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Ecological and genetic evidence for cryptic ecotypes in a rare sexually deceptive orchid, *Drakaea elastica*

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Species with specialized ecological interactions present significant conservation challenges. In plants that attract pollinators with pollinator-specific chemical signals, geographical variation in pollinator species may indicate the presence of cryptic plant taxa. We investigated this phenomenon in the rare sexually deceptive orchid *Drakaea elastica* using a molecular phylogenetic analysis to resolve pollinator species boundaries, pollinator choice experiments and a population genetic study of the orchid. Pollinator choice experiments demonstrated the existence of two ecotypes within *D. elastica*, each attracting their own related but phylogenetically distinct pollinator species. Despite the presence of ecotypes, population genetic differentiation was low across populations at six microsatellite loci (F_{ST} = 0.026). However, Bayesian STRUCTURE analysis revealed two genetic clusters, broadly congruent with the ecotype distributions. These ecotypes may represent adaptation to regional variation in pollinator availability and perhaps the early stages of speciation, with pronounced morphological and genetic differences yet to evolve. Resolution of the taxonomic status of the *D. elastica* ecotypes is required as this has implications for conservation efforts and allocation of management funding. Furthermore, any reintroduction programmes must incorporate knowledge of ecotype distribution and pollinator availability to ensure reproductive success in restored populations. *Botanical Journal of the Linnean Society*, 2015, **177**, 124–140.

ADDITIONAL KEYWORDS: clonality – conservation – microsatellites – Orchidaceae – pollination – thynnine wasp.

INTRODUCTION

Plants are predicted to show adaptations to geographical variation in the most effective pollinator, particularly those with specialized pollination systems (van der Niet, Peakall & Johnson, 2014). This phenomenon has been most frequently demonstrated in plant species with ecotypic variation in colour or morphology that reflect regional differences in the preferences and morphology of the local pollinator fauna (Robertson & Wyatt, 1990; Johnson, 1997; Anderson & Johnson, 2008; Pauw, Stofberg & Waterman, 2009; Anderson *et al.*, 2010; Newman, Anderson & Johnson, 2012; Boberg *et al.*, 2014; Sun, Gross & Schiestl, 2014). However, in plants that attract their pollinators primarily via chemical cues, morphological evidence for local adaptation to pollinators may not exist. In rare species, the presence of cryptic variation presents a unique set of conservation challenges, as the newly recognized entities will

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require a re-assessment of their conservation status and potentially different management strategies (Schönrogge *et al*., 2002; Bickford *et al*., 2007).

Orchidaceae are one of the most diverse plant families, estimated to contain > 26 000 species worldwide (Joppa, Roberts & Pimm, 2011). Of the plant families studied to date, orchids have a high frequency of specialized pollination strategies involving chemical-mediated attraction (Gaskett, 2011; Ramírez *et al*., 2011; van der Niet, Hansen & Johnson, 2011), suggesting that this family may contain numerous cases of cryptic species, particularly in recently diverged groups, with many potentially of conservation concern. The threatening processes facing orchids are primarily anthropogenic, most often resulting directly from habitat destruction, modification and fragmentation, and in some regions over-collection (Swarts & Dixon, 2009). However, unique features of orchid biology may also predispose them to rarity. In particular, the tendency to exist in naturally patchy populations (Tremblay *et al*., 2005; Phillips *et al*., 2011b) and form specialized ecological interactions (Schiestl & Schlüter, 2009; McCormick & Jacquemyn, 2014) make orchids particularly susceptible to disturbance and can pose conservation challenges (Swarts & Dixon, 2009; Menz *et al*., 2011). For example, lower levels of reproduction and subsequent local extinction in a community of oil-producing orchid species has been linked to a decline in the abundance of their single pollinator species, the oil-collecting bee *Rediviva peringueyi* (Pauw & Bond, 2011; Pauw & Hawkins, 2011). Therefore, consideration of the pollinator in conservation programmes for rare plants with specialized pollination interactions is a key issue.

Pollination by sexual deception is one of the most specialized pollination systems in Orchidaceae. Sexual deception involves the attraction of male insects (usually Hymenoptera) to flowers by the mimicry of the species-specific sex pheromones released by female insects (Schiestl *et al*., 1999, 2003; Ayasse, Stökl & Francke, 2011; Bohman *et al*., 2014). Pollination is typically achieved when male insects are sexually attracted to and attempt to copulate with the flower (pseudocopulation), whereby they come into contact with the pollinium and stigma (Stoutamire, 1975; Paulus & Gack, 1990; Peakall, 1990). Australia is recognized as a centre of diversity of sexually deceptive orchids, involving > 150 species from at least 11 genera (Phillips *et al*., 2009, 2014b; Gaskett, 2011). However, sexual deception is also known in orchids from other continents including Europe, Central and South America and southern Africa (Kullenberg, 1961; Singer, 2002; Blanco & Barboza, 2005; Gaskett, 2011).

This study focuses on *Drakaea elastica* Lindl., a rare and threatened sexually deceptive terrestrial orchid, reported to be pollinated solely by males of the thynnine wasp *Zaspilothynnus gilesi* (Hymenoptera, Thynnidae) (Menz *et al*., 2013; Phillips *et al*., 2014a). However, *Z. gilesi* is a variable taxon reported to contain three forms of uncertain taxonomic rank, which are distinguished by minor differences in morphology and coloration (Turner, 1910). While *D. elastica* is morphologically similar across its range (Hopper & Brown, 2007), during investigations of the mate-searching behaviour of male *Z. gilesi* (Menz *et al*., 2013), preliminary evidence indicated that *D. elastica* may consist of a northern and southern ecotype that each exclusively attracts a different morphological form of *Z. gilesi*. From these initial observations, it appeared that northern plants attract the form of *Z. gilesi* with a red femur and large spots on the abdomen (hereafter red-legged form), and southern plants attract the form of *Z. gilesi* with a black femur and small spots on the abdomen (hereafter black-legged form).

Consistent differences in pollinator attraction between *D. elastica* flowers may signify the presence of ecotypes, probably underpinned by differences in floral odour (Peakall *et al*., 2010; Bohman *et al*., 2012a, b, 2014). Differences in pollinator attraction are likely to generate reproductive isolation, allowing the accumulation of genetic divergence between ecotypes (Peakall *et al*., 2010; Xu *et al*., 2011; Peakall & Whitehead, 2014; Whitehead & Peakall, 2014). As such, combining pollinator choice trials with studies of population genetic structure represents a powerful approach to detect the presence of cryptic ecotypes (Peakall & Whitehead, 2014).

Given the possibility in *D. elastica* of cryptic ecotypes attracting pollinators of uncertain taxonomic status, our study had the following objectives. First, we conducted phylogenetic analysis of the pollinators of *D. elastica* to test if these morphological forms represent different species. Secondly, we investigated population-level and regional differences in pollinator attraction by *D. elastica* to assess the possibility of ecotypic variation in pollinator attraction. Thirdly, using microsatellite markers, we investigated the pattern and extent of population genetic variation within *D. elastica* to assess whether there is genetic evidence for cryptic ecotypes. Finally, we evaluated the consequences of the ecological and genetic findings for the conservation of *D. elastica* and cryptic species more broadly.

METHODS

Study species

Drakaea elastica is endemic to the Swan Coastal Plain in south-western Western Australia (Brown,

Thomson-Dans & Marchant, 1998) and is listed as critically endangered under the Australian Federal Environmental Protection and Biodiversity Conservation Act (EPBC). The species persists at approximately 42 locations (1–1500 plants), comprising seven major populations (Department of Environment and Conservation, 2008). Approximately 60% of the known locations contain < 50 plants (Department of Environment and Conservation, 2008). *Drakaea elastica* produces only a single leaf, but often forms small colonies that may include clonally produced daughter plants, as seen in other species of *Drakaea* Lindl. (Peakall, 1990). However, as population counts treat each of these leaves as individual genets, this may lead to overestimation of true effective population sizes and consequently an underestimation of the degree of threat to the species (Hogbin, Peakall & Sydes, 2000).

The main populations are located in two regions, *c.* 110 km apart, with four near the town of Mandurah [Carrabungup (Ca), Lakes Road (LR), Paganoni (Pa) and Serpentine River (SR)]; and three near the town of Capel [Capel (C), Lindburg Road (Li) and south Capel (SC)] (Fig. 1). Although most major *D. elastica* populations are situated in conservation reserves, continued land clearing still threatens the species by reducing and fragmenting suitable habitat (Brown *et al.*, 1998; Department of Environment and Conservation, 2008). Across its range *D. elastica* forms scattered populations in areas of open, grey sandy soil and fine leaf litter in mixed stands of *Kunzea glabrescens* Toelken and banksia woodland

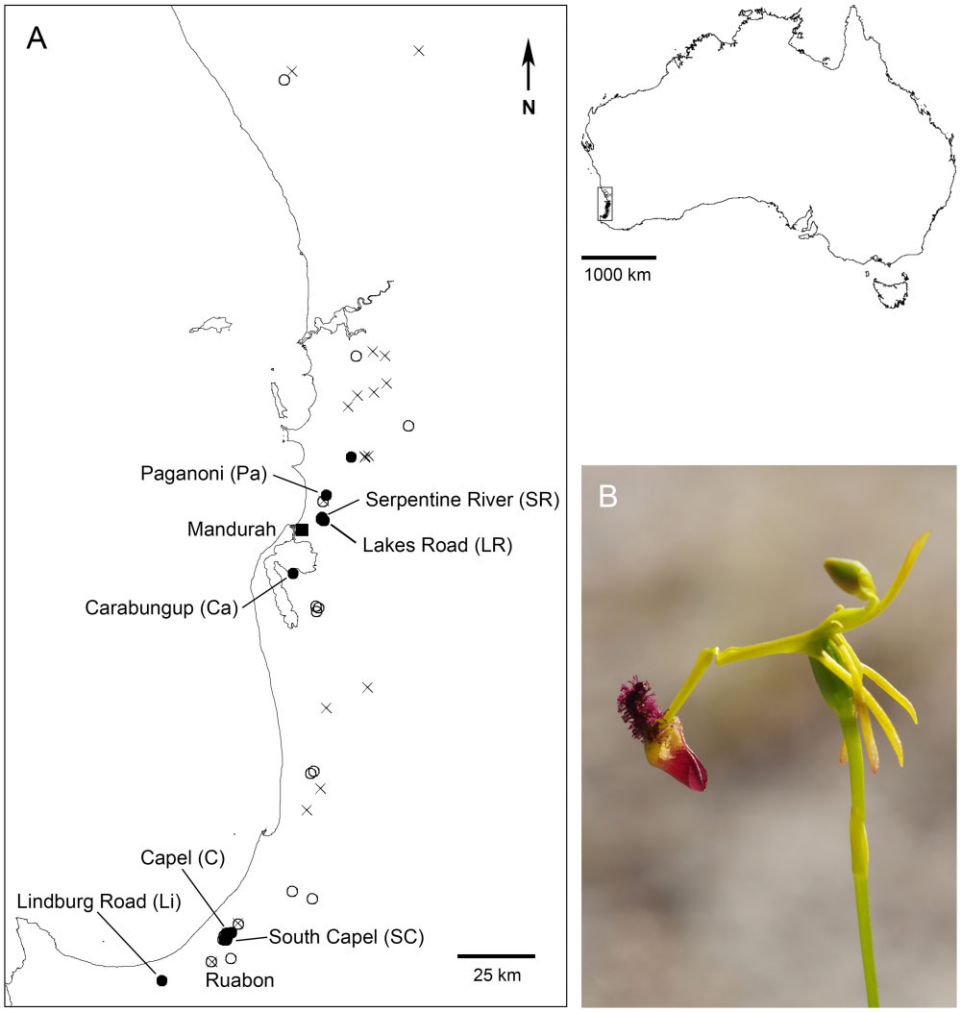

Figure 1. A, location of all known populations of *Drakaea elastica* and place names referred to in the text. Solid circles indicate populations with > 50 plants (including juveniles); open circles indicate populations with < 50 plants (including juveniles), crosses indicate extinct populations; and solid squares indicate place names referred to in the text. B, flower of *D. elastica* showing the hinged, insectiform labellum, typical of *Drakaea*. Image: R. Peakall.

(Brown *et al*., 1998; Department of Environment and Conservation, 2008; Menz, 2013), leading to a naturally patchy distribution (Phillips *et al*., 2011a).

All *Drakaea* spp. exploit the courtship behaviour of thynnine wasps to achieve pollination (Phillips *et al*., 2014a). The wingless females spend most of their adult lives underground where they search for beetle larvae on which to lay their eggs (Ridsdill Smith, 1970). In contrast, the winged adult males regularly make patrolling flights to locate the 'calling' wingless females that emerge periodically from below ground (Alcock, 1981). Calling females typically perch on low vegetation where they release sex pheromones and are quickly located by males (often within seconds; for a video, see Bohman *et al*., 2014). The successful male grasps the female in flight before carrying her, *in copula*, to a food source to feed and mate (Ridsdill Smith, 1970; Alcock, 1981; Peakall, 1990).

The solitary flower of *D. elastica* is highly reduced, consisting primarily of the hinged semiochemical-releasing labellum (Phillips *et al*., 2013; Bohman *et al*., 2014) and the column (combined stigma and anther of orchids). As the male wasp pollinator attempts to fly off with the insectiform labellum (pseudo female), the pollinator is tipped upside down by its own momentum and brought into contact with the column where pollen removal/pollination occurs. Although *D. elastica* requires a thynnine wasp as a pollen vector, it is self-compatible (Phillips, 2010). Pollinator-mediated self-pollination may occur occasionally, as observed in other species of sexually deceptive orchids pollinated by thynnine wasps (Peakall & Beattie, 1996).

Identification and phylogenetic analysis of pollinators

We used phylogenetic analysis of the mitochondrial CO1 region to investigate if the two forms of *Z. gilesi* responding to *D. elastica* represent different species. The CO1 region has previously been shown to be informative for resolving species-level variation in thynnine wasps (Mant *et al*., 2002; Griffiths *et al*., 2011; Phillips *et al*., 2013). In addition to specimens collected from *D. elastica*, we also included opportunistic collections of *Z. gilesi* to assist in resolving the number of taxa within *Z. gilesi s.l.* Other members of the genus *Zaspilothynnus* were also included to gauge the level of genetic variation within versus among species at the CO1 locus (Table S1).

Laboratory procedures followed Griffiths *et al*. (2011). A multiple sequence alignment was constructed in Geneious Pro version 7.1.4 (Drummond *et al*., 2011). For phylogenetic analyses, *Zaspilothynnus trilobatus* was used as the outgroup based on previous analyses of the CO1 region indicating this taxon is divergent from the main clade in the genus (Phillips *et al*., 2013). A phylogenetic tree was produced with a maximum-likelihood (ML) analysis using the Geneious plugin of PhyML version 3.0 (Guindon & Gascuel, 2003; Guindon *et al*., 2010). Support for nodes was assessed for ML trees using 100 pseudoreplicates of bootstrapping. DNA sequence data were also analysed under a Bayesian framework using the MrBayes version 2.1.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) plugin in Geneious Pro. Bayesian phylogeny estimation used default priors and was run for 1 100 000 generations with a sampling frequency of 200, which was sufficient to lead to strong convergence and split frequencies of < 0.03. A GTR+G+I model was used for all analyses as all other models are nested inside this model. Phylogenetic trees were displayed using FigTree version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/). The percentage of maximum sequence divergence within and among taxa was calculated in Geneious. For comparison, the minimum and maximum sequence divergence was calculated between the forms of *Z. gilesi*.

Pollinator behavioural experiments

Pollinator baiting experiments were designed to test for differences in pollinator attraction between orchid individuals. Baiting for pollinators involves the artificial presentation of picked orchid flowers, which readily attract male thynnine wasps, if present (Stoutamire, 1983; Peakall, 1990). Picked orchid flowers were stored in a portable refrigerator held at *c*. 4 °C to maintain freshness. Flowers stored in this way are known to maintain attractiveness for at least 3 weeks (Phillips, 2010), but for this study, individual bait flowers were typically used for no longer than 2 weeks. For all baiting experiments, flowers were kept in a sealed container between trials. Subsequent baiting locations were separated by at least 20 m to avoid the decrease in wasp responses over time documented by Peakall (1990) and Whitehead & Peakall (2013). Experiments were restricted to warm (> 18 °C) and sunny days between 09:00 and 16:00 h when thynnine wasps are most active (Peakall, 1990). All flowers used in the baiting trials were collected from wild populations, except for population LR, for which we used flowers from a population maintained in the Botanic Gardens and Parks Authority (BGPA) glasshouse (established from wild plants from the LR population in 2008) in Perth, Western Australia. All *Z. gilesi* wasps responding to orchids in the behavioural experiments were assigned to one of the morphological forms described by Turner (1910).

Initially, baiting experiments were undertaken at both Mandurah and Capel in 2009 and 2010. In a

survey designed to detect the presence of pollinators in the Mandurah area in 2009, the red-legged form of *Z. gilesi* was detected at only three of the 30 sites surveyed (6 × 2 min per site), amounting to only four wasp responses (our unpubl. data). In 2010, a return to the Mandurah sites where *Z. gilesi* had been previously detected also resulted in few responses ($N = 4$). Consequently, due to the rarity of the red-legged form of *Z. gilesi*, experiments in subsequent years were only conducted using populations of the black-legged form at Capel.

To test for differences in the pollinator attracted to *D. elastica* flowers sourced from multiple populations, we conducted baiting experiments in a habitat remnant at Ruabon, near Capel, in 2010 and 2011 (Fig. 1), where the black-legged form of *Z. gilesi* occurs reliably. Furthermore, *D. elastica* does not grow at the site, preventing interference with wasp responses to artificially presented flowers (Menz *et al.*, 2013). Due to the conservation significance of *D. elastica*, obtaining multiple orchid samples from some populations was not possible. Nonetheless, at least six individual flowers were tested from one population from each region (LR and C), and single flowers from three other Mandurah populations (Pa, Ca and SR). Orchid samples were selected to evaluate potential regional variation in the pollinator attracted. Baiting was undertaken following a similar method to the sequential experimental design as described by Bower (1996). In 2010, flowers from LR and Capel were compared in a series of 27 trials (6 min) over five field days. Initially, the 'foreign' LR orchid was presented for a period of 3 min, followed by the presentation of the 'local' Capel orchid for a further 3 min as a control for pollinator availability, as these flowers were known to attract the black-legged form. In 2011, the 'foreign' Mandurah orchids (LR, Ca, Pa and SR) were presented for a period of 5 min. Subsequently, the 'local' Capel orchids were presented alongside the 'foreign' orchids for a further 5 min. Flowers from three 'foreign' populations were presented simultaneously in each of the 12 trials undertaken in 2011. All flowers were presented 50 cm apart and perpendicular to any prevailing wind. The number of male wasps attempting to copulate with the flowers was recorded over the whole period for each of the choice trials in 2010 and 2011.

In 2012, baiting at Ruabon was undertaken in a sequential fashion as described above, using 3-min trials, with flowers from Mandurah (SR) and Capel. Here, we compared the hierarchy of behaviours necessary for pollination for the black-legged form of *Z. gilesi* (Peakall, 1990) with single flowers from the two regions. Wasps were recorded as approaching the flower (to within 5 cm), landing on the flower or attempting copulation with the flower.

Population genetic sample collection and study sites

Leaf samples for genetic analysis were collected from the seven major extant populations (including the BGPA glasshouse population) spanning the core of the range of *D. elastica* (Fig. 1). Exact location details have been withheld here as a requirement of permits to study threatened flora. Only populations of > 50 plants were included in the sampling, with populations defined as patches of plants that were growing within 500 m of one another. Samples were collected across the full spatial extent of each population. To avoid sampling potential clones, a single sample consisting of a section of leaf material was taken from each colony, with samples taken from colonies at least 20 cm apart. Samples were kept in the field at 4 °C in a portable refrigerator and stored at –80 °C on returning to the laboratory.

Microsatellite cross-transferability validation

DNA extraction and amplification of microsatellite loci were performed as per Anthony *et al*. (2010). A set of 15 microsatellite markers originally isolated from *Drakaea glyptodon* Fitzg. were tested for cross-transferability to *D. elastica* (Anthony *et al*., 2010). Only those markers that amplified, were repeatable and easy to score were used. As there are potential issues with cross-transferability of markers between species (Peakall *et al*., 1998), the following steps were taken to validate the successful transfer of markers from *D. glyptodon* to *D. elastica*. First, a search was undertaken for outlier loci with a high likelihood of null alleles using MICRO-CHECKER version 2.2.3 (van Oosterhout *et al*., 2004). Secondly, GENEPOP version 4.1.1 (Raymond & Rousset, 1995; Rousset, 2008) was used to perform exact tests (Guo & Thompson, 1992) for deviation from Hardy–Weinberg equilibrium (HWE), at each locus across all sampled populations, and to test for linkage disequilibrium between loci (Slatkin, 1994). A Bonferroni correction was applied to significance levels for multiple tests (Rice, 1989).

Population genetic variability

GenAlEx 6.501 (Peakall & Smouse, 2006, 2012) was used for all analyses unless otherwise stated. The number of alleles (N_a), effective number of alleles (N_e), fixation index (F), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated for each locus and population. The program STRUCTURE version 2.3.3 was used to assign individuals to genetic clusters based on multilocus genotype data (Pritchard, Stephens & Donnelly, 2000). This

approach provides an assessment of the possibility of co-occurring, genetically distinct ecotypes, independently of the source population of a sample. STRUCTURE implements the Bayesian Markov chain Monte Carlo (MCMC) method to assign individuals to k clusters. We used the admixture model, with allele frequencies correlated among populations as recommended by Falush, Stephens & Pritchard (2003) for populations that may exhibit subtle structure. Burn-in was set to 10 000 iterations, followed by 100 000 MCMC iterations and replicated ten times for each value of k, from one to eight. The program STRUCTURE HARVESTER (Earl & von Holdt, 2012) was used to determine the optimal value of k, based on the level at which the mean logarithm of the probability of the data [ln P(k)] reached a plateau, and the second-order rate of change of the likelihood function (Δk) as per Evanno, Regnaut & Goudet (2005).

The analysis of molecular variance (AMOVA) framework was used to partition genetic variation within and among populations and regions, estimate overall and pairwise population genetic differentiation (F_{ST}), estimate differentiation among regions (F_{RT}) and estimate differentiation among populations within regions (F_{SR}) following Wright (1965), Excoffier, Smouse & Quattro (1992) and Peakall, Smouse & Huff (1995). Tests for departure from the null hypothesis of no genetic differentiation were performed by random permutation (1000 permutations). Regions were initially defined geographically to coincide with the remaining strongholds of *D. elastica* situated in the Mandurah and Capel areas (Fig. 1). Subsequently, regions were redefined to reflect the STRUCTURE results.

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

Isolation by distance across the geographical range of *D. elastica* was tested for using a Mantel test (Mantel, 1967), performed in GenAlEx, with departure from the null hypothesis of no significant relationship between genetic and geographical distances tested by random permutation (10 000 permutations) (Smouse, Long & Sokal, 1986; Smouse & Long, 1992).

Clonality

To investigate the presence of clonality in *D. elastica*, 15 colonies were randomly selected from wild populations. In addition, 14 colonies from the BGPA glasshouse collection were also sampled. Colonies consisted of two to ten leaves (median 3.0) in the wild populations and two to eight leaves (median 2.5) in the glasshouse population. All leaves from each cluster were sampled and genotyped.

RESULTS

Phylogenetic analysis of *Z. gilesi*

Phylogenetic analysis provided strong support that the red-legged and black-legged forms of *Z. gilesi* represent two species (Fig. 2). Within the red-legged and black-legged forms of *Z. gilesi*, maximum genetic divergence between individuals was 2.3 and 0.7%, respectively. The level of genetic divergence between individuals of the two forms of *Z. gilesi* ranged from 7.3 to 8.1%, which is higher than recorded within other *Zaspilothynnus* spp. Within *Zaspilothynnus* spp. sampled from more than one site, the maximum genetic divergence between individuals ranged from 0.6 to 5.8% (*Z. dilatatus* 0.6%; *Z. nigripes* 5.8%; *Z.* sp. A 1.5%; *Z.* sp. B 2.6%; *Z. seductor* 1.1%). The species with the broadest geographical range (*Z. nigripes*) showed the highest intraspecific genetic divergence and the species with the smallest geographical range (*Z. dilatatus* and *Z. gilesi* – black-legged) had the lowest intraspecific genetic divergence.

Pollinator behavioural experiments

During the course of the study, the full set of behavioural responses that characterize a genuine pollinator of *Drakaea* (*sensu* Peakall, 1990; Phillips *et al.*, 2014a) was observed for three pollinator–orchid combinations: black-legged *Z. gilesi* responding to Capel orchids, black-legged *Z. gilesi* responding to Carrabungup (Mandurah) orchids and red-legged *Z. gilesi* responding to orchids from other Mandurah sites. In each of these cases, wasps were observed alighting on the labellum, attempting to carry the labellum away and making contact with the stigma during attempted copulation. Wild wasps were also observed visiting *D. elastica* flowers at Mandurah (SR) and Capel.

The 23 sequential trials in the Mandurah region in 2010, using local (LR) and Capel flowers, resulted in only four responses from male *Z. gilesi* wasps, all of which were identified as the red-legged form. Of these, three responses were to *D. elastica* flowers from Mandurah (LR), and one was to the flowers from Capel. The sole wasp approaching the Capel flowers did not land. Conversely, all three responses to

Figure 2. A phylogenetic analysis of *Zaspilothynnus* based on a maximum-likelihood analysis of the mitochondrial CO1 region. Numbers above the line are bootstrap support from a maximum-likelihood analysis in PHYML, and numbers below the line are posterior probabilities from a Bayesian analysis in MRBAYES. Numbers in parentheses indicate the number of identical sequences.

Table 1. Behavioural responses of male *Zaspilothynnus gilesi* wasps to *Drakaea elastica* orchids from Mandurah (SR) and Capel (C)

	No. of responses	
Behaviour	Mandurah orchid (%)	Capel orchid (%)
Approach decoy (within 5 cm)	1 (100)	113 (100)
Alight on decoy (labellum)	1 (100)	83 (73.5)
Attempt to carry decoy (labellum) away	1 (100)	60 (72.3)

All experiments were conducted in Capel. Modified hierarchy of attraction from Peakall (1990).

Mandurah orchids resulted in attempted copulation with the flower. At the Ruabon site, the 27 sequential trials in 2010, comparing Mandurah (LR) and Capel orchids, resulted in a total of 52 responses, all to the local Capel *D. elastica*. All wasps responding were identified as the black-legged form of *Z. gilesi*. Subsequent quantification of pollinator behaviour in 2012 showed that 74% of the males attracted to the Capel orchids alighted on the flower and 72% of these males attempted to carry the decoy (labellum) away (Table 1).

In all sequential choice trials conducted at Ruabon, flowers from Mandurah were exposed prior to the introduction of local flowers. As seen in 2010, the experiments conducted in 2011 showed that the black-legged form of *Z. gilesi* from Ruabon approached [0.32 ± 0.11 wasps min^{-1} (mean ± SE), $N = 19$], and alighted on (84.2%, $N = 16$) the *D. elastica* from Capel. In contrast, *D. elastica* from the three Mandurah populations tested (Pa, SR and LR) elicited no response from the black-legged form of *Z. gilesi* (Fig. 3). Similarly, in pollinator baiting experiments at Ruabon in 2012, nearly all responses were to the Capel *D. elastica* flower (99%, $N = 113$), with only 0.9% ($N = 1$) of responses to the Mandurah (SR) *D. elastica* flower (Table 1). This single wasp responding to the Mandurah *D. elastica* attempted copulation with the flower, but this was probably a consequence of the presentation of orchids in close proximity during a period of high wasp activity, rather than a genuine pollinator response. Across all years of the study, 187 black-legged *Z. gilesi*

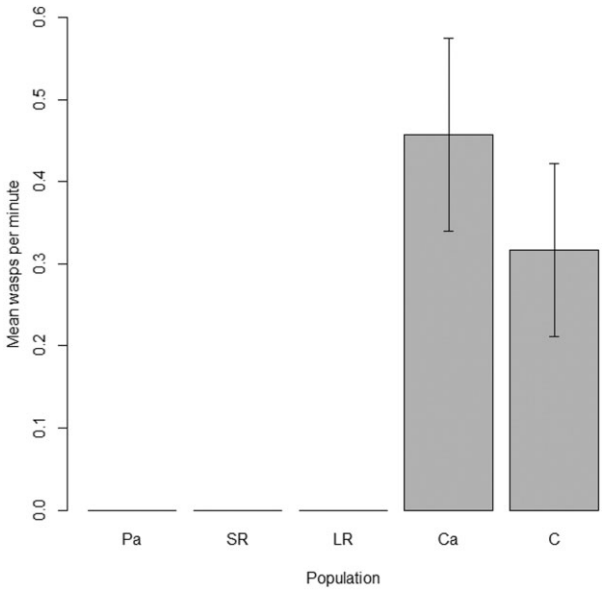

Figure 3. Mean responses per minute of male *Zaspilothynnus gilesi* wasps to artificially presented fresh *Drakaea elastica* flowers from five populations. Mean responses (± SE) per minute during sequential trials in which 'foreign' orchids from four locations were presented for the first 5 min, followed by addition of the 'local' Capel orchid as a control. Populations are ordered geographically from north to south: Paganoni (Pa, *N* trials = 12), Serpentine River (SR, *N* trials = 9), Lakes Road (LR, *N* trials = 10), Carrabungup (Ca, *N* trials = 7) and Capel (C, *N* trials = 12).

responded to Capel *D. elastica*, whereas only one black-legged *Z. gilesi* responded to orchids from Mandurah (Pa, SR or LR). The *Z. gilesi* at Ruabon (black-legged form) approached (0.46 ± 0.12 wasps min^{-1}, $N = 32$) and alighted on (87.5%, $N = 28$) the single flower tested from Carrabungup in comparable quantities to the Capel flower (Fig. 3).

Microsatellite cross-transferability validation

Of the 15 microsatellite loci isolated from *D. glyptodon* (Anthony *et al*., 2010), ten amplified with polymorphic alleles, one was monomorphic, one was unable to be scored and three did not amplify for all the individuals used in the trial. Eight of the remaining ten loci were selected for the study. Two of these loci (A116 and A108) showed evidence of null alleles at most populations, high fixation index (*F*) values (0.294 and 0.618, respectively) and evidence of linkage disequilibrium. Consequently, these two loci were excluded from all subsequent analyses. This resulted in six loci being retained for further analysis.

In *D. glyptodon*, these loci are known to exhibit genotypes typical of diploids (Anthony *et al*., 2010). However, more than two alleles were identified in some individuals. This was observed at each of the eight loci, but at different loci for different individuals (8.54%, $N = 492$ ramets sampled across populations). Triple banding (three alleles) appeared in at least one individual in six of the seven populations. These patterns of triple banding were repeatable, present in all individuals of a clonal collection and not due to accidental mixture of DNA or PCR cross-contamination. Triple bands may represent triploid individuals, or alternatively, this may be due to genetic mosaicism (Reusch & Boström, 2010). As such, samples displaying triple banding patterns were excluded from further analysis.

Population genetic variability in *D. elastica*

The final population genetic analyses were based on the 331 individual *D. elastica* samples that exhibited diploid banding patterns. At the locus level, the number of alleles (N_a) ranged from seven (C110) to 28 (B102) with a mean of 10.4 (Table 2). Expected heterozygosity (H_E) ranged from 0.543 (C110) to 0.876 (B102) (mean 0.780, Table 2). Following a Bonferroni correction, significant linkage disequilibrium ($P < 0.005$) was detected in only five out of 105 tests, with no consistent pairs of linked loci. Across loci and within populations, the mean number of alleles (N_a) ranged from five to 23 (Table 3). Mean levels of H_O for populations ranged from 0.673 to 0.827 and levels of H_E ranged from 0.741 to 0.828 (Table 3). Lower H_O than H_E was observed at all seven populations giving positive *F* values (Table 3).

The STRUCTURE analysis indicated the optimal number of clusters at $k = 2$, where ln $P(k)$ reached a plateau (Fig. S1). Additionally, Δk peaked at $k = 2$ (Fig. S2), and minimal additional information was gained by increasing k (Figs 4, S2, S3). Mean membership values (q-mean) for Cluster 1 ranged from 0.814 to 0.861 for the Mandurah populations, and from 0.164 to 0.272 for the Capel populations. Conversely, the q-mean for Cluster 2 ranged from 0.139 to 0.187 for the Mandurah populations and 0.728 to 0.836 for the Capel populations (Fig. 4). The Carrabungup population, although geographically closer to Mandurah, showed a pattern of admixture, with a q-mean of 0.549 and 0.451 for Clusters 1 and 2, respectively (Fig. 4).

Pairwise estimates of population genetic differentiation were low, but significant in all but two cases (Table 4; $F_{ST} = 0.026$, $F'_{ST} = 0.126$, $D_{est} = 0.106$). A principal coordinates analysis (PCoA) at the population level revealed north–south population groupings with one exception: the population at Carrabungup

Table 2. Average genetic variation of six polymorphic microsatellite loci for *Drakaea elastica*

Locus	N_a	Mean N_e	H_O	H_E	F
D3 (GU560054)	14	4.325	0.742* (2)	0.763	0.031
B109 (GU560051)	24	6.363	0.820* (1)	0.837	0.019
B102 (GU560048)	28	8.347	0.776* (3)	0.876	0.114
A114 (GU560046)	13	5.125	0.734* (2)	0.802	0.082
C110 (GU560053)	7	2.383	0.532* (2)	0.540	0.025
D104 (GU560057)	23	7.251	0.844* (3)	0.860	0.019
Mean	10.357	5.632	0.741	0.780	0.048

Presented are locus code and GenBank accession number, number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F).
*Significant ($P < 0.05$) departure from HWE. The number of populations is given in parentheses.

Table 3. Genetic variation from seven populations of *Drakaea elastica* for six polymorphic microsatellite loci

Population (CODE)†	N	N est.	N_a range	Mean N_e	H_O	H_E	F
Paganoni (Pa)	22	1476	5–14	5.307	0.717	0.741	0.028
Serpentine River (SR)	69	420	5–17	5.979	0.732* (1)	0.765	0.051
Lakes Road (LR)	43	64	6–15	5.643	0.760* (1)	0.771	0.009
Carrabungup (Ca)	22	819	5–12	4.979	0.736* (1)	0.778	0.061
Capel (C)	135	1284	7–23	6.013	0.753* (4)	0.811	0.071
South Capel (SC)	22	210	7–13	6.374	0.827* (4)	0.828	–0.006
Lindberg Road (Li)	17	119	10–17	5.174	0.673* (3)	0.769	0.120

Number of individuals sampled (N), estimated population size (number of individuals including juveniles and clones) (N est.), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F) are given. Populations are arranged from north to south.
*Significant ($P < 0.05$) departure from HWE. Number of loci given in parentheses.
†Exact locations are withheld for conservation reasons.

Table 4. Pairwise comparisons of F_{ST} (below diagonal) and D_{est} (above diagonal) between populations of *Drakaea elastica*; populations are in geographical order from north to south

	Pa	SR	LR	Ca	C	SC	Li
Pa		*0.064*	0.022	*0.051*	*0.125*	*0.169*	*0.236*
SR	*0.019*		*0.034*	*0.114*	*0.131*	*0.126*	*0.180*
LR	0.008	*0.011*		*0.070*	*0.113*	*0.114*	*0.225*
Ca	*0.016*	*0.029*	*0.021*		*0.050*	*0.092*	*0.137*
C	*0.030*	*0.031*	*0.028*	*0.010*		0.014	*0.109*
SC	*0.039*	*0.029*	*0.028*	*0.016*	0.003		*0.066*
Li	*0.078*	*0.061*	*0.075*	*0.039*	*0.036*	*0.024*	

Significant ($P < 0.05$) comparisons are italicized.

(Ca), which clustered with the southern (Capel) populations of *D. elastica* and not with those from the north (Mandurah), to which it is geographically close (Fig. S4). In light of the STRUCTURE, PCoA and the pollinator behavioural experiments, the Ca population was repositioned within the southern region for a subsequent second AMOVA. The resulting AMOVA attributed 2% of the variation among regions ($F_{RT} = 0.019$, $F'_{RT} = 0.1$, $P = 0.001$), 1% among populations within regions ($F_{RT} = 0.019$, $F'_{RT} = 0.1$, $P = 0.001$) and 97% within populations. (the first regional AMOVA with populations grouped geographically attributed 1% of the variation among regions, 2% among populations within regions and 97% within populations). The Mantel test revealed no evidence of isolation by distance among populations

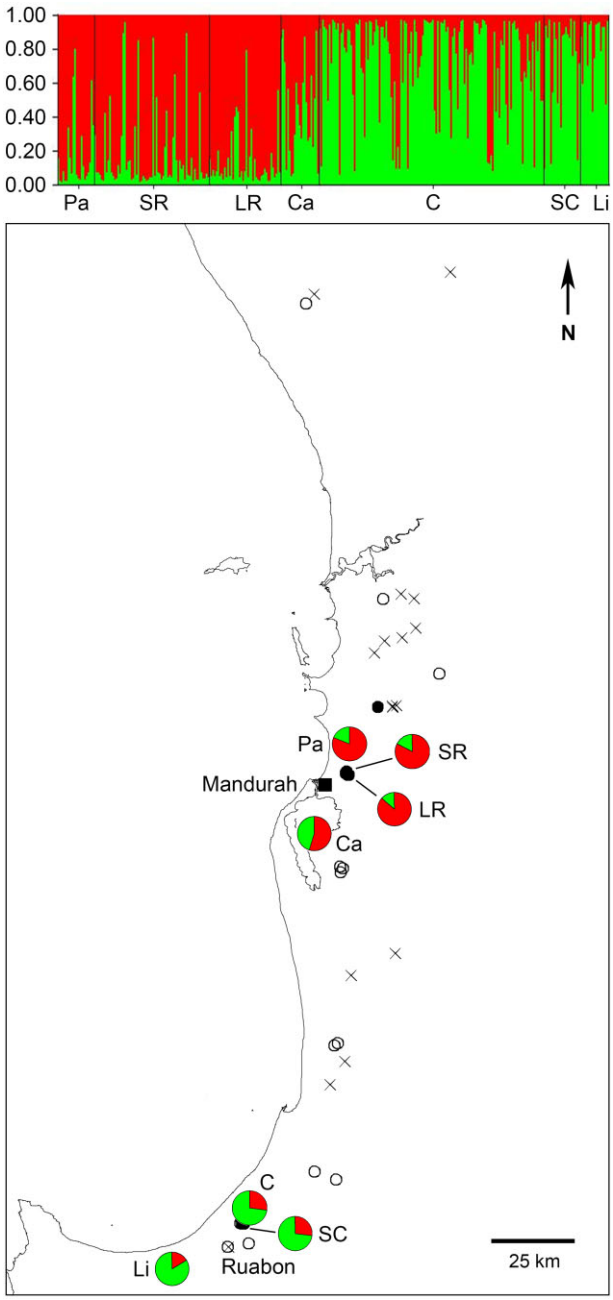

Figure 4. Genetic clustering of seven study populations of the rare orchid *Drakaea elastica* based on STRUCTURE analysis. Coloured bars show the assigned proportion of membership of each individual multilocus genotype to each cluster ($k = 2$). Pie charts visualize membership of each population to each of the two clusters.

(F_{ST}, $R_{xy} = -0.134$, $P = 0.251$), despite evidence for some regional structure.

Clonality

There were no cases in which discrete multilocus genotypes were shared among populations and no cases of genotype sharing among samples > 20 cm apart. Of the 110 leaves sampled within colonies (< 20 cm apart) as potential clones, 87.3% ($N = 96$) shared multilocus genotypes indicative of clonality. Furthermore, no individuals sampled > 12.5 cm apart were identified as clones. Due to the possibility that the optimized growing conditions in the glasshouse could lead to an increased incidence of clonality, we tested if the proportion of clones was higher in the BGPA glasshouse population. Contrary to expectations, the median proportion of clones across colonies was significantly higher in the wild samples than in the glasshouse samples (Mann–Whitney *U*-test, $Z = 3.457$, $P = 0.001$).

DISCUSSION

Is there evidence for cryptic ecotypes in *D. elastica*?

The pollinators attracted to *D. elastica* represented two distinct morphological forms of the wasp *Z. gilesi*, originally recognized by Turner (1910) as morphological variants of uncertain taxonomic status. Sequencing of the CO1 region revealed consistent base pair divergence between the two wasp forms, within the range of species-level differences for thynnine wasps (Mant *et al*., 2002; Griffiths *et al*., 2011). Furthermore, our morphologically based field assignment of the specimens to the different forms was fully congruent with the findings of the phylogenetic analysis. The orchids tested from the Mandurah region attracted the red-legged form of *Z. gilesi*, but were not attractive to the black-legged form of the wasp. Conversely, the orchids from the Capel region were attractive to the black-legged form of the wasp and elicited only a minor response from the red-legged form, the wasps approaching the orchid but not alighting or attempting copulation. Thus, the collective evidence supports our prediction that *D. elastica* contains two ecotypes, each pollinated by a different species within the *Z. gilesi* complex.

A recent taxonomic revision of *Drakaea* detected no morphological differences between populations of *D. elastica* across its range (Hopper & Brown, 2007). As long-range pollinator attraction in sexually deceptive orchids is achieved by floral odour (Schiestl *et al*., 1999, 2003; Ayasse & Dötterl, 2014), we propose that the differences in pollinator attraction indicate the presence of ecotypes within *D. elastica*, most likely underpinned by differences in floral odour. We are in the process of identifying the active compounds in the floral odour of *D. elastica*, but progress is impeded by the low quantities of the compounds present in the flowers, and the limited amount of floral material that we can source from this endangered species. However,

the compounds responsible for pollinator attraction are likely to include pyrazines, as recently confirmed for *D. glyptodon* (Bohman *et al*., 2014) and implicated in the pollination of *D. livida* J.Drumm. (Bohman *et al*., 2012a, b).

The presence of ecotypic variation in floral odour (referred to as chemotypes) within *D. livida* has recently been reported (Bohman *et al*., 2012a, b). Similarly, there is strong evidence for multiple chemotypes based on pollinator choice experiments and chemical analysis of floral odour in other sexually deceptive orchids. These studies include European *Ophrys* L. (Paulus & Gack, 1990; Breitkopf *et al*., 2013), and Australian *Chiloglottis* R.Br. (Bower, 1996, 2006; Bower & Brown, 2009; Peakall *et al*., 2010; Peakall & Whitehead, 2014) and *Caladenia* R.Br. (Bower, 2001). In some cases, morphologically similar chemotypes have been shown to represent different orchid species (Bower, 2006; Bower & Brown, 2009; Peakall *et al*., 2010). The emerging evidence suggests that pollinator switching in sexually deceptive orchids can be achieved simply via changes in floral odour (Peakall *et al*., 2010; Xu, Schlüter & Schiestl, 2012; Whitehead & Peakall, 2014). Therefore, such entities are likely to remain morphologically and even genetically cryptic during the early stages of speciation (Bower, 1996; Schiestl & Schlüter, 2009; Xu *et al*., 2012; Peakall & Whitehead, 2014).

Genetic divergence between ecotypes

The genetic evidence provided support for the results from the pollinator baiting experiments. Although the overall population genetic differentiation was low (F_{ST} = 0.026), STRUCTURE analysis revealed two clusters, which broadly corresponded to the ecotypes as defined by the pollinator responses. The overall low level of genetic differentiation, despite the likelihood of at least some pollinator-mediated reproductive isolation (given the pollinator choice test results in the south), is consistent with other sexually deceptive orchids. For example, Peakall & Whitehead (2014) recently evaluated the patterns of chemical, morphological and genetic variation among multiple closely related *Chiloglottis* orchids at varying stages of evolutionary divergence. As predicted, they found that plastid DNA divergence preceded nuclear divergence, with only trivial nuclear differentiation detected among chemically distinct but morphologically cryptic taxa. Even among morphologically divergent taxa, in which plastid DNA divergence was strong, nuclear differentiation (as measured by F_{ST}) still falls well within the range of population-level genetic variation within mature species (Phillips, Dixon & Peakall, 2012).

Despite the apparent north–south clustering of *D. elastica* ecotypes, the PCoA clustered the population at Carrabungup with the Capel populations *c*. 100 km to the south and not with Mandurah populations to which it is geographically closer (13 km apart). The genetic similarity of the Carrabungup and Capel populations was paralleled by the pollinator baiting experiments, which revealed that the *D. elastica* flower tested from Carrabungup was strongly attractive to the black-legged form of *Z. gilesi*, with frequent pseudocopulation observed. This observation suggests that despite the tendency for separate north–south distributions, both ecotypes could potentially co-occur at some sites. The possibility of sympatric orchid ecotypes is further supported by the phylogenetic analysis of *Z. gilesi*, which revealed that the two pollinator taxa are sympatric in the Mandurah region.

If mixed populations do occur with little or no hybridization between ecotypes, as might be expected given that the pollinator choice experiments indicated a low probability of pollinator sharing, pollinator-mediated reproductive isolation may already be established in this system. For example, previous studies on sexually deceptive orchids in *Chiloglottis* and *Ophrys* have shown virtually complete reproductive isolation based only on differences in the floral odour compounds that mediate pollinator attraction and specificity (Xu *et al*., 2011; Peakall & Whitehead, 2014; Whitehead & Peakall, 2014). However, we note that red-legged *Z. gilesi*, which responds to the Mandurah orchids, also partially responded to Capel orchids. Furthermore, STRUCTURE analysis showed that some individuals from the Carrabungup population appear to be intermediate between the two genetic clusters delimited. It is not clear whether these intermediate forms are hybrids between the two ecotypes, but this warrants further investigation. Given the limited opportunities to conduct pollinator choice tests with the red-legged form of *Z. gilesi*, we do not yet know if pollinator specificity is as strong as in the case of the black-legged form.

The question of whether the evolution of pollination ecotypes can in turn promote reproductive isolation remains a key question in the field of speciation research (van der Niet *et al*., 2014). However, in *D. elastica* more work is needed to assess whether these ecotypes are fully reproductively isolated. The local sympatry of the two forms of *Z. gilesi*, but with strong levels of genetic differentiation, suggests strong reproductive isolation between the pollinators, which may have driven reproductive isolation in *D. elastica*. The analysis of hypervariable plastid DNA markers, as conducted in the closely related genus *Chiloglottis* by Peakall & Whitehead (2014), should prove particularly informative in evaluating this hypothesis. Methods such as tracking the transfer of marked pollen (Peakall, 1989; Peakall &

Beattie, 1996; Xu *et al*., 2011), paternity analysis and intensive genetic analysis in search of hybrids (e.g. Whitehead & Peakall, 2014) would shed further light on the degree of reproductive isolation. Resolving the chemical basis of pollinator specificity, and working more extensively with the northern form of *D. elastica*, which is pollinated by the rare red-legged form of *Z. gilesi*, will also be important. If further research does support strong bidirectional reproductive isolation between the ecotypes, *D. elastica* may well be in the early stages of speciation, with defined morphological and genetic differences yet to be evolved. If this is the case, we suggest that the pollination ecotypes we have discovered probably represent a case of adaptation to local variation in the availability of pollinators.

Genetic divergence within ecotypes

Although significant regional genetic structure was detected, which was broadly consistent with the north–south ecotype distributions, only low levels of population genetic differentiation were observed within ecotypes. Low levels of population genetic differentiation may be typical in orchids, as illustrated in a recent meta-analysis showing that mean genetic differentiation in Orchidaceae (F_{ST} = 0.146 based on allozyme loci) is the third lowest reported for well-studied plant families (Phillips *et al*., 2012). The low level of population genetic differentiation in *D. elastica* is similar to that reported in microsatellite studies for two other sexually deceptive orchids from south-western Australia, *D. glyptodon* (F_{ST} = 0.045, Phillips *et al*., 2012) and *Caladenia huegelii* Rchb.f. (F_{ST} = 0.047, Swarts *et al*., 2009). Furthermore, both of these studies reported a lack of evidence for isolation-by-distance. Population genetic studies on orchids tend to only detect isolation-by-distance across geographical scales > 250 km (Phillips *et al*., 2012). It is hypothesized that low levels of differentiation and lack of isolation-by-distance arise through long-distance dispersal of the dust-like seed maintaining a level of population connectivity (Arditti & Ghani, 2000; Phillips *et al*., 2012). In *D. elastica*, the low levels of genetic differentiation and lack of isolation-by-distance are consistent with this hypothesis.

Implications for conservation and management

Given the evidence for cryptic ecotypes in the rare *D. elastica*, it is highly desirable to understand the abundance and geographical range of these ecotypes better and to document any cases of mixed populations fully. The strong genetic evidence for some local clonality indicates that failure to account for clonal structure has led to an overestimation of the effective population size of *D. elastica*. High levels of clonality in orchids could potentially confer some resilience to low pollinator abundance (Bond, 1994; Pauw & Bond, 2011; Pauw & Hawkins, 2011), but may also mask demographic processes that could ultimately lead to population extirpation (Kuussaari *et al*., 2009). The discovery of cryptic ecotypes adds a further complication, as even partial pollinator-mediated reproductive isolation could impact on population size estimates. Here, we may have uncovered two cryptic ecotypes with total population sizes that are far smaller than the present estimates.

In terms of managing the orchid–pollinator relationship, managers must now consider the requirements of two pollinator species. Our findings suggest that the red-legged form of *Z. gilesi*, which is responsible for pollination of the northern *D. elastica* ecotype, is rare. Furthermore, the northern range of *D. elastica* appears to be on the extreme southern edge of the geographical range of the red-legged form of *Z. gilesi* (R. D. Phillips, unpubl. data). Given the rarity and distribution of this species, populations of *D. elastica* reliant on this pollinator may be particularly susceptible to pollinator loss through environmental changes such as habitat alteration and fragmentation. Furthermore, research priority needs to be given to understanding the ecological requirements of the red-legged form of *Z. gilesi* and identifying areas of suitable habitat for protection. Despite the huge diversity of thynnine wasps, little is known about their complex life cycles outside of just a few species (Ridsdill Smith, 1970; Brown & Phillips, 2014). Pollinator baiting could be used to identify sites that support populations of pollinators and consequently potential sites for orchid reintroduction.

Given that there are at least several hundred sexually deceptive orchid species worldwide (Gaskett, 2011; Phillips *et al*., 2014b) and that cryptic ecotypes have been recorded in multiple genera (Bower, 1996, 2006; Bower & Brown, 2009; Peakall *et al*., 2010; Breitkopf *et al*., 2013), cryptic variation may occur much more widely than originally considered. The species status of these entities needs to be assessed using multiple lines of evidence. Of particular importance is an assessment of the presence of reproductive isolation, potentially complementing experimental approaches with the use of alternative genetic markers, such as those isolated from plastid DNA (Peakall & Whitehead, 2014). Among the practical challenges of managing cryptic entities is recognition of the taxa of concern. If species without recognizable morphological differences are to be accepted, conservation practitioners need a way of rapidly identifying the relevant species. Recently, there has been discussion regarding DNA-based species description, whereby a distinct sequence could be defined as a

distinct entity (Cook *et al*., 2010). However, given the low levels of genetic differentiation characterizing many sexually deceptive orchids, further refinement of suitable molecular markers will be required.

The importance of cryptic ecotypes for conservation priorities

A thorough knowledge of the co-occurrence of ecotypes, which is likely in at least one population (Ca), may also have implications for which populations receive the maximum conservation priority when funding is limited. For example, mixed populations of ecotypes, with evidence for the presence of both pollinators, might be deemed a high priority for management. Such mixed populations may exhibit the highest evolutionary potential for responding to the unpredictable changes in pollinator availability that may occur as a result of habitat loss and fragmentation, and climate change.

Critically, the formal recognition of ecotypes as taxonomic units has important consequences for the allocation of often limited funding and capacity for management actions. The status of cryptic entities/variants, particularly in orchids, has been a topic of intense debate. A classic example is the genus *Ophrys*, where the number of species recognized ranges from 19 (Pedersen & Faurholdt, 2007) to > 250 (Delforge, 2006). The debate lies with how the individual authors interpret variation in chemotype and pollinator use despite morphological and genetic similarity (e.g. Bateman *et al*., 2003, 2011; Devey *et al*., 2008; Bradshaw *et al*., 2010; Vereecken *et al*., 2011; Swarts *et al*., 2014). Given the large number of rare sexually deceptive orchids, the manner in which we treat cryptic entities will potentially impact a large number of taxa. In this case, combining molecular, ecological, chemical and pollinator evidence may assist in resolving some of these issues.

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SUPPORTING INFORMATION

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

Figure S1. Mean log likelihood of k [ln $P(K)$] for k clusters.
Figure S2. Second-order rate of change of the likelihood distribution (Δk) as a function of k clusters.
Figure S3. Results of STRUCTURE analysis for *Drakaea elastica* for $k = 2$ to 8.
Figure S4. PCoA of genetic differentiation (F_{ST}, six loci) for the seven major populations of *Drakaea elastica*. The first three factors explained 96.06% of the variation.
Table S1. Collection details of wasp specimens used in the phylogenetic analysis.