



Molecular genetic analysis and ecological evidence reveals multiple cryptic species among thynnine wasp pollinators of sexually deceptive orchids

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ARTICLE INFO

Article history:

Received 29 March 2010

Revised 18 January 2011

Accepted 2 February 2011

Available online 15 February 2011

Keywords:

Chiloglottis

Cryptic species

Neozeleboria

Orchidaceae

Thynninae

Semiochemicals

Sexual deception

Molecular phylogenetic analysis

Population genetic analysis

Statistical parsimony

ABSTRACT

Sexually deceptive *Chiloglottis* orchids lure their male thynnine wasp pollinators to the flower by emitting semiochemicals that mimic the specific sex pheromone of the wasp. Sexual deception is possible because chemical rather than visual cues play the key role in wasp mate search, suggesting that cryptic wasp species may be frequent. We investigated this prospect among *Neozeleboria* wasp pollinators of *Chiloglottis* orchids, drawing on evidence from molecular phylogenetic analysis at three genes (CO1, *rhodopsin* and *wingless*), population genetic and statistical parsimony analysis at CO1, orchid associations and their semiochemicals, and geographic ranges. We found a compelling relationship between genetically defined wasp groups, orchid associations, semiochemicals and geographic range, despite a frequent lack of detectable morphological differences. Our findings reveal multiple cryptic species among orchid pollinators and indicate that chemical changes are important for wasp reproductive isolation and speciation. The diversity of *Neozeleboria* may have enabled, rather than constrained, pollinator-driven speciation in these orchids.

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1. Introduction

Cryptic species are predicted to be frequent in those taxa where non-visual (e.g., chemical or acoustic) signals mediate mate recognition or mate choice. Indeed, cryptic species do appear to be particularly common in marine environments where non-visual communication systems predominate (Knowlton, 1993). On land, insects are also likely to exhibit much cryptic diversity (Bickford et al., 2007) because chemical signalling for mate search and recognition is common (Ayasse et al., 2001; Symonds and Elgar, 2008). Molecular analysis has revealed evidence for cryptic species among flies (Condon et al., 2008), beetles (Monaghan et al., 2005; Pons et al., 2006), moths and butterflies (Hebert et al., 2004, but see Brower, 2006; Hajibabaei et al., 2006), and among ants (Moder et al., 2006; Schlick-Steiner et al., 2006), bees (Ellis et al., 2006) and wasps (Haine et al., 2006).

In this study we investigate a group of insects, the thynnine wasp pollinators of Australian sexually deceptive orchids, where cryptic species may be frequent. In this exceptional pollination system the orchids sexually lure male wasps primarily by mimicry of

the sex pheromone, with pollination occurring during a pre-mating routine or attempted mating with the flower (Stoutamire, 1974; Peakall, 1990; Schiestl et al., 2003; Whitehead and Peakall, 2009; Peakall et al., 2010). Pollination by sexual deception has evolved in multiple orchid lineages on at least four continents. A diverse array of male insects is exploited including ants, bees, wasps, sawflies, beetles and flies (Peakall, 1990; Paulus and Gack, 1990; Cozzolino and Widmer, 2005; Schiestl, 2005; Gaskett, 2011).

Within Australia, more than 150 species of terrestrial orchid sexually exploit male wasps from the parasitic Australasian subfamily Thynninae (Thynnidae) as pollinators (Peakall, 1990; Peakall and Beattie, 1996; Phillips et al., 2009; Peakall et al., 2010). Male thynnines are strong and active flyers, while the flightless females live most of their lives underground (Ridsdill Smith, 1970; Stoutamire, 1974). When ready to mate, females emerge from the ground and emit a volatile sex pheromone to attract a mate. Males typically respond to the female in seconds, approaching from downwind. Experiments show that male thynnine wasps can readily locate hidden females, confirming the primary role of chemical cues in attraction (Peakall, 1990).

In a pivotal breakthrough, the sex pheromone of the thynnine wasp *Neozeleboria cryptoides* has been found to be a single compound representing a new class of natural products, 2-ethyl-5-propylcyclohexan-1,3-dione, called 'chiloglottone 1' (Schiestl

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et al., 2003; Schiestl and Peakall, 2005; Franke et al., 2009). The same novel compound is produced by the orchid *Chiloglottis trapeziformis* to attract male *N. cryptoides*, its specific pollinator. Chiloglottone 1 is an example of a 'semiochemical' – being a chemical involved in both intra and interspecies communication. In choice experiments male *N. cryptoides* exhibited an equal preference for the synthetic compound (presented on a round bead) and the females, further demonstrating the importance of non-visual communication between the sexes in this wasp (Schiestl et al., 2003).

The orchid genus *Chiloglottis*, with some 30 species, is the largest exclusively sexually deceptive genus in Australia. Field experiments using artificially presented flowers have confirmed pollination in this genus is highly specific with an average of 1.1 pollinator species per orchid (Peakall et al., 2010). The majority of the pollinators belong to *Neozeleboria* or closely related wasp genera (Mant et al., 2002, 2005; Peakall et al., 2010). The specific interaction between *Chiloglottis* orchids and their wasp pollinators is known to involve one, two or three compounds from a pool of six related chemical variants, all 2,5-dialkylcyclohexan-1,3-diones or 'chiloglottones' (Schiestl et al., 2003; Franke et al., 2009; Peakall et al., 2010). Bioassays with synthetic chiloglottones indicate two mechanisms for controlling the extreme orchid-pollinator specificity: (1) a single specific compound is required for pollinator attraction, e.g. *C. trapeziformis* and chiloglottone 1, with different compounds attracting different specific pollinators; (2) two or more compounds in a particular blend trigger specific attraction. Co-flowering, sympatric *Chiloglottis* species are always characterised by quantitative or qualitative semiochemical differences. By contrast, some allopatric *Chiloglottis* orchids are known to use the same semiochemical but attract different, non-overlapping pollinator species (Schiestl and Peakall, 2005; Franke et al., 2009; Peakall et al., 2010). When the semiochemicals involved are mapped onto a phylogeny of *Chiloglottis* orchids it is evident that orchid speciation is always associated with pollinator switching and usually is underpinned by chemical change (Peakall et al., 2010).

Notwithstanding some progress, our understanding of chemical communication between male and female thynnine wasps lags behind our growing understanding of the orchid-pollinator interaction. This is in large part because the female wasps are extremely difficult to find (Franke et al., 2009). Nonetheless, we predict that sex pheromone differences will frequently mediate reproductive isolation in these thynnine wasps since they appear to be heavily dependent on chemical rather than visual cues for sexual reproduction. Furthermore, given the extreme orchid-pollinator specificity and its strong chemical basis we predict that pollinator pheromones will be either identical to the orchid semiochemical(s) as in *C. trapeziformis/N. cryptoides* (Schiestl et al., 2003), or will share the same major components in similar proportions. If these hypotheses are true, then wasp-orchid associations, and the semiochemicals that underpin them, should provide strong independent ecological evidence for delimiting wasp species boundaries even in the absence of apparent morphological differences in the wasps. In addition, the mapping of orchid-pollinator-semiochemical interactions onto a robust phylogeny of the pollinators will offer insights into the role of chemical change in wasp speciation.

Neozeleboria wasps appear to be prime candidates for cryptic speciation associated with chemical rather than morphological change. We take an integrated approach to investigate this prospect among those species of this genus that are involved in orchid pollination. We draw on evidence from molecular phylogenetic analysis at three genes (CO1, *rhodopsin* and *wingless*), population genetic and statistical parsimony analysis at the mtDNA gene (CO1), knowledge of orchid associations and their semiochemicals, and data on geographic ranges. Specifically we test the *a priori* hypothesis that orchid-pollinator relationships will be strongly

congruent with wasp species boundaries even when morphological evidence is lacking. Conclusions are drawn concerning the phylogenetic relationships of the wasps, the prevalence of cryptic species, and the evolution and diversification of both wasps and orchids.

2. Materials and methods

Since female thynnine wasps are rarely collected, species identification is based on male morphology, with male genitalia providing the critical diagnostic features used in species-level identification (Brown, 1998, 2005). There are currently around 600 described species in 45–50 genera. However, Kimsey (1996) anticipates at least three times as many undescribed species, while Brown (2009) has independently estimated that a further 1000 undescribed species are present in existing collections.

The wasp genus *Neozeleboria* presently includes 22 named and approximately 52 undescribed species (Brown, unpublished), mostly found in south-eastern Australia. Previous genetic and morphological analysis divided the genus into three clades: 'Proxima', 'Cryptoides', and 'Monticola', and a sub-clade 'Impatiens' within the Monticola clade (Mant et al., 2002, 2005), but there has been little further phylogenetic resolution. While only a subset of the described *Neozeleboria* species are known to be *Chiloglottis* orchid pollinators, these species represent a broad cross-section over all three known clades (Mant et al., 2005; Bower and Brown, 1997, 2009).

DNA was taken from a collection of dry or ethanol-preserved thynnine wasps obtained during 6 years of studies of the semiochemicals involved in orchid-pollinator interactions. All wasp samples were associated with orchids in one of three ways: (1) as specific orchid pollinators, being species whose behaviour at orchid flowers included attempted copulation, pollen removal and pollen deposition; (2) as minor responders, being species that occasionally responded to orchid flowers but whose behaviour at the flower did not result in pollination (e.g. approach only); (3) as responders to synthetic semiochemicals, but not yet known to be orchid pollinators or minor responders. All samples were obtained from baiting experiments (baited with either orchids or semiochemicals) as described in detail in Peakall et al. (2010). This approach provides a very efficient method for sampling *Neozeleboria* wasps, which are otherwise extremely difficult to find due to their limited flight times, patchy distribution and diffuse feeding habits. Three wasps were chosen for sequencing from each site in the collection, except where fewer individuals were available. The sample represents 18 sites across south-eastern Australia, from northern NSW to Tasmania (Table 1). Outgroup taxa, also representing orchid pollinators, were selected from the thynnine genera *Chilothynnus*, *Phymatothynnus*, and *Zaspilothynnus*.

Sequencing was performed at one mitochondrial locus (cytochrome *c* oxidase 1, CO1) and two nuclear loci (*rhodopsin* and *wingless*). CO1, *rhodopsin* and *wingless* have provided robust results in previous phylogenetic studies of Hymenoptera (e.g. Mardulyn and Cameron, 1999; Banks and Whitfield, 2006; Danforth et al., 2004; Almeida and Danforth, 2009; Wild, 2009) and both CO1 and *wingless* were sequenced in a previous study of *Neozeleboria* (Mant et al., 2005).

2.1. DNA extraction, PCR and sequencing

In order to retain the taxonomically most informative parts of the wasp, tissue samples were taken from the head of the specimen. Each sample was macerated in a 1.5 mL tube with Digsol buffer (pH 8.0) and Proteinase K (10 mg/mL); incubated at 55 °C for 1 h; and extracted with 4 M ammonium acetate solution. DNA

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