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Chemical communication in the sexually deceptive orchid genus *Cryptostylis*

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Pollination by sexual deception is among the most intriguing of orchid pollination syndromes. Odours are well established as the primary stimuli for sexually attracting the male insect pollinators in these orchids. We applied gas chromatography with electroantennographic detection (GC-EAD) to investigate chemical communication between the sympatric, but morphologically distinct, orchids *Cryptostylis erecta* and *C. subulata* and their pollinators. *Cryptostylis* is unusual among sexually deceptive orchid genera in that all five Australian species share the same pollinator, the ichneumonid wasp *Lissopimpla excelsa*, but hybrids are unknown. We show that volatile odour compounds are not produced in detectable amounts in either species. Floral extracts containing many low-volatility compounds showed considerable differences in composition between *C. erecta* and *C. subulata*. By contrast, GC-EAD revealed the male wasp pollinators are electrophysiologically responsive to the same GC peak on two different kinds of GC column in both orchids. This leads us to predict that a single compound is the sexual attractant in all Australian *Cryptostylis*. The apparent conservation of chemical signals among distinct species contrasts with that of other sexually deceptive orchids that are often morphologically similar but reproductively isolated by their different chemical signals.

ADDITIONAL KEYWORDS: chemical signals – Diurideae – floral odour – GC-EAD – Ichneumonidae – pollination.

INTRODUCTION

Orchids are renowned for their diversity of pollination syndromes. Among the most intriguing syndrome is pollination by sexual deception, in which orchids sexually attract male insects as pollinators. At long range the attraction of the male pollinators is achieved by mimicking the sex pheromones of the pollinator species. At short range, visual and tactile mimicry of the female insect may also become important. At the flower, pollination occurs during either a precopulatory routine, or attempted copulation – so-called pseudocopulation (Peakall, 1990; Nilsson, 1992; Schiestl *et al*., 1999).

Sexual deception is a pollination strategy found in at least two orchid subtribes. It is well known in the European genus *Ophrys* (Orchideae), in which all but one or two selfing species in the genus (*c*. 80 species) employ the mechanism (Kullenberg, 1961; Paulus & Gack, 1990; Schiestl *et al*., 1999). However, Australia is the centre of diversity for sexual deception with more than 150 species in nine genera of terrestrial Diurideae involved. Equally diverse in Australia is the array of male insects exploited, with representatives of five different subfamilies of Hymenoptera present: parasitic thynnine wasps (Tiphideae) pollinate species of *Arthrochilus*, *Caladenia s.s.*, *Chiloglottis*, *Drakaea*, *Paracaleana* and *Spiculaea*; scoliid wasps (Scoliidae) pollinate *Calochilus*; saw flies (Pergidae) pollinate *Caleana*; male ants (Myrmecidae) pollinate *Leporella*; and ichneumonid wasps (Ichneumonidae) pollinate *Cryptostylis* (Coleman, 1928a; Stoutamire, 1975, 1983; Peakall, Beattie & James, 1987; Peakall, 1990; Bower, 1996).

Recent phylogenetic studies confirm that sexual deception has multiple evolutionary origins across the Orchidaceae, but also within the Australian Diurideae

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itself (Kores *et al*., 2000, 2001). Thynnine wasp pollination, the dominant mode of sexual deception with more than 100 species, has evolved at least twice, once in the clade containing *Caladenia s.s.* and once in a clade containing *Arthrochilus*, *Chiloglottis*, *Drakaea*, *Paracaleana* and *Spiculaea*. Pollination by male ants, scoliid wasps and ichneumonid wasps may all have independent origins, although there has been a likely switch from thynnine wasp to sawflies in the case of *Caleana major* (Kores *et al*., 2001; Mant *et al*., 2002).

The discovery of pollination by pseudocopulation was made independently on two continents in the early 1900s. In 1917, Pouyanne reported his observations of pollination by pseudocopulation in a French journal (Pouyanne, 1917), but his work lay largely unknown until his observations were confirmed and brought to light by Godfery (1925). In Australia, Edith Coleman, a prominent naturalist of her time, confirmed in a series of publications (e.g. Coleman, 1927, 1928a,b, 1929a,b, 1930a,b, 1938) that pollination of *Cryptostylis* was achieved by male *Lissopimpla excelsa* (Casta) (formerly *L. semipunctata*) wasps during pseudocopulation with the flower. Her detailed observations and experimental approach generated considerable excitement and her Australian reports were reproduced in *Transactions of the Entomological Society of London* (Coleman, 1928b), *The Journal of Botany* (Coleman, 1929b) and *Proceedings of the Royal Entomological Society of London* (Coleman, 1938). Despite this prestigious start to our understanding of pollination in *Cryptostylis*, there has been little further study of this pollination system. By contrast, we know far more about the genera pollinated by thynnine wasps (e.g. Stoutamire, 1974, 1975, 1983; Peakall, 1990; Peakall & Handel, 1993; Bower, 1996; Peakall & Beattie, 1996; Alcock, 2000; Mant *et al*., 2002; Wong & Schiestl, 2002), and *Leporella* pollinated by male ants (Peakall, 1989; Peakall *et al*., 1987, 1990).

In addition to its historical significance, the pollination of *Cryptostylis* by sexual deception is of particular interest because it appears to differ in several important respects from other sexually deceptive clades. Sexually deceptive orchids achieve a remarkable pollinator specificity, yet *Cryptostylis* has many species using the one pollinator, whereas in *Ophrys* and the thynnine-pollinated diurids, many species each use a different pollinator. *Cryptostylis* species are morphologically disparate, whereas many species within thynnine-pollinated genera can be morphologically cryptic. The ichneumonid wasp *Lissopimpla excelsa* is reported as the sole pollinator of *Cryptostylis*, at least amongst the five Australian species for which there are published studies (Jones, 1988). In Eastern Australia, up to three species of *Cryptostylis* may be sympatric at any one site, yet no hybrids are known.

Despite the absence of a detectable scent in *Cryptostylis* to humans, the role of scent as the primary attractant of the male pollinators soon became apparent to Coleman (1928a), who later demonstrated that flowers covered in muslin cloth still attracted male wasps (Coleman, 1930a). However, a major problem for the investigation of chemical communication between orchid and pollinator is that although floral odours may consist of many tens or hundreds of compounds, only a fraction of those may play a role in attracting pollinators (Schiestl *et al*., 1999). A powerful technique, developed in the 1950s by the German researcher Schneider, allows the recording of minute electric potentials that are created when an insect smells an odour compound (Schneider, 1957). Such a recording, called an electroantennogram (EAG), when combined with gas chromatography to separate the blend of compounds into single constituents, allows those compounds that are detected by insects to be determined. Compounds eliciting the appropriate response are thus identified to be the biologically active compounds involved in chemical communication (Schiestl *et al*., 1999; Schiestl & Marion-Poll, 2002). This combined technique is called gas chromatography with electroantennographic detection (GC-EAD). Once an electrophysiologically active compound has been identified, it may be tested in synthetic form for its behavioural impact on a pollinator insect. Despite its power, GC-EAD has been largely ignored as a tool in pollination studies.

In this study, we report the first investigation of chemical communication between *Cryptostyli*s and its ichneumonid wasp pollinator, *Lissopimpla excelsa*. Specifically we ask:

1. How similar are the odour bouquets of the sympatric orchids *Cryptostylis subulata* (Labill.) Rchb.f and *C. erecta* R.Br.?
2. How many electrophysiologically active compounds within the odour bouquets are detected by the wasp pollinators?
3. Are the same electrophysiologically active compounds found in the two orchids species?
4. What are the implications of our findings for the evolution of this pollination system?

MATERIAL AND METHODS

ORCHID TAXA

The genus *Cryptostylis*, once classified in the Cranichideae in its own subtribe, the Cryptostylidinae (Dressler, 1981), has been shown by recent molecular phylogenetic analyses to belong to the tribe Diurideae (Kores *et al*., 2001). There are 20 species in the genus, distributed in south-east Asia, the Pacific, Australia and New Zealand. Except for one rare saprophytic spe-

cies, all are evergreen terrestrials characterized by upside-down flowers dominated by a large (20–30 mm long), often reddish labellum, with the remaining floral parts inconspicuous (Jones, 1988). Multiple flowers are presented in succession over a period of several weeks on inflorescences that in some species can exceed 1 m in height. There are five species in Australia, some of which are widespread and often locally common. We focus this study on *C. subulata* and *C. erecta*, both of which are widespread in coastal New South Wales, and are commonly found growing and flowering together. The floral morphology of *C. erecta* is the most distinctive of the Australian species, differing from the other species in having a hooded labellum rather than the more typical narrow elongated form.

Pollinators

The genus *Lissopimpla* (Ichneumonidae: Pimplinae) contains only four Australian species, of which one species (*L. excelsa* (Casta)) is the pollinator of all *Cryptostylis* in Australia. *Lissopimpla* wasps are parasitic and females may be seen probing around grass tussocks to search for prepupae and pupae of Lepidoptera to parasitize (Naumann, 1991). Males and females are both winged and may be seen feeding on nectar-producing flowers but are not reported as pollinators for any other plants. *Lissopimpla excelsa* is a common and widespread species, occurring throughout Australia, New Zealand and the Pacific, in urban areas as well as native vegetation.

Collection of orchid odour samples

Flowers of *Cryptostylis erecta* and *C. subulata* were collected in January 2000 in the Blue Mountains, west of Sydney, Australia, for the extraction of floral odour. Highly volatile odour compounds were sampled by headspace sorption. Flowers were enclosed in a glass chamber and the air in the chamber was drawn over an adsorbent material (Porpak Q) for 4 h. Subsequently, the trapped volatile compounds were eluted using pentane. To extract less volatile components, flower labella were extracted in *c*. 2 mL pentane for 12 h and then removed from the solvent. A standard mixture of straight chain alkanes (C9–C18) was added and the samples were concentrated to a total volume of about 200 μL. All samples were stored at –20°C for subsequent analysis.

Collection of wasp males for GC-EAD

In order to obtain live wasp pollinators for GC-EAD, three flowers of *C. erecta* and one flower of *C. subulata* were offered simultaneously in the field. Pollinators were readily attracted to the flowers, with a total of ten male wasps caught while pseudocopulating on *C. erecta* flowers and seven males on *C. subulata* flowers. These captured males were transported live to the laboratory. There was no detectable morphological difference between the two groups of males visiting the two different orchid species. Despite differences in the floral morphology of the two orchid species, the pollinators of both removed pollen on the 5th–7th abdominal tergits. Nonetheless, for the GC-EAD analyses the males were used in tests only involving the orchids species on which they were captured.

Gas chromatography and electrophysiology (GC-EAD)

GC-EAD experiments were performed according to Schiestl *et al*. (2000) and Schiestl & Ayasse (2000). One microlitre of each odour sample was injected splitless at 50°C (1 min) into a gas chromatograph (HP 6890) followed by opening the split valve and programming to 310°C (DB-5) or 230°C (DB-FFAP) at a rate of 10°C/min. The GC was equipped with a DB-5 column (30 m × 0.25 mm) or a DB-FFAP column (30 m × 0.32 mm); helium was used as carrier gas. A GC effluent splitter (press-fit-connection; split ratio 1 : 1) was used and the outlet was placed in a purified and humidified airstream. This air was directed over a male wasp's antenna prepared as follows: the tip of the excised antenna was cut off and the antenna mounted between two glass electrodes filled with insect Ringer's solution. The electrode holding the base of the antenna was connected to grounded Ag–AgCl wire. The distal end of the antenna was connected in the same way via an interface box to a signal acquisition interface board (IDAC; Syntech, Hilversum) for signal transfer to a PC. EAD signals and flame ionization detector (FID) responses were simultaneously recorded.

RESULTS

Collection of floral odour

Headspace sorption sampling, a method by which volatile compounds are sampled from the air surrounding the orchid flower, did not contain detectable amounts of any compounds for either orchid species, indicating volatile components of the odour are emitted in very small amounts. By contrast, extracts from labella contained many compounds in considerable quantities (Fig. 1). Inspection of the same portion of a GC trace for both species of orchid (Fig. 1) clearly shows that there was considerable difference in the composition of the respective odours, particularly for compounds with GC retention times in excess of 15 min. These differences in the GC traces reflect both the composition of

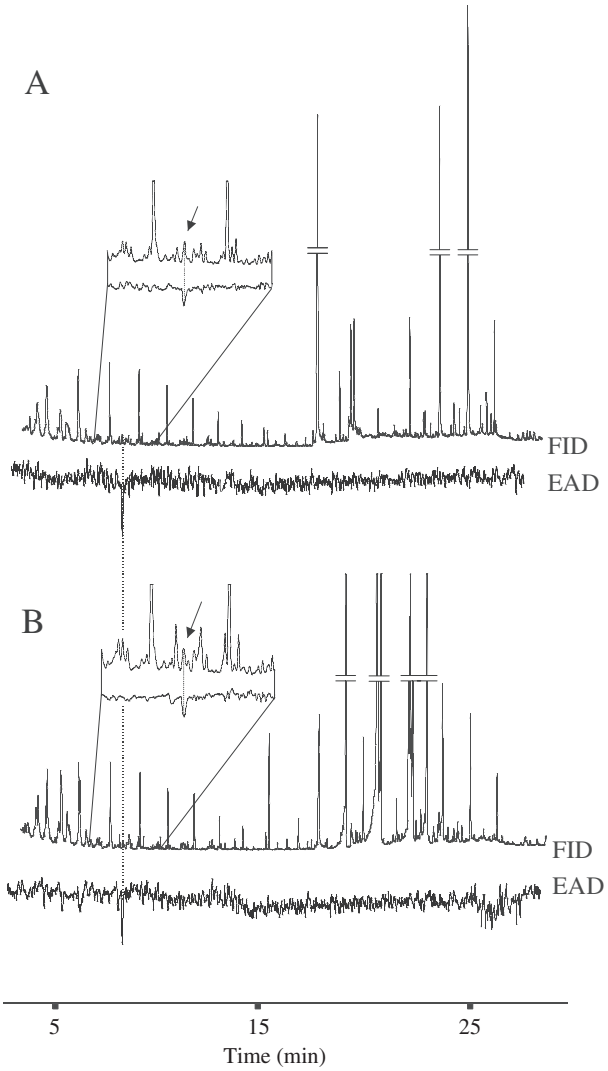

Figure 1. Gas chromatographic analysis with flame ionization detector (FID) of (A) *Cryptostylis erecta* labella extract (pool of three labella) and (B) *C. subulata* labellum extract on a non-polar DB-5 column. Reactions from a male *Lissopimpla excelsa* antenna (electroantennographic detection, EAD) were recorded simultaneously. One peak (arrow) with identical retention time in both samples elicits an EAD reaction in male antennae.

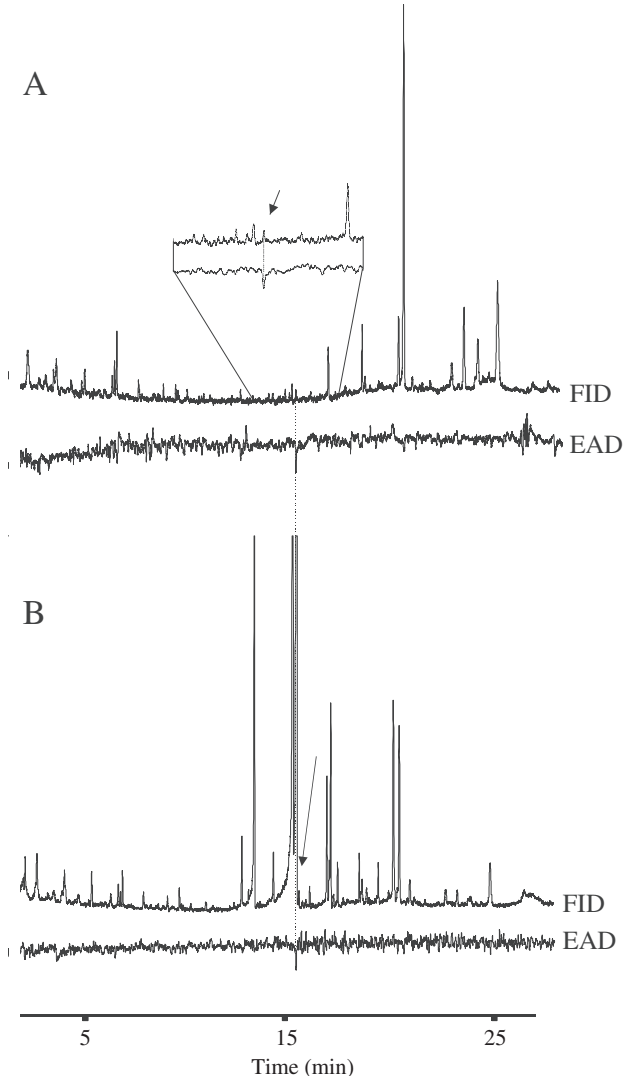

Figure 2. Gas chromatographic analysis with flame ionization detector (FID) of (A) *Cryptostylis erecta* labella extract (pool of three labella) and (B) *C. subulata* labellum extract on a polar DB-FFAP column. Reactions from a male *Lissopimpla excelsa* antenna (electroantennographic detection, EAD) were recorded simultaneously. One peak (arrow) with identical retention time in both samples elicits an EAD reaction in male antennae.

the compounds and the quantities of shared compounds.

Electrophysiologically active compounds

Although the odour samples of both *Cryptostylis* species contained numerous compounds, in all GC-EAD analyses using *Lissopimpla excelsa* antennae, only one minor peak elicited an EAD reaction (Figs 1, 2). This peak had exactly the same retention time in both *Cryptostylis* species. Although it is possible that two different active compounds may elute at the same time (i.e. have identical retention times), this is highly unlikely to be the case with two different GC columns. Two compounds that co-elute on one column are very likely to separate on a different column. In analyses on a non-polar DB5 column, the peak had a retention time of 8.8 min (Fig. 1); on a polar DB-FFAP column the retention time was 15.7 min (Fig. 2). Given the identical retention times on two different types of GC column for both orchid species, we conclude that it is

highly likely that the same single compound is electrophysiologically active in both orchids.

DISCUSSION

Although fragrances are well established as the primary stimuli for attracting and eliciting pseudocopulatory behaviour in the pollinators of sexually deceptive orchids (Coleman, 1928a; Kullenberg, 1961; Stoutamire, 1983; Peakall, 1990; Schiestl *et al*., 1999), we have only recently begun to discover how many and which of the many odour compounds produced by the flower are involved in sexual attraction. In this study we have shown that volatile odour compounds do not occur in detectable amounts in headspace samples of *Cryptostylis*. This suggests that *Cryptostylis* does not produce typical floral odour compounds in large amounts, consistent with the observation that the flowers have no detectable scent to humans (Coleman, 1928a).

Odour composition of *C. erecta* and *C. subulata*

Headspace samples of both orchids did not trap any compounds from the flowers; floral extracts, which contain odour compounds of low volatility, contained numerous substances. Comparisons of the GC traces from the floral extracts, however, revealed considerable differences between *C. erecta* and *C. subulata* (Figs 1, 2). Thus, the floral odour bouquets of these species are different, consistent with their marked differences in floral morphology.

Electrophysiologically active compounds in *C. erecta* and *C. subulata*

In contrast to the diversity of compounds found in floral extracts, our GC-EAD analysis revealed that male wasp pollinators are electrophysiologically responsive to the same single compound in both *C. erecta* and *C. subulata*. Although GC-EAD does not prove the behavioural activity of an odour compound, this relationship has been established in all other investigations with sexually deceptive orchids (Schiestl *et al*., 1999; Schiestl & Ayasse, 2000; Ayasse *et al*., 2003; Schiestl *et al.*, 2003). However, we are now in the process of identifying and synthesizing this compound, which we predict will also match the sex pheromone of the wasp pollinators of *Cryptostylis*. Once synthesized, the compound will be used in bioassays to confirm its biologically activity in both *C. erecta* and *C. subulata*.

These GC-EAD results support our own pollinator visitation observations and previous studies (Coleman, 1928a, 1930b, 1938; Stoutamire, 1975) that report a single ichneumonid species pollinating *Cryptostylis*. We further anticipate that this same single compound will be found in the remaining *Cryptostylis* species.

Chemical communication among sexually deceptive orchids

The presence of a single compound as the sexual attractant in *Cryptostylis* accords with similar recent findings in Australian orchids, but contrasts with the European *Ophrys.* Recent GC-EAD results suggest chemical mimicry in Australian species is achieved with only one to three active compounds. In the thynnine-pollinated *Chiloglottis trapeziformis* Fitzg, *C. valida* D.L.Jones and *C. seminuda* D.L.Jones one compound is involved, whereas in *C. trilabra* Fitzg, *C. reflexa* (Labill.) Druce, *Arthrochilus huntianus* (F.Muell.) Blaxell, two or three active compounds have been found (Mant *et al*., 2002; Schiestl *et al.*, 2003). In *C. trapeziformis*, a single active compound has been identified and its behavioural activity verified by field trials with the synthesized compound. It has further been confirmed that the pollinator of *C. trapeziformis*, the thynnine wasp *Neozeleboria cryptoides* Smith, uses the same single compound as its sex pheromone (Schiestl *et al.*, 2003). By contrast, a blend of 14 electrophysiologically active compounds has been identified in *Ophrys sphegodes* Mill. These compounds also attracted the pollinator, the solitary bee *Andrena nigroaenea* Kirby, and released mating behaviour (Schiestl *et al*., 1999, 2000). The same compounds, in similar relative amounts, constitute the sex pheromone of the pollinator.

Evolution of *Cryptostylis* pollination

Despite the use of the same pollinator in the sympatric *Cryptstylis erecta* and *C. subulata*, natural hybrids are unknown (Coleman, 1929a, 1930a; Stoutamire, 1975; Jones, 1988). Even in early studies the lack of hybrids was perplexing, with Coleman (1929a) noting that both orchid species can simultaneously flower and set fruit within metres of each other, yet hybrids are absent. Pollination success can exceed 80% per plant. A survey of *C. subulata* at two sites by RP showed a mean of $85 \pm 0.2\%$ (mean ± SD, n plants = 55) plants set one or more fruits, with mean fruit set per flower of $27.2 \pm 0.09\%$ (mean ± SD, n flowers = 561). If similar rates of pollination occur in sympatric populations, the likelihood of hybridization appears high. Given high pollinator availability and no evidence for mechanical isolation, it seems likely that reproductive isolation among *Cryptostylis* species is achieved by some form of genetic incompatibility. Stoutamire (1975) reported that artificial crosses between species fail to set 'mature embryos'. In a more detailed study, Lloyd

(2003) found that artificial crosses between *C. erecta* and *C. subulata* produce fruits despite slower pollen tube growth than occurs within species. Seed viability remains untested.

Although both *C. erecta* and *C. subulata* share pollinators and a single active compound for pollinator attraction, they differ greatly in morphology and non-volatile floral chemistry. Molecular phylogenetic studies indicate the two species are genetically dissimilar, at least in comparison with species within thynnine-pollinated genera (Kores *et al*., 2001; Mant *et al*., 2002). The other Australian species of *Cryptostylis* exhibit a similar morphological disparity. These observations suggest allopatric divergence predominated in *Cryptostylis*, but with conservation in floral chemistry, such that when secondary contact occurred, retention of the same pollinator species was possible. Whether barriers to gene-flow are due in response to a build up of genetic divergence in allopatry or relate to an incompatibility mechanism, or both, awaits further research.

The contrast with other sexually deceptive diurids is striking, where ethological isolation owing to highly specific pollinators is typically the sole barrier to gene flow among interfertile close relatives (Bower, 1996). Although there are rare suggestions of hybrids forming among thynnine-pollinated species, only one has been examined in detail. *Chiloglottis* x *pescottiana* R.S.Rogers has been confirmed by morphometric and genetic analysis to be a natural hybrid between *C. trapeziformis* and *C. valida* (Peakall *et al*., 1997). However, whereas viable F_1 hybrid seed is produced by artificial pollination, genetic factors limit F_2 and back-cross formation such that only F_1 hybrids are found in the wild (Peakall *et al*., 1997). Hybridization between *C. valida* and *C. trapeziformis* is possible because both can share the pollinator of the other when they come together on the extreme edges of their range (Peakall *et al*., 1997, 2002). GC-EAD analysis shows both orchid species produce the same single compound that has been confirmed as the sex pheromone of *Neozeleboria cryptoides*, the usual pollinator of *C. trapeziformis* (Schiestl *et al.*, 2003). However, as the two parent species are found in different clades within the genus (Mant *et al*., 2002), it is likely that the post-zygotic barriers evolved in isolation, but either conservative or convergent evolution in floral scent chemistry prevents prezygotic isolation on secondary contact.

These comparisons among sexually deceptive orchid lineages suggest that pollinator specialization via sexual mimicry can lead to quite different evolutionary outcomes. In sexually deceptive orchids, such as the thynnine-pollinated *Chiloglottis* and *Caladenia*, pollinator specialization has been linked to adaptive radiations to different pollinators, mediated probably by minor changes in floral chemistry (Mant *et al*., 2002). In the case of *Chiloglottis*, this radiation has occurred conservatively, such that related orchids tend to use related thynnines as pollinators. In the European *Ophrys*, a similar radiation is evident while pollinator specificity is maintained through differences in the relative amounts of a few components of a complex floral odour blend (Schiestl & Ayasse, 2002; Soliva, Kocyan & Widmer, 2001). In these orchids, the evolution of reproductive isolation may occur rapidly without major divergence in morphology. By contrast, pollinator specialization in *Cryptostylis* has not been accompanied by pollinator diversification. Instead, floral morphological divergence has occurred in parallel with conservation of the floral chemistry used to attract the same pollinator.

Much remains to be learned about the evolution of pollination by sexual deception, both in *Cryptostylis* and more generally. Understanding chemical communication in sexually deceptive species is undoubtedly one key to unlocking the secrets of this intriguing pollination system, particularly when combined with ecological, genetic and phylogenetic investigations.

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