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Defining conservation units in a complex of morphologically similar, sexually deceptive, highly endangered orchids

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ABSTRACT

In plants, rapid speciation attributed to pollination strategy is unlikely to lead to obvious morphological traits for identification or large genetic differences among populations. The species-rich Australian orchid genus *Caladenia* contains numerous taxonomically difficult species complexes, including the reticulata complex comprising 37 species, many of which are highly endangered with narrow-range geographic distributions as a consequence of severe habitat loss. Here, we test morphological species delimitation in the reticulata complex with a multigene phylogenetic analysis, detailed population genetic analysis and comprehensive data on specific sexually deceived pollinators of species within the complex. Phylogenetic analysis revealed limited species-level resolution within the reticulata complex and no difference between species with different pollinators. Microsatellite data confirmed this result for Victorian populations of the reticulata complex but revealed significant isolation by distance between Victorian and South Australian populations. Pollinator preference tests indicated that 10 reticulata complex morphospecies all shared the thynnid wasp pollinator *Phymatothynnus nitidus*, yet the microsatellite analysis revealed only limited differentiation between those and three species within the reticulata complex shown to employ different pollinators. The combined molecular and ecological approach revealed that morphospecies that share the same pollinator are likely to be the same biological species. Conservation and taxonomic focus should shift from a 'microspecies' approach to capturing key populations across the geographic range of the *Caladenia reticulata* biological species which will greatly assist the allocation of limited conservation resources.

1. Introduction

Conservation practitioners face an ongoing struggle to identify populations of organisms for conservation purposes (i.e. conservation units Funk et al., 2012) in taxonomically difficult species groups. Contributing to this sensu struggle are the facts that (i) the word 'species' may be used as a taxonomic rank or, as a natural lineage (Hey et al., 2003) and (ii) it is difficult to select the most appropriate method for delimitating species boundaries with limited available empirical data (Sites and Marshall, 2003). This problem is further compounded by the ongoing debate across disciplines over which species concept or concepts best apply (de Queiroz, 2005a). A general theme that links species concepts is that species are evolving lineages (de Queiroz, 2005b) however, this insight does little to resolve the 'problem' of species' ambiguity in their natural environments (Hey et al., 2003) or how best to develop policy to conserve them. There is therefore a need to 'diagnose' conservation units, taking into consideration established taxonomy, molecular evidence, biogeography and conservation status of the species group in question (Green, 2005). The delineation of conservation units should facilitate the preservation of unique genetic lineages as well as the protection of extant genetic diversity required for future adaptation (Funk et al., 2012). Yet, it is well understood that population structure, life history and genetic variability differ greatly among taxonomic groups. Thus, compiling and interpreting data for distinguishing conservation units should be completed case by case (Green, 2005).

Pollinator-driven speciation in plants, a phenomenon that is reputedly prevalent in the Orchidaceae (Ayasse et al., 2011), is a key mechanism of angiosperm diversification (Johnson, 2007), yet it does not always lead to morphological or genetic distinction

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between taxa. Indeed, with over 25,000 species (Dressler, 2005), the Orchidaceae is famous for its diversity in floral morphology but infamous for its taxonomic difficulties. This issue is particularly evident in orchids with sexually deceptive pollination, where male insects are deceived into attempting copulation with a flower (Schiestl, 2005). Orchid flowers attract their pollinators via the release of chemical signals that mimic their female counterpart's sex-pheromones (Schiestl et al., 2003). Research has shown that sexual deception, employed by hundreds of species that together encompass numerous origins might be determined by minor chemical differences in floral scent (Xu et al., 2012). Consequently, reproductive isolation in sexually deceptive orchids precedes morphological differentiation leading to taxonomic problems, most famously in *Ophrys,* where there is frequent hybridisation and considerable infraspecific floral variation (Devey et al., 2008).

Orchid species with highly specific environmental interactions such as sexual deception are often intrinsically rare (Swarts and Dixon, 2009) and often show geographically disjunct distributions (Phillips et al., 2014). This rarity, coupled with habitat loss, has led to the listing of many such species as endangered at risk of extinction. Habitat loss significantly complicates understanding of natural rarity because many species now occur as scattered populations in isolated, highly fragmented remnants of native vegetation, making it difficult to determine whether the species was naturally rare in the landscape or originally much more widespread but recently declined through habitat loss. When listed, these species are given high levels of statutory protection and in many cases resources are allocated for their conservation (Bateman et al., 2011).

Most of our taxonomic knowledge of orchids is generated from traditional circumscriptions based on perceived morphological discontinuities. More recently, molecular and ethological (pollinator behaviour) data have provided significant insights into species' relationships, offering a more comprehensive and complimentary approach to circumscribing species. This is typical in the Orchidaceae and perhaps no more dramatically exemplified than in the European orchid genus *Ophrys*, (Bateman et al., 2011; Bradshaw et al., 2010; Devey et al., 2008; Vereecken et al., 2011). However, the criteria for species delimitation in *Ophrys*, and the weight given to the diagnosability of characters (be they morphological, genetic or chemical) by different research groups greatly influences the number of species recognised (Bateman, 2012). A more traditional herbarium based morphological approach identifies 16 species broadly (Pedersen and Faurholdt, 2000), 10 species are recognised when DNA sequencing and AFLP data are considered (Devey et al., 2008) whereas more than 250 species are recognised when a more ethological approach is adopted (Delforge, 2005). Therefore, there is strong incentive to develop a stable taxonomy and agreement between scientists and conservation practitioners on diagnosable conservation units *in situ* complemented by empirical support.

The reticulata complex of Australia's most conspicuous orchid genus *Caladenia* is a large group of 37 sexually deceptive species (Jones et al., 2006), most described in the last decade based on subtle differences in floral morphology, geographic distribution and phenology (Jones, 2006). These plants remain difficult to identify to species because the morphological character states overlap between sympatric taxa. The reticulata complex reputedly contains more threatened species than any orchid group in Australia; seven species are nationally classified as critically endangered and another 20 species are recognised for their significant conservation concern. The taxonomic uncertainty, and the high incidence of rarity that can include extinction threats, mean that conservation planning and allocation of resources is difficult but urgently required.

Here, we use a complimentary molecular and ecological approach to identify diagnosable conservation units for key taxa within the reticulata morphospecies' complex. We posit that reproductive cohesion is necessary for maintaining a species' integrity and thus we are testing for quantitative divergence among populations/species. Specifically we: (1) estimate patterns of genetic diversity and population structure of a subset of 'reticulata' complex morphospecies across their distribution range with microsatellite markers; (2) attempt to delimit species boundaries within the reticulata complex through phylogenetic analysis; and (3) test the hypothesis of a one-to-one relationship between morphospecies in the reticulata complex and unique thynnine wasp pollinator species, and question whether such relationships are reflected in the microsatellite and phylogenetic data sets. We discuss the implications of diagnosing conservation units within taxonomically difficult species groups and the future conservation of species within the reticulata complex.

2. Materials and methods

2.1. Description of Caladenia reticulata and associated taxa used in this study

Members of the reticulata complex, like all caladenias, have a single linear-lanceolate hirsute leaf and stem arising from a (deeply buried), underground tuber and like almost all orchids, their flowers are characterised by a modified petal known as the labellum. The *Caladenia* labellum is either fringed or toothed and lined with small projections called calli. The three sepals and two remaining petals are elongated giving the orchid its 'spider-like' appearance and hence its common name. Members of the reticulata complex are known for their prominent osmophores (clubbing) at the tips of their petals, often varying in shape, size and colour. The phenology of growth and development is based on a cycle of winter (wet season) active growth and summer (dry season) dormancy. Members of the reticulata complex used in the molecular and pollinator trials are shown in Fig. 1, and a description of their taxonomic distinctions and conservation status is provided in Supplementary Section 1.

2.2. Population genetics

For the microsatellite analysis, 23 populations (20–60 plants per population) were sampled from 11 species within the reticulata complex (Fig. 1) from their type locations where possible (Table 1, Fig. 2a). To capture maximum genetic diversity within the complex we included range-wide sampling of *C. reticulata* (6 populations), populations of recent circumscriptions with narrow-range endemic distributions (*C. ampla, C. lowanensis*, *C. cruciformis, C. xanthochila* and *C. douglassiorum*), single populations of geographically isolated species (*C.* sp. Raymond Island, *C. valida* and *C. rigida*), a morphologically distinct species (*C. clavigera*), and a species with a distinctly later flowering time (*C. hastata*).

Six polymorphic microsatellite loci (Swarts et al., 2007) were used to investigate genetic differences among populations. Leaves were sampled from plants not less than 50 cm apart in the spring of 2010. DNA extraction followed the methods described above for phylogenetic analysis. Microsatellite reaction followed Swarts et al. (2007). Genotyping was performed on an ABI 3130xl Fragment Analyser. Data were captured using the software Applied Biosystems GeneMapper(r) Software v4.0 and independently checked for genotyping errors.

2.3. Phylogenetics

DNA sequences for the nuclear ribosomal DNA internal transcribed spacers (ITS) and three plastid regions (*psb*A-*trn*H, *trn*Q-5′*rps*16, and *psb*D-*trn*T) were obtained from 34 specimens