HIDDEN BIODIVERSITY: DETECTION OF CRYPTIC THYNNINE WASP SPECIES USING SEXUALLY DECEPTIVE, FEMALE-MIMICKING ORCHIDS

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Abstract

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Cryptic or sibling species are difficult to detect by external morphological characters alone, and represent hidden biodiversity. One group in which such species commonly occur is the large and predominantly Australian flower wasp subfamily Thynninae (Hymenoptera: Tiphiidae). Many thynnine species are pollinators of sexually deceptive orchids in the subtribe Caladeniinae. For example, male wasps of the genus *Neozeleboria* Rohwer are attracted by pseudopheromones produced by flowers of the genus *Chiloglottis* R. Br. Pollination occurs when males attempt to copulate with structures on the orchid labellum that mimic the wingless, ant-like female. Such wasp-orchid relationships may be species specific within their normal environmental range, and can be used to supplement classical alpha taxonomy. Field baiting experiments (using picked flowers to attract wasps) have provided large numbers of wasp specimens and evidence of numerous unexpected cryptic species. An example is discussed involving three closely related orchids, *Chiloglottis trilabra* Fitzg., *C. reflexa* Labill and *C. seminuda*, D.L. Jones and their respective pollinators, *Neozeleboria proxima* (Turner) and two new cryptic species of *Neozeleboria*.

Introduction

Cryptic or sibling species are notoriously difficult to detect (Paterson, 1990) and represent hidden biodiversity. By definition cryptic species cannot be separated easily by traditional alpha taxonomic methods. They are usually revealed by the presence of biological discontinuities (Paterson, 1990), such that some populations behave differently to others thought to be the same species. This is often interpreted as intraspecific variability, when in fact a complex of sibling species may be present. When biological discontinuities in complexes of sibling species are recognised, close examination of the morphology will often, but not always, reveal discrete, consistent differences amenable to circumscription by alpha taxonomic methods (Drew and Hardy, 1981; Whittle et al., 1987). Unequivocal proof of the existence of sibling species requires evidence of genetic or reproductive isolation in sympatric populations (Mahon et al., 1982).

In this paper we report on studies which have led to the recognition of cryptic species in thynnine (Tiphiidae: Thynninae) wasps that pollinate sexually deceptive orchids. The subtribe Caladeniinae of the Orchidaceae contains several genera with species exclusively pollinated by male thynnine wasps (Stoutamire, 1974,

1975, 1983). The males are attracted to the orchids by odours that appear to mimic the sex pheromones of the female. Flowers are then pollinated when the males attempt to mate with the orchid labellum, which mimics the shape and/or colour of the female.

Various studies have suggested that a specific relationship exists between each orchid species and a single thynnine species (Stoutamire, 1983, 1986; Peakall, 1990; Handel and Peakall, 1993; Peakall and Handel, 1993). However, recent studies (Bower, 1992, 1996) have shown that sexually deceptive species of Caladeniinae may attract more than one thynnine visitor, but only one, or occasionally two, of these is capable of effecting pollination.

One of us (CCB) has been using this specific relationship to identify cryptic species of orchids (Bower, 1992, 1996). Samples of flowers from two or more orchid populations thought to represent different taxa are exposed simultaneously in the field in pollinator choice tests. If orchid samples consistently attract different thynnine species they are reproductively isolated and represent different species (Paulus and Gack, 1990). Several new cryptic species of orchids in the genus *Chiloglottis* R. Br. have been detected by these methods (Bower, 1996). In the course of this work it became apparent

there were also cryptic species of thynnines among the pollinators of *Chiloglottis*.

This paper presents the methodology we have used to demonstrate the existence of cryptic thynnine species. An example is presented of the use of pollinator choice tests in the field to detect two undescribed sibling wasp species among the pollinators of a group of three cryptic orchid species in the *Chiloglottis reflexa* (Labill) Druce group.

Methods

Orchids studied

Chiloglottis are small terrestrial herbs that occur in sheltered locations in open forests on the coast and ranges of eastern Australia. Plants consist of a small underground tuber, twin lanceolate, prostrate leaves and a single small flower on a slender stem about 5–12 cm high. The labella of *Chiloglottis reflexa* group species are about 10 mm long and have prominent clusters of dark, glandular calli which collectively mimic the ant-like female of the thynnine wasp pollinator. Males are attracted to the flower and attempt to mate with this pseudofemale. In so doing the dorsal thorax of the male contacts the overhanging anther and removes the pollinia which are subsequently wholly or partially transferred to the stigmas of other flowers he may visit.

Three similar, sympatric, autumn flowering species in the *Chiloglottis reflexa* group from the Blue Mountains region of the NSW central tablelands and the Wollongong area on the south coast were studied. *Chiloglottis reflexa* and *C. seminuda* D. Jones frequently occur together on sandy soils, while *C. trilabra* Fitzg. prefers heavier soils at high altitudes and may overlap on shales with *C. seminuda*. In areas with complex geology all three species may occur in close proximity, e.g., at Mt Wilson where basalt overlies sandstone. *Chiloglottis reflexa* and *C. trilabra* are difficult to separate taxonomically (Jones, 1988). Samples of *C. trilabra* from the northern (Serpentine Nature Reserve) and southern (Batlow) tablelands were used in some tests.

Pollinators of Chiloglottis

Chiloglottis species are pollinated by small thynnine wasps of the genus *Neozeleboria* (Stoutamire, 1975; Bower, 1992, 1996; Handel and Peakall, 1993; Peakall and Handel, 1993), and two recently recognised undescribed genera (Brown, unpublished). The *Chiloglottis reflexa* group is pollinated by *Neozeleboria proxima* and its allies, the males of which are 8–13 mm long,

and black with orange legs and yellow markings on the head and body.

Field techniques

Chiloglottis flowers were exposed in the field with their stems in small glass vials (13 × 50 mm) of water mounted in a wood block placed on the ground in suitable habitat. Three flowers from the same population of each taxon were placed in each vial. Up to 5 sets of flowers placed in a line 35–40 cm apart could be monitored simultaneously in choice experiments. As far as possible the line of vials was oriented perpendicular to the wind direction to minimise overlap of the pheromone trails close to the blocks.

Flowers were exposed for a standard 3 min at each site since most males arrived within the first few minutes of an exposure (Peakall, 1990). By moving flowers to a new site 20 m or more away, a renewed response could be obtained by sampling a new segment of the wasp population. The number of exposure sites used at each locality depended on wasp abundance. New exposure sites were selected until a sample of 20 or more wasps was obtained for each orchid taxon eliciting a response. For scarce or patchily distributed wasps it was not always possible to obtain samples of this size.

Insects were only considered to be attracted to a set of flowers if they:
1. landed on or within 5 cm of the flowers;
2. approached flowers directly in flight to within about 10 cm; and/or
3. circled flowers closely in flight.

Thynnine wasps or other insects which made general approaches to the area, and did not clearly single out flowers were ignored. Wasps meeting the above criteria were captured by rapidly lowering an insect net over them and the flowers, with the mesh held clear of the flowers to prevent damage.

Experimental design

Three designs of field tests have been used to establish the preferences among thynnine pollinators for specific orchid taxa (Bower, 1996).
1. **Sympatric choice test:** Two or more sets of flowers of different or suspected different taxa from the same geographic region are exposed together.
2. **Allopatric choice test:** Samples of closely related orchid taxa from different geographic regions are exposed together in each of their regions and habitats.
3. **Sequential test:** This tests for variation in attractiveness to wasps of different sets of flowers of the same species. It aims to deter-

mine whether nil responses to flowers in previous choice tests were due to the greater attractiveness of other conspecific sets of flowers. It consists of two parts: (a) a 3 min exposure of sets of flowers with low or nil responses in a previous choice test, followed at the same site by (b) a further 3 min in which previously attractive flowers are added to the array as controls.

Responses by wasps in the first 3 min to previously unattractive flowers show they have similar pseudopheromones and may be the same species as the more attractive samples. If the flowers remain unattractive and there is a response to the control, they are different taxa.

The above tests enable cryptic orchid species to be distinguished, provided the pollinators are readily separable (Bower, 1996). However, if the pollinators also belong to a complex of cryptic taxa, further tests are needed to isolate both the orchid and wasp species. This is done by conducting choice tests with the same orchid samples at a number of localities in appropriate habitats. By chance, populations of each cryptic wasp taxon will be absent from some locations when others are present. Two orchid taxa with different cryptic pollinators will each exclusively attract wasps at some locations. When such data are obtained, samples of the sibling wasp species are subjected to detailed examination of external morphology and genitalia by one of us (GRB) to determine whether any consistent differences in morphological characters occur.

Results

Initial pollinator choice tests attracted wasps, which appeared to conform with *Neozeleboria proxima* (Turner), to each of *Chiloglottis reflexa*, *C. trilabra* and *C. seminuda* (e.g., Experiment 1, Table 1). However, subsequent testing at a number of localities found that the *Neozeleboria proxima* sensu lato populations at some localities did not respond to one or two of the orchid taxa present (Experiments 4, 5, 6, 9 and 12, Table 1). This showed there were populations within *Neozeleboria proxima* s.l. which responded differentially to the odours emitted by the three orchid species, and suggested there may be cryptic taxa within *N. proxima*.

In addition, at two localities outside the normal range and habitats of *Chiloglottis seminuda* and *C. reflexa*, there were specific responses to each of the orchids by other easily distinguishable thynnine taxa. At Mullion Creek and Mt

Canobolas (Experiments 2 and 3, Table 1) *Asthenothynnus westwoodi* (Guérin-Méneville) was uniquely attracted to *Chiloglottis seminuda*. On Mt Canobolas, *Neozeleboria* sp. nov. 25 was attracted to *Chiloglottis trilabra* and a species of *Eirone* Westwood to *C. reflexa* (Experiment 3, Table 1). These results showed the three orchid taxa had different pseudopheromones and supported their recognition as different species.

Close examination of the *Neozeleboria proxima* s.l. collections revealed consistent morphological differences among the populations responding to the three orchids. These differences are summarised in Table 2. The three wasp species are almost identical externally, with slightly more yellow on the two undescribed species, and morphological differences limited to the shape of the fore coxae and hypopygium (last visible sternite). The genitalia however, are distinctive and the three species are most readily and accurately determined from these.

In summary, the data in Table 1 show that *Chiloglottis trilabra* specifically attracted *Neozeleboria proxima* s.s. (131 exposures, 87 wasps caught) and *N.* sp. nov. 25 (Mt Canobolas only, 5 wasps caught). *Chiloglottis seminuda* attracted *Neozeleboria* sp. nov. 29 (173 exposures, 74 wasps caught), but also attracted *Asthenothynnus westwoodi* outside its normal range (59 exposures, 29 wasps caught). *Chiloglottis reflexa* attracted *Neozeleboria* sp. nov. 30 (192 exposures, 124 wasps caught) in its normal habitats and an *Eirone* sp. outside its range at Mt Canobolas (14 exposures, 5 wasps caught).

Occasionally *Neozeleboria proxima* s.s. may respond weakly to *Chiloglottis reflexa* (Experiment 10, Table 1) (17 exposures, 2 wasps caught) and *C. seminuda* (unpublished data). These minor responses do not result in pollination (Bower, 1996) and indicate a slight similarity in pseudopheromones among the three orchid species, and possibly also the wasps.

Discussion

The experimental data presented above show that cryptic thynnine species can be detected by their specific attraction to particular sexually deceptive orchid species. The differential attraction is almost certainly based on differences in pseudopheromone chemistry between orchid species rather than appearance. Sexually deceptive orchids are therefore convenient sources of pheromones for studies of cryptic thynnine species. Due to their high attractiveness to

Table 1. Pattern of attraction of *Neozeleboria proxima* and its cryptic allies to *Chiloglottis reflexa*, *C. trilabra* and *C. seminuda* on the central coast and tablelands of NSW.

Chiloglottis species	Source of orchid	Thynnine species[1]	No. of wasps caught
Experiment 1: Mt. Wilson, 27 Feb 1993, 59 exposures, Sympatric test			
C. trilabra	Sunny Corner	*N. proxima*	51
C. seminuda	Mt Wilson	*N.* sp. nov.29	26
C. reflexa	Mt Wilson	*N.* sp. nov.30	14
Experiment 2: Mullion Creek, 13–14 Mar 1993, 45 exposures, Allopatric Test			
C. trilabra	Batlow	—	0
C. seminuda	Mt. Wilson	*A. westwoodi*	27
C. reflexa	Mt. Wilson	—	0
Experiment 3: Mt Canobolas, 13 Mar 1993, 14 exposures, Allopatric test			
C. trilabra	Batlow	*N. proxima*	24
		N. sp. nov.25	5
C. seminuda	Mt Wilson	*A. westwoodi*	2
C. reflexa	Mt Wilson	*Eirone* sp.	5
Experiment 4: Nullo Mountain, 12 Apr 1993, 38 exposures, Allopatric Test			
C. trilabra	Serpentine NR	*N. proxima*	2
C. seminuda	Olinda	*N.* sp. nov. 29	24
C. reflexa	Nullo Mt.	—	0
Experiment 5: Mt Wilson, 18 Ap 1993, 21 exposures, Sympatric test			
C. seminuda	Nullo Mt	—	0
C. seminuda	Olinda	—	0
C. reflexa	Nullo Mt	*N.* sp. nov. 30	23
Experiment 6: Hartley Pass, 18 Ap 1993, 10 exposures, Sympatric test			
C. seminuda	Nullo Mt	—	0
C. seminuda	Olinda	—	0
C. reflexa	Nullo Mt	*N.* sp. nov. 30	6
Experiment 7: Mt Keira, Wollongong, 13 Feb 1994, 20 exposures, Sympatric test			
C. seminuda	Mt Keira	*N.* sp. nov. 29	2
C. reflexa	Mt Keira	*N.* sp. nov. 30	21
Experiment 8: Mt Keira, Wollongong, 13 Feb 1994. 15 exposures, Sequential test			
A[2]. *C. seminuda*	Mt Keira	*N.* sp. nov. 29	7
B. *C. reflexa*	Mt Keira	*N.* sp. nov. 30	17

Table 1. Continued

Experiment 9: Jamberoo Mt, 14 Feb 1994, 6 exposures, Sympatric test

C. seminuda	Mt Keira	—	0
C. reflexa	Mt Keira	*N.* sp. nov. 30	9

Experiment 10: Macquarie Pass, Robertson, 14 Feb 1994, 17 exposures, Sympatric Test

C. seminuda	Mt Keira	*N.* sp.nov.29	2
C. reflexa	Mt Keira	*N.* sp.nov.30	27
		N. proxima	2

Experiment 11: Fitzroy Falls, 14 Feb 1994, 24 exposures, Sympatric Test

C. seminuda	Mt Keira	*N.* sp. nov. 29	13
C. reflexa	Mt Keira	*N.* sp. nov. 30	1

Experiment 12: Hartley Pass, 27 Feb 1994, 20 exposures, Sympatric Test

C. trilabra	Kanangra	*N. proxima*	10
C. seminuda	Mt Keira	—	0
C. reflexa	Mt Keira	*N.* sp. nov. 30	6

[1] N. = *Neozeleboria,* A. = *Asthenothynnus*
[2] A = Orchid species exposed alone in first 3 min. B = Orchid species added to the baiting array for the second 3 min.

Table 2. External morphological differences among *Neozeleboria proxima* and its sibling species.

Character	*Neozeleboria proxima*	*N.* sp. nov. 29	*N.* sp. nov. 30
fore coxa emarginate	weakly	not	strongly
hypopygium	triangular, not truncate	triangular, not truncate	subtriangular, truncate
parameres	long, curved	normal	normal
aedeagus	long	short	short
base of femora	black	orange	orange
anterior pronotum	black laterally	yellow laterally	yellow laterally
metasomal spots	reduced, yellow	not reduced, pale yellow	not reduced, pale yellow

thynnine males, they facilitate the collection of large samples for taxonomic study.

The apparent chemical basis for reproductive isolation in both cryptic *Chiloglottis* and pollinator species suggests that morphological and visual differences are of little or no importance in mate selection in thynnines and in their behaviour on the orchids they pollinate. Similarly, Paterson (1990) concluded that any group of organisms relying mainly on chemical or other non-visual signals for mate recognition is likely to contain cryptic species.

The selective attraction of thynnines by orchids has revealed significant diversity which may not have been detected by the alpha taxonomic approaches traditionally applied alone. Similar work still in progress has revealed other complexes of cryptic species in the Thynninae; among *Neozeleboria monticola* Turner and its allies, and in *Phymatothynnus monilicornis* (Smith). It is likely that a great deal more pheromone-based hidden biodiversity is present in thynnine wasps and, indeed, in other insect groups, e.g., moths (Mahon et al., 1982).

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