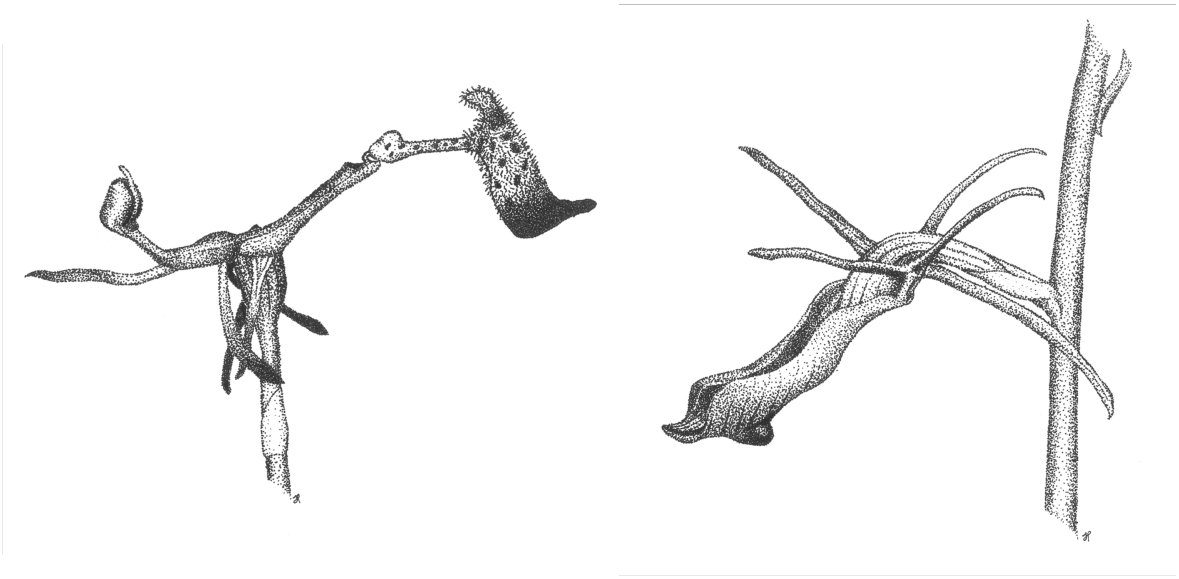
Pollination ecology of Australian sexually deceptive orchids with contrasting patterns of pollinator exploitation



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DECLARATION

The research presented in this thesis is my own original work. This thesis contains published work and work prepared for publication that has been co-authored with collaborating researchers. As such, these Chapters can be read independently and some duplication of concepts may arise. The author contributions are declared at the start of each Chapter. No part of this thesis has been submitted for any previous degree.

Alyssa M. Weinstein, February 2020

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ABSTRACT

Sexual deception, entailing the pollination of flowers through chemical and/or morphological mimicry of female insects, is one of the most remarkable pollination strategies to have evolved. This thesis explores two Australian sexually deceptive orchid systems with contrasting patterns of pollinator exploitation.

The first three chapters focus on the orchid genus *Cryptostylis*, a system with a unique case of pollinator sharing - five Australian species, four of which are largely sympatric, all deceive the same male ichneumonid wasp pollinator *Lissopimpla excelsa*. In Chapter One (published in *Biological Journal of the Linnaean Society*), mark-recapture experiments were used to investigate the consequences of ichneumonid pollination on pollen movement in *C. ovata*. A high pollinator revisitation rate indicated some potential for self-pollination. In Chapter Two, reproductive barriers contributing to the absence of hybrids between *Cryptostylis* species were investigated. Pre-pollination barriers, assessed in field experiments, did not prevent hybridisation. Hand cross-pollinations conducted among the four common *Cryptostylis* species in a greenhouse all produced fruits, however seed mass and the percentage of formed embryos were reduced in hybrids. Major differences in ploidy and chromosome number likely explain this post-pollination fitness reduction. Two species of *Cryptostylis* were found to be self-incompatible, marking the first case of self-incompatibility in the Diurideae. The unique reproductive biology of Australian *Cryptostylis*, encompassing pollinator sharing, self-incompatibility, and post-pollination reproductive isolation driven by large ploidy differences, may indicate that its mode of diversification may differ greatly to those in other sexually deceptive genera. Chapter Three presents the first phylogeny to encompass both Australian and Asiatic *Cryptostylis*. An Australian origin of *Cryptostylis* is supported, with a likely single subsequent dispersal event to Asia. Ploidy variation and geographic barriers appear to have played a role in diversification across *Cryptostylis*.

In Chapter Four, the potential presence of pollination ecotypes in the sexually deceptive *Drakaea livida* was tested for. Patterns of chemical diversity and pollinator availability across the distribution of the species were investigated. Pollinator choice trials revealed the presence of three discrete ecotypes each attracting its own pollinator species. Patterns of pollinator availability did not correlate with ecotype distribution. Each ecotype possessed a significantly different floral volatile composition. Using Partial Least Squares Discriminant Analysis

(PLS-DA), the presence-absence of a subset of taxonomically informative compounds could be used to accurately predict the ecotype of a flower. Different classes of electrophysiologically active compounds were present in different ecotypes. These marked differences in chemical composition between the ecotypes suggest either a long time since their divergence and may hint at a scenario of convergent evolution of floral morphology. In Chapter Five, the ecotype geographic ranges and methods of identifying the ecotypes were investigated. Species distribution modelling predicted each ecotype to have a different core range. Two ecotypes were widespread, while one had a limited distribution within extensively cleared agricultural land, raising conservation concerns. PLS-DA correctly identified the ecotype of a flower when labella extracts were made from pollinated flowers, thereby providing a non-destructive identification technique. The pollinator specificity, morphology, floral chemistry, and ranges of the ecotypes supported them as Evolutionary Significant Units.

In conclusion, the ecological and evolutionary consequences of pollination by sexual deception may vary extensively between plant taxa in accordance with their different patterns of pollinator exploitation. The taxonomy, species richness of the pollinator group, and the plant species to pollinator species ratio all influence the evolution and diversification of sexually deceptive orchids.

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GENERAL INTRODUCTION

Angiosperms are the most diverse and species rich group of terrestrial plants, comprising over 300,000 species and representing approximately 90% of terrestrial plant diversity (Vamosi et al., 2018; Hernández-Hernández & Wiens, 2020). Their amazing diversity has shaped ecosystems and may have even promoted diversification in other plant lineages (Magallón & Castillo, 2009), for example in ferns through the provision of a complex habitat (Schneider et al., 2004). Among the vascular plant clades, the angiosperms have the most recent origin, making patterns of diversification easier to infer than in older lineages (Silvestro et al., 2015; Vamosi et al., 2018). Angiosperm diversification has been influenced by an interaction of intrinsic morphological characters and extrinsic environmental conditions (Vamosi & Vamosi, 2011). Such a interaction is evident in biotic pollination, where specific floral traits interact with pollinator availability to determine the pollinators employed and their mode of attraction (Olesen & Jordano, 2002; Sargent & Otto, 2006; Vamosi & Vamosi, 2011). Biotic pollination is hypothesised to have played a major role in driving the diversification of the angiosperms, (Stebbins, 1970; Grant, 1994; Johnson, 2006; van der Niet & Johnson, 2012; Hernández-Hernández & Wiens, 2020), particularly in cases of specialised pollination where only one or few pollinator species are involved (Kiester et al., 1984; Kay & Sargent, 2009).

Lineages that have zygomorphic (bilaterally symmetrical) flowers are hypothesised to be more likely to evolve specialised floral morphologies, and thereby more likely to evolve specialised pollination systems (Johnson & Edwards, 2000; Sargent, 2004; Hernández-Hernández & Wiens, 2020). An example of this process is suggested in the Orchidaceae - the plant family with the highest incidence of specialised pollination systems (Tremblay, 1992; Schiestl & Schlüter, 2009). Having a bilaterally symmetrical flower can restrict the movement of the pollinator on the flower, and direct its approach, thereby allowing precise placement of pollen on its body (Sargent, 2004). In orchids, the unique orchid morphology of the column and pollinia further enable precise pollinator positioning. The column is comprised of a fused style and stamens, which support the pollen filled pollinia. Pollen being presented in pollinia (pollen grains bound together into a single mass), allows a large amount of pollen to be transferred from pollinator to flower in a single visit, thus lowering the number of pollinator visits required for successful pollination (Johnson & Edwards, 2000). Precise pollinator positioning, with efficient pollen transfer mechanisms, can lead to

pollinator specificity and thereby to reproductive isolation and speciation in orchids (Sargent, 2004; Pauw, 2006; Waterman et al., 2011).

Orchids account for approximately 8% of the vascular plant flora, occurring on all continents with the exception of Antarctica (Pridgeon, 1999-2014; Givnish et al., 2016). Orchid distribution is potentially limited by that of their mycorrhizal symbionts (McCormick et al., 2018), on which they are reliant for nutrient provision during germination and protocorm formation (McCormick et al., 2012). Several mechanisms have contributed to the extreme diversity of the Orchidaceae; evolution of pollinia, epiphytism, CAM photosynthesis, a tropical distribution encompassing several cordilleras, the use of Lepidoptera and euglossine bees as pollinators, and deceptive pollination strategies (Givnish et al., 2015). Approximately one third of orchids achieve pollination through deceit and do not offer pollinators a nectar reward (Ackerman, 1986; Tremblay et al., 2005). The mechanisms of deception are as intriguing as they are varied, encompassing food deception, brood-site mimicry, shelter imitation, pseudoantagonism, rendezvous attraction, and perhaps most remarkably, sexual deception (Jersáková et al., 2006).

Pollination by sexual deception occurs in several hundred orchid species across Europe, Australia, southern Africa, and South America (Gaskett, 2011; Xu et al., 2012; Bohman et al., 2016). Almost 50% of known sexually deceptive orchid species occur in Australia, with this pollination strategy having independently evolved at least six times (Kores et al., 2001; Gaskett, 2011). Sexually deceptive orchids achieve pollination by visually and chemically mimicking female insects (Schiestl et al., 2000; Gaskett & Herberstein, 2010; Ayasse et al., 2011). Sexual behaviour is induced in pollinators that brings them into contact with the orchids' reproductive structures (Correvon & Pouyanne, 1916; Coleman, 1927; Schiestl et al., 2003; Mant et al., 2005). At long range, pollinators are attracted to flowers by chemical mimicry of female sex pheromones using floral semiochemicals (Kullenberg, 1961; Schiestl et al., 1999; Ayasse et al., 2011; Bohman et al., 2014; Bohman et al., 2016). The unique floral morphology of orchids has also enabled mimicry of pollinator shape. While orchids possess the three petals and three sepals typical of a monocotyledon, the third petal is usually highly modified and forms a lip-like insectiform labellum, which is also typically the site of semiochemical release (Schiestl et al., 2000; Schiestl et al., 2003; Peakall et al., 2010; Falara et al., 2013; Phillips et al., 2013; Bohman et al., 2014; Phillips et al., 2014b).

Typically, sexually deceptive orchids have a high degree of pollinator specificity, with each orchid species usually attracting its own unique pollinator (Paulus & Gack, 1990; Peakall et al., 2010; Phillips et al., 2017), though exceptions of pollinator sharing are noted (Cortis et al., 2008; Gögler et al., 2009; Phillips et al., 2013; Bohman et al., 2017; Phillips et al., 2017). This use of different pollinator species is a common barrier to hybridisation and introgression in sexually deceptive orchids (Xu et al., 2011; Xu et al., 2012; Whitehead & Peakall, 2014).

While gnats (Blanco & Barboza, 2005; Phillips et al., 2014b), bee-flies (Ellis and Johnson, 2010), beetles (Tyteca et al., 2006), and ants (Peakall et al., 1987) can function as sexually deceived pollinators, the majority of known sexually deceptive orchid pollinators are solitary haplodiploid bees and wasps (Gaskett, 2011). Haploploidy may provide a level of resilience to exploitation by orchids in that if females do not mate they can still produce male offspring (King, 1987; Hardy, 1994; Gaskett et al., 2008). Therefore, a scenario where female mating is inhibited by orchid exploitation of available males could result in the production of even more naïve males that could pollinate orchids in subsequent flowering seasons (Gaskett et al., 2008). As long as some male-female pollinator mating still occurred allowing pollinator persistence, this mechanism may potentially lead to improved orchid pollination rates over evolutionary time (Gaskett et al., 2008). Pollinator mating is likely to occur despite the presence of orchids given that males may learn to avoid dense patches of orchids (Wong & Schiestl, 2002), females themselves may move out of orchid patches (Wong & Schiestl, 2002), and pollinator flight distances typically exceed the size of orchid colonies (Weinstein et al., 2016).

For successful pollination, most sexually deceptive flowers only require the pollinator to exhibit pre-copulatory behaviour, and not actual copulation (Gaskett, 2011). In Australian sexually deceptive genera a large portion of wasps approach the flowers in their characteristic zig-zag flight pattern but do not alight on the flower, and of those that do alight an even smaller percentage attempt to copulate with and/or fly away with the labellum (Peakall, 1990; Bower, 2006; Phillips et al., 2009; Phillips et al., 2013; Weinstein et al., 2016). This observed difference in pollinator response strength is likely due to variation in the sensitivity of insect sensory perceptions, as well as variation in the attractiveness of each orchid (Peakall, 1990). Only in rare cases, such as that of Australian *Cryptostylis*, is the pollinator known to actually ejaculate onto the flower (Gaskett et al., 2008).

This thesis explores two Australian sexually deceptive orchid systems with contrasting patterns of pollinator exploitation, with a view to providing insights relevant both to evolution and conservation. The first three chapters focus on Australian *Cryptostylis*. *Cryptostylis* has an atypical pattern of pollinator exploitation, being renowned for its unusual pollinator sharing - with five species all sexually deceiving the same ichneumonid pollinator *Lissopimpla excelsa*. *Cryptostylis* is further unique in being the only known ichneumonid-pollinated sexually deceptive orchid genus, with the majority of Australian sexually deceptive orchids being pollinated by thynnine wasps. In Chapter One (published in *The Biological Journal of the Linnean Society*) pollinator behaviour and movement are examined to investigate the consequences of pollination by sexual deception of ichneumonids. In Chapter Two, an assessment of the reproductive barriers contributing to the absence of hybrids between *Cryptostylis* species, in spite of their shared pollinator and sympatry, is conducted. In Chapter Three, the first phylogeny including both Australian and Asiatic *Cryptostylis* species is presented to explore the evolutionary history of this unique genus.

Chapters Four and Five focus on the south-west Australian *Drakaea livida*, the Warty Hammer Orchid, and its pollinators. This system was selected as its pattern of pollinator exploitation deviates from the base expectation for sexually deceptive orchids of a one-to-one plant pollinator relationship: the presence of multiple pollinator species in *D. livida* has been reported (Bohman et al., 2012a; Bohman et al., 2012b; Phillips et al., 2014a; Phillips et al., 2017). In Chapter Four, the presence of pollination ecotypes in *D. livida* is tested for. Patterns of pollinator response and availability, and floral chemical variation across the range of *D. livida*, are investigated. In Chapter Five, the conservation of the newly discovered *D. livida* ecotype is addressed. Species distribution modelling, floral morphometrics, chemotaxonomy, and genome size estimation are employed to define conservation units and determine effective methods of identifying them.

One appendix is presented - "2-(Tetrahydrofuran-2-yl)acetic Acid and Ester Derivatives as Long- Range Pollinator Attractants in the Sexually Deceptive Orchid *Cryptostylis ovata*", published in *Journal of Natural Products*. This paper describes the elucidation of an attractant semiochemical present in *Cryptostylis ovata* that attracts its *Lissopimpla excelsa* pollinator.

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CHAPTER ONE

Behaviour of sexually deceived ichneumonid wasps and its implications for pollination in *Cryptostylis* (Orchidaceae)

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Behaviour of sexually deceived ichneumonid wasps and its implications for pollination in *Cryptostylis* (Orchidaceae)

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Pollination via sexual deception is hypothesised to be associated with more frequent outcrossing and greater pollen dispersal distances than strategies involving food-foraging behaviour. In this study, we investigated the behaviour and movement distances of *Lissopimpla excelsa* (Hymenoptera: Ichneumonidae), and their implications for the pollination of the sexually deceptive *Cryptostylis ovata* (Orchidaceae). Pollinator observations revealed that while *L. excelsa* will alight on multiple flowers within a single visit to a patch of orchids, the frequency of attempted copulation decreases with successive visits, suggesting that pollinator learning may inhibit within-patch pollen transfer. Mark-recapture demonstrated that 25% of wasps revisited inflorescences within a day and 50% revisited within a week. Despite the apparent site fidelity of some individuals, *L. excelsa* often move over large distances (maximum = 625 m), and are capable of dispersing pollen between patches. To resolve the consequences of pollination by sexual deception of ichneumonids, we compared our results with those from studies of other sexually deceptive systems. While pollination rates were comparable with other sexually deceptive orchids, *L. excelsa* showed high rates of column contact and moved over large distances relative to other sexually deceived pollinators. Among sexually deceptive orchids in general, the frequency of column contact was not correlated either with the frequency of pseudocopulation or with pollination rate. These results suggest that the consequences of pollination by sexual deception may vary extensively between plant taxa due to variation in floral traits, and behavioural differences between pollinator groups. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **119**, 283–298.

KEYWORDS: *Cryptostylis ovata* – *Lissopimpla excelsa* – mark-recapture – pollen dispersal – pollination – pollinator learning – sexual deception.

INTRODUCTION

Deceived pollinators typically avoid repeat visits to rewardless plants, potentially leading to higher rates of outcrossing and greater offspring fitness (Dressler, 1981; Peakall & Beattie, 1996; Johnson & Nilsson, 1999; Jersáková, Johnson & Kindlmann, 2006; Ellis & Johnson, 2010). In sexually deceptive orchids, which effect pollination through floral mimicry of female insects (Coleman, 1927b; Kullenberg, 1961; Stoutamire, 1974; Schiestl *et al.*, 1999; Gaskett & Herberstein, 2010), outcrossing and pollen dispersal may be greater because the insects are searching for mates rather than moving between nearby plants while foraging (Peakall & Beattie, 1996; Peakall & Schiestl, 2004). These predictions have received some empirical support, with high outcrossing rates reported for the thynnine-pollinated

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sexually deceptive orchids *Caladenia tentaculata* (Peakall & Beattie, 1996), *Chiloglottis valida*, and *Chiloglottis* aff. *jeansii* (Whitehead, Linde & Peakall, 2015). In contrast, in the ant-pollinated *Leporella fimbriata*, outcrossing is low due to both extensive pollen transfer within large clonal patches and occasional revisitation to flowers (Peakall & James, 1989).

In sexually deceptive orchids the primary trait involved in pollinator attraction is chemical mimicry of the sex pheromones of female insects (Kullenberg, 1961; Schiestl *et al.*, 1999, 2003; Bohman *et al.*, 2012, 2014). Due to the high specificity of insect mating signals, sexually deceptive systems typically involve specialised plant–pollinator relationships, in which each orchid species has one or few pollinator species (Paulus & Gack, 1990; Phillips *et al.*, 2009, 2014a; Peakall *et al.*, 2010; Gaskett, 2011). The reproductive success and offspring fitness of a sexually deceptive orchid will therefore be strongly dependent upon its efficacy at eliciting pollinator sexual attraction, and upon the mate search behaviour and home range of its specific pollinator species. Surprisingly, some elements of the behaviour of sexually deceived pollinators remain poorly studied, despite their importance for plant fitness. For example, detailed studies of the extent and temporal period of pollinator revisitation are mostly lacking (though see Whitehead & Peakall, 2013). Further, most detailed studies of the behaviour of sexually deceived pollinators have focused on thynnine wasps (e.g. Peakall (1990), Peakall & Beattie (1996), Alcock (2000), Menz *et al.* (2013), Whitehead & Peakall (2013), De Jager & Peakall (2015) but see Peakall (1989), Peakall & Schiestl (2004), De Jager & Ellis (2014)).

A diversity of families of solitary wasps, bees and flies is known to be involved in pollination by sexual deception (Paulus & Gack, 1990; Ellis & Johnson, 2010; Gaskett, 2011; Phillips *et al.*, 2014b). The contrasting life histories of these groups may have different consequences for pollen movement and reproductive success. In an unusual case of pollinator sharing, the ichneumonid wasp *Lissopimpla excelsa* Costa pollinates all five Australian species of *Cryptostylis*, and is the only member of the Ichneumonidae known to visit sexually deceptive flowers (Coleman, 1927a, b, 1929, 1930a, b, 1938; Nicholls, 1938). The *Cryptostylis–L. excelsa* system is further unusual in that it is one of only two documented cases in which the sexual attraction of deceived pollinators to flowers can lead to pollinator ejaculation (Coleman, 1931; Erickson, 1951; Blanco & Barboza, 2005; Gaskett, Winnick & Herberstein, 2008). While it is likely that floral odour plays a role in pollinator attraction to *Cryptostylis* flowers (Coleman, 1930a; Schiestl, Peakall & Mant, 2004), recent evidence suggests that colour mimicry but not shape mimicry may also be important for pollinator sexual attraction or detection of the flower upon approach (Gaskett & Herberstein, 2010; Gaskett, 2012).

At present, the courtship and mate searching behaviour of *L. excelsa* and its consequences for the pollination of *Cryptostylis* remains poorly known. In the majority of studied ichneumonid species, male courtship behaviour involves antennal vibration and wing fanning displays followed by anntenation with females, with subsequent copulation if the male is accepted (Fisher, 1959; Juillet, 1959; Kugler & Wollberg, 1967; Spradbery, 1969; Cole, 1970; Morey, 1971; Vinson, 1972; Slobodchikoff, 1973; Dowell & Horn, 1975; Barrows, 1976; Gordh & Hendrickson, 1976; Crankshaw & Matthews, 1981; Rotheray, 1981; van Veen, 1982; Dyer & Landis, 1997). If rejected by the female, males often continue to exhibit courtship behaviour (Spradbery, 1969; Gordh & Hendrickson, 1976; Crankshaw & Matthews, 1981; Rotheray, 1981; van Veen, 1982). There is strong experimental evidence that male ichneumonids are attracted via a sex pheromone (Heatwole, Davis & Wenner, 1964; Cole, 1970; Vinson, 1972; Rotheray, 1981; Jewett & Carpenter, 1999). However, while antennal glands have been suggested to play a role in courtship via transfer of a contact pheromone (Bin *et al.*, 1999; Kolarov & Gürbüz, 2007; Klopfstein, Quicke & Kropf, 2010; Steiner *et al.*, 2010), the sex pheromone has yet to be resolved for any ichneumonid. There have been few studies investigating ichneumonid mate searching behaviour, with movement distances reported for just five species (Juillet, 1959; Heatwole & Davis, 1965), ranging from a maximum of 37 m for *Eulimneria rufifemur* (Juillet, 1959), to a maximum of 107 m for *Ephialtes ruficollis* (Juillet, 1959).

The present study aimed to investigate the behaviour and spatial movement patterns of *Lissopimpla excelsa*, and their implications for the pollination of *Cryptostylis ovata* R.Br. The behaviour of *L. excelsa* was investigated at multiple spatial scales relevant to pollen movement: on a *C. ovata* inflorescence, within *C. ovata* patches, and between *C. ovata* patches. A combination of observations of artificially presented *C. ovata* patches, and mark-recapture techniques were implemented. Further, we aimed to investigate the consequences of ichneumonid pollination by comparing pollinator movement distances, pollinator sexual attraction to a flower, and pollination rate between the *Cryptostylis* system and other sexually deceptive systems in the literature.

METHODS

Study system

The genus *Cryptostylis* is comprised of 25 species, found through south-east Asia, Australia, and New Guinea, and extending to New Zealand (Pridgeon, Cribb & Chase, 2001). Five species are present in Australia, with *Cryptostylis ovata* R.Br. occurring exclusively in southwestern Australia (Brown *et al.*, 2008), and *C. erecta*, *C. hunteriana*, *C. leptochila*, and *C. subulata* occurring in eastern Australia, sometimes in sympatry (Gaskett, 2012). *Cryptostylis ovata* flowers between November and March, and relies on reserves of moisture in its tubers during this period (Pate & Dixon, 1982). This long flowering period is due to the sequential opening of flowers along the raceme (Pridgeon *et al.*, 2001). Racemes typically have 10–15 flowers (maximum observed = 24), one to three of which are usually open at any given time. Plants typically form clonal patches (Dodd *et al.*, 1984) that in some cases can have over 100 leaves and support more than 10 racemes, although they also occur as isolated individuals (single leaf).

Cryptostylis ovata is pollinated solely by the ichneumonid wasp *Lissopimpla excelsa* Costa (Coleman, 1929, 1930b). Visiting wasps land on the resupinate labellum and reverse into the flower until their abdomen comes into contact with the column (Fig. 1). This reversing behaviour is essential for pollination – removal of pollinia is effected as the dorsal side of the apex of the abdomen contacts the viscidium (Van der Pijl & Dodson, 1966). Once positioned, the wasp forms a falcate curve with its abdomen and begins copulatory probing (Coleman, 1927b), although this behaviour is not required to effect pollination. The typical ichneumonid courtship behaviours of wing and antennal movement have not been observed in *L. excelsa* individuals on flowers. In some species of *Cryptostylis*, including *C. ovata*, male *L. excelsa* have been observed to ejaculate onto the flowers (Coleman, 1928; Erickson, 1951; Gaskett *et al.*, 2008). Artificial self-pollination of *C. ovata* usually results in seed set (A. Weinstein & R. Phillips, unpubl. data).

Figure 1. A male *Lissopimpla excelsa* on a flower of *Cryptostylis ovata*. The insect has reversed into the flower, bringing pollinia from another flower into contact with the stigma.

Floral presentations

Lissopimpla excelsa males were attracted using the baiting method developed by Stoutamire (1983) and Peakall (1990), in which the experimental presentation of picked flowers at a new position within the landscape leads to the rapid attraction of their pollinators. Similar to the behaviour observed for thynnine species (Peakall, 1990; Peakall & Beattie, 1996; Alcock, 2000; Peakall *et al.*, 2010; Whitehead & Peakall, 2013), the greatest number of responses of *L. excelsa* to presented flowers occurs within the first few minutes of the presentation (Tomlinson & Phillips, 2012). Flowers used for baiting were collected from populations in Capel (33°35′29.69″S, 115°32′31.77″E) and Margaret River (33°58′2.21″S, 115°0′58.37″E), southwest Western Australia. All experiments were undertaken in a site approximately 400 × 700 m within Kings Park and Botanic Garden (31°57′48.92″S, 115°50′18.21″E), an urban bushland remnant in Perth, Western Australia. *Lissopimpla excelsa* occurs in abundance at this site, while *C. ovata* is absent. Experiments were conducted during December 2011–January 2012, and December 2013–February 2014. All experiments were conducted between 06:00 and 12:00 h to coincide with the period of maximum wasp activity (Tomlinson & Phillips, 2012).

Pollinator behaviour: on an inflorescence and within a patch

To quantify pollinator behaviour on an inflorescence, and pollinator movement between inflorescences within a patch, an artificial patch was created using inflorescences with a minimum of two open flowers. Following the method of Peakall & Beattie (1996), an inflorescence was placed on each corner of a 1 × 1 m quadrant, and pollinator behaviour within this artificial patch was recorded by two observers. Observations were conducted for 10-min periods, after which the patch was relocated a minimum distance of 20 m to achieve a renewed pollinator response (Bower, 1996; Tomlinson & Phillips, 2012). For each wasp that approached flowers in the patch it was recorded whenever the wasp: (1) landed on an inflorescence, (2) contacted the column, or (3) attempted copulation

with a flower. Due to the large number of responding wasps, it was not possible to examine flowers for ejaculate as confirmation of attempted copulation, because we did not have sufficient flowers to introduce a fresh flower after every wasp. Behaviour was recorded from initial approach through multiple floral visitations until the individual left the patch, noting which inflorescence(s) the individual landed on. To compare the proportion of wasps alighting, contacting the column, and copulating with the flowers over multiple visitations, *G*-tests were conducted in GenAlEx v6.5 (Peakall & Smouse, 2006, 2012).

Pollinator revisitation: within a day and within a 7-day period

To determine the rate of pollinator revisitation to an inflorescence within a day and within a week, two mark-recapture experiments, that each comprised of 4 days of captures, were conducted during the week 27 December 2013–2 January 2014, and 14–20 January 2014. Every second day during the week, an inflorescence was presented at the exact same location from 06:00 until 10:30 h. All wasps observed contacting the column were caught and marked with an individual colour code using nail varnish (Peakall & Schiestl, 2004), which was applied to the thorax and hind leg to allow easy identification following recapture. The colour ID of all recaptured wasps was recorded along with the date and time of their recapture. Two people were involved in wasp capture and identification during all experiments, which ensured that all wasps contacting the column were captured. Wasps that alighted but did not contact the column were not interfered with because: (1) our aims relate to the consequences of the behaviour of *L. excelsa* for pollen movement, for which column contact is the only relevant behaviour; (2) pollinator behaviour within a patch, including the full sequence of behavioural responses, was quantified in a previous experiment; and (3) it is implausible to capture wasps without altering their subsequent behaviour (for example, after alighting only). Recapture rates were calculated for both within a day and within a week using pooled data from the two mark-recapture studies (data were pooled after returning $P = 0.5631$ (within day) and $P = 0.3775$ (between days) in a Fligner–Killeen test for homogeneity of variance of the number of recaptures per wasp). These recapture rate data were collected and analysed separately to the within-patch behavioural experiments. None of the wasps caught in the first mark-recapture experiment was recaptured in the second (repeat) experiment, demonstrating that the behaviour of pollinators was not affected by encounters with orchids in the previous experiment.

Long-distance pollinator movement

To approximate pollinator movement between patches of *C. ovata*, a larger scale mark-recapture experiment was implemented. Male *L. excelsa* wasps were attracted by baiting with fresh *C. ovata* flowers between the 9 and the 16 January 2012. A 20 × 20 m grid was overlain onto the study site (600 × 200 m) and baiting locations determined in a random, stratified fashion, so that baiting was only undertaken within patches of vegetation. Baiting was undertaken at 112 locations within the study site for 10-min periods, with a minimum of 40 m distance between consecutive locations. Orchid flowers were transported in a sealed container between baiting locations. Wasps were marked on the thorax using coloured paint pens (Uniball Posca) and nail varnish. A unique colour combination was used for each location. Recaptured wasps were marked on the femur of the right hind leg with a colour unique to the recapture location, and were given an additional mark upon first recapture. Wasps were released at the point of capture. Potential movement distances were calculated from UTM points using GenAlEx v6.5 (Peakall & Smouse, 2006, 2012). Observed mark-recapture distances are presented as raw frequencies, and as frequencies adjusted for the probability of a recapture according to their distance class (calculated from the distribution of possible movement distances).

Comparison with other sexually deceptive systems

To investigate the consequences of ichneumonid pollination relative to other sexually deceptive pollination systems, we compared data from the present study with the literature. Data were gathered for orchid pollination rate, frequency of pollinator column contact, frequency of pollinator pseudocopulation with a flower, and pollinator movement distances. Species were only included if data were available for two out of the three variables of pollination rate, rate of pseudocopulation, and rate of column contact. When behavioural data were available from multiple studies, data were selected from the study providing both pseudocopulation and column contact. Fruit set data were included for *Pterostylis sanguinea*, as this species is known to be primarily pollen limited (Phillips *et al.*, 2014b). When pollination rate data were available from multiple years, the rate was calculated by taking the mean of all yearly values. When multiple sampling strategies (e.g. sweep netting and baiting) had been implemented in wasp movement studies, data from the baiting method only were compared, to be consistent with our own sampling strategy.

For *C. ovata*, data for fruit set rather than pollination rate were gathered. In *C. ovata* fruit set is primarily pollen limited (A. Weinstein & R. Phillips, unpubl. data), and given the long 3-month flowering period and sequential opening of the flowers, it was not possible to monitor populations regularly to obtain reliable pollination rate data. Fruit set for *C. ovata* was recorded from 16 populations (for locations see also Supporting Information, Tables S1) at the end of the flowering season. For a total of 242 flowering stems it was recorded whether or not capsules developed. Fruit set was calculated as the percentage of flowers that developed capsules per inflorescence, from which population means were calculated. The final value for *C. ovata* was calculated by averaging the fruit set across the 16 populations.

We aimed to understand the effect of pollinator behaviour at a flower on reproductive success. Firstly, we tested for a correlation between the frequency of pseudocopulation and column contact. We then tested the expectation that a high frequency of column contact would result in a high pollination rate. Correlations were tested for using R v 3.1.3 (R Development Core Team, 2015). To test if the size of the study site affects estimates of movement distances, we tested for a correlation between the maximum observed movement distances and the size of the study sites. Further, we present the maximum movement distances both as a raw distances and as percentages of the largest movement distances possible within the study sites.

RESULTS

Pollinator behaviour: on an inflorescence and within a patch

In total, 240 wasps were observed visiting *C. ovata* flowers in artificially presented patches. Varying degrees of sexual attraction were displayed by individual wasps – in their first visitation to a flower 214 (89.2%) wasps alighted on the flower, 108 (50.5% of alighting wasps) contacted the column, and 72 (33.6% of alighting wasps) attempted copulation (Table 1). Over the course of the study, both pollinia removal and deposition were observed during the reversing-in behaviour, both with and without subsequent pseudocopulation. Approximately 10% of copulating wasps did not initially assume the reversed in position, and instead moved over the surface of the labellum. Typically, these wasps eventually reversed in to the flower, with the delay in reaching this position often being caused by the presence of another male already occupying the flower.

Of the wasps visiting the experimental patch, 59 (24.6%) engaged in a second floral visitation, 18 (7.5%) in a third, and 3 (1.3%) in a fourth. In the second visitation, 49.1% of wasps moved to a new inflorescence, 17.5% moved to a different flower on the same inflorescence, and 33.3% returned to the same flower. In the third visitation, 44.4% of wasps moved to a new inflorescence, while the remaining 55.6% returned to the same flower as in their first or second visitation. During the course of our experiment, 13 wasps (5.4%) contacted the column on at least two visitations to flowers within a single encounter with the patch. A significant difference in pollinator behaviour was observed between the initial and subsequent visitations, with a significant decrease being observed for both the percentage of wasps contacting the column ($P = 0.004$, $G = 11.20$, d.f. = 2) and copulating with flowers ($P = 0.036$, $G = 6.643$, d.f. = 2) as the number of visitations increased (Table 1).

Table 1. Number of *Lissopimpla excelsa* alighting on, contacting the column of, and copulating with *Cryptostylis ovata* flowers in artificially presented patches over multiple visitations

	First visitation	Second visitation	Third visitation	*G*-test *P*-value
N	240	59	18	
Alight (%)	89.2	91.5	100	0.128
Column contact (%)	50.5	35.2	16.7	0.004*
Copulation (%)	33.6	22.2	11.11	0.036*

Values for column contact and copulation are given as a percentage of animals that alighted, while those alighting were calculated as a percentage of total responses.
*$P < 0.05$.

Pollinator revisitation rate: within a day

In total, 94 observations of marked wasps visiting and contacting the column were made. Of these wasps, 24 (25.5%) returned and contacted the column again during the same 5½ h presentation period (Fig. 2). Of the returning wasps, 18 (19.1%) returned and contacted the column once, and six (6.4%) returned and contacted the column two to four times (Fig. 2).

Pollinator revisitation rate: within a 7-day period

Within the two 7-day presentation periods, 31 (50.0%) of the 62 marked wasps observed contacting the column of artificially presented flowers returned and contacted the column of the presented flower again (Fig. 3). Sixteen wasps (26.2%) returned and

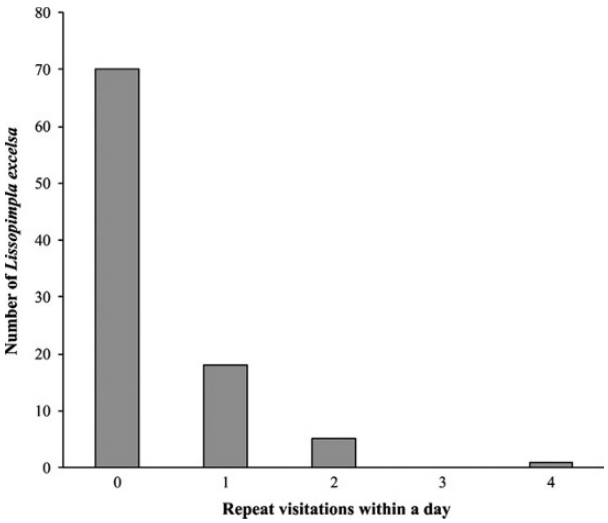

Figure 2. Total number of *Lissopimpla excelsa* returning to a *Cryptostylis ovata* flower within the daily 5½ h observation period across the eight sampling periods.

contacted the column once, eight wasps (13.11%) twice, and seven wasps (11.45%) returned and contacted the column three to eight times (Fig. 3).

In total, 64 revisitation events were observed within the two 7-day trials. Fifty per cent of these revisitation events were contributed by 11.3% (7) of the individual wasps, which revisited flowers on between three to eight occasions (Fig. 4). A further 38.7% (24) of individuals revisited once or twice and contributed the remaining revisitations. Fifty per cent (31) of wasps did not revisit the infloresence (Fig. 4).

Long-distance pollinator movement

In total, 224 male *L. excelsa* wasps were marked at 78 locations (Fig. 5). Wasps were not detected at 33 locations. Thirty-two individuals were recaptured (14.3% of those captured), with 30 of these being recaptured once, and four recaptured twice, totalling 40 recapture events. The median time between capture and recapture was 1 day (range = 0–4 days). Recapture distances ranged from 16.1 to 625.0 m, with a mean of 99.2 ± 21.4 m (median = 49.0 m, $N = 40$; Fig. 6). Observed and adjusted pollinator movement distances approximated a leptokurtic distribution (Fig. 6). These distributions were more positively skewed than the distribution of possible movement distances, and as such a greater number of movement distances < 50 m were observed than would be expected in a random movement pattern.

Comparison with other sexually deceptive systems

Across the 16 populations of *C. ovata* surveyed (a total of 242 plants), fruit set averaged 27.8 ± 1.3%. The mean number of flowering stems per population was 13.7 ± 5.2 (range = 1–79). The mean number of flowers per raceme was 11.21 ± 0.28 (range = 1–24). Across the other species previously investigated and

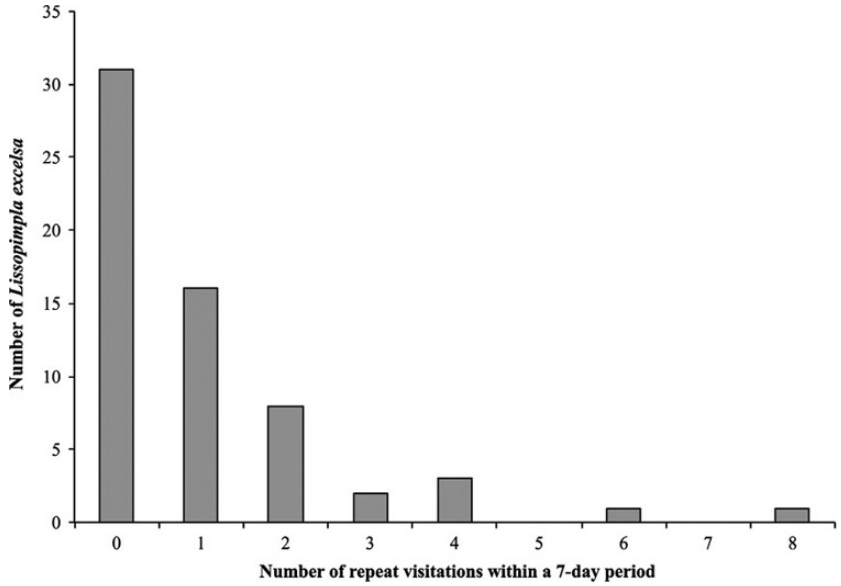

Figure 3. Total number of *Lissopimpla excelsa* that returned and reversed in to a *Cryptostylis ovata* flower over the two 7-day observation periods (totalled from two periods).

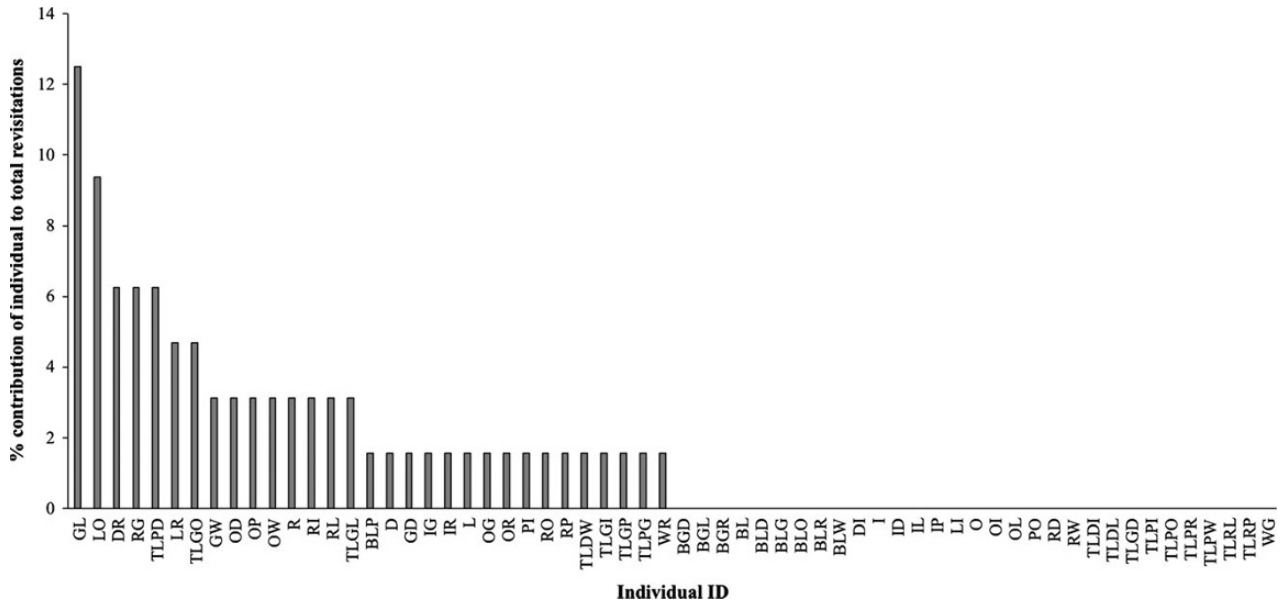

Figure 4. Percentage contribution of each individual *Lissompimpla excelsa* wasp to total revisitations on artificially presented *Cryptostylis ovata* flowers across two 7-day experimental periods.

Figure 5. Map of the study site in Kings Park and Botanic Garden, showing movements of male *Lissopimpla excelsa* wasps attracted to *Cryptostylis ovata* bait flowers. Red dots are points where baiting for pollinators was conducted.

reported in the literature, pollination rate was lowest in *Caladenia* species (mean = 16.9%; minimum 5.9%, *Caladenia ferruginea*), and highest in *Drakaea* species (mean = 59.4; maximum 63.2%, *D. glyptodon*; Table 2). The rate of pseudocopulation was lowest for *Caladenia tentaculata* (7.5%), and greatest in *Caladenia attingens* (63.1%). Column contact ranged from 1.2% in *Caladenia* sp. Moora to 50.5% in *C. ovata*. There was no significant correlation between the frequency of pseudocopulation and the frequency of column contact ($R = 0.278$, $P > 0.05$, $N = 11$), or between the frequency of column contact and pollination rate ($R = 0.270$, $P > 0.05$, $N = 9$).

Across the mark-recapture studies, both the mean (99.2 m) and maximum (625 m) recapture distances were greater for *L. excelsa* than the other species in the literature (Table 3). The shortest mean recapture distance was for *Colletes cunicularius* (5 m) (Colletidae), and the shortest maximum recapture distance for *Thynnoides pugionatus* (40 m) (Tiphiidae). A significant positive correlation between the maximum observed pollinator movement distance and the maximum possible recapture distance in the study site was observed ($R = 0.924$, $P < 0.001$). However, this result is likely to arise through sites being selected to match the greater predicted pollinator movement distances of larger species. Critically, *Thynnoides* sp. A was the only species for which the maximum observed recapture distance was within 10% of the maximum possible distance, with the observed movement distances for all species ranging from 40.0% to

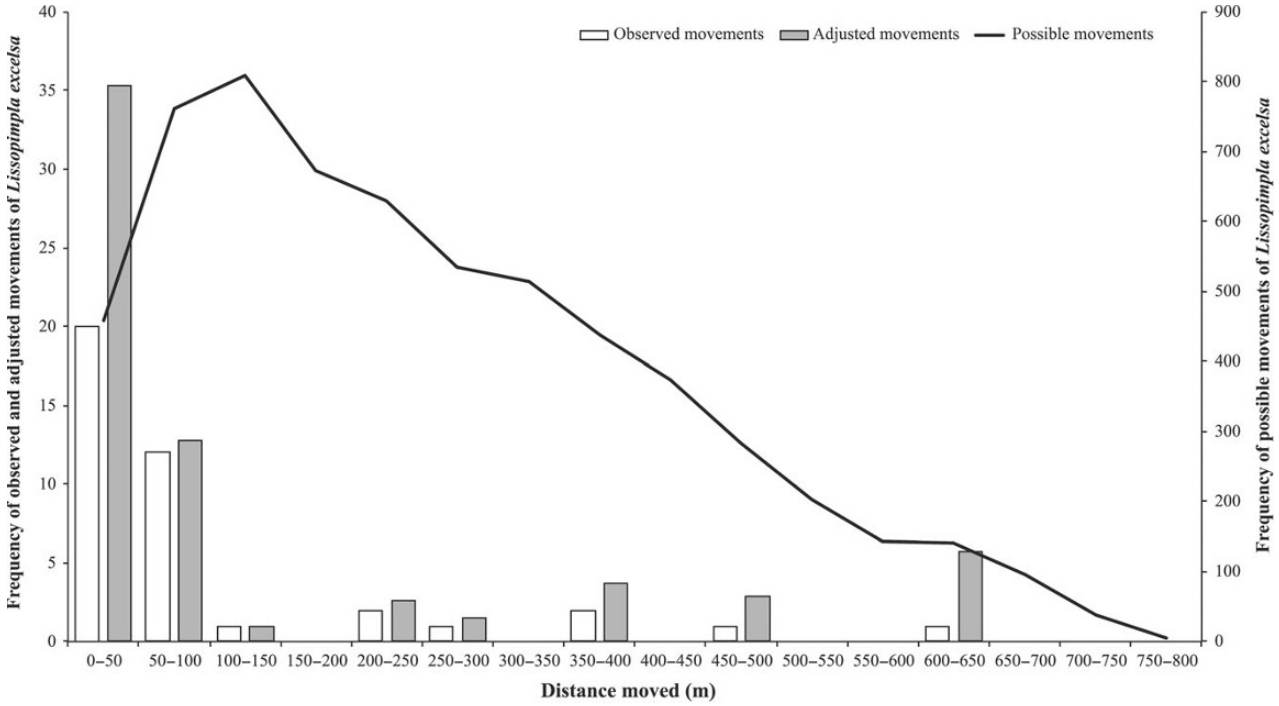

Figure 6. Frequency distribution of movement distances of *Lissopimpla excelsa* males (movement distance categories: observed, adjusted for the probability of a recapture according to distance class, and all possible).

98.7% of the maximum possible movement distances (Table 3).

DISCUSSION

Behaviour of *Lissopimpla excelsa* within a visit to a patch

Within a visit to a patch, pollinators that interacted with multiple flowers exhibited a decrease in sexual attraction to the flower with subsequent floral visits. As a result, while 25% of pollinators engaged in multiple floral visitations within a single visit to the patch, only a small portion (5.4%) contacted the column twice or more. A decrease in the degree of sexual attraction to stimuli over time has been observed in plant-pollinator interactions and laboratory experiments (Robacker, Weaver & Hendry, 1976; Ayasse *et al.*, 2000; Gaskett *et al.*, 2008; Whitehead & Peakall, 2013; De Jager & Ellis, 2014). Data from the present study suggest that pollinator learning is responsible for the decrease in sexual response observed in *L. excelsa*, as with each encounter the attraction of individual wasps to the flower decreased. Studies of ichneumonids in other contexts have demonstrated a capacity for learning. *Itoplectis conquisitor* (which, like *L. excelsa* is in subfamily Pimplinae) males were observed to respond to blanks in a laboratory bioassay after, but not before, they had been exposed to female extracts presented in an identical manner to the blanks (Robacker *et al.*, 1976). Further, female ichneumonids can learn to associate artificial host shelters with the presence of hosts (Wardle & Borden, 1985), and more specifically to associate colour (Arthur, 1966; Wardle, 1990; Schmidt, Cardé & Vet, 1993), size (Arthur, 1967), and odour cues (Arthur, 1971; Iizuka & Takasu, 1998) with the presence of hosts.

While learning is likely to explain the observed decline in sexual response over time, pollinators revisiting *Cryptostylis* flowers within a patch may experience a post-mating refractory period, potentially caused by sperm depletion (Gordh & Hendrickson, 1976). Although the presence or absence of ejaculate on visited *C. ovata* flowers was not assessed, this phenomenon is known in the genus (Coleman, 1931; Erickson, 1951; Gaskett *et al.*, 2008). There have been only a few studies of mating behaviour within the Ichneumonidae, making it impossible to generalise to the family as a whole and determine if this refractory period is related to a shortage of sperm. Within the Ichneumonidae both the presence (Gordh & Hendrickson, 1976) and absence (Vinson, 1972) of post-mating refractory periods have been recorded. Male *Bathyplectes anurus* (Ichneumonidae) typically ignore females for 1 h

Table 2. Rates of pollination, column contact, and pseudocopulation for sexually deceptive orchids and their pollinators

Orchid	Pollinator	Column contact (%)	Pseudocopulation (%)	*N* (visitors)	Pollination rate (%)	*N* (sites)	Reference
Caladenia attingens	*Thynnoides* sp. C (Tiphiidae)	21.4	63.1	130	13.4	4	R. Phillips, unpubl. data
Caladenia crebra	*Camplyothynnus flavipictus* (Tiphiidae)	12.8	60	113	–	–	R. Phillips, unpubl. data
Caladenia ferruginea	Thynnid new genus 3 sp. B (Tiphiidae)	2.8	52.1	142	5.9	7	R. Phillips, unpubl. data
Caladenia pectinata	*Zaspilothynnus nigripes* (Tiphiidae)	4	10	233	12.3	8	Phillips *et al.* (2013)
Caladenia sp. Moora	*Thynnoides* sp. J (Tiphiidae)	1.2	30.6	114	–	–	R. Phillips, unpubl. data
Caladenia tentaculata	*Thynnoides pugionatus* (Tiphiidae)	7.5	7.5	287	36	3	Peakall & Beattie (1996)
Chiloglottis trapeziformis	*Neozeleboria cryptoides* (Tiphiidae)	–	40	200	24.7*	2	De Jager & Peakall (2015), M. de Jager, unpubl. data*
Chiloglottis trilabra	*Neozeleboria proxima* (Tiphiidae)	25	25	2411	41	1	Peakall & Handel (1993)
Chiloglottis valida	*Neozeleboria monticola* (Tiphiidae)	–	28.6†	48	21.7*	1	Whitehead & Peakall (2014), Whitehead *et al.* (2015)*
Cryptostylis ovata	*Lissopimpla excelsa* (Ichneumonidae)	50.5	30	240	28‡	16	Present study
Drakaea glyptodon	*Zaspilothynnus trilobatus* (Tiphiidae)	16	5.9	618	63.2*	51	Peakall (1990), Phillips *et al.* (2014a)*
Drakaea livida	*Zaspilothynnus nigripes* (Tiphiidae)	43	35	313	55.5	19	Phillips *et al.* (2013)
Leporella fimbriata	*Myrmecia urens* (Formicidae)	58	58	57	27.18	7	Peakall (1989)
Pterostylis sanguinea	*Mycomya* sp. (Mycetophilidae)	–	57.8	135	23.7‡	19	Phillips *et al.* (2014b)

The rates of column contact and pseudocopulation are given as a percentage of the animals that alighted. Letters (e.g. sp. A) refer to species recognised in previous papers such as Phillips *et al.* (2014a) and an unpublished dataset on the pollinators of *Caladenia*.

*The origin of data where data for the same species has been sourced from two different studies.

Data marked †, while being part of the referenced studies, were not explicitly stated and were obtained in personal communication with the authors.

‡Data is for fruit set in place of pollination rate, as fruit set is in these systems known to be primarily pollen limited and collection of pollen deposition data was not feasible.

Table 3. Mean and maximum recapture distances for pollinator species of sexually deceptive orchids for which data were available

Pollinator species	Family	Orchid species pollinated	*N*	Mean recapture distance (m)	Maximum recapture distance (m)	Maximum distance in study site (m)	Maximum recapture distance as a % of maximum possible distance	Reference
Colletes cunicularius	Colletidae	*Ophrys fusca*, *O. integra*, *O. exaltata*, *O. archipelagi*	577	5	50	120*	41.7	Peakall & Schiestl (2004)
Lissopimpla excelsa	Ichneumonidae	*Cryptostylis ovata*, *C. erecta*, *C. subulata*, *C. leptochila*, *C. hunteriana*	224	99.2	625	775	80.1	Present study
Macrothynnus sp. A	Tiphiidae	*Caladenia thinicola*	120	56.3	470	707	66.5	M. H. M. Menz, unpubl. data
Neozeleboria cryptoides	Tiphiidae	*Chiloglottis trapeziformis*	505	15.5	161	280*	57.5	Whitehead & Peakall (2013)
Thynnoides pugionatus	Tiphiidae	*Caladenia tentaculata*	20	17	40	100	40.0	Peakall & Beattie (1996)
Thynnoides sp. A	Tiphiidae	*Drakaea gracilis*	148	51.4	293	297	98.7	M. H. M. Menz, unpubl. data
Zaspilothynnus gilesi	Tiphiidae	*Drakaea elastica*	147	55.2*	150	457	32.8	Menz *et al.* (2013)
Zaspilothynnus nigripes	Tiphiidae	*Drakaea livida*	506	46.5*	300	457	65.6	Menz *et al.* (2013)
Zaspilothynnus trilobatus	Tiphiidae	*Drakaea glyptodon*	21	32	132	160†	82.5	Peakall (1990, 1987)†

Letters (e.g. sp. A) refer to species recognised in previous papers such as Phillips *et al.* (2014a) and an unpublished dataset on the pollinators of *Caladenia*.
Data marked *, while being part of the referenced studies, were not explicitly stated and were obtained by personal communication with the authors.
†The origin of data where data for the same species has been sourced from two different studies.

following copulation (Gordh & Hendrickson, 1976). In contrast, male *Campoletis sonorensis* (Ichneumonidae) have been observed to copulate a second time within minutes of the first copulation (Vinson, 1972). We predict that in *L. excelsa* a degree of learning is likely to be occurring within a visit to a patch, possibly in concert with the effect of sperm depletion in some individuals.

Revisitation behaviour of *Lissopimpla excelsa*

During a mark-recapture study of the wasps that contacted the column of a single flowering plant over a 7-day period, 50% of marked *L. excelsa* were recaptured at least once – twice the recapture rate observed during a single day. Similarly, in previous studies of sexually deceptive orchids, the thynnine wasps *Neozeleboria cryptoides* and *Zaspilothynnus trilobatus* were observed not to revisit a site within the course of a day, yet to revisit the same site after a 24 h period had elapsed (Peakall, 1990; Whitehead & Peakall, 2013). Given the evidence for learning within a single visitation to a patch in *L. excelsa*, it is plausible that learning could also be functioning over a 24-h time period, potentially explaining the greater revisitation rate observed over several days. As this avoidance occurs for a 24-h period only, it is unlikely to be a strategy to avoid deceptive orchids, which flower for several weeks. Instead, such learning could have evolved via selection for males to avoid sites where they had recently encountered a female, thereby optimising the discovery of newly emerged females. In *Alabagrus texanus* (Braconidae), following initial scramble competition, males avoid the site where a female has eclosed for 1 h, after which they will again be attracted to females at the site (Goh & Morse, 2010). Only rarely does more than one female eclose within a five 5 m area on the same day, making avoiding the eclosure site advantageous for increasing the efficacy of mate searching (Goh & Morse, 2010). Importantly, this explanation is likely to apply regardless of whether or not the encounter with a female resulted in a successful mating. It remains unknown whether a similar phenomenon may be operating in *L. excelsa*, as patterns of female eclosure for both *L. excelsa* and ichneumonid wasps in general have not been described.

Implications for the pollination of *Cryptostylis ovata*

Given the clonal nature of *C. ovata* (Dodd *et al.*, 1984), and the prevalence of fine-scale genetic structure in orchids (Peakall & Beattie, 1996; Trapnell, Hamrick & Nason, 2004; Chung, Nason & Chung, 2005; Mant *et al.*, 2005), pollen transfer within a patch of *C. ovata* inflorescences is likely to contribute to inbreeding. The analysis of pollinator behaviour over multiple time scales revealed that any occurrence of pollen transfer within a patch of *C. ovata* flowers is most likely to be due to pollinator revisitation across several days, and not within a day or within a single encounter with a patch. Despite pollinators alighting on flowers multiple times within a single visit to a patch, only 5.4% of pollinators exhibited behaviour that could lead to the transfer of pollen within the patch (contacting the column two or more times). While 25% of pollinators were observed to revisit within a day, the highest rate of revisitation, and thereby the greatest potential for pollen transfer within a patch, occurred over a 7-day period (50% revisitation rate). It must be noted that these estimates of pollen transfer within a patch do not take into account that it is unlikely that all pollinator visits will involve both pollinia removal and deposition, and that pollinators may visit additional patches between revisits to the monitored patch.

Lissopimpla excelsa had a mean recapture distance of 99 m and a capacity to travel distances of at least 625 m, which far exceeds the size of a *C. ovata* patch (typically < 5 m in diameter). Despite these large movement distances, 50% of pollinators were observed to revisit patches multiple times, suggesting that *L. excelsa* males are patrolling a home range. A similar degree of site fidelity has also been reported for other ichneumonid species, along with the potential to move distances in excess of 100 m (Heatwole & Davis, 1965). Considering the observed movement distances of *L. excelsa*, and that *C. ovata* patches are usually located within a few hundred metres of one another, it is likely that frequent pollen dispersal between patches or subpopulations of *C. ovata* is occurring.

It is of interest that despite the large number of wasps visiting the *C. ovata* flowers, proportionally few were responsible for the high rate of revisitation. This result may indicate that pollen movement within patches of *C. ovata* could be caused by only a small portion of the population of visiting wasps. A skew in the rate of revisitations could possibly be related to a variation in male fitness or in the stimulus threshold required to respond to flowers. A low stimulus threshold could be attributed to either time since emergence, or a lack of sexual experience. However, the one study to address the role of sexual experience in ichneumonid mating behaviour (Vinson, 1972) reported no difference in the response rates of mated and virgin *Campoletis sonorensis* (Ichneumonidae) males to females. Alternately, the wasps making repeat visits may have a different

mate search strategy or home range than the remainder of the population.

Behaviour of *L. excelsa* in the context of other sexually deceptive systems

Pollinator behaviour at a flower and its impact on fruit set

Despite being one of only two genera in which the pollinator has been observed to ejaculate onto the flower (Coleman, 1931; Erickson, 1951; Blanco & Barboza, 2005; Gaskett *et al.*, 2008), the rate of pollinator pseudocopulation was low relative to other sexually deceptive species included in the analyses. However, the efficacy of *C. ovata* at converting pollinator attraction into column contact was high when compared with other orchids.

While it could be generally predicted that more frequent pseudocopulation would increase contact with the column, this relationship was not observed in our analysis across sexually deceptive systems. Such a relationship would be dependent on pseudocopulation being with the part of the flower where pseudocopulatory movements bring the pollinator into contact with the column. While in most sexually deceptive orchids the sexual attractant is released from the labellum (Schiestl *et al.*, 2000; Phillips *et al.*, 2013, 2014b; Bohman *et al.*, 2014), in *Caladenia* pseudocopulation frequently occurs with the glandular sepal tips, leading to a comparatively inefficient pollination mechanism (Phillips *et al.*, 2013). As demonstrated in *C. ovata*, the converse may also apply, where column contact can occur without pseudocopulatory behaviour. Therefore, the frequency of pseudocopulation and column contact may not be correlated across systems due to taxonomic variation in floral traits and pollinator behaviour.

It is often presumed that a high efficacy of converting pollinator attraction into contact with the reproductive structures would be correlated with a high pollination rate. However, this trend was not observed in our analyses. While a high efficacy of converting attraction into column contact contributes towards pollination rate, the abundance and fidelity of the pollinator species, and the spatial distribution of the plants, also have an effect. For example, a small number of effective pollinator visits could lead to a similar pollination rate as could many less effective visits. For a direct comparison of the efficiency of pollinators between systems, it may be informative to focus on the ratio of pollen deposition relative to removal, as this measure is independent of the number of pollinator visitations. Unfortunately, these data are currently only available for two out of the 14 study species, suggesting that this will be an important future area of study.

The lack of a correlation between pseudocopulation and column contact, and between column contact and pollination rate is in contrast with the finding of Gaskett *et al.* (2008) that orchids provoking more intense pollinator behaviour have higher pollination success. As a measure of pollinator behaviour, Gaskett *et al.* (2008) assigned orchids to discrete categories of observed pollinator sexual behaviour (ejaculation, copulation, gripping a hinged petal, and entrapment). It was found that more intense pollinator behaviour was associated with a higher pollination rate. However, this result was largely driven by the high frequency of pollinated plants reported for *Cryptostylis*, the only genus in the highest category of pollinator sexual response. Since the analysis of Gaskett *et al.* (2008), a much larger dataset has become available allowing our more detailed investigation, in which pollination rate is assessed on a per flower basis. While our comparison across genera does not support the hypothesis of Gaskett *et al.* (2008) that greater sexual attraction leads to a higher pollination rate, a comparison of closely related species with similar floral traits may support this prediction.

Pollinator movement distances

Here we compare the movement distances of an ichneumonid (*Lissopimpla excelsa*) to those of other sexually deceived pollinators. The sole bee species in this comparison, *Colletes cunicularius*, has a considerably shorter mean recapture distance than the other species, which is due to the different mating behaviours of solitary bees and thynnine wasps (which represent the majority of species in the comparison). Male solitary bees patrol specific rendezvous sites (Alcock *et al.*, 1978; Ayasse, Paxton & Tengö, 2001), whereas thynnine males patrol comparatively large home ranges (Alcock, 1981). Amongst the thynnine species there was a high degree of variability in maximum movement distance, ranging from *T. pugionatus* (40 m) to *M. insignis* (470 m). However, *L. excelsa* has even greater mean and maximum recapture distances, with its mean recapture distance being almost double that of all the other compared species (Table 3). The difference in movement distances between *L. excelsa* and the other studied sexually deceived pollinators may be attributable to differences in the mate searching or foraging behaviour between insect families. Previous studies of ichneumonid wasps, however, report movement distances of no more than 107 m (Juillet, 1959; Heatwole & Davis, 1965). While these studies were not consistent in methodology, these results suggest that the home range of *L.*

excelsa may also exceed those of some other ichneumonids.

CONCLUSIONS

Combining the observation of experimental *C. ovata* patches with mark-recapture studies demonstrated that pollen transfer within a patch of orchids is likely to arise most frequently through pollinator revisitation over a period of several days, rather than within a day or within a single visit. However, *L. excelsa* are also capable of moving distances in excess of half a kilometre, demonstrating their ability to transfer pollen between subpopulations or farther. These movement distances are the largest reported to date for sexually deceived pollinators, and the first reported for a sexually deceived ichneumonid, highlighting the unexplored potential for variation in pollinator behaviour between taxonomic groups. As such, to comprehensively understand the consequences of pollination by sexual deception, detailed investigations of the natural history of other pollinator groups and their interaction with sexually deceptive flowers are required. Such studies should encompass aspects of pollinator behaviour responsible for pollen transfer, and how these behaviours are influenced by floral traits.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Locations of populations of *Cryptostylis ovata* from which fruit set data were collected.

CHAPTER TWO

Post-pollination barriers, including extreme differences in ploidy, impede hybridisation in Australian *Cryptostylis* (Diurideae, Orchidaceae) species that share a sexually deceived pollinator

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This study was conceptualised by A Weinstein on consultation with all authors.. Experiments and data analyses were conducted by A Weinstein. Original draft preparation was conducted by A Weinstein. Review and editing was conducted by all authors. Funding was acquired by A Weinstein.

ABSTRACT

Hybridisation in plants is prevented by pre- and post-pollination reproductive barriers. In sexually deceptive orchids, different orchid species typically have their own specific pollinator species, conferring a strong pre-pollination barrier. Unusually, all five Australian *Cryptostylis* species, some of which occur in sympatry, sexually deceive the same ichneumonid pollinator. These species do not appear to form hybrids. The present study investigated barriers that may be preventing hybridisation in Australian *Cryptostylis*: phenology, site co-occurrence, bioclimatic niche, pollinator behaviour, and differences in genome size and ploidy. Hand pollinations within and between species were used to calculate self-incompatibility and inter-species reproductive isolation values, which were compared to phylogenetic distance. Geographic and phenological surveys revealed an overlap in flowering time and distribution among the sympatric species. Mark-recapture experiments demonstrated that pollinators moved between experimentally presented sympatric species. Hand cross-pollinations conducted among the four common *Cryptostylis* species in a greenhouse revealed seed mass and the percentage of formed embryos to be lower in the hybrid treatments than in the intraspecific control. Linear mixed effects models demonstrated that these differences were significant in over half of the hybrid treatments. Flow cytometry showed three consistently different ploidy levels, within which there was aneuploidy, in Australian *Cryptostylis* species. Existing and newly collected chromosome count data supported this result. *Cryptostylis leptochila* was found to have the highest chromosome count thus far reported among the Orchidaceae. *Cryptostylis subulata* and *C. erecta* were found to be self-incompatible, marking first case of self-incompatibility in the Diurideae. There was stronger reproductive isolation between sympatric than allopatric species pairs, potentially explained by reinforcement. It is evident that post, not pre-pollination barriers are preventing hybridisation in Australian *Cryptostylis*, with differences in ploidy likely explaining the inter-species incompatibility. The unique reproductive biology of Australian *Cryptostylis*, encompassing pollinator sharing, self-incompatibility, and post-pollination reproductive isolation driven by large ploidy differences, may indicate that its mode of diversification may differ greatly to those in other sexually deceptive genera, inviting further investigation of its evolutionary history.

INTRODUCTION

In plants, hybridisation can be prevented by both pre- and post-pollination reproductive barriers. Pre-pollination barriers include pollinator specificity (Cozzolino & Scopece, 2008; Xu et al., 2011; Peakall & Whitehead, 2014), mechanical barriers such as pollen placement location (Pauw, 2006), and temporal (Lennartsson, 1997; Lowry et al., 2008b) and ecogeographic (Peakall et al., 2002; Glennon et al., 2012; Sobel, 2014) barriers. Post-pollination barriers, which can be pre- or post- fertilisation, also contribute to reproductive isolation. Pre-fertilisation barriers include pollen tube growth (Ascher & Peloquin, 1968; Carney & Arnold, 1997; Dresselhaus et al., 2011) and gametic incompatibility (de Nettancourt, 1977; Seavey & Bawa, 1986; Howard, 1999). Post-fertilisation barriers include embryo abortion and hybrid inviability (Stebbins, 1958; Drake, 1975; Scopece et al., 2008), which may be due to differences in ploidy or chromosome number (Valentine & Woodell, 1963; Levin, 1978; Amich et al., 2007). Late acting self-incompatibility can also function as a post-fertilisation barrier (Seavey & Bawa, 1986). Pre-pollination barriers are generally thought to play a more important role in the formation of reproductive isolation in angiosperm speciation than are post-pollination barriers (Grant, 1994; Coyne & Orr, 2004; Rieseberg & Willis, 2007). Pre-pollination barriers are particularly important in systems with ecologically specialised pollination strategies (Hodges, 1997; Johnson et al., 1998; Johnson & Steiner, 2000; Lowry et al., 2008a), such as the Orchidaceae, which has an exceptionally high incidence of species with one or few pollinator species (Tremblay, 1992; Schiestl & Schlüter, 2009; Joffard et al., 2019).

In sexually deceptive orchids, whose pollination strategies are amongst the most specialised known in plants (Schiestl, 2005; Xu et al., 2012), pollinator-mediated floral isolation is the major reproductive barrier, while post-zygotic barriers are typically absent or weak (Peakall et al., 2010; Xu et al., 2011; Whitehead & Peakall, 2014). Sexually deceptive orchids mimic female insects, inducing sexual behaviour in pollinators that brings them into contact with the orchids' reproductive structures (Correvon & Pouyanne, 1916; Coleman, 1927; Schiestl et al., 2003; Mant et al., 2005b). Pollen transfer is effected as males are deceived by multiple plants, which they attempt to mate with and in doing so inadvertently pollinate. Typically, sexually-deceptive

orchids do not share pollinator species, with each orchid species usually attracting its own unique pollinator (Paulus & Gack, 1990; Peakall et al., 2010; Phillips et al., 2017a), though exceptions are noted (Cortis et al., 2008; Gögler et al., 2009; Breitkopf et al., 2015; Bohman et al., 2017; Phillips et al., 2017a). As such, the use of different pollinator species is commonly a major reproductive barrier in sexually deceptive orchids (Xu et al., 2011; Xu et al., 2012; Whitehead & Peakall, 2014). Hand cross pollination experiments between orchid species reproductively isolated by their use of different specific pollinator species have shown an absence of post-pollination barriers (Xu et al., 2011; Whitehead & Peakall, 2014).

An unusual exception among sexually deceptive orchids is the genus *Cryptostylis*, where all five Australian species are pollinated by the same sexually deceived ichneumonid wasp, *Lissopimpla excelsa* (Coleman, 1927; Coleman, 1929, 1930b, a; Nicholls, 1938). Despite pollinator sharing, it remains untested whether individual wasps will visit multiple species of *Cryptostylis*. While the extent of species co-occurrence remains uninvestigated at both a continent and a site level, visits by pollinators to multiple *Cryptostylis* species are spatially feasible as the species are known to occur in sympatry (Gaskett & Herberstein, 2006) and pollinators have been recorded moving distances of up to 625 m (Weinstein et al., 2016). If wasps do move between multiple *Cryptostylis* species, interspecies pollination is likely to occur: in all *Cryptostylis* species pollination occurs when *L. excelsa* reverse into a flower bringing the dorsal apex of their abdomen into contact with the column, thereby attaching pollen in the same position across species (Coleman, 1927; Coleman, 1929, 1930b; Van der Pijl & Dodson, 1966; Lloyd, 2003).

Despite hybridisation often being a common process in some orchid genera (Adams & Lawson, 1993; Neiland & Wilcock, 1998; Backhouse et al., 2019), in the almost 100 years of study since Coleman's initial pollination observations (Coleman, 1927, 1928; Coleman, 1929, 1930b, a), no observations of hybrid Australian *Cryptostylis* have been recorded. This absence is not due to a lack of survey effort - Australian *Cryptostylis* have been the focus of multiple research papers (> 10 in past 30 years), surveys are regularly conducted for the rare *C. hunteriana*, and the other species are common and frequently sought out by amateur naturalists. While the Australian *Cryptostylis* species are frequently referred to as being isolated by the post pollination barrier of genetic incompatibility (Silva-Pereira et al., 2007; Scopece et al., 2008; Gaskett, 2012), no systematic investigation of a potential role of pre-pollination barriers, nor a quantification of the

role of post-pollination barriers, has been conducted.

Different chromosome numbers have been reported for three of the five Australian *Cryptostylis* species: 2n = 56 in *C. erecta*, 2n = 64 in *C. subulata*, and indicative of a higher ploidy level - 2n ≈ 187 in *C. ovata* (Peakall & James, 1989; De Lange et al., 2004; Dawson et al., 2007). Of the 23 Asiatic species of *Cryptostylis* (for which the pollination strategy is unknown) a single chromosome count of 2n = 42 exists (Larsen, 1966). Differences in ploidy level and/or chromosome number are a major barrier to reproduction that typically manifests in severely reduced hybrid fitness (Stebbins, 1958; Levin, 1978; Murfett et al., 1996; Watanabe et al., 1996; Tong & Bosland, 2003), however it is not always a complete barrier to gene flow (Kolář et al., 2017). The ploidy remains un-assessed for two Australian *Cryptostylis* species, and for no species has variation within and among populations been examined. While differences in mycorrhizal associations can play a role in the formation of reproductive isolation (Jacquemyn et al., 2018), a minimum of one OTU is shared between all Australian *Cryptostylis* species, indicating that specificity in mycorrhizal partners does not prevent hybridisation (Arifin et al., in prep.).

Given the sympatry and shared pollinator in eastern Australian *Cryptostylis* the absence of hybridisation is somewhat puzzling, however it is clear that a strong pre-pollination barrier - geography - prevents hybridisation with the allopatric western Australian *C. ovata*. In this scenario, it becomes interesting to examine the reproductive barriers present if the species were brought into secondary contact. Reinforcing selection for the formation of reproductive isolation occurs in zones of sympatry where the opportunity for hybridisation exists (Hopkins, 2013). To avoid a reduction in fitness through the production of unfit hybrids, increased reproductive isolation between sympatric taxa is selected for (Dobzhansky, 1937; Coyne, 1992). Contrastingly, in the presence of a strong geographic barrier, there may be little selection for the formation of reproductive isolation. An increase in reproductive isolation between sympatric species explained by reinforcement has been found in species of plants (Paterniani, 1969; Kay & Schemske, 2008) and fungi (Dettman et al., 2003).

There is some evidence that *Cryptostylis* species may be self incompatible and not produce seed with mature embryos (Stoutamire, 1975) though successful seed set from self-pollinations have

been reported for *C. subulata* (Johns & Molloy, 1983). Self-incompatibility is a widespread mechanism in angiosperms that promotes outcrossing, and is proposed to have increased the efficiency of biotic pollination strategies (Franklin-Tong, 2008). Self-incompatibility is based on a process of self-recognition controlled by the *S*-locus, and can occur either pre- or post-zygotically (Seavey & Bawa, 1986; Takayama & Isogai, 2005). In pre-zygotic self-incompatibility, self-recognition occurs between proteins produced in the pollen and stigma, inhibiting pollen tube growth and subsequent seed set (Takayama & Isogai, 2005). This inhibition can either occur on the stigmatic surface to prevent the germination of the pollen tube, conferring sporophytic incompatibility, or it can occur in the style through which the pollen tube grows, conferring gametophytic incompatibility (Seavey & Bawa, 1986). The *S* gene may also function later in the pollination process: the growth of pollen tubes may be inhibited upon reaching the ovary, or zygotes may abort during the early stages of development - cases termed as late-acting or ovarian self-incompatibility (Barrett, 1988; Nilsson, 1992; Franklin-Tong, 2008). Self-incompatibility manifests in a reduced or absent seed set and the abortion of embryos (Pandey, 1981; Barrett, 1988; Richards, 1997). Self-compatibility and self-incompatibility are not discrete states, but are two ends of a spectrum, between which many intermediates may exist (Schemske & Lande, 1985; Borba et al., 2001).

The majority of orchids are self compatible, with self pollination being prevented by herkogamy (spatial separation of pollinia and stigma), pollinarium reconfiguration (Peter & Johnson, 2005), or pollinator behaviour (Van der Pijl & Dodson, 1966; Tremblay et al., 2005). However, some examples of self-incompatibility, such as the failure of pod or seed set, in the Orchidaceae do exist (Singer & Koehler, 2003; Tremblay et al., 2005), being most extensively studied in *Dendrobium* (Johansen, 1990; Pinheiro et al., 2015), in the Vandoideae (Agnew, 1986), and the Pleurothallidinae (Christensen, 1992; Borba et al., 2001; Barbosa et al., 2009; Cheng et al., 2009; Gontijo et al., 2010; Borba et al., 2011). There are presently no examples of self-incompatibility within the Diurideae tribe (Pridgeon et al., 2001), to which *Cryptostylis* belongs. However, a sole example of self-incompatibility has been reported in the Orchidoideae subfamily (containing the Diurideae), in the sexually deceptive *Bipinnula pennicillata* (formerly *Geoblasta*) (Ciotek et al., 2006).

The present study investigated barriers that may be preventing hybridisation in Australian *Cryptostylis*, encompassing relevant aspects of the species' ecology and mating system, and potential ploidy differences. Specifically, (1) the presence of pre- pollination barriers to hybridisation in the four sympatric eastern-Australian *Cryptostylis* species, (2) the presence of post-pollination barriers between and among the allopatric *C. ovata* and the three common eastern *Cryptostylis* species, (3) ploidy of Australian *Cryptostylis*, and (4) the degree of self-compatibility in Australian *Cryptostylis,* were examined. In addressing (1), the following pre-pollination barriers were assessed: bioclimatic niche overlap, site level species co-occurrence, flowering phenology, and inter-species pollinator movement. Given the known sympatry and pollinator sharing, it was hypothesised that pre-pollination barriers do not prevent hybridisation in eastern Australian *Cryptostylis*. In addressing (2) and (4), controlled hand cross-pollinations were conducted in a greenhouse, with fruit, seed, and embryo development being measured and reproductive isolation and self-incompatibility values calculated. It was hypothesised that the fruit, seed, and embryo development would be greater in the intra-species cross-pollinations than in the hybrid treatments, and that in the self-pollination treatments fruit, seed, and embryo development would not differ to those in the intra-species controls. It was further hypothesised that when accounting for phylogenetic distance, there would be greater reproductive isolation between sympatric species than allopatric species. To examine (3), flow cytometry was conducted to infer ploidy level within and between populations of *Cryptostylis*. Genome sizes were compared with existing chromosome counts for *Cryptostylis* species from the literature, and with those conducted for *C. leptochila* in the present study.

METHODS

Study species

Four of the five Australian *Cryptostylis* species (*C. erecta, C. hunteriana, C. leptochila,* and *C. subulata*) occur in eastern Australia, while *C. ovata* occurs in allopatry in Western Australia. *Cryptostylis hunteriana*, which is federally listed as vulnerable (Energy), is leafless, while the other species are evergreen. *Cryptostylis* forms clonal patches that can support multiple inflorescences, however plants can also occur as isolated single-leafed plants. Inflorescences are multi-flowered, with flowers opening sequentially one to three at a time over a summer (November - March) flowering period. All five Australian *Cryptostylis* species are pollinated by the ichneumonid wasp *Lissopimpla excela* (Coleman, 1927; Coleman, 1929, 1930b, a; Nicholls, 1938), the attraction of which to *C. ovata* is known to be mediated by Tetrahydrofuran-2-yl)acetic acid (Bohman et al., 2019). In addition to the five Australian species, 18 species of *Cryptostylis* are broadly distributed throughout South-East Asia, the pollinator and pollination strategy of which are unknown (Pridgeon et al., 2001; Royal Botanic Gardens Kew, 2020).

Pre-pollination barriers

Species bioclimatic niche overlap

To determine the degree of overlap between the bioclimatic niches of the four sympatric eastern Australian *Cryptostylis* species, species distribution modelling was conducted (Phillips et al., 2017b). This method was selected in preference to comparing extant species distributions, as extant distributions may not be representative of the original area of occupancy prior to European settlement. Maximum Entropy (MaxEnt) species distribution modelling was conducted as this method is suitable for presence only data such as open-source species occurrence records, and has a high predictive accuracy in comparison to other available methods (Elith et al., 2011; Merow et al., 2013). Species occurrence data was sourced from the Atlas of Living Australia (Belbin, 2011), and records with a spatial uncertainly greater than one kilometre were excluded. A final set of 702 presence records for *C. erecta*, 74 presences for *C. hunteriana*, 337 presence

records for *C. leptochila*, and 1320 presence records for *C. subulata*, were used. Analyses were undertaken in R v 3.5.1 (R Core Team, 2018) using the package 'dismo' (Hijmans et al., 2017), with bioclimatic variables calculated to a 1 x 1 km scale in ANUCLIM v 6.1 (Xu & Hutchinson, 2011). Bioclimatic variables likely to influence orchid growth and persistence were selected; BIO 01: annual mean temperature; BIO 08: mean temperature of the wettest quarter; BIO 09: mean temperature of the driest quarter; BIO 18: precipitation of the warmest quarter; BIO 19: precipitation of coldest quarter; BIO 25: radiation of the driest quarter; BIO 32: mean moisture index of wettest quarter; and BIO 33 mean moisture index of driest quarter. The highest Pearson correlation between these variables was 0.83, which falls below the exclusion cut off of 0.85 recommended in Elith et al. (2006), however above the exclusion cut off of 0.7 recommended by Dormann et al. (2013). Whether or not to exclude highly correlated variables in MaxEnt modelling, and if so at what cut off, has long been a point of contention (Elith et al., 2006; Phillips & Dudík, 2008; Elith et al., 2011; Dormann et al., 2013; Merow et al., 2013; Shcheglovitova & Anderson, 2013). A recent study by Feng et al. (2019) concluded that Maxent is capable of regulating contributions from redundant variables and is robust to predictor collinearity, and that therefore the strategy of removing highly correlated variables has little impact in Maxent model performance. As such, variables with a Pearson correlation greater than 0.7 were not excluded from analyses. For each *Cryptostylis* species, the model training area was set using the terrestrial ecoregions defined by Olson et al. (2001) and Dinerstein et al. (2017), using the ecoregions that the species occurs in. The same model projection area was used for all species, being all the ecoregions that the eastern Australian species occur in. Twenty percent of species occurrence data was randomly selected and withheld for model cross-validation. Default model settings were used: a betamultiplier of one, maximum background points of 10 000, convergence threshold of 1.0E-5, and a default prevalence of 0.5. Niche overlap was calculated using the 'nicheOverlap' function in dismo (Hijmans et al., 2017). Niche overlap values (Warren et al., 2008) are a similarity statistic (*I*) calculated from predicted species' distributions and range from zero (no niche overlap) to one (identical niches).

Site level species co-occurrence

Given that the ranges of eastern *Cryptostylis* species overlap, an investigation of whether this overlap also occurs at a site level, thus allowing inter-specific pollen transfer, was conducted. Specifically, the percentage of populations where interspecific pollen transfer could occur (species co-occurring) versus those where only intraspecific pollen transfer could occur (species occurring alone), was determined. Population level data on species occurrence was collected both through field surveys, and also by distributing a citizen science survey to local orchid groups for two flowering seasons. Volunteers were asked to report the species observed (only including flowering and thus easily identifiable plants), the latitude and longitude, and the spatial extent surveyed. Given the highly distinctive floral morphologies of the eastern *Cryptostylis* species, each with different shapes, positions, and colouration of the labellum (Brown, 1810; Reichenbach, 1871; Bentham & Mueller, 1873; Nicholls, 1938), volunteer identifications were able to be accurately carried out. Populations greater than one kilometre (40% more than the longest reported pollinator flight distance (Weinstein et al., 2016)) apart from each other were classed as discrete.

Flowering phenology

To determine the degree to which the eastern Australian *Cryptostylis* species co-flower, the number of open flowers of *C. erecta, C. hunteriana, C. leptochila,* and *C. subulata* were compared at seven sites where a minimum of two *Cryptostylis* species co-occur. To capture variation across the season from early to late flowering, sites were surveyed a minimum of three (maximum five) times at two week intervals. Plants were individually labelled using jewellers' tags, and at each survey the number of buds, open flowers, and dead flowers per plant were recorded, including for plants that had no open flowers.

Potential for interspecific pollen transfer

The potential for interspecific pollen transfer at different time points within the surveyed populations for species in flower was calculated as

$$1 - \frac{\text{number of focus species flowers open}}{\text{total number of flowers open of all species}}$$

Potential interspecific pollen transfer values range from zero to one, with zero indicating no chance of interspecific pollen transfer, and one indicating the maximum possibility of interspecific pollen transfer.

Inter-species pollinator movement

To test whether *L. excelsa* could function as a vector for pollen flow between sympatric species of *Cryptostylis*, and to rule out the possibility of cryptic species or races of *L. excelsa*, each pollinating a different species of *Cryptostylis*, a mark recapture experiment was conducted. An artificial patch of sympatric *Cryptostylis* species was created using picked inflorescences, with one flowering stem of each of *C. erecta, C. leptochila,* and *C. subulata*, which naturally co-occur. These picked flowering stems were placed in a triangle approximately 5 m apart from

each other (to mimic natural clump spacing) in native bushland (35°16'37.5"S 149°06'41.7"E). Three observers continuously monitored the stems between 6:45 and 10am, the time of day with peak wasp activity (Tomlinson & Phillips, 2012). All wasps reversing into flowers (behaviour necessary for pollination) were caught and marked with nail polish (1 unique colour for each *Cryptostylis* species). To account for the recapture of individual wasps, each time a wasp was captured reversing into a flower an additional mark was added to its thorax or hind leg (Weinstein et al., 2016). At each capture, all previous markings (or lack thereof) were recorded, legs enabling identification of individual revisiting wasps. The experiment was conducted over four days (24 - 27 December 2016), with flowering stems being replaced after two days of presentations. Significant differences between the observed ratio of wasps visiting different species and the expected (equal) ratio were tested for using a *G*-test in GenAlEx (Peakall & Smouse, 2006, 2012).

Post-pollination barriers

A minimum of ten different clonal plants of the four common *Cryptostylis* species (*C. erecta, C. leptochila, C. ovata,* and *C. subulata*), each with a minimum of two inflorescences, were maintained in a greenhouse under equal temperature and watering conditions. For each plant, five hand-pollination treatments were conducted in a random order: 1) self-pollination (within flower), 2) intra-species cross (always between plants of different populations to ensure different genotypes), 3-5) inter-species crosses with each of the three other *Cryptostylis* species included in the experiment. The intended design had exactly 10 flowers per pollination treatment, however the number was reduced due to the unforeseen death of some flowering stems. Where possible, extra (in some cases precautionary) pollinations were made on additional plants, resulting in the range of flowers pollinated per treatment being eight to twelve. Hand pollinations were conducted using toothpicks to transfer pollinia. To remove the risk of wasps entering the glasshouse and pollinating experimental flowers, immediately following hand pollination a piece of masking tape was stuck across the labellum and over (though not in direct contact with) the stigma to prevent wasp access. Fruits were picked when they showed signs of imminent dehiscence (brown colouration), and their length and width measured with digital callipers. Some fruits were inadvertently collected after dehiscence, resulting in potential seed loss, and as such

these fruits were not included in subsequent seed mass and embryo counts. Fruits were dried in individual tubes housed in a container with silica gel until fully dehisced, after which point the seeds and fruit were weighed separately on a AG204 DeltaRange balance. Where available (as some fruits contained very few seeds), a subset of 290-300 seeds from each fruit was scored in two categories: embryo present and fully formed, and embryo malformed or absent. Seeds were mounted on slides in a water/tween mixture and examined using a compound microscope. To determine whether *Cryptostylis* plants can set fruit without a pollinator, teabags were placed around 10 flowers of each species to exclude pollinators that may enter the glass house, and plants were checked for fruit set.

Differences in the number of developed fruits between pollination treatments were tested using Fisher's exact test. To test for differences between pollination treatments within a maternal species in mean total seed mass, linear mixed effects modelling was conducted using the packages 'lme4' (Bates et al., 2015) and 'emmeans' (Lenth, 2018) in Rv3.5.1 (R Core Team, 2018). The pollination treatment, number of fruits set on the inflorescence, and the sequential position of the fruit within the set fruits on the inflorescence were set as fixed effects, and the plant that the fruit was grown on was set as a random effect. Residuals were plotted and visually checked for non-random patterns. To test for differences between pollination treatments within a maternal species in the mean percentage of fully formed embryos present, a binomial generalised linear mixed effects model was conducted. To account for overdispersion, an observation level random effect was included in the models, where each data point is allocated a unique level of a random effect (Harrison, 2014). As for the linear models, the pollination treatment, number of fruits set on the inflorescence, and the sequential position of the fruit within the set fruits on the inflorescence were treated as fixed effects, and the plant that the fruit was grown on was treated as a random effect. All pairwise differences were calculated using Tukey's pairwise contrasts.

To test for differences in the direction of the cross (species 1 x species 2, compared to species 2 x species 1) in the proportion of fruits developed, *G*-tests were conducted in GenAlEx (Peakall & Smouse, 2006, 2012). To test for differences in the direction of the cross in seed mass, data in each maternal species were centered so that the mean of the intra-species control was zero, and linear modelling was conducted. To test for differences in the direction of the cross in the

percentage of formed embryos, a binomial generalised linear mixed effects model was conducted. To account for overdispersion, an observation level random effect was included in the models, where each data point is allocated a unique level of a random effect (Harrison, 2014). The pollination treatment was treated as a fixed effect, and the plant that the fruit was grown on and the maternal species was treated as a random effect. All pairwise differences were calculated using Tukey's pairwise contrasts.

Reproductive isolation

Reproductive isolation values (Coyne & Orr, 1989; Ramsey et al., 2003; Coyne & Orr, 2004) were calculated for: fruit development, seed mass, and percentage of embryos formed according to the formula:

$$RI_{trait} = 1 - \frac{\text{(mean hybrid trait value)}}{\text{(mean intraspecific trait value)}}$$

For each trait, a value ranging from zero (no reproductive isolation) to one (complete reproductive isolation) was returned. The $RI_{fruitdevelopment}$ was calculated using the percentage of developed fruits as the trait value. In the few instances where negative *RI* values were returned, they were set to zero (Gervasi et al., 2017) before calculating RI_{total}.

Phylogenetic distance and reproductive isolation

To examine the phylogenetic relationship among Australian *Cryptostylis* species, a phylogeny was generated for *C. erecta* (*N* = 1), *C. hunteriana* (*N* = 2), *C. leptochila* (*N* = 2), *C. ovata* (*N* = 2), and *C. subulata* (*N* = 1), with *Rimacola elliptica, Leporella fimbriata*, *Epiblema grandiflorum*, and *Diuris orientis* as outgroups. An exome-capture approach using the 315 single-copy orthologous genes identified in Deng et al. (2015) was used following the methodology of Peakall et al. (in prep.). Of the 315 orthologous genes, 211 were successfully sequenced for thirteen individuals of Australian *Cryptostylis* and the additional five outgroup

species. Loci were manually checked in Geneious 9.1.8 (Kearse et al., 2012) and alignment errors were corrected, resulting in a total of 186,827 base pairs. A phylogenetic tree was inferred by Maximum Likelihood analysis in IQTREE 2.0 (Nguyen et al., 2014), using the best-fit substitution model (GTR+F+R2) automatically selected by ModelFinder according to the Bayesian Information Criterion (Kalyaanamoorthy et al., 2017). Branch supports were obtained with the built-in ultrafast bootstrap algorithm (Hoang et al., 2018) from 10000 iterations. The phylogeny was visualised and midpoint rooted in R v 3.5.1 using the 'ape' (Paradis et al., 2004) and 'phytools' (Revell, 2012) packages.

Phylogenetic distances between a representative individual of the species used in the cross pollination experiment were calculated using the 'distTips' function in the 'adephylo' package (Jombart & Dray, 2010) in Rv3.5.1 (R Core Team, 2018). To test for an association between phylogenetic distance values and the reproductive isolation values for each direction of each cross (Kay & Schemske, 2008), Kendall's τ rank correlation was calculated (Pinheiro et al., 2015). To allow visual interpretation of phylogenetic distance and reproductive isolation values simultaneously, averaged pairwise reproductive isolation values for species pairs were calculated by taking the average of the two RI_{total} values (one from each direction of the cross) and presented on the relevant branches of a phylogeny along with the relevant phylogenetic distance values.

Ploidy

Genome size

To investigate whether there were differences in genome size between *Cryptostylis* species, which can reflect differences in chromosome number and ploidy level, flow cytometry was conducted on a Attune NxT acoustic focusing flow cytometer as per Doležel & Bartoš (2005) with a Tris.$MgCl_2$ buffer. Pollen was used as this tissue is not prone to progressive partial endoreplication, a potential problem in orchid flow cytometric analyses (Trávníček et al., 2015). To test for the presence of different ploidy levels within and between populations, a minimum of fifteen individuals were sampled per species across at least three different populations. Seed samples from the greenhouse cross-pollination experiment were analysed to investigate their

genome size and thereby confirm their hybrid origin. While these seeds were hand cross-pollinated, the possibility of apomictic seed formation, which is known in the Orchidaceae (Sorensen et al., 2009; Hojsgaard & Hörandl, 2019), remains. Seed from three fruits of both directions of each hybrid combination were analysed, with the exception of *C. subulata* x *C. erecta* and *C. ovata* x *C. subulata*, for which seed from two fruits were analysed. For all samples, one of *Vicia faba* (2C = 26.90 pg), *Triodia wiseana* (2C = 5.307 pg), or *Triodia longiceps* (2C = 2.928 pg) were chopped with samples as standards depending on the genome size of the sample. Data were analysed in Flowing Software v2.5.1, freely accessible fromhttp://flowingsoftware.btk.fi/index.php?page=3, and genome sizes calculated using the standards as per Doležel & Bartoš (2005).

Chromosome counts

To confirm whether patterns observed in the flow cytometric analyses were indicative of ploidy, chromosome counts were conducted for *C. leptochila*, which does not have a published chromosome number. While the chromosome number of *C. hunteriana* is also unknown, due to its protected status and the destructive nature of the counts they were not attempted - inferences were drawn from flow cytometry data alone. In conducting chromosome counts for *C. leptochila*, root tips several millimeters long were excised from plants and refrigerated for 48 hours, after which they were transferred to 3:1 ethanol acetic acid at room temperature for three hours. Root tips were stored frozen in the ethanol acetic acid until squash preparation. Root tips were hydrolysed in 1M HCl at 60º for 12 minutes as per (Peakall & James, 1989) before being briefly rinsed in 45% acetic acid. The terminal millimeter of the root tips were excised under a dissecting microscope and placed on a slide in a drop of aceto-orcein. Preparations were then squashed, and the cover slip tapped with a metal rod.

Self-incompatibility

Self-incompatibility values were calculated for: fruit development, seed mass, and percentage of embryos formed according to the formula:

$$SI_{trait} = 1 - \frac{\text{(mean self trait value)}}{\text{(mean intraspecific trait value)}}$$

For each trait, a value ranging from zero (no self-incompatibility) to one (complete self-incompatibility) was returned. The $SI_{fruitdevelopment}$ was calculated using the percentage of developed fruits as the trait value. All negative *SI* values were set to zero before calculating $SI_{total,}$ which was calculated by summing the trait values.

RESULTS

Pre-pollination barriers

Species niche overlap

MaxEnt modelling revealed extensive niche overlap between the eastern Australian *Cryptostylis* species. All MaxEnt models (Supplementary Figure A) returned area under the curve (AUC, common indicator of model performance) values greater than 0.9 (*C. erecta* 0.99, *C. hunteriana* 0.99, *C. leptochila* 0.97, *C. subulata* 0.94), indicating a good discrimination ability of the model (Pearce & Ferrier, 2000). Thus, the predicted geographic niches are plausible. The difference between the model calibration and evaluation AUC scores was low, as were the model omission rate presence thresholds, indicating that the models were not overfitted (Radosavljevic & Anderson, 2014). Niche overlap values (Supplementary Table A) ranged from 0.62 (*C. erecta* and *C. leptochila*, least similar) to 0.88 (*C. erecta* and *C. hunteriana*, and *C. leptochila* and *C. subulata*, most similar).

Site level species co-occurrence

Approximately half of the surveyed populations contained two or more *Cryptostylis* species. Of the total 43 populations spanning a distance of over 1,300km that were included in the analysis, 23 populations (53.5%) contained one species only, and the remaining 20 populations (46.5 %) contained two or more species. *Cryptostylis leptochila* had the highest percentage of sole-species populations (54.5 %, 6/11), and *C. subulata* the highest percentage of multi-species populations (75 %, 18/24, Supplementary Figure B). Of the 20 populations with two or more species present, all possible species pairs except *C. leptochila* and *C. hunteriana* were recorded (Supplementary Figure B). Three populations had three species present, in all three cases being *C. erecta, C. hunteriana,* and *C. subulata* (Supplementary Figure B).

Flowering phenology

Across the seven sites, 268 *C. erecta* plants, 87 *C. hunteriana* plants, 78 *C. leptochila* plants, and 205 *C. subulata* plants were surveyed. At all sites, flowering phenology of the co-occurring species showed extensive overlap (Supplementary Figure C). Only two instances were observed where a species growing in a sympatric site was flowering at a time that did not overlap at all with the co-occurring species (T1, *C. leptochila* flowering but not the co-occurring *C. subulata*, Fitzroy Falls, and T4, *C. erecta* flowering but not the co-occurring *C. subulata*, Wogamia, Supplementary Figure C). Of the plants with open flowers, there was an average of 1.44 (max 4) flowers open per inflorescence for *C. erecta*, 1.23 (max 3) flowers open per inflorescence for *C. hunteriana*, 1.43 (max 4) flowers open per inflorescence for *C. leptochila*, and 1.26 (max 4) flowers open per inflorescence for *C. subulata*. For *C. erecta*, the average number of flowers per raceme was 6.10 (range 1-14), for *C. hunteriana* average 8.34 flowers per raceme (range 3-16), for *C. leptochila* average 11.79 flowers per raceme (range 3-21), and *C. subulata* average 6.35 flowers per raceme (range 2-16).

Potential for interspecific pollen transfer

Across the seven sympatric sites measured at specific time internals, the potential for interspecific pollen transfer (value ranges 0-1) due to overlapping flowering time for *C. erecta* ranged from 0 (Time period 4 (T4), South Nowra) to 0.91 (T4, Bombaderry Creek), for *C. hunteriana* ranged from 0.40 (T4, Meroo) to 0.83 (T4, Erowal Bay), for *C. leptochila* ranged from 0.03 (T5, Fitzroy Falls) to 0.68 (T2, Fitzroy Falls), and for *C. subulata* ranged from 0 (T1, Fitzroy Falls) to 0.97 (T5, Fitzroy Falls, S3).

Inter-species pollinator movement

Wasps were observed to move between and reverse into (the position required for pollination) all three artificially presented species of *Cryptostylis*. In total, 84 wasps were marked over the experimental period, 31 of which were recaptured at least once. There was no significant difference in the number of wasps visiting each species ($G = 5.39$, $P = 0.07$). Overall, 20.24 %

(17) of the observed 84 wasps visited two or more species of *Cryptostylis*. These 17 wasps represented 54.84 % of the wasps recaptured at least once. Of the total 31 recaptured wasps, five (16.13%) visited all three species, 12 wasps (38.71) visited two species, and 14 (45.16%) wasps revisited the same species only.

Post-pollination barriers

No flowers where pollinators were excluded set fruit, demonstrating fruit set to be dependent on a vector in *C. erecta, C. leptochila, C. subulata,* and *C. ovata*.

Comparisons among hybrid treatments and with intra-species controls within a maternal species

Fruit development

All pollination treatments with *C. erecta* and *C. leptochila* as the mother plant developed into fruits (Supplementary Table B). For the treatments with *C. subulata* as the mother plant, a minimum of one fruit did not develop, with *C. subulata* x *ovata* having two undeveloped fruits (out of 11), and *C. subulata* x *C. leptochila* having six undeveloped fruits (out of 11), (Supplementary Table B). There was no significant difference in the proportion of developed fruits between pollination treatments (Fischer's Exact Test, $P = 0.12$).

Seed mass

In all species, the hybrid pollination treatments had lower average seed masses than did the control intra-species crosses (Figure 1). This difference was significant in seven of the twelve hybrid treatments, In *C. erecta* the seed mass of the hybrid treatments was significantly lower than those of the control intra-species treatments ($P < 0.001$, Figure 1). In *C. subulata*, two hybrid treatments had significantly lower seed masses than the control intra-speciess treatments and the hybrid cross *C. subulata* x *C. ovata* ($P < 0.001$, Figure 1). In *C. ovata,* two hybrid treatments *C. ovata* x *C. erecta* ($P = 0.001$) and *C. ovata* x *C. leptochila* ($P = 0.026$) had

significantly lower seed masses than the control intra-species treatment (Figure 1). In contrast to the other species, while hybrid seed masses were lower in the hybrid treatments than in the intra-species controls in *C. leptochila*, these differences were not significant.

Embryo formation

In all species, the hybrid pollination treatments had a lower proportion of formed embryos than did the control intra-species treatments. This difference was significant in eight of the eleven assessed hybrid treatments (the twelfth treatment, *C. subulata* x *C. leptochila* did not produce enough seeds for embryos to be counted). In *C. erecta*, the hybrid treatments had a significantly lower proportion of formed embryos than did the intra-species cross treatment ($P < 0.001$, Figure 2). In *C. subulata*, no embryo formation data was able to be collected for the treatment *C. subulata* x *C. leptochila* due to the low number of seeds set. The hybrid treatment *C. subulata* x *C. erecta* had a significantly lower proportion of formed embryos than did the intra-species control treatment and the hybrid treatment *C. subulata* x *C. ovata* ($P < 0.001$, Figure 2). In *C. leptochila*, two hybrid treatments *C. leptochila* x *C. ovata* ($P = 0.003$) and *C. leptochila* x *C. subulata* ($P = 0.015$) had significantly lower proportions of formed embryos than did the intra-species control treatments (Figure 2). In *C. ovata*, two hybrid treatments had significantly lower proportions of formed embryos than did the intra-species control treatments and the hybrid cross *C. ovata* x *C. subulata* (*C. ovata* x *C. leptochila* vs. control $P = 0.002$, vs. *C. ovata* x *C. subulata* $P = 0.008$; *C. ovata* x *C. erecta* vs. both treatments $P < 0.001$, Figure 2).

Post-pollination reproductive isolation

Varying degrees of reproductive isolation was observed in all interspecies hybrid pairs, with *C. subulata* x *C. ovata* having the lowest RI_{total} of 0.24, and *C. subulata* x *C. erecta* having the greatest RI_{total} of 1.62 (Supplementary Table B)

Reciprocal comparisons between pairwise maternal and paternal species

Fruit development

There was a significant difference in the proportion of fruits developed depending on the direction of the cross in *C. leptochila* x *C. subulata* (all 10 developed) and *C. subulata* x *C. leptochila* (five developed, six did not, $P = 0.003$). There were no other significant reciprocal differences in fruit development.

Seed mass

Significant reciprocal differences in seed mass were observed for all hybrid crosses that included *C. leptochila* ($P < 0.001$), in all three of which seed masses were greater with *C. leptochila* as the maternal not paternal species, reflected in the lower maternal and higher paternal RI_{seed} values (Table 1). A significant reciprocal difference in seed mass was also observed between *C. subulata* x *C. erecta* and *C. erecta* x *C. subulata* ($P < 0.001$), in which higher seed masses, and a lower RI_{seed} was observed with *C. erecta* as the maternal species (Table 1). No significant reciprocal differences in seed mass were observed in the other pairwise crosses.

Embryo formation

For only one reciprocal pairwise cross was a significant difference in the percentage of embryos formed observed: *C. subulata* x *C. erecta* and *C. erecta* x *C. subulata*, where the percentage of embryos formed was greater with *C. erecta* as the paternal species (Table 1).

Phylogenetic distance and reproductive isolation

Two clades with high bootstrap support were present in the Australian *Cryptostylis* phylogeny - one containing *C. erecta* and *C. leptochila*, and the other *C. ovata, C. hunteriana* and *C. subulata* (Supplementary Figure E). There was a significant positive correlation between the total amount of reproductive isolation between species and their phylogenetic distance (Kendall's $\tau = 0.75$, *P*

< 0.001). The sister species pair *C. ovata* - *C. subulata* (allopatric) had an RI_{total} of 0.32, while the sister species pair *C. leptochila* - *C. erecta* (sympatric) had a higher RI_{total} of 0.72. In species pairs of similar phylogenetic distance, where one was allopatric and the other pair sympatric, the sympatric pair always had higher reproductive isolation (Figure 3).

Ploidy

Genome size

All Australian *Cryptostylis* species had different genome sizes. Genome size was smallest in *C. hunteriana* (1C = 3.34 ± 0.04 pg), slightly larger in *C. subulata* (1C = 4.66 ± 0.03) and *C. erecta* (1C = 5.39 ± 0.05), followed by *C. ovata*, which was approximately double this size (1C = 11.35 ± 0.13), and the even larger *C. leptochila* (1C = 29.6 ± 0.67, Table 2), which was approximately six times larger than *C. erecta* and *C. subulata*. Genome sizes were consistent within and between populations of the same species, as exemplified in the low standard errors that are based on a minimum of eighteen individuals and two populations (Table 2). Genome sizes of hybrid seeds were approximately an average of the genome sizes of the parents (Supplementary Table C), confirming that seeds were not of an apomictic origin.

Chromosome counts

Flow cytometry demonstrated *C. leptochila* to have a genome size approximately six times that of *C. erecta* and *C. subulata*, indicating a major difference in ploidy level between the species. Chromosome counts supported the status of *C. leptochila* as a high polyploid, with approximately 492 chromosomes (Supplementary Figure D), and several similar cells with this magnitude of chromosomes present being observed. Due to the large number of chromosomes present, this count may not be precise, however, it does indicate a difference in ploidy level. This count is several times greater than existing chromosome counts for other Australian *Cryptostylis* species (*C. erecta* = 56, *C. subulata* = 64, *C. ovata* ≈ 187, (Peakall & James, 1989; Dawson et al., 2007), Table 2).

Self-pollination treatments

Fruit development

All self-pollinated flowers developed fruit with two exceptions: in *C. subulata*, for which the self-pollination treatment produced three undeveloped fruits (out of 11), and in *C. ovata,* for which the self-pollination treatment produced one undeveloped fruits (out of eight, Supplementary Table B). There was no significant difference in the proportion of developed/undeveloped fruits between intra-species control and self-pollination treatments across all tested species.

Seed mass

The seed masses in the self-pollination treatment were significantly lower than in the intraspecific crosses for *C. subulata* ($P < 0.001$) and *C. erecta* ($P = 0.01$, Figure 1). The seed masses in the self-pollination treatments in *C. leptochila* and *C. ovata* were not significantly different to those of their respective intra-species controls.

Embryo formation

For *C. subulata* and *C. erecta*, the proportion of formed embryos in the self-pollination treatments were significantly lower than in the intraspecific crosses ($P < 0.001$, Figure 2). The proportion of formed embryos in the self-pollination treatments in *C. leptochila* and *C. ovata* were not significantly different to those of their respective intra-species controls. The variance in embryo formation was significantly greater in the self-pollination treatment than in the intra-species control for *C. erecta* ($P = 0.028$) and *C. ovata* ($P = 0.037$, Table 3).

Self-incompatibility values

Cryptostylis leptochila and *C. ovata* had lower SI_{total} values (0.12 and 0.33 respectively) than did *C. subulata* and *C. erecta* (1.54 and 0.94 respectively, Supplementary Table B).

DISCUSSION

Pre-pollination barriers to hybridisation

The hypothesis that pre-pollination barriers do not prevent hybridisation in eastern Australian *Cryptostylis* was supported. MaxEnt modelling indicated an extensive overlap in climatic niche between the species, further reflected in that approximately half the surveyed sites containing more than one *Cryptostylis* species. Niche overlap and sympatry are not uncommon between sexually deceptive orchid taxa, being prevalent within both the European sexually deceptive *Ophrys* (Schlüter et al., 2007; Xu et al., 2011; Gervasi et al., 2017; Tsiftsis & Djordjević, 2020) and Australian sexually deceptive genera (Mant et al., 2005a; Peakall et al., 2010; Whitehead & Peakall, 2014; Phillips et al., 2017a). *Cryptostylis* flowering times were also found to extensively overlap. Finally, while naturally occurring intra-species pollen transfer was not explicitly observed, a mark recapture experiment demonstrated that pollinators were capable of transferring pollen between the different *Cryptostylis* species that may be present at a single site. This result that pre-pollination barriers do not prevent hybridisation is unusual among sexually deceptive orchids, in which reproductive isolation is normally formed by pre pollination barriers such as floral isolation, which is of particular importance in sympatric scenarios (Ayasse et al., 2010; Xu et al., 2012; Whitehead & Peakall, 2014; Breitkopf et al., 2015).

Post-pollination barriers to hybridisation

Post-pollination barriers were found to contribute to reduced hybrid fitness, indicating that they play a major role in preventing hybridisation. While it was hypothesised that fruit, seed, and embryo development would be reduced in the hybrid treatments compared to the intraspecific control, this prediction was only supported for seed and embryo development, with fruit development not being found to differ between pollination treatments. For *C. subulata* the number of fruits was reduced in some intra-specific treatments, and the lack of significant differences may be due to low sample size. However in *C. erecta*, *C. leptochila*, and *C. ovata*, the numbers of fruits set were equal to that of the intraspecies control, indicating that post-

pollination incompatibilities do not affect the swelling of the ovary in *Cryptostylis* - congruent with the self-incompatibility results.

The reduced seed and embryo development in the hybrid treatments indicate that both pre-zygotic (reflected in reduced seed set) and post-zygotic (reflected in the reduced proportion of formed embryos) post-pollination barriers are impeding hybridisation between Australian *Cryptostylis* species. However, this reduction in hybrid fitness does not fully explain the absence of hybrids in natural populations - fully formed hybrid embryos were observed, which should they not subsequently succumb to hybrid lethality, may potentially germinate. As was predicted in a similar scenario in food-deceptive orchids (Scopece et al., 2008), it is likely that hybridisation is impeded by later acting hybrid lethality, however it remains unknown whether this occurs at an early stage of development, or whether adult plants incapable of reproduction may exist undetected in natural populations. The latter scenario is feasible as *Cryptostylis* species can only be conclusively identified based on floral morphology, so non-flowering hybrid plants would remain undetected. To investigate the stage at which hybrid lethality potentially occurs, germination trials could be implemented, which would require the capability to culture the mycorrhizal fungi of *Cryptostylis*. Although mycorrhizal fungi that associate with the five Australian *Cryptostylis* species have recently been identified (Arifin *et al.,* in prep.), further research would be required to develop culture techniques suitable to conduct germination trials.

The patterns of reproductive isolation observed in the interspecies crosses were complex, with extensive variation in the degree of reproductive isolation observed within and between treatments. This complex variation may indicate that different mechanisms contribute to the formation of post-pollination reproductive isolation, potentially including ploidy, self-incompatibility, and phylogenetic distance and reinforcement, which are addressed individually below.

Ploidy

Different ploidy levels and chromosome numbers are present within Australian *Cryptostylis*. Flow cytometry demonstrated that ploidy levels remained constant within and between

populations. *Cryptostylis erecta* and *C. subulata* had both similar genome sizes and chromosome counts, and are likely the same ploidy level with a gain or loss in some chromosomes explaining the difference in counts (56 cf. 64 (Peakall & James, 1989; Dawson et al., 2007)). *Crypostylis ovata* and *C. leptochila* both showed evidence of polyploidy - with genome sizes and chromosome counts several times greater than those for both *C. erecta* and *C. subulata*. *Cryptostylis leptochila* had the highest ploidy level, with a genome size six times greater, and a chromosome count ten times greater, than that of *C. erecta* and *C. subulata*. Based on genome size comparisons to *C. erecta* and *C. subulata*, this indicates that *C. leptochila* is at minimum a dodecaploid (12n), or if based on the lowest chromosome count in the genus *Cryptostylis* of $2n$ = 42 (x=21) (*C. arachnites* (Larsen, 1966)), gives 24n. For *C. ovata* the difference with *C. erecta* and *C. subulata* was two (genome size) to three (chromosome count) times greater. Based on genome size comparisons within Australian *Cryptostylis*, *C. ovata* is indicated to be at minimum a tetraploid, or using the genus minimum chromosome count of $2n$ = 42 as a base number, an octaploid. While no chromosome counts were conducted in the present study or prior for the rare *C. hunteriana*, its genome size indicates its ploidy level to be similar to that of *C. erecta* and *C. subulata*. Within the Diurideae there is a precedent for multiple ploidy levels occurring within a genus, as demonstrated in *Acianthus*, *Diuris*, *Microtis*, *Prasophyllum*, and *Thelymitra* (Peakall & James, 1989; Dawson et al., 2007).

While higher numbers of chromosomes have been reported outside of the Orchidaceae: (2n = 1440 in the fern *Ophioglossum reticulatum* (Ghatak, 1977), 2n = 596 in the monocotyledon *Voanioala gerardii* palm (Johnson et al., 1989), and 2n = 640 in the dicotyledon *Sedum suaveolens* (Uhl, 1978), the present count for *C. leptochila* far exceeds the thus far largest known in the Orchidaceae, which is 2n = 240 in the dodecaploid *Epidendrum cinnabarium* (da Conceição et al., 2006). Other high extremes of ploidy levels in orchids include dodecaploid species in *Eulophia* 2n = 84 (Poggio et al., 1986), decaploids and dodecaploids 2n = 100 and 120 in the *Gymnadenia conopsea* aggregate (Trávníček et al., 2011), and the decaploid Zeuxine strateumatica 2n = 100 (Mehra & Vij, 1972). Within the Diurideae, 2n = 93 has been reported in *Thelymitra* (Dawson et al., 2007). In this context, *C. leptochila* has one of highest ploidy levels observed in an orchid, an unsurprising result given it has the largest chromosome count known among the Orchidaceae - being approximately twice the previous highest count. Polyploidy may

offer a selective advantage through its association with a high level of heterozygosity and genetic diversity, low rates of inbreeding depression and an associated high tolerance to selfing, and through gene duplication fostering biochemical diversity and the evolution of new functions (Levin, 1983; Soltis & Soltis, 2000). These factors may allow polyploids to survive in a broader ecological niche than their diploid progenitors (Levin, 1983; Ramsey & Schemske, 1998), which in combination with a tolerance for self-pollination and the ability to develop novel interactions (eg. with pollinators) makes polyploids ideal colonisers (Soltis & Soltis, 2000; Te Beest et al., 2012). Indeed, a review by Pandit et al. (2011) found that polyploid plants are more likely to be invasive, while diploid plants are more likely to be endangered. It is of interest that the only threatened Australian *Cryptostylis* species, *C. hunteriana*, also has the lowest genome size, though a chromosome count (not conducted in the present study due to its destructive nature) would be needed to draw further conclusions.

It is evident that polyploidy and chromosome number is a major barrier to hybridisation in *Cryptostylis* - all crosses between species of different chromosome numbers and/or ploidy levels displayed a reduced level of fitness in comparison to the intra-species controls. This observed reduction in hybrid fitness is congruent with expectations - differences in chromosome number and/or ploidy level are known to result in interspecific incompatibility and manifest in reduced fitness of hybrid seed, if it is formed (Stebbins, 1958; Levin, 1978; Murfett et al., 1996; Watanabe et al., 1996; Tong & Bosland, 2003). However, while a reduction in hybrid fitness may occur, ploidy differences are an imperfect barrier to gene flow (Kolář et al., 2017), as reflected in the formation of some seeds and embryos in hybrid treatments of differing ploidy levels. In some cases, inter-ploidy crosses can produce seed and/or further result in the production of fertile hybrid plants (Zillinsky, 1956; Asker, 1971; Evans, 1974; Castro et al., 2011; Kolář et al., 2017). Specifically, in orchids, fertile hybrids occur between a hexadecaploid and an octaploid in *Zygopetalum* (Gomes et al., 2018), between diploid and tetraploid *Dactylorhiza* (Aagaard et al., 2005; Ståhlberg, 2007; De Hert et al., 2011), and between diploid and tetraploid *Epidendrum* (Pinheiro et al., 2010; Moraes et al., 2013; Marques et al., 2014; Pinheiro et al., 2016). Within the Diurideae, chromosomal and molecular evidence supports the presence of allopolyploid hybrids in *Thelymitra* (Dawson et al., 2007; Nauheimer et al., 2018). While no studies have yet investigated crosses between diploids and higher ploidy levels in

orchids, outside of the Orchidaceae examples of seed set and sometimes the production of mature adults between diploids crossed with hexaploids and octaploids exist, however in all cases fertility is reduced (Asker, 1971; Evans, 1974; Castro et al., 2011).

While genetic studies have revealed the presence of several hybrids between orchids of different ploidy levels, only two studies, both between different species of tetraploid and diploid *Epidendrum*, have hand cross-pollinated wild orchids of different ploidy levels (Pinheiro et al., 2010; Pinheiro et al., 2016). Neither study measured seed mass, nor the related measure of pollen tube growth, however in both cases inter-species seed viability was found to be similar to that of the parental intra-species control, and also similar in interspecies reciprocal crosses between ploidy levels (Pinheiro et al., 2010; Pinheiro et al., 2016). It was subsequently confirmed that there was no significant difference in seed viability between these treatments (Pinheiro et al., 2016). The results in *Cryptostylis* are congruent with these findings, in that in crosses between plants of different ploidy levels (all except *C. subulata* x *C. erecta*), there was no difference in the percentage of formed embryos depending on the paternal/maternal order of the cross. However, in contrast to the results from *Epidendrum*, in six out of the nine interploidy crosses, the percentage of formed embryos was lower in the hybrid treatment than in the pure-species controls. This effect may be due to the greater inter-species differences in ploidy observed in *Cryptostylis* compared to the diploid x tetraploid crosses conducted in *Epidendrum* (Pinheiro et al., 2010; Pinheiro et al., 2016). Alternately, differences in ploidy may not provide the largest contribution to reproductive isolation, or its effect may be eclipsed by other factors. Indeed, the lowest percentage of embryos formed was found in a cross within the same ploidy level, while the highest percentages of embryos formed were found in crosses between different ploidy levels. Similarly, between ploidy crosses often had greater seed masses than did within-ploidy crosses. These results indicate that differences in inter-species compatibility are not controlled by ploidy differences alone, and other factors may have an influence, such as unilateral incompatibility, phylogenetic placement, and reinforcement.

Self-(in)compatibility

Contrary to the hypothesis of there being equal fruit formation, seed mass, and percentage of embryos formed in the self-pollination and control intra-species treatments, in some *Cryptostylis* species self-incompatibility was observed. In *C. erecta* and *C. subulata*, seed masses and the proportion of formed embryos were reduced in the self-pollination treatments, demonstrating self-incompatibility. *Cryptostylis erecta* and *C. subulata* are therefore the first known examples of self-incompatibility in the Diurideae tribe, and only the second within the Orchidoideae subfamily. Contrastingly, in *C. leptochila,* for none of the measured traits did the self-pollination treatment display a significant difference in fitness relative to the intra-species control, demonstrating a high degree of self-compatibility. In many cases, polyploidy is associated with a breakdown of self-incompatibility systems due to the presence of additional *S* alleles that may provide the requisite recognition factor (de Nettancourt, 1977; Richards, 1997; Entani et al., 1999), though see (Mable, 2004). It is plausible that *C. leptochila* may have lost its SI mechanism as a result of its polyploidy. *Cryptostylis ovata* is suggested to be a midpoint on the spectrum of self(in)-compatibility. While no significant reduction in fitness was observed in the self-pollination treatment compared to the control intraspecies cross treatment in *C. ovata*, *C. ovata* had a greater SI_{total} value than did the self-compatible *C. leptochila*, driven by its lower mean seed mass and embryo formation values. Further, the variance in the mean seed mass and percentage of fully formed embryos in *C. ovata* was greater in the self-treatment than in the control intraspecies cross treatment, and the variance in the percentage of fully formed embryos differed significantly to the control. This variance potentially indicates the presence of both self-compatible and self-incompatible individuals. Similar results were obtained in species of the distantly related genus *Acianthera* (subfamily Epidendroideae), which compared to other species in the genus had intermediate levels of fruit set and embryo formation (Borba et al., 2011). Some of these species were suggested to have a mixed mating system comprising both self-incompatible and self-compatible individuals (Borba et al., 2011). In three out of the four species tested in the present study (all except *C. subulata*), the variance in the seed mass and percentage of fully formed embryos was greater in the self-treatments than in the intraspecies control. This result indicates a high degree of variation in self-compatibility within species, consistent with the expectation that self(in)-compatibility operates as a spectrum encompassing a range of states

from completely self-compatible to completely self-incompatible (Schemske & Lande, 1985). A similar degree of variation in self-incompatibility within a species was observed in the Epidendroideae (Borba et al., 2001; Borba et al., 2011).

Hand cross-pollinations indicated both the presence of pre- and post-zygotic self-incompatibility systems in the four tested *Cryptostylis* species. While differences in chromosome number can be expected to result in eventual hybrid failure (Levin, 1978; Watanabe et al., 1996; Tong & Bosland, 2003), the functioning of pre-zygotic incompatibility systems may not be affected (Murfett et al., 1996). Reduced seed masses, which are indicative of a failure of pollen tube growth and pre-zygotic self-incompatibility, were found in the self-pollination treatments for *C. erecta* and *C. subulata*. Similar to the fruit development observed in the inter-specific crosses, there was no significant reduction in the number of fruits that developed in the self-pollination treatments compared to the intra-species control. Ovary development in orchids is induced by the recognition of compatible pollen on the stigma (Zhang & O'Neill, 1993), a process that is inhibited in sporophytic incompatibility when incompatible pollen grains are detected on the stigmatic surface and pollen germination is prevented (Seavey & Bawa, 1986). The result of fruit development in the inter-species and self-pollination treatments may indicate that it is gametophytic incompatibility (inhibition of pollen tube growth in style, post germination), and not sporophytic incompatibility that is contributing to the observed reduced seed masses in *C. erecta* and *C. subulata* self-pollination treatments. Similar gametophytic mechanisms of self-incompatibility may be operating in the only other example of self-incompatibility in the Orchidoideae subfamily, *Bipinnula pennicillata* (Ciotek et al., 2006), suggested in the result of fruit formation with reduced seed mass in self-pollination treatments. In self-incompatible species that demonstrate sporophytic incompatibility in the Epidendroideae subfamily, fruits did not develop (Singer & Koehler, 2003; Barbosa et al., 2009; Gontijo et al., 2010; Borba et al., 2011). Reduced percentages of fully formed embryos (indicative of embryo abortion in early development stages and post-zygotic incompatibility) were also found in the self-pollination treatments for *C. erecta* and *C. subulata*. The presence of both reduced seed mass and embryo formation in individual plants indicates that self-incompatibility in *Cryptostylis* is not controlled wholly by one of either pre- zygotic (sporophytic or gametophytic incompatibility) or post-zygotic (late-acting self-incompatibility) mechanisms. Similarly, species of *Anathallis*

(Epidendroideae) were found to have different self-incompatibility reactions (Gontijo et al., 2010), indicating that the evolution of different self-incompatibility mechanisms within a single genus is not unique to *Cryptostylis*.

In the present study, cross-pollinations with *C. leptochila* as the maternal species resulted in significantly greater seed masses than did the reciprocal crosses were *C. leptochila* was the paternal species. This trend was not observed in the percentage of formed embryos. It is of interest that this pattern of maternal vs paternal parent influencing seed set was observed in *C. leptochila* - the only self-compatible species, and may suggest the presence of unilateral incompatibility. Unilateral incompatibility functions through the pollen/stigma self-(in)compatibility recognition factors affecting whether interspecific pollen is accepted (de Nettancourt, 1977). It is expected that when self-compatible pollen is deposited on a self-incompatible stigma, pollen tube growth is inhibited (Lewis & Crowe, 1958), whereas on the contrasting reciprocal cross (self-incompatible pollen on a self-compatible stigma), no inhibition is expected and a higher seed set is predicted. Through this mechanism, the self(in)compatibility of a species may affect its interspecific compatibility (de Nettancourt, 1977). Patterns of unilateral incompatibility are congruent with those observed in *C. leptochila*. When *C. leptochila* was the mother species, $RI_{seedmass}$ was lower than when it was the father species (Table 1). It is likely that the observed lower seed set when *C. leptochila* is the father species could be due to failure of pollen tube growth due to rejection of the self-compatible pollen by the self-incompatible stigma in the absence of a recognition factor. Similar patterns of unilateral incompatibility in orchids have been observed in *Dendrobium* (Pinheiro et al., 2015).

Interestingly, *C. ovata*, which demonstrated a degree of self-compatibility, did not show evidence for unilateral incompatibility when crossed with self-incompatible species - in these crosses RI_{seed} values were not lower when *C. ovata* was the mother instead of father species. Further, a pattern of unilateral incompatibility in the self-compatible *C. leptochila* was observed when *C. ovata* was crossed with it, as was also observed in crosses with the self-incompatible *C. erecta* and *C. subulata*. As unilateral compatibility manifests in reciprocal crosses between self-compatible and self-incompatible species, this result indicates a degree of self-incompatibility is

operating in *C. ovata*. This result provides further evidence of *C. ovata* being a mid point on the spectrum of self-(in)compatibility.

While inbreeding can result in reduced fitness and, similar to self-incompatibility, manifest in a reduced seed set and percentage of formed embryos (Seavey & Bawa, 1986; Barrett, 1988), the presence of unilateral incompatibility (discussed below) in *C. erecta, C. subulata,* and *C. ovata* when crossed with the self-compatible *C. leptochila* suggests that the reduced fitness of self crosses in these three species is at least partially due to the presence of an incompatibility system.

Evolution of self-incompatibility

A high incidence of self-pollination causes a reduction in genetic variation, (Charlesworth & Charlesworth, 1995), which can result in inbreeding depression and thereby decreased offspring fitness (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). Self-incompatibility may serve to avoid this reduction in fitness associated with self-pollination. However, self-incompatibility may provide little advantage in taxa that are predominantly outcrossing. Sexually deceptive orchids may have greater pollen dispersal distances and outcrossing rates than rewarding pollination strategies, because deceived pollinators patrol for mates rather than moving between nearby flowers while foraging (Peakall & Beattie, 1996; Peakall & Schiestl, 2004; Whitehead et al., 2015). This outcrossing advantage is thought to have contributed to the evolution of sexually deceptive flowers (Dressler, 1981; Nilsson, 1992).

In the present multispecies mark-recapture study, almost half of the recaptured *L. excelsa* pollinators returned to the same *Cryptostylis* inflorescence and contacted the column. Further, in a study of pollinator movement, the degree of pollinator revisitation to experimentally presented *C. ovata* flowers indicated that geitonogamous pollen transfer within clonal patches may contribute to inbreeding (Weinstein et al., 2016). The occurrence of a high rate of pollinator revisitation in the *L. excelsa* - *C. ovata* system could reflect its unusually high degree of pollinator sexual attraction to the flower, being the only Australian sexually deceptive orchid genus in which pollinator ejaculation has been observed (Coleman, 1927, 1928; Gaskett et al., 2008). Therefore, it follows that the highly attractive cues operating in the *L. excelsa* -

Cryptostylis system could encourage more frequent revisitation than in other sexually deceptive systems.

It is known that pollinator behaviour may play a role in driving transitions between pollination strategies, by selection favouring strategies that provide beneficial patterns of pollen flow that increase offspring fitness and reproductive success (Stebbins, 1970; Dressler, 1981; Devaux et al., 2014). Indeed, self-incompatibility has evolved in several other orchid species in scenarios that promote geitonogamous pollen transfer, such as pollinators that remain on plants for a long period of time and the presence of multi-flowered inflorescences (Christensen, 1992; Borba & Semir, 1999; Borba et al., 2001; Singer & Koehler, 2003; Borba et al., 2011). Given its clonality, multi-flowered inflorescences, and the high revisitation rate of its pollinator, Australian *Cryptostylis* may have a higher rate of geitonogamous pollen transfer than is typical for a sexually deceptive orchid. The evolution of self-incompatibility in Australian *Cryptostylis* may therefore be an adaptation to promote outcrossing and reduce inbreeding depression.

Phylogenetic distance and reproductive isolation: evidence for reinforcing selection

The positive correlation between the total amount of post-pollination reproductive isolation between species and their phylogenetic distance indicates an effect of relatedness on inter-species compatibility, where more closely related species have a higher compatibility. With greater time since divergence, post-zygotic isolation increases, likely through the gradual accumulation of genetic differences. This theory is supported by similar findings of a correlation between post-pollination reproductive isolation and genetic distance in *Dendrobium* (Pinheiro et al., 2015) and food deceptive Mediterranean orchids (Scopece et al., 2007; Scopece et al., 2008), and by the correlation of hybridisation potential and genetic similarity in *Orchis* species (Scacchi et al., 1990). It is noteworthy that the only hybrid treatment that failed to set any seed, *C. subulata* x *C. leptochila*, was not only between plants of very different ploidy level, but was also between the two clades within Australian *Cryptostylis*.

While overall there was a correlation between post-pollination reproductive isolation and phylogenetic distance, when comparing species pairs of similar phylogenetic distance, the

sympatry or allopatry of a species pair also played a role in determining the degree of total post-pollination reproductive isolation observed. The hypothesis that there would be greater reproductive isolation between sympatric species than allopatric species was supported. Reinforcing selection, or reinforcement, occurs in zones of sympatry where two taxa have the opportunity to hybridise (Hopkins, 2013). In the present study, when comparing between species pairs of similar phylogenetic distance, reproductive isolation was higher in hybrids of sympatric species pairs than in hybrids of allopatric species pairs. Such a finding is evidence of reinforcing selection for reproductive isolation between sympatric *Cryptostylis* species in eastern Australia, though is limited in that it was only possible to investigate four species. Due to the geographic barrier isolating *C. ovata* from its relatives, there is predicted to be no increased selection for reproductive isolation between *C. ovata* and the eastern species. Hence, when brought into artificial secondary contact, reproductive success between *C. ovata* and the eastern Australian species was comparatively high, most notably between *C. ovata* and its closest relative *C. subulata*. While the initial mechanism of speciation between the sympatric *Cryptostylis* species cannot be determined, reinforcement appears to have played a role in their divergence.

Function of pre- and post- pollination barriers in preventing hybridisation in Cryptostylis

The result that pre-pollination barriers did not prevent hybridisation in sympatric *Cryptostylis* species is in stark contrast to the trends observed in the majority of investigated sexually deceptive systems. In sexually deceptive orchids, pre-pollination barriers are typically the main reproductive barriers, with pollinator-mediated floral isolation likely to be an important driver of speciation (Ayasse et al., 2010; Xu et al., 2012; Whitehead & Peakall, 2014; Breitkopf et al., 2015). In the sexually deceptive *Chiloglottis*, differences in floral chemistry drive pollinator isolation, leading to strong pre-pollination barriers, and an absence of post pollination barriers (Peakall et al., 2010; Peakall & Whitehead, 2014; Whitehead & Peakall, 2014). Similarly, in sympatric sexually deceptive *Ophrys* species, the pre-pollination barrier of floral isolation is very strong, while later-acting post-pollination barriers are effectively absent (Mant et al., 2005b; Scopece et al., 2007; Xu et al., 2011; Gervasi et al., 2017). Pollinator isolation also appears to be functioning in *Drakaea* and clades of *Caladenia,* in which many species occur in sympatry yet attract different pollinator species (Hopper & Brown, 2007; Phillips et al., 2009; Phillips et al.,

2014). Pollinator-mediated speciation, such as is predicted to be occurring in *Chiloglottis*, *Caladenia*, *Drakaea*, and *Ophrys*, can occur very rapidly, potentially only involving one or a few genes and as a result is not associated with deep divergences between taxa (Schemske & Bradshaw, 1999; Bleiweiss, 2001; Bradshaw & Schemske, 2003; Lowry et al., 2008a; Breitkopf et al., 2013). Congruently, patterns of divergence in these taxa show rapid radiations (Supplementary Figure F, modified from Peakall et al. (in prep.), (Breitkopf et al., 2015)), and further these Australian clades contain more than twice as many species than are shown on the phylogeny.

In contrast to the pollinator specificity typical of the sexually deceptive *Ophrys*, *Chiloglottis*, *Drakaea*, and *Caladenia*, Australian *Cryptostylis* species share a pollinator. While atypical instances of pollinator sharing have been reported in some species of sexually-deceptive *Ophrys* (Paulus & Gack, 1990; Cortis et al., 2008; Gögler et al., 2009), *Drakaea*, *Chiloglottis*, and *Caladenia* (Phillips et al., 2013; Phillips et al., 2017a), in the majority of cases species sharing a pollinator occur allopatrically (Phillips et al., 2017a). The pollinator sharing in *Cryptostylis* removes the possibility for pollinator isolation and pollinator-mediated speciation. Congruently, the patterns of diversification in Australian *Cryptostylis* species show deeper branch divergences than do clades of rapidly radiated sexually deceptive orchids, which show patterns typical of pollinator mediated speciation ((Mant et al., 2005a), Supplementary Figure F, modified from Peakall et al. (in prep.)). In the absence of pollinator isolation and thereby pollinator-mediated speciation, it is likely that alternate mechanisms contribute to diversification in Australian *Cryptostylis*. A role for both geographic barriers (present for *C. ovata*, and potentially historically prior to secondary contact for other species) and changes in chromosome number in the diversification of *Cryptostylis* are suggested.

In a similar scenario to *Cryptostylis*, differences in karyotype have been found to act as a post-zygotic reproductive barrier in species of Mediterranean orchids with low-levels of pollinator specificity, where they may be responsible for driving diversification (Cozzolino et al., 2004; Cozzolino & Widmer, 2005; Moccia et al., 2007). It was proposed that karyotype differences may potentially have (a) evolved in some sympatric species, leading to sympatric speciation with retention of the ancestral pollinator species, or (b) evolved as a byproduct of allopatric

speciation, yet ensured the maintenance of species boundaries upon instances of secondary contact (Cozzolino et al., 2004). These two evolutionary scenarios could both potentially explain the pattern of pollinator-sharing and differences in chromosome number in Australian *Cryptostylis*.

Patterns of reproductive isolation in the sexually deceptive Australian *Cryptostylis* more closely resemble those observed in food deceptive orchids, where post-pollination barriers impede hybridisation (Scopece et al., 2007; Cozzolino & Scopece, 2008; Scopece et al., 2008; Pellegrino et al., 2010), than in other sexually deceptive orchids. Similar to in *Cryptostylis*, pollinator sharing and the potential for heterospecific pollen transfer also exist in food deceptive *Ophrys* (Neiland & Wilcock, 1999; Cozzolino et al., 2005), suggesting that this pollination scenario may favour the evolution of post-pollination reproductive isolation. In *Cryptostylis*, the unusual instance of pollinator sharing has likely led to the observed patterns of post-pollination reproductive isolation, which are atypical for a sexually deceptive orchid.

Conclusions and future directions

Investigation of the reproductive barriers preventing hybridisation in *Cryptostylis* has revealed this genus to be even more unique among sexually deceptive orchids than previously anticipated. Upon first recording the bizarre pollinator sharing scenario among Australian *Cryptostylis* species, Coleman suspected a role for post-pollination barriers in preventing hybridisation (Coleman, 1930a). Almost 100 years later, this predicted result has now been confirmed. In the process, this investigation of post pollination barriers has revealed some unexpected and fascinating aspects of the mating system of *Cryptostylis*. Firstly, it contains a remarkable degree of polyploidy. *Cryptostylis leptochila* was found to have the greatest known chromosome number in the Orchidaceae, being approximately twice the previous highest number of chromosomes, and further it has the second highest chromosome number in the monocotyledons. *Cryptostylis* is further unique among orchids in that it is one of few cases of confirmed self-incompatibility - in fact it is the first orchid in the Diurideae tribe known to be self-incompatible. This self-incompatibility is potentially driven by *Cryptostylis*' clonality, multi-flowered habit, and exploitation of a unique ichneumonid pollinator with a high revisitation rate. Given its

unique characteristics of post-pollination reproductive isolation, high ploidy variation, and self-incompatibility, it is likely that different mechanisms underlie the evolution and diversification of *Cryptostylis* compared to those operating in other sexually deceptive genera. Investigating the relationship between the Australian and Asiatic *Cryptostylis* species (which outnumber the Australian species) may aid in further inferring the evolutionary history of this unique genus.

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FIGURES AND TABLES

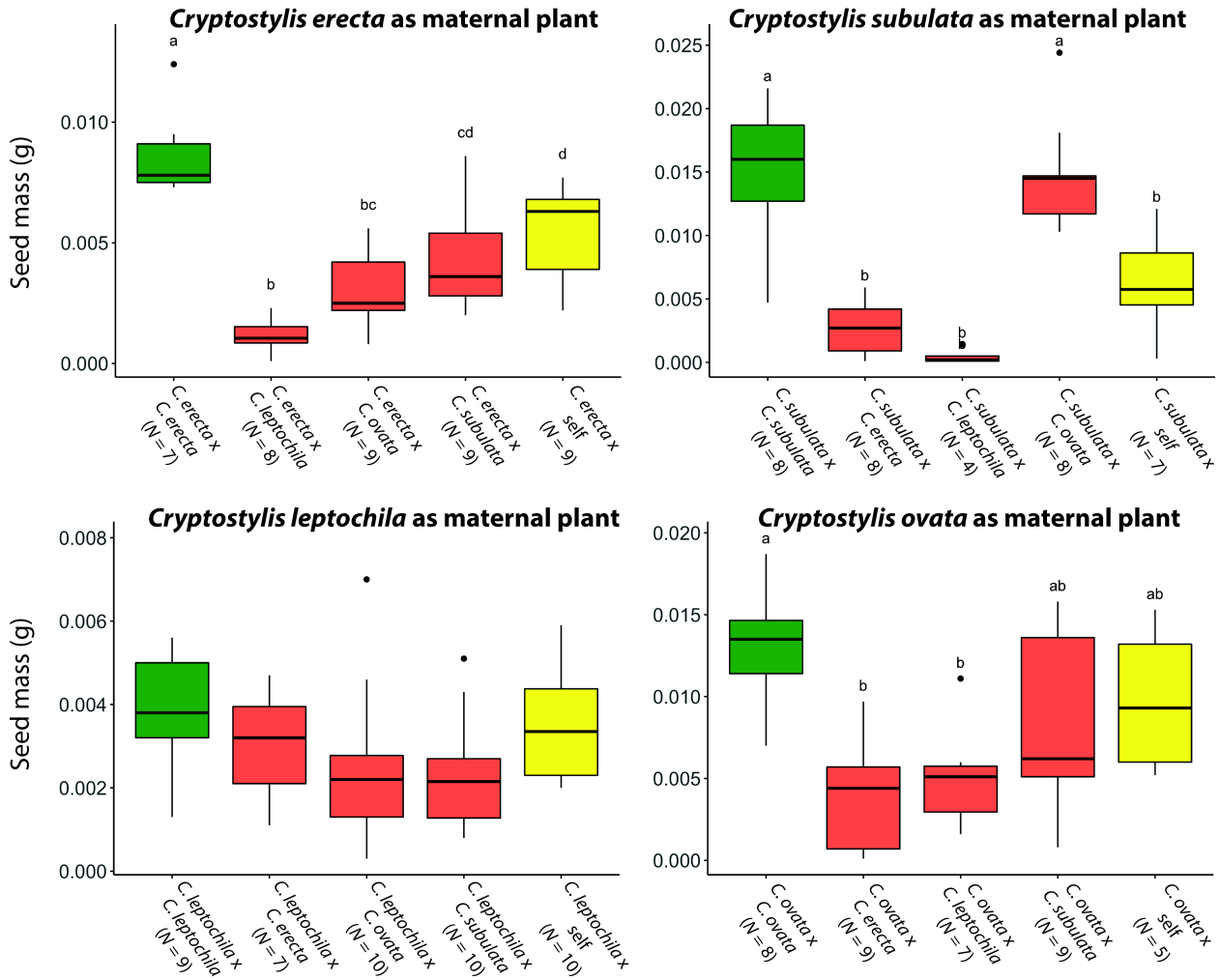

Figure 1: The mass of seeds (g) by pollination treatment. Letters denote between treatment differences (P<0.05). Boxes indicate interquartile ranges with the inner line denoting the median value. Control intraspecies treatments are shown in green, hybrid treatments in red, and self pollination treatments in yellow.

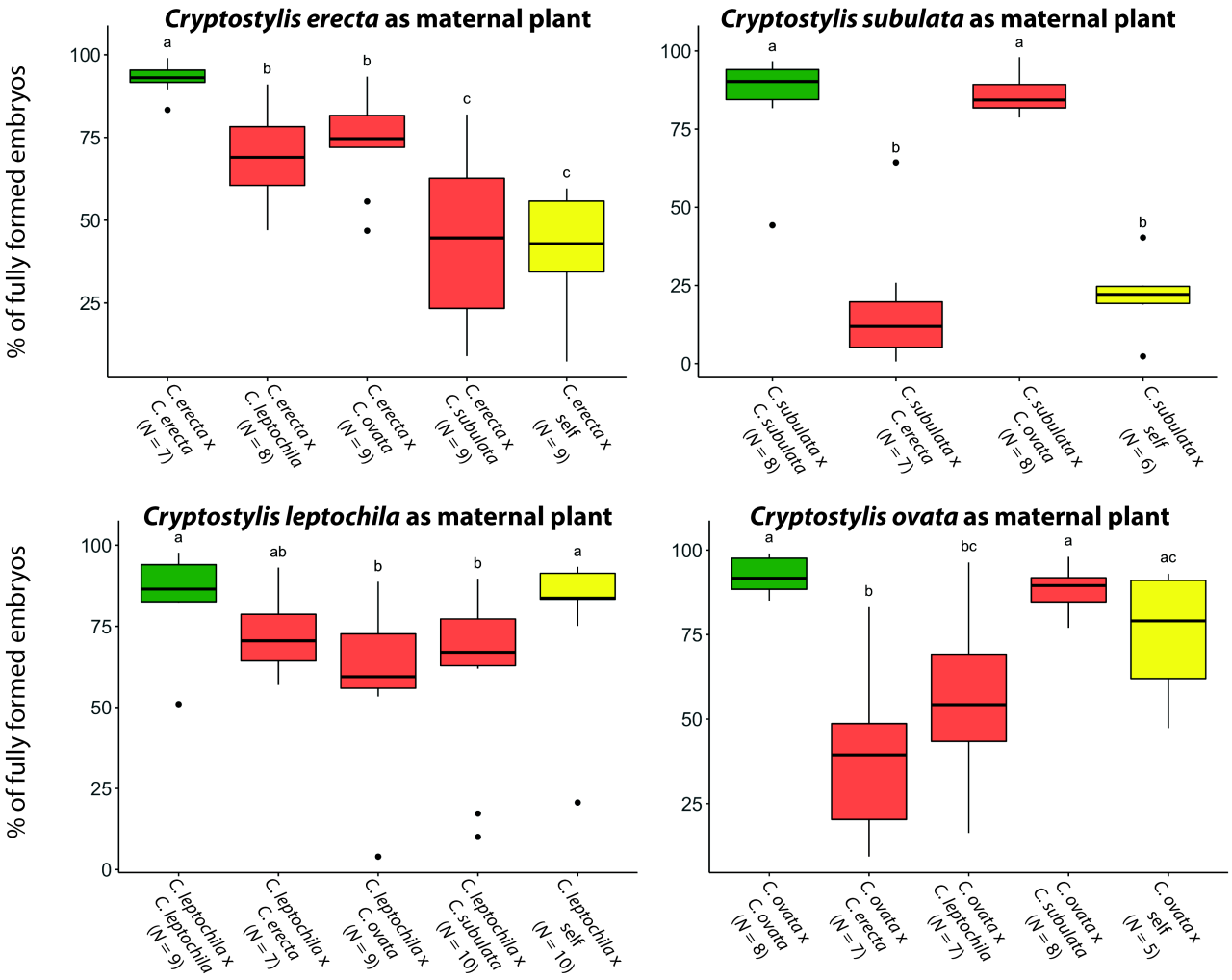

Figure 2: The median percentage of formed embryos by pollination treatment. Letters denote between treatment differences ($P < 0.05$). Boxes indicate interquartile ranges with the inner line denoting the median value. Control intraspecies treatments are shown in green, hybrid treatments in red, and self pollination treatments in yellow.

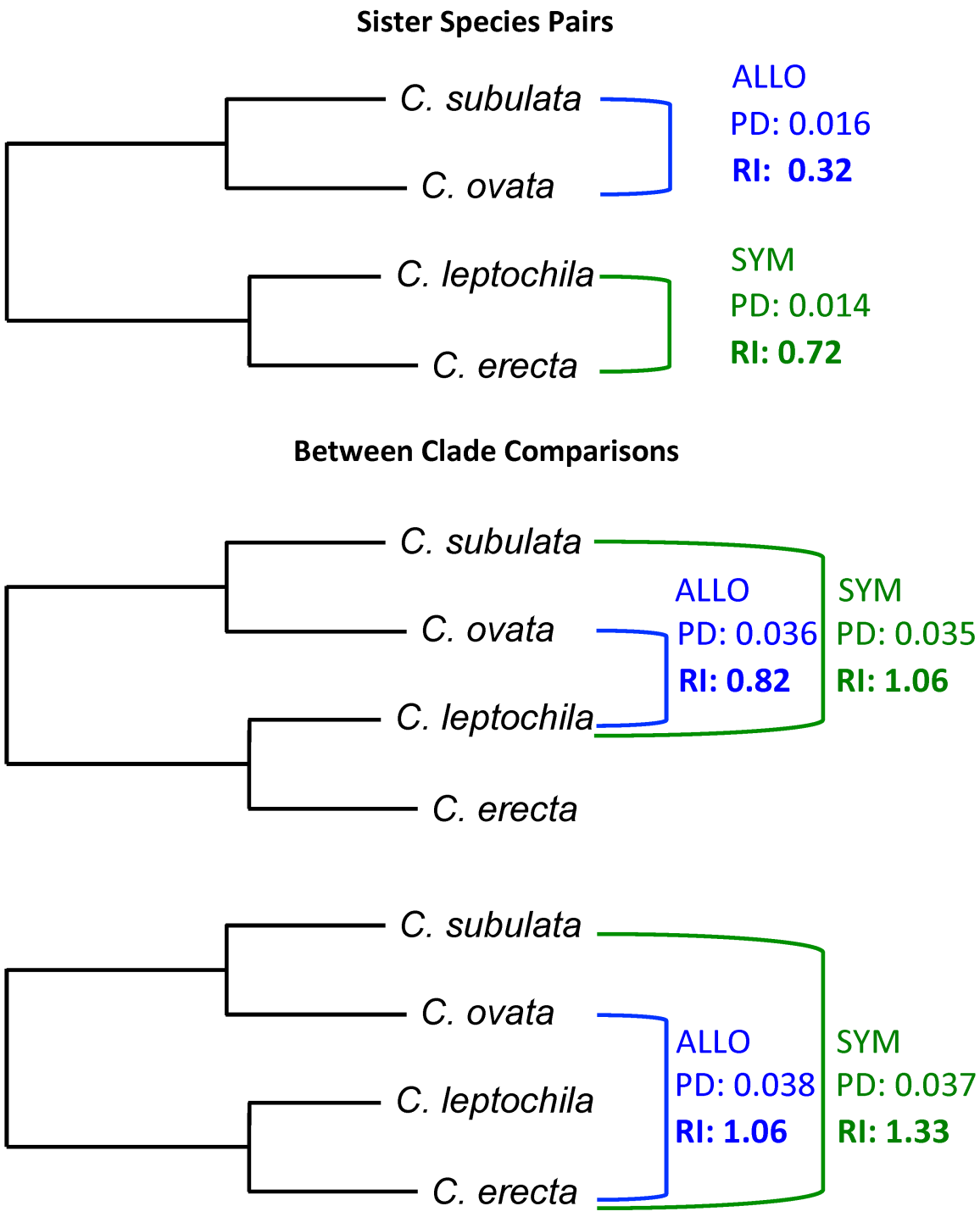

Figure 3: Averaged pairwise reproductive isolation values (RI) for allopatric (blue, ALLO) and sympatric (green, SYM) *Cryptostylis* sister species pairs and between clade pairs of similar phylogenetic distance (PD).

Table 1: Mean centered average seed masses, RI_{seed} values, percentage of formed embryos, and RI_{embryo} values for reciprocal crosses of pairs of *Cryptostylis* species. All crosses are written as *Maternal x paternal* species. * denotes a significant reciprocal difference between cross directions in a linear model ($P < 0.05$).

Species cross direction 1	**Zero-centred seed mass ± SE**	***RI seedmass***	**Species cross opposite direction**	**Zero-centred seed mass ± SE**	***RI seedmass***	**Significant reciprocal difference in average centered seed mass**
C. leptochila x C. erecta	-0.00073 ± 0.00055	0.19	*C. erecta x C. leptochila*	-0.0075 ± 0.00024	0.86	*
C. leptochila x C. subulata	-0.0014 ± 0.00044	0.36	*C. subulata x C. leptochila*	-0.016 ± 0.00009	0.99	*
C. leptochila x C. ovata	-0.0012 ± 0.00063	0.33	*C. ovata x C. leptochila*	-0.0078 ± 0.00119	0.61	*
C. ovata x C. subulata	-0.0046 ± 0.00182	0.36	*C. subulata x C. ovata*	-0.0026 ± 0.00092	0.14	
C. ovata x C. erecta	-0.0088 ± 0.00108	0.69	*C. erecta x C. ovata*	-0.0055 ± 0.00052	0.64	
C. subulata x C. erecta	-0.013 ± 0.00078	0.82	*C. erecta x C. subulata*	-0.0043 ± 0.00073	0.5	*
Species cross direction 1	**Percentage of formed embryos ± SE**	***RI embryo***	**Species cross opposite direction**	**Percentage of formed embryos ± SE**	***RI embryo***	**Significant reciprocal difference in percentage of formed embryos**
C. leptochila x C. erecta	72.37 ± 4.70	0.14	*C. erecta x C. leptochila*	69.94 ± 5.24	0.24	
C. leptochila x C. subulata	61.78 ± 8.56	0.27	*C. subulata x C. leptochila*	NA	NA	
C. leptochila x C. ovata	59.06 ± 7.85	0.3	*C. ovata x C. leptochila*	55.73 ± 10.73	0.39	
C. ovata x C. subulata	88.33 ± 2.33	0.04	*C. subulata x C. ovata*	86.04 ± 2.42	0	
C. ovata x C. erecta	38.54 ± 2.33	0.58	*C. erecta x C. ovata*	73.74 ± 4.88	0.2	
C. subulata x C. erecta	18.11 ± 8.30	0.79	*C. erecta x C. subulata*	42.86 ± 8.57	0.54	*

Table 2: Genome sizes with standard error and chromosome counts of Australian *Cryptostylis* species, with the number of populations and individuals sampled for flow cytometry

Cryptostylis Species	No populations	No individuals	Haploid (pollen) genome size (pg)	Chromosome number (2N)	Reference
C. erecta	8	43	5.39 ± 0.05	56	Peakall & James (1989)
C. hunteriana	2	23	3.34 ± 0.04	-	-
C. leptochila	5	18	29.6 ± 0.67	492	Present study
C. ovata	3	20	11.35 ± 0.13	187	Peakall & James (1989)
C. subulata	8	40	4.66 ± 0.03	64	Dawson et al. (2007)

Table 3: Mean and variance of seed weights and embryo formation percentages for intraspecies control crosses and self-pollination treatments (with SI values). Bold indicates a significant difference ($P < 0.05$) between the intraspecies cross and its corresponding self-pollination treatment.

Pollination treatment	***Seed***			***Embryo***			
	Mean value	variance	SI_{seed}	Mean value	variance	SI_{embryo}	SI_{total}
C. leptochila x *C. leptochila*	0.004	2E-06		84.52	189.27		
C. leptochila self	0.003	2E-06	0.07	79.12	513.52	0.06	0.13
C. erecta x *C. erecta*	0.009	3E-06		92.58	26.26		
C. erecta self	**0.006**	4E-06	0.36	**38.94**	**316.85**	0.58	0.94
C. subulata x *C. subulata*	0.016	2E-05		84.63	294.27		
C. subulata self	**0.006**	2E-05	0.6	**21.79**	149.78	0.74	1.34
C. ovata x C. ovata	0.013	2E-05		91.65	25.97		
C. ovata self	0.010	2E-05	0.23	82.30	**225.79**	0.1	0.33

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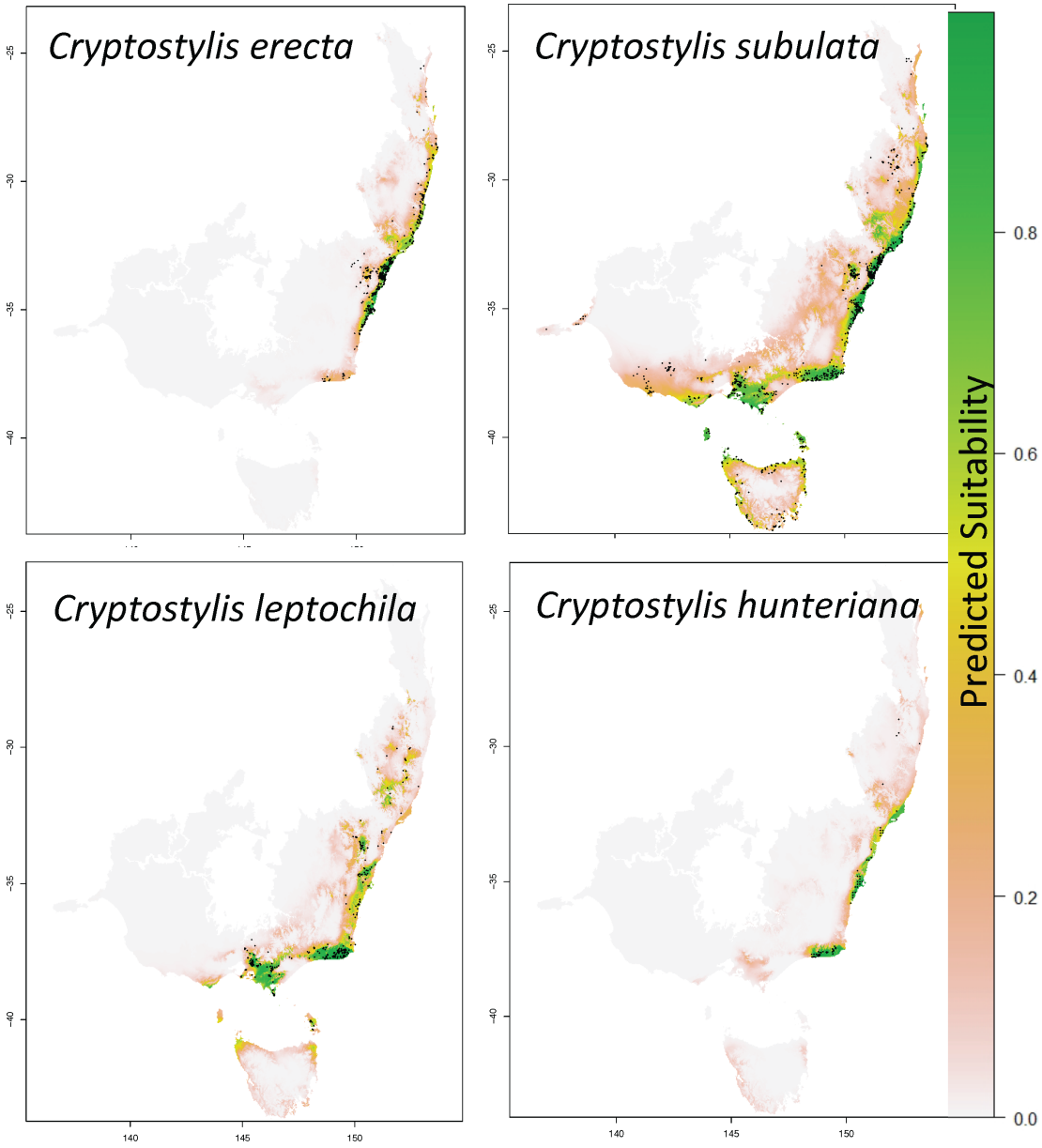
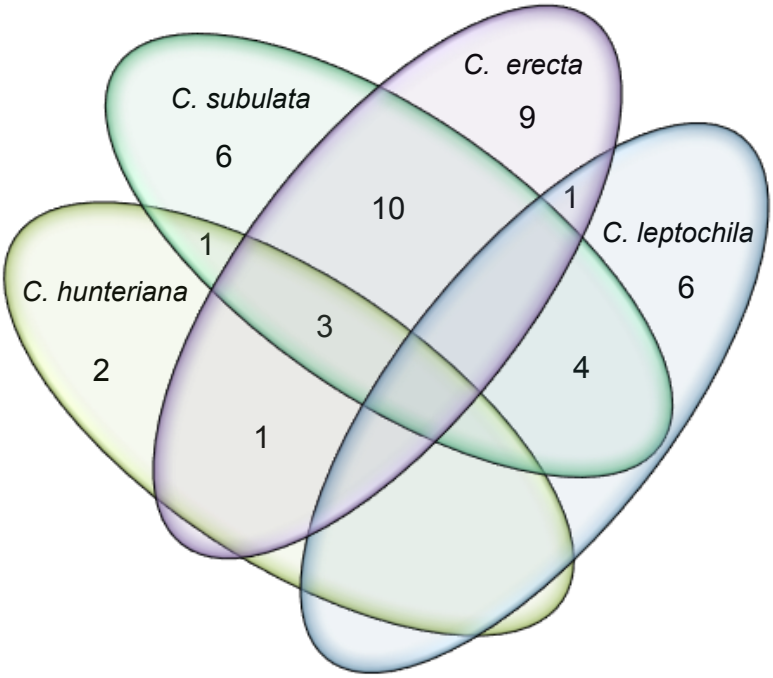
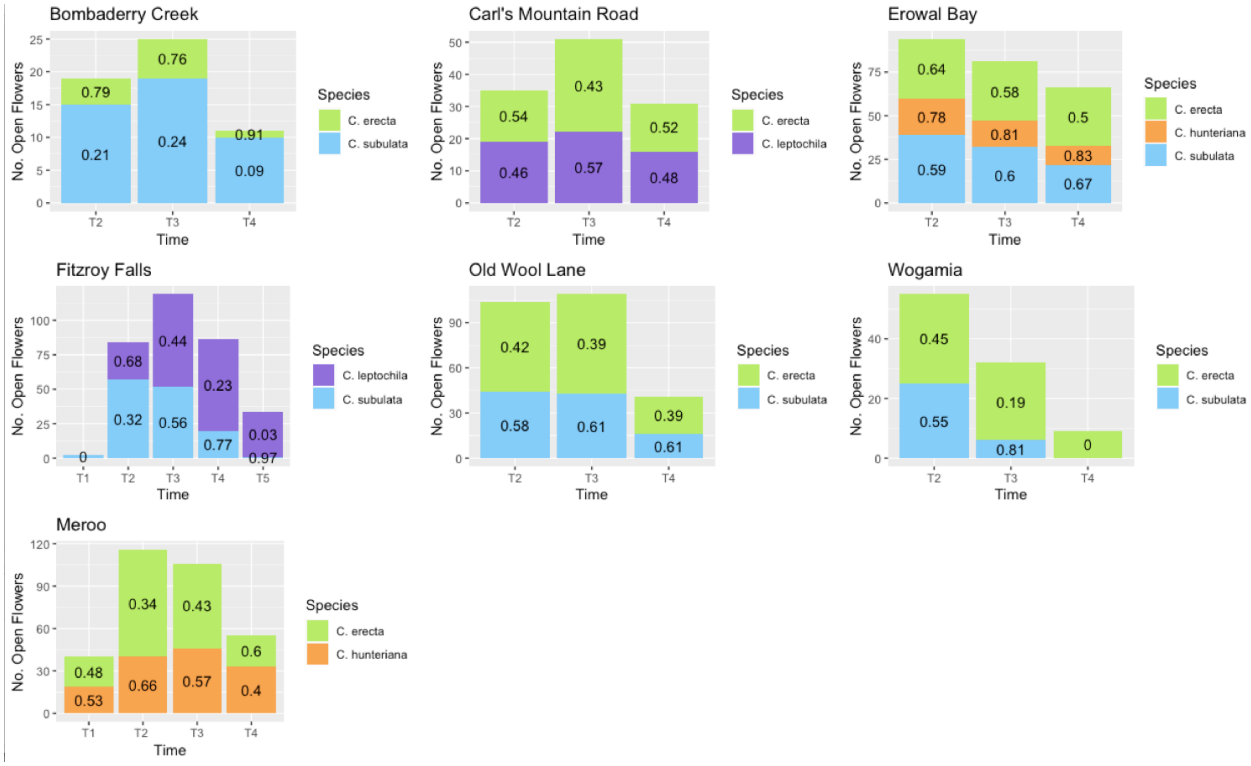
SUPPPLEMENTARY FIGURES AND TABLES



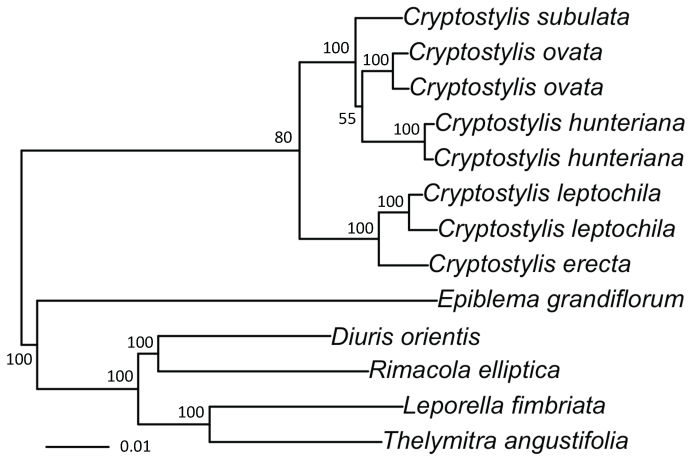
Supplementary Figure A: Predicted bioclimatic niches for the four eastern Australian *Cryptostylis* species from MaxEnt modelling. Species occurrence records are shown in black.



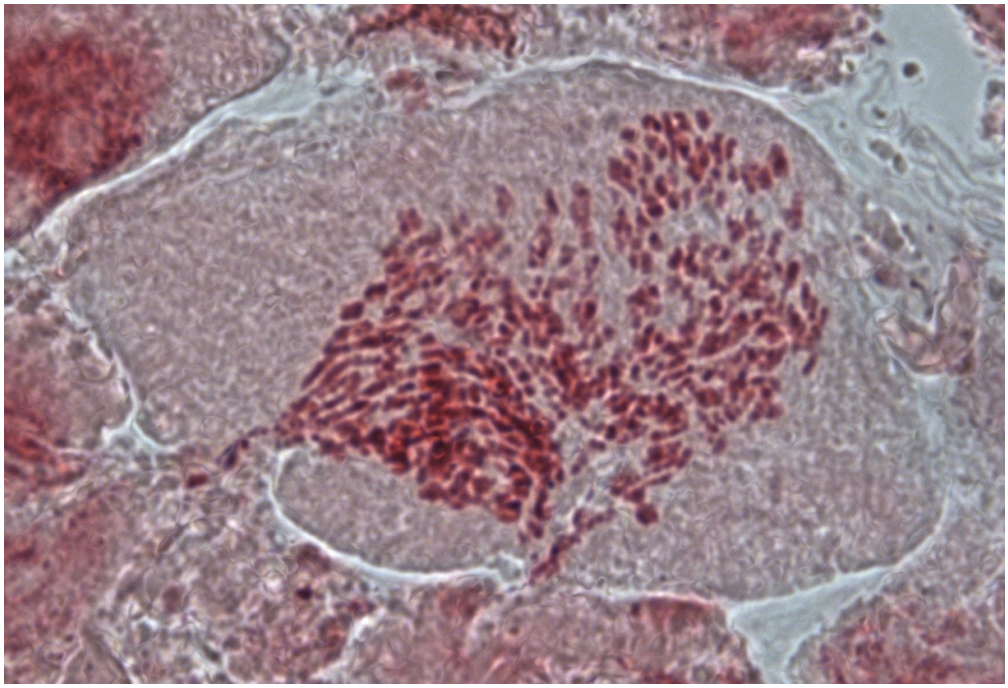
Supplementary Figure B: Four-way Venn diagram showing the number of sites at which different *Cryptostylis* species were found alone or in sympatry with one another.



Supplementary Figure C: Number of open flowers of *C. erecta, C. hunteriana, C. leptochila,* and C. *subulata* at seven sites where T1 (time period 1) is 4-6/12/17, T2 is 26-28/12/17, T3 is 9-11/01/18, T4 is 30/01/18 - 01/02/18, and T5 is 20/02/18. Interspecific pollen transfer risk values (via co-flowering) for each species at each time period are shown in the bars.



Supplementary Figure D: Mid-point rooted IQTREE phylogeny of Australian *Cryptostylis* with outgroups based on 186,827 base pairs of exon capture data. Nodes display UFBoot support values. The scale bar indicates the number of base pairs substitutions per site.



Supplementary Figure E: Chromosome spread from a root tip of *Cryptostylis leptochila* showing a minimum of ≈ 492 chromosomes

Supplementary Figure F (modified from Peakall *et al.* 2020, *in* prep): IQTREE phylogeny of representative members of terrestrial orchids in Australia. The loci for the alignment were generated using an exon capture approach of orthologues designed Deng et *al.* (2015). The concatenated alignment consists of 200935 bp and has 70011 parsimony-informative sites. The phylogenetic tree was rooted to *Pterostylis* in Figtree v 1.4.3. Sexually deceptive clades referred to in text are highlighted in green boxes.

Supplementary Table A: Niche overlap values (*I*) between pairs of *Cryptostylis* species calculated from their predicted bioclimatic niches.

	Cryptostylis erecta	*Cryptostylis hunteriana*	*Cryptostylis leptochila*	*Cryptostylis subulata*
Cryptostylis erecta	-			
Cryptostylis hunteriana	0.88	-		
Cryptostylis leptochila	0.62	0.78	-	
Cryptostylis subulata	0.72	0.79	0.88	-

Supplementary Table B: Total reproductive isolation (RI) values for intra-species crosses between sympatric (green) and allopatric species (blue), and self-incompatibility (SI) values for self-pollination treatments (purple). Crosses are writtten *Maternal x paternal* species.

	C. leptochila x C. subulata	***C. leptochila x C. erecta***	***C. leptochila x C. ovata***	***C. leptochila* self**
RI/SI pod development	0 (10/10)	0 (10/10)	0 (10/10)	0 (12/12)
RI/SI seed mass	0.36	0.19	0.33	0.07
RI/SI embryo	0.27	0.14	0.3	0.06
RI/SI total	**0.63**	**0.33**	**0.63**	**0.13**
	C. erecta x C. leptochila	***C. erecta x C. subulata***	***C. erecta x C. ovata***	***C. erecta* x self**
RI/SI pod development	0 (9/9)	0 (10/10)	0 (10/10)	0 (11/11)
RI/SI seed mass	0.86	0.5	0.64	0.36
RI/SI embryo	0.24	0.54	0.2	0.58
RI/SI total	**1.1**	**1.04**	**0.84**	**0.94**
	C. subulata x C. erecta	***C. subulata x C. leptochila***	***C. subulata x C. ovata***	***C. subulata* x self**
RI/SI pod development	0.01 (9/10)	0.5 (5/11)	0.1 (9/11)	0.2 (8/11)
RI/SI seed mass	0.82	0.99	0.14	0.6
RI/SI embryo	0.79	NA	-0.01	0.74
RI total	**1.62**	**1.49**	**0.24**	**1.54**
	C. ovata x C. erecta	***C. ovata x C. leptochila***	***C. ovata x C. subulata***	***C. ovata* x self**
RI/SI pod development	0 (9/9)	0 (8/8)	0 (9/9)	0 (7/8)
RI/SI seed mass	0.69	0.61	0.36	0.23
RI/SI embryo	0.58	0.39	0.04	0.1
RI/SI **total**	**1.27**	**1**	**0.4**	**0.33**

Supplementary Table C: Genome sizes of *Cryptostylis* hybrid seed grown in a greenhouse cross-pollination with standard error. Genome sizes of parent species are calculated from Table 1 (haploid sizes) multiplied by two.

	Father Species	*Cryptostylis subulata*	*Cryptostylis erecta*	*Cryptostylis ovata*	*Cryptostylis leptochila*
Mother species	**Diploid genome size**	**9.32**	**10.78**	**22.7**	**59.2**
Cryptostylis subulata	**9.32**	-	10.13 ± 0.09	15.9 ± 0.03	36.99 ± 1.53
Cryptostylis erecta	**10.78**	10.08 ± 0.02	-	16.54 ± 0.11	38.37 ± 1.80
Cryptostylis ovata	**22.7**	15.84 ± 0.1	16.55 ± 0.07	-	43.44 ± 1.18
Cryptostylis leptochila	**59.2**	35.8 ± 1.0	38.26 ± 1.56	43.76 ± 1.47	-

CHAPTER THREE

The phylogenetic relationship between Australian and Asiatic *Cryptostylis* (Diurideae, Orchidaceae) with taxonomic implications for the Asiatic species

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This study was conceptualised by A Weinstein. Samples were collected by A Weinstein and S Gale. Bioinformatics were conducted by D Wong and R Peakall. Phylogenetic Analyses were conducted by A Weinstein and C Linde. Interpretation of the taxonomic literature was conducted by A Weinstein and M Clements. Original draft preparation was conducted by A Weinstein. Review and editing was conducted by A Weinstein, R Peakall, and C Linde. Funding was acquired by A Weinstein.

ABSTRACT

Cryptostylis is unique in being the only sexually deceptive genus in the orchid tribe Diurideae to have a greater diversity in Asia than in Australia, and is renowned for its five Australian species all sharing the sexually deceived pollinator *Lissopimpla excelsa*. Differences in ploidy level play a major role in preventing hybridisation among these species. Comparatively little is known about the 18 described Asiatic *Cryptostylis* species, whose pollinator species are unknown, and among which there is a high degree of taxonomic uncertainty regarding species boundaries. In recently published literature, many Asiatic species are referred to as *C. arachnites* irrespective of their morphology. The present study aimed to investigate the phylogenetic relationships in *Cryptostylis*, for the first time encompassing both Australian and some Asiatic species to allow inference about their evolutionary history. Accompanying flow cytometric and chemical analyses were conducted to allow discussion of genome size and semiochemical presence in a phylogenetic context. An exome-capture approach using concatenation of 211 single-copy orthologous genes identified for the Orchidaceae, revealed *Cryptostylis* to be comprised of three clades, two of which diverged in Australia. These observed patterns of divergence indicate an Australian origin for *Cryptostylis,* with a single dispersal event from Australia and subsequent diversification giving rise to the Asiatic clade. The current distribution of the Asiatic *Cryptostylis* may potentially be explained by the complexity of the cordillera habitat driving New Guinean diversification, and long distance dispersal events explaining the large distribution of *Cryptostylis*. Flow cytometry revealed different genome sizes to be present in two Asiatic species, suggesting ploidy variation to have played a role in diversification across the genus. 2-(Tetrahydrofuran-2-yl)acetic acid, which has previously been found to attract *L. excela* to *C. ovata*, was discovered to be present in all Australian *Cryptostylis* species, suggesting the most recent common ancestor of *Cryptostylis* may have attracted *L. excelsa*. Taxonomic treatment of Asiatic *Cryptostylis* must be amended as it is presently an impediment to both their effective conservation and their inclusion in evolutionary studies.

INTRODUCTION

The Orchidaceae family accounts for approximately 8% of the angiosperm diversity globally, with its distribution spanning all continents except Antarctica (Pridgeon, 1999-2014; Royal Botanic Gardens, 2013; Givnish et al., 2016). This diverse family originated in Australia (Givnish et al., 2015), where it has a high degree of endemism (Royal Botanic Gardens, 2013; Nargar et al., 2019). The Diurideae tribe dominates the Australian orchid flora, comprising ~ 60% of its diversity (Royal Botanic Gardens, 2013; Nargar et al., 2019). This tribe comprises mostly geophytic perennials, which are classified into nine subtribes (Pridgeon et al., 2001; Chase et al., 2015). The center of Diurideae diversity is Australia, with seven of its nine subtribes having a predominantly Australian distribution (Pridgeon et al., 2001). Two subtribes, *Acianthinae* and *Cryptostylidinae*, have a greater diversity in Asia than Australia, while of those two only *Cryptostylidinae* has a greater Asiatic diversity in all its constituent genera (Pridgeon et al., 2001).

Cryptostylidinae contains two genera: the monotypic *Coilochilus* endemic to New Caledonia, and *Cryptostylis*, which is comprised of five Australian species and 18 Asiatic species whose distribution ranges from Sri Lanka to Samoa (Pridgeon et al., 2001; Weston et al., 2014). The centre of diversity of *Cryptostylis* is New Guinea, where nine of the eleven species are considered to be endemic (O'Byrne & Schneider, 2015). All species in the *Cryptostylidinae* are unique among the terrestrial Diurideae in having evergreen leaves, with the exception of the Australian *C. hunteriana* which is mycoheterotrophic (Nicholls, 1938; Pridgeon et al., 2001). *Cryptostylidinae* are further characterised by their multi-flowered inflorescences (Pridgeon et al., 2001). Historically, the placement of the *Cryptostylidinae* has been uncertain, with various authors placing it in the Spiranthoideae or Orchidoideae prior to molecular data confirming its position in the Diurideae (Dressler & Dodson, 1960; Freudenstein, 1991; Kores, 1997; Cameron et al., 1999; Pridgeon et al., 2001). While the placement of the sister genera *Cryptostylis* and *Coilochilus* are now well supported, the relationship between the Asiatic and Australian *Cryptostylis* species remains uninvestigated - across all the published phylogenies none have included any Asiatic *Cryptostylis* species (Cameron et al., 1999; Kores et al., 2001; Pridgeon et al., 2001; Miller & Clements, 2014; Weston et al., 2014). Given that the diversity of the Asiatic *Cryptostylis* exceeds that of the Australian *Cryptostylis*, their omission is striking, and may potentially be explained by rarity and taxonomic uncertainty in this group.

Taxonomic uncertainty amongst the Asiatic *Cryptostylis* taxa is rife, with poor separation of taxa and confusion between morphological ecotypic variation and species boundaries (Hunt, 1970; O'Byrne & Schneider, 2015; Robinson et al., 2016). The Asiatic *Cryptostylis* species were described between 1859 and 1935 based on variation in floral morphology, with one species, *C. taiwaniana*, also having characteristic variegated leaves (Lin, 1976). Since these original descriptions, only seven of the Asiatic species have been recorded - mainly as part of flora surveys eg. (Fernando & Ormerod, 2008; Ghollasimood et al., 2011; Go et al., 2011; Jin et al., 2012; Majit et al., 2015). In 1970 ten species of Asiatic *Cryptostylis* were merged into *C. arachnites* on grounds of morphological similarity (Hunt, 1970). While these species are not currently formally recognised, it is worth noting that the decision to merge these species was based on morphological comparison of the material from the original species' descriptions - no genetic analyses were conducted and all taxa had not been recollected from the wild and used in comparisons (Hunt, 1970). This rarity in observation of Asiatic *Cryptostylis* may partly be explained by their typically high elevation habitats, and the unpredictable timing of their flowering (Dassanayake, 1981; Wood et al., 1993; Pridgeon et al., 2001). Despite 18 Asiatic species being formally recognised today (Royal Botanic Gardens, 2013), it appears that the majority of publications and field guides refer to all Asiatic *Cryptostylis* as *C. arachnites*, resulting in a variety of morphologically diverse plants being termed '*C. arachnites*'. For example, photos of *C. arachnites* in three different field guides show three different floral morphologies, which vary in labellum shape (some recurved and some straight) and colouration (pale pink, orange, and a mixture thereof) (Comber, 1990; O'Byrne, 2008; Gale, 2014). This taxonomic uncertainty and ambiguous treatment of '*C. arachnites*' extends far beyond these highlighted books and is pervasive throughout the literature (though see Robinson et al. (2016), O'Byrne & Schneider (2015), and Comber (1990)). It can be concluded that references to *C. arachnites* are circular, with authors not referring back to the original species descriptions to assign species names. Two scenarios are possible: (1) morphological variation in Asiatic *Cryptostylis* represents discrete species and the original nomenclature has been largely ignored in modern literature, or (2) morphological variation in Asiatic *Cryptostylis* do not reflect species boundaries, and as concluded by Hunt (1970), and merging of morphologically similar taxa is appropriate.

In comparison to the elusive Asiatic taxa, the Australian *Cryptostylis* are well studied. These five species are renowned for being one of the first reported cases of sexual deception, where

five Australian species sexually deceive the ichneumonid pollinator *Lissopimpla excelsa* (Coleman, 1927; Coleman, 1929, 1930b, a; Nicholls, 1938). This degree of pollinator sharing is unusual among sexually deceptive orchids, which typically attract a sole pollinator species (Paulus & Gack, 1990; Gaskett, 2011; Phillips et al., 2017). While other cases of pollinator sharing are known in sexually deceptive orchids, they typically occur between only two species, and often represent cases of convergent evolution of the use of the same pollinator between different orchid groups (Cortis et al., 2008; Gögler et al., 2009; Phillips et al., 2013; Bohman et al., 2017; Phillips et al., 2017). Conversely, in Australian *Cryptostylis*, pollinator sharing occurs between closely related species (Chapter Three).

Australian *Cryptostylis* are the only known sexually deceptive orchids to employ an ichneumonid pollinator, which has a high rate of revisitation to individual inflorescences (Gaskett, 2011; Weinstein et al., 2016). This high revisitation rate, in combination with the multiflowered inflorescences and clonality of *Cryptostylis*, may have contributed to the evolution of self-incompatibility in Australian *Cryptostylis* (Chapter Two). *Cryptostylis* is the only known genus in the Diurideae to contain self incompatible species (Chapter Two), while in the Orchidoideae more broadly only one other example of self-incompatibility has been reported, that in *Geoblasta pennicillata* (Ciotek et al., 2006). The self-compatibility and pollination strategy of the Asiatic species is unknown (Pridgeon et al., 2001).

The interaction between *L. excelsa* and *Cryptostylis* is further unusual in being only one of two instances in which sexually deceived pollinators are known to ejaculate (Blanco & Barboza, 2005; Gaskett et al., 2008). In *Cryptostylis ovata,* this strong attraction of *L. excelsa* is known to be mediated in part by 2-(Tetrahydrofuran-2-yl)acetic acid (Bohman et al., 2019). However, this compound alone does not induce pollinator copulation, indicating it may function as part of a blend (Bohman et al., 2019). It is further unknown whether 2-(Tetrahydrofuran-2-yl)acetic acid is present in the four other Australian *Cryptostylis* species. Phylogenetic analyses in combination with these chemical data may elucidate the evolutionary history of this important trait.

Four species of Australian *Cryptostylis* occur sympatrically in eastern Australia, while one occurs allopatrically in south-west Western Australia (Jones, 1988). Due to their pollinator sharing and the occurrence of sympatry (with the exception of the disjunct *C. ovata*), it is

post-pollination barriers that control reproductive isolation in Australian *Cryptostylis* - an unusual scenario for sexually deceptive orchids (Chapter Two). A major component of this reproductive isolation is driven by differences in chromosome number between species - several polyploidy events have occurred in Australian *Cryptostylis* - with three different ploidy levels present, and those species sharing a ploidy level showing evidence of aneuploidy (Chapter 2). Only one chromosome count exists for Asiatic *Cryptostylis* - 2n = 42 from *C. arachnites* collected in Thailand (Larsen, 1966), which is lower than the lowest chromosome count in an Australian *Crypostylis* - 2n = 56 in *C. erecta* (Peakall & James, 1989).

The present study aimed to examine the phylogenetic relationships among Australian and Asiatic *Cryptostylis* by incorporating all Australian species in addition to some available Asiatic species in creating the first molecular phylogeny for the genus. A phylogeny of *Cryptostylis* will allow an exploration of the evolutionary history of the genus, including inferences about its biogeographic origin and drivers of evolution and diversification. To complement existing genome size data available for the Australian *Cryptostylis* (Chapter Two) and enable a phylogenetic comparison of genome sizes in *Cryptostylis*, it was aimed to measure genome sizes of available Asiatic *Cryptostylis* species. To explore the chemical basis of the shared attraction of *L. excelsa* in Australian *Cryptostylis* in a phylogenetic context, gas-chromatography mass-spectrometry data from floral extracts of Australian *Cryptostylis* were screened for the *L. excelsa* attractant 2-(Tetrahydrofuran-2-yl)acetic acid (Bohman et al., 2019).

METHODS

Sample collection

Silica dried leaf and floral material from *C. acutata* and *C. filiformis* were collected from a wild population on Mt. Alab, Borneo. Voucher specimens SAN 165625 and SAN 165626 respectively were deposited at the Forest Research Center Herbarium (SAN). Silica dried leaf and floral material of *C. javanica* were collected from a wild population in Lantau, Hong Kong (voucher SW Gale SG1380, lodged at Kadoorie Farm and Botanic Garden). Silica dried leaf material of *C. taiwaniana* originating from Tianti Scenic Area, Zhushan Township, Nantou County, Taiwan, and *C. stenochila* originating from Kolombangara Island in the Solomon Islands were obtained from a glasshouse collection at the Dr Cecilia Koo Botanic Conservation Center in Taiwan (*C. taiwaniana* accession no K211137 and *C. stenochila* accession no K209250).

Species identifications for the Asiatic *Cryptostylis* samples were derived on consultation of the original species descriptions. As a result, in some cases the species names used in this study do not reflect the species names provided with the voucher specimens: the voucher for *C. javanica* is recorded as *C. arachnites*, the voucher for *C. taiwaniana* is recorded as *C. arachnites*, and the voucher for *C. stenochila* is recorded as *C. taiwaniana*.

Australian *Cryptostylis* from New South Wales were collected under permit no SL102019: *C. erecta* (voucher ORG 7811, Ulladulla), *C. hunteriana* (CANB 882130.1, Buladelah), *C. leptochila* (CBG 9004747.1, Fitzroy Falls), and *C. subulata* (ORG 7808, Comberton). *Cryptostylis ovata* was collected from Western Australia under permit no SW018906 (voucher PERTH 06731481, Boyanup).

Phylogenetics

To examine the phylogenetic relationships among *Cryptostylis* species, a phylogeny was generated that encompassed the Australian species, the available Asiatic species, and relevant outgroups. Specifically, the following samples were included; Australia: *C. erecta* ($N = 1$), *C. hunteriana* ($N = 2$), *C. leptochila* ($N = 2$), *C. ovata* ($N = 2$), and *C. subulata* ($N = 1$); Asia: *C. acutata* (Borneo, $N = 2$), *C. filiformis* (Borneo, $N = 1$), *C. javanica* (Hong Kong, $N = 2$), *C.*

taiwaniana (Taiwan, *N* = 1), and *C. stenochila* (Solomon Islands, *N* = 2); outgroups: *Rimacola elliptica, Leporella fimbriata*, *Epiblema grandiflorum*, and *Diuris orientis* (all *N* = 1).

An exome-capture approach using single-copy orthologous genes identified in Deng et al. (2015) was used following the methodology of Peakall et al. (in prep.). 211 genes were sequenced for the *Cryptostylis* species and the additional five outgroup species. Loci were manually checked in Geneious 9.1.8 (Kearse et al., 2012) for alignment errors. A phylogenetic tree was inferred by Maximum Likelihood analysis in IQTREE 2.0 (Nguyen et al., 2014), using the best-fit substitution model (GTR+F+R2) automatically selected by ModelFinder according to the Bayesian Information Criterion (Kalyaanamoorthy et al., 2017). Branch supports were obtained with the built-in ultrafast bootstrap algorithm (Hoang et al., 2018) from 10000 iterations. The phylogeny was visualised and midpoint rooted in R v 3.5.1 using the 'ape' (Paradis et al., 2004) and 'phytools' (Revell, 2012) packages.

Genome size of Asiatic taxa

To investigate genome sizes in Asiatic *Cryptostylis* taxa, material for flow cytometric analyses was collected from two available species (using the same populations of the species as for the phylogenetic analyses). Pollen and seed material were targeted, as these tissues are not susceptible to progressive partial endoreplication (a potential problem in orchid flow cytometric analyses (Trávníček et al., 2015)), were successfully used in (Chapter Two), and further are desiccation resistant and suitable for international transport. Due to unpredictable flowering and fruiting occurrences, tissue was only able to be collected for two species. For *C. javanica* from Hong Kong pollen tissue was obtained, and for *C. acutata* from Borneo seed was obtained. Flow cytometry was conducted on a Attune NxT acoustic focusing flow cytometer using a Tris.$MgCl_2$ buffer as per (Chapter Two). To estimate a comparable haploid genome size value from the diploid seed data, the diploid genome size value was halved.

Gas-chromatography mass-spectrometry

Individual labella of *Cryptostylis* flowers were collected and extracted in field for 24 hours in 200 µl dichloromethane with 20 µl 10 ng/µl tert-butyl benzene (Sigma-Aldrich, 99.8%) as an internal standard. For *C. erecta* 15 individual labella were collected from three populations,

C. hunteriana 11 samples from two populations, *C. leptochila* 14 samples from three populations, *C. ovata* 14 samples from three populations, and *C. subulata* 14 samples from three populations. Samples were stored at −20°C until analysis. GC-MS analysis of the floral extracts were conducted using an Agilent 5973 Network Mass Selective Detector connected to an Agilent 6890N Network GC system equipped with an HP5MS-UI column [30 m × 0.25 mm × 0.25 μm film thickness, Agilent], using helium as a carrier gas at 1 mL/min. The mass spectra of 2-(Tetrahydrofuran-2-yl)acetic acid, which attracts *L. excelsa to C. ovata* (Bohman et al., 2019), was added to an AMDIS target library (Davies, 1998). This target library was used to individually screen each extract for the presence of 2-(Tetrahydrofuran-2-yl)acetic acid. Mass spectra and retention times were manually checked when a library hit occurred. The default AMDIS search settings were used with the exception of 'Sensitivity', which was set to 'High'.

RESULTS

Phylogenetics

In total 186,827 base pairs were used to construct an IQTREE (Nguyen et al., 2014) phylogeny of Australian and Asiatic *Cryptostylis*. Two main *Cryptostylis* clades were identified (Figure 1). The first clade consisted of Australian species only (Australian clade 1: *C. huntereiana*, *C. ovata*, and *C. subulata*). The second clade consisted of two separate diversifications, one containing Australian species (Australian clade 2: *C. erecta* and *C. leptochila*) and the other containing all the sampled Asiatic taxa. Diversification of species in Australian clade 1 marginally predates that of species in Australian clade 2, followed (again marginally) by the diversification of the Asiatic taxa. All taxa were placed on separate branches with 100% bootstrap support.

Genome size

Flow cytometric analysis of six pollinia from one clone *C. javanica* (Hong Kong) gave a haploid genome size of 4.71 ± 0.01. Analysis of two seed pods from different *C. acutata* plants (Sabah) gave a diploid genome size of 14.37 ± 0.04, from which a haploid genome size of 7.19 was estimated.

Gas-chromatography mass-spectrometry

2-(Tetrahydrofuran-2-yl)acetic acid was found to be present in all Australian *Cryptostylis* species. Specifically, it was found in 15/15 *C. erecta* extracts (three populations), 11/11 *C. hunteriana* extracts (two populations), 14/14 *C. leptochila extracts* (three populations), 14/14 *C. ovata* extracts (three populations), and 13/14 *C. subulata* extracts (three populations), indicating consistency within and between populations.

DISCUSSION

Phylogenetic relationship between Australian and Asiatic Cryptostylis

The observed phylogenetic relationships between Australian and Asiatic *Cryptotylis* support an Australian origin for *Cryptostylis*. Two of the three clades of *Cryptotylis* diverged in Australia, indicating an initial Australian diversification of *Cryptostylis*. Comprehensive sampling of the Asiatic taxa and subsequent ancestral trait reconstruction would be required to provide additional support for this result. An Australian origin has been supported for other genera within the Diurideae whose distributions span Australia and Asia such as *Calochilus* (Nargar et al., 2019) and *Thelymitra* (Nauheimer et al., 2018). These results, in combination with the high level of Australian endemism within the other Diurideae (Pridgeon et al., 2001), support the Australian origin of the Diurideae proposed by (Givnish et al., 2016).

All the sampled Asiatic taxa belonged to a single clade, indicating a single dispersal event from Australia to Asia and thereafter a subsequent Asiatic diversification. While this conclusion cannot be confidently extended to Asiatic species not included in the phylogeny, it is possible that it may apply to them given their morphological similarity. As exemplified in the taxonomic uncertainty surrounding them, the majority of the Asiatic species have very similar morphologies, and thus may likely form part of the present Asiatic Clade predicted to have arisen from a single dispersal event. An exception may lie in the Philippine *C. carinata*, which bears a very similar floral morphology to the Australian *C. erecta* (Jones, 1988; O'Byrne & Schneider, 2015; Robinson et al., 2016). Further analyses would be required to determine the evolutionary and biogeographic origin of *C. carinata*.

Taxonomic implications for Asiatic Cryptostylis

The phylogenetic placement of the Asiatic taxa supports the species names derived in the present study using the original species descriptions, and not those used on the voucher specimens. The species sampled from Hong Kong and Taiwan (both *C. arachnites* vouchers) were placed on different branches of the phylogeny, with the Hong Kong species being more closely related to *C. filiformis* from Borneo, and the species from Taiwan being more closely related to *C. stenochila* from the Solomon Islands, thereby supporting them as different species. While these two species, *C. javaniva* and *C. taiwaniana*, are broadly

morphologically similar, they can be distinguished based on differences in the width and curvature of the labella, which is flatter and broader in *C. taiwaniana*. The fact that these minor morphological variations are indicative of different species contradicts the conclusion of Hunt (1970) that minor variation of labellum coloration and morphology does not reflect the presence of discrete species. Indeed, in the present study the original description of *C. stenochila* (merged by Hunt) was found to best match the Solomon Islands specimen included in the present study. The other nine species merged by Hunt (1970) on grounds of morphological similarity may warrant reinvestigation. In order to resolve the taxonomic uncertainty in *Cryptostylis*, additional genetic analyses of a wider variety of taxa (ideally the recollection of all the originally described taxa) is required. These data would be crucial for a well-informed revision of the genus.

The present study highlights that the current use of inaccurate and inconsistent species nomenclature to refer to the Asiatic *Cryptostylis* species is a barrier to their effective conservation. Given the lack of observations of the majority of the described Asiatic *Cryptostylis*, the taxonomic uncertainty surrounding those that are recorded, and the clearing of large areas of habitat in the last century, it is likely that some of the Asiatic *Cryptostylis* species may qualify as endangered given proper evaluation. Such an evaluation should include taxonomic assessment - it is impossible to conserve biodiversity if it has not first been accurately quantified (Bickford et al., 2007). Further, taxonomic uncertainty can impede evolutionary studies.

A scenario for the evolutionary history of Cryptostylis

The combination of a phylogeny for *Cryptostylis* and the recent work on mechanisms of reproductive isolation in Australian *Cryptostylis* (Chapter Two) provides a framework to explore the evolutionary history of this unique genus. Models for the evolution of the key traits of (1) sexual deception of *Lissopimpla excelsa,* and (2) self-incompatibility, are proposed. Factors influencing the diversification of the genus as a whole, being ploidy and geographic barriers, are explored.

Evolution of sexual deception of Lissopimpla excelsa

Given the present phylogeny, the most parsimonious prediction for the evolution of sexual deception of *L. excelsa* is that this pollination strategy occurred in the most recent common ancestor of *Cryptostylis*. This conclusion is supported by the presence of the *L. excelsa* attractant 2-(Tetrahydrofuran-2-yl)acetic acid (Bohman et al., 2019) in all five Australian *Cryptostylis* species. It is likely that the most recent common ancestor of Australian *Cryptostylis*, which given the Australian origin of the genus would be the common ancestor to the whole genus, also produced 2-(Tetrahydrofuran-2-yl)acetic acid and therefore likely sexually deceived *L. excelsa*.

Evolution of self-incompatibility

The evolution of self incompatibility in Australian *Cryptostylis* is proposed to have been driven by it being a clonal plant with multi-flowered inflorescences and having a pollinator with a high revisitation rate (Chapter Two, (Weinstein et al., 2016). All Asiatic *Cryptostylis* species are clonal and also have multi-flowered inflorescences, as does the sister genus *Coilochilus* (Pridgeon et al., 2001). It is therefore likely that the most common recent ancestor of *Cryptostylis* occurred in Australia, had multi-flowered inflorescences, and was pollinated by *L. excelsa*. This common ancestor may consequently have evolved self-incompatibility to prevent inbreeding through geitonogamous pollen transfer. Self-incompatibility as an ancestral trait in *Cryptostylis* is supported in that two of the four Australian species examined (*C. erecta* and *C. subulata*) showed evidence of strong self-incompatibility, and a third species (*C. ovata*) showed a lesser degree of self-incompatibility (Chapter Two). *Cryptostylis subulata* is placed in Australian clade 1, while *C. erecta* is placed in Australian clade 2. It is therefore more likely that the two most self compatible Australian species (*C. leptochila* and *C. ovata*) have, to varying degrees, lost their self-incompatibility through polyploidy, than that self-incompatibility evolved convergently in the two Australian *Cryptostylis* clades. In many cases, polyploidy is associated with a breakdown of self-incompatibility systems due to the presence of additional *S* alleles that may provide the requisite recognition factor to overcome self-incompatibility (de Nettancourt, 1977; Richards, 1997; Entani et al., 1999, though see Mable (2004)). This breakdown may have occurred in the polyploid *C. leptochila,* explaining its high degree of self-compatibility

(Chapter 2). Similarly, the polyploid *C. ovata* may be in the process of experiencing a breakdown of self-incompatibility (de Nettancourt, 1977), which would explain its mixed state between self-compatible and self-incompatible (Chapter Two). It remains unknown whether Asiatic *Cryptostylis* have retained their self-incompatibility.

The current phylogeny supports the loss of ancestral self-incompatibility in *C. leptochila* as the most likely evolutionary scenario for this trait. With the loss of self-incompatibility, the potential for unilateral incompatibility is enabled. Unilateral incompatibility is a difference in cross-compatibility between two species, one self-compatible and one self-incompatible, depending on the order in which they are crossed (Lewis & Crowe, 1958; de Nettancourt, 1977). It is expected that when self-compatible pollen is deposited on a self-incompatible stigma, pollen tube growth and thereby seed set is inhibited. In the contrasting reciprocal cross (self-incompatible pollen on a self-compatible stigma), no inhibition is expected and a higher seed set is predicted (Lewis & Crowe, 1958; de Nettancourt, 1977). Indeed, when the self-compatible *C. leptochila* was crossed with the self-incompatible *C. erecta* and *C. subulata*, patterns consistent with unilateral incompatibility were observed in the resultant seed set (Chapter Two). Unilateral incompatibilty may be another isolating mechanism that may have contributed to driving divergence between Australian *Cryptostylis* species after the polyploidisation of *C. leptochila*.

Geographic barriers and ploidy variation in *Australian* Cryptostylis

Both ploidy and geographic barriers are suggested to have played a role in the diversification of the Australian *Cryptostylis*. *Cryptostylis ovata* has diverged both through dispersal to Western Australia rendering it allopatric, and polyploidisation, though it cannot be determined in which order these speciation mechanisms occurred. The biogeographic barrier of the arid Nullabor Plain, which separates eastern and western Australia, is known to have to led to the vicariant divergence of multiple plant lineages (Crisp & Cook, 2007). However, long distance dispersal out of eastern Australia may also explain the presence of *C. ovata* in Western Australia - as is predicted to have occurred in another diurid genus - *Calochilus* (Nargar et al., 2019). *Cryptostylis leptochila* has also diverged through polyploidy, while *C. erecta*, *C. hunteriana*, and *C. subulata* show evidence for aneuploidy, thus differences in chromosome number could have driven speciation in these eastern Australian taxa. While these four eastern Australian species are presently sympatric, it cannot be ruled out that

historical allopatry may have played a role in their divergence. Reinforcing selection is also suggested to have contributed to the maintenance of species boundaries in sympatric eastern Australian *Cryptostylis* (Chapter Two). Accounting for phylogenetic distance, in comparisons of allopatric and sympatric species pairs, sympatric species pairs were found to have a greater degree of reproductive isolation (Chapter Two). As historical patterns of sympatry and allopatry remain unknown, it cannot be determined at what stage in the evolution of the genus reinforcement began to play a role.

Ploidy variation in Asiatic Cryptostylis

The present study suggests that differences in ploidy may have played a role in the diversification of Asiatic *Cryptostylis*. The different genome sizes of *C. acutata* and *C. javanica* provide evidence for aneuploidy or polyploidy between the species. Given that the two genome sizes are almost double of one another (4.71 and 7.19), they may represent a difference in ploidy level, potentially diploid and tetraploid. Differences in ploidy playing a role in the diversification of Asiatic *Cryptostylis* would not be unexpected given its major role in the Australian species (Chapter 2). Further, other genera in the Diurideae such as *Acianthus*, *Diuris*, *Microtis*, *Prasophyllum*, and *Thelymitra* have been found to comprise multiple ploidy levels (Peakall & James, 1989; Dawson et al., 2007), suggesting polyploidy may play a role in the diversification of the Diurideae more broadly.

Geographic barriers in Asiatic Cryptostylis

The diversification of the New Guinean *Cryptostylis* may have been driven by the topographic complexity of their habitat. Growing at an elevation of 1000m or greater in a tropical cordillera (group of parallel mountain ranges), such as the New Guinea Highlands (the center of diversity for *Cryptostylis*) or the Andes was found to be associated with orchid diversification (Givnish et al., 2015; Givnish et al., 2016). The New Guinea Highlands are a well known center of plant diversity, where the combination of complex geology and topology, the equatorial wet climate, and isolation on an island created an environment conducive to extensive speciation (Rafiqpoor et al., 2005). While dust-like orchid seed typically has a high dispersal ability (Arditti & Ghani, 2000), this ability is suggested to be reduced in wet tropical montane areas, potentially due to rapid seed rainout, allowing localised diversification without subsequent gene flow (Givnish et al., 2015; Givnish et al.,

2016).

Long distance dispersal most likely explains the presence of *Cryptostylis* on several oceanic islands such as the Andaman and Nicobar Islands (Karthigeyan et al., 2014), the Ryukyus Archipelago (Fujita et al., 2015), the Solomon Islands (Lewis & Cribb, 1991), Vanuatu (Lewis & Cribb, 1989), Fiji (Keppel et al., 2005), and Samoa (Cribb & Whistler, 1996). Long distance dispersal events may also potentially explain instances where phylogeny does not correlate with geography - e.g. the two sympatric Borneo species are placed on separate branches, as are the geographically close *C. taiwaniana* (Taiwan) and *C. javanica* (Hong Kong). There is a precedent for successful long distance dispersal events in the Diurideae, which has likely occurred in *Calochilus* (Burns-Balogh & Bernhardt, 1988; Nargar et al., 2019), *Corybas* (Clements et al., 2007; Lehnebach et al., 2016), and *Thelymitra* (Burns-Balogh & Bernhardt, 1988; Nargar et al., 2019).

While *L. excelsa* is endemic to Australia and New Zealand and does not occur in Asia (Parrott, 1952), the sexual deception strategy could have been retained in Asiatic *Cryptostylis* through switching to a new pollinator species. Pollinator switching in sexually deceptive orchids may only require minor (if any) genetic changes, as a small change can result in the production of different chemical compounds that may attract a novel pollinator (Haynes & Hunt, 1990; Ayasse et al., 2011; Breitkopf et al., 2013). Switching to another pollinator within the Pimplini subfamily is feasible given their diversity in New Guinea and Continental South East Asia (13 genera) (Gauld, 1984). Observations of racemes with irregular fruit set suggest the involvement of a pollinator (A. Weinstein, pers. obvs.), while the red and green colouration of the labella (Comber, 1990; Cheam et al., 2009; O'Byrne & Schneider, 2015) could be suggestive of sexual deception (Phillips et al., 2009). Further investigation is required to determine potential pollinator species involved and their method of attraction. Assuming the most recent common ancestor of *Cryptostylis* to have been self-incompatible, a loss of self-incompatibility in the Asiatic species would have further aided their establishment. Self-compatibility enables a sexually reproducing colony to form from a single individual, and thereby offers a fitness advantage to colonising species (Baker, 1955; Grossenbacher et al., 2017).

Conclusions and future directions

The present study resolves the first phylogeny for the genus *Cryptostylis*, and highlights a potential role for ploidy variation and geographic barriers in its diversification. Additional investigation of the Asiatic species will further elucidate patterns of divergence. It would be informative to resolve the pollination strategy of the Asiatic *Cryptostylis* - incorporating the pollinator species involved, their method of attraction, and their specificity. Further, it would be of interest to determine whether these species are self-compatible, or even autogamous. Such investigations are also lacking for the monotypic *Coilochilus*, which being the only other genus in *Cryptostylidinae*, could offer insights into the evolutionary history of the subtribe.

The proposed investigations would be limited by the unresolved taxonomy of *Cryptostylis*. It is recommended that all originally described species be recollected, and their taxonomic status reassessed. To ensure the conservation of these elusive Asiatic taxa, and to aid future evolutionary studies, a well-informed revision of the genus that incorporates genetic analyses, and updated use of nomenclature in the literature, is apposite.

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FIGURES AND TABLES

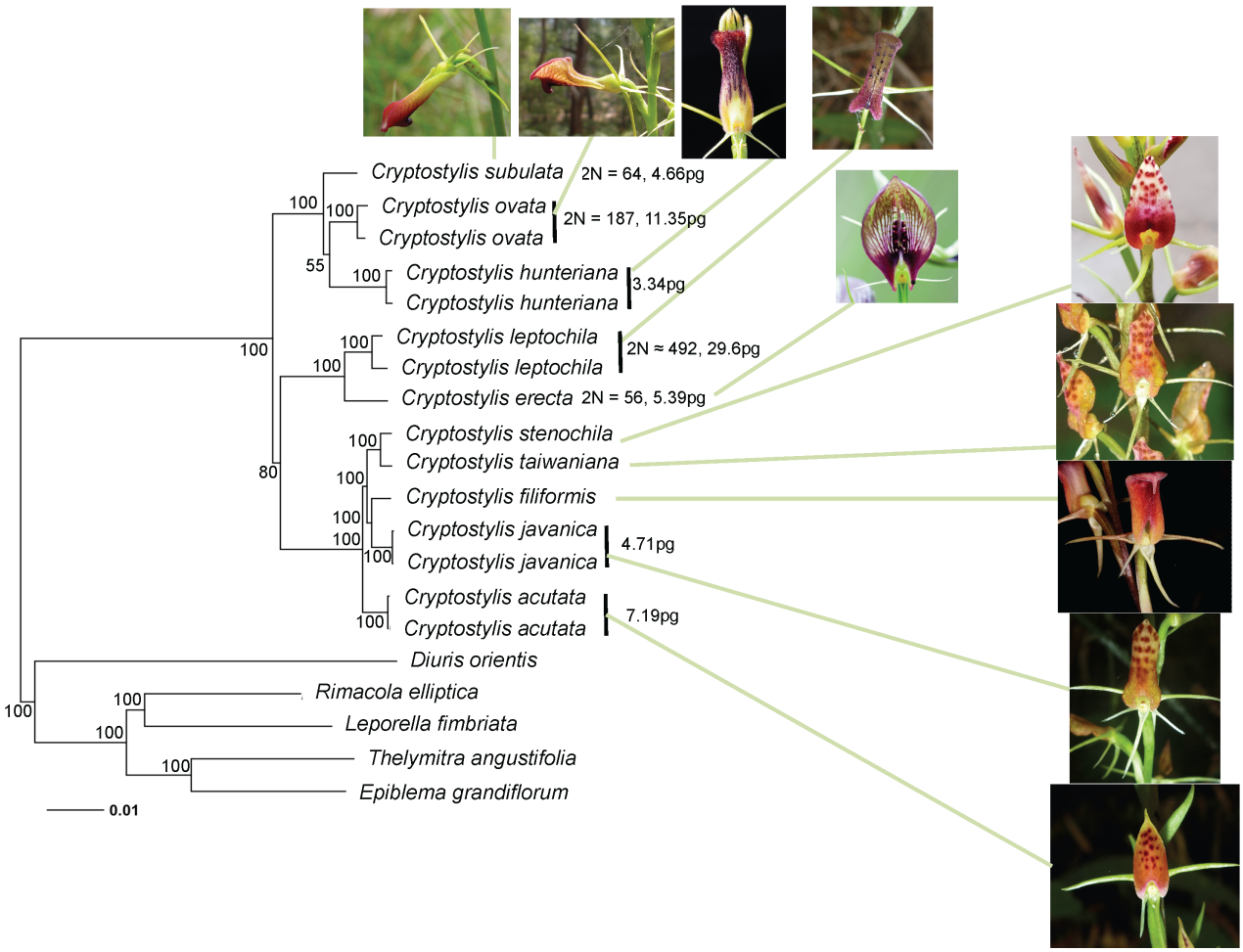

Figure 1: Mid-point rooted IQTREE phylogeny of *Cryptostylis* with outgroups based on 186,827 base pairs of exon capture data. Nodes display UFBoot support values. The scale bar indicates the number of base pairs substitutions per site. Where available, chromosome counts and haploid genome size data from Chapter Two, Peakall & James (1989), Dawson et al. (2007), and the present study were mapped onto the phylogeny.

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CHAPTER FOUR

Discovery of three chemically distinct pollination ecotypes in the sexually deceptive *Drakaea livida* (Orchidaceae)

Weinstein AM, Bohman B, Linde CC, Flematti GR, Peakall R, & Phillips RD

This study was conceptualised by A Weinstein in consultation with R Phillips. Experiments and data analyses were conducted by A Weinstein. Synthesis of semiochemicals was conducted by B Bohman and G Flematti. Identification of semiochemicals was conducted by A Weinstein, B Bohman, and G Flematti. Original draft preparation was conducted by A Weinstein. Review and editing was conducted by all authors. Funding was acquired by A Weinstein.

ABSTRACT

Sexually deceptive orchids are unusual among plants in their ability to attract specific novel pollinators through minor differences in floral volatile composition. These differences are underpinned by simple genetic changes such as random mutations. A random mutation that changes floral volatile composition may lead to pollinator switching, potentially in association with adaptation to locally available pollinator species. While both floral chemistry and local adaptation to differences in pollinator availability have been explored in sexually deceptive orchids, they are rarely studied in combination. The present study tested for the potential presence of pollination ecotypes in the sexually deceptive *Drakaea livida* and investigated patterns of pollinator availability and chemical divergence across the distribution of the species. Pollinator choice trials revealed the presence of three pollination ecotypes within *D. livida*, each attracting a specific thynnine wasp pollinator species. Surveys of pollinator distribution revealed one pollinator species to be present throughout the range of all three ecotypes, two of which it was not attracted to, demonstrating patterns of pollinator availability to not correlate with ecotype distribution. Each ecotype possessed a significantly different floral volatile composition with a high degree of separation evident in multivariate space. Partial Least Squares Discriminant Analysis based on the presence-absence of a subset of informative compounds could be used to accurately predict the ecotype of a flower. Some of these compounds are known to electrophysiologically active based on previous studies, while additional compounds were found to be active in the present study. Different classes of electrophysiologically active compounds (pyrazines and (methylthio)phenols), that likely have different biosynthetic pathways, were present in different ecotypes. These marked differences in chemical composition between the ecotypes may suggest a long time since their divergence, and could potentially hint at a scenario of convergent evolution of floral morphology. The ecotypes represent distinct evolutionary entities and should be treated as such in conservation management. Further investigation of ecotype geographic ranges and additional potentially discriminating traits is recommended to enable effective conservation management of the ecotypes.

INTRODUCTION

In specialised pollination systems, pollinator switching (where populations of a plant adopt a novel pollinator or group of pollinators) is predicted to occur in response to variation in the regional availability and efficacy of local pollinator species (Stebbins, 1970; Johnson, 2010). Differences in pollinator geographic availability and efficiency create an adaptive landscape, within which local adaptation to the most effective pollinator species may occur (Stebbins, 1970; Johnson, 2010; Duffy & Johnson, 2017). Local adaptation to pollinators is predicted to occur most frequently in broadly-distributed species that span multiple different habitats - each with different locally abundant pollinators (Johnson, 2010; Van der Niet et al., 2014).

One of the most specialised pollination strategies is that of sexual deception (Ayasse et al., 2011; Xu et al., 2012), in which pollination occurs via sexual attraction of male insects to a flower through chemical mimicry of females (Coleman, 1928, Kullenberg, 1961, Stoutamire, 1974). While sexual deception has been reported in the Asteraceae (Ellis & Johnson, 2010) and Iridaceae (Vereecken et al., 2012), it is most prevalent among the Orchidaceae. Due to the high specificity of insect sex pheromones, sexually deceptive orchids frequently have only a sole pollinator species, with closely related orchids typically exploiting different pollinator species (Paulus & Gack, 1990; Bower & Brown, 2009; Peakall et al., 2010; Gaskett, 2011; Phillips et al., 2017).

Closely related sexually deceptive orchids often attract their pollinators using structurally similar specific semiochemicals (pheromones or other inter-organism signalling compounds), though blends of structurally diverse semiochemicals can be found within a genus. For example, *Chiloglottis* species use combinations of cyclohexanediones (chiloglottones) (Schiestl et al., 2003; Peakall et al., 2010) and *Drakaea* uses blends containing pyrazines (Bohman & Peakall, 2014; Bohman et al., 2014), though one species uses pyrazines in combination with a ß-hydroxylactone (Bohman et al., 2019a), to attract different thynnine wasp pollinator species. In *Caladenia,* two species use (methylthio)phenols to attract *Campylothynnus* pollinators (Bohman et al., 2017a; Bohman et al., 2017b), while another species attracts a different genus of wasp, *Zeleboria*, using a terpene and an acetophenone (Xu et al., 2017). European *Ophrys* species use blends of alkanes and alkenes to attract bee pollinators (Schiestl et al., 1999; Schiestl et al., 2000; Mant et al., 2005a; Gervasi et al.,

2017), though one species uses carboxylic acids to attract a scoliid wasp pollinator (Ayasse et al., 2003).

Given the pivotal role of floral chemistry in pollinator attraction in sexually deceptive orchids, minor shifts in floral chemistry may attract novel pollinator species (Ayasse et al., 2011; Bohman et al., 2016). Pheromone production can be altered with only one or few genes (Haynes & Hunt, 1990; Schlüter & Schiestl, 2008), therefore random mutations can lead to the attraction of novel pollinator species (Ayasse et al., 2011; Breitkopf et al., 2013; Peakall & Whitehead, 2014). At least four likely evolutionary scenarios are possible following a mutation leading to the attraction of a novel pollinator to individuals in a population of sexually deceptive orchids, as reviewed in Peakall & Whitehead (2014). Two scenarios are based on the plants and both pollinator species (original and novel) sharing a sympatric distribution. In the first sympatric scenario, the production of the chemical shift does not prevent the attraction of the original pollinator, leading to the attraction of two pollinator species in the population. In this scenario, floral semiochemicals perceived by pollinator species would be expected to be present in all flowers. This dual pollinator attraction scenario may occur in an ecotype of *D. concolor* that attracts two different wasps that both function as effective pollinators, although the semiochemicals underlying the interaction remain unknown (Phillips et al., 2015a). In the alternate sympatric scenario, the chemical shift that attracts the novel pollinator species impairs the attraction of the original pollinator, leading to selection against intermediate phenotypes and reproductive isolation between the plants attracting the original and novel pollinator species through pollinator mediated speciation (Cozzolino & Scopece, 2008; Ayasse et al., 2011; Peakall & Whitehead, 2014). In this scenario, following pollinator mediated speciation, plants attracting different pollinator species would be expected to contain different semiochemicals.

A further two evolutionary scenarios are based on the two pollinator species occurring primarily in allopatry, where attraction of the novel pollinator leads to range expansion of the plant into the distribution of the novel pollinator species. In one scenario semiochemistry remains constant, while in the other it differs between plants attracting different pollinators. In the first of these allopatric scenarios, all plants have the capability to attract both pollinator species, however differences in the geographic distribution of the pollinators mean that most plants are only visited by one pollinator species. Under this scenario of geographic pollinator replacement, the semiochemistry of all flowers is expected to be constant. This scenario has

been demonstrated in *Chiloglottis* that contain the same semiochemicals yet attract different pollinators at different populations (Peakall et al., 2010). In the final allopatric scenario, attraction of the novel pollinator species leads to isolation from the original pollinator species and pollinator-mediated speciation in allopatry. In this scenario, plants that attract different pollinators are expected to contain different semiochemicals. While the historic geographic patterns of speciation are difficult to infer, this scenario may be operating in closely related *Chiloglottis* taxa that attract different pollinator species with different semiochemicals, some of which occur allopatrically (Peakall & Whitehead, 2014). In instances of allopatric speciation, floral volatile composition could further be expected to vary in response to different environmental selection pressures.

One method of investigating pollinator switching is through conducting choice trials, in which flowers from different populations are presented to multiple pollinator species to test for potential differences in pollinator response (Bower, 1996). Application of this methodology has led to the detection of several incipient species and pollination ecotypes in sexually deceptive orchids (Bower, 2006; Bower & Brown, 2009; Peakall & Whitehead, 2014; Menz et al., 2015; Phillips et al., 2015a). An ecotype is an ecological unit within a species that displays adaptation to its particular environmental conditions (Turesson, 1922; Lowry, 2012). A pollination ecotype possesses specific traits that attract different pollinator species (Newman et al., 2014; Van der Niet et al., 2014). In sexually deceptive orchids it is predicted that the first floral trait to diverge will be the chemical traits associated with pollinator attraction (Xu et al., 2012; Peakall & Whitehead, 2014). Accordingly, the chemical composition of ecotypes and/or species of sexually deceptive orchids are expected to vary due to their use of different pollinator attractant compounds (Mant et al., 2005b; Xu et al., 2012), and also potentially due to differences in associated by-products or intermediates formed in the biosynthesis of the attractants. Many pollination ecotypes and incipient species have been found to be morphologically cryptic, thus differences in chemical composition may be of use in identifying them (Bower, 2006; Bower & Brown, 2009; Peakall & Whitehead, 2014; Menz et al., 2015; Phillips et al., 2015a).

Chemical composition of floral volatiles has proved an informative trait in distinguishing morphologically cryptic taxa in a number of plant species (Li et al., 1995; Goffman et al., 1999; Velasco & Goffman, 1999; Velasco et al., 2000; Özcan, 2008; Coutinho et al., 2015). Sexually deceptive *Chiloglottis* taxa, which co-occur and are morphologically cryptic, can be

differentiated based on a combination of one or two specific pollinator attractant compounds (Peakall & Whitehead, 2014). These taxa are also supported based on analyses of chloroplast DNA (Peakall & Whitehead, 2014). Similarly, morphologically similar *Ophrys* taxa could be discriminated using bioactive alkanes and alkenes (Mant et al., 2005b).

A variety of methods have been implemented to detect pollinator perceived semiochemicals in sexually deceptive orchids. Electroantennographic detection coupled with gas chromatography has been successfully implemented as a part of the discovery of the semiochemicals involved in the attraction of orchid pollinators (Schiestl et al., 1999; Schiestl et al., 2000; Schiestl et al., 2003; Mant et al., 2005b; Peakall et al., 2010; Bohman et al., 2012a; Bohman & Peakall, 2014). In some systems electroantennographic detection has proved unsuccessful, and instead methods such as the screening of extracts from active versus non-active tissues (Bohman et al., 2017a), and bioassay guided fractionation (Bohman et al., 2019b) have been implemented.

An alternate method of distinguishing between potentially cryptic sexually deceptive orchid taxa is analysing the entire chemical composition of a flower using multivariate analyses of gas chromatography mass spectrometry (GC-MS) data from floral extracts. The use of such multivariate analyses can provide a high degree of discriminatory power between taxa - as has been successfully demonstrated in *Ophrys*. For example, Joffard et al. (2016) found that three *Ophrys* taxa of uncertain taxonomic rank, previously distinguished by their attraction of different pollinator species, could be distinguished with 94% accuracy using Partial Least Squares Discriminant Analysis (PLS-DA) of floral chemical composition, supporting their status as separate species. Similarly, Véla et al. (2007) found clear discrimination between the chemical composition of labellum extracts of *Ophrys* taxa in multivariate analyses, in some cases resolving taxon relationships that could not be differentiated using microsatellite analyses (Mant et al., 2005b).

Two of the ten species of sexually deceptive *Drakaea* have been found to contain morphologically cryptic ecotypes. In *D. elastica*, a northern and a southern form attract different pollinator species (Menz et al., 2015). In *D. concolor*, populations nested within the middle of the species distribution attract a second pollinator species in addition to the primary pollinator attracted by the other populations (Phillips et al., 2015a). A third case of ecotypes may potentially be present in *D. livida*, in which different populations attract different

pollinator species (Bohman et al., 2012a; Bohman et al., 2012b; Phillips et al., 2014; Phillips et al., 2017). While the chemical composition of *Drakaea* floral ecotypes is predicted to be important (Menz et al., 2015), floral chemical composition has not been investigated as a tool for distinguishing between them.

Existing methods for distinguishing sexually deceptive orchid taxa based on their chemical composition, i.e. the use of GC-MS data from a small subset of identified attractant semiochemicals, or the use of all components of the total ion chromatogram from GC-MS analysis, have their advantages and disadvantages. The use of more compounds in discriminant analyses counteracts any issue of variability in compound presence between extracts, and delivers more discriminatory power and confidence in the allocated taxon than using a very small subset of compounds. However, due to the difficulty in comparing such large datasets (particularly in quantitative analyses) that may span different time periods, and have different sampling conditions and GC-MS equipment, analysing chemical composition of entire extracts works optimally within a single study, beyond which it has limited applicability. An intermediate solution, combining the best of using pollinator attractant compounds and whole extract composition to define taxa, may lie in defining a set of discriminatory compounds whose presence/absence can be used to discriminate taxa. These discriminatory compounds may include both pollinator perceived or attractant compounds, and compounds of unknown function. An ideal system to test this methodology is *Drakaea livida*, in which two of the pollinator species have been found to respond to different electophysiologically active compounds, already suggesting the potential presence of chemical variation between populations (Bohman et al., 2012a; Bohman et al., 2012b).

The thynnine wasp *Zaspilothynnus nigripes* was the first species recorded as a pollinator of *D. livida* (Hopper & Brown, 2007), however more recent studies have reported two additional pollinator species, an undescribed species of *Catocheilus* (Bohman et al., 2012a; Phillips et al., 2014) and *Zaspilothynnus dilatatus* (Phillips et al., 2017), to be attracted to particular populations of *D. livida*. The attraction of *Catocheilus* sp. to *D. livida* is mediated by a blend of pyrazine compounds found in the labellum (Bohman et al., 2012a; Bohman & Peakall, 2014). A different pyrazine found both in flowers attracting male *Z. nigripes* and in sexually calling female *Z. nigripes* was found to be electrophysiologically active to males of this pollinator species (Bohman et al., 2012b). The last revision of *Drakaea* noted qualitative

morphological differences at some populations of *D. livida*, and suggested that further investigation of their taxonomic status was warranted (Hopper & Brown, 2007).

Given the observation of multiple pollinator species in *Drakaea livida*, the present study tested for the presence of pollination ecotypes, investigated patterns of pollinator availability and chemical divergence across the range of *D. livida*, and determined which floral compounds were electrophysiologically active to *Z. dilatatus*. It was hypothesized that: (1) *D. livida* is comprised of pollination ecotypes, (2) the distribution of plants attracting different pollinator species (potential ecotypes) correlates with the availability of their pollinator species, (3) floral volatile composition of plants attracting different pollinator species (potential ecotypes) differs, (4) the presence of electrophysiologically active compounds will vary according to the pollinator species attracted (potential ecotypes), and (5) the pollinator species (potential ecotype) of a plant can be predicted based on the presence absence of a subset of informative floral compounds.

METHODS

Study species

Drakaea plants do not flower every flowering season (spring), and when they do they produce only a single scape bearing a single flower (Hopper & Brown, 2007). *Drakaea livida* is endemic to South-West Western Australia, where it is almost entirely restricted to well-drained, grey sandy soils (Hopper & Brown, 2007). All *Drakaea* species are reliant on the same species of symbiotic *Tuslasnella* fungi for germination and annual growth (Phillips et al., 2014; Linde et al., 2017). *Drakaea livida*, the Warty Hammer orchid, derives its name from the purple wart-like spots present on its labellum.

Drakaea achieves pollination by chemical and visual mimicry of flightless female thynnine wasps (Bohman et al., 2014). Male wasps attempt to pick up and fly off with odour-producing labella *in copula*. Due to the presence of the unique hinge structure in *Drakaea*, in attempting to copulate and fly off with the orchid labellum, the wasps' momentum causes the hinge to swing the wasp upside down, bringing its thorax into contact with the column, where the pollinia and stigma are housed (Stoutamire, 1974). This flipping of the hinge by the wasp is required for pollination to occur.

Testing for the presence of pollination ecotypes in *D. livida*

In testing the hypothesis that *D. livida* is comprised of pollination ecotypes, two experiments were implemented using floral baiting. Baiting for pollinators entails the artificial presentation of picked flowers in natural bushland, which typically leads to the attraction of the pollinator species within minutes if they are present (Stoutamire, 1974; Peakall, 1990). To achieve new pollinator responses, flowers are relocated a minimum of 10 meters following each three-minute presentation. The first experiment comprised a survey to determine which wasp species pollinate different populations of *D. livida* across its range. Using this knowledge of the distribution of pollinator usage, pollinator choice experiments were then conducted to determine the response of different pollinator species to different populations of orchids. Baiting was conducted on sunny days ≥ 20ºC when thynnine wasps are most active (Stoutamire, 1974). Flowers were kept at 4 ºC in a portable refrigerator between baiting experiments.

Floral baiting survey to determine the pollinator species of **D. livida** *populations*

To determine which species of wasp are attracted to *D. livida* flowers across its distribution, flowers from 33 populations across the range of *D. livida* were individually 'baited with' (for populations and samples sizes see Supplementary Table A). Flowers were baited with in areas of natural bush within the range of *D. livida* that were either in the vicinity of *D. livida* populations or where pollinator species were known to be abundant. Wasps observed flipping the hinge of the flower (as required for pollination) were caught in an insect net for identification. Where possible, wasps were captured in cases where they closely approached flowers yet did not land. Voucher specimens of *Drakaea livida* have been deposited at the West Australian Herbarium (voucher numbers in Supplementary Table B). Locations of the populations and pollinator species attracted were mapped, with the addition of a population attracting *Catocheilus* sp. discovered by Phillips et al. (2014) for which no pollination data were collected during the present survey.

Pollinator choice experiments

To determine if the pollinator species of a given population of *D. livida* respond to flowers from populations that attract different pollinators, which may provide evidence of ecotypic variation between populations, pollinator choice experiments consisting of a series of sequential trials were conducted based on the methodology of Bower (1996). Each trial was conducted at one location and consisted of two sequential phases. In the first phase, a foreign flower is presented alone to test if the local pollinator species respond to the foreign flower, and in the second phase a local flower is presented alongside the foreign flower to confirm the presence of the local pollinator species. While not being presented, bait flowers were kept in an airtight cooler box. Choice trials could not be conducted for *Catocheilus* sp. due to its infrequent response to flowers compared to other *Drakaea* pollinators - *Catocheilus* sp visits flowers at a very low frequency, and when they do the behaviour necessary for pollination is not frequently displayed (R. Phillips personal observation, corroborated in the results of present study). Due to differences in pollinator abundance and behaviour between study sites, the methodology of the experiments varies slightly, so is explicitly stated below.

Response of Zaspilothynnus dilatatus *to flowers from populations that attract other pollinator species*

To test whether *Z. dilatatus* was attracted to flowers from populations attracting *Z. nigripes* and *Catocheilus* sp., 29 sequential two-phase choice trials (Bower, 1996) were conducted at a site in Yalgorup National Park (-32.68946, 115.63824) where *Z. dilatatus* is common and *Z. nigripes* does not occur. In phase one, flowers from populations that attract *Z. nigripes* (11 flowers from four populations) and flowers from populations that attract *Catocheilus* sp. (10 flowers presented from one population) were presented for five minutes while flowers from populations that attract *Z. dilatatus* were kept in an airtight cooler box. In phase two, flowers from populations that attract *Z. dilatatus* (nine flowers presented from four populations) were brought out to confirm the presence of *Z. dilatatus,* and were presented at a minimum distance of one meter from the phase one flowers. Four categories of wasp responses were scored, modified from Peakall (1990): 1) approach only to the flower (within 30 cm), 2) landing on the flower with an absence of hinge flipping, 3) landing on the flower and subsequently flipping the hinge, and 4) attempting copulation with the flower.

Response of Zaspilothynnus nigripes *to flowers from populations that attract* Zaspilothynnus dilatatus

To test whether *Z. nigripes* is attracted to flowers from populations attracting *Z. dilatatus*, sequential choice trials were conducted at Island Point Reserve (-32.757339, 115.690028), a site in the middle of the geographic range of the populations attracting *Z. dilatatus*, where *Z. nigripes* is known to be abundant. In phase one, flowers from populations attracting *Z. dilatatus* were presented alone for a three-minute period, and in phase two (three-minute presentation) flowers from populations attracting *Z. nigripes* were presented alongside flowers from populations attracting *Z. dilatatus* as a control to confirm the presence of *Z. nigripes*. As *Z. dilatatus* also occurs at Island Point Reserve and is indistinguishable from *Z. nigripes* in flight, only wasps that landed on flowers were recorded as these could be caught and identified in the field using differences in the colour of the underside of the leg and the shape of the clypeus.

To determine the response of *Z. nigripes* to flowers from populations that attract *Z. dilatatus* outside their natural distribution (based on museum records of *Z. dilatatus*), choice trials

presenting flowers from populations that attract *Z. dilatatus* were conducted outside the range of *Z. dilatatus* at Ruabon Nature Reserve, where *Z. nigripes* is abundant (Menz et al., 2013). Flowers from populations attracting each pollinator species (flowers from populations attracting *Z. dilatatus*: eight flowers from four populations, flowers from populations attracting *Z. nigripes*: four flowers from three populations) were presented alternately in three-minute trials. Wasp behaviour was scored according to the three categories 'approach', 'land', and 'hinge flip'. A *G*-test was conducted to compare the responses categories of *Z. nigripes* to the populations attracting the two different pollinator species.

Response of Zaspilothynnus nigripes *to flowers from populations that attract* Catocheilus *sp.*

To test whether *Z. nigripes* functions as a pollinator of flowers from populations that attract *Catocheilus* sp., sequential choice trials were conducted at Perup Road (-34.300155, 116.432782), a site in the middle of the geographic range of populations attracting *Catocheilus* sp., where *Z. nigripes* is has been found to be abundant in previous trials. In phase one (three minutes), flowers from populations attracting *Catocheilus* sp. (four flowers from two populations) were presented alone, and in phase two (three minutes) flowers from populations attracting *Z. nigripes* (six flowers from six populations presented) were presented alongside the flowers from populations attracting *Catocheilus* sp. as a control to confirm the presence of *Z. nigripes*.

To determine the response of *Z. nigripes* to flowers from populations that attract *Catocheilus* sp. outside the currently known distribution of the ecotype, sequential choice trials were conducted at Ruabon Nature Reserve. In phase one, flowers from populations attracting *Catocheilus* sp. were presented alone, and in phase two flowers from populations attracting *Z. nigripes* were removed from a sealed container and presented alongside the flowers from populations that attract *Catocheilus* sp. as a control to confirm the presence of *Z. nigripes*.

Correlation of plant distribution and pollinator availability

To test whether the distribution of plants attracting different pollinator species correlated with the availability of their pollinator species, a pollinator survey was conducted at populations that had attracted different pollinator species either in the initial floral baiting survey in the present study, or in previous studies. In total, 28 different populations across the geographic

range of *D. livida* were surveyed for pollinator abundance between 2015-2017. Nine populations that had attracted *Z. nigripes* either in the baiting survey or in earlier studies (e.g. Phillips et al. (2013); Phillips et al. (2014)), and seven populations that had attracted *Z. dilatatus,* were included. Due to the infrequent response of *Catocheilus* sp., in addition to including populations that attracted *Catocheilus* sp. in the baiting survey, an additional eight populations containing the known *Catocheilus* sp. attractants (3,5,6-trimethylpyrazin-2-yl)methyl3-methylbutanoate and 2-(3-methylbutyl)-3,5,6-trimethylpyrazine (Bohman & Peakall, 2014) were included. At each population, six two-minute baiting trials were conducted and the number and species of wasp landing on bait orchids were recorded (Phillips et al., 2014). Due to the slightly earlier flowering period of populations from the northern end of the geographic range of *D. livida*, only populations attracting *Z. dilatatus* and *Z. nigripes* were presented at the early-flowering Swan Coastal Plain populations. Populations attracting each of the three pollinators were presented at the later flowering southern populations. Differences in the number of responding wasps detected at populations attracting different pollinators were tested using a Wilcoxon rank sum test in Rv3.5.1 (R Core Team, 2018).

Floral volatile composition of plants attracting different pollinator species

In testing the hypothesis that the floral volatile composition of plants attracting different pollinator species differs, multivariate analyses of GC-MS data from floral extracts of flowers from populations that attract different pollinator species were conducted. For all extractions, individual labella were extracted for 24 hours at room temperature in 100 µL of dichloromethane containing 10 µl 10 ng/µl tert-butyl benzene (Sigma-Aldrich, 99.8%) as an internal standard, after which period they were stored at −20 °C until analysis.

For populations attracting *Z. nigripes* and *Z. dilatatus,* picked flowers were baited with to confirm the attractiveness of flowers to their specific pollinator species before extraction. To ensure that extracts were made from fresh flowers, flowers were presented to pollinators within an hour of collection, and were baited with for a maximum period of one hour prior to extraction. Flowers attracting the same pollinator species (predicted from pollinator survey) were sampled on different days to remove any temporal effect of the sampling conditions, eg. the effect of sunlight (Falara et al., 2013). For all floral extracts, only the labellum was used, as previous dissection experiments have shown that the labellum is the source of the

pollinator attractants in *D. livida* (Phillips et al., 2013). Three populations attracting *Z. nigripes* and three populations attracting *Z. dilatatus* were sampled on different days to give a total of ten fresh flowers collected per pollinator species across the season. For these populations, as soon as a pollinator landed on a flower, it was caught for subsequent identification and the flower was immediately extracted. Only flowers to which pollinators responded were extracted.

For populations that attract *Catocheilus* sp., where pollinator responses are rare, 10 flowers were sampled from the Frosty Road population, which was used in the chemical studies that originally identified that tetrasubstituted pyrazines underlie the attraction of *Catocheilus* sp. (Bohman et al., 2012a; Bohman & Peakall, 2014). Despite the infrequent pollinator responses by *Catocheilus* sp. in floral baiting trials (see results), prior to extraction Frosty Road flowers were presented to any potential pollinators for a matching period of time to flowers from other populations to ensure comparable treatment of flowers.

GC-MS analysis of the floral extracts were conducted using an Agilent 5973 Network Mass Selective Detector connected to an Agilent 6890N Network GC system equipped with an HP5MS-UI column [30 m × 0.25 mm × 0.25 μm film thickness, Agilent], using helium as a carrier gas at 1 mL/min. Peak detection and deconvolution was conducted using the EasyGC python pipeline (https://libraries.io/github/dkainer/easyGC, based on PyMS python library (O'Callaghan et al., 2012)) with the default parameters. Compounds that occurred in less than three of the thirty flowers were excluded from the analyses. Data were analysed qualitatively by assessing differences in the suite of compounds present in flowers. Data were presence-absence transformed, with all values greater than zero being set to one, prior to analyses. To visualise the difference in qualitative chemical composition between samples, a Jaccard distance matrix (Finch, 2005; Hervé et al., 2018) was calculated using the package 'vegan' (Oksanen et al., 2018), from which a principle coordinate analysis (PCoA) was conducted using the package 'ape' (Paradis et al., 2004) in R v 3.5.1 (R Core Team, 2018). To test for differences between groups of flowers attracting different pollinator species, Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted using the vegan 'adonis' function. Pairwise comparisons between groups were calculated for 100,000 permuations using a Holm correction for multiple comparisons in the R package 'funfuns' (https://github.com/Jtrachsel/funfuns).

Presence of electrophysiologically active compounds

Previous studies have already detected compounds electrophysiologically active to the two pollinator species *Z. nigripes* (Bohman et al., 2012b) and *Catocheilus* sp. (Bohman et al., 2012a). To complement this data, gas chromatography/mass spectrometry-electroantennographic detection (GC/MS-EAD) was conducted for the third pollinator species *Z. dilatatus* using floral extracts from populations attracting this pollinator species. To test if the presence of electrophysiologically active compounds varied according to the pollinator species attracted, extracts from populations across the range of *D. livida* with known pollinator species were screened for the presence of all known electrophysiologically active compounds.

Gas chromatography/mass spectrometry-electroantennographic detection of Z. dilatatus

Male *Z. dilatatus* were caught with insect nets to flowers from populations of *D. livida* known to attract this species, and kept in a refrigerator at 4 ºC until use in GC/MS-EAD experiments. Single antennae were run against pooled single labella extracts from populations attracting *Z. dilatatus* that were concentrated under a nitrogen stream, and synthetic compounds (prepared as per Bohman et al. (2017b)). GC/MS-EAD data were recorded using a HP GCD 1800A equipped with a BPX5 column [(5% phenyl dimethylpolysiloxane), 30 m × 0.25 mm × 0.25 μm film thickness, SGE Australia], using helium as a carrier gas. A GC effluent splitter was used to split the flow to the MS and EAD. The split for EAD was passed through a Syntech effluent conditioner (Syntech, Kirchzarten, Germany) containing a heated transfer line, with the outlet placed in a purified and humidified airstream, where the stainless steel electrodes holding the antenna were contained in a glass tube. For each EAD run, an excised antenna with the tip cut off was mounted on the holder (consisting of two electrodes) using electrode gel. The electrodes were connected to a PC via a Syntech Intelligent Data Acquisition Controller (IDAC2) for recording of EAD signals in the Syntech software package GC-EAD/2014 (freely available from http://gcead.sourceforge.net/download.html). For all observed EAD responses, Kovát's retention indices were calculated to enable comparison of data across instruments and experimental conditions. Retention indices and mass spectra of compounds that elicited an electrophysiological response were compared to those of matches returned by searching: (i) the National Institute of Standards and Technology (NIST) database, and (ii) a custom library

compiled within the laboratory group of orchid semiochemicals and candidate semiochemicals. Where synthetic standards were available, co-injections of candidate compounds were conducted on two columns (column 1: VF5-MS column (30 m × 0.25 mm × 0.25 µm film thickness, Varian, USA), column 2: AT™ WAX MS column (30 m × 0.25 mm × 0.25 µm film thickness, Grace, USA)) to confirm their identities.

Screening of floral extracts for electrophysiologically active compounds

347 single flower extracts from 28 populations for which pollinator response data was collected in the present or previous studies were screened for the presence of the electrophysiologically active compounds both detected in Bohman et al. (2012a) and Bohman et al. (2012b) and those detected for *Z. dilatatus* in the present study. Floral extractions and GC-MS were conducted as described previously, with the exception of not including the pre-extraction baiting step. The mass spectra of electrophysiologically active compounds were added to an AMDIS (Davies, 1998) target library, which was used to individually screen each extract. Mass spectra and retention times were manually checked when a library hit occurred. The default AMDIS search settings were used with the exception of 'Sensitivity', which was set to 'High'.

Predicting the pollinator species of a plant

To test the hypothesis that the pollinator species of a plant can be predicted based on the presence absence of a subset of informative floral compounds, a candidate subset of informative floral compounds was compiled. This subset comprised (a) all compounds electrophysiologically active to *D. livida* pollinators, and, to increase the number of compounds used and therefore potentially the confidence in predicted pollinator species, (b) compounds found to be associated with the attraction of one or two, but not all three, pollinator species.

Selection of informative compounds independent of electrophysiological activity

To detect compounds consistently present in populations attracting one pollinator species only, or in populations attracting one pollinator species and populations attracting an alternate pollinator species but not populations attracting the third pollinator species, the 347 single

labellum extracts used in the screening for electrophysiologically active compounds were further analysed. Peak detection and deconvolution was conducted using the EasyGC python pipeline (https://libraries.io/github/dkainer/easyGC, based on PyMS python library (O'Callaghan et al., 2012)) with the default parameters. Data were analysed qualitatively by assessing differences in the suite of compounds present in flowers. Data were presence-absence transformed, with all values greater than zero being set to one, prior to analyses. Compounds were sorted according to the pollinator species of the population the compounds occurred in using the R packages 'data.table' (Dowle & Srinivasan, 2018) and 'reshape' (Wickham, 2007). Compounds that occurred in populations that attracted each of the three pollinator species were removed, leaving only compounds associated with the attraction of a sole, or two, pollinator species. These candidate informative compounds were manually checked using AMDIS and any peaks called incorrectly by the software were removed. Candidate compounds were identified using the protocol previously described for electrophysiologically active compounds.

Retention indices and mass spectra of the candidate compounds were compared to those of matches returned by searching: (i) the National Institute of Standards and Technology (NIST) database, and (ii) a custom library compiled within the laboratory group of orchid semiochemicals and candidate semiochemicals from thynnine-pollinated systems. Where synthetic standards were available, co-injections of candidate compounds were conducted on two columns (column 1: VF5-MS column (30 m × 0.25 mm × 0.25 μm film thickness, Varian, USA), column 2: AT™ WAX MS column (30 m × 0.25 mm × 0.25 μm film thickness, Grace, USA)) to confirm their identities.

Partial Least Squares Discriminant Analysis

The set of 347 single-flower extracts were manually checked and screened for the final candidate set of informative compounds comprised of electrophysiologically active compounds and compounds associated with the attraction of a sole, or two, pollinator species. The spectra of candidate informative compounds were added to an AMDIS target library, which was used to individually screen and check each extract. Mass spectra and retention times were manually checked when a library hit occurred. The default AMDIS search settings were used with the exception of 'Sensitivity', which was set to 'High'. To predict the pollinator of the flowers, data from this manual screening were collated in a binary presence-

absence matrix of candidate informative compounds from which a Partial Least Squares-Discriminant Analysis was conducted using the R package 'mixOmics' (Rohart et al., 2017). Leave-one-out cross validation of the predicted groups was implemented, where the model is run *N* times, with *N*-1 as the training set and each sample point being predicted individually in a single iteration of the model. This method avoids issues with manually assigning a pollinator based on a potentially low number of informative compounds by having a model statistically determine the cut-off for inclusion.

RESULTS

Testing for the presence of pollination ecotypes in *D. livida*

Floral baiting survey to determine the pollinator species of D. livida *populations*

All three previously recorded pollinator species of *D. livida* were detected in the floral baiting survey. Wasps were caught to flowers from 28 populations of *D. livida* from across its distribution. All flowers tested from within a single population attracted the same pollinator species as one another (average 3.93 ± 0.88 SE flowers tested per population). Flowers from fifteen populations were found to attract the pollinator *Z. nigripes*, which displayed copulatory behaviour with the flowers (Supplementary Table A, Figure 1). Interestingly, despite *Catocheilus* sp. being a confirmed pollinator species (Bohman et al., 2012a), no wasps of this species (or other) were observed flipping the hinge of flowers in this baiting survey, yet they were observed to closely approach (within 5 cm) flowers from five populations (Supplementary Table A, Figure 2). Flowers from seven populations, all on the Swan Coastal Plain, were found to attract *Z. dilatatus* (Supplementary Table A, Figure 1). *Zaspilothynnus dilatatus* displayed copulatory behaviour with the flowers and was observed to flip the floral hinge as is required for pollination.

Pollinator choice experiments

Response of Zaspilothynnus dilatatus *to flowers from populations attracting* Z. nigripes *and* Catocheilus sp.

Zaspilothynnus dilatatus was only attracted to flowers from local populations, and ignored flowers from populations attracting alternate pollinator species. In phase one, no *Z. dilatatus* approached or landed on flowers from populations that attract *Z. nigripes* or *Catocheilus* sp., demonstrating these flowers to be unattractive to *Z. dilatatus*. In phase two (presentation of local flowers from populations attracting *Z. dilatatus*), *Z. dilatatus* was confirmed as present by its response to known attractive flowers in 24 out of the 29 trials. Of the 60 *Z. dilatatus* observed approaching flowers, 45% landed, 42% contacted the column by flipping the hinge, and 30% attempted copulation with the flower.

Response of Zaspilothynnus nigripes *to flowers from populations that attract* Zaspilothynnus dilatatus

Within the distribution of *Z. dilatatus*, the co-occurring *Z. nigripes* did not respond to flowers from populations attracting *Z. dilatatus*. No *Z. nigripes* landed on flowers from populations attracting *Z. dilatatus* in either phase one or two of the 20 trials conducted at Island Point Reserve. In 17 of these trials, *Z. nigripes* was confirmed as present by landing on the flowers from populations that attract *Z. nigripes* that were added as a control in phase two (N = 81, 4.05 responses per trial). While not the focus of this specific sequential choice experiment, it is noteworthy that 36 *Z. dilatatus* were caught to the local flowers expected to attract this species, corroborating the results of the choice trials conducted at Yalgorup National Park.

When trials were conducted outside of the known geographic range of *Z. dilatatus* (based on museum records) and the populations of *D. livida* that it is attracted to, 142 *Z. nigripes* (15.78 wasps per trial) responded to flowers from populations attracting *Z. nigripes*, while 116 (5.52 wasps per trial) were attracted to flowers from populations that typically only attract *Z. dilatatus* (Supplementary Figure A). When landing on flowers from populations that attract *Z. nigripes*, 27.5% of *Z. nigripes* conducted the hinge flip behaviour necessary for pollination. When attracted to flowers from populations that normally attract *Z. dilatatus* only, 0.9% of *Z. nigripes* exhibited the hinge flip behaviour. A G-test comparing the proportion of each response category (approach, land, hinge flip) in flowers from populations attracting *Z. dilatatus* and in flowers from populations attracting *Z. nigripes* revealed a significant difference (G = 44.3 and $P < 0.001$).

Response of Zaspilothynnus nigripes to flowers from populations that attract Catocheilus sp.

No *Z. nigripes* responded to flowers from populations that attract *Catocheilus* sp. in phase one of the 25 choice trials conducted at a population attracting *Catocheilus* sp.. In phase two, 81 *Z. nigripes* responded to flowers from populations attracting *Z. nigripes*, confirming their presence at the site. One *Z. nigripes* was observed flipping the hinge of a flower from a population attracting *Catocheilus* sp. during phase two (simultaneous presentation of flowers from populations attracting *Z. nigripes* and from populations attracting *Catocheilus* sp).

When the experiment was repeated outside of the geographic area where *Catocheilus* sp. are involved in pollination, no *Z. nigripes* responded to the *Catocheilus sp*. attracting flowers presented in phase one. In phase two, 355 *Z. nigripes* responded to the control flowers from populations that attract *Z. nigripes*, with 24.9 % flipping the hinge. In phase two, where flowers from both *Catocheilus* sp. attracting and *Z. nigripes* attracting populations were simultaneously presented, one *Z. nigripes* was observed flipping the hinge of a flower from a *Catocheilus* sp. attracting population.

Correlation of plant distribution and pollinator availability

Populations attracting different pollinator species each occupied a largely discrete geographic region (Figure 1). *Zaspilothynnus nigripes* was recorded at populations of *D. livida* across its geographic range: at populations where local orchids attracted *Z. nigripes*, and also at populations where local orchids attracted only *Z. dilatatus* or *Catocheilus* sp. (Figure 1). A Wilcoxon rank sum test revealed there to be significantly more *Z. nigripes* recorded at populations known to attract *Z. nigripes* (12 ± 2.49 wasps per trial) than at populations known to attract *Catocheilus* sp. (4.4 ± 1.58 wasps per trial, $P = 0.029$, $W = 68$) and populations known to attract *Z. dilatatus* (3 ± 1.11, $P = 0.021$, $W = 43$). There was no significant difference between the number of *Z. nigripes* recorded at populations attracting *Catocheilus* sp. and populations attracting *Z. dilatatus* ($P = 0.864$, $W = 39.5$, Wilcoxon rank sum test). There was no significant difference between the number of *Z. dilatatus* and *Z. nigripes* recorded at populations attracting *Z. dilatatus* ($P = 0.895$, $W = 26$, Wilcoxon rank sum test). *Zaspilothynnus dilatatus* was recorded at six out of the seven (85.7 %) populations known to attract *Z. dilatatus*, and was not recorded at any populations known to attract *Z. nigripes* or *Catocheilus* sp. (Table 1). While observed in other experiments, no *Catocheilus* sp. were observed during the pollinator distribution survey.

Floral volatile composition of plants attracting different pollinator species

Flowers that attracted different pollinator species were found to possess different floral volatile compositions. Using floral extracts from specimens with a confirmed pollinator response, 66 compounds met the criteria for inclusion in the multivariate extract analysis. The principle coordinate analysis showed three distinct clusters (Figure 2), each comprised of samples from populations attracting a single pollinator species. Samples from populations

attracting *Catocheilus sp.* separated along the first axis, while all three pollinator species groups displayed some separation along the second axis. Cumulatively, the first three axes contribute 52.5% of the total variation (Axis 1: 26.9%, Axis 2: 16.9%, Axis 3: 8.7%). There was a significant global difference between flowers attracting different pollinator species (PERMANOVA, $R^2 = 0.48$, $P = 0.001$). Pairwise comparisons revealed significant differences between all groups of flowers as defined by pollinator response (*Z. nigripes* vs. *Catocheilus* sp. attracting, $R^2 = 0.46$, $P < 0.001$; *Z . nigripes* vs. *Z. dilatatus* attracting, $R^2 = 0.31$, $P < 0.001$; *Catocheilus* sp. vs. *Z. dilatatus* attracting, $R^2 = 0.44$, $P < 0.001$).

Presence of electrophysiologically active compounds

Gas chromatography/mass spectrometry-electroantennographic detection of Z. dilatatus

In addition to the six compounds already found to be electrophysiologically active in *D. livida* (**1**, **8-9**, **12-14**) (Bohman et al., 2012a; Bohman et al., 2012b), analysis of GC/MS-EAD data revealed electroantennographic responses of *Z. dilatatus* to two compounds present in floral extracts from populations attracting this species. These compounds were identified by comparisons of retention times and mass spectra, and confirmed by co-injection to be 2-(methylthio)benzene-1,4-diol (**18**) and 4-hydroxy-3-(methylthio)benzaldehyde (**19**), both available from previous studies (Bohman et al., 2017) (Supplementary Figure B, Table 2).

Screening of floral extracts for electrophysiologically active compounds

Sixteen percent (55/347) of floral extracts did not contain any electrophysiologically active compounds. Compounds electrophysiologically active to a specific pollinator species were only present in plants attracting those pollinator species. Compound **1** (electrophysiologically active to *Z. nigripes* in Bohman et al. (2012b)) was found in 78 flowers, all of which came from populations that attracted *Z. nigripes* (total 111 flowers). Compounds **8-9** and **12-14** (electrophysiologically active to *Catocheilus* sp. in Bohman et al. (2012a)) were found exclusively in populations known to attract *Catocheilus* sp.. Compound **14** was found in 100% (113) of flowers from populations known to attract *Catocheilus* sp. Compounds **18** and **19** (electrophysiologically active to *Z. dilatatus* in the present study) were found exclusively in populations attracting *Z. dilatatus*. While compound **18** was not detected in the automated extract analyses due to co-elution with **19**, manual screening enabled its detection.

Compounds **18** and **19** were found in 46% (57) and 80% (98) of the flowers from populations known to attract *Z. dilatatus* (123). Each of the 28 populations with replicate individuals (mean samples per population = 7.94 ± 1.6) was composed of flowers containing compounds electrophysiologically active to a single pollinator species only.

Predicting the pollinator species of a plant

Selection of informative compounds independent of electrophysiological activity

Of the 12 candidate informative compounds detected only by floral solvent extract analyses, three were identified, one was tentatively identified based on NIST library matches, and eight remain unknown. Compound **20** was identified by co-injection of a synthetic standard as 4-(hydroxymethyl)-2-(methylthio)phenol. Compound **4** was identified by co-injection as 4-(2-hydroxyethyl)-2-methoxyphenol (homovanillyl alcohol). Compound **7** was identified as 3,5,6-trimethylpyrazine-2-carbaldehyde, which probably is an artefact from the analysis, formed in the GC-inlet by oxidation (Bohman & Flematti, 2015) and was therefore not included in the subsequent analyses. Compound **6** was tentatively identified as a heneicosene isomer (double bond position not confirmed). The mass spectra and retention indices of the unidentified compounds **2-3**, **5**, **10-11**, and **15-17** did not match any of those in the NIST database, nor those of any orchid semiochemicals or candidate semiochemicals known to the laboratory group. All compounds **1-20** could be reliably detected in extracts using their characteristic mass fragments and RIs presented in Table 2. Full mass spectra of **1-20** are presented in Supplementary Figure C.

Partial Least Squares Discriminant Analysis

The suite of informative compounds present in a population remained constant across years (mean number of years sampled per population = 2.7 ± 0.3 SE). Excluding the seven extracts that did not contain any of **1-6** & **8-20**, Partial Least Squares-Discriminant Analysis based of the presence/absence of **1-6** & **8-20** with leave-one-out cross validation correctly assigned the pollinator with 100% accuracy - for all 338 extracts the predicted pollinator species matched that known from baiting data for the population. The matrix of presence absence data for compounds **1-20** used in the model is provided in Supplementary Table C.

DISCUSSION

Presence of pollination ecotypes in Drakaea livida

In accordance with the hypothesis that *D. livida* is comprised of pollination ecotypes, the results of the floral baiting survey and pollinator choice trials indicate that three distinct ecotypes, each defined by differences in pollinator response and species, are present within *D. livida*. One ecotype is visited exclusively by *Z. nigripes* across its broad geographic range (Ecotype One). Another ecotype (Ecotype Two) consists of populations known to attract *Catocheilus sp*., which were not attractive to *Z. dilatatus*. While flowers from these populations elicited rare responses from *Z. nigripes* (less than 0.01% of the *Z. nigripes* to which Ecotype Two flowers were presented in this study), the behaviour of *Z. nigripes* when responding to Ecotype Two flowers differed markedly to the behaviour of *Z. nigripes* responding to Ecotype One flowers. A third ecotype was found to occur on the Swan Coastal Plain, which exclusively employs *Z. dilatatus* as a pollinator (Ecotype Three). *Zaspilothynnus dilatatus* displayed a similar rate of column contact to *Z. nigripes* (42% and 43%, (Phillips et al., 2013)). In summary, there were clear quantitative differences in the species and/or behaviour of pollinator attracted to each ecotype, allowing the definition of three discrete ecotypes of *D. livida* based on pollinator response.

While Ecotype Two is clearly supported as a different ecological entity to Ecotypes One and Three, aspects of its pollination are not yet fully resolved by the current data. While *Catocheilus* sp. has been observed to conduct the behaviour necessary for pollination (Bohman et al., 2012a; Phillips et al., 2014), given its propensity to approach flowers without landing or flipping the hinge (behaviour that results in a low pollination efficiency) it is plausible that Ecotype Two has an additional undetected pollinator species that also contributes to fruit set. If additional pollinator species are present, they are likely to occur in low abundance, or potentially differ in life history to the pollinator species successfully detected with the baiting methodology. Already, the disjunct northernmost population of this ecotype has been found to attract a different, yet closely related species of *Catocheilus* (Phillips et al., 2017), potentially indicating undetected variation within this ecotype. To this end, it may be possible that populations of *D. livida* from populations at the margins of its range not included in this study may potentially attract different pollinator species and represent additional ecotypic diversity.

Of further interest is that *Z. dilatatus* was the sole pollinator species of Ecotype Three, despite the presence of *Z. nigripes* at sites where it grows. Interestingly, when tested outside the geographic range of *Z. dilatatus*/Ecotype Three, infrequent responses of *Z. nigripes* were recorded to Ecotype 3 flowers, which could be due to the greater abundance of *Z. nigripes* at these sites. Outside the range of Ecotype Three, 15.78 *Z. nigripes* were recorded per three-minute trial, while inside the range of Ecotype Three only 4.05 *Z. nigripes* were recorded per three-minute trial. It is possible that when male thynnines are in greater abundance, thereby experiencing a higher operational sex ratio (Gaskett et al., 2008), they may be more likely to respond to a broader range of mate signals (Bretman et al., 2011). Alternatively, within the geographic range of *Z. dilatatus* there may be selection pressure for *Z. nigripes* to recognise and avoid females of *Z. dilatatus*, while this may not be the case outside of the range of *Z. dilatatus*.

No population attracted more than one pollinator species, indicating that the ecotypes do not occur sympatrically within a population. Further, based on the populations sampled, each ecotype appeared to occupy a largely distinct geographic area: Ecotype One predominantly on the south coast, Ecotype Two in inland areas of Jarrah forest, and Ecotype Three on the Swan Coastal Plain (Figure 1). The allopatric distribution of the ecotypes indicates that pollinator switching has likely occurred in allopatry.

The differences in pollinator response to different ecotypes (with each ecotype having its own specific pollinator species), and the co-occurrence of pollinator species in similar abundances, demonstrate that geographic pollinator replacement does not explain the presence of multiple pollinator species across the range of *D. livida*. Instead, it is likely that pollinator mediated speciation in allopatry explains pollinator switching to different pollinator species.

Correlation of ecotype distribution and pollinator availability

The hypothesis that the distribution of the ecotypes correlates with the availability of their pollinator species was not supported across the *D. livida* ecotypes. The pollinator survey revealed the distribution of the Ecotype One pollinator *Z. nigripes* to be much broader than that of the Ecotype One orchids (Figure 1), suggesting a potential role for abiotic factors in

limiting the distribution of Ecotype One. The ability to infer patterns of pollinator efficacy are limited in Ecotype Two, given its *Catocheilus* sp. pollinator has a much lower pollination efficiency than Ecotypes One and Three, and that its distribution could not be quantified. The pollinator survey revealed the distribution of Ecotype Three to be strongly correlated with the distribution of its pollinator, the Swan Coastal Plain endemic *Z. dilatatus*. However, despite this correlation, the alternative Ecotype One pollinator species *Z. nigripes* was present throughout the distribution of Ecotype Three. Similarly, the Ecotype One pollinator occurred throughout the distribution of Ecotype Two. The presence of the Ecotype One pollinator throughout the distributions of all three Ecotypes (two of which do not attract it) is in contrast to other systems where floral ecotypes have evolved in adaptation to the regionally available pollinator species (Robertson & Wyatt, 1990; Johnson, 1997). The present availability of pollinators, and the present ecotype distributions, indicate that recent pollinator switching to the most locally effective pollinator is not supported. Assuming a correlation between historic and present patterns of pollinator availability, pollinator mediated speciation in *D. livida* does has not appear to have been driven by local availability of pollinators.

A similar scenario of mismatched pollinator and ecotype distributions was found in ecotypes of *D. concolor* (Phillips et al., 2015a). An explanation for these distributions may lie in that due to the high diversity of thynnine wasps (Mackerras, 1970), random mutations may lead to differences in floral scent that may attract novel pollinators anywhere within the distribution of an ecotype (Phillips et al., 2015a). Minor genetic changes can cause a difference in the attractant compounds produced (Haynes & Hunt, 1990; Schlüter & Schiestl, 2008), and may be sufficient to induce pollinator switching between species with similar sex pheromone chemistry (Ayasse et al., 2011; Breitkopf et al., 2013). Therefore, pollinator switching may not necessarily occur in response to local pollinator efficacy, and could potentially be more dependent on the availability of wasp species with similar sex pheromone chemistry. The *D. livida* ecotype distributions may have been influenced by potential differences in bioclimatic habitat preference, supported by their allopatric distributions. Further investigation of the ecotype geographic ranges and bioclimatic niches would be required to explore this alternative.

Floral volatile composition of the ecotypes

The hypothesis that the floral volatile composition of the ecotypes differs was supported. Analyses of the volatile composition of labellum extracts of *D. livida* flowers were congruent with the results of the pollinator baiting experiments in showing three discrete groups within *D. livida*. In multivariate space three significantly different clusters were found, each that correlated with the attraction of a different specific pollinator. The high degree of separation between the ecotypes in chemical multivariate space does not appear to be congruent with a model of recent pollinator switching underpinned by minor genetic and therefore chemical changes. This result suggests that an alternate evolutionary scenario potentially applies in the *D. livida* ecotypes, as further implicated in electrophysiology results.

Presence of electrophysiologically active compounds in the ecotypes

The hypothesis that the presence of electrophysiologically active compounds would vary according to the pollinator species attracted/ecotype was supported. In addition to the known pyrazine and tetrasubstituted pyrazine compounds electrophysiologically active to Ecotypes One and Two respectively (Bohman et al., 2012a; Bohman et al., 2012b), in the present study two (methylthio)phenol compounds were found to be electrophysiologically active to the Ecotype Three pollinator *Z. dilatatus*.

The discovery of (methylthio)phenols in *D. livida* adds a new class of pollinator-perceived compounds known to be present in *Drakaea*, in addition to the previously reported pyrazines and β-hydroxylactone (drakolide) (Bohman et al., 2012a; Bohman et al., 2012b; Bohman & Peakall, 2014; Bohman et al., 2014; Bohman et al., 2019a). The discovery that (methylthio)phenols are present in Ecotype Three and are perceived by the pollinator *Z. dilatatus* presents an interesting case of convergent evolution of floral volatiles. In two species of *Caladenia*, the (methylthio)phenols perceived by *Z. dilatatus* underlie the attraction of sexually-deceived pollinators in the thynnine wasp genus *Campylothynnus* (in one case as part of a blend) (Bohman et al., 2017a; Bohman et al., 2017b). While compound sharing of a pyrazine in *Drakaea* and *Caladenia* has been previously reported (Bohman et al., 2013), this example in *D. livida* represents the first case where the shared compound(s) are known to be perceived by pollinators in both orchid genera. These (methylthio)phenol compounds are not currently known as semiochemicals in any other organisms, yet given that

they are perceived by two different genera of thynnine wasps, they may represent an important class of semiochemicals within the clade containing *Zaspilothynnus* and *Campylothynnus,* see (Phillips et al., 2017), or perhaps more broadly in Australian thynnines. The presumed convergent evolution of the production of (methylthio)phenol and pyrazine compounds in the distantly related *Drakaea* and *Caladenia* (Weston et al., 2014) likely occurred as a result of their shared pollination strategy - sexual deception of thynnine wasps.

All electrophysiologically active compounds were found exclusively in flowers of the ecotype pollinated by the pollinator they were perceived by. No extracts displayed a mixed phenotype, as would be characterised by containing multiple different electrophysiologically active compounds associated with the attraction of different pollinator species. While a large number of floral extracts were sampled and results were consistent across years, mixed phenotype individuals may yet occur at a low abundance in some populations of *D. livida*, potentially where the ranges of the ecotypes adjoin. Despite this concession, the ecotype specificity of the electrophysiologically active compounds is suggestive of reproductive isolation between the ecotypes, as would be congruent with the model of pollinator-mediated speciation explaining pollinator switching in the *D. livida* ecotypes.

Predicting the ecotype of a plant based on a subset of informative floral compounds

The hypothesis that the ecotype of a plant could be predicted based on the presence absence of a subset of informative floral compounds was supported. Of the total 347 individual floral extracts, all samples containing informative compounds (338) were correctly assigned a pollinator species/ecotype in the PLS-DA. It is possible for subsequent investigators to assign ecotypes to additional *D. livida* samples by presence absence screening extracts for the provided spectra of the informative compounds (Supplementary Figure C). Assignment can be conducted based on manual matching of compound presence-absences (identified using spectra and retention indices provided in Supplementary Figure C) with those diagnostic of each ecotype (Table 2), or by running a PLS-DA or similar model using the provided presence-absence data matrix from the present study (Supplementary Y) as a training set.

While a subset of compounds was effective as an ecotype-discriminant tool, many of which were specific to a single ecotype, these compounds are not all assumed to be pollinator attractants. Further investigation including field bioassays would be required to determine

whether these informative compounds are pollinator attractants, non-active by-products of the synthesis of the attractant semiochemicals, or other unrelated compounds. It is of interest that all compounds found to be electrophysiologically active either in the present or previous studies (Bohman et al., 2012a; Bohman et al., 2012b), with the exception of one compound that co-eluted, were also detected in the extract analyses. While not the focus of the present study, extract analyses may prove a complementary method to electrophysiology in finding candidate pollinator attractant compounds, particularly in systems where pollinator availability is limited.

Evolutionary origin of the D. livida ecotypes

Remarkably, structurally diverse compounds ((methylthio)phenols and pyrazines) occur in different ecotypes - an unexpected situation for plants that are ostensibly each others' closest relatives. While there is a precedent for the use of structurally diverse compounds within a genus (Ayasse et al., 2003; Xu et al., 2017; Bohman et al., 2019a), it is remarkable that within a single species, two different wasp genera are attracted, which respond to compounds with very different structures. The production of structurally distinct compounds is expected to occur through different biosynthetic pathways that are associated with different suites of by-products and intermediates. Each ecotype using different biosynthetic pathways may therefore explain the high degree of chemical differentiation between the ecotypes in the principal co-ordinates analysis, through the presence of different biosynthetic by-products and intermediates in each ecotype. This potential use of different biosynthetic pathways in different ecotypes is not the expected scenario for closely related taxa, and hints at an interesting evolutionary origin.

Of the four speciation models presented in Peakall & Whitehead (2014), only one fits with the patterns of geographic distribution, pollinator specificity, and chemical differences present in the *D. livida* ecotypes. The apparent allopatry of the ecotypes rules out the first two sympatric scenarios. Further, the observed pollinator specificity, and the ecotype-specificity of the electrophysiologically active compounds, rule out the possibility of the allopatric geographic pollinator replacement scenario. This leaves only one possible scenario that is congruent with the patterns observed in *D. livida* - that of pollinator switching and subsequent pollinator mediated speciation in allopatry. Under an allopatric speciation scenario, significant differences in overall floral volatile composition could be expected to

occur over time in response to local environmental selection pressures following pollinator-mediated speciation. If this model is operating in *D. livida*, the divergence event was likely not recent given the large degree of chemical differentiation between ecotypes that would be expected to take a time to accumulate. However, given that the pollinator perceived compounds themselves (those hypothesised to have driven pollinator mediated speciation) display a high degree of structural diversity, an alternate fifth model of convergent evolution may be proposed.

This fifth model of speciation would entail the ecotypes having convergently evolved the warty floral morphology. Evolutionary flexibility of the warty phenotype is plausible given that occasional warty individuals occur in *D. gracilis* and *D. isolata*, while populations of the warty *D. confluens* sometimes contain individuals with a smooth labellum (Hopper & Brown, 2007). Furthermore, some *D. livida* individuals with wartless labella containing ecotype diagnostic compounds have been found (A. Weinstein unpublished data). This hypothesised evolutionary scenario of convergent morphology may explain the presence of different chemical classes, and the significant differences in overall floral chemical composition, in the *D. livida* ecotypes. It could be postulated that the pyrazine-containing Ecotypes One and Two are closely related to the pyrazine-containing *D. glyptodon* and *D. micrantha*, while Ecotype Three may have a separate evolutionary origin.

Further analyses would be required to conclusively distinguish between these two evolutionary scenarios of convergence of floral morphology and pollinator switching from a common ancestor with a long time since divergence. Phylogenetic analyses, such as the exome-capture next generation sequencing implemented in Chapter Three may be informative in distinguishing between the two evolutionary scenarios.

Conservation implications of the presence of ecotypes

The present study found strong evidence for three chemically distinct pollination ecotypes of *D. livida* that each occupy a different geographic region. The three ecotypes are ecologically distinct, may represent different evolutionary lineages, and potentially could be considered discrete taxa under the biological species concept (Mayr, 1942). As such, it is recommended that the three ecotypes be treated as distinct entities in conservation management. If not treated separately there may be detrimental effects for the ecotypes, such as the potential

unnatural mixing of ecotypes or mismatch with pollinator distribution that could occur in a poorly planned translocation (Hufford & Mazer, 2003; Weeks et al., 2011; Reiter et al., 2016).

Conservation concerns may stand for Ecotype Three, which is thus far known to occur only in nine remnant bushland reserves on the Swan Coastal Plain, where it grows in *Kunzea ericifolia* thickets among mixed *Eucalyptus* and *Banksia* woodland. The Swan Coastal Plain is a known hotspot for orchid rarity, where regional endemics have become rare through extensive habitat clearing for agriculture and development (Horwitz et al., 2008; Phillips et al., 2011; Phillips et al., 2015b). As such, it is likely that Ecotype Three may be rare and threatened by habitat loss. It is recommended that further research is conducted to determine the geographic extent of the ecotypes. A critical component of such an investigation will be determining reliable method/s of identifying the ecotypes - pollinator baiting is not ideal as it is destructive in that it requires the picking of fresh flowers. While the recent revision of the genus did not recognise three distinct groupings in *D. livida* (Hopper & Brown, 2007), more targeted analyses focusing on populations known to be different ecotypes may uncover undiscovered morphological differences.

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TABLES AND FIGURES

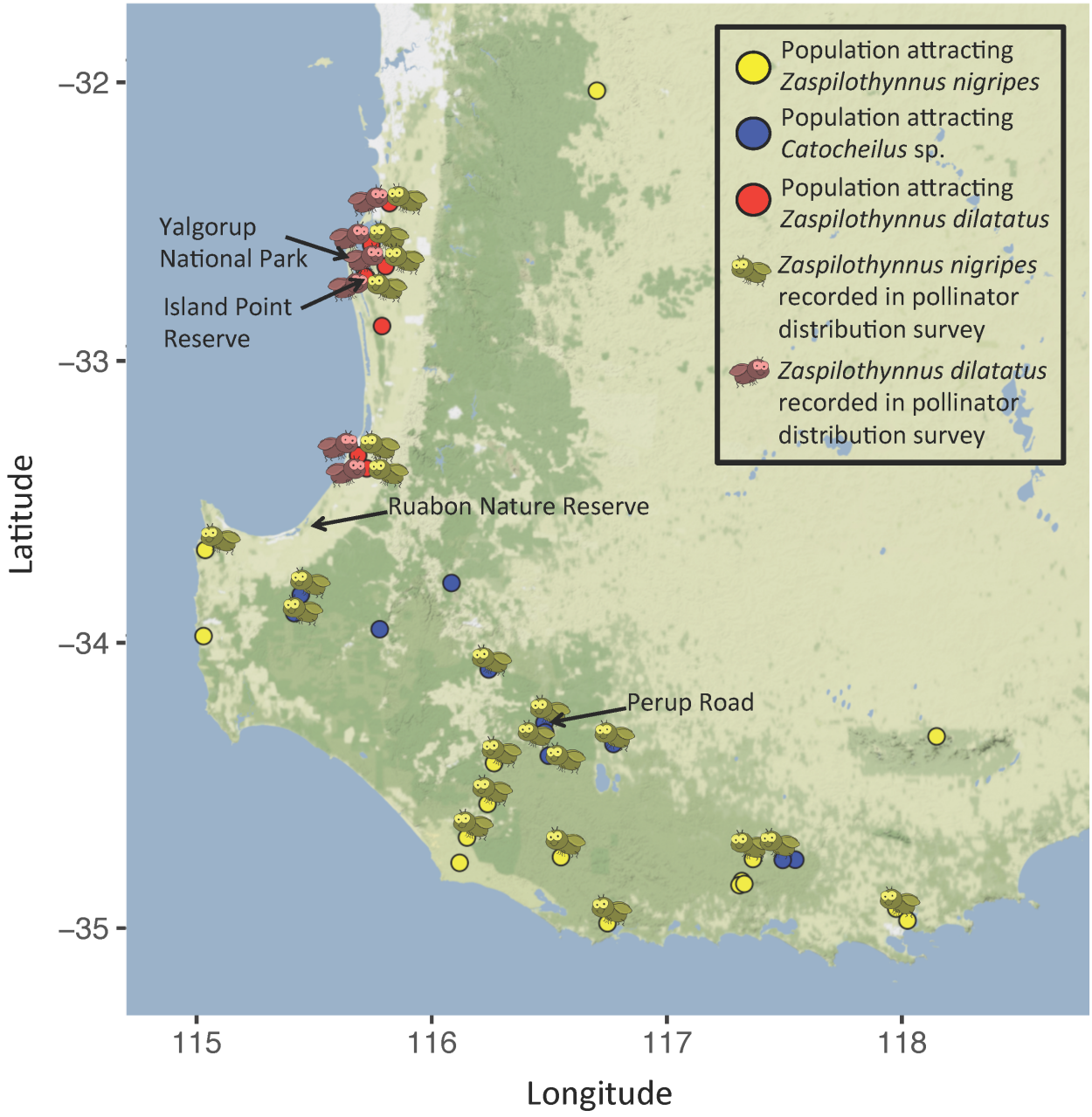

Figure 1: Distribution of populations of *Drakaea livida* attracting *Zaspilothynnus nigripes* (yellow circles), *Catocheilus* sp. (blue circles), and *Zaspilothynnus dilatatus* (red circles) showing which pollinator species were detected present in the pollinator survey: *Z. nigripes* present (yellow wasp), *Z. dilatatus* present (red wasp).

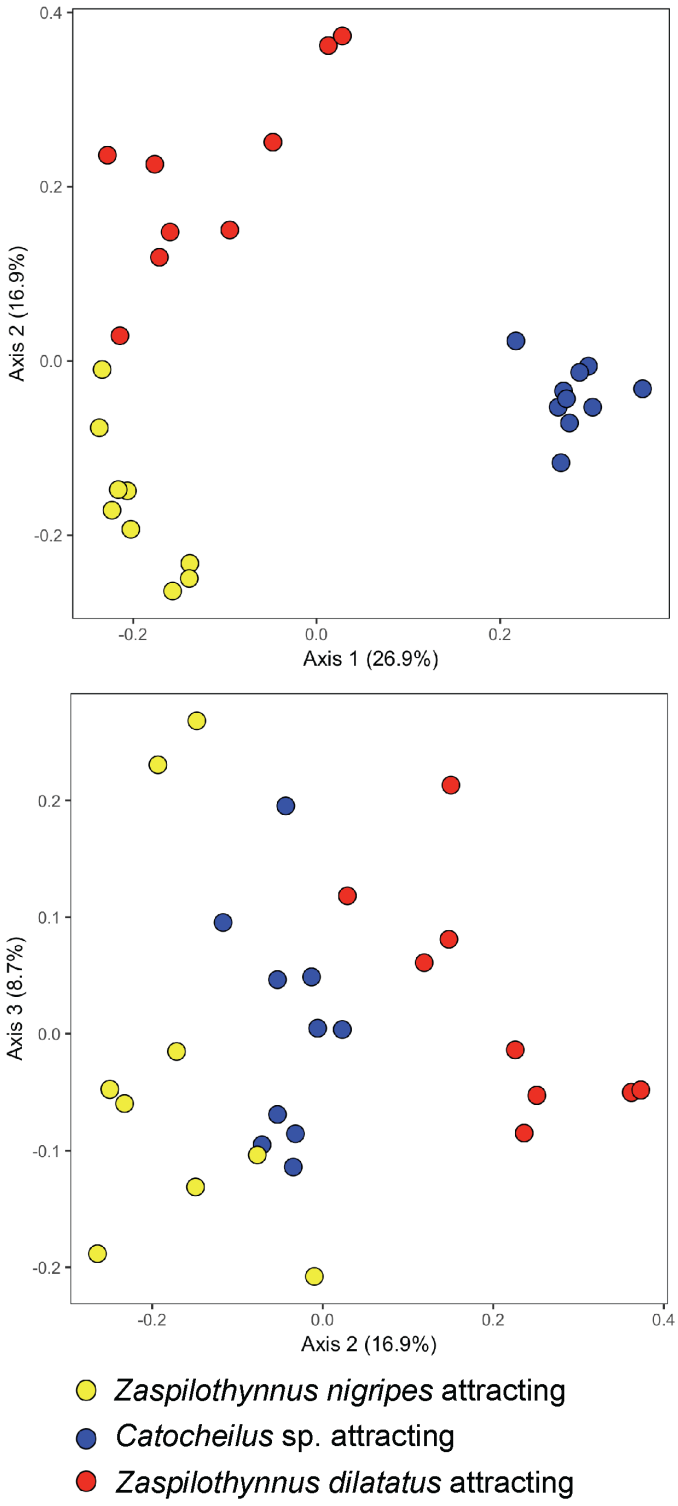

Figure 2: Principle coordinate analysis of the presence-absence of 66 compounds detected in the *Drakaea livida* extracts (flowers that attracted *Zaspilothynnus nigripes* = yellow, flowers from populations attracting *Catocheilus* sp. = blue, flowers that attracted *Zaspilothynnus dilatatus* = red). The relative corrected Eigen values denoting the percentage contribution of each axis to the total variation is displayed in the axes titles

Table 1: Number of wasps of each species of pollinator recorded at populations attracting different pollinator species. Bolded lines indicate the local populations attracting the responding pollinator species. *denotes differences in the number of pollinators observed at populations attracting different pollinator species $P<0.05$.

Zaspilothynnus nigripes responses	*N* sites surveyed	% sites present	Average number of wasps per survey	Total wasps observed
***Zaspilothynnus nigripes* pollinated populations**	**9**	**100**	**12 ± 2.49***	**108**
Catocheilus sp. pollinated populations	12	66.7	4.4 ± 1.58	53
Zaspilothynnus dilatatus pollinated populations	7	71.4	3 ± 1.11	21
Zaspilothynnus dilatatus responses				
Zaspilothynnus nigripes pollinated populations	9	0	0	0
Catocheilus sp. pollinated populations	12	0	0	0
***Zaspilothynnus dilatatus* pollinated populations**	**7**	**85.7**	**2.14 ± 0.46***	**15**
Catocheilus sp. responses				
Zaspilothynnus nigripes pollinated populations	9	0	0	0
***Catocheilus* sp. pollinated populations**	**12**	**0**	**0**	**0**
Zaspilothynnus dilatatus pollinated populations	not surveyed	not surveyed	not surveyed	not surveyed

Table 2: Characteristic mass fragments, molecular weights, and retention indices (RI) of informative compounds detected by gas chromatography/mass spectrometry - electroantennographic detection and extract analyses.

No	Pollinator association	Name	Characteristic mass fragments	RI	Detection Method
1	*Zaspilothynnus nigripes*	2-hydroxymethyl- 3-(3-methylbutyl)- 5-methylpyrazine	194, 163, 138, 109	1532	EAD*/Extract analyses
2	*Zaspilothynnis nigripes*	Unknown 1	168, 150, 139, 122	1557	Extract analyses
3	*Zaspilothynnus nigripes*	Unknown 2	196, 154, 136, 108	1804	Extract analyses
4	*Zaspilothynnus nigripes /Cateocheilus* sp.	4-(2-hydroxyethyl)-2-methoxyphenol (homovanillyl alcohol)	168, 150, 137, 122	1547	Extract analyses
5	*Zaspilothynnis nigripes /Cateocheilus* sp.	Unknown 8	208, 124, 107, 77	1722	Extract analyses
6	*Zaspilothynnis nigripes /Z. dilatatus*	Heneicosene (unknown isomer)	294, 11, 97, 83, 55	2086	Extract analyses
7	*Cateocheilus* sp.	3,5,6-trimethylpyrazine-2-carbaldehyde	150, 122, 121, 107	1207	Extract analyses
8	*Cateocheilus* sp.	2-hydroxymethyl-3,5,6-trimethylpyrazine	152, 151, 134, 123	1299	EAD$^{+/}$Extract analyses
9	*Cateocheilus* sp.	2-(3-methylbutyl)-3,5,6-trimethylpyrazine	191, 177, 149, 136	1389	EAD^{+}/Extract analyses
10	*Cateocheilus* sp.	Unknown 3	168, 151, 139, 121	1538	Extract analyses
11	*Cateocheilus* sp.	Unknown 4	208, 193, 175, 149	1568	Extract analyses
12	*Cateocheilus* sp.	(3,6-dimethylpyrazin-2-yl)methyl 3-methylbutanoate	222, 180, 138, 121	1580	EAD^{+}/Extract analyses
13	*Cateocheilus* sp.	(3,5,6-trimethylpyrazin-2- yl)methyl-3-methylbutanoate	236, 208, 152, 151	1660	EAD^{+}/Extract analyses
14	*Cateocheilus* sp.	(3,5,6-trimethylpyrazin-2-yl)methyl(2*S*)-methylbutanoate	236, 194, 152, 151	1667	EAD^{+}/Extract analyses
15	*Cateocheilus* sp.	Unknown 5	252, 168, 151, 138	1899	Extract analyses
16	*Cateocheilus* sp.	Unknown 7	253, 168, 151, 121	2001	Extract analyses
17	*Cateocheilus* sp.	Unknown 6	210, 168, 151, 122	2022	Extract analyses
18	*Zaspilothynnus dilatatus*	2-(methylthio)benzene-1,4-diol	156, 141, 113, 97	1507	EAD^
19	*Zaspilothynnus dilatatus*	4-hydroxy-3-(methylthio)benzaldehyde	168, 167, 139, 97	1507	EAD^/Extract analyses
20	*Zaspilothynnus dilatatus*	4-(hydroxymethyl)-2-(methylthio)phenol	170, 153, 141, 123	1560	Extract analyses

*EAD data from Bohman et al (2012a), $^{+}$ EAD data from Bohman et al (2012b), ^ EAD data from present study

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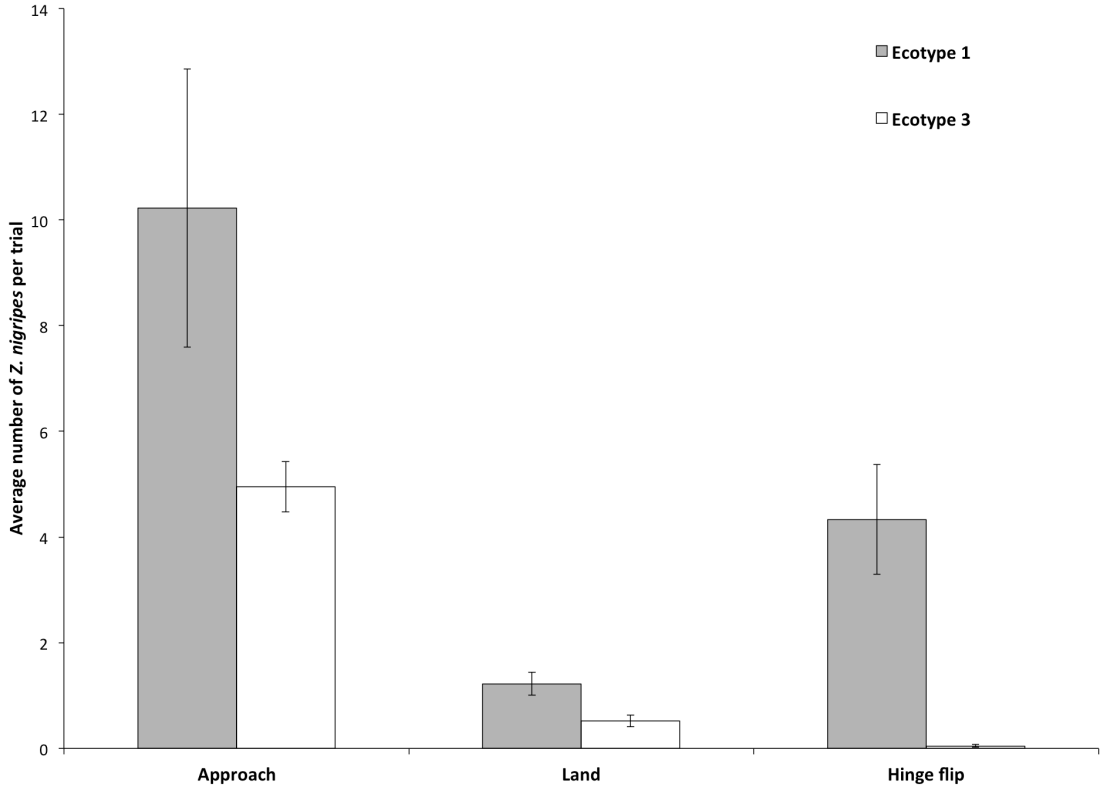
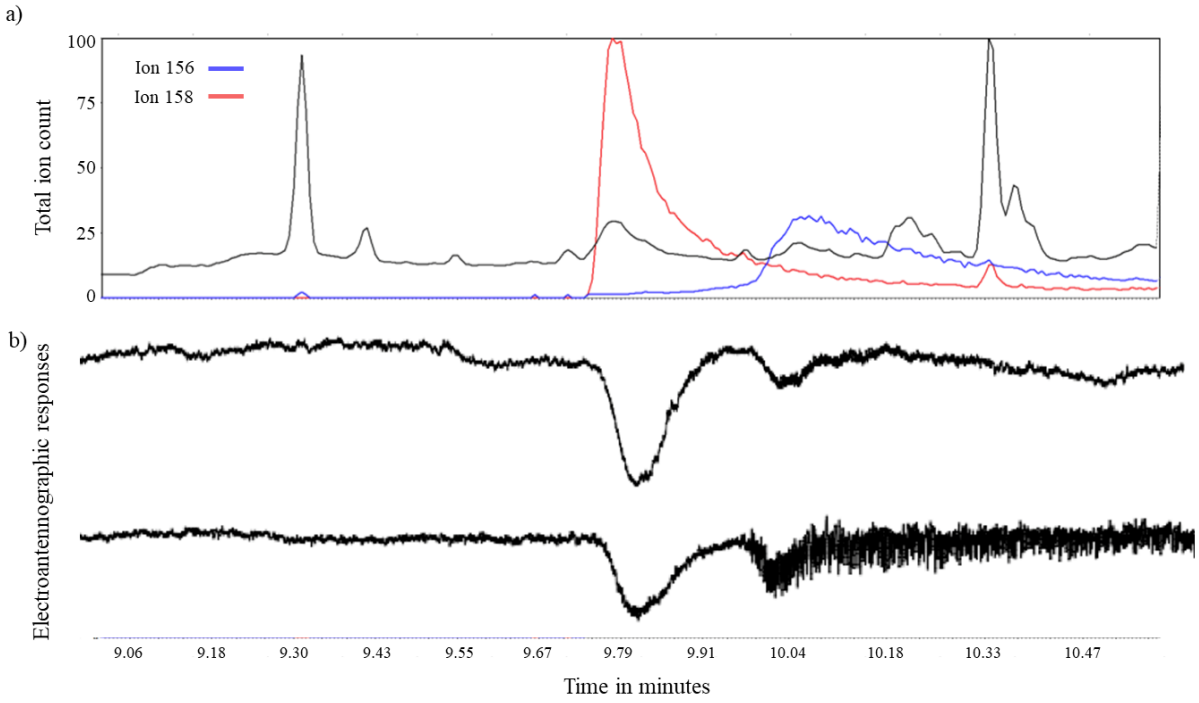
SUPPLEMENTARY INFORMATION



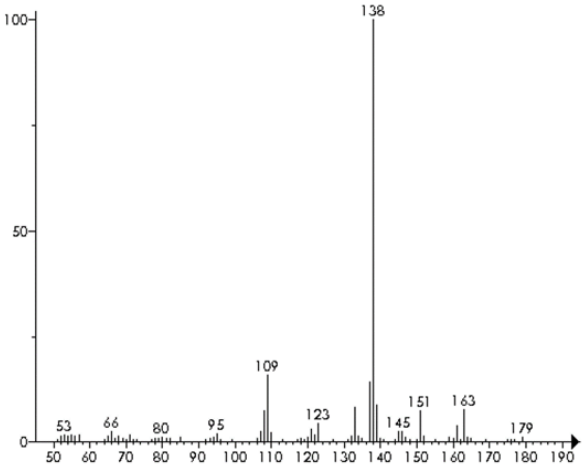
Supplementary Figure A: Average number and behaviour of *Zaspilothynnus nigripes* responding to flowers from populations attracting *Z. nigripes* and populations attracting *Z. dilatatus* at Ruabon Nature Reserve per trial. Error bars denote standard error. Each wasp is included in one category only - the approach category includes only wasps that approached but did not land nor flip the hinge, the land cateogry includes only wasps that approached and landed but did not flip the hinge, and the hinge flip category includes only wasps that approached, landed, and flipped the hinge.



Supplementary Figure B: (a) Total ion count of synthetic-spiked extract of flowers from populations attracting *Zaspilothynnuz dilatatus* with ion 168 (indicating the presence of 4-hydroxy-3-(methylthio)benzaldehyde) shown in red, and ion 156 (indicating the presence of 2-(methylthio)benzene-1,4-diol) shown in blue, with (b) two responses from different Z. dilatatus antennae beneath.

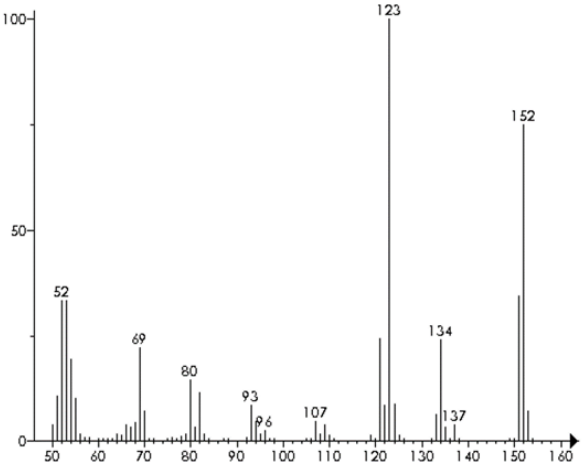
Supplementary Figure C

(1)



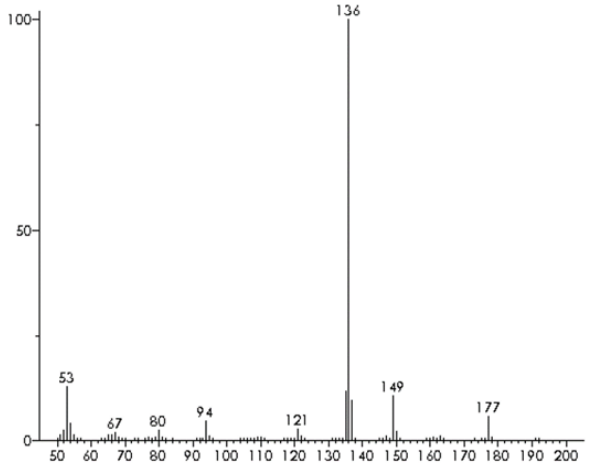
2-hydroxymethyl- 3-(3-methylbutyl)- 5-methylpyrazine (RI 1532)

(2)



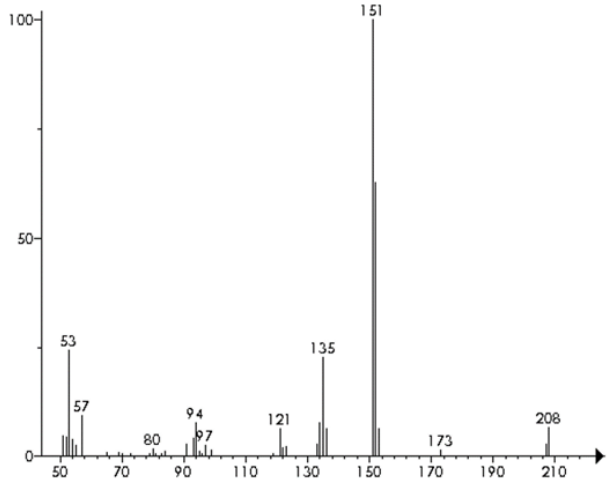
2-hydroxymethyl-3,5,6-trimethylpyrazine (RI 1299)

(3)



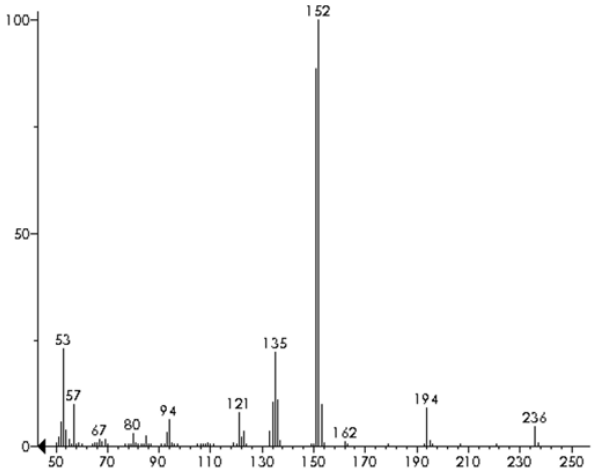
2-(3-methylbutyl)-3,5,6-trimethylpyrazine (RI 1389)

(4)



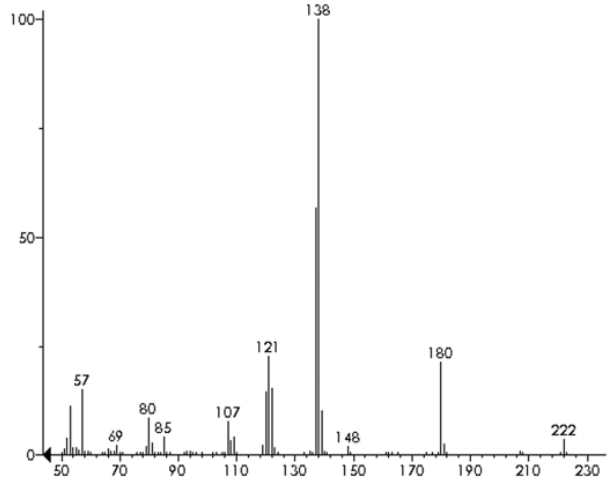
(3,5,6-trimethylpyrazin-2- yl)methyl-3-methylbutanoate (RI 1660)

(5)



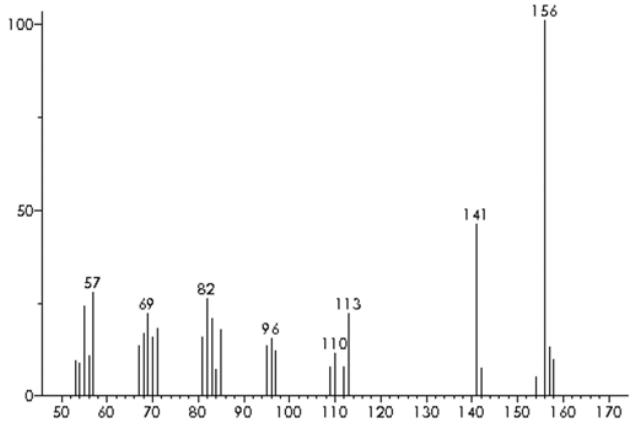
(3,5,6-trimethylpyrazin-2-yl)methyl(2S)-methylbutanoate (RI 1667)

(6)



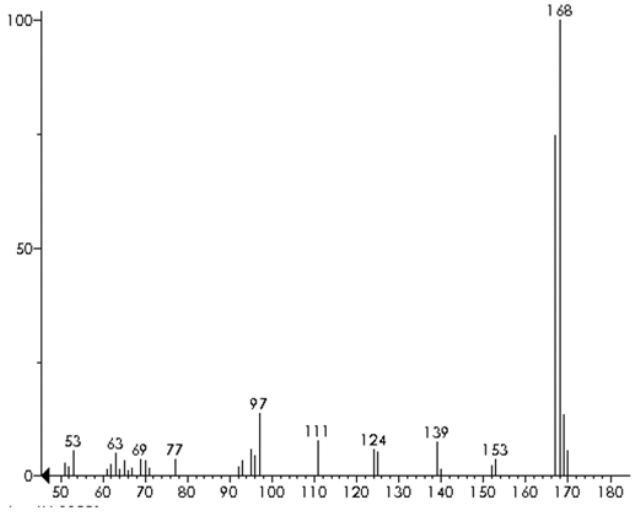
(3,6-dimethylpyrazin-2-yl)methyl 3-methylbutanoate (RI 1580)

(7)



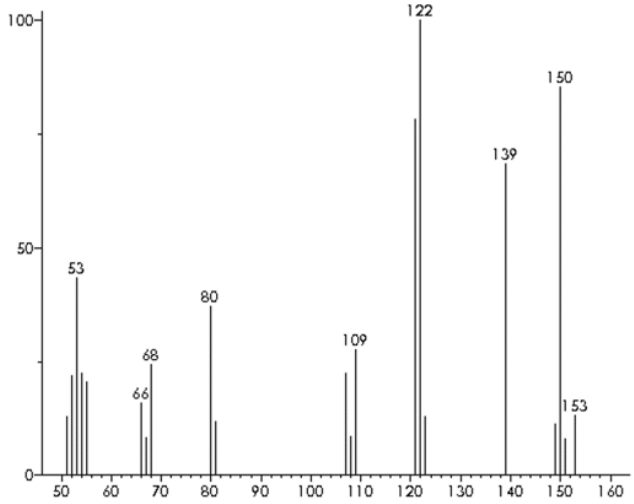
2-(methylthio)benzene-1,4-diol (RI 1507)

(8)



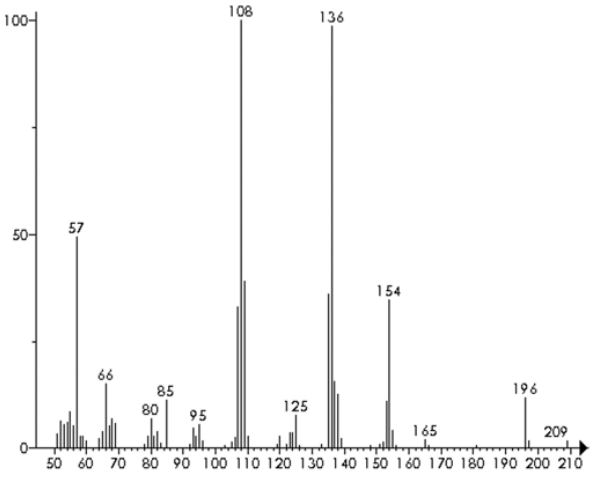
4-hydroxy-3-(methylthio)benzaldehyde (RI 1507)

(9)



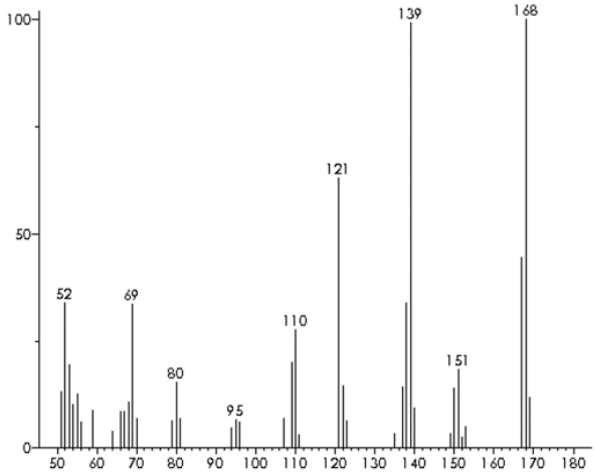
Unknown 1 (RI 1557)

(10)



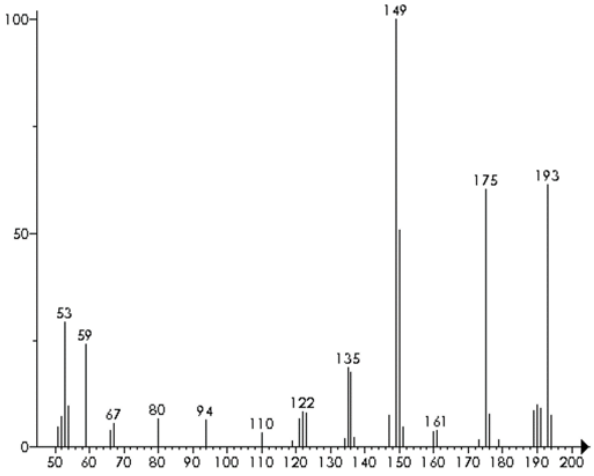
Unknown 2 (RI 1804)

(11)



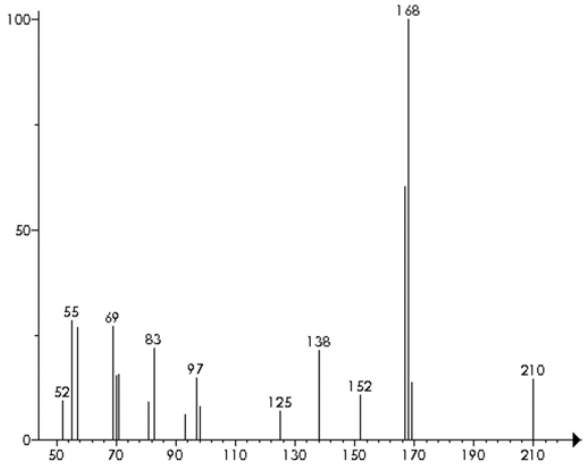
Unknown 3 (RI 1538)

(12)



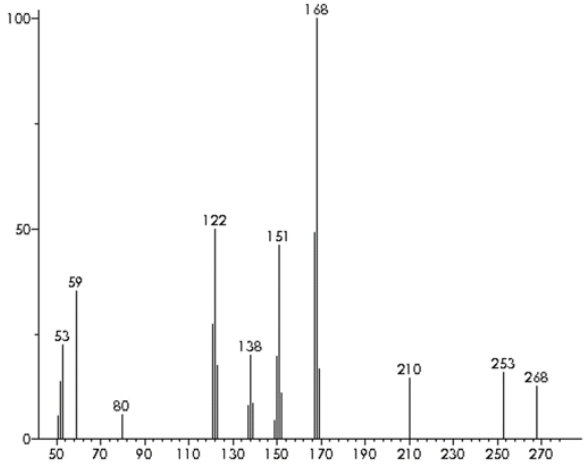
Unknown 4 (RI 1568)

(13)



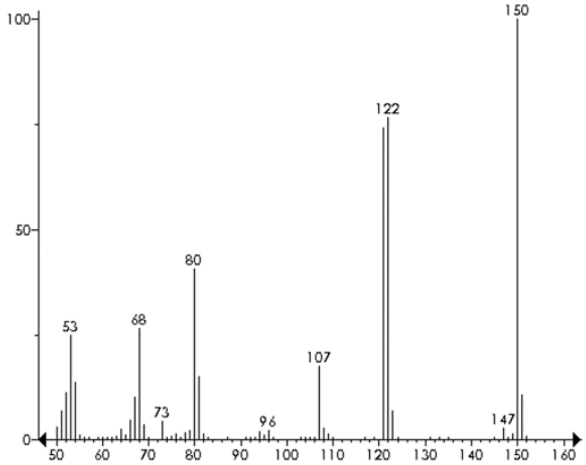
Unknown 5 (RI 1899)

(14)



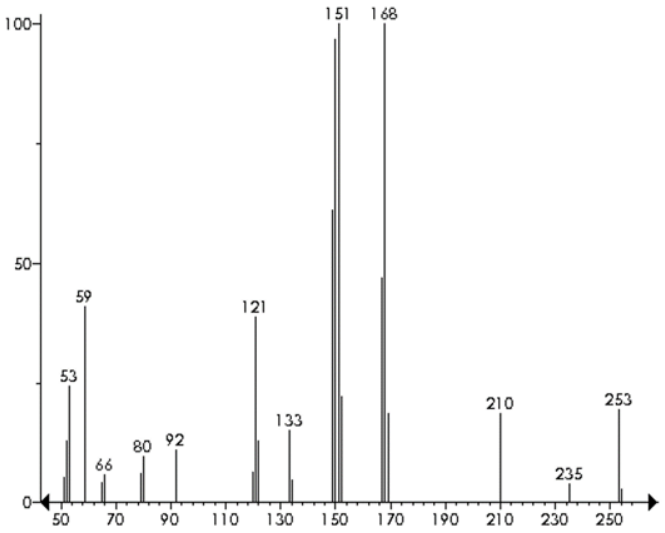
Unknown 6 (RI 2022)

(15)



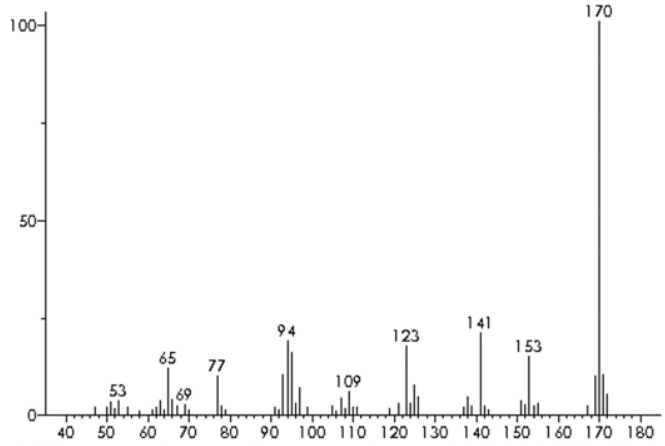
3,5,6-trimethylpyrazine-2-carbaldehyde (RI 1207)

(16)



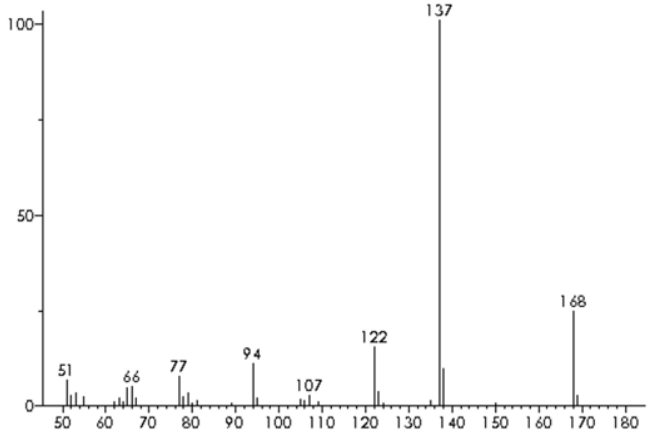
Unknown 7 (RI 2001)

(17)

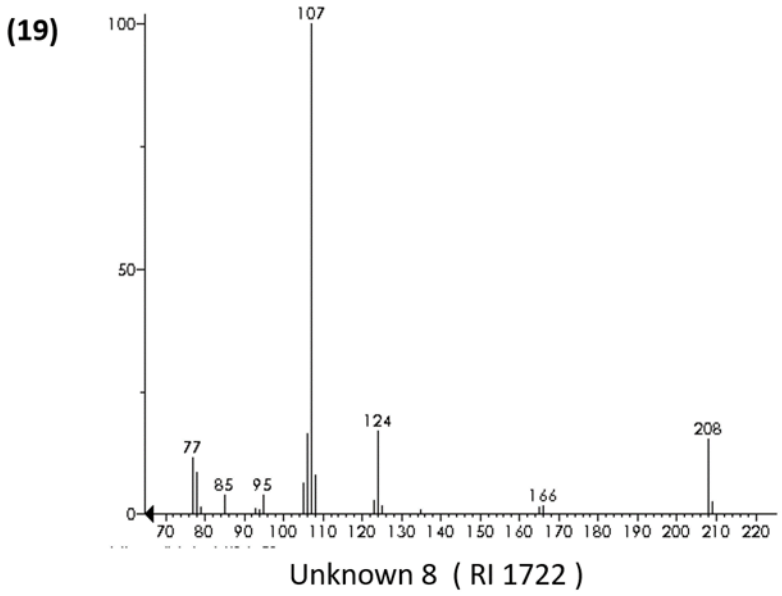


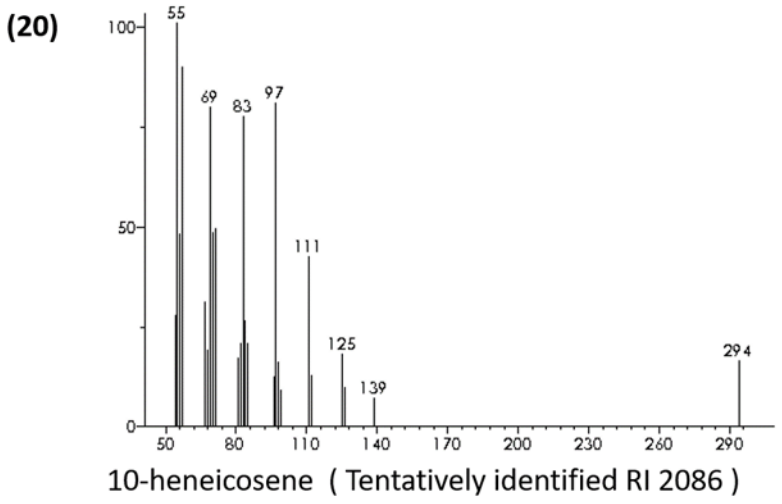
4-(hydroxymethyl)-2-(methylthio)phenol (RI 1560)

(18)



4-(2-hydroxyethyl)-2-methoxyphenol (homovanillyl alcohol) (RI 1547)





Supplementary Figure C: Mass spectra and retention indices of the 20 compounds from Table 2 that were identified to be taxonomically informative in distinguishing the three ecotypes of *Drakaea livida*

Supplementary Table A: Species and behaviour of wasps attracted to flowers from different populations of *Drakaea livida* and the number of flowers of each population that were baited with.

Population	Species caught	Wasp behaviour observed	No. of flowers baited with	IBRA Subregion
Albany King River	*Zaspilothynnus nigripes*	Hinge flip	1	Southern Jarrah Forest
Bayonet Head	*Zaspilothynnus nigripes*	Hinge flip	1	Southern Jarrah Forest
Blue Lake Road Clearing	*Zaspilothynnus nigripes*	Hinge flip	4	Southern Jarrah Forest
Chesapeake track	*Zaspilothynnus nigripes*	Hinge flip	1	Warren
Granite Road	*Zaspilothynnus nigripes*	Hinge flip	1	Southern Jarrah Forest
Grays Road	*Zaspilothynnus nigripes*	Hinge flip	1	Warren
Isle Road	*Zaspilothynnus nigripes*	Hinge flip	2	Warren
Lane Poole Road	*Zaspilothynnus nigripes*	Hinge flip	5	Warren
Mabinup Track	*Zaspilothynnus nigripes*	Hinge flip	1	Esperance Plains
Mount Lindsey	*Zaspilothynnus nigripes*	Hinge flip	1	Southern Jarrah Forest
Northcliffe Outcrop	*Zaspilothynnus nigripes*	Hinge flip	1	Warren
Qualen Road	*Zaspilothynnus nigripes*	Hinge flip	1	Northern Jarrah Forest
Rainbow Cave Road	*Zaspilothynnus nigripes*	Hinge flip	7	Warren
Scotsdale Outcrop	*Zaspilothynnus nigripes*	Hinge flip	1	Warren
Spencer Road	*Zaspilothynnus nigripes*	Hinge flip	20	Warren
SW Hwy Outcrop	*Zaspilothynnus nigripes*	Hinge flip	2	Warren
Blue Lake Road	*Catocheilus* sp.	Close approach (<5cm)	3	Southern Jarrah Forest
Blue Lake Road Sand Patch	*Catocheilus* sp.	Close approach (<5cm)	1	Southern Jarrah Forest
Frosty Road	*Catocheilus* sp.	Close approach (<5cm)	9	Southern Jarrah Forest
Greenbushes	*Catocheilus* sp.	Close approach (<5cm)	2	Southern Jarrah Forest
Mowen Road	*Catocheilus* sp.	Close approach (<5cm)	2	Southern Jarrah Forest
Carrabungup Nature Reserve	*Zaspilothynnus dilatatus*	Hinge flip	5	Swan Coastal Plain
Franklandia Nature Reserve	*Zaspilothynnus dilatatus*	Hinge flip	3	Swan Coastal Plain
Goodale Sanctuary	*Zaspilothynnus dilatatus*	Hinge flip	5	Swan Coastal Plain
Island Point Nature Reserve	*Zaspilothynnus dilatatus*	Hinge flip	14	Swan Coastal Plain
Johnston Road	*Zaspilothynnus dilatatus*	Hinge flip	1	Swan Coastal Plain
Manea Park	*Zaspilothynnus dilatatus*	Hinge flip	3	Swan Coastal Plain
Serpentine River Nature Reserve	*Zaspilothynnus dilatatus*	Hinge flip	12	Swan Coastal Plain

Supplementary Table B: Voucher specimens numbers, ecotypes, and locations of populations of *Drakaea livida* included in the present study

Population Location	Ecotype	Voucher Number	Latitude & Longitude
Albany King River	Ecotype One	PERTH 09005633	-34.933°, 117.891°
Bayonet Head	Ecotype One	PERTH 09005579	-34.974°, 117.937°
Lane Poole Northcliffe	Ecotype One	PERTH 09005765	-34.577°, 116.197°
Mount Lindsey	Ecotype One	PERTH 09005757	-34.854°, 117.241°
Northcliffe Outcrop	Ecotype One	PERTH 09005730	-34.778°, 116.081°
Qualen Road	Ecotype One	PERTH 05493714	-32.108°, 116.652°
Rainbow Cave Road	Ecotype One	PERTH 08605254	-34.004°, 115.022°
Scotsdale Outcrop	Ecotype One	PERTH 09005587	-34.850°, 117.261°
Spencer Road	Ecotype One	PERTH 09005773	-33.706°, 115.027°
SW Hwy Outrcrop	Ecotype One	PERTH 08604584	-34.758°, 116.501°
Isle Road	Ecotype One	PERTH 08603561	-34.988°, 116.695°
Granite Road	Ecotype One	PERTH 09005749	-34.840°, 117.251°
Frosty Road	Ecotype Two	PERTH 09005609	-34.357°, 116.385°
Mowen Road	Ecotype Two	PERTH 09005714	-33.924°, 115.396°
Carrabungup Nature Reserve	Ecotype Three	PERTH 09048014	-32.647°, 115.715°
Franklandia Nature Reserve	Ecotype Three	PERTH 09005692	-33.425°, 115.697°
Goodale Sanctuary	Ecotype Three	PERTH 09005668	-32.722°, 115.775°
Island Point Nature Reserve	Ecotype Three	PERTH 09005706	-32.757°, 115.690°
Manea Park	Ecotype Three	PERTH 09005595	-33.382°, 115.657°
Serpentine River Nature Reserve	Ecotype Three	PERTH 08739889	-32.335°, 115.791°

Supplementary Table C: Presence absence matrix of the 20 compounds found to be taxonomically informative in the *Drakaea livida* ecotypes

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
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Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
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Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
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Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
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Two	0	1	0	1	1	1	0	0	0	0	1	0	0	1	1	0	0	1	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
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Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1
Three	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
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Three	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
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Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16	17	18	19	20
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Three	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0

CHAPTER FIVE

Conservation assessment and identification of the *Drakaea livida* (Orchidaceae) ecotypes: evidence for three evolutionary significant units

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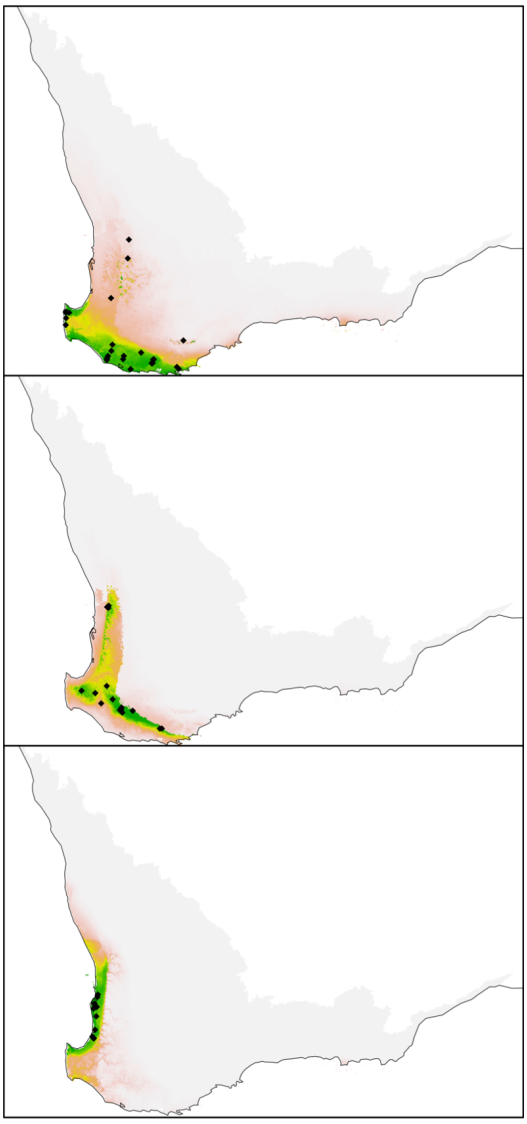
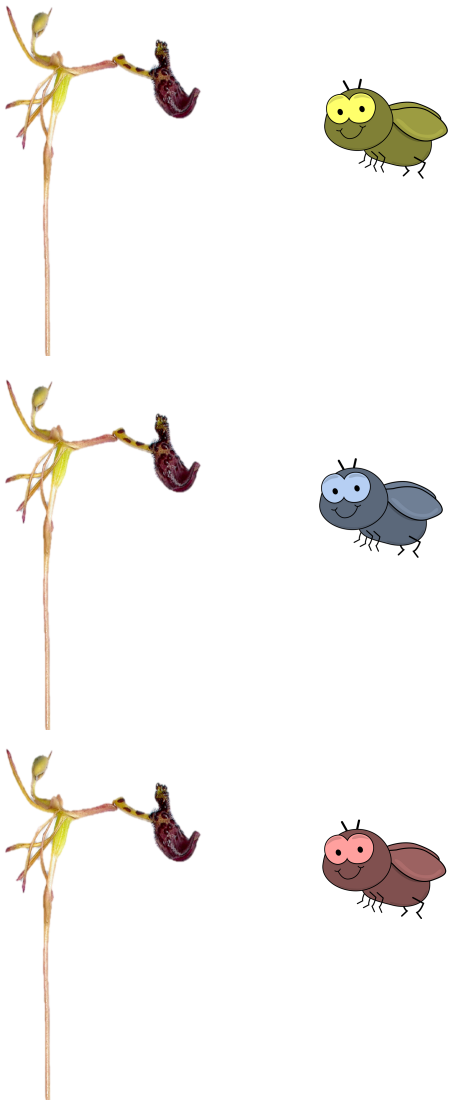
This study was conceptualised by A Weinstein in consultation with B Bohman, and R Phillips. Experiments and data analyses were conducted by A Weinstein. Original draft preparation was conducted by A Weinstein. Review and editing was conducted by all authors. Funding was acquired by A Weinstein.





ABSTRACT

Morphologically cryptic taxa form an unrecognised component of the biota that must be accounted for in both understanding the full extent of Earth's biodiversity and in implementing effective conservation measures. Many sexually deceptive orchids have been found to contain morphologically cryptic taxa, such as the Warty Hammer Orchid, *Drakaea livida*. This species is comprised of three cryptic pollination ecotypes, which can be distinguished using components of their floral odour. As ecologically distinct entities, the ecotypes merit inclusion in conservation planning. The present study aimed to: *a)* investigate the geographic range of the three *D. livida* ecotypes, enabling both assessment of their conservation status and their effective inclusion in management planning; and *b)* to test the efficacy of different methods of identifying the *D. livida* ecotypes. Ecotype distribution data were used in MaxEnt species distribution models, which revealed each ecotype to have a different predicted geographic range with small areas of overlap predicted at the ecotype margins. One ecotype, which only has ten known populations, was found to have a very limited geographic range, the majority of which has been cleared for agriculture and housing. It is likely that this ecotype qualifies for listing as endangered under the IUCN Red List criteria. Three methods of ecotype identification were assessed: morphometric analysis, genome size comparison, and the novel methodology of analysis of chemical composition of labella extracts from pollinated flowers. Chemical analyses returned a 96.5% correct assignment rate, providing an accurate non-destructive identification method that may be of wider applicability in distinguishing rare sexually deceptive orchid taxa. While there was broad overlap between the ecotypes in individual morphological traits, multivariate analyses delivered an 87% correct assignment rate, suggesting that further investigation of floral morphology may identify discriminating traits. Given their different pollinators, floral chemistry, distributions, and divergent morphology that reflect different evolutionary trajectories, the ecotypes represent discrete evolutionary significant units and should be treated as such in conservation management.

INTRODUCTION

Cryptic taxa, being two or more morphologically indistinguishable taxa classified as a single taxon, form an unrecognised component of the biota that must be catalogued and accounted for in order to understand the full extent of Earth's biodiversity (Bickford et al., 2007). The presence of cryptic taxa can pose a major challenge for conservation efforts (Hebert et al., 2004; Bickford et al., 2007; Angulo & Icochea, 2010) either through the challenge of identification in the field, or because newly recognised taxa have a smaller population size and likely a smaller geographic range than the original species complex (Bickford et al., 2007; Niemiller et al., 2013). Following the recognition of cryptic taxa, conservation efforts must adapt accordingly – populations that were not thought to be of high conservation value can become an immediate priority if they represent a rare, newly defined taxon. Further, the strategies employed to conserve populations between which gene flow may occur will differ to those required to conserve populations between which there is little or no gene flow (Hufford & Mazer, 2003; Brown et al., 2014). Further, the ecological requirements of the cryptic species may differ (Schönrogge et al., 2002).

In taxa where there is evidence of potential crypsis, this possibility must be fully investigated before appropriate conservation measures can be implemented. Such studies may reveal cryptic entities of several taxonomic ranks, including species, subspecies, and ecotypes. Some of these entities may qualify as evolutionary significant units (ESUs) - a grouping specifically developed to allow entities that have not been designated a taxonomic rank to be included in conservation management. An ESU may be designated at the population, sub-species, or species level, and represents a separate evolutionary lineage for which existing taxonomy does recognise current knowledge of the ESU evolutionary history (Ryder, 1986; Karl & Bowen, 1999; Moritz, 1999; Fraser & Bernatchez, 2001). Many ESUs are not distinguished based on morphological characters and therefore cannot be identified in field (Guschanski et al., 2007; Morgan et al., 2016; Ferreira et al., 2017; Gray et al., 2018).

Speciation without obvious morphological divergence can occur in animals that employ non-visual mating signals, such as acoustic (Narins, 1983; Henry, 1994) or chemical (Byers & Struble, 1990; Kozlov et al., 1996) signals. Some plants may have a high incidence of cryptic variation more akin to that expected in animals, such as sexually deceptive plants that use

non-visual animal mating signals to achieve pollination. Pollination via sexual deception is effected as male insect pollinators are sexually attracted to a flower through chemical and visual mimicry of conspecific females (Coleman, 1928; Kullenberg, 1961; Stoutamire, 1974). Sexual-deception occurs in the Asteraceae (Ellis & Johnson, 2010) and Iridaceae (Vereecken et al., 2012), but is most prevalent among the Orchidaceae, with several hundred species of orchid employing this pollination strategy (Schiestl, 2005; Gaskett, 2011). Due to the high specificity of insect sex pheromones, sexually deceptive orchids often have a single specific pollinator species (Paulus & Gack, 1990; Blanco & Barboza, 2005; Bower & Brown, 2009; Peakall et al., 2010; Gaskett, 2011; Phillips et al., 2017a). Due to the pivotal role of floral chemistry in pollinator attraction, novel pollinators can be attracted via a change in floral odour, which is not necessarily accompanied by morphological divergence (Bower & Brown, 2009; Breitkopf et al., 2013; Peakall & Whitehead, 2014). In some cases, the attraction of novel pollinators can lead to the formation of distinct cryptic taxa through pollinator-mediated speciation.

Research on the pollination of sexually deceptive orchids has uncovered a growing number of morphologically cryptic ecotypes, some of which may be worthy of taxonomic recognition (Bower, 2006; Bower & Brown, 2009; Breitkopf et al., 2013; Peakall & Whitehead, 2014; Menz et al., 2015; Phillips et al., 2015). This prevalence of crypsis poses difficulties for orchid conservation management, in which cryptic entities would ideally be treated as distinct, with knowledge of their specific pollinators being incorporated in management planning, a process complicated by the difficulty of identifying them. Species identification is a critical component of environmental impact assessments, which in many countries are frequently conducted by consultants as a condition of approval for development (mining, housing, etc) (Munn, 1979).

A range of techniques has been used to differentiate orchid ecotypes and taxa that are morphologically challenging to distinguish. Pollinator choice trials, where flowers are presented sequentially to multiple pollinator species, have proved a reliable method of testing differences in pollinator response that can indicate the presence of ecologically distinct entities. In cases where discrete entities have been found, the pollinator species attracted is diagnostic of the entities (Bower, 2006; Bower & Brown, 2009; Peakall et al., 2010; Swarts et al., 2014; Menz et al., 2015; Phillips et al., 2015). Further support for differences between entities can come from analysis of chloroplast DNA (Peakall & Whitehead, 2014), or floral

chemistry - either of pollinator attractants (Peakall & Whitehead, 2014), or of overall chemical composition (Véla et al., 2007; Joffard et al., 2016). In some cases, cryptic orchid taxa may also differ in ploidy level, which is reflected in their genome size (Trávníček et al., 2010; Gale et al., 2015). In some cases, genome size determination using flow cytometry can provide a cost-efficient and reliable tool for distinguishing taxa (Trávníček et al., 2010).

A recent study on the Warty Hammer Orchid, *Drakaea livida*, discovered three ecotypes, each attracting a different specific pollinator species, by employing pollinator baiting in the form of pollinator surveys and choice trials (Chapter Four). These ecotypes were not detected in the most recent taxonomic revision of the genus (Hopper & Brown, 2007), and appear to lack obvious differences in morphology. However, this revision was unaware of the ecotypes existence, and as such did not specifically target populations of each ecotype. Morphological differences between the ecotypes are yet to be formally assessed.

The labella (modified petal that mimics female wasps) of Warty Hammer Orchid flowers from different ecotypes differed in floral volatile composition (Chapter Four). Ecotypes could be reliably distinguished using Partial Least Squares Discriminant Analysis (PLS-DA) of the presence-absence of a subset of floral odour components diagnostic of particular ecotypes (Chapter Four), some of which are known to be bioactive to pollinator species (Bohman et al., 2012a; Bohman et al., 2012b; Bohman & Peakall, 2014). Two ecotypes are characterised by containing different sets of pyrazines compounds, and these pyrazine ecotypes both also contain homovanillyl alcohol (Chapter Four). The third ecotype contains neither pyrazines nor homovanillyl alcohol, and is instead characterised by (methylthio)phenol compounds (Chapter Four). In addition to these pyrazines, (methylthio)phenols, and homovanillyl alcohol, each ecotype also contained diagnostic unidentified compounds that likely represent new natural products. These compounds can be readily identified based on their characteristic mass spectra and retention times (available in Chapter Four).

As discrete ecological entities, the three ecotypes were recommended to be recognised and accounted for in conservation planning (Chapter Four). The initial investigation of the *D. livida* ecotypes suggested that each ecotype may have a different geographic range (Chapter Four), however, their distribution has thus far not been fully investigated. Knowledge of the geographic range and location of populations of a species is a key component in conservation assessment and will affect management strategies (Ferreira et al., 2013). For example, in the

cases of the *D. livida* ecotypes, this knowledge is crucial in avoiding the mixing of genotypes from different ecotypes, as may inadvertently occur in translocations (Hufford & Mazer, 2003; Weeks et al., 2011). Further, knowledge of geographic range is required in determining conservation status. Sub-specific taxa and ESUs can be listed under Australia's federal conservation legislation, the Environment Protection and Biodiversity (EPBC) Act. Conservation status under both this federal legislation and state equivalents is determined using the International Union for Conservation of Nature (IUCN) Red List Categories assessment. This assessment method is an internationally recognised standard established by the IUCN.

A further impediment to the effective conservation of the ecotypes is the difficulty of identifying them. The ecotypes were initially identified based on pollinator response, and chemical analyses were also found to be able to distinguish the ecotypes. However, both of these methods are destructive in that they entail the picking of fresh flowers. An ideal identification method for the ecotypes would not impact their reproductive success.

The present study aimed to: *a)* investigate the geographic range of the three *D. livida* ecotypes, enabling both assessment of their conservation status and their effective inclusion in management planning; and *b)* to test the efficacy of different methods in identifying the *D. livida* ecotypes. In addressing *a)*, the chemical identification methodology of Chapter Four was applied to determine the ecotype of additional populations across the range of *D. livida.* This larger distribution dataset was then used to generate species distribution models to identify the predicted geographic ranges of the ecotypes. To address *b)*, three methods of identifying ecotypes were tested. Firstly, morphometric analyses of the traits used in the most recent revision of the genus were conducted (Hopper & Brown, 2007). Secondly, it was tested whether the destructive chemical identification method described in Chapter Four could be successfully applied to field sampling of pollinated flowers, rendering it non-destructive. Lastly, it was investigated whether potential differences in genome size between species could be used to distinguish the ecotypes. Cryptic variation in genome size has been found in other orchids (Trávníček et al., 2010; Gale et al., 2015), and within the Drakaeinae subtribe existing data show variation in chromosome number (Peakall & James, 1989).

METHODS

Study species

Drakaea plants are dormant in summer, developing a single leaf during the winter growth period, and flowering in spring. Individual plants do not flower every flowering season, and when they do they produce only a single scape bearing a single flower (Hopper & Brown, 2007). While populations sizes are often small, fruit set is typically high (> 30%), with greater per-plant reproduction at small population sizes (Phillips et al., 2014). All *Drakaea* species are reliant on the same species of symbiotic *Tuslasnella* fungi for germination and annual growth (Phillips et al., 2014; Linde et al., 2017). *Drakaea* achieve pollination by sexually luring male thynnine wasps into attempting copulation with their labella, which mimic a female wasp (Peakall, 1990). A hinge located mid-way along the labellum enables pollination by causing the wasp's upper thorax to be brought into contact with the column where pollinia are transferred by his own momentum as he attempts to fly off with the labellum (Peakall, 1990). Three pollination ecotypes have been recognised in *D. livida*: Ecotype One, pollinated by *Zaspilothynnus nigripes*; Ecotype Two, pollinated by *Catocheilus* sp.; and Ecotype Three, pollinated by *Z. dilatatus* (Chapter Four). Ecotypes One and Two are each characterised by specific pyrazine compounds, while Ecotype Three is characterised by (methylthio)phenol compounds (Chapter Four).

Drakaea livida is endemic to South-West Western Australia, where it spans a 500km distribution encompassing a variety of different habitats (Hopper & Brown, 2007). Ecotypes occur in the same soil type (Supplementary Information 1), being almost entirely restricted to well-drained grey sandy soils, though there is evidence that they have separate geographic ranges where they are associated with different vegetation communities (Chapter Four).

Investigating the geographic range of the ecotypes

Determining the ecotype of populations of D. livida

Previous studies have successfully used pollinator choice experiments to determine the pollinator species attracted to, and thus the ecotype of, *Drakaea* flowers (Menz et al., 2015;

Phillips et al., 2015). However, the ability to conduct pollinator-baiting experiments is constrained by the weather conditions, with male thynnine wasps being most active on sunny days ≥ 20ºC (Stoutamire, 1974). Chapter Four demonstrated that ecotypes of *D. livida* could be accurately assigned based on their floral chemistry using diagnostic marker compounds, with Partial Least Squares Discriminant Analysis providing a robust method for assigning the ecotypes. In the present study, this methodology is implemented to assign ecotypes to floral extracts from populations of unknown ecotype. Doing so will provide a greater understanding of the location of populations of different ecotypes, which is required to conduct species distribution modeling.

Drakaea livida flowers were opportunistically collected from 22 previously un-sampled populations of unknown ecotype between 2011-2018, broadly following the methodology of Chapter Four. In brief, labella were extracted in 100 µL of dichloromethane and kept at -20ºC before being analysed by gas chromatography-mass spectrometry (GC-MS). Peak detection and deconvolution were conducted using the EasyGC python pipeline (https://libraries.io/github/dkainer/easyGC, based on PyMS python library (O'Callaghan et al., 2012)) with the default parameters. The mass spectra of the 20 detected ecotype-diagnostic compounds from Chapter Four were added to an AMDIS target library, which was used to individually screen each floral extract for library hits, with all extracts being manually checked when a hit occurred. The default AMDIS search settings were applied with the exception of 'Sensitivity' which was set to 'High'. Screening data was collated in a binary presence-absence matrix. For floral extracts that contained one or more ecotype-diagnostic compound (Chapter Four), ecotypes were predicted using a Partial Least Squares-Discriminant Analysis with the R package 'mixOmics' (Rohart et al., 2017). As a training dataset (dataset used for model learning), the matrix of ecotype-diagnostic compound presence-absences for 345 extracts with pollinator data from Chapter Four was used. Populations assigned ecotypes were checked on a map to see if newly assigned ecotypes made geographic sense, i.e. fell within or nearby the pre-established ranges of the ecotypes.

Predicting ecotype geographic range

To predict the geographic range of the ecotypes, MaxEnt species distribution modelling was conducted (Phillips et al., 2017b). The analysis was undertaken in R v 3.5.1 (R Core Team,

2018) using the package 'dismo' (Hijmans et al., 2017), with bioclimatic variables calculated to a 1 x 1 km scale in ANUCLIM v 6.1 (Xu & Hutchinson, 2011). Default model settings were used: a betamultiplier of one, maximum background points of 10 000, convergence threshold of 1.0E-5, and a default prevalence of 0.5. In addition to abiotic factors, pollinator presence is potentially an important predictor of flowering plant distributions in species with specialist pollination strategies (Duffy & Johnson, 2017). For Ecotypes One and Three, where pollinator distribution data were available, bioclimatic suitability (continuous) for the pollinator, generated in a separate MaxEnt model, was also included as an explanatory variable in addition to bioclimatic variables. For Ecotype Two, where the pollinator species is not represented in museum collections and shows an infrequent response to orchids, no suitable data were available to model pollinator distribution.

Distribution records for the Ecotype One (*Z. nigripes*) and Three (*Z. dilatatus*) pollinators were obtained from the Western Australian Museum records and from field records from other publications (Phillips et al., 2009; Menz et al., 2013; Phillips et al., 2013; Tomlinson & Phillips, 2015; Phillips et al., 2017a; Phillips & Peakall, 2018, Chapter Four). To model pollinator distributions, bioclimatic layers likely to influence pollinator habitat suitability were used. Whether or not to exclude highly correlated variables in MaxEnt modelling, and if so at what cut off, has long been a point of contention (Elith et al., 2006; Phillips & Dudík, 2008; Elith et al., 2011; Dormann et al., 2013; Merow et al., 2013; Shcheglovitova & Anderson, 2013). A recent study by Feng et al. (2019) concluded that Maxent is capable of regulating contributions from redundant variables and is robust to predictor collinearity, and that therefore the strategy of removing highly correlated variables has little impact in Maxent model performance. In the present study, an initial run was conducted, and a final set of variables was selected using those that had predictive power in the initial run, namely: Bio01 annual mean temperature, Bio05 maximum temperature of warmest week, Bio06 minimum temperature of coldest week, Bio10 mean temperature of warmest quarter, Bio11 mean temperature of coldest quarter, Bio12 annual precipitation, bio18 precipitation of warmest quarter, Bio19 precipitation of coldest quarter, Bio20 annual mean radiation, and Bio28 annual mean moisture index.

The resultant bioclimatic pollinator suitability layers were included as an explanatory variable in the models for Ecotypes One (*Z. nigripes* suitability) and Three (*Z. dilatatus* suitability). Presence records for each ecotype were based on ecotypes assigned by pollinator

response (Chapter Four), and from populations assigned to ecotypes based on floral chemistry in the present study. Bioclimatic layers were selected for modeling orchid ecotype distributions that influenced the habitat generally, and that were specific to the winter growth months (critical for spring-flowering *Drakaea*). Following an initial run, layers that had shown predictive power in the initial run were selected: Bio01 annual mean temperature, Bio08 mean temperature of wettest quarter, Bio11 mean temperature of coldest quarter, Bio12 annual precipitation, Bio16 precipitation of wettest quarter, Bio18 precipitation of warmest quarter, Bio24 radiation of wettest quarter, Bio28 annual mean moisture index, and Bio32 mean moisture index of wettest quarter, were used.

Model training regions (geographic area which model learning is restricted to) for pollinators and orchids were set as the Interim Biogeographic Regionalisation for Australia (IBRA) (Thackway & Cresswell, 1995) bioregions where the species occurred, and any bioregions directly adjoining to bioregions where the species occurred (for map of relevant bioregions see Supplementary Figure A). For species/ecotypes that occurred on the Swan Coastal Plain only, Warren was also included as an adjoining bioregion due to its close proximity to the Swan Coastal Plain bioregion. The same background projection region was used for all orchid ecotypes (and thus also their pollinator species), being set as the IBRA bioregions that contained records of one or more of the ecotypes, and the bioregions directly adjoining the bioregions containing ecotype records. For *Z. nigripes* (N = 126 unique locations), 20% of the presence data were withheld for subsequent use in model testing. Data were not withheld for model testing for the other taxa due to their lower number of presence records (*Z. dilatatus* N = 19, Ecotype One N = 25, Ecotype Two N = 15, Ecotype Three N = 10). With no data being witheld, and considering the narrow-range of Ecotype Three, the sample sizes are appropriate for MaxEnt modeling (van Proosdij et al., 2016).

Determining an effective identification method for the ecotypes

Morphometrics

To determine whether the ecotypes differed in their floral morphology, a minimum of 13 flowers from each ecotype were measured (Ecotype One, N = 13, five populations; Ecotype Two, N = 19, four populations, Ecotype Three, N = 14, six populations) with digital callipers

according to 17 traits, including those used in the most recent revision of *Drakaea* (Supplementary Table A)(Hopper & Brown, 2007). Significant differences among ecotype trait means were tested for using pairwise Holm-corrected t-tests in R v 3.5.1 (R Core Team, 2018). To visualise these data, a PCA was generated from the trait data. The ecotypes of flowers were predicted using Linear Discriminant Analysis (LDA) with leave one out cross validation, where the model is run *N* times, with *N*-1 as the training set and each sample point being predicted individually in a single iteration of the model, using the R package 'MASS' (Ripley et al., 2013).

Chemical analysis of pollinated flowers

Screening for ecotype-diagnostic compounds in extracts of unpollinated *D. livida* flowers can be used to determine their ecotype (Chapter Four). To test whether this methodology could also be successfully applied in pollinated flowers, extracts were made from populations of known ecotype (either identified in this study or in Chapter Four) at various extents of post-pollination withering. The removal of the labellum naturally occurs in instances when wasps vigorously attempt to copulate and fly off with it. The artificial removal of the labellum does not adversely affect fruit set - fruit set has been observed on *Drakaea* with missing labella (A. Weinstein, pers. obvs.). Two metrics were calculated for each flower: the extent of post-pollination withering, and the proportion of ecotype-diagnostic compounds detected out of the maximum number of diagnostic compounds for the ecotype recognised in Chapter Four.

Extent of post-pollination withering

After being pollinated, *Drakaea* flowers begin to wither. This withering is visible in the deflation of the labellum, and changes in the stigmatic surface, which loses its shiny and sticky appearance and becomes opaque. On initial deposition, the pollen mass remains largely intact and retains its bright yellow colouration. However, as time progresses the original mass loses shape and takes on a white appearance. At the same time as these changes to the labellum and stigma, the ovary begins to swell as the plant nears fruit set. The present study investigated at which stage during this post pollination withering process diagnostic chemical compounds were still present, with a view to pollinated labella being collected from populations of unknown ecotype for identification purposes. It is aimed to determine at which extent of post pollination withering ecotypes can still be reliably identified.

Floral extracts were made in the field from pollinated flowers at populations of known ecotype (for full methods see below). Before extracts were made, photos were taken of the labellum, ovary, and stigmatic surface of the flower. To ensure consistency across the dataset, all the photos were assessed for their degree of withering in one batch at the end of the flowering season. To do so, four traits were assessed on a scale of three sequential mutually exclusive stages and a score from zero to two awarded (Figure 1, Table 1). The total extent of post-pollination withering score was calculated by summing the scores from each trait to give a total score out of eight, where zero represents freshly pollinated and eight represents the most advanced stage of withering.

Proportion of the total possible diagnostic compounds present in pollinated labella

Extracts of pollinated flowers of each ecotype were made in the field immediately after the plants were photographed, following the methodology of Chapter Four. GC-MS and screening for ecotype diagnostic compounds was conducted as outlined earlier for the assignment of unknown populations to ecotypes. To test the effect of extent of post-pollination withering score on the proportion of ecotype-diagnostic compounds detected out of the maximum number of detected compounds for the ecotype, a generalised linear mixed effects model with the binomial family was conducted using the R package ‘lme4’ (Bates et al., 2015). To account for overdispersion, an observation level random effect was included in

the model, where each data point was allocated a unique level of a random effect (Harrison, 2014). The ecotype of the plant was included in the model as a fixed effect. PLS-DA was then implemented to predict the ecotype of the pollinated labella using the data from Chapter Four as a training set, as was done in the assignment of ecotypes to unknown populations.

Genome size of *D. livida* ecotypes

To investigate whether the ecotypes differed in genome size, flow cytometry was conducted on pollinia from 45 *D. livida* plants from 20 populations attracting different pollinator species. Pollen was used as this tissue is a reliable standard not prone to progressive partial endoreplication, a major problem in orchid flow cytometric analyses (Trávníček et al., 2015). Data was acquired using a Attune NxT acoustic focusing flow cytometer as per Doležel & Bartoš (2005) with a Tris.$MgCl_2$ buffer. For all samples, one of *Pisum sativum* (2C = 9.09 pg), or *Triodia longiceps* (2C = 2.928 pg) were chopped with samples as standards, depending on availability. Data were analysed in Flowing Software v2.5.1 (freely accessible from http://flowingsoftware.btk.fi/index.php?page=3), and genome sizes calculated using standards as per (Doležel & Bartoš, 2005). Both distinct 1C and 2C peaks were returned in our analyses (from haploid vegetative nuclei and 2C generative nuclei), as is common in orchid species (Trávníček et al., 2015). Genome sizes were calculated using the 2C values, as these peaks had a lower CV (calculated as the standard deviation of the peak divided by the mean channel position of the peak, multiplied by 100) error value. Differences in genome size were tested for with an ANOVA in R v 3.5.1.

RESULTS

Investigating the geographic range of the ecotypes

Determining the ecotype of populations of D. livida

Analysis of chemical composition enabled the assignment of ecotypes to 22 populations for which the ecotype was previously unknown. Of the 74 floral extracts analysed from 22 populations that had no associated pollinator response data, 95% (70) contained compounds previously identified as ecotype-diagnostic. Each sampled population had at least one representative flower containing a pollinator-specific compound. Ten of the analysed populations contained compounds indicative of the attraction of *Z. nigripes* only (assigned Ecotype One in PLS-DA), nine populations contained compounds indicative of the attraction of *Catocheilus* sp. only (assigned Ecotype Two in PLS-DA), and three populations contained compounds indicative of the attraction of *Z. dilatatus* only (assigned Ecotype Three in PLS-DA). The locations of these newly identified populations were all congruent with the previously identified ecotype ranges, in that no newly identified populations were located disjunct from the known ecotype ranges.

One population situated at the southernmost extent of the range of Ecotype Three, and near the range margins of Ecotypes One and Two, contained compounds indicative of multiple ecotypes. Flowers from this population contained two (methylthio)phenol compounds, which occur uniquely in Ecotype Three (Chapter Four). Homovanillyl alcohol, thus far indicative of Ecotype One or Two (not Three), was also detected in this population. Of the ten samples from this population, eight single-flower extracts contained one or more ecotype diagnostic compounds identified in Chapter One, while two did not. Four of these eight extracts contained 2-(methylthio)benzene-1,4-diol, one of which also contained 4-hydroxy-3-(methylthio)benzaldehyde (both indicative of Ecotype Three). All eight extracts contained homovanillyl alcohol, indicative of Ecotype One or Two.

Predicting ecotype geographic range

MaxEnt modeling revealed each of the three ecotypes to occupy its own geographic range. All MaxEnt models returned area under the curve (AUC, a common indicator of model performance) values greater than 0.95 within the training area. The *Z. nigripes* model testing with withheld data returned an AUC value of 0.96. AUC values greater than 0.9 indicate a good discrimination ability of the model (Pearce & Ferrier, 2000), and thus that the predicted geographic ranges are plausible.

When considering the model for Ecotype One (Figure 1), the habitat suitability for *Z. nigripes* was the explanatory variable with the highest percentage contribution, followed by Bio32 - mean moisture index of driest quarter. In a jackknife test of variable importance, habitat suitability for *Z. nigripes* had the highest gain when used in isolation, and decreased the gain the most when omitted, indicating that this variable both contains the most useful information of the single variables, and also has the most information not present in other variables (Supplementary Figure B). The predicted distribution of Ecotype One was coastal, comprising most of the Warren IBRA bioregion, and extending along the south coast east of Albany into the Jarrah Forest bioregion, and at the northern limit of the predicted distribution extending into the Swan Coastal Plain bioregion at Geographe Bay (see IBRA regions in Supplementary Figure A). The habitat suitability for *Z. dilatatus* and Bio32 were the two variables with the highest percentage contribution in the Ecotype Three model, which predicted Ecotype Three to occur on the Swan Coastal Plain. In a jackknife test of variable importance, Bio 16 was the variable with the highest gain (making it the most informative alone), and Bio 24 showed the largest decrease in gain when omitted (making it the variable containing the most information not present in other variables, Supplementary Figure B). Bio 32 was the variable that explained the most variation in the Ecotype two model, followed by Bio08 mean temperature of wettest quarter, and Bio28 annual mean moisture index (no pollinator data were available for this ecotype). These two layers were also shown to be important in a jackknife text of variable importance: Bio 28 had the highest gain (most useful information alone) and Bio 32 showed the largest decrease in gain when omitted (most information not present in other variables, Supplementary Figure B). The predicted distribution for Ecotype Two was restricted to the western side of the Jarrah Forest bioregion. While there were some areas of predicted overlap between the ecotypes, each ecotype

occupied a distinct core geographic range - Ecotype One on the south coast, Ecotype Three on the Swan Coastal Plain, and Ecotype Two on the western side of the inland Jarrah forest.

Determining an effective identification method for the ecotypes

Morphometrics

While no single trait could differentiate the ecotypes, they appear to have divergent floral morphology. Significant differences were observed between ecotypes in the majority of traits measured, with Ecotype One typically having larger trait values than Ecotypes Two and Three (Supplementary Table A). For three traits (labellum length, proximal hinge length, and column wing length), the three ecotypes differed significantly from one another ($P<0.05$, Figure 3). There was a degree of overlap in the size ranges of the traits between ecotypes (Figure 3, Supplementary Table A). The ecotypes did not form discrete clusters in a PCA but there was some separation on PC2 of Ecotypes Two and Three, and on PC3 of Ecotype One from Ecotypes Two and Three (Figure 4). In the Linear Discriminant Analysis with leave one out cross validation, six samples (13%) were miss-assigned (one Ecotype One, three Ecotype Two, and two Ecotype Three), in that the ecotype assigned did not match that of the population, giving a 87% accuracy of assignment.

Chemical analysis of pollinated flowers

The sampling of pollinated flowers detected ecotype specific compounds and allowed the assignment of ecotypes. The labella of 85 pollinated flowers were sampled across known populations of the three ecotypes (Ecotype One $N = 21$, Ecotype Two $N = 41$, Ecotype Three $N = 23$). The average post-pollination withering score was 3.38 ± 0.27 SE out of eight (Ecotype One 2.62 ± 0.52 SE, Ecotype Two 3.54 ± 0.41 SE, Ecotype Three 3.83 ± 0.49 SE). The average percentage of ecotype diagnostic compounds detected per flower was 46.89% ± 2.23 SE (Ecotype One 45.67% ± 5.53 SE, Ecotype Two 45.15% ± 2.73 SE, Ecotype Three 50.00% ± 4.45 SE). Of the 85 flowers analysed, 82 (96%) were correctly assigned their ecotype in the Partial Least Squares Discriminant Analysis. Extracts from two flowers contained zero diagnostic compounds (2.35%, one Ecotype Two, withering score eight; one Ecotype Three, withering score seven). Of the remaining 83 flowers that contained diagnostic

compounds, 82 (98%) were correctly assigned. The miss-assigned sample (Ecotype Two) only contained one diagnostic compound and had the highest possible withering score of eight. There was a significant effect of the extent of post-pollination withering score on the proportion of detected ecotype-diagnostic compounds ($P < 0.05$) in the generalised linear mixed effects model, with a model estimate of -0.15 and R^2 of 0.05 indicating a weak negative correlation. All flowers with a withering score of six or less were correctly assigned (Figure 5).

Genome size of *D. livida* ecotypes

No significant differences in genome size were detected between the ecotypes (Ecotype One 5.17pg ± 0.006 SE, Ecotype Two 5.24pg ± 0.009 SE, Ecotype Three 5.26pg ± 0.011 SE, $P = 0.08$, Supplementary Table B). There was extensive overlap in the genome sizes of each of the ecotypes (Figure 6).

DISCUSSION

Investigating the geographic range of the ecotypes

Species distribution modeling predicted each ecotype to have a different core geographic range, with small areas of range overlap predicted at the ecotype margins (Figure 1). Ecotype One had a broad range, predominantly occupying the south coast, but also with isolated patches of suitability occurring in the Stirling Ranges and woodlands east of the Darling Scarp. Ecotype Two also had a broad inland distribution, occurring on the western edge of the Jarrah Forest bioregions. In contrast to Ecotypes One and Two, which both have large areas of bushland within their predicted geographic ranges, the majority of the much smaller distribution of Ecotype Three has been cleared for agriculture, raising conservation concerns for this ecotype. Ecotype Three has been found to occur in only ten bushland remnants on the Swan Coastal Plain, which is a known hotspot for orchid rarity, where regional endemics have become rare through extensive habitat clearing for agriculture and development (Shepherd et al., 2002; Horwitz et al., 2008; Phillips et al., 2011). Given that there were areas of predicted range overlap, and that other *Drakaea* species frequently grow in sympatry (Hopper & Brown, 2007), it is unusual that no populations were found to contain sympatric ecotypes. The extensive clearing of Ecotype Three habitat may have removed much of the habitat within the potential sympatry zone, though rare sympatric populations may yet be revealed in more extensive surveys of the ecotypes' distributions.

Potential hybridisation between ecotypes

While 49 of the 50 populations of *D. livida* analysed to date have contained compounds diagnostic of a single ecotype only, in the present study four flowers from a population in the area of predicted geographic range overlap of Ecotype One and Three contained (methylthio)phenols (thus far exclusively found in Ecotype Three), but also contained homovanillyl alcohol (thus far only found in Ecotype One and Two). Given the location of the population where the mixed phenotype flowers were located at the predicted range margin of Ecotypes Three and One, these flowers may potentially indicate a rare case of hybridisation between these two ecotypes. This hybridisation scenario is more likely than the flowers representing an undiscovered fourth ecotype given the geographic distribution of the population, the absence of other habitat differences such as soil type and vegetation at the

population, and the behaviour of *Z. nigripes*. The possibility of additional undiscovered ecotypes of *D. livida* may remain at other locations, particularly the range margins of the species. *Zaspilothynnus nigripes*, the pollinator of Ecotype One, is known to display a partial attraction to Ecotype Two and Three flowers when presented outside their core geographic range (Chapter Four), which may in rare cases potentially lead to pollen transfer. For pollen transfer to occur between ecotypes, two scenarios are possible. Firstly, *Zaspilothynnus nigripes* individuals may travel between populations of different ecotypes, or alternately seed of one ecotype may blow into a population of another ecotype, germinate, and *Z. nigripes* could then move pollen between the co-occurring ecotype within the population. The former scenario is not supported by the maximum observed movement distance for *Z. nigripes* being only 267m (Menz et al., 2013), however dispersal potential of wasps can be increased by winds (Ahmed et al., 2009). The latter scenario would entail non-hybrid plants of two ecotypes occurring sympatrically within a population, a scenario that was not observed at the mixed-phenotype population, however this could potentially be due to the small sample size. In either scenario, if mixed phenotype flowers were to be found, it is unsurprising that it has occurred in this population that occurs at the intersection of the ranges of the ecotypes. Hybridisation between the ecotypes, assuming successful pollen transfer, is plausible given the similar genome sizes of the ecotypes and rare records of *D. livida* hybridising with other *Drakaea* species (Hopper & Brown, 2007). Irrespective of potential hybridisation at the range margins, the ecotypes still stand as distinct from one another, with each having their own separate ecological niche, as reflected in their differing core geographic ranges, different pollinator species, and diagnostic chemical compositions.

Ecotype identification

Irrespective of taxonomic recognition, to enable identification of unknown populations and thereby the potential for their effective inclusion in conservation management, a practical method for determining the ecotype of a population is required. A previous study on the sexually deceptive orchid *Ophrys sphegodes* showed that pollinated flowers had a different volatile composition to un-pollinated flowers (Schiestl et al., 1997). While in the present study differences in composition may be present, of relevance is that the ecotype diagnostic compounds were found in quantities suitable to allow the identification of the ecotypes. Of the 85 labella of pollinated flowers that were analysed from populations of known ecotype, 82 (96.5%) were correctly assigned to their ecotype using PLS-DA. The three miss-assigned

samples all had a pollination score of seven or eight. Therefore, if samples with a pollination score greater than six are excluded, analysis of the chemical composition of extracts from labella of pollinated *D. livida* flowers is an effective, and impact free, method of identifying the ecotype, which demonstrated 100% accuracy in the present set of pollinated flowers with a pollination score of six or less. Analysis of chemical composition of pollinated flowers is a novel approach in chemotaxonomy that builds upon existing applications of chemical composition in discerning cryptic taxa (Véla et al., 2007; Peakall & Whitehead, 2014; Joffard et al., 2016, Chapter Four), and improves them by reducing their impact on the sampled populations. This methodology is well suited for application to taxa that are rare or of unknown conservation status. It is predicted that the use of pollinated flowers in chemotaxonomy may have broader applicability beyond *D. livida* and sexually deceptive orchids to other systems where floral chemical composition is taxonomically informative.

In the *D. livida* system, one limitation of using PLS-DA is that potential hybrid populations, such as that detected at the boundary of the Ecotype One and three predicted ranges, will not be identified as atypical. PLS-DA is trained off three groups and will thus only classify samples into these three groups without identifying patterns that may suggest a fourth hybrid grouping. To negate this limitation, it must be ensured that extract data is manually checked in order to identify samples containing compounds diagnostic of more than one ecotype.

There was broad overlap between the ecotypes in individual floral morphological traits, meaning that ecotypes cannot be distinguished based on trait measurements in field. However, trait means were often different between ecotypes, and in multivariate analyses the ecotypes displayed semi-distinct, though not discrete, clusters. These results suggest morphological differentiation between the ecotypes, reflected in the 87% correct assignment rate in the LDA. Further investigation using additional traits or methodologies may reveal discriminating traits. The most recent revision of the genus (Hopper & Brown, 2007) noted that some populations of *D. livida* (now recognised as within the geographic range of Ecotype Two) displayed darker colouration and more inflated labella than is typical. Analyses of colouration and or labellum shape, which may require techniques such as 3D geometric morphometrics (van der Niet et al., 2010), may uncover discrete differences in ecotype floral morphology. Genome size did not prove an informative trait in identifying the ecotypes, which appear to be all of the same ploidy level with no variation therein.

Baiting with fresh flowers to determine the pollinator species attracted, which was used in the initial discovery of the ecotypes (Chapter Four), remains an effective method of ecotype identification. However, this method is destructive in that it entails the picking of fresh flowers, is weather dependant, and is more time intensive than the chemical analysis of pollinated labella. For the *D. livida* system, when considering impact on the population, time investment, and the accuracy of the prediction, chemical analysis of pollinated labella proved the most effective method of identifying the ecotypes. Given that pollinator switching effected through shifts in floral chemistry is typically associated with speciation in sexually-deceptive orchids (Cozzolino & Scopece, 2008; Xu et al., 2012; Peakall & Whitehead, 2014), it is likely that the use of ecotype diagnostic chemical compounds could be implemented to aid in the identification of other morphologically challenging sexually-deceptive orchid taxa.

Conservation status of the D. livida *ecotypes*

Only ten populations of Ecotype Three have been located, which occupy the majority of bushland remnants with suitable habitat on the Swan Coastal Plain. Ecotype Three habitat is highly distinctive and readily identifiable - consisting of *Banksia* woodland with *Kunzea ericifolia* thickets and an open understorey, growing on well drained grey sandy soils that are typically low in the landscape. Each population of Ecotype Three comprises no more than 150 individual plants (A. Weinstein & R. Phillips, unpublished data). At five of the ten known populations, *D. livida* was found to co-occur with *Drakaea elastica* (endangered under the EPBC Act), however, at four of these populations, *D. livida* was the less numerous of the two species. Using the IUCN Red List Categories assessment, Ecotype Three could be classed as endangered under Criterion C2 - *Population size estimated to number fewer than 2,500 mature individuals and a continuing decline, observed, [estimated,] projected, or inferred, in numbers of mature individuals*, condition a) (i) *no subpopulation estimated to contain more than 250 mature individuals*. A history of land clearing for agriculture and development of *Drakaea* habitat (as recent as 2009) has incontestably reduced the habitat range and thus population size of both Ecotype Three and the co-occurring *D. elastica* (Shepherd et al., 2002; Horwitz et al., 2008). The remaining populations of Ecotype Three are currently threatened by grazing, weeds, salinity, and rubbish dumping due to their semi-rural location (Conservation, 2009). Many of these threats are exacerbated by edge effects due to the small size of the remnant bushland (Harrison & Bruna, 1999) and by reduced winter rainfall under climate change (McFarlane et al., 2012). Considering the much larger areas of

bushland within the predicted ranges of Ecotypes One and Two, where suitable unexplored habitat occurs, it is unlikely that these two ecotypes would qualify for listing as threatened. For effective conservation management to be implemented, such as a formalised recognition as endangered for Ecotype Three, assessment of the taxonomic status of the ecotypes is pivotal.

Taxonomic status of the ecotypes

Within the current Western Australian government framework, evolutionary significant units can and have been included in conservation management (Coates & Hamley, 1999; Shepherd et al., 2015; Rosauer et al., 2018). ESUs are identified based on a concordance of datasets such as "natural history information, morphometrics, range and distribution data, protein electrophoresis, cytogenetic analysis, and restriction mapping of nuclear and mitochondrial DNA" (Ryder, 1986). In Chapter Four and this present study, four such datasets are presented. In three of them, natural history information (specific pollination), morphometrics, and range data, distinct differences are observed between the three ecotypes, while in the fourth (genome size) no differences were found. While not one of the methods of distinguishing ESUs originally proposed by Ryder (1986), an additional line of evidence lies in the distinct floral chemical compositions of the three ecotypes (Chapter Four), giving a total of four congruent datasets supporting the presence of three distinct evolutionary entities within *D. livida*. As such, it is proposed that each *D. livida* ecotype be defined as an ESU. Further investigation into potentially discriminating floral traits and genetic analyses (such as the chloroplast DNA markers implemented in Peakall & Whitehead (2014)) may potentially support the *D. livida* ecotypes' status as subspecies or species. An official taxonomic rank is likely to improve the long-term conservation outcomes of the ecotypes (Coates et al., 2018), however such a conclusion is beyond the scope of the present study.

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FIGURES AND TABLES

Figure 1: Flowers displaying different pollination stages (stage one being the first state of pollination, and stage three being the last/oldest) of the labellum, ovary, stigma, and pollen at which chemical sampling was conducted

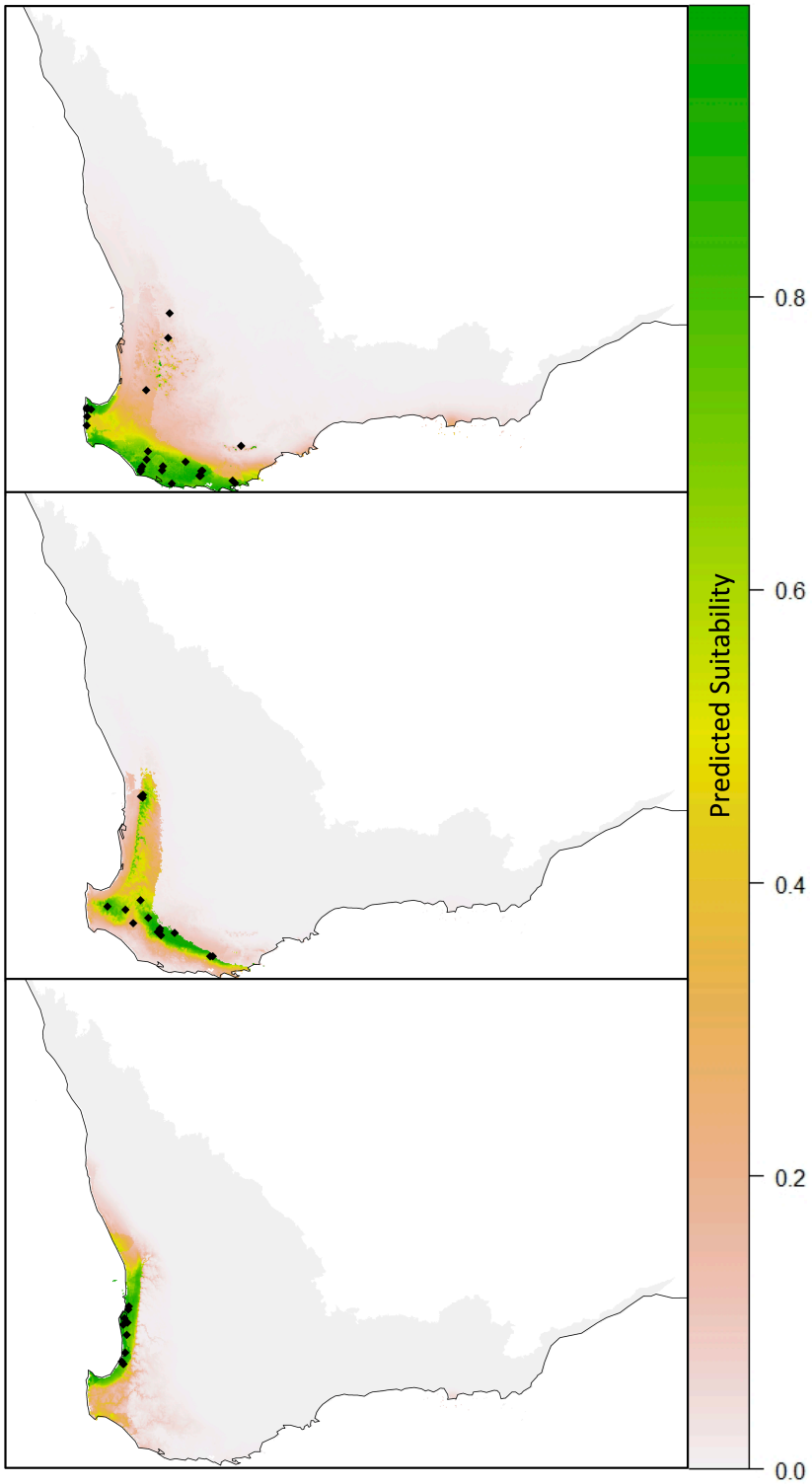

Figure 2: MaxEnt species distribution models for each of the ecotypes of *Drakaea livida*. Presence points are represented by black dots. Grey denotes the extent of the model prediction area.

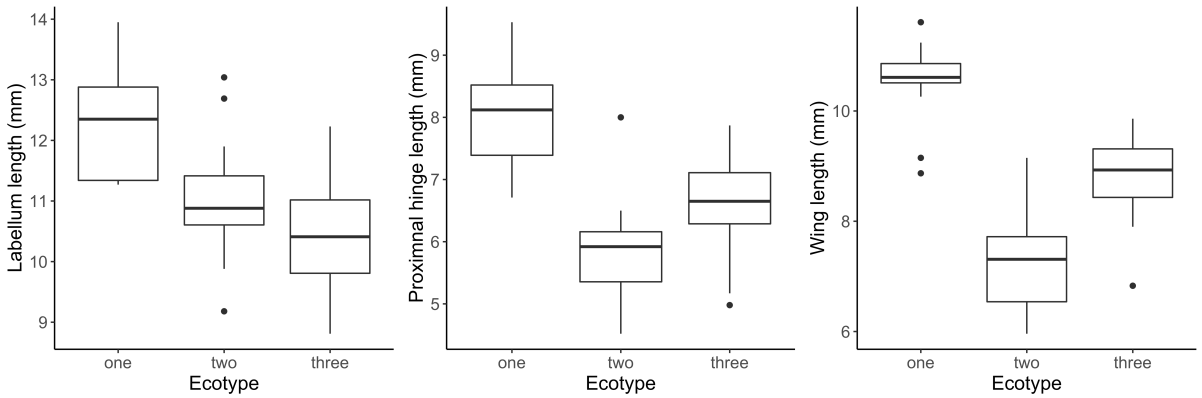

Figure 3: Ecotype means for traits that displayed significant differences between all three ecotypes ($P < 0.05$); labellum length, proximal hinge length, and column wing length (all in mm).

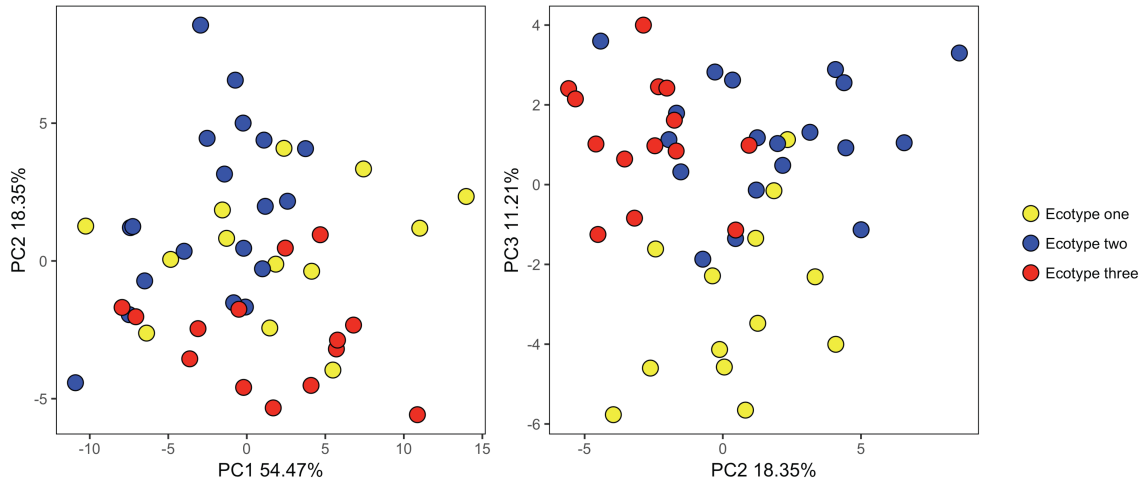

Figure 4: Principal component analysis based on the 17 morphological traits measured for individuals of each ecotype of *Drakaea livida*

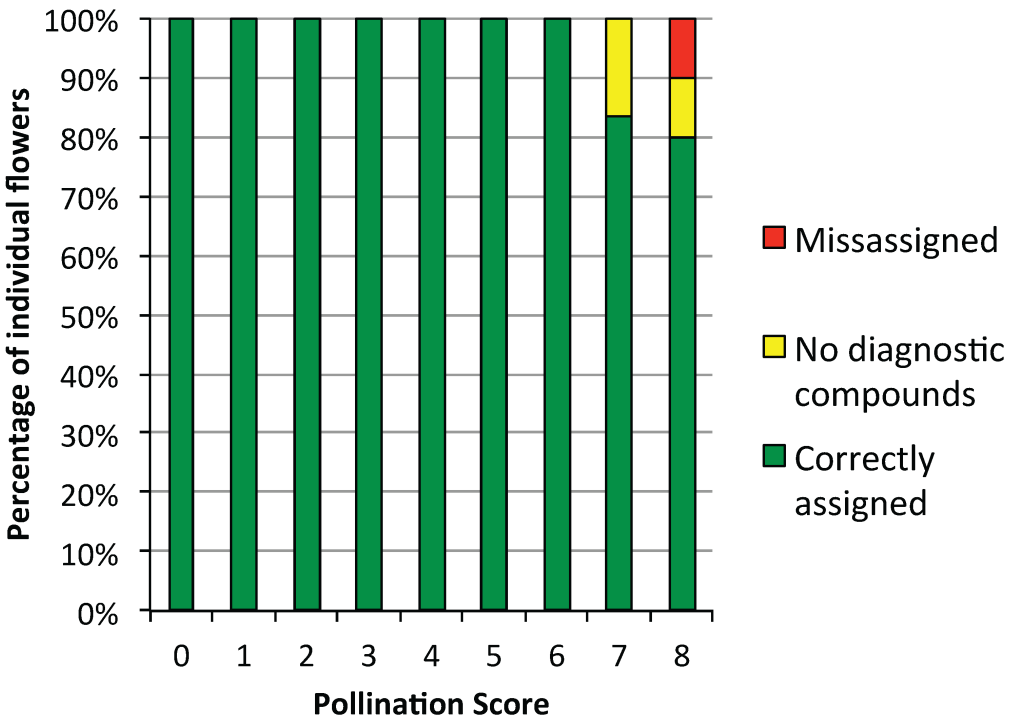

Figure 5: Percentage of individual extracts correctly assigned (green), containing no ecotype diagnostic compounds (yellow), and miss-assigned (red) by pollination score.

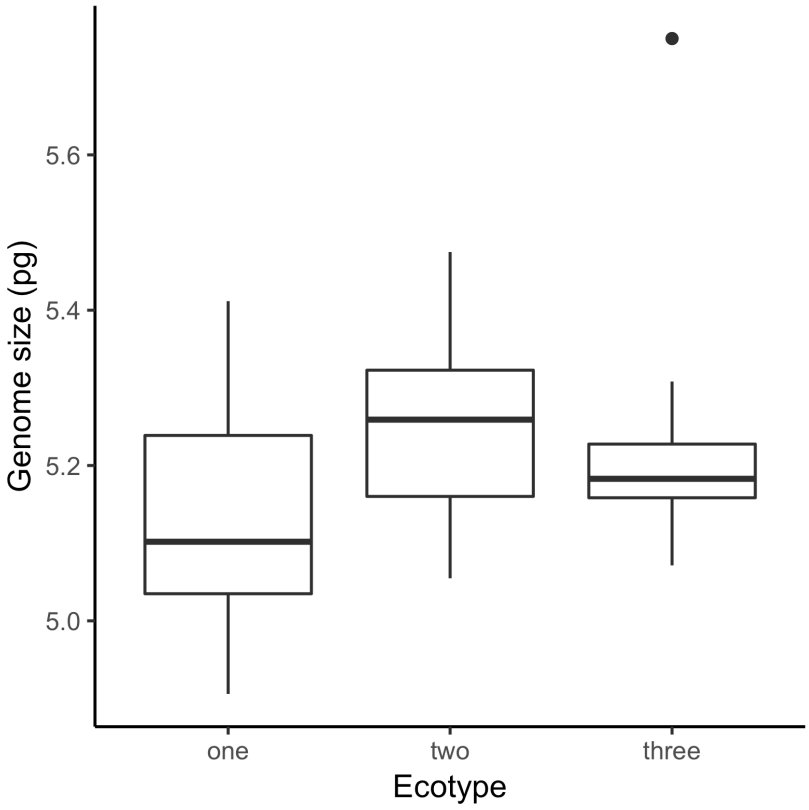

Figure 6: Genome sizes of the *Drakaea livida* ecotypes in picograms

Table 1: Description of the pollination stages of the labellum, ovary, stigma, and pollen that were used to assess degree of post pollination withering, and the corresponding scores awarded.

	Stage 1	Stage 2	Stage 3
Score awarded	0	1	2
Labellum	Fresh and fully inflated	Partially withered: no longer fully inflated	Withered: shrunken and dry
Ovary	Not swollen	Swollen less than twice unpollinated girth	Swollen two times or more unpollinated girth
Stigma	Stigma shiny with pollen clumped in pollinia	Stigma shiny with pollen spread out on stigma	Stigma unreceptive and matt, pollen spread out on stigma
Pollen	Pollen yellow	Pollen yellow/brown	Pollen brown

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Supplementary Information 1 - Soil analyses

Methods

To determine whether the ecotypes grow in different soil types, analysis of the soil in close proximity to *D. livida* individuals were conducted at five populations generally spanning the distribution of each ecotype (total individuals sampled = 45, 15 per ecotype, Figure S1). Soil was sampled from within 30 cm of flowering *D. livida* individuals at a 10cm depth (depth of *Drakaea* tubers). CSBP Laboratories Perth performed testing for the soil characters: ammonium nitrogen, nitrate nitrogen, Colwell phosphorus, Colwell potassium, sulphur, organic carbon, conductivity, pH ($CaCl_2$), and pH (H_2O).

To investigate whether the fire history of the sites sampled was a confounding factor in subsequent analyses, each soil character was plotted against the number of years since the last fire at the sampling site and trends were visually checked for with the aid of smoothed conditional means. Fire history data was provided by the Department of Biodiversity, Conservation and Attractions as the shapefile 'DEC_Fire_History' last updated 26/10/2018, and in the case of one site (Goodale Sanctuary) fire history data was provided through consultation with the landowners.

To test for differences in each individual soil character between ecotypes, either t-tests or Wilcoxon Rank Sum tests were conducted where appropriate after data was tested for normality (Shapiro-Wilk test) and equality of variances (Levene's test).

To detect any potential grouping of samples based on soil characters, multivariate analysis was conducted. Characters for which no significant differences nor any between-ecotype trends were observed (pH level(H_2O) and conductivity) were removed prior to analyses to reduce noise levels. Data were normalised using the R package 'clusterSim' (Walesiak and Dudek 2017). A Euclidean distance matrix was calculated in the 'vegan' package (Jari Oksanen et al. 2018), from which a principle coordinate analysis was conducted using the 'ape' package (Paradis et al. 2004). To test for a global difference in the variation of soil characters between ecotypes, a PERMANOVA was conducted using the vegan 'adonis' function. Pairwise adonis

was conducted (Holm correction for multiple comparisons, 100,000 permutations) to determine which ecotypes differed significantly according to their soil characters.

Results

Fire history data was available for 11 out of the 15 sampled sites. There was no observed effect of time since fire on the soil characters.

Ecotype two was found to differ from ecotypes one and/or three for four of the eight presented soil characters, namely ammonium nitrogen, organic carbon, sulphur, and pH level $CaCl_2$ (Table S1). However, there was a large degree of overlap in the range of values observed between ecotypes for all soil characters (Table S1). Organic carbon, pH level ($CaCl_2$), and pH level (H_2O) were found to be normally distributed and to have equal variances, and hence were analysed using t-tests. All other soil characters were analysed with a Wilcoxon Rank Sum test. Nitrate nitrogen was not included in analyses as all samples returned a value of less than one mg/Kg (detectability limit of instrument).

In the principle coordinate analysis axes one and two cumulatively explained 77.2% of the observed variation (Figure S2). While there was a large degree of overlap between ecotypes with no discrete clusters being observed, the placement of the points when considering their ecotype differed to random placement (adonis PERMANOVA, $R^2 = 0.17$, $P = 0.005$). Pairwise differences revealed ecotype two to differ in soil characters to both ecotype one ($R^2 = 0.20$, $P = 0.004$) and to ecotype three ($R^2 = 0.14$, $P = 0.02$).

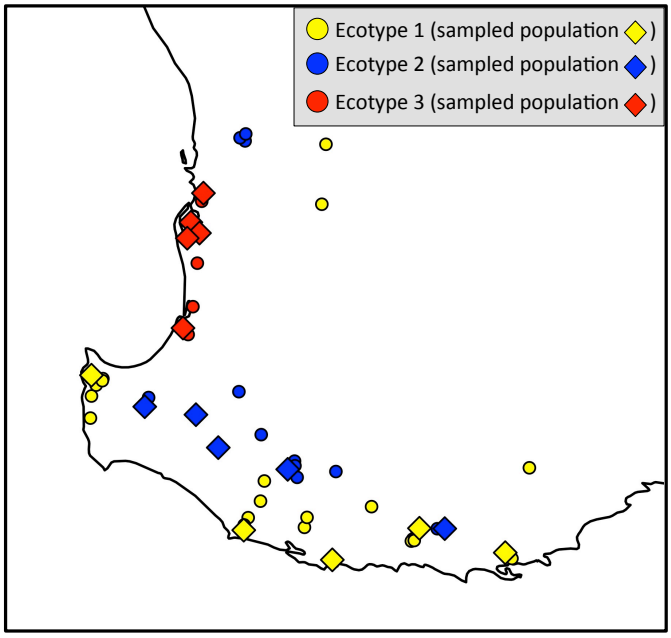


Figure S1: Map of the distribution of each ecotype of *Drakaea livida*, with populations where soil sampling was conducted denoted with diamonds.

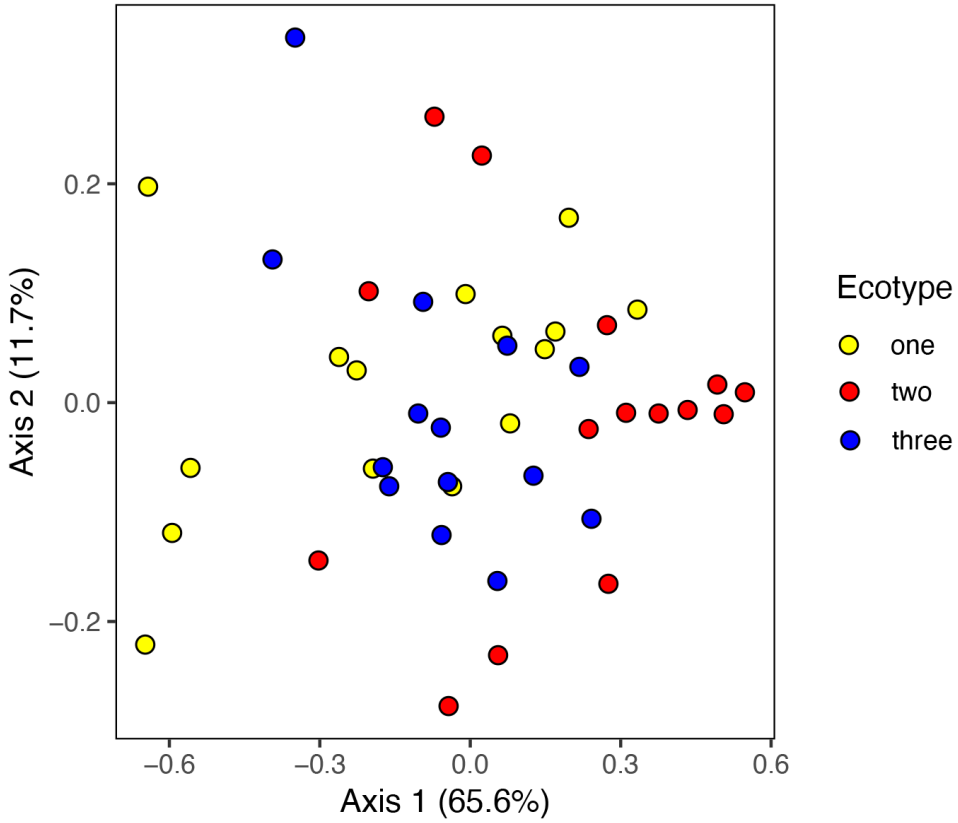
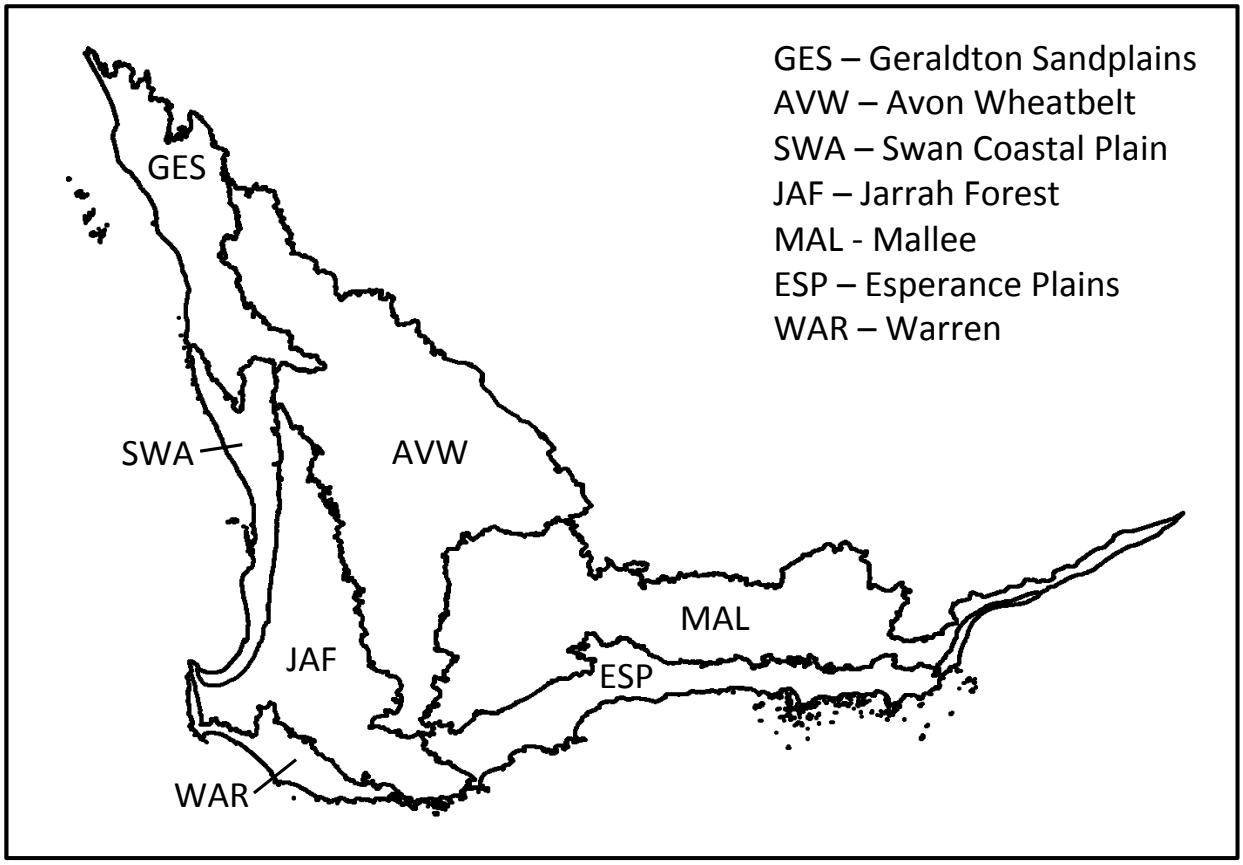


Figure S2: Principle coordinate analysis of soil characters by ecotype generated from a Euclidean distance matrix calculated from normalised soil character data. Axis 1 and 2 cumulatively explain 77.2 % of the variation.

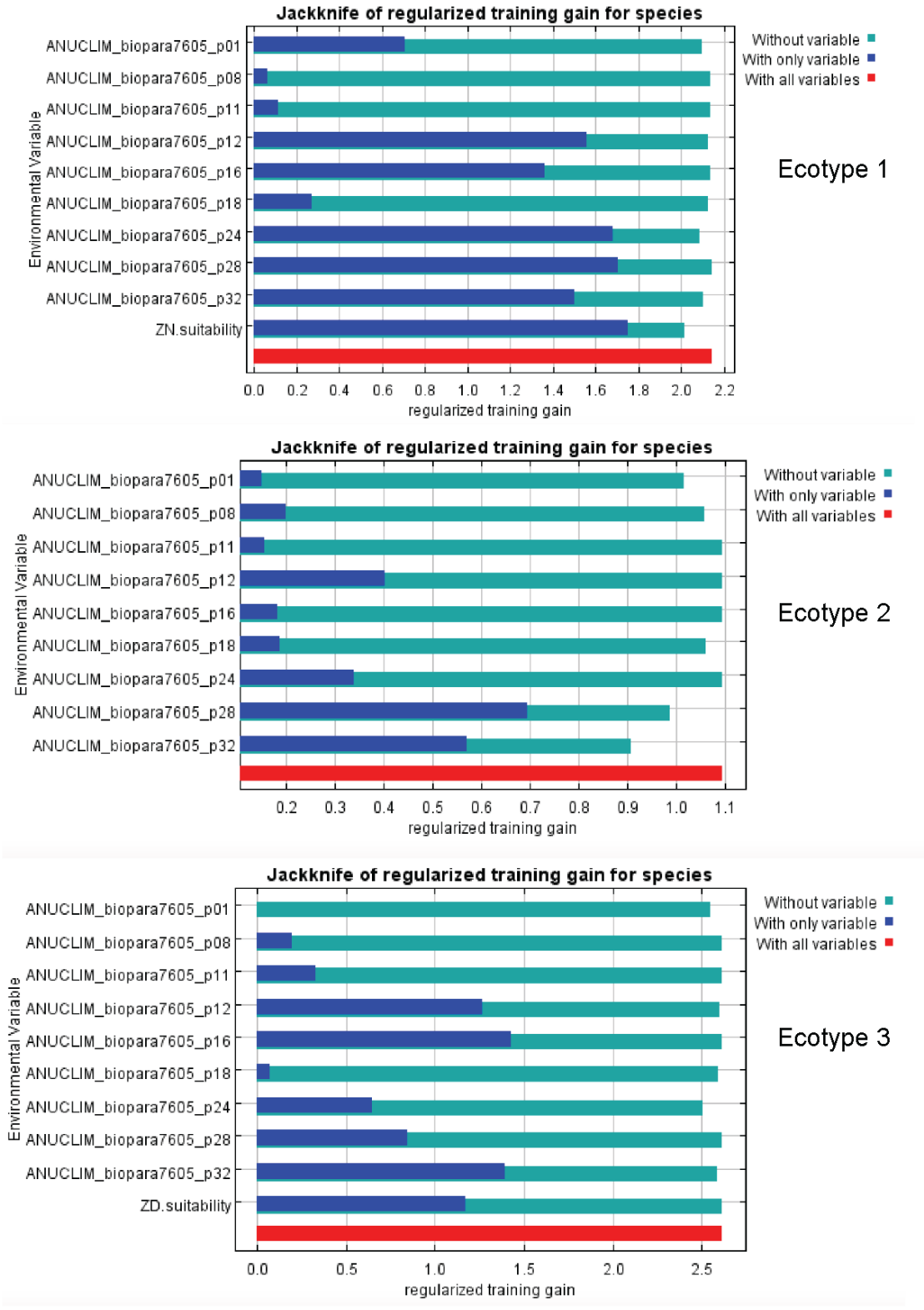
Table S1: Ecotype means, standard errors, and ranges for eight soil characters analysed for populations of *Drakaea livida*.

Ecotype	**1**	**2**	**3**
N sites sampled	5	5	5
N soil samples	15	15	15
Ammonium Nitrogen (mg/Kg)	1.73 ± 0.21[a] (1-4)	0.67 ± 0.25[b] (0-3)	1.36 ± 0.37 (0-5)
Organic Carbon (%)	3.16 ± 0.31[a] (1.25-4.98)	1.39 ± 0.24[b] (0.24-3.23)	2.55 ± 0.21[a] (1.25-3.96)
Phosphorus Colwell (mg/Kg)	1.87 ± 0.4 (0-4)	1.07 ± 0.43 (0-5)	1.47 ± 0.45 (0-6)
Potassium Colwell (mg/Kg)	23.13 ± 5.78 (0-73)	12.67 ± 4.3 (0-49)	23.13 ± 2.73 (0-48)
Sulphur (mg/Kg)	1.95 ± 0.29[a] (0.6-4.0)	1.14 ± 0.29[b] (0.0-4.3)	2.01 ± 0.18[a] (1.1-3.7)
pH Level ($CaCl_2$)	3.85 ± 0.09[a] (3.3-4.4)	4.23 ± 0.09[b] (3.7-4.8)	3.85 ± 0.07[a] (3.5-4.4)
pH Level (H_2O)	5.43 ± 0.08 (4.9-5.9)	5.51 ± 0.1 (4.7-6.1)	5.29 ± 0.1 (4.5-5.9)
Conductivity (dS/m)	0.02 ± 0.003 (0.00-0.04)	0.01 ± 0.004 (0.0-0.05)	0.02 ± 0.004 (0.0-0.5)

SUPPLEMENTARY FIGURES AND TABLES



Supplementary Figure A: Interim Biogeographic Regionalisation for Australia (IBRA) regions referred to in MaxEnt modelling



Supplementary Figure B: Jackknife tests of variable importance from the MaxEnt models conducted for each of the three Ecotypes of *Drakaea livida*

Supplementary Table A: Floral trait means with standard error and ranges for the three ecotypes of *Drakaea livida.* Letters denote significant differences in pairwise Holm-corrected t-tests *(P < 0.05)*.

Ecotype	**One**	**Two**	**Three**
N flowers	13	19	14
N populations	5	4	6
Labellum-column distance (mm)	4.04 ± 0.22 (2.94 - 5.46)[a]	3.73 ± 0.19 (1.93 - 5.45)[a]	3.00 ± 0.17 (2.19 - 3.93)[b]
Labellum width (mm)	4.50 ± 0.15 (3.77 - 5.63)[a]	4.58 ± 0.14 (3.33 - 5.45)[a]	3.88 ± 0.12 (3.03 - 4.77)[b]
Labellum length (mm)	12.39 ± 0.28 (11.27 - 13.95)[a]	11.01 ± 0.21 (9.18 - 13.04)[b]	10.40 ± 0.25 (8.81 - 12.23)[c]
Dorsal sepal length (mm)	14.26 ± 0.31 (12.37 - 16.10)[a]	12.58 ± 0.26 (10.91 - 14.77)[b]	12.74 ± 0.39 (10.57 - 15.27)[b]
Lateral sepal length (mm)	13.70 ± 0.36 (11.33 - 15.67)[a]	12.37 ± 0.27 (9.32 - 14.14)[b]	13.40 ± 0.32 (11.83 - 15.60)[a]
Petal length (mm)	14.25 ± 0.28 (12.78 - 16.41)[a]	12.42 ± 0.34 (8.73 - 13.95)[b]	12.82 ± 0.31 (11.21 - 15.11)[b]
Proximal hinge length (mm)	8.13 ± 0.23 (6.71 - 9.53)[a]	5.85 ± 0.17 (4.52 - 8.00)[b]	6.60 ± 0.21 (4.98 - 7.87)[c]
Distal hinge length (mm)	7.55 ± 0.25 (6.77 - 9.92)[a]	6.01 ± 0.21 (3.88 - 7.97)[b]	6.25 ± 0.12 (5.70 - 7.25)[b]
Labellum head length (mm)	3.12 ± 2.96 (2.66 - 3.84)	2.96 ± 0.12 (2.13 - 3.96)	2.85 ± 0.10 (2.11 - 3.49)
Column length (mm)	11.87 ± 0.26 (10.26 - 13.31)[a]	11.03 ± 0.27 (8.59 - 12.84)[ab]	10.79 ± 0.21 (9.33 - 12.55)[b]
Wing length (mm)	10.52 ± 0.21 (8.87 - 11.61)[a]	7.34 ± 0.23 (5.96 - 9.15)[b]	8.79 ± 0.22 (6.83 - 9.86)[c]
Length of mucro (mm)	1.97 ± 0.08 (1.46 - 2.74)	2.07 ± 0.08 (1.26 - 2.56)	2.07 ± 0.09 (1.62 - 2.60)
Callus width (mm)	2.00 ± 0.07 (1.64 - 2.48)	1.97 ± 0.06 (1.50 - 2.39)	2.04 ± 0.12 (1.15 - 2.76)
Stem width (mm)	0.97 ± 0.05 (0.76 - 1.39)[ab]	0.91 ± 0.03 (0.72 - 1.12)[a]	1.07 ± 0.4 (0.79 - 1.30)[b]
Scape 1 (cm)	4.83 ± 0.54 (2.50 - 8.30)[a]	4.64 ± 0.28 (2.10 - 6.97)[a]	7.21 ± 0.74 (3.78 - 12.60)[b]
Scape 2 (cm)	15.11 ± 1.63 (4.50 - 24.80)[ab]	12.10 ± 0.73 (7.18 ± 16.34)[a]	16.52 ± 1.27 (8.40 ± 24.70)[b]
Pedicel (mm)	15.72 ± 1.18 (9.03 - 23.69)	16.08 ± 0.89 (8.12 - 22.60)	14.09 ± 0.67 (10.99 - 18.42)

Supplementary Table B: Genome size (2C, pg) and number of individual *Drakaea* flowers sampled from different populations, with the number of events sampled, and the sample CV (error measure).

Species average 2C genome size (pg)	Ecotype	Population	2C genome size (pg)	No individuals	Sample events	Sample CV	Total events
5.17	One	King River Hall	5.32	1	270	2.79	1630
		Bayonet Head	5.13	1	473	2.8	1966
		Blue Lake	5.10	1	152	2.68	1329
		Blue Lake Road Open Area	5.06 ± 0.03	3	531 ± 127	2.87 ± 0.48	1950 ± 284
		Granite Road	5.2 ± 0.05	2	561 ± 46	2.58 ± 0.06	2432 ± 409
		Grays Road	5.02	1	289	2.89	1276
		Mount Lindsey	5.32 ± 0.08	2	326 ± 23	3.25 ± 0.11	1490 ± 218
		Lane Poole Road (Northcliffe)	5.07 ± 0.05	5	416 ± 77	2.78 ± 0.14	2559 ± 493
		Windy Harbour Road	5.03 ± 0.01	2	145 ± 54	2.89 ± 0.21	1725 ± 510
		Rainbow Cave Road	5.41	1	1353	2.40	4071
		Scotsdale Outcrop	5.1	1	846	2.57	2579
		Spencer Road	5.32	1	306	2.31	1280
		Stirling Ranges	5.1	1	580	2.12	1327
5.24	Two	Frosty Road	5.2 ± 0.04	7	326 ± 33	2.88 ± 0.25	1674 ± 179
		Mowen Road	5.19	1	613	3.04	2158
		Nannup	5.33 ± 0.05	5	2085 ± 420	2.31 ± 0.17	2991 ± 496
5.26	Three	Franklandia Nature Reserve	5.11 ± 0.02	4	168 ± 33	2.73 ± 0.17	1401 ± 229
		Island Point Nature Reserve	5.28 ± 0.03	2	222 ± 14	2.96 ± 0.23	927 ± 239
		Manea Park	5.38 ± 0.19	3	530 ± 148	2.36 ± 0.05	2103 ± 697
		Serpentine River Nature Reserve	5.27	1	98	2.87	1114

THESIS CONCLUSION

From the examination of two sexually deceptive systems with contrasting patterns of pollinator exploitation, it is evident that different patterns of pollinator exploitation confer different ecological and evolutionary consequences upon the plants they pollinate. The taxonomy, species richness of the pollinator group, and the plant species to pollinator species ratio (described below) all influence the evolution and diversification of a sexually deceptive orchid, as here exemplified in *Cryptostylis* and *Drakaea*.

Pollinator taxonomy

Different taxonomic groups may have different behavioural patterns that may influence pollen movement. *Cryptostylis* is the only known sexually deceptive orchid with an ichneumonid pollinator (Gaskett, 2011). As suggested in Chapters One and Two, *L. excelsa* may have a high rate of revisitation to a flower that potentially contributes to inbreeding through geitonogamous pollen transfer. The potential for geitonogamous pollen transfer may be further augmented by the clonal multi-flowered growth habit of *Cryptostylis* (Chapter Two). These factors have potentially had consequences for the mating system of *Cryptostylis* - it is possible that the evolution of self-incompatibility may have occurred in response to this geitonogamous pollen transfer. *Cryptostylis* is the only example of self-incompatibility in the Diurideae (Chapter Two).

Pollinator movement distances also have consequences for plant mating through their role in pollen dispersal (Harder & Barrett, 1996; Brunet et al., 2019). As demonstrated by the literature review in Chapter One, it is evident that the adoption of different taxonomic groups of pollinator species may confer different pollen dispersal distances. Different taxonomic groups may vary in mating strategy, which influences pollinator movement distance and thereby pollen dispersal and gene flow.

Pollinator species richness

The species richness of a pollinator group likely has an effect on the rate of pollinator switching and pollinator mediated speciation experienced within a lineage. As suggested in Chapter Four, pollinator switching may be most likely to happen in scenarios where there is a high availability of pollinator species with similar sex pheromone chemistry. There is a high diversity of thynnine wasps (Thynnidae) in Australia (Mackerras, 1970), and congruently thynnine pollinated Australian sexually deceptive genera show rapid radiations predicted to be achieved through pollinator isolation and pollinator mediated speciation (Chapter Two). Conversely, there is a low diversity of species in the Pimplini subfamily (Gauld, 1984), to which *L. excelsa* belongs, limiting the potential for pollinator switching and pollinator isolation. In the absence of pollinator isolation in Australian *Cryptostylis*, alternate post-pollination mechanisms are responsible for reproductive isolation and diversification (Chapters Two & Three). Perhaps consequently, Australian *Cryptostylis* have a much lower diversity than the thynnine pollinated sexually deceptive orchids. Through its influence on pollinator switching, the species richness of a pollinator group may potentially influence the species richness of the orchids they pollinate.

Pollinator species to plant species ratio

As summarised above, the more potential pollinators there are, the more likely it is that a random mutation may match with a sex pheromone of a pollinator species and lead to its attraction. Similarly, if there are more orchid species each with slight differences in floral chemistry, there are more random mutations occurring from a variety of origins, potentially increasing the chance that a novel pollinator species will be attracted. Therefore, a highly speciose a plant lineage may have a greater likelihood of adopting new pollinators and further diversifying than a less speciose plant lineage. Diversification is therefore limited by both the richness of plant and, perhaps to a greater extent, by pollinator lineages. In Australian *Cryptostylis*, only one pollinator species is attracted to few species of plants, potentially representing a dead end for pollinator switching. Given that the attraction of *L. excelsa* is proposed to be an ancestral trait (Chapter 3), it follows that if random chemical variation in *Cryptostylis* were sufficient to produce pheromones of related wasp species (of which there are few), pollinator switching may potentially have already occurred. At the opposite end of the spectrum, *D. livida* attracted three pollinator species within a single species, leading to its

presumed incipient divergence into three separate evolutionary significant units (Chapter Five).

Conclusions

The consequences of pollination by sexual deception may vary extensively between plant taxa due to their patterns of pollinator exploitation. The present thesis examined two contrasting systems that have vastly different plant to pollinator ratios, and in which the pollinators are from different wasp families that have different degrees of local species richness. These factors may help explain the observed different proposed mechanisms for the formation of reproductive isolation in the two systems (pre- vs post-pollination), which have likely contributed to overall patterns of diversification in these genera.

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APPENDIX I

2-(Tetrahydrofuran-2-yl)acetic Acid and Ester Derivatives as Long-Range Pollinator Attractants in the Sexually Deceptive Orchid *Cryptostylis ovata*

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AM Weinstein contributed to this work through conducting the column fractionation of extracts, the field bioassays for the column and prep-GC fractions, the statistical analysis, and through contribution to the manuscript preparation and funding acquisition (AOF grant 319.17 to A. Weinstein).

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2-(Tetrahydrofuran-2-yl)acetic Acid and Ester Derivatives as Long-Range Pollinator Attractants in the Sexually Deceptive Orchid *Cryptostylis ovata*

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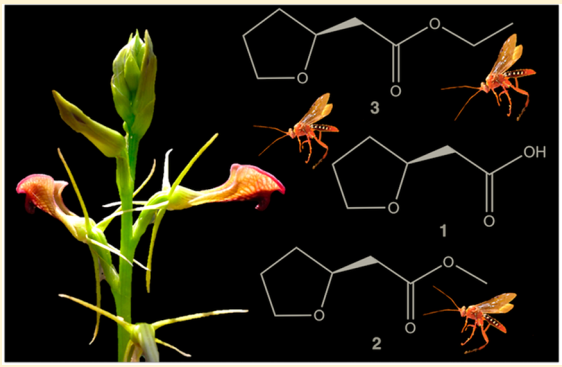
Supporting Information

ABSTRACT: Sexually deceptive orchids achieve pollination by luring male insects to flowers through chemical and sometimes visual mimicry of females. An extreme example of this deception occurs in *Cryptostylis*, one of only two genera where sexual deception is known to induce pollinator ejaculation. In the present study, bioassay-guided fractionations of *Cryptostylis* solvent extracts in combination with field bioassays were implemented to isolate and identify floral volatiles attractive to the pollinator *Lissopimpla excelsa*. (*S*)-2-(Tetrahydrofuran-2-yl)acetic acid [(*S*)-**1**] and the ester derivatives methyl (*S*)-2-(tetrahydrofuran-2-yl)acetate [(*S*)-**2**] and ethyl (*S*)-2-(tetrahydrofuran-2-yl)acetate [(*S*)-**3**], all previously unknown semiochemicals, were confirmed to attract *L. excelsa* males in field bioassays. Chiral-phase GC and HPLC showed that the natural product **1** comprised a single enantiomer, its *S*-configuration being confirmed by synthesis of the two enantiomers from known enantiomers of tetrahydrofuran-2-carboxylic acid.



Pollination via sexual deception is achieved when male insects display copulatory or precopulatory behavior with flowers mimicking female insects.[1] This pollination strategy is most widely employed in the Orchidaceae, where several hundred plant species are known to be involved.[2] In orchids, the sexual attraction of male pollinators is usually achieved by species-specific blends of semiochemicals.[2] Thus, each orchid species is typically pollinated by only one pollinator species, although some cases of multiple pollinators or pollinator sharing between orchids are known.[3,4] Members of the Hymenoptera are the most widely exploited pollinators, with well-known cases involving male bees, wasps, sawflies, and winged ants.[5−7] Pollination by sexually attracted male fungus gnats (Diptera) has also been recorded[8,9] and may be widespread in some orchid genera.

Cryptostylis is unique among sexually deceptive orchids as the only genus where pollination by male ichneumonid wasps has been recorded.[5,10] Furthermore, in an unusual case of pollinator sharing, *Lissopimpla excelsa* (Costa) (Ichneumonidae) is exploited by all five Australian species of *Cryptostylis*.[11−15] Additionally, *Cryptostylis* is one of only two genera in which sexually deceived pollinators have been observed to ejaculate during attempted copulation at the flowers.[8,16,17]

To date, most studies on the semiochemicals involved in the pollination of Australian orchids have focused on thynnine wasp pollinators.[4,18−24] Despite the unusual pollination biology of Australian *Cryptostylis*,[10,25−31] only one study has investigated the chemical signals mediating pollinator attraction.[32] This study by Schiestl et al. focused on detecting electrophysiologically active compounds from *C. subulata* and *C. erecta*. While the two species emitted different floral odor bouquets, they were found to share an unidentified compound that was electrophysiologically active to *L. excelsa* males.[32] In other chemical studies, unrelated to pollinator attraction, multiple alkaloids known as cryptostylines have been extracted from the leaves of several Asiatic species of *Cryptostylis*.[33−35]

There have been few investigations into the sexual pheromones used by members of the Ichneumonidae, despite being one of the most diverse families in the Hymenoptera.[36−38] There are only two species of ichneumonids where the identification of sexual pheromone constituents have been confirmed by bioassays. In the first case, Robacker and Hendry[38] applied chemical methods to characterize the

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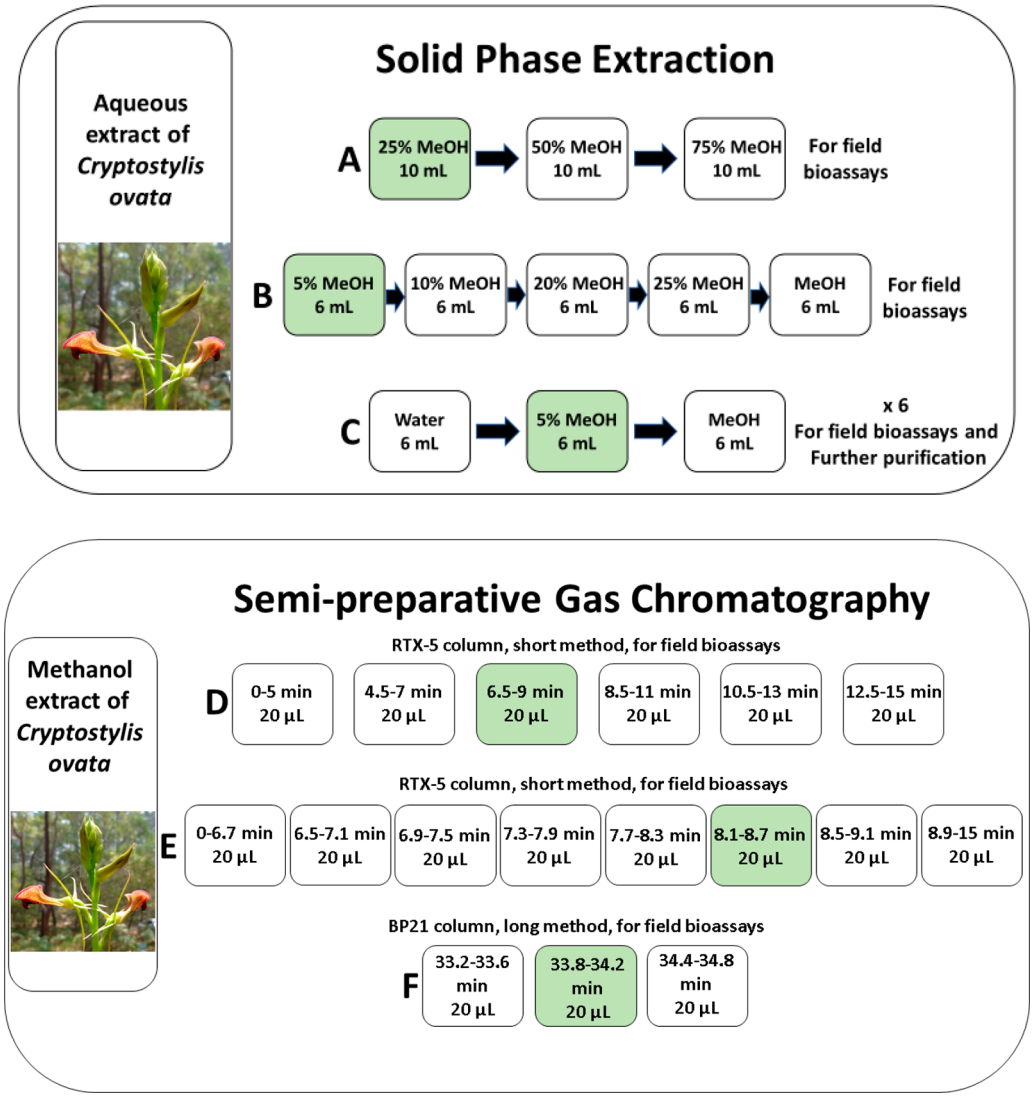

Figure 1. Bioassay-guided fractionation of solvent extracts of *Cryptostylis ovata*. Bioactive fractions are indicated as shaded boxes. Top: SPE fractionation; protocols A, B, C, eluent composition (MeOH/water), and volume displayed in boxes. Bottom: Semipreparative GC; protocols D, E, F, retention time, and solvent extract volume (MeOH) displayed in boxes.

functional groups of extract constituents of female ichnuemonids. They found the sex pheromone of *Itoplectis conquisitor* to be composed of several unsaturated aldehydes or ketones and showed that both neral and geranial elicited male sexual activity in field bioassays. In the second case, Eller et al. identified the sex pheromone of *Syndipnus rubiginosus* by using large-scale extraction of females, column chromatography, and microderivatization, to identify a single compound, ethyl (*Z*)-9-hexadecenoate, as an attractant for conspecific males.[39] Interestingly, in another ichneumonid, *Pimpla disparis*, instead of using a sex pheromone, the males locate mates by co-opting non-sex-specific eclosion pheromones (pheromones accompanying emergence), relying on the 50% likelihood that an emerging wasp will be female.[40]

Herein, more than 90 years after the landmark discovery of sexual deception in *Cryptostylis*,[12] we investigated the semiochemicals used by *Cryptostylis ovata* R.Br. to attract *L. excelsa*. Two parallel methodologies, semipreparative gas chromatography and liquid chromatography, both in combination with field bioassays, were employed to identify floral compounds mediating long-range attraction of pollinators. NMR spectroscopy and GC-MS were used to confirm the structure of the isolated compound and two additional bioactive derivatives. Synthesis of authentic standards, and comparison of their retention times and spectra, was used to determine the absolute configuration of the main attractant.

RESULTS AND DISCUSSION

By conducting an experiment where orchid flowers were hidden from the pollinators' view with a screen, but where volatiles were still able to disperse, it was confirmed that long-range pollinator attraction to *C. ovata* flowers is mediated by chemical cues. No wasps approached the screen in the absence of the orchid (as a negative control), and there was no significant difference between the total number of wasps responding to the screened flower (84 responding wasps, 15 trials, 5.6 ± 1.2 responses per trial) and the total number of wasps responding to the flower alone (108 responding wasps, 15 trials, 7.2 ± 1.5 responses per trial, Mann−Whitney U-test, $W = 135.5$, $P = 0.35$). These results are in agreement with the experiment reported in 1930 for the related *C. erecta*, where muslin cloth was used to obscure visual signals.[15]

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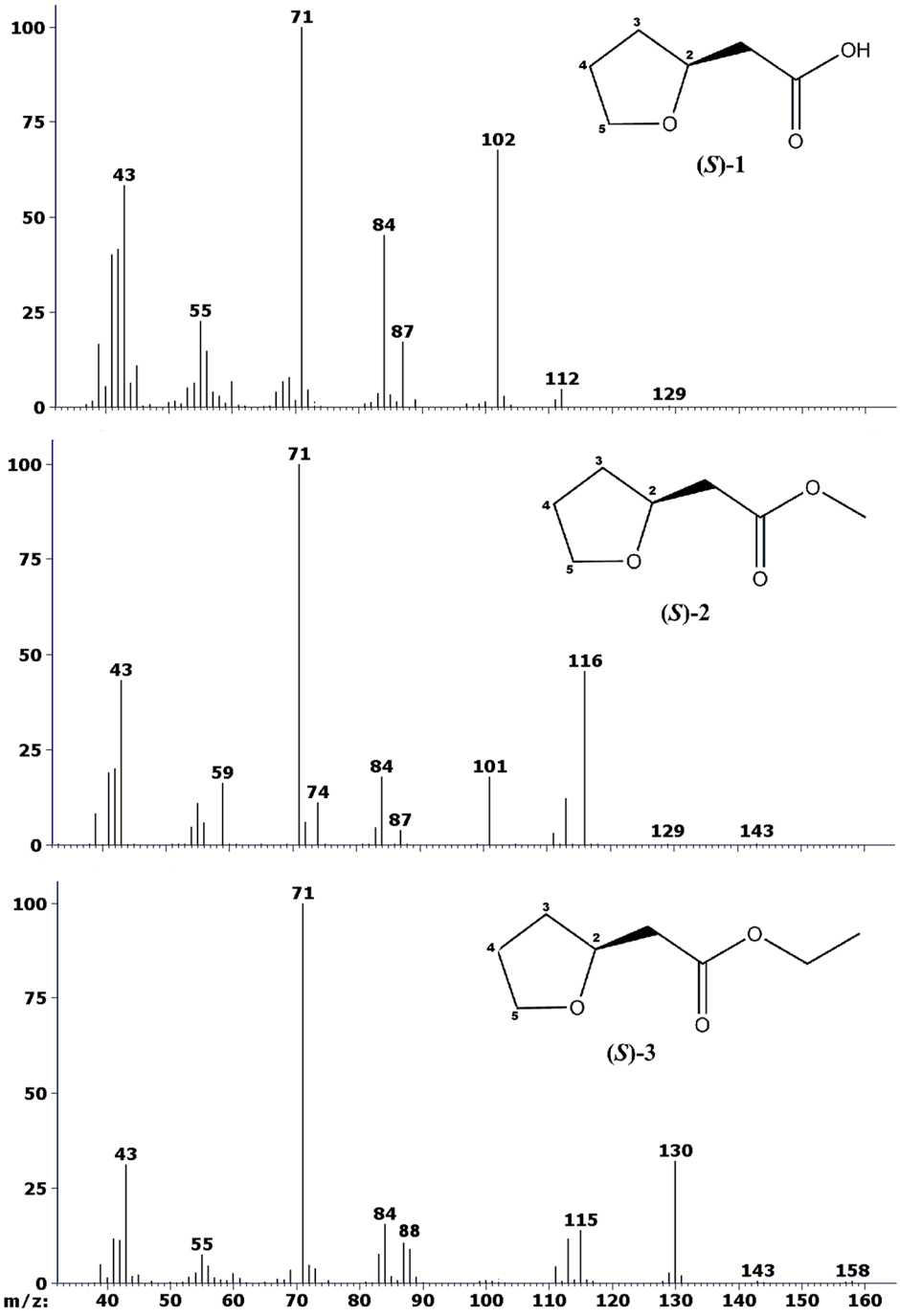

Figure 2. Mass spectra of selected peaks from GC-MS analysis of a methanol extract of *C. ovata*, with the corresponding identified bioactive compounds **1**, **2**, and **3**.

In preliminary experiments (Supporting Information, Table S1), flowers were extracted using solvents of different polarity, ranging from water to hexanes. These experiments showed that extracts made with polar or semipolar solvents were more attractive than nonpolar hexane extracts. Based on these findings, bioassay-guided fractionation was conducted using two separate methods in parallel: solid-phase extraction (SPE, C_{18}, from floral extract in water) and semipreparative GC (from floral extract in MeOH) (Figure 1). Both methods independently led to the isolation of a single pollinator-attracting fraction, which when compared by GC was shown to contain the same main compound. Since the amount of material obtained in the GC-purified fraction was too low for further spectroscopic analysis, semipreparative HPLC was used to purify the active compound from the bioactive SPE fraction.

The GC retention time and mass spectra of the purified compound from semipreparative HPLC were confirmed to match those of the active compound that was isolated through semipreparative GC. The active compound was analyzed by HRMS and NMR, including 2D experiments (COSY, HSQC, and HMBC, see Supporting Information, Figures S1–S6). From the HRMS data, the molecular formula was indicated as $C_6H_{10}O_3$, which was supported by the ^{13}C NMR spectrum showing the presence of six unique carbon environments (Supporting Information, Figure S3). A carbonyl signal was observed at δ_C 175.1, which showed HMBC correlations to a

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methylene group at δ_H 2.49 and a methine at δ_H 4.23, which were connected due to the observation of a $^1H-^1H$ COSY correlation (Supporting Information, Figure S6). Further consideration of the remaining alkyl signals, including a second methylene group at δ_C 68.8, suggested a tetrahydrofuran system was present with substitution at C-2. The MS fragments, m/z = 60 and m/z = 112 (M − H_2O, Figure 2), in conjunction with the GC peak shape, and the presence of the compound in aqueous extracts were consistent with a substituted acetic acid assignment. Hence the active compound was tentatively identified as 2-(tetrahydrofuran-2-yl)acetic acid (**1**). Co-injection with the commercially available racemate of **1** confirmed this identification. When presented in the field, the racemic synthetic compound **1** attracted a total of 98 male *L. excelsa* wasps to within 5 cm of the pin, across two field experiments of four 2 min trials each.

Analysis of floral extracts by chiral-phase GC-MS (Supporting Information, Figure S12) showed only the *S*-enantiomer of **1** to be present in *C. ovata* flowers. Field tests of (*R*)-**1** and(*S*)-**1** (separated using chiral-phase HPLC) revealed that the naturally occurring (*S*)-**1** was significantly more attractive than (*R*)-**1** (Mann−Whitney U-test, W = 100, p = 0.0001). Over 10 trials across 3 days and at two different sites, only three *L. excelsa* males approached (*R*)-**1**, while 53 approaches were observed to the naturally occurring (*S*)-**1**.

It should be noted that while the presentation of (*S*)-**1** across multiple trials of 2 min duration regularly led to the rapid attraction of male *L excelsa* to within 5 cm of the compound, only one individual landed on the pin, and no copulatory behavior (as regularly observed on flowers) was observed. Dose−response experiments (Supporting Information, Table S1) showed that (*S*)-**1** elicited close approaches in amounts from 20 ng to 100 μg, which is in the same range as measured in the floral extracts and applied on the pins in the bioassays.

To further explore the possibility that additional compounds were required to elicit pseudocopulation (a step essential for pollination at the flower), solvent extracts of *C. ovata* were screened for related compounds, as experience from other sexually deceptive orchids and pollinators suggests that it is common for the floral attractants and sex pheromones to contain a series of related active compounds.[2] Indeed, the methyl and ethyl esters **2** and **3** of (*S*)-**1** were found in small amounts when floral extracts (MeOH and CH_2Cl_2) were analyzed in detail. Chiral-phase GC showed that, as with **1**, only the *S*-enantiomers of **2** and **3** were present in the flower. The new semiochemicals (*S*)-**2** and (*S*)-**3** were prepared by Fischer esterification of (*S*)-**1**. The *S*-enantiomers of **1**, **2**, and **3** were compared in seven field trials conducted across 3 days at two sites. Across the trials, at 2 μg, (*S*)-**1** attracted 29, (*S*)-**2** 15, and (*S*)-**3** 25 wasps. There was no significant difference between the mean rank of wasp responses to each compound (Kruskal−Wallis rank sum test, H = 1.15, df = 2, p = 0.56). While low wasp availability meant that only two combinations could be tested, it is worth noting that in additional trials neither lands nor attempted copulations were observed when combinations of **1**, **2**, and **3** were tested in 50:0:0, 50:50:50, and 50:5:5 (μg). In 10 trials over 2 days, a total of 63, 64, and 65 wasps were attracted per treatment, with no significant difference between treatments observed (Kruskal−Wallis rank sum test, H = 0.64, df = 2, p = 0.73).

Despite the use of various extraction and chromatography methods, none of the isolated fractions from these protocols led to sexual attraction as strong as the whole crude extracts (Supporting Information, Table S1), possibly indicating that some active compounds are lost in the separation process. Furthermore, none of the crude extracts, despite the use of different solvents (water, MeOH, CH_2Cl_2, and hexanes) and doses, were comparable to the flower in attracting *L excelsa*.[10] Additionally, when aliquots of all four extracts were combined on the same pin, no significant enhancement of attraction was achieved (Supporting Information, Table S1). These findings are in contrast to our earlier studies of Australian hammer and spider orchids,[2,23] where using similar methodology we have successfully isolated semiochemicals that induce strong sexual behavior, including frequent attempted copulation at rates similar to that observed with the flowers. For *C. ovata*, while it is clear that we have successfully isolated long-range pollinator attractants, further work is required to elucidate the missing piece of the puzzle: what triggers pseudocopulation in *L. excelsa*.

To rule out the possibility that (*S*)-**2** and (*S*)-**3** were simply artifacts of using MeOH or EtOH as solvents, it was confirmed that extracts prepared using only CH_2Cl_2, without any exposure to alcohols, still contained similar levels of **2** and **3**. Some discrepancies were noted in the spectroscopic data reported for **1**, **2**, and **3** prepared by organic and chemoenzymatic synthesis compared with our data.[41,42] For example, Laxmi and Iyengar reported the ^1H NMR spectra for compounds **1** and **2**, where in **1** the H-2 proton was reported as δ_H 4.10[41] compared with δ_H 4.23 in this study (both in $CDCl_3$), while the corresponding signal in **2** was in agreement (δ_H 4.24 vs 4.25).[41] Bellur et al. later reported ^1H and ^{13}C NMR spectra for **1**−**3**, although neither the NMR nor the MS data are in agreement with this study.[42] For example, in **1**, C-2 was reported at δ_C 75.0 (vs δ_C 76.9) and the protons on the α-carbon to the carbonyl were reported at δ_H 2.58−2.60 (vs δ_H 2.49). For compound **3**, the carbonyl carbon was reported at δ_C 166.7 (vs δ_C 171.3). All EIMS spectra were fundamentally different from ours, suggesting different compounds. In this study, both enantiomers of **1** were prepared from enantiopure tetrahydrofuran-2-carboxylic acid, where the absolute configuration has been assigned.[43] NMR data of the isolated natural products, purchased *rac*-**1**, and the synthetically prepared products are identical and in full agreement with the most recent studies.[44,45]

Despite their structural simplicity, there are only a few examples of oxygenated tetrahydrofuran derivatives as floral volatiles or pheromone components. One example is pityol, which was originally identified from bark beetles[46] and later found to be present in various other beetle species (for example Birgersson et al.[47] and Pierce et al.[48]). Additional examples of tetrahydrofuran derivatives are linalool oxides and lilac alcohols/aldehydes, which are known to attract moth[49] and fungus gnat pollinators.[50] Thus far, there are no examples of tetrahydrofuran compounds as orchid semiochemicals; hence our discovery adds another compound class to a growing list of semiochemicals used by orchids to achieve pollination by sexual deception.[2]

The attraction of male *L. excelsa* to the tetrahydrofuran derivatives **1**−**3** marks the first identification of semiochemicals in the genus *Cryptostylis* and the first identification of floral semiochemicals that attract an ichneumonid wasp. To date, pollinator attractant compounds have only been experimentally confirmed from four other genera of sexually deceptive orchid: alkenes, and acyclic hydroxy acids in *Ophrys*,[51−54] cyclo-

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hexanediones (chiloglottones) in *Chiloglottis*,[4] pyrazines in *Drakaea*,[22,55] and methylthiophenols, acetophenones, and monoterpenes in *Caladenia*.[18,24,56] The discovery of **1**−**3** as pollinator attractants in *Cryptostylis* highlights the diversity of chemical systems employed by sexually deceptive orchids.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were acquired on a Kruss Optronic P-8000 polarimeter. Electronic circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (using the collected fractions from the chiral-phase HPLC separation, i.e., in 4% 2-propanol/hexanes at ca. 0.5 mg/mL). NMR spectra were acquired on a Bruker Avance 500 or 600 MHz (with a 1.7 mm TXI microprobe) spectrometer with either $CDCl_3$ or methanol-d_4 as solvent. Chemical shifts were calibrated to resonances attributed to residual solvent signals. HR-MS (EI, 70 eV) were recorded on a Waters GCT Premier TOF-MS equipped with a BPX5 column [(5% phenyl polysilphenylene-siloxane), 30 m × 0.25 mm × 0.25 μm film thickness, SGE Australia], using helium as a carrier gas. EIMS (70 eV) were recorded on an Agilent 5973 mass detector connected to an Agilent 6890 GC also equipped with a BPX5 column (30 m × 0.25 mm × 0.25 μm) or an HP 5972 mass detector connected to an HP5890 GC equipped with a Restek Rt-GammaDex sa column (30 m × 0.25 mm × 0.25 μm) using helium as a carrier gas. The scan range was *m*/*z* 33−300. High-performance liquid chromatography (HPLC) was performed on an Agilent 1200 HPLC system, equipped with a photodiode array detector (PDA) and fraction collector. Solvents for extractions and purifications were of HPLC grade unless otherwise stated.

Plant Materials and Insects. *Cryptostylis ovata* flowers were sourced from populations in southwest Western Australia near Margaret River (33°58′02.21″ S, 115°00′58.37″ E), Boyanup (33°28′30.9″ S 115°45′26.2″ E), and Capel (33°35′29.69″ S, 115°32′31.77″ E) in November 2015 to January 2019. Flowers were kept on ice in cooler boxes (ca. 4 °C) during transportation to the laboratory, where they were extracted in either MeOH or CH_2Cl_2 for semipreparative gas chromatography or frozen within 24 h of collection for subsequent liquid chromatography separations. Additional small-scale extracts of three flowers were conducted individually in four solvents (water, MeOH, CH_2Cl_2, and hexanes) for preliminary studies comparing pollinator attraction between solvent extracts. Preparations were presented to *L. excelsa* wasps at two sites in suburban Perth: Mosman Park (32°01′02.3″ S 115°45′18.0″ E) and Kings Park and Botanic Garden (31°57′44.5″ S 115°50′18.5″ E), where wasps are known to occur in suitable numbers for experiments.[10,57] Bioassays were conducted between 6 A.M. and 10 A.M. to coincide with the period of highest wasp activity.[57] Voucher specimens of *C. ovata* are held at the Western Australian Herbarium (voucher number PERTH 06731481).

Extraction and Isolation. All bioassay-guided fractionation methods were based on the results from preliminary experiments (Supporting Information, Table S1), showing that *C. ovata* extracts in polar and semipolar solvents were more attractive to *L. excelsa* males than nonpolar extracts. Two independent methods were implemented in order to maximize the likelihood of discovering multiple semiochemicals. To target polar compounds in the aqueous floral extracts, reverse-phase SPE in combination with HPLC was employed. For semipolar compounds detected in the MeOH extract, semipreparative gas chromatography was used. Three fractionations (below A, B, and C) of *C. ovata* crude water extracts were conducted with a C_{18} solid-phase extraction column (Waters Sep-Pak Classic C18, WAT051910 [360 mg, 55−105 μm, SPE]) according to the following procedure: For each SPE column, 15 frozen flowers were defrosted in a 5 mL conical extraction vial, after which they were crushed with a glass rod. The resulting floral extract (ca. 1 mL) was separated from the floral debris with a pipet and transferred to a new vial. Each column was preconditioned with MeOH (5 mL) followed by water (10 mL). The aqueous floral extract was loaded onto the column, and fractions were eluted with a set of solvents of decreasing polarity (Figure 1).

For fractionations A and B, all fractions were field tested, while in C subsamples were field tested and the remains of the active fraction were retained for further purification and instrumental analysis. Each eluted fraction was concentrated to ca. 0.5 mL by a gentle stream of nitrogen at room temperature and stored at 4 °C for subsequent analysis or bioassays. For semipreparative HPLC and subsequent NMR analysis, fractionation C was scaled up to obtain a pooled sample from six columns in parallel.

Semipreparative Gas Chromatography. All semipreparative gas chromatography experiments were performed on an HP 5890 GC, equipped with a three-way glass splitter separating the gas flow post column into the FID and the collector. An Rtx-5 column, 30 m × 0.53 mm id × 5 μm film (Restek, USA), or a BP21 column, 30 m × 0.32 mm id × 0.25 μm film (SGE, USA), was used. Samples of 3 μL were injected in splitless mode (1 min), and helium was used as carrier gas. A manual fraction collector was used, with samples collected in glass capillaries (100 × 1.55 mm i.d., Hirschmann Laborgeräte, Eberstadt, Germany) positioned in an aluminum holder submerged in a dry ice/acetone bath. All fractions were eluted with CH_2Cl_2 or MeOH (as appropriate) and stored at −20 °C until field-tested or further analyzed.

In the initial fractionation of the crude MeOH extract (for bioassay methods, see below), a short GC method (1 min 50 °C, then programmed to 280 °C at a rate of 15 °C/min, and held for 3 min) was used with the Rtx-5 column (see above). A sample of 48 flowers was extracted in MeOH (5 mL) for 24 h, and the extract was concentrated to 0.5 mL under a gentle stream of nitrogen. Aliquots of this concentrated extract were injected (3 μL), and six fractions, each with 30 s overlap (i.e., two injections per complete set of fractions were performed, allowing overlapping fractions to be collected per pair of runs), were collected to ensure that no bioactive compounds would be lost (Figure 1). The fractions were subsequently eluted with MeOH (20 μL). In total, eight injections (24 μL) were conducted for each set of fractions for field bioassays (i.e., in total 16 injections). The activity within the first fractionation series was confined to the fraction eluting at 6.5−9 min. Therefore, this fraction was subfractionated to create a further eight 0.6 min fractions. Field tests revealed that the fraction at 8.1−8.7 min retained activity. This fraction contained two distinct peaks, which could not be separated on this column, even with a longer method. However, the two peaks could be separated using the more polar BP21 column (5 min 40 °C, then programmed to 200 °C at a rate of 5 °C/min, then to 230 °C at a rate of 15 °C/min and held for 1 min). Field bioassays confirmed the active compound to be present in the fraction at 33.8−34.2 min, which contained the main peak from the nonpolar column. The minor peak from the nonpolar column was not active in field bioassays and was discarded.

Semipreparative HPLC Purification. The 5% MeOH SPE fraction (C, Figure 1, 36 mL combined) was concentrated to ca. 2 mL under reduced pressure and purified further by semipreparative HPLC. Separation was achieved using a 250 × 10 mm i.d., 5 μm, Apollo C_{18} reversed-phase column (Grace-Davison Discovery Sciences, Melbourne, VIC, Australia) with a 33 mm × 7 mm guard column of the same material. The column was eluted at 4 mL/min with 5% (v/v) MeOH/water, increasing to 40% (v/v) MeOH/water over 30 min and then to 100% MeOH at 35 min and held for 5 min. Injection volumes of 500 μL were used (× 4), and UV absorbance was monitored at wavelengths of 220, 254, and 280 nm. Fractions were collected every minute for 40 min, and these were monitored by GC-MS for the main active compound isolated by semipreparative GC. The active compound eluted in the fractions collected between 16 and 18 min retention time, which were combined and evaporated to dryness under reduced pressure. This purified sample was sufficiently pure for NMR studies (Supporting Information, Figures S1−S6).

Enantiomer Separation and Determination of Absolute Configuration. As the preparation of **1** from tetrahydrofuran-2-carboxylic acid by Arndt-Eistert homologation (see below) unavoid-

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ably resulted in some epimerization, chiral-phase HPLC was used to obtain (*R*)-**1** and (*S*)-**1** in >99% ee for field bioassays. Separation of the two enantiomers of **1** was achieved using semipreparative HPLC with an Astec Cellulose DMP chiral-phase HPLC column (250 mm × 10 mm × 5 μm, Supelco, Bellefonte, PA, USA). An isocratic solvent mixture of 4% 2-propanol/hexanes at a flow rate of 2 mL/min with 200 μL injection volumes of 10 mg/mL **1** (in 1:1 2-propanol/hexanes) provided enantiopure samples of (*R*)-**1** (t_R = 25.5 min) and (*S*)-**1** (t_R = 29.2 min).

The absolute configuration of the natural products was confirmed by preparing (*R*)-**1** and (*S*)-**1** from tetrahydrofuran-2-carboxylic acid[58−60] of known configuration,[43] purchased from Enamine Ltd., Ukraine. The specific rotations of the *R*- and *S*-enantiomers of tetrahydrofuran-2-carboxylic acid, respectively were confirmed beforehand: $[\alpha]^{22}_D$ +16.0 and −15.6 ($CHCl_3$) respectively. As the optical rotation of (*R*)-**1** and (*S*)-**1** was weak, and we only had access to limited amounts of these compounds in pure form, electronic circular dichroism (ECD) spectra were recorded rather than optical rotation (Supporting Information, Figure S11).

Chemicals. Racemic **1** was purchased from Princeton Bio (NJ, USA), and the enantiomers were separated by chiral-phase HPLC (see above). The methyl and ethyl esters (*S*)-**2** and (*S*)-**3** were prepared from (*S*)-**1** on a small scale (ca. 3 mg) by Fischer esterification with MeOH and EtOH, respectively.[61] The chemical purity was confirmed to be >95% by GC-MS.

*(S)-2-(Tetrahydrofuran-2-yl)acetic acid ((S)-**1**):* ^{1}H NMR (600 MHz) δ 4.23 (m, 1H), 3.85 (m, 1H), 3.73 (m, 1H), 2.49 (m, 2H), 2.10 (m, 1H), 1.93 (m, 2H), 1.58 (m, 1H); ^{13}C NMR (150 MHz) δ 175.1, 76.9, 68.8, 41.3, 32.2, 26.4; HREIMS found 130.0627 ($C_6H_{10}O_3$ calcd 130.0630).

*Methyl (S)-2-(tetrahydrofuran-2-yl)acetate ((S)-**2**):* ^{1}H NMR (500 MHz) δ 4.24 (m, 1H), 3.87 (m, 1H), 3.75 (m, 1H), 3.69 (s, 3H), 2.59 (m, 1H), 2.48 (m, 1H), 2.08 (m, 1H), 1.90 (m, 2H), 1.55 (m, 1H); ^{13}C NMR (125 MHz) δ 171.8, 75.3, 68.0, 51.7, 40.5, 31.3, 25.6; HREIMS found 144.0788 ($C_6H_{10}O_3$ calcd 144.0786).

*Ethyl (S)-2-(tetrahydrofuran-2-yl)acetate ((S)-**3**):* ^{1}H NMR (500 MHz) δ 4.24 (m, 1H), 4.15 (q, *J* = 7.1 Hz, 2H), 3.87 (m, 1H), 3.74 (m, 1H), 2.58 (m, 1H), 2.45 (m, 1H), 2.08 (m, 1H), 1.90 (m, 2H), 1.55 (m, 1H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz) δ 171.3, 75.3, 68.0, 60.5, 40.7, 31.2, 25.6, 14.2; HREIMS found 158.0949 ($C_6H_{10}O_3$ calcd 158.0943).

Field Bioassays. To determine whether long-distance pollinator attraction in *C. ovata* is chemically mediated, experiments were conducted with picked flowers hidden from the view of the pollinator. The flowers were concealed by a nonporous black screen, which had a small opening at the top to allow floral volatiles to disperse. The total number of wasp approaches to within 5 cm for each of three treatments (screen alone, flower alone, and flower concealed inside screen) was recorded. Treatments were presented individually in random order for trials of 3 min duration until a total of 15 trials had been completed per treatment. Owing to the data being non-normally distributed (Shapiro−Wilk normality test, $p < 0.001$), the non-parametric Mann−Whitney U-test was conducted to test for differences in responses between treatments in R v3.4.0 (R Core Team, 2017).[62]

The field bioassays using fractions or synthetic compounds broadly followed the experimental "wasp baiting" bioassay methods of Bohman et al.,[63] with the exception that the standard 4 mm diameter black-colored pin head was replaced by a larger 6 × 10 mm red-colored map pin to increase similarity with the color and dimension of the *C. ovata* flower and the female wasp. Each baiting trial was conducted at least 10 m from the previous baiting location to renew the pollinator response.[1] For GC fractions, the solvent (10 μL) was allowed to evaporate on the map pin before fractions were tested in trials of 2 min duration. Experiments tested multiple fractions from SPE or GC and synthetic (*R*)-**1**, (*S*)-**1**, (*S*)-**2**, and (*S*)-**3**, with each experiment consisting of a series of trials in which a single fraction or a synthetic compound was presented for 2 min, with the test fractions or synthetic compounds presented in random order within each experiment, with the exception of the enantiomeric comparison experiment. In this experiment, where the two enantiomers of **1** were tested, the aim was to test whether the *R*-enantiomer was comparable with the naturally occurring *S*-enantiomer. Therefore, (*R*)-**1** was presented for 2 min, before being replaced with (*S*)-**1** as the positive control.

In a preliminary experiment (Supporting Information, Table S1), flowers (n = 3 for each solvent) were extracted in four separate solvents: water, MeOH, CH_2Cl_2, and hexanes. Each set of flowers was extracted in 2 mL of solvent for 24 h before the extracts were concentrated to ca. 100 μL under a gentle stream of nitrogen at room temperature. For each solvent, 10 μL of each extract was suspended on a pin. In addition to testing the individual solvent extracts, the combination of all four solvents on a single pin was tested (3 μL of each solvent). In total eight trials were conducted over 2 days.

In the experiment evaluating SPE fractions (Figure 1), 10 μL of each fraction (500 μL) from 15 flowers was suspended on a pin (representing ca. a 1/50 flower extract equivalent per pin). In the experiment testing GC fractions, 10 μL of each fraction (20 μL eluted, from 25 μL injected of 500 μL extract) from 48 flowers was suspended on a pin (representing ca. one flower extract equivalent per pin).

In experiments testing synthetic compounds, doses of 2−50 μg were used (see the results for individual experiments). These doses were based on preliminary dose−response experiments (Supporting Information, Table S1), where doses from 0.4 ng to 100 μg were tested, confirming that doses from 20 ng to 100 μg elicited close approaches to the pins.

Throughout the study, trials where no responses were observed were not included in analyses. Across all experiments, neither lands on nor attempted copulation with the map pin was observed. Therefore, the number of wasp approaches to within 5 cm of the map pin was recorded as our response variable. The outcome of each trial was recorded by the same researcher. The number of wasps attracted to each treatment was compared using Mann−Whitney *U*-tests (two treatments) and the Kruskal−Wallis rank sum test (three treatments) in Rv3.4.0, as the data were non-normally distributed (Shapiro−Wilk normality test, $p < 0.01$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00772.

NMR spectra of **1**−**3**, ECD spectrum of **1**, GC-MS traces of floral extracts and enantiomeric separation of (*R*)-**1** and (*S*)-**1**, and results of additional field bioassays (PDF)

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Notes

The authors declare no competing financial interest.

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