pollinator fidelity under an extreme version of sympatry. Floral choice tests showed patterns of attraction similar to the synthetic choice trials.

When flowers of both taxa were artificially presented side-by-side, we did observe some breakdown in pollinator specificity for *N. monticola*. To assess the likelihood of this occurrence in nature, we calculated all possible heterospecific pairwise distances between samples from our fine-scale population map. The results showed that just 0.13% of all possible heterospecific interindividual pairwise distances ($n = 72,664$) were less than 2 m (the limit of accuracy in our mapping of the site). In other words, when plants occurred within 2 m of one another, the neighbor was almost always a plant of the same species. This is no doubt driven by patchiness associated with clonality and shows that the natural coflowering of both species within centimeters of each other is exceedingly rare, but not impossible. Therefore, the fine-scale (centimeters to meters) spatial separation of the two taxa reinforces the chemically mediated pollinator fidelity in this system.

WHAT ARE THE RELATIVE CONTRIBUTIONS OF THE ISOLATING BARRIERS?

Chiloglottis aff. jeanesii's known range is entirely embedded within the larger range of *C. valida* and coflowering populations are the norm. Geography, habitat, and phenology are therefore not necessary isolating mechanisms. By far, the strongest reproductive barrier is floral isolation driven by the specific behavioral response of pollinators to floral odor. We calculated $RI_{pollinator}$ to be in the range of 0.83–1.00 depending on the criteria we used (Table 3). Under experimental conditions best approximating a naturally occurring scenario, we should estimate isolation based on interplant sympatry scale of 20–40 cm separation and require that pollinators attempt copulation to achieve pollination. Under these conditions, the result is $RI_{pollinator} = 1$, or complete isolation due to pollinator fidelity.

It is important to note that our $RI_{pollinator}$ estimate is a proxy calculated on probabilities derived from wasp visits rather than observed matings between flowers per se (see Martin and Willis 2007; Lowry et al. 2008; Xu et al. 2011). Recent paternity analysis of natural pollination within the same study population (Whitehead 2012) found no evidence for interspecific pollination and if we used these observed paternity results in the same calculation, $RI_{pollinator}$ would still be 1. Thus, to our knowledge, *Chiloglottis* orchids represent one of the strongest known cases of floral

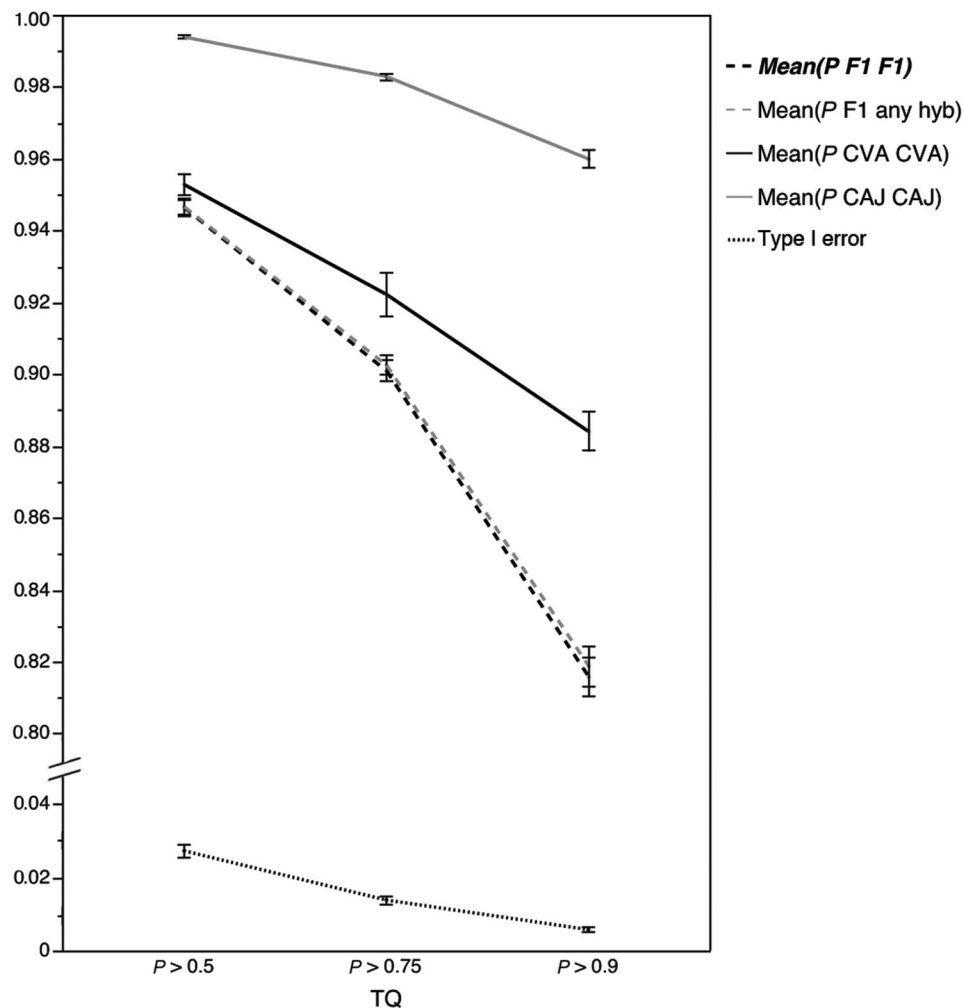


Figure 5. The proportion of accurate assignment of simulated genotypes by NEWHYBRIDS under different threshold values of Q (TQ). Dashed lines show the proportion of simulated hybrids successfully assigned to the F1 genotype category (black dashed) or any nonpure parental genotype category (gray dashed), equal to the inverse of type II error. Solid lines show the proportion of simulated *C. valida* (gray) and *C. aff. jeanesii* (black) successfully assigned to their respective pure parental genotype category. The proportion of simulated pure parental population being assigned to a nonpure genotype category (type I error) is shown by the dotted gray line. Error bars represent SE.

isolation due to pollinator specificity alone, with *Ophrys* providing the only other system in which similar results have been found (Xu et al. 2011).

The observed absence of isolating mechanisms in the post-pollination barriers we assessed is consistent with other studies of sexually deceptive orchids (Scopece et al. 2007; Xu et al. 2011) and is perhaps not surprising given that *C. valida* can successfully hybridize with the more phylogenetically distant *C. trapeziformis* (Peakall et al. 1997, 2010). However, discovering hybrid vigor in seedling growth of F1 hybrids was unexpected and if sustained through the life cycle, should facilitate interspecific gene flow (Lowry et al. 2008). Presently, it is difficult to infer what the effects of heterosis might be for the overall fitness of hybrids, be-

cause this will depend on their viability across the full life cycle and how they respond to selection pressures in nature. Additionally, the fitness of hybrid genotypes cannot be inferred solely from F1s, as hybrid breakdown can manifest with backcrossing in later generations (Milne et al. 2003; Taylor et al. 2009). F1 hybrids of *C. valida* and *C. trapeziformis* are capable of both flowering and vegetative reproduction in the field, however no backcrossing or F2s have been detected (Peakall et al. 1997). It is therefore likely that *C. valida* × *C. aff. jeanesii* hybrids could grow to maturity. Furthermore, *C. valida* × *C. trapeziformis* F1 hybrids form extensive clones indicating that even rare hybrids, once established, should persist for a very long time. Our failure to detect a single hybrid colony is then perhaps a more stark result in light of this fact.

Table 4. Proportion of samples assigned to a nonparental genotype class from two NEWHYBRIDS analyses of a mixed *C. valida* and *C. aff. jeanesii* sample ($n = 571$). α Levels for the various Q thresholds (TQ) are determined from simulations. Analyses were run with and without chemically defined samples being assigned a priori to a taxon.

	TQ		
	0.5	0.75	0.9
α	0.027	0.014	0.006
Chemistry prior	0.005	0.000	0.000
No chemistry prior	0.037	0.016	0.009

IS THERE EVIDENCE FOR INTERSPECIFIC GENE FLOW IN THE POPULATION?

Despite demonstrating the potential for rare heterospecific pollination and the absence of postpollination barriers, rigorous population genetic analysis found no evidence for hybridization between these species. Bayesian admixture analysis failed to assign a single sample to the F1 genotype class out of a population sample of 571 unique multilocus genotypes. Simulations using real allele frequencies showed that even under the most relaxed assignment criteria with highest type I error, we should expect close to a 95% chance of detecting hybrids. Even under these relaxed criteria, we found that the number of genetic samples attributed to nonpure genotypes to be within the range of type I error when using a chemistry prior, and barely above type I error when offering no such information. We therefore cannot find any evidence for natural hybridization at a site where both species grow intermingled in close sympatry.

Conclusion

A thorough study of pre- and postpollination barriers to reproduction has shown that genetic boundaries in these sympatric orchid taxa are maintained by the specific attraction of distinct pollinators. We have attempted to address the challenge of measuring postzygotic hybrid fitness beyond the seed stage by carrying out the first study to our knowledge to include symbiotic germination trials in quantification of orchid reproductive isolation. Postzygotic barriers often require time to accumulate (Dobzhansky 1937; Lowry et al. 2008) and despite some genetic divergence between these two very closely related lineages, we find that intrinsic postzygotic isolation barriers have yet to form between these taxa. It is however possible that postpollination barriers may manifest in later life stages not assessed in this study.

Among studies of individual isolating mechanisms in closely related species, this system conforms to the general observation that prezygotic barriers exert a greater influence on the strength

of reproductive isolation (Ramsey et al. 2003; Kay 2006; Lowry et al. 2008; Dell'Olivo et al. 2011). However, *Chiloglottis*, along with *Ophrys* (Xu et al. 2011), stands out as an extreme example of the pre-eminence of pollinator isolation.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.13n29.

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Supporting Information

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

Table S1. Distribution of sampling effort during the study.