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Floral odour chemistry defines species boundaries and underpins strong reproductive isolation in sexually deceptive orchids

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• *Background and Aims* The events leading to speciation are best investigated in systems where speciation is ongoing or incomplete, such as incipient species. By examining reproductive barriers among incipient sister taxa and their congeners we can gain valuable insights into the relative timing and importance of the various barriers involved in the speciation process. The aim of this study was to identify the reproductive barriers among sexually deceptive orchid taxa in the genus *Chiloglottis*.
• *Methods* The study targeted four closely related taxa with varying degrees of geographic overlap. Chemical, morphological and genetic evidence was combined to explore the basis of reproductive isolation. Of primary interest was the degree of genetic differentiation among taxa at both nuclear and chloroplast DNA markers. To objectively test whether or not species boundaries are defined by the chemistry that controls pollinator specificity, genetic analysis was restricted to samples of known odour chemistry.
• *Key Results* Floral odour chemical analysis was performed for 600+ flowers. The three sympatric taxa were defined by their specific chiloglottones, the semiochemicals responsible for pollinator attraction, and were found to be fully cross-compatible. Multivariate morphometric analysis could not reliably distinguish among the four taxa. Although varying from very low to moderate, significant levels of genetic differentiation were detected among all pairwise combinations of taxa at both nuclear and chloroplast loci. However, the levels of genetic differentiation were lower than expected for mature species. Critically, a lack of chloroplast DNA haplotype sharing among the morphologically indistinguishable and most closely related taxon pair confirmed that chemistry alone can define taxon boundaries.
• *Conclusions* The results confirmed that pollinator isolation, mediated by specific pollinator attraction, underpins strong reproductive isolation in these taxa. A combination of large effective population sizes, initial neutral mutations in the genes controlling floral scent, and a pool of available pollinators likely drives diversity in this system.

Key words: *Chiloglottis*, orchid, Orchidaceae, sexual deception, floral odour, pollination, 2,5-dialkylcyclohexane-1,3-diones, pollinator-driven speciation, population genetic analysis, microsatellites, chloroplast DNA, cpSSRs.

INTRODUCTION

The extraordinary diversity of flowering plants has long been attributed, at least in part, to the role animal pollinators have played as drivers of plant speciation (Stebbins, 1970; Grant, 1994; Schemske and Bradshaw, 1999; Johnson, 2006). Indeed, a recent review of the phylogenetic evidence reveals that pollinator shifts have been frequent and important in the diversification of some angiosperm lineages (van der Niet and Johnson, 2012). However, the full extent to which pollinator-driven speciation has contributed to the diversification of the flowering plants as a whole remains unclear. In large part this is because multiple reproductive barriers, both prezygotic and postzygotic, typically isolate plant species (Rieseberg and Willis, 2007; Lowry *et al.*, 2008; Widmer *et al.*, 2009). Therefore, even in those cases where strong pollinator isolation is now evident, it is difficult to determine whether this originated as an early reproductive barrier or developed secondarily after speciation (Johnson, 2006, 2010, van der Niet and Johnson, 2012).

A key goal of plant speciation research is identifying the early reproductive barriers that initiated speciation (Lowry *et al.*, 2008; Widmer *et al.*, 2009). Support for the hypothesis of pollinator-mediated speciation further requires establishing whether or not pollinator isolation is responsible for speciation or merely facilitates coexistence following speciation via other reproductive barriers (Johnson, 2006, van der Niet and Johnson, 2012). It follows that these first steps in speciation are best investigated within systems where the speciation process is ongoing or likely incomplete, such as divergent ecotypes and incipient species (Johnson, 2006; Lexer and Widmer, 2008; Sobel *et al.*, 2009; Rosenblum and Harmon, 2011; Van der Niet *et al.*, 2014).

Population genetic tools offer a powerful approach for testing hypotheses on gene flow and reproductive barriers among diverging or recently diverged lineages. For example, hypervariable nuclear markers can reveal cryptic taxa (Bickford *et al.*, 2007; Griffiths *et al.*, 2011) or detect very subtle divergence among lineages, while in plants chloroplast markers can be used to confirm or refute introgression (Ebert and Peakall, 2009*a*). By studying a range of closely related species, genetic differentiation between allopatric congeneric species can provide context to the levels of differentiation found in sympatric congeners. Combining population genetic data with corresponding data on variation in traits important to pollinator attraction, such as morphology (Rymer *et al.*, 2010) or odour (Whitehead

and Peakall, 2009), can further offer new insights into the micro-evolutionary outcomes of pollinator-mediated selection.

Since Grant's (1994) seminal review of floral isolation, sexually deceptive orchids have been recognized as potential examples of strong reproductive isolation by pollinators. In this pollination strategy, male insects are sexually attracted to the orchid flower, with pollination occurring during either a pre-copulatory routine or attempted copulation with the flower—so called pseudocopulation (Peakall, 1990; Schiestl, 2005). Sexual deception is employed by several hundred orchid species, with multiple independent evolutionary events in Australia, Europe and South Africa as well as South and Central America (Paulus and Gack, 1990; Peakall, 1990; Steiner *et al.*, 1994; Singer, 2002; Singer *et al.*, 2004; Schiestl, 2005; Gaskett, 2011). Long thought to be restricted to the Orchidaceae (Peakall, 1990), this pollination strategy has recently been discovered in a South African daisy (Asteraceae) (Ellis and Johnson, 2010) and a Eurasian iris (Vereecken *et al.*, 2012). Thus, pollination by sexual deception may be more widespread among plants than presently reported.

The last decade, in particular, has seen much progress in our understanding of how sexual deception operates in two parallel but unrelated systems: *Chiloglottis* orchids in Australia and *Ophrys* orchids in Europe (for reviews see Schiestl, 2005; Ayasse *et al.*, 2011; Xu *et al.*, 2012). Orchids in both systems have been shown to attract their specific pollinators by specific volatile chemical signals that mimic female-released sex pheromones (Schiestl *et al.*, 1999, 2003; Mant *et al.*, 2005*b*; Stokl *et al.*, 2007; Franke *et al.*, 2009). Thus, these orchids may offer cases where strong pollinator-mediated reproductive isolation can be achieved by minor chemical differences in floral scent (Schiestl and Ayasse, 2002; Stökl *et al.*, 2009; Xu *et al.*, 2011). If there is simple genetic control of the chemical differences, then genic speciation, likely coupled with pollinator-mediated selection, seems highly plausible in these systems (Peakall *et al.*, 2010; Xu *et al.*, 2012). Sexually deceptive orchids may thus represent a new model system for exploring questions about the evolution of floral reproductive isolation.

Furthermore, there is strong evidence in some lineages of sexually deceptive orchids that speciation is an ongoing process. Within *Ophrys* there is considerable intraspecific variation, frequent hybridization and evidence for chemically but not genetically distinct cryptic taxa (Mant *et al.*, 2005*c*; Stökl *et al.*, 2009). Perhaps as a consequence, the taxonomy of *Ophrys* is particularly controversial (Devey *et al.*, 2008; Bateman *et al.*, 2011; Vereecken *et al.*, 2011). By contrast, within *Chiloglottis* hybridization between recognized species is very rare (Peakall *et al.*, 2002; Whitehead, 2012) but putative cryptic and incipient species are evident (Bower, 2006; Bower and Brown, 2009). Thus, these sexually deceptive orchids offer unique opportunities for studying speciation in action. In this paper we report the outcomes of a study of Australian sexually deceptive orchids that offer an ideal system for exploring the early phases of speciation.

Within Australia, more than 150 species of terrestrial orchid sexually exploit male wasps from the parasitic Australasian subfamily Thynninae (Thynnidae) as pollinators (Peakall, 1990; Peakall and Beattie, 1996; Phillips *et al.*, 2009). The orchid genus *Chiloglottis*, with some 30 species, is the largest exclusively sexually deceptive genus in Australia. Field experiments using artificially presented flowers have confirmed pollination in this genus is highly specific, with an average of 1·1 pollinator species per orchid (Peakall *et al.*, 2010). 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 chemical variants representing a new class of natural products, all 2,5-dialkylcyclohexane-1,3-diones or 'chiloglottones' (Schiestl *et al.*, 2003; Franke *et al.*, 2009; Peakall *et al.*, 2010). When the chiloglottones are mapped onto the phylogeny it is evident that orchid speciation is always associated with pollinator switching and usually underpinned by chemical change (Peakall *et al.*, 2010).

Our study targeted four closely related *Chiloglottis* taxa with varying degrees of geographic overlap. By combining ecological, chemical, morphological and molecular genetic analyses we explore the basis of reproductive isolation and the role of floral odour chemistry in defining species boundaries. We conclude by exploring the implications of our findings for pollinator-driven speciation.

MATERIALS AND METHODS

Overview of the genus Chiloglottis

Chiloglottis orchids are small terrestrial herbs that grow as clonal colonies in moist locations in the forests and swamps of eastern Australia. Plants consist of just two opposite leaves, usually borne prostrate on the substrate. While colonies may consist of hundreds to thousands of plants, only a few plants produce a single dull-coloured flower in any one year (Peakall *et al.*, 1997; Peakall *et al.*, 2002; Fig. 1).

Most *Chiloglottis* orchids are pollinated by single species of thynnine wasp from the genus *Neozeleboria* or closely related genera (Mant *et al.*, 2002, 2005*c*; Peakall *et al.*, 2010; Griffiths *et al.*, 2011). However, combined molecular genetic and ecological analysis has confirmed that many of the orchid pollinators represent cryptic species that cannot be distinguished by morphological analysis. In this study, only one pollinator taxon is a described species, hence the nomenclature follows that of Griffiths *et al.* (2011), which was developed specifically for these undescribed cryptic taxa.

Study taxa and their floral odour chemistry

Four closely related putative *Chiloglottis* taxa within the *valida* clade (Fig. 2) were the target of this study. Note that we use the word 'taxa' as an explicit acknowledgement of the taxonomic uncertainty. Table 1 introduces these taxa, their distribution, range overlap, proposed diagnostic floral odour chemistry and specific pollinators. See Franke *et al.* (2009) and Peakall *et al.* (2010) for chemical details. *Chiloglottis pluricallata* (CPL) was chosen to represent an allopatric taxon, being known only from the Northern Tablelands of New South Wales. CPL co-flowers with an undescribed cryptic taxon (Mant *et al.*, 2005*a*; Peakall *et al.*, 2010) not included in this present study. The other three taxa were sympatric and co-flowering. *Chiloglottis valida sensu stricto* (CVA) shares an extensive range with *C.* affinity (aff.) *valida* (CAV) across southern New South Wales and south-eastern Victoria, with co-flowering occurring at many sites (Fig. 2). The fourth taxon,

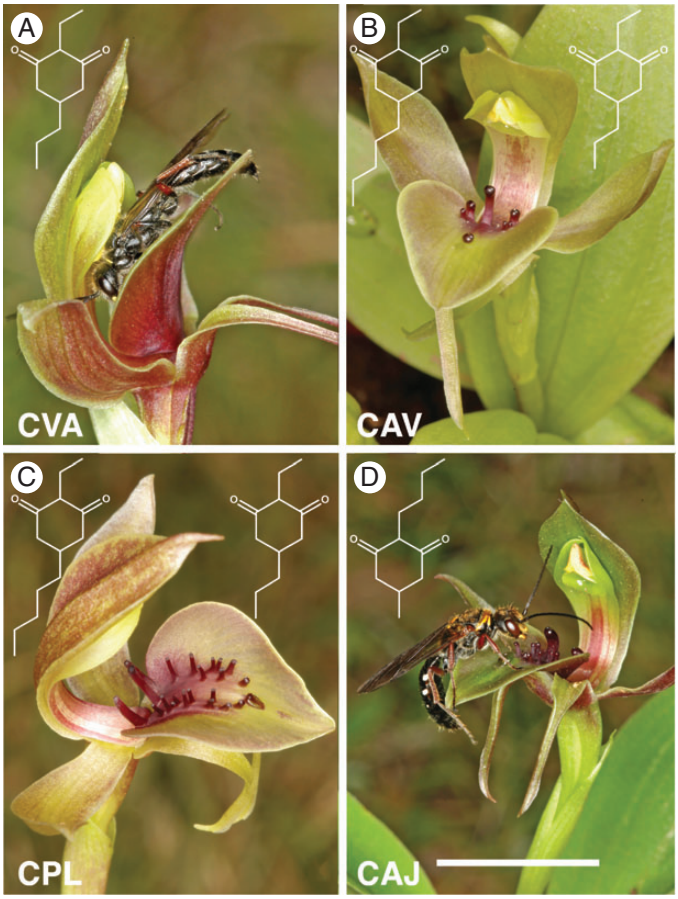

FIG. 1. Photographs of the four *Chiloglottis* study taxa showing their flowers and the chemical structures of the chiloglottone semiochemicals used to attract their respective pollinators. (A) *C. valida* (CVA) and pollinator *Neozeleboria monticola*, chiloglottone 1; (B) *C.* aff. *valida* (CAV), chiloglottones 1 and 2; (C) *C. pluricallata* (CPL), chiloglottones 1 and 2; (D) *C.* aff. *jeanesii* (CAJ) and pollinator *N.* sp. (impatiens2), chiloglottone 3. Key to chiloglottones: 1 = 2-ethyl-5-propylcyclohexane-1,3-dione; 2 = 2-ethyl-5-pentylcyclohexane-1,3-dione; 3 = 2-butyl-5-methylcyclohexane-1,3-dione. Scale bar = 10 mm.

C. aff. *jeanesii* (CAJ), is known only from two regions embedded within the range of the *C. valida* complex (CVA/CAV).

Field sampling

Populations of the four study taxa were broadly sampled across their known ranges (Table 1). Figure 2 shows approximate distributions, while Supplementary Table S1 provides details of the study site locations. Where possible, samples were provisionally identified in the field by their floral morphology (noting that CVA and CAV are indistinguishable and that some CAJ can also be confused with these taxa). The final taxon determination was based on the chemistry of the sample (as outlined in Table 1) with the exception that CAV could not be chemically distinguished from the allopatric CPL. Thus, CPL samples were defined by their chemistry in combination with location. To minimize sampling within clones, we avoided collecting multiple individuals within colonies (<5 m).

Chemical analysis

Flowers were processed on the day of collection (<6 h after sampling) by washing the labellum in 100 μl of HPLC grade dichloromethane. Gas chromatographic analysis with mass spectrometry was performed for each extract following Peakall *et al.* (2010). This method employed selective ion monitoring designed specifically to target chiloglottones from single labellum extracts.

Morphological analysis

Samples for the morphological analysis were drawn from across the range of each taxon and were identified chemically. Floral material was preserved in 80 % ethanol until required. Using digital callipers, 11 floral characters were measured under a dissecting microscope, including the length and width of sepals, petals, labellum and column and the number of calli on the labellum (see Supplementary Table S2 for full details).

Genetic compatibility

We made hand-pollinations to assess the compatibility of selfed pollen as well as inter- and intra-taxon crosses among the three sympatric taxa (CAJ, CAV and CVA). These were carried out on virgin flowers (determined by inspection of stigma and anthers) collected from the field and matured in a growth cabinet. On dehiscence of the capsule, seeds were collected and stored desiccated at 4 °C. As one measure of seed viability, estimates of the percentage of seeds with embryos was assessed for each fruit by acetocarmine staining and the counting of 400 seeds, following Peakall *et al.* (1997). Embryos were considered to be normal if they were ovoid in shape, while abnormal embryos were recognized by their shrivelled state and irregular shape.

Genetic markers

We employed both nuclear and chloroplast microsatellites [or simple sequence repeats (SSRs)] as markers for genetic analysis. The value of combining nuclear and chloroplast genetic markers has long been recognized. However, the application of chloroplast DNA (cpDNA) markers in population genetic studies is often hampered by a lack of variation at the individual and population levels (see review by Ebert and Peakall, 2009*a*). It is for this reason that we targeted chloroplast SSRs (cpSSRs), which have been largely overlooked as powerful population level markers (Ebert and Peakall, 2009*a*). While superficially similar to nuclear microsatellites, cpSSRs offer several important and unique characteristics, including haploidy, a lack of recombination and uniparental inheritance. Thus, unlike nuclear loci, even when described by multiple polymorphic loci across the genome, the combined set of polymorphisms in an individual represent one haplotype, which, barring mutation, is identical to that of the mother (Ebert and Peakall, 2009*a*).

Our set of 13 nuclear loci were drawn from the 16 loci developed for *Chiloglottis* by Flanagan *et al.* (2006), who also describe the DNA extractions, PCR and genotyping methods followed in this study. For cpDNA analysis we used the set of 41 cpDNA genetic markers designed by Ebert *et al.* (2009) to

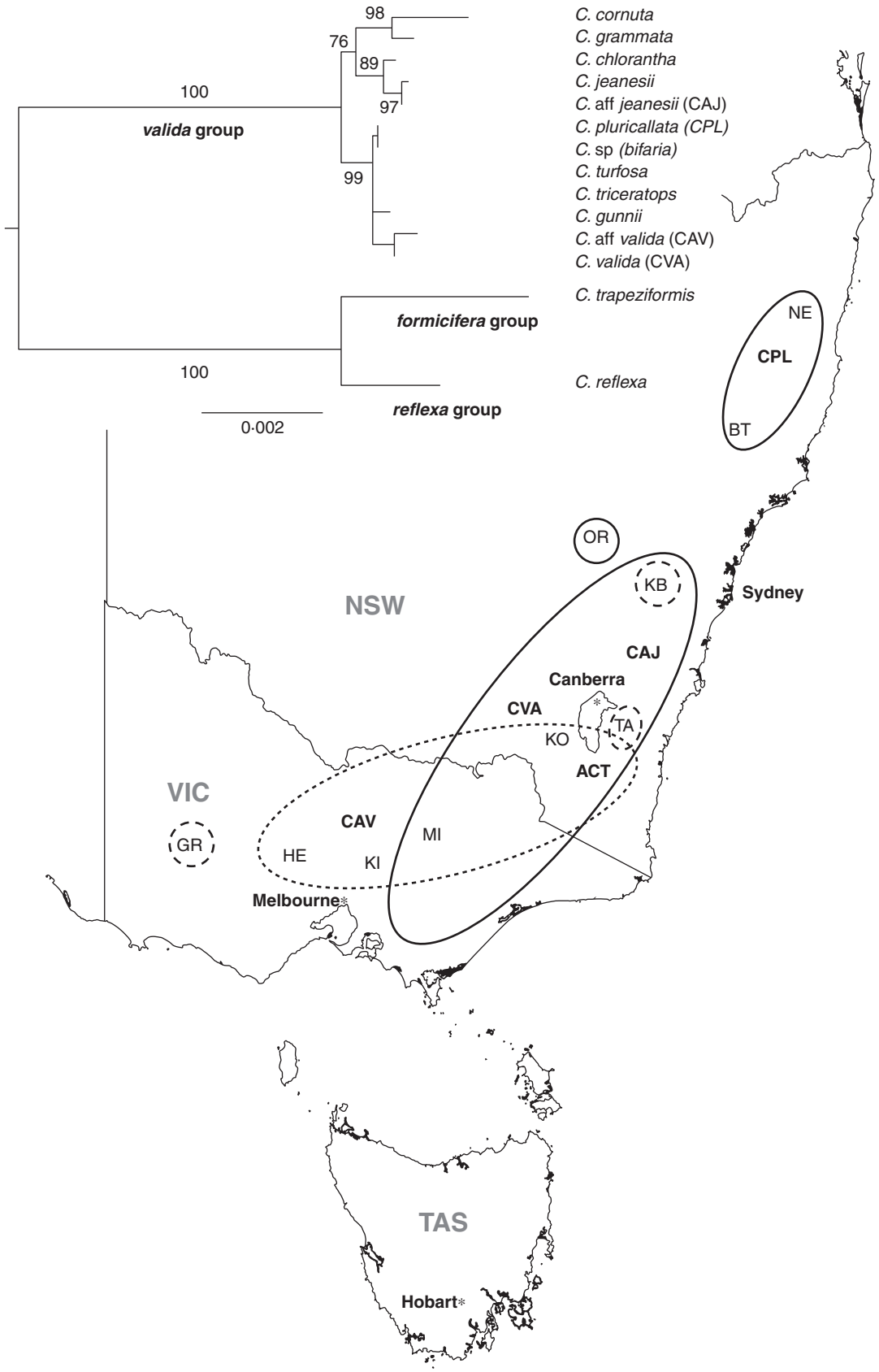

FIG. 2. Map of south-eastern Australia showing the approximate distributions of the four taxa (see Table 1 for abbreviations) and the field sites (two-letter abbreviations), together with a phylogeny of the *valida* clade within *Chiloglottis* (adapted from Peakall *et al.* 2010).

TABLE 1. *Summary of the* Chiloglottis *study taxa showing their regional locations, range overlap, diagnostic chemistry and* Neozeleboria *pollinators*

Taxon	Code	Distribution	Range overlap	Chiloglottone chemistry	Pollinators
C. valida s.s.	CVA	OR, KB, TA, KO, MI, KI, HE	CAV, CAJ	1	*N. monticola*, *N. cryptoides*
C. aff. *valida*	CAV	TA, KO, MI, KI, HE, GR	CVA, CAJ	1, 2	*N.* sp. (impatiens4), *N.* sp. (unknown3)
C. aff. *jeanesii*	CAJ	KB, TA	CAV, CVA	3	*N.* sp. (impatiens1), *N.* sp. (impatiens2)
C. pluricallata	CPL	BT, NE	None	1, 2	*N.* sp. (impatiens3)

Refer to Table S1 for site and region details. Structural diagrams for chiloglottones 1–3 appear in Fig. 1. Note that a third compound, yet to be identified, is required for pollinator attraction in *C. pluricallata*. See Jones (1991) for descriptions of CVA and CPL. For more details of taxon phylogeny, nomenclature and chemistry, see Peakall *et al.* (2010). Pollinator nomenclature follows Griffiths *et al.* (2011).

target both intra- and interspecific polymorphic cpSSRs and chloroplast indels (cpIndels) in *Chiloglottis*. These genetic markers were discovered within *Chiloglottis* by first employing the universal set of cpDNA sequencing primers of Ebert and Peakall (2009*b*) with the final set of markers spread across more than 19 kb of non-coding sequence within the large single-copy region of the chloroplast. The cpDNA laboratory methods followed Ebert *et al.* (2009).

Genetic analysis

We conducted population genetic analysis of both the nuclear and chloroplast genetic markers in the software package GenAlEx 6·5 (Peakall and Smouse, 2006, 2012). For the nuclear SSRs (nSSRs) this included calculation of allele frequencies and subsequent codominant genetic marker summary statistics, such as observed and expected heterozygosity. For the haploid cpDNA markers, the software was used to investigate haplotype number, haplotype frequencies and haplotype sharing and to calculate haploid diversity and other haploid summary statistics.

For both marker types, we also performed analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) to investigate the partitioning of genetic variation within and among various *a priori* groupings of the samples and taxa. For the codominant nSSRs we estimated F_{ST} following Peakall *et al.* (1995) and Michalakis and Excoffier (1996). For the cpDNA, we followed Ebert *et al.* (2009) to estimate Φ_{PT} (as the haploid analogue of F_{ST}) at both the haplotype (treating haplotypes as equidistant, either 0 or 1) and the locus level (taking into account the haploid genetic distance across all loci). Tests for departure from the null hypothesis of no genetic differentiation among groups were performed by random permutation (999 permutations per run).

There has been much recent debate about the utility of F_{ST} (and analogues) as a measure of population genetic structure (Jost, 2008; Ryman and Leimar, 2009; Meirmans and Hedrick, 2011; Whitlock, 2011). Therefore, we also employed new AMOVA routines in GenAlEx 6·5 to estimate standardized F'_{ST} and Φ'_{PT} (range 0–1), following Meirmans (2006). These new estimators avoid the downward bias associated with highly polymorphic loci (Hedrick, 2005; Meirmans and Hedrick, 2011), ensuring a range of 0–1, with the upper limit of 1 reached when populations have non-overlapping sets of alleles or haplotypes.

By virtue of maternal inheritance and the lack of recombination, chloroplast haplotypes will invariably be shared with other maternally related individuals (Ebert and Peakall, 2009*a*). Chloroplast haplotype sharing (or lack thereof) can thus provide important clues about taxon distinctiveness and the extent of hybridization (if any), which complements and extends other genetic analyses, such as estimates of genetic differentiation among taxa. To facilitate this analysis we extended the capability of GenAlEx 6·5 to allow haplotype-sharing analysis among the multiple levels of populations, regions and taxa. In addition, we employed statistical testing by random permutation, using procedures analogous to those used in AMOVA, to enable non-parametric statistical tests of the null hypothesis of no difference in haplotype sharing patterns, as predicted in the case of no reproductive isolation (i.e. complete bi-directional gene flow).

GenAlEx was also used to format chloroplast haploid data for the software package Network 4·6 (Fluxus Technology, 2010). This package was used to reconstruct median-joining networks of the non-recombining haploid chloroplast data, following the manufacturer's guidelines. For background to the statistical procedures that underpin this analysis see Bandelt *et al.* (1999) and Forster *et al.* (2000).

RESULTS

Chemical and genetic sampling

Chemical analysis was successfully performed for more than 600 flowers sampled from across the range of the four taxa. Samples for the genetic analysis were drawn from this set of chemically identified samples. After the exclusion of putative clones (based on shared nSSR genotypes and cpDNA haplotypes), cpSSR haplotypes constructed on 64 loci were obtained for 470 samples, while nSSR genotypes were obtained across the 13 loci for 400 samples.

Chemical variation within and among the taxa

The taxa CVA and CAJ were defined by the presence of chiloglottone 1 and chiloglottone 3, respectively. CAV and CPL were distinguished from CVA and CAJ by the presence of both chiloglottone 1 and chiloglottone 2, with CVA and CPL deemed to have non-overlapping ranges (Table 1).

Unlike the genetic samples, our chemical analysis included some replication within putative clones (all with matching nuclear and cpDNA loci), to test the stability of the chemistry.

In total, a set of 80 samples drawn from across the four taxa contained genetic replicates. In all cases, putative clones had identical chiloglottone chemistry (CAJ, $N_c = 11, N = 27$; CPL, $N_c = 4, N = 8$; CAV, $N_c = 6, N = 14$; CVA, $N_c = 15, N = 34$, where N_c is the number of clones and N is the number of samples). Furthermore, as expected, chemistry was stable over 3 years in the one clone repeatedly sampled in CAJ.

Morphometric analysis

Consistent with their overall morphological similarity (Fig. 1), no taxon formed a discrete cluster in a canonical multivariate analysis (Fig. 3). Similarly, linear discriminant analysis misclassified eight of the samples (11 %, $P < 0{\cdot}05$), with a further 18 samples with plausible alternative classifications (25 %, $0{\cdot}05 > P < 0{\cdot}95$), most of these being CAV/CVA. Outcomes of a univariate morphometric analysis are summarized in Table S2. It is evident that it is not possible to fully discriminate among the four taxa by either method.

Genetic compatibility

Across the three sympatric taxa (CVA, CAV, CAJ) estimates of seed viability were obtained from 44 inter- and intra-taxon crosses and 19 selfs, with the number of replicates per treatment varying from 2 to 10. All three taxa were self-compatible, with no difference in the percentage of seeds with normal embryos detected among self versus intra-taxon crosses [CVA, self $72{\cdot}2 \pm 4{\cdot}3$, cross $85{\cdot}7 \pm 5{\cdot}3$ (mean $\pm$ s.e.), $F_{1,8} = 3{\cdot}9$, $P = 0{\cdot}08$; CAV, self $68{\cdot}9 \pm 5{\cdot}4$, cross $79{\cdot}0 \pm 10{\cdot}7$ (mean $\pm$ s.e.), $F_{1,8} = 0{\cdot}69$, $P = 0{\cdot}43$; CAJ, self $82{\cdot}3 \pm 4{\cdot}7$, cross $88{\cdot}1 \pm 3{\cdot}7$ (mean $\pm$ s.e.), $F_{1,11} = 0{\cdot}9$, $P = 0{\cdot}36$]. Similarly, no evidence of inter-taxon incompatibility was detected, with the mean percentage of seeds with normal embryos varying from 79 to 94 % across the nine combinations of intra- and inter-taxon crosses (overall mean = 87 %, $F_{8,34} = 1{\cdot}28$, $P = 0{\cdot}28$, after exclusion of one extreme outlier).

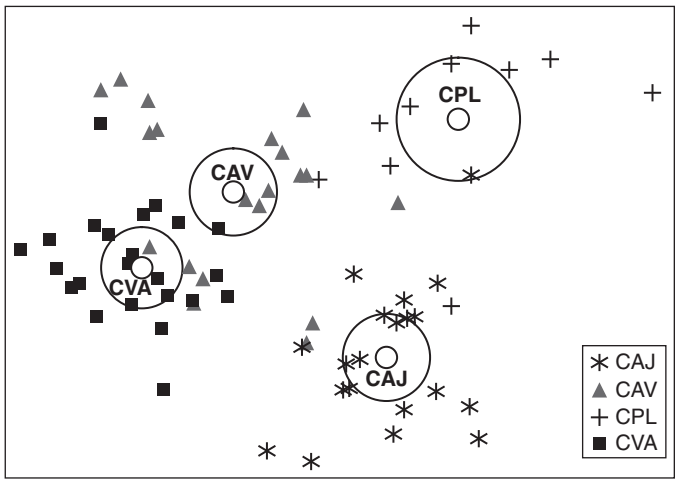

FIG. 3. Canonical plot depicting the outcome of multivariate morphological analysis of floral characters across the four taxa (see Table 1 for abbreviations).

Genetic analysis at nSSR loci

Across the 13 nSSR loci, the three taxa CAV, CPL and CVA were very similar at the allelic level. For example, the total number of higher-frequency alleles (frequency $>0{\cdot}05$) ranged from 54 to 57, with genetic diversity (H_e) ranging from 0·58 to 0·68. By contrast, CAJ exhibited less genetic diversity than the other taxa. One locus was monomorphic, while allelic diversities and heterozygosities were also lower (Table 2).

Many nSSR alleles were shared across the taxa, with the number of private alleles per taxon varying from 0 (CVA) to 15 (CAV). However, all private alleles were at very low frequencies ($\geq 0{\cdot}06$), and thus none was diagnostic (Table 2). Despite the extensive allele sharing, significant genetic differentiation was detected among the taxa (Table 3). Pairwise F_{ST} values estimated across the combined 13 nSSR loci varied from 0·150 to 0·363 among CAJ, CPL and CAV/CVA contrasts. Furthermore, standardized F'_{ST} values were considerably higher (0·427–0·730) for these same contrasts, reflecting the expected downward bias of F_{ST}. For the morphologically indistinguishable taxa, CAV and CVA, the magnitude of the differentiation was much smaller ($F_{ST} = 0{\cdot}008$; $F'_{ST} = 0{\cdot}026$), although significantly different from zero ($P \leq 0{\cdot}001$) (Table 3).

Genetic analysis of cpDNA

Following Ebert *et al.* (2009), our analysis of cpDNA variation split the polymorphisms into two types: cpIndels and cpSSRs. Here we report the analysis outcomes for a set of 23 cpIndels and for a combined set of 64 loci (23 cpIndels + 41 cpSSRs). Representative haplotype networks across the four taxa for both the indel only and combined locus sets are shown in Fig. 4. Associated summary statistics are summarized in Table 4.

A total of 17 haplotypes were detected across the 23 indels, with the number of haplotypes per taxon varying from two to eight (Fig. 4, Table 4). One haplotype predominated in three of the four taxa (frequency $>0{\cdot}7$). In the exception, two haplotypes, each with frequency $>0{\cdot}4$, were found in CAV. There was no haplotype sharing across the four taxa, with CAJ characterized by two unique haplotypes. One haplotype was shared across CPL/CAV/CVA, while four haplotypes were shared between CAV/CVA (Table 4, Fig. 4).

Across the combined set of 64 loci, 213 haplotypes were detected with a total haplotype diversity of 0·90 (range 0·91–0·98). Given this extreme diversity, no one haplotype predominated, with the maximum haplotype frequency varying from 0·04 in CAV to 0·26 in CPL. CAJ exhibited a unique set of haplotypes (Table 4), with the network analysis revealing a long branch separated this taxon from the other three taxa (Fig. 4). CPL also exhibited a unique set of haplotypes, but these formed a cluster close to the intermixed CAV and CVA haplotypes in the network (Fig. 4). Despite clustering together, only seven out of the total of 170 haplotypes were shared between CAV and CVA (Fig. 4, Table 4). Frequency distributions of pairwise haploid genetic distances closely reflect the haplotype network. Being distinct, CAJ showed a non-overlapping distribution of haploid distances from the other taxa. The remaining three taxa exhibited overlapping distributions, with CPL the most distinct of the trio. It is evident that genetic distance

TABLE 2. *Summary genetic statistics across the four taxa based on the 13 nSSR loci*

	Taxon			
	CAJ	CPL	CAV	CVA
Number of samples	55	50	189	109
N_a	68	92	138	122
$N_{a0\cdot05}$	37	57	54	57
P_i	6	10	15	0
Max P_i frequency	0·045	0·060	0·024	0·000
N_a range over loci	1–13	2–19	3–21	3–19
N_a, mean ± s.e.	5·23 ± 0·93	7·08 ± 1·33	10·62 ± 1·42	9·38 ± 1·29
$N_{a0\cdot05}$, mean ± s.e.	2·85 ± 0·52	4·38 ± 0·69	4·15 ± 0·52	4·38 ± 0·49
N_e, mean ± s.e.	2·38 ± 0·42	3·61 ± 0·89	4·07 ± 0·69	4·01 ± 0·74
P_i, mean ± s.e.	0·46 ± 0·18	0·77 ± 0·36	1·15 ± 0·25	0·00 ± 0·00
H_e, mean ± s.e.	0·42 ± 0·09	0·58 ± 0·07	0·68 ± 0·04	0·68 ± 0·04

N_a, number of alleles (determined by direct count); $N_{a0\cdot05}$, number of alleles with frequency greater than 0·05; N_e, effective number of alleles; P_i, number of private alleles; H_e, expected heterozygosity.

TABLE 3. *AMOVA-based estimates of pairwise differentiation within and among the four taxa across nuclear and chloroplast DNA genetic markers*

		nSSRs (13 loci)		cpIndels (23 loci)				cpIndels + cpSSRs (64 loci)			
		F_{ST}	F'_{ST}	Φ_{PT} H_{23}	Φ'_{PT} H_{23}	Φ_{PT} L_{23}	Φ'_{PT} L_{23}	Φ_{PT} H_{64}	Φ'_{PT} H_{64}	Φ_{PT} L_{64}	Φ'_{PT} L_{64}
Within taxon											
CAJ		0·099	0·165	*0·054*	*0·074*	*0·054*	*0·055*	0·097	0·792	0·218	0·235
CPL		0·049	0·115	*0·008*	*0·013*	*0·042*	*0·043*	0·054	0·716	0·197	0·213
CAV		0·014	0·044	0·076	0·176	0·081	0·084	0·041	0·943	0·083	0·096
CVA		0·050	0·144	*0·033*	*0·120*	*0·046*	*0·047*	0·079	0·860	0·111	0·127
Mean		0·053	0·117	0·043	0·096	0·056	0·057	0·068	0·828	0·152	0·168
s.e.		0·017	0·026	0·015	0·035	0·009	0·009	0·013	0·048	0·033	0·033
Among taxa											
CAJ	CPL	0·363	0·730	0·685	1·000	0·917	0·930	0·075	1·000	0·831	0·902
CAJ	CAV	0·244	0·588	0·508	1·000	0·861	0·886	0·041	1·000	0·752	0·861
CPL	CAV	0·150	0·427	0·202	0·415	0·274	0·282	0·042	1·000	0·444	0·508
CAJ	CVA	0·231	0·536	0·622	1·000	0·901	0·919	0·050	1·000	0·771	0·873
CPL	CVA	0·161	0·447	0·519	0·868	0·541	0·553	0·051	1·000	0·519	0·588
CAV	CVA	0·008	0·026	0·133	0·285	0·101	0·104	0·018	0·928	0·062	0·072
Total		0·144	0·388	0·383	0·728	0·715	0·734	0·036	0·986	0·571	0·652

Separate estimates of differentiation are shown for the 13 nSSR loci (nSSRs), 23 chloroplast indels (cpIndels) and 23 chloroplast indels combined with 41 chloroplast SSRs (cpSSRs). For the within-taxon analysis, cases that were not significantly different at $P \leq 0{\cdot}01$ are shown in italics. For the among-taxa analysis, all cases were significantly different at $P \leq 0{\cdot}001$.

F'_{ST} and Φ'_{PT} were calculated using AMOVA following Meirmans (2006) and provide 0 to 1 standardized estimators of differentiation (Peakall and Smouse, 2012). Φ_{PT} is an analogue of F_{ST} for haploid and haplotype data (for calculation details see Peakall *et al.*, 1995 and Ebert *et al.*, 2009), where Φ_{PT} H_{23} and Φ_{PT} H_{64} represent the differentiation as estimated across haplotypes for the 23 indel and 64 combined locus sets, respectively. Conversely, Φ_{PT} L_{23} and Φ_{PT} L_{64} represent the differentiation as estimated across the haploid loci for the respective locus sets.

per se cannot be used to distinguish among these three taxa (Fig. 5).

Table 3 allows comparisons of the AMOVA estimates of genetic differentiation across both the nuclear and cpDNA markers, and for within- and among-taxon comparisons. While the magnitude of Φ_{PT} and Φ'_{PT} values varied across sets and treatments, the patterns were broadly congruent with the nuclear results, revealing CAJ as the most distinct taxon. With the exception of the haplotype analysis for the combined set of 64 cpDNA loci (Φ_{PT} H_{64}), the levels of differentiation were consistently higher for the chloroplast than for the nuclear loci (Table 3), consistent with theoretical expectations (Ebert and Peakall, 2009*a*).

Among taxa, standardized estimates of Φ'_{PT} were also substantially larger than Φ_{PT} for the haplotype (e.g. Φ_{PT} H_{64} range 0·018–0·075 cf. Φ'_{PT} H_{64} range 0·928–1·00), but not the haploid analyses (Φ_{PT} L_{64} range 0·062–0·831; Φ'_{PT} L_{64} range 0·072–0·902). These large differences reflecting the strong downward bias in Φ_{PT} associated with the extreme haplotype diversity uncovered in this study.

The various estimates of genetic differentiation among the taxa CAJ, CPL and CAV/CVA were typically stronger than their respective within-taxon equivalents, with the exception of estimates of Φ'_{PT} H_{64} (Table 3). Of note was the finding of no detectable significant differentiation among populations within CAJ, CPL and CVA, as estimated across the 23 cpIndels.

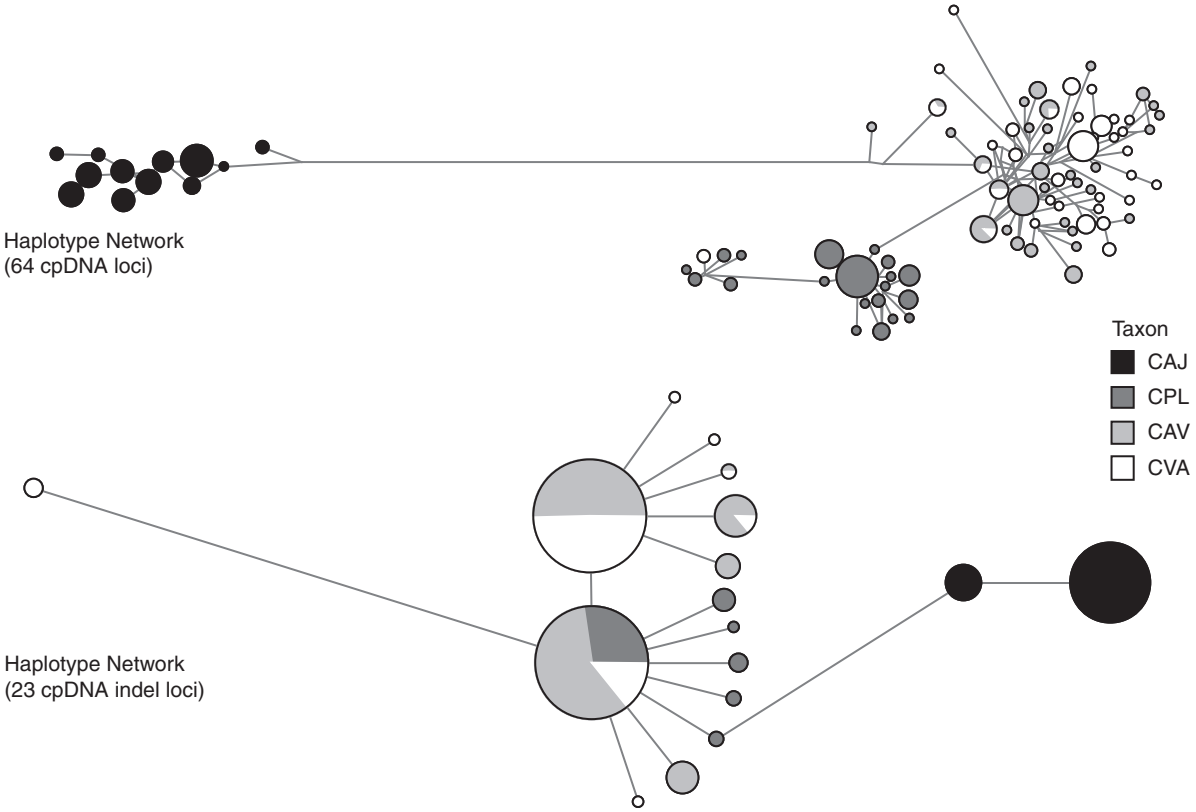

FIG. 4. Representative cpDNA haplotype networks across the four taxa (see Table 1 for abbreviations). To simplify the presentation, a representative subset of samples was randomly selected from each taxon. Two different networks are shown based on the haplotypes defined by the 23 indel cpDNA loci, and the 64 cpDNA loci (23 indels + 41 cpSSRs).

By contrast, among-taxon genetic differentiation at these loci was significantly different for all taxon contrasts and typically much higher in magnitude (Table 3). Thus, substantial genetic differences among the taxa are indicated by these cpIndels.

For the CAV and CVA contrast, which exhibited low differentiation at nSSRs ($F'_{ST} = 0{\cdot}026$), marginally larger differences were detected for the haploid cpDNA analysis ($\Phi'_{PT}\ L_{23} = 0{\cdot}104$; $\Phi'_{PT}\ L_{64} = 0{\cdot}072$). By contrast, the estimates of standardized differentiation were particularly large at the haplotype level ($\Phi'_{PT}\ H_{23} = 0{\cdot}285$; $\Phi'_{PT}\ H_{64} = 0{\cdot}928$). Furthermore, all analyses involving the cpIndels revealed more differentiation between these two taxa than among populations within each taxon, confirming substantive differences between the taxa at this set of markers (Table 3).

Table 4 includes a summary of the outcomes of cpDNA haplotype sharing at the levels of populations and regions across the four taxa (based on the full set of 64 cpDNA loci). Over the four taxa the majority of samples (65–90 %) were characterized by a haplotype that was shared with one or more other samples. Proportionally fewer singletons (haplotypes found in only one sample) were found in CAJ (10 %) than the other three taxa (35–24 %). Across all four taxa, most of the haplotype sharing occurred within populations, with fewer cases of haplotype sharing extending to samples among populations or regions. Thus, haplotype sharing tended to be strongly localized (Table 4). It is this localization of haplotypes that likely explains the high mean values of $\Phi'_{PT}\ H$ in both the within-taxon and the among-taxon comparisons (mean $\Phi'_{PT}\ H_{64} = 0{\cdot}828$ within taxon; total $\Phi'_{PT}\ H_{64} = 0{\cdot}986$ among taxa; Table 3). It follows that the detection of any local haplotype sharing between taxa might indicate strong evidence for extant hybridization. It is also apparent that any search for haplotype sharing (due to hybridization) will be constrained by the availability of suitable local samples. Thus, any statistical tests must take sample sizes into account.

Figure 6 summarizes the outcomes of the novel haplotype sharing analysis we conducted between CAV and CVA. As already noted, only seven haplotypes, represented by 35 samples, were shared between CAV and CVA, being substantially fewer than the 57 haplotypes predicted (representing 175 individuals). Similarly, at three regions of sympatry (MI, TA and KI, see Fig. 2), both the number of observed shared haplotypes and the number of samples with shared haplotypes were significantly fewer than predicted.

In summary, by contrast with expectations under the null hypothesis, not only was the level of haplotype sharing substantially less than expected, but also the patterns of haplotype sharing were contrary to expectations. For example, six of the seven haplotypes were shared only among geographically distant regions with the most abundant haplotype ($n = 8$) only shared between the taxa at the two extremes of the range. Thus, there is compelling evidence that the patterns of observed haplotype sharing depart significantly from expectations under the null hypothesis of no reproductive isolation.

TABLE 4. *Summary genetic statistics across the four taxa based on the chloroplast DNA analysis, including the patterns of haplotype sharing within each taxon*

	CAJ	CPL	CAV	CVA	Total	s.e.
Number of samples	60	62	225	126	473	
H_{23} haplotypes (cpIndels 23 loci)						
#H	2	6	6	8	17	
$\#H_P$	2	5	2	4		
#HAT	0	1	4	4		
eH	1·38	1·52	2·51	1·77	1·81	0·20
Max frequency	0·83	0·81	0·48	0·72		
H	0·28	0·34	0·60	0·43	0·43	0·06
H_{64} haplotypes (cpIndels + cpSSRs 64 loci)						
#H	18	32	108	62	220	
$\#H_P$	18	32	101	55		
#HAT	0	0	7	7		
eH	11·32	10·98	65·15	28·45	23·97	11·06
MaxF	0·15	0·26	0·04	0·12		
H	0·91	0·91	0·98	0·96	0·90	0·04
Patterns of H_{64} haplotype sharing within taxa						
$\#H_S$	6	22	55	40	123	
$\#H_{WP}$	5	7	35	15	62	
$\#H_{AP}$	3	2	6	3	14	
$\#H_{AR}$	4	1	12	4	21	
N_{SH}	54	40	170	86	350	
$N_{SH\%}$	90%	65%	76%	68%	74%	

N, number of samples; *#H*, number of haplotypes; $\#H_P$, number of private haplotypes; *#HAT*, number of haplotypes shared among taxa; *eH*, effective number of haplotypes; *MaxF*, maximum haplotype frequency; *H*, unbiased haplotype diversity.

For haplotype sharing within taxa: $\#H_S$, number of haplotypes found only once (singletons); $\#H_{WP}$, number of haplotypes shared within populations; $\#H_{AP}$, number of haplotypes shared among populations; $\#H_{AR}$, number of haplotypes shared among regions; N_{SH}, number of samples sharing a haplotype; $N_{SH\%}$, number of samples sharing a haplotype as percentage of total.

Note that the counts allocated to the categories $\#H_{WP}$, $\#H_{AP}$ and $\#H_{AR}$ are mutually exclusive. For example, a haplotype counted in category $\#H_{WP}$ cannot also be represented in the count of either $\#H_{AP}$ or $\#H_{AR}$. See Ebert *et al.* (2009) for formulae used in the calculation of *eH* and *H*.

DISCUSSION

Overview

A key challenge in evolutionary biology is elucidating the order of development and the relative importance of different reproductive isolating mechanisms during speciation. The possibility that sexually deceptive orchids may offer examples of rapid pollinator-driven speciation, mediated by minor chemical changes in floral scent, has been widely discussed (Grant, 1994; Schiestl, 2005; Ayasse *et al.*, 2011; Xu *et al.*, 2012). Yet the integrated ecological, chemical and genetic analysis essential for a comprehensive assessment of the relative importance and strength of different reproductive isolation mechanisms is generally lacking (but see Xu *et al.*, 2011; Whitehead, 2012).

In this study we evaluated the patterns of chemical, morphological and genetic variation within and among *Chiloglottis* orchids representing varying degrees of genetic divergence, and include one allopatric taxon (CPL) and three sympatric taxa (Fig. 1). Among the three sympatric taxa, we also tested for genetic compatibility by hand-pollination. At the outset of the study we defined taxa based solely on their chiloglottone floral odour chemistry, chiloglottones being the chemicals responsible for pollinator attraction (Schiestl *et al.*, 2003; Franke *et al.*, 2009). We chose this *a priori* classification in order to test objectively whether or not species boundaries are defined by the chemistry that controls pollinator specificity.

Which barriers are important for reproductive isolation?

Our data show that phenological and geographic isolation appears unnecessary for reproductive isolation. The distribution of CAJ was entirely contained within the larger range of CVA, while CAV and CVA exhibited partial overlap. Co-flowering of these three taxa was observed at several sites (within TA), while CAJ/CVA and CAV/CVA commonly co-flowered (Table 1). Thus the allopatric CPL is the only taxon for which geographic isolation might play a role in reproductive isolation. While it is possible that the currently sympatric taxa were more geographically isolated in the past, a complex secondary contact scenario is not required to explain reproductive isolation, given the confirmed role of floral volatiles in mediating pollinator specificity (Peakall *et al.*, 2010).

We detected some morphological differences among the study taxa, with most CAJ distinguished by their smaller size. There was considerable overlap in morphometric space for the remaining three taxa, with the number of calli being a divergent trait in CPL versus CAV/CVA. All pollinators in this study carried pollinia on their thorax and broadly overlapped in size (data not shown), suggesting no mechanical isolation via differential pollen placement. Additionally, the tendency of synthetic pheromones to elicit sexual behaviours in the absence of floral traits leads us to conclude there is no behavioural isolation being driven by differences in morphology.

No post-zygotic genetic incompatibility, as assessed by the percentage of seeds with embryos, was evident for any combination of artificial crosses among the three sympatric taxa. This finding was not surprising since more phylogenetically distant crosses, such as between CVA and *Chiloglottis trapeziformis*, are known to yield viable F_1 seed (Peakall *et al.*, 1997).

In the absence of evidence for any other reproductive barriers, it is likely that pollinator isolation as a consequence of specific pollinator attraction is the strongest isolating mechanism among the sympatric taxa in this study. A finding of genetic differentiation among chemically defined taxa would provide strong support for this hypothesis.

Genetic differentiation

The degree of nuclear genetic differentiation detected among the taxa varied from very low to moderately high (F_{ST} = 0·008–0·363, F'_{ST} = 0·026–0·730), with CAJ the most distinct taxon. Consistent with expectations, the patterns of cpDNA differentiation were generally stronger but followed similar patterns to the nuclear markers (Table 3). Furthermore, the patterns of genetic differentiation broadly matched the patterns of morphological similarity, suggesting a lack of pollinator-mediated selection on the morphological traits measured.

One challenge in interpreting the patterns of genetic differentiation among recently evolving taxa is distinguishing ancestral polymorphism from extant gene flow (Funk and Omland, 2003). In this study, the exceptional cpDNA haplotype diversity

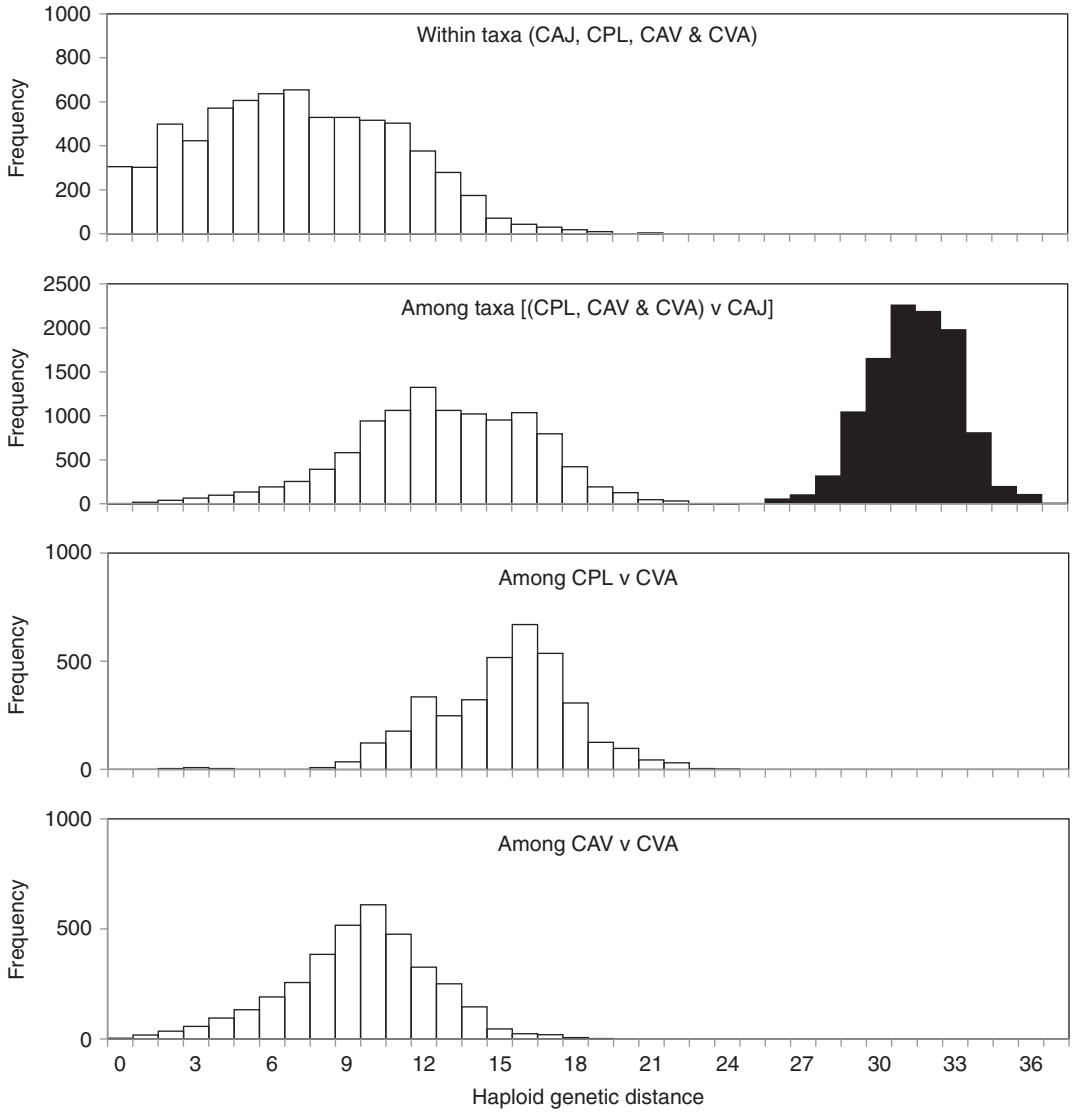

FIG. 5. Frequency distributions of cpDNA haploid distances among the haplotypes representing the four taxa (see Table 1 for abbreviations). Distributions are shown as follows: within taxa; among taxa with the differences between CAJ and the other taxa highlighted in black; among CPL versus CVA; and among CVA versus CAV. The haploid distances were calculated as the tally of pairwise differences across the 64 cpDNA loci (23 indels + 41 cpSSRs).

($H = 0{\cdot}90$, range 0·91–0·98) offered a unique opportunity to aid resolution of this problem. Our novel haplotype-sharing analysis built on the routines used within the widely applied AMOVA framework, allowing us to compare the observed patterns of haplotype sharing with that expected under the null model of no reproductive isolation (complete bi-directional gene flow).

Our haplotype-sharing analysis was particularly important for the morphologically indistinguishable and genetically very similar CAV and CVA. Under the null hypothesis, substantially more sharing of haplotypes was predicted at every level of the analysis than was observed (Fig. 6). Furthermore, not only was the overall level of sharing low (seven out of 170), but also the geographic patterns of sharing were inconsistent with the null expectation, with haplotype sharing entirely absent at the population level and virtually absent even at the regional level. Thus, despite the undisputed genetic similarity of the taxa in both nuclear and cpDNA, the haplotype-sharing analysis provide compelling evidence for complete or very nearly complete pollinator-mediated reproductive isolation between CAV and CVA. Thus, chemistry defines this subtle but critical genetic difference between these taxa.

The evolution of genetic divergence

Our inclusion of multiple closely related taxa at suspected varying stages of divergence allows us to infer the evolution of genetic differentiation. As expected theoretically (Ebert and Peakall, 2009*a*), cpDNA divergence preceded nuclear divergence. The initial impact on cpDNA variation arising from restrictions on gene flow, such as in the case of CAV/CVA, appears to be reduced haplotype sharing among the taxa. At this early stage, taxon-specific haplotypes remain intermixed within the haplotype network (paraphyletic) and genetic distances among haplotypes are not diagnostic (Figs 4 and 5). The

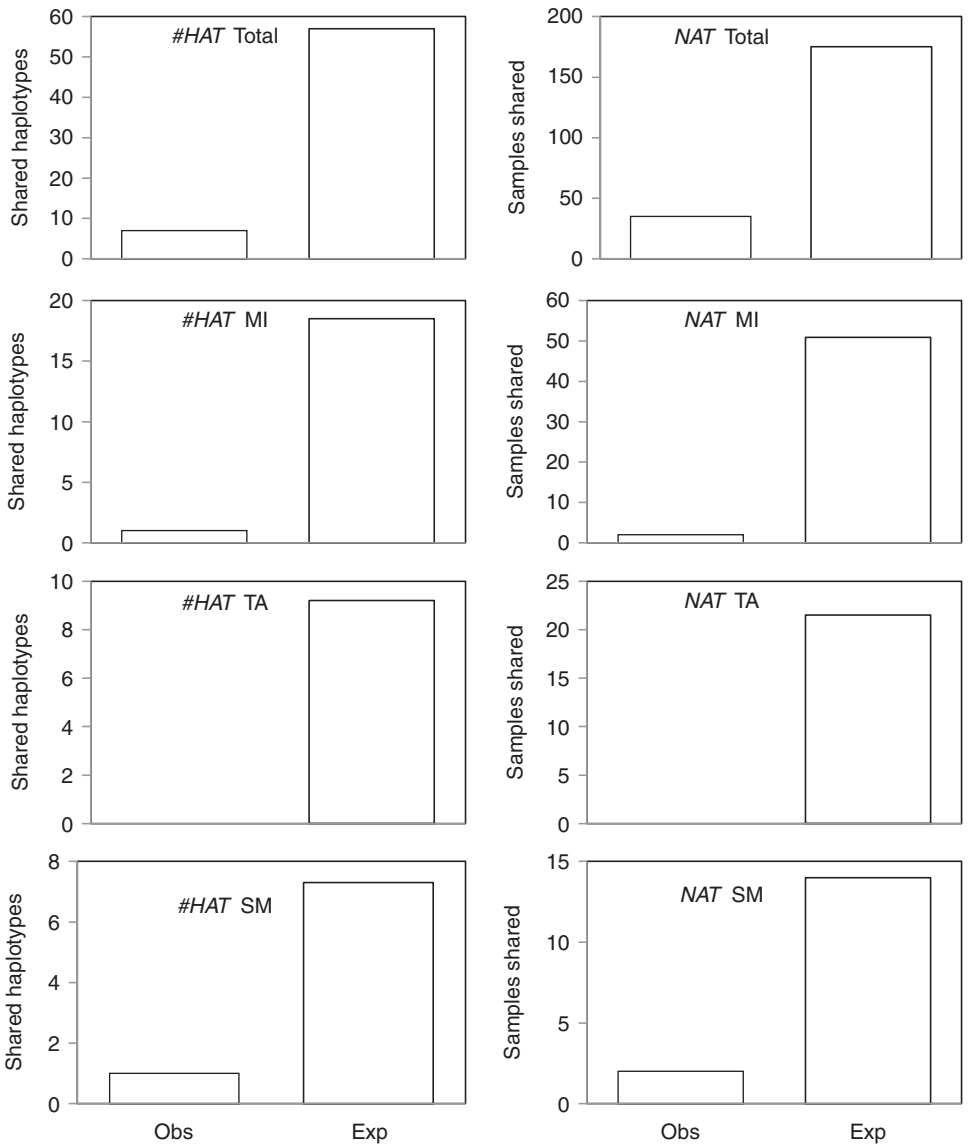

FIG. 6. Observed patterns of cpDNA haplotype sharing between CAV and CVA compared with that expected under the null hypothesis of no reproductive isolation between the taxa. Results are shown for the total and three regions of sympatry (MI, TA and KI, see Fig. 2). In all cases the observed and expected values were significantly different ($P < 0{\cdot}001$), based on statistical tests by random permutation (999 permutations). See Supplementary Table S3 for additional statistics and analyses. #HAT, number of haplotypes shared among taxa; NAT, number of samples containing haplotypes shared among taxa.

next stage, exemplified by CPL, reveals the development of taxon structure within the haplotype network (monophyletic on a short branch), but genetic distances among haplotypes are still not diagnostic. Next, as exemplified by CAJ, stronger separation is evident in the haplotype network, genetic distances are now diagnostic of the taxon, and genetic divergences become larger. Meanwhile, even at this later stage of divergence there is considerable nuclear allele sharing and overall levels of nuclear differentiation (as measured by F_{ST}) still fall well within the range of population-level genetic variation within mature species (Phillips *et al.*, 2012).

Maintenance of low differentiation

Given the chloroplast haplotype evidence for a lack of ongoing gene flow, how can one explain the apparent maintenance of such low levels of nuclear genetic differentiation between CAV and CVA? We suggest two factors are relevant in *Chiloglottis*: orchid longevity and large effective population sizes. *Chiloglottis* orchids form colonies by vegetative reproduction with genets consisting of hundreds of ramets (R. Peakall, unpubl. res.; Fig 1); thus, clones could be hundreds, perhaps even thousands, of years old. Consistent with theoretical expectations, we predict that the combination of longevity combined with large effective population size is likely to impede the development of genetic divergence by genetic drift, even in the absence of extant gene flow. In this study, it is notable that the differentiation among even the geographically isolated taxa (CAV/CVA and CPL) falls well within the range of within-species differentiation (Phillips *et al.*, 2012). Thus, in *Chiloglottis* we suggest large effective sizes are the norm.

Cases of very weak population and species level genetic differentiation at nuclear microsatellite loci are also known in *Ophrys* orchids. Soliva and Widmer (2003) interpreted their finding of lower genetic differentiation among pairs of sympatric compared with allopatric taxa as evidence for contemporary gene flow across species boundaries. Mant *et al.* (2005*c*) found similarly low levels of genetic differentiation (mean F_{ST} of 0·075) among putative *Ophrys* taxa, but strong differences in the biologically active chemicals involved in specific pollinator attraction. This led them to caution against automatically interpreting low differentiation as evidence for ongoing gene flow, with size homoplasy, divergence underestimation at hypervariable markers, and ancestral polymorphism possible alternative explanations (Mant *et al.*, 2005*c*). Xu *et al.* (2012) also concluded that low genetic differentiation among *Ophyrs* species could reflect ancestral polymorphism rather than gene flow. Findings of low nuclear genetic differentiation among evolving taxa are not restricted to orchids. Cooper *et al.* (2010) report the finding of very low nuclear genetic differentiation among morphologically distinct species of *Aquilegia*, and noted that incipient species can appear almost identically at many loci, even in the complete absence of gene flow.

Implications of low genetic differentiation for orchids generally

Beyond *Chiloglottis* and *Ophrys*, there is mounting evidence that low levels of population genetic differentiation are a characteristic of orchids. In a recent meta-analysis of 58 allozyme-based population genetic studies spanning the family (including both terrestrial and epiphytic species), Phillips *et al.* (2012) reported that the Orchidaceae had the lowest mean F_{ST} of all herbaceous families (mean $F_{ST} = 0{\cdot}146$). Only the primarily wind-pollinated and wind-dispersed Pinaceae and Fagaceae exhibited lower mean F_{ST} values in allozyme studies. Thus, despite their tendency to have small and isolated populations, orchids appear to maintain high levels of gene flow (probably via their wind-dispersed seed) and therefore likely exhibit much larger effective population sizes than previously thought (Phillips *et al.*, 2012). We know little about orchid longevity, but it is also possible that many orchids are relatively long-lived, providing more opportunity for occasional long-distance gene flow per generation. Certainly, orchids frequently have the capacity to vegetatively replace underground parts (terrestrial) and pseudobulbs (epiphytes), making it difficult, if not impossible, to age a plant (Dressler, 1993).

The overlooked combination of large effective population size supported by potential long-distance seed dispersal and orchid longevity warrant further research attention as factors potentially aiding orchid diversification. The combination of these two factors will strongly impede divergence via genetic drift, providing an explanation for the low levels of genetic differentiation that characterize the orchids. More importantly, large effective population sizes enhance opportunities for selection for advantageous alleles, while promoting the efficient removal of deleterious ones (Slatkin, 1987; Ellegren, 2008). It follows that large effective population sizes may offer optimal conditions for facilitating pollinator-driven speciation.

A model for speciation

In the present study of sexually deceptive *Chiloglottis* orchids we have provided compelling new genetic evidence that minor chemical differences define species boundaries. The subtle chemical differences between species probably have a simple genetic basis, with both gene duplication and allelic variation likely playing a role in the evolution of chiloglottone variation (Peakall *et al.*, 2010; Xu *et al.*, 2012). However, a plausible mechanism for how the evolutionary process of chemical change and pollinator switching might operate has been lacking.

In the light of this study, we speculate that in *Chiloglottis* orchids the first step might involve the evolution of neutral chemical mutations (Fig. 7A). We emphasize that neutrality or near neutrality is important, since in combination with the large effective population sizes this will allow the mutation to spread rapidly and harmlessly throughout the ancestral species (Fig. 7B). In a taxon such as CVA that employs chiloglottone 1 to secure pollination, such a mutation might produce low levels of chiloglottone 2, while chiloglottone 1 production is maintained. Sex pheromone receivers need not automatically react to small amounts of novel compounds in an established pheromone signal (Niehuis *et al.*, 2013) and in this way minor components of a volatile blend may remain selectively neutral. High penetrance combined with pollen limitation [pollination rates of 0·15 and 0·23 for CAJ and CVA respectively (Whitehead, 2012)] then maximize the opportunity for pollinator-mediated selection when a second potential pollinator is encountered.

Contact with a second pollinator (Fig. 7C, D) now enables four evolutionary scenarios, depending on the interplay between geography, gene flow and pollinator-mediated selection. The first scenario is that dual pollination becomes a stable strategy within sympatric populations (Fig. 7E). Second, pollinator switching may occur in part of the range without speciation (Fig. 7G). As in other systems (Peter and Johnson, 2014; Sun *et al.*, 2014; Van der Niet *et al.*, 2014), this may give rise to distinct intraspecific pollination ecotypes. The third and fourth scenarios involve pollinator switching that leads to reproductive isolation followed by either sympatric (Fig. 7F) or allopatric (Fig. 7H) speciation, respectively. Note that in the case of speciation, 'transfer of function via an intermediate stage of double function' would ensue, as predicted by Stebbins (1970) as a general feature of pollinator-mediated speciation. Van der Niet *et al.* (2014) provide a compelling example of this double function, where apparently diverging and specialized pollination ecotypes still attract a less effective ancestral pollinator species.

Conclusions

In sexually deceptive *Chiloglottis* orchids, it is clear that floral odour chemistry, which in turn controls pollinator specificity, underpins strong reproductive isolation between incipient species. We predict that the combination of initial neutral mutations and large effective population sizes has been important in the evolution of *Chiloglottis* orchids. Moreover, these features may be a general characteristic of pollinator-driven speciation that has been overlooked until now. These characteristics would facilitate the rapid spread of mutations and maximize opportunities for selection under conditions of pollinator limitation. Certainly, such a scenario in combination with a geographic mosaic of pollinator availability (Johnson, 2006; Harder and Johnson, 2009) warrants closer scrutiny in future studies.

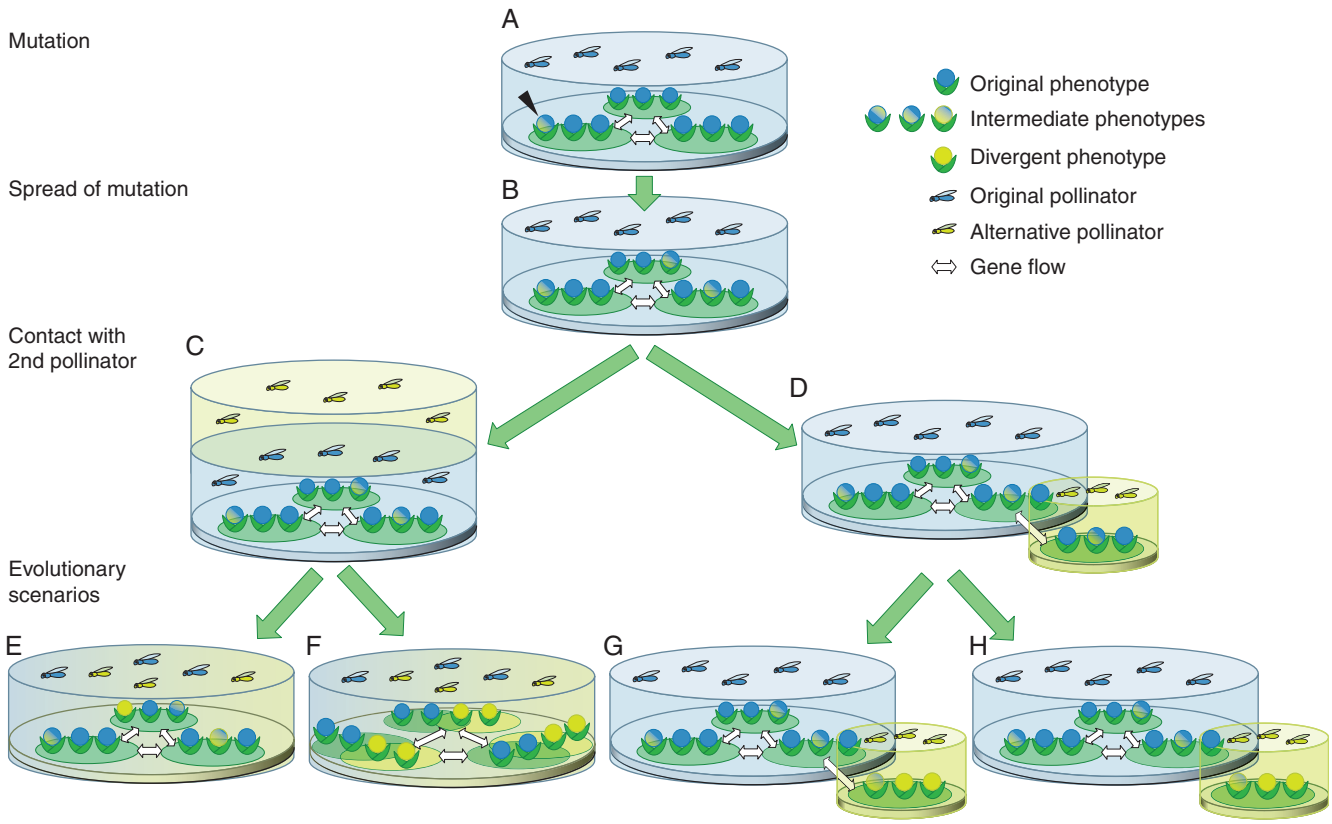

FIG. 7. Flow diagram showing a speciation model for *Chiloglottis* orchids. The model predicts that a mutation in one population of orchids results in phenotypic variation for floral volatile chemistry (A). Extensive gene flow and large effective population size promote the spread of the mutant (B). Contact with a new pollinator via range expansion of either pollinator (C) or orchid (D), coupled with pollinator-mediated selection, enables four evolutionary scenarios: dual pollination becomes a stable strategy in sympatry (E) or allopatry (G); pollinator switching leads to reproductive isolation and sympatric speciation (F), or speciation in allopatry (H).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: orchid population and regional location details including state, district, geographic coordinates and sample sizes; Table S2: outcomes of univariate morphometric analysis of 11 floral characters across the four taxa; Table S3: observed haplotype sharing between the taxa CAV and CVA, compared with that predicted under the null hypothesis of no genetic difference.

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LITERATURE CITED

Ayasse M, Stokl J, Francke W. 2011. Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* **72**: 1667–1677.

Bandelt HJ, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37–48.

Bateman RM, Bradshaw E, Devey DS, *et al*. 2011. Species arguments: clarifying competing concepts of species delimitation in the pseudo-copulatory orchid genus *Ophrys*. *Botanical Journal of the Linnean Society* **165**: 336–347.

Bickford D, Lohman DJ, Sodhi NS, *et al*. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* **22**: 148–155.

Bower CC. 2006. Specific pollinators reveal a cryptic taxon in the bird orchid, *Chiloglottis valida sensu lato* (Orchidaceae) in south-eastern Australia. *Australian Journal of Botany* **54**: 53–64.

Bower CC, Brown GR. 2009. Pollinator specificity, cryptic species and geographical patterns in pollinator responses to sexually deceptive orchids in the genus *Chiloglottis*: the *Chiloglottis gunnii* complex. *Australian Journal of Botany* **57**: 37–55.

Cooper EA, Whittall JB, Hodges SA, Nordborg M. 2010. Genetic variation at nuclear loci fails to distinguish two morphologically distinct species of *Aquilegia*. *PLoS One* **5**: e8655.

Devey DS, Bateman RM, Fay MF, Hawkins JA. 2008. Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid genus *Ophrys*. *Annals of Botany* **101**: 385–402.

Dressler RL. 1993. *Phylogeny and classification of the orchid family*. Cambridge: Cambridge University Press.

Ebert D, Peakall R. 2009*a*. Chloroplast simple sequence repeats (cpSSRs): technical resources and recommendations for expanding cpSSR discovery and applications to a wide array of plant species. *Molecular Ecology Resources* **9**: 673–690.

Ebert D, Peakall R. 2009*b*. A new set of universal de novo sequencing primers for extensive coverage of non-coding chloroplast DNA: new opportunities for phylogenetic studies and cpSSR discovery. *Molecular Ecology Resources* **9**: 777–783.

Ebert D, Hayes C, Peakall R. 2009. Chloroplast simple sequence repeat markers for evolutionary studies in the sexually deceptive orchid genus *Chiloglottis*. *Molecular Ecology Resources* **9**: 784–789.

Ellegren H. 2008. A selection model of molecular evolution incorporating the effective population size. *Evolution* **63**: 301–305.

Ellis AG, Johnson SD. 2010. Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of the Orchidaceae. *American Naturalist* **176**: E143–E151.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitocondrial DNA restriction sites. *Genetics* **131**: 479–491.

Flanagan NS, Ebert D, Porter C, Rossetto M, Peakall R. 2006. Microsatellite markers for evolutionary studies in the sexually deceptive orchid genus *Chiloglottis*. *Molecular Ecology Notes* **6**: 123–126.

Fluxus Technology Ltd. 2010. *Network 4.6. 0.0*. http://www.fluxus-engineering.com/ (last accessed August 24, 2013).

Forster P, Röhl A, Lünnemann P, *et al*. 2000. A short tandem repeat-based phylogeny for the human Y chromosome. *American Journal of Human Genetics* **67**: 182–196.

Franke S, Ibarra F, Schulz CM, *et al*. 2009. The discovery of 2,5-dialkylcyclohexan-1,3-diones as a new class of natural products. *Proceedings of the National Academy of Sciences of the USA* **106**: 8877–8882.

Funk DJ, Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology Evolution and Systematics* **34**: 397–423.

Gaskett AC. 2011. Orchid pollination by sexual deception: pollinator perspectives. *Biological Reviews* **86**: 33–75.

Grant V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. *Proceedings of the National Academy of Sciences of the USA* **91**: 3–10.

Griffiths KE, Trueman JWH, Brown GR, Peakall R. 2011. Molecular genetic analysis and ecological evidence reveals multiple cryptic species among thynnine wasp pollinators of sexually deceptive orchids. *Molecular Phylogenetics and Evolution* **59**: 195–205.

Harder LD, Johnson SD. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for adaptation. *New Phytologist* **183**: 530–545.

Hedrick PW. 2005. A standardized genetic differentiation measure. *Evolution* **59**: 1633–1638.

Johnson SD. 2006. Pollinator-driven speciation in plants. In: Harder LD, Barrett SCH. eds. *Ecology and evolution of flowers.* Oxford: Oxford University Press, 295–310.

Johnson SD. 2010. The pollination niche and its role in the diversification and maintenance of the southern African flora. *Philosophical Transactions of the Royal Society B Biological Sciences* **365**: 499–516.

Jones DL. 1991. New taxa of Australian Orchidaceae. *Australian Orchid Research* **2**: 1–208.

Jost L. 2008. GST and its relatives do not measure differentiation. *Molecular Ecology* **17**: 4015–4026.

Lexer C, Widmer A. 2008. The genic view of plant speciation: recent progress and emerging questions. *Philosophical Transactions of the Royal Society B Biological Sciences* **363**: 3023–3036.

Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH. 2008. The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B Biological Sciences* **363**: 3009–3021.

Mant J, Bower CC, Weston PH, Peakall R. 2005*a*. Phylogeography of pollinator-specific sexually deceptive *Chiloglottis* taxa (Orchidaceae): evidence for sympatric divergence? *Molecular Ecology* **14**: 3067–3076.

Mant J, Brandli C, Vereecken NJ, Schulz CM, Francke W, Schiestl FP. 2005*b*. Cuticular hydrocarbons as sex pheromone of the bee *Colletes cunicularius* and the key to its mimicry by the sexually deceptive orchid, *Ophrys exaltata*. *Journal of Chemical Ecology* **31**: 1765–1787.

Mant J, Peakall R, Schiestl FP. 2005*c*. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus *Ophrys*? *Evolution* **59**: 1449–1463.

Mant JG, Schiestl FP, Peakall R, Weston PH. 2002. A phylogenetic study of pollinator conservatism among sexually deceptive orchids. *Evolution* **56**: 888–898.

Meirmans PG. 2006. Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution* **60**: 2399–2402.

Meirmans PG, Hedrick PW. 2011. Assessing population structure: FST and related measures. *Molecular Ecology Resources* **11**: 5–18.

Michalakis Y, Excoffier L. 1996. A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics* **142**: 1061–1064.

Niehuis O, Buellesbach J, Gibson JD, *et al*. 2013. Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* **494**: 345–348.

Paulus HF, Gack C. 1990. Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidaceae). *Israel Journal of Botany* **39**: 43–79.

Peakall R. 1990. Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. *Functional Ecology* **4**: 159–167.

Peakall R, Beattie AJ. 1996. Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentactulata*. *Evolution* **50**: 2207–2220.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**: 288–295.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* **28**: 2537–2539.

Peakall R, Smouse PE, Huff DR. 1995. Evolutionary implications of allozyme and RAPD variation in diploid populations of dioecious buffalograss *Buchloe dactyloides*. *Molecular Ecology* **4**: 135–147.

Peakall R, Bower CC, Logan AE, Nicol HI. 1997. Confirmation of the hybrid origin of *Chiloglottis*×*pescottiana* (Orchidaceae: Diurideae). 1. Genetic and morphometric evidence. *Australian Journal of Botany* **45**: 839–855.

Peakall R, Ebert D, Poldy J, Barrow R, Francke W, Bower C, Schiestl F. 2010. Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. *New Phytologist* **188**: 437–450.

Peakall R, Jones L, Bower CC, Mackey BG. 2002. Bioclimatic assessment of the geographic and climatic limits to hybridisation in a sexually deceptive orchid system. *Australian Journal of Botany* **50**: 21–30.

Peter CI, Johnson SD. 2014. A pollinator shift explains floral divergence in an orchid species complex in South Africa. *Annals of Botany* **113**: doi:10.1093/aob/mct216.

Phillips RD, Faast R, Bower CC, Brown GR, Peakall R. 2009. Implications of pollination by food and sexual deception for pollinator specificity, fruit set, population genetics and conservation of *Caladenia* (Orchidaceae). *Australian Journal of Botany* **57**: 287–306.

Phillips RD, Dixon KW, Peakall R. 2012. Low population genetic differentiation in the Orchidaceae: implications for the diversification of the family. *Molecular Ecology* **21**: 5208–20.

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

Rosenblum EB, Harmon LJ. 2011. 'Same same but different': replicated ecological speciation at White Sands. *Evolution* **65**: 946–960.

Ryman N, Leimar O. 2009. GST is still a useful measure of genetic differentiation – a comment on Jost's D. *Molecular Ecology* **18**: 2084–2087.

Rymer PD, Manning JC, Goldblatt P, Powell MP, Savolainen V. 2010. Evidence of recent and continuous speciation in a biodiversity hotspot: a population genetic approach in southern African gladioli (*Gladiolus*; Iridaceae). *Molecular Ecology* **19**: 4765–4782.

Schemske DW, Bradshaw HD. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Acadademy of Sciences of the USA* **96**: 11910–11915.

Schiestl FP. 2005. On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften* **92**: 255–264.

Schiestl FP, Ayasse M. 2002. Do changes in floral odor cause speciation in sexually deceptive orchids? *Plant Systematics and Evolution* **234**: 111–119.

Schiestl FP, Ayasse M, Paulus HF, *et al*. 1999. Orchid pollination by sexual swindle. *Nature* **399**: 421–422.

Schiestl FP, Peakall R, Mant JG, *et al*. 2003. The chemistry of sexual deception in an orchid-wasp pollination system. *Science* **302**: 437–438.

Singer RB. 2002. The pollination mechanism in *Trigonidium obtusum* Lindl (Orchidaceae: Maxillariinae): sexual mimicry and trap-flowers. *Annals of Botany* **89**: 157–163.

Singer RB, Flach A, Koehler S, Marsaioli AJ, Amaral MDE. 2004. Sexual mimicry in *Mormolyca ringens* (Lindl.) Schltr. (Orchidaceae: Maxillariinae). *Annals of Botany* **93**: 755–762.

Slatkin M. 1987. Gene flow and the geographic structure of natural populations. *Science* **236**: 787–792.

Sobel JM, Chen GF, Watt LR, Schemske DW. 2009. The biology of speciation. *Evolution* **64**: 295–315.

Soliva M, Widmer A. 2003. Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. *Evolution* **57**: 2252–2261.

Stebbins GL. 1970. Adaptive radiation of reproductive characteristics in angiosperms I. Pollination mechanisms. *Annual Review of Ecology and Systematics* **1**: 307–326.

Steiner KE, Whitehead VB, Johnson SD. 1994. Floral and pollinator divergence in 2 sexually deceptive South African orchids. *American Journal of Botany* **81**: 185–194.

Stökl J, Schluter PM, Stuessy TF, *et al*. 2009. Speciation in sexually deceptive orchids: pollinator-driven selection maintains discrete odour phenotypes in hybridizing species. *Biological Journal of the Linnean Society* **98**: 439–451.

Stokl J, Twele R, Erdmann DH, Francke W, Ayasse M. 2007. Comparison of the flower scent of the sexually deceptive orchid *Ophrys iricolor* and the female sex pheromone of its pollinator *Andrena morio*. *Chemoecology* **17**: 231–233.

Sun M, Gross K, Schiestl FP. 2014. Floral adaptation to local pollinator guilds in a terrestrial orchid. *Annals of Botany*, **113**: doi:10.1093/aob/mct219.

Van der Niet T, Johnson SD. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology and Evolution* **27**: 353–361.

Van der Niet T, Pririe MD, Shuttleworth A, Johnson SD, Midgley JJ. 2014. The evolution of three pollination ecotypes in the Cape flora shrub *Erica plukenetii*. *Annals of Botany* **113**: doi:10.1093/aob/mct193.

Vereecken NJ, Streinzer M, Ayasse M, *et al*. 2011. Integrating past and present studies on *Ophrys* pollination – a comment on Bradshaw *et al.* *Botanical Journal of the Linnean Society* **165**: 329–335.

Vereecken NJ, Wilson CA, Hötling S, Schulz S, Banketov SA, Mardulyn P. 2012. Pre-adaptations and the evolution of pollination by sexual deception: Cope's rule of specialization revisited. *Proceedings of the Royal Society B Biological Sciences* **279**: 4786–4794.

Whitehead MR. 2012. *The evolutionary biology of pollination: studies in a genus of Australian sexually deceptive orchids*. PhD Thesis, Australian National University, Australia. http://hdl.handle.net/1885/10260 (last accessed August 24, 2013).

Whitehead MR, Peakall R. 2009. Integrating floral scent, pollination ecology and population genetics. *Functional Ecology* **23**: 863–874.

Whitlock MC. 2011. G'ST and D do not replace FST. *Molecular Ecology* **20**: 1083–1091.

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

Xu SQ, Schluter PM, Scopece G, *et al*. 2011. Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution* **65**: 2606–2620.

Xu S, Schluter PM, Schiestl FP. 2012. Pollinator-driven speciation in sexually deceptive orchids. *International Journal of Ecology* **2012**: ID 285081.