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Specific pollinator attraction and the diversification of sexually deceptive *Chiloglottis* (Orchidaceae)

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Abstract. Evidence indicates that sexually deceptive *Chiloglottis* R.Br. (Orchidaceae) taxa specifically attract their thynnine wasp (Tiphiidae) pollinators through the floral odour mimicry of female wasp sex pheromones. We use amplified fragment length polymorphisms (AFLPs) to reconstruct the species-level phylogeny of *Chiloglottis*, make a preliminary evaluation of genetic distinctions between species, and compare the historical association among orchids and their pollinators using wasp sequence data from a previous study. AFLPs show large differences between three subgeneric clades relative to that found among species within each clade. Interspecific genetic barriers are indicated by AFLP discontinuities among species unlike in previously reported DNA sequence data. However, such barriers are demonstrated clearly in only one of the two pairs of sympatric species sampled more intensively. We interpret these patterns as indicating either (i) a rapid and recent radiation of species within each clade following histories of stasis or extinction, or (ii) alternating cycles of divergence and gene flow acting to homogenize genetic differences among species within each of the three clades.

Key words: Adaptive radiation, AFLP, cospeciation, cryptic species, pseudocopulation, sexual deception, ethological isolation.

One of the most enduring questions in evolutionary biology is how the association between adaptive change and reproductive isolation can drive ecological divergence and in turn lead to evolutionary radiation (Schluter 2000). Closely related, interfertile sexually deceptive orchid species are thought to be isolated, at least in sympatric comparisons, by the specific attraction of pollinators (Ehrendorfer 1980, Paulus and Gack 1990, Bower 1996). Orchids employing this mode of pollination provide no reward to their male hymenopteran pollinators but deceive them by mimicking the female insects. In particular, the orchids employ a chemical mimicry of the female insect's sex pheromone (Schiestl et al. 1999, 2003). Floral odour is thought to be the key trait responsible for pollinator attraction and is therefore likely to be under strong pollinator-mediated selection (Ayasse et al. 2000, Schiestl 2004, Mant et al. in press). Crucially, it is also floral odour, by mimicking species-specific sex pheromones, that mediates pollinator specialisation and establishes pre-zygotic ethological isolation (Mant et al. 2002, Schiestl and Ayasse 2002). For these reasons, it has been proposed that the highly specialized nature of pollination by

sexual deception has led to adaptive radiations driven by changes in floral scent (Stebbins and Ferlan 1956, Stebbins 1970, Paulus and Gack 1990, Nilsson 1992, Grant 1994, Soliva et al. 2001, Mant et al. 2002).

Phylogenetic investigations into three independently derived sexually deceptive lineages reveal strikingly similar evolutionary histories. Nuclear and/or chloroplast DNA sequence data on *Ophrys* (Soliva et al. 2001, Bateman et al. 2003), *Chiloglottis* (Mant et al. 2002) and *Caladenia* (Jones et al. 2001) all show broad agreement in the tempo of cladogenesis. The low or non-existent DNA sequence variation between species within each of these genera could be attributed to rapid species diversification via pollinator shifts. However, the evolutionary stability of pollinator-mediated reproductive isolation has only recently begun to be evaluated using genetic data. The observed lack of DNA sequence divergence may be the result of persistent or even episodic gene flow among closely related species. Genetic differentiation among species could be hampered by a breakdown of pollinator specificity in sympatry, or where range expansion leads to pollinator replacement and hybridisation with neighbouring orchid populations. Recent microsatellite studies in *Ophrys* (Soliva and Widmer 2003, Mant et al. in press) argue along these lines by suggesting that low genetic differentiation among sympatric species is evidence of gene flow occurring across species boundaries.

A complication in studying sexually deceptive orchids is that taxonomic boundaries at the species-level are notoriously difficult to establish from morphology alone. This is perhaps demonstrated by the degree of synonymy found in the approximately 140 species of *Ophrys* (Delforge 1995), not to mention the large number of postulated *Ophrys* hybrids. However, a long history of poorly co-ordinated taxonomic contributions has no doubt contributed to the confusion. In Australia, there has been a dramatic increase in the number of species with *Chiloglottis* expanding from nine known species (Clements 1989) to the present 22 described and several undescribed species (Jones 1991, 1997, 1998; Bower and Brown 1997). Differences among sexually deceptive species in physiologically active floral odour compounds have been found, including qualitative differences between *C. trilabra* and *C. seminuda* (Mant et al. 2002) and quantitative odour differences between *Ophrys fusca* and *O. bilunulata* (Schiestl and Ayasse 2002). However, diagnostic morphological characters for species are often minor and open to question, as in the cryptic *C. reflexa* and *C. trilabra*. As a consequence, it is difficult to ascertain the level of gene flow or hybridisation that might be occurring among closely related sexually deceptive taxa attracting distinct pollinators.

Pollinator specificity has been demonstrated to be the rule among *Chiloglottis* species. Bower (1996), following early observations by Stoutamire (1975), employed a series of behavioural choice tests among translocated flowers from different *Chiloglottis* populations and species to demonstrate the specificity of response by thynnine wasps (Hymenoptera: Tiphiidae). A single *Neozeleboria* species was shown to be the confirmed or potential pollinator of eight *Chiloglottis* species (Bower 1996). Stoutamire (1975) also documented the pollinators of different forms of *C. gunnii* from Tasmania that have subsequently been described as separate taxa (*C. gunnii*, *C. grammata* and *C. triceratops,* Jones 1998). Thus, this experimental approach has enabled morphologically similar *Chiloglottis* taxa to be differentiated by their attraction of distinct pollinators (Bower and Brown 1997). The pollinator status of the remainder of the *Chiloglottis* species sampled in this paper have been documented in a series of unpublished reports (C. C. Bower, unpublished data) and in Bower (2001a, unpubl. data). Thus, although published accounts for all species are at present lacking, the evidence published to date is indicative of a pollination system in which pollinator responses are specialised. Observations of pollinator specificity have also been

made in the other sexually deceptive genera of the Diurideae (Bower 2001a,b; Stoutamire 1975, 1983; Peakall 1989, 1990; Peakall and Beattie 1996; Alcock 2000).

A caveat to the use of pollinators in the diagnosis of cryptic orchid taxa is that unambiguous species boundaries should also be demonstrated in the pollinators themselves. For *Chiloglottis*, the majority of wasp pollinators sampled in this paper are morphologically distinct, although many await formal taxonomic treatment (G. R. Brown, unpubl. data). For an example of species differences in a *Neozeleboria* Rohwer species group that includes pollinating and non-pollinating taxa see Brown (1998). In lieu of a full taxonomic treatment of the primary pollinating genus, *Neozeleboria* (Brown, unpubl. data), we present, by way of an example, a tabulation of the morphological differences among three closely related pollinator species (see Table 2).

In some cases, however, a real problem of morphological crypsis among wasp taxa is as evident as that indicated in putative *Chiloglottis* taxa. Examples include different geographically separated forms of *N. monticola*, which show divergent responses to orchid taxa indicative of pheromonal-races (Bower 2001a, unpubl. data; Mant, Peakall and Schiestl, unpublished data). Gas chromatography with electroantennogram (GC-EAD) experiments support the recognition of wasp taxa by demonstrating species-specific pheromone responses (Mant et al. 2002, Schiestl et al. 2003) and further studies using GC-EAD and synthetic compounds are underway to test indications of morphologically cryptic wasp taxa.

The genus *Chiloglottis* shows a highly specialized association with the thynnine wasp genus, *Neozeleboria*. Subgeneric groups within *Chiloglottis* also display a degree of specialization, such that related orchids tend to use related *Neozeleboria* as pollinators (Mant et al. 2002). Taxonomic associations of this nature in plant-pollinator interactions are more usually found in mutualistic systems, such as the fig–fig wasp and the yucca–yucca moth interactions. At least in the case of *Ficus*, the association with pollinators shows patterns consistent with cospeciation (Weibes 1979). In sexually deceptive orchids, however, pollination is achieved in an essentially "parasitic" or deceptive interaction based on sexual lures. The orchid is wholly reliant on the wasp for sexual reproduction, whereas the insect may be disadvantaged by disruptions to its mating system (Wong and Schiestl 2002). Notwithstanding the differences from mutualistic systems, orchid species may still track divergences occurring in the pollinators to which they are specialized. Such a process may lead to matching phylogenetic patterns consistent with cocladogenesis. By contrast, non-matching orchid and wasp phylogenies would suggest a lack of cospeciation where, instead, orchids frequently colonize or switch onto novel pollinators. An alternative model suggests the historical association among *Chiloglottis* and *Neozeleboria* reflects a process of "preferential pollinator switching" among a limited taxonomic pool of pollinators. Under this model, phylogenetic constraints in thynnine wasp sex pheromones and emergence phenology may account for the tendency of related orchids to use related wasps as pollinators (Mant et al. 2002). To examine these competing hypotheses, we use the enhanced phylogenetic power offered by more variable AFLP markers (over DNA sequences) to test for congruence among *Chiloglottis* and its *Neozeleboria* pollinators.

In this paper, we examine amplified fragment length polymorphisms (AFLPs) within *Chiloglottis* for molecular markers that are informative among closely related species and populations within species. The following questions are posed: Are observations of ethological isolation among *Chiloglottis* taxa correlated with genetic discontinuities? Are molecular phylogenetic patterns consistent with the adaptive radiation of *Chiloglottis* through shifts in pollinators? Does the association between *Chiloglottis* and its wasp pollinators reflect a history of cocladogenesis?

Materials and methods

Taxon sampling. All the *Chiloglottis* species sampled in Mant et al. (2002) were included in this AFLP phylogenetic study (see Table 1 for voucher details). In addition, several other taxa were included for which pollinator information is either lacking or, in the case of the selfing *C. cornuta*, for which pollinators are absent. To make some account of intraspecific variation we have included multiple samples from each species. In the majority of cases (except *C. anaticeps*, *C. platyptera*, *C. truncata*, *C. trullata* and *C. diphylla*) more than one population of each species was sampled, preferably across a wide geographical scale. The first four of these exceptions are geographically restricted which limited our capacity for intraspecific sampling. To test for species differences in AFLPs, we have sampled more intensively two species pairs that have overlapping geographic ranges. These are *C. trilabra* (9 individuals, 4 locations) and *C. reflexa* (8 individuals, 4 locations) from NSW and *C. grammata* (7 individuals, 5 locations) and *C. triceratops* (5 individuals, 5 locations) from Tasmania. Pollinators for the DNA sequence study (Mant et al. 2002) were collected following established methods (Stoutamire 1975; Peakall 1990; Bower 1996, 2001). Orchids flowers were collected and offered as 'baits' to patrolling wasps. Testing and collection of wasp responders occurred in areas of close proximity to where orchids were sampled. Wasps were identified by Graham Brown (Museum and Art Gallery of Northern Territory). Morphological differences among three closely related (Mant et al. 2002) pollinator taxa were recorded and tabulated (Table 2) with reference to Bower and Brown (1997).

AFLP procedures. The (AFLP) procedure was performed using a modified version of the methods of Vos et al. (1995). Conditions followed the general protocol described by Invitrogen Life Technologies using fluorescently-labelled EcoRI primers. Total DNA was extracted from silica-dried leaf material using Qiagen Kits, as described in Mant et al. (2002).

Each DNA sample was cut with restriction endonucleases, *Eco*RI and *Mse*I, and the resulting fragments ligated to known double-stranded adapters using reagents purchased from Invitrogen™ (Cat. No. 10482-016). To approximately 200 ng of DNA were added 5 μl 5X reaction buffer, 13 μl H2O and the 2 μl of *Eco*RI/*Mse*I as directed in the Invitrogen AFLP Core Reagent Kit. Digestion was carried out at 37°C for 6 hours. Ligation products were subjected to pre-amplification using primers that match the adapter sequence with the addition of an additional base at the 3′ end. PCR reactions were performed on 5 μl undiluted ligation product in 28 μl reactions containing pre-amplification primer-mix I (Invitrogen™, Australia), BSA, 10X PCR Buffer (Sigma-Aldrich P-2192: 100 mM Tris-HCl, ph 8.3; 500 mM KCl; 15 mM MgCl; 0.01% gelatin) and 0.5 units Taq (Promega, Australia). Pre-amplification reactions were performed on a Hybaid OMN-E thermal sequencer using the following temperature profile: 20 cycles of (94°C 30 s, 56°C 1 min; 72°C 1 min).

After a two-fold dilution with TE buffer, the PCR products were amplified using *Mse*I and *Eco*RI primers with three additional selective bases. PCR reactions contained the following: 10X PCR Buffer, BSA, *Mse*I primer, *Eco*RI primer, 0.05 units Taq (Promega, Australia). PCR conditions used a touch down procedure starting with (94°C 30 s, 66°C 30 s; 72°C 2 min) with the annealing temperature reduced by 1°C over each of the next ten cycles, followed by 20 cycles at (94°C 20 s, 56°C 30 s; 72°C 2 min), and finished with 72°C for 30 min. Six different combinations of two *Mse*I primers and three *Eco*RI primers were chosen because they had a suitable number of fragments: (*Mse*CAT/*Eco*ACT, *Mse*CTA/*Eco*ACT, *Mse*CAT/*Eco*AGC, *Mse*CTA/*Eco*AGC; *Mse*CAT/*Eco*AGC; *Mse*CTA/*Eco*ACA). Fluorescent labels (FAM or HEX) were added to the EcoRI-primers allowing accurate sizing using an ABI 3700 capillary electrophoresis system with internal size markers in each reaction. Primers were tested for reproducibility across different restriction-ligation digests of the same individuals, in addition to different pre-amplifications and selective amplifications.

AFLP data analysis. Fragments between 70 and 450 base pairs were scored as either present or absent using Genotyper® Version 2.5 (Perkin Elmer Applied Biosystems). Each size class was treated as a dominant binary character in the recognition that AFLP heterozygotes are not usually detected. The calculation of genetic distances for the AFLP data set and subsequent Analysis of Molecular Variance (AMOVA) followed the method of Peakall et al. (1995) and Maguire et al. (2002) using the software GenAlEx V5 (Peakall and Smouse 2004). Individual pairwise genetic distance was calculated as:

Table 1. AFLP vouchers. Species are arranged alphabetically within each of the three main *Chiloglottis* clades (F: Formicifera clade; R: Reflexa clade; V: Valida clade). NP: National Park, NR: Nature Reserve. Undescribed geographical forms of *C. formicifera* and *C. pluricallata* that have distinct pollinators are listed with an abbreviated geographical location

Clade	Taxon	Voucher & species code	Lat/Long	Locality
F	*C. formicifera*	CF119	33 52S 151 02E	NSW: Royal NP
F	*C. formicifera*	CF124	31 18 13S 149 02 16E	NSW: Warrumbungles NP
F	*C. formicifera*-TENT	CFF120	28 58 00S 152 04 52E	NSW: Tenterfield region
F	*C. formicifera*-TENT	CFF122	28 51 00S 151 58 00E	NSW: Giraween NP
F	*C. platyptera*	CPT188	31 55 48E 151 20 45S	NSW: Barrington Tops
F	*C. trullata*	CTA123	23 51 04S 149 05 35E	QLD: Blackdown NP
F	*C. trullata*	CTA126	23 51 04S 149 05 35E	QLD: Blackdown NP
F	*C. truncata*	CTU121	27 14 47S 152 03 37E	QLD: Crows Nest
F	*C. truncata*	CTU121	27 14 47S 152 03 37E	QLD: Crows Nest
F	*C. truncata*	CTU125	27 14 47S 152 03 37E	QLD: Crows Nest
F	*C. trapeziformis*	CTZ118	33 06 16S 145 04 34E	NSW: Orange
F	*C. trapeziformis*	CTZ189	33 34 12S 150 14 27E	NSW: Mt York
R	*C. anaticeps*	CA105	31 07 19S 152 20 20E	NSW: Stokes Mt
R	*C. anaticeps*	CA112	31 07 19S 152 20 20E	NSW: Stokes Mt
R	*C. anaticeps*	CA113	31 07 19S 152 20 20E	NSW: Stokes Mt
R	*C. diphylla*	CD156	33 30 56S 150 29 19E	NSW: Bilpin
R	*C. diphylla*	CD157	33 30 56S 150 29 19E	NSW: Bilpin
R	*C. reflexa*	CR158	33 35 35S 150 16 02E	NSW: Mt Victoria
R	*C. reflexa*	CR159	33 35 35S 150 16 02E	NSW: Mt Victoria
R	*C. reflexa*	CR160	34 24 02S 150 50 26E	NSW: Robertsons Lookout
R	*C. reflexa*	CR168	34 24 02S 150 50 26E	NSW: Robertsons Lookout
R	*C. reflexa*	CR169	34 40 09S 150 42 39E	NSW: Barren Grounds
R	*C. reflexa*	CR170	34 21 00S 150 47 00E	NSW: Macquarie Pass
R	*C. reflexa*	CR171	34 40 09S 150 42 39E	NSW: Barren Grounds
R	*C. reflexa*	CR173	34 24 02S 150 50 26E	NSW: Robertsons Lookout
R	*C. seminuda*	CSM153	33 32 26S 150 38 05E	NSW: Kurrajong Heights
R	*C. seminuda*	CSM154	33 43 45S 150 26 34E	NSW: Hazelbrook
R	*C. sphyrnoides*	CSP109	31 39 60S 151 48 44E	NSW: Giro SF
R	*C. sphyrnoides*	CSP110	31 37 20S 152 11 11E	NSW: Styx State Forest
R	*C. sylvestris*	CSY108	29 28 24S 152 19 07E	NSW: Washpool NP
R	*C. sylvestris*	CSY152	33 32 26S 150 38 05E	NSW: Kurrajong Heights
R	*C. trilabra*	CT107	30 27 07S 152 18 47E	NSW: Cathedral Rocks
R	*C. trilabra*	CT116	30 27 07S 152 18 47E	NSW: Cathedral Rocks
R	*C. trilabra*	CT161	33 29 39S 150 24 14E	NSW: Mt Wilson
R	*C. trilabra*	CT162	33 30 40S 150 22 24E	NSW: Mt Wilson
R	*C. trilabra*	CT163	33 30 40S 150 22 24E	NSW: Mt Wilson
R	*C. trilabra*	CT164	35 21 26S 148 40 02E	ACT: Namadgi NP
R	*C. trilabra*	CT165	35 21 26S 148 40 02E	ACT: Namadgi NP
R	*C. trilabra*	CT166	35 21 26S 148 40 02E	ACT: Namadgi NP
R	*C. trilabra*	CT172	33 26 34S 149 50 40E	NSW: Sunny Corner NR
V	*C. chlorantha*	CCH128	33 51 16S 150 01 52E	NSW: Kanangra-Boyd NP
V	*C. chlorantha*	CCH130	34 39 S 150 46 E	NSW: Jamberoo
V	*C. cornuta*	CCO142	41 16 23S 145 36 58E	TAS: Hellyer Gorge

Table 1. (Continued)

Clade	Taxon	Voucher & species code	Lat/Long	Locality
V	*C. cornuta*	CCO144	41 41 43S 146 46 24E	TAS: Liffey Falls
V	*C. grammata*	CG136	42 54 52S 147 15 34E	TAS: Mt Wellington
V	*C. grammata*	CG137	41 21 33S 147 24 00E	TAS: Mt Barrow
V	*C. grammata*	CG138	41 20 55S 147 22 46E	TAS: Mt Barrow
V	*C. grammata*	CG139	41 42 06S 146 43 24E	TAS: Liffey Falls
V	*C. grammata*	CG143	42 40 47S 146 41 17E	TAS: Mt Field NP
V	*C. grammata*	CG146	42 13 34S 147 07 04E	TAS: Table Mt
V	*C. grammata*	CG150	42 53 34S 147 15 38E	TAS: Mt Wellington
V	*C. gunnii*	CGU148	42 17 29S 147 04 26E	TAS: Bothwell
V	*C. gunnii*	CGU151	42 54 52S 147 15 34E	TAS: Mt Wellington
V	*C. triceratops*	CTP134	42 40 47S 146 41 17E	TAS: Mt Field NP
V	*C. triceratops*	CTP135	42 54 52S 147 15 34E	TAS: Mt Wellington
V	*C. triceratops*	CTP141	42 13 34S 147 07 44E	TAS: Table Mt
V	*C. triceratops*	CTP147	41 20 55S 147 22 46E	TAS: Mt Barrow
V	*C. triceratops*	CTP149	43 02 54S 147 07 48E	TAS: Pelverata Falls
V	*C. valida*	CV127	35 21 26S 148 40 02E	ACT: Namadgi NP
V	*C. valida*	CV129	33 26 34S 149 50 40E	NSW: Sunny Corner NR
V	*C. valida*	CV131	35 20 41S 148 49 21E	ACT: Namadgi NP
V	*C. valida*	CV133	33 26 34S 149 50 40E	NSW: Sunny Corner NR
V	*C. pluricallata*	CP005	31 56 40S 151 26 58E	NSW: Barrington Tops
V	*C. pluricallata*	CP026	31 57 20S 151 25 36E	NSW: Barrington Tops
V	*C. pluricallata*	CP040	31 55 42S 151 26 40E	NSW: Barrington Tops
V	*C. bifaria*	CB063	31 58 56S 151 27 33E	NSW: Barrington Tops
V	*C. bifaria*	CB069	31 55 05S 151 33 33E	NSW: Barrington Tops
V	*C. bifaria*	CB093	32 05 23S 151 35 00E	NSW: Gloucester Tops
V	*C. pluricallata*	CP111	32 31S 151 12E	NSW: Hanging Rock NR
V	*C. pluricallata*	CP112	32 31S 151 12E	NSW: Hanging Rock NR
V	*C. pluricallata*-NE	CAP119	31 29S 152 25E	NSW: New England NP
V	*C. pluricallata*-NE	CAP120	31 29S 152 25E	NSW: New England NP
V	*C. pluricallata*	CP124	31 29S 152 25E	NSW: New England NP
V	*C. pluricallata*	CP125	31 29S 152 25E	NSW: New England NP

Table 2. Morphological differences among *Neozeleboria proxima* and its sibling species (reproduced with modification from Bower and Brown 1997)

Morphological character	*N. proxima*	*N.* sp. nov. 29	*N.* sp. nov. 30
Fore coax emarginate	weakly	not	strongly
Hypopygium	triangular, not truncate	triangular, not truncate	subtriangular, truncate
Parameres	long, curved	normal?	normal?
Aedeagus length	0.75 mm	0.525 mm	0.5 mm
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

$$E = n(1 - (2nxy/2n)),$$

where n is the total number of polymorphic bands and 2nxy is the number of markers shared by two individuals. Both band presence and band absence are considered informative in this analysis. This is a true Euclidean metric as required for AMOVA. Total genetic variation was partitioned by AMOVA into three levels: among the three main clades (c), among species within clades (s) and within species. Variation was summarized both as the proportion of the total variance and as ϕ-Statistics or F-Statistic analogues, ϕ_{SC}, ϕ_{CT} and ϕ_{ST}. In the statistic, ϕ_{SC} represents the correlation of species within a clade, relative to the clade, while ϕ_{CT} is the correlation of species from the same clade, relative to the total, and ϕ_{ST} is the correlation of species within a clade, relative to the total. In this context, ϕ_{ST} is the AFLP equivalent of Fst among species. Statistical significance tests were performed by random permutation, with the number of permutations set to 1000.

Otherwise, the AFLP data were analyzed by maximum parsimony and UPGMA and neighbor joining (NJ) clustering using PAUP* version 4.0b (Swofford 2001). NJ and UPGMA trees were constructed using Nei and Li distance measure under the restriction site option in PAUP (Nei and Li 1979). Support for branches was assessed using the bootstrap method with 100 replicates (Felsenstein 1985). Outgroups were not included in this study due to difficulties in interpreting AFLP band homologies between *Chiloglottis* and related genera. The AFLP trees were left as unrooted networks and redrawn according to the root found using DNA sequence data (Mant et al. 2002).

Testing congruence between orchid and pollinator topologies. Two statistical tests comparing the phylogenies of *Chiloglottis* and its thynnine wasp pollinators were performed to examine the null hypothesis that the orchids have undergone cocladogenesis with their pollinators. For the wasps, we used the combined DNA sequence data of three genes (nuclear *wingless* and mitochondrial 16S and cyt*b*) published in Mant et al. (2002, GenBank numbers and voucher details available therein). The most parsimonious (MP) tree(s) and its length were calculated for the orchid and wasp data sets separately using PAUP* heuristic searches using TBR branch swapping and 50 random addition sequence starting trees. The MP tree of one data set was then forced onto the other data set and the tree lengths recalculated. The two trees were then compared using the Templeton test (Templeton 1983). Significantly different scores were interpreted as a rejection of the null hypothesis. The second test followed that used in Mant et al. (2002) by using a partition homogeneity test as implemented in PAUP*. To examine congruence in phylogenetic signal, orchid (AFLP) and wasp (DNA sequence) datasets were treated as two separate partitions of the one data set, in which the associated orchid-wasp species pairs were assigned as terminals. A significant difference between the tree length of the combined data set and the summed tree lengths of partitioned AFLP and DNA sequence data sets was again interpreted as a rejection of the null hypothesis.

Results

AFLP variation within and among *Chiloglottis* clades. The AFLP method produced presence/absence characters that were highly reproducible between treatments where it was examined in replicated samples. A total of 251 characters was scored across the entire genus (63, 48, 40, 39, 42, 19 for each primer combination of mCAT-eACA, -eACT, -eAGC and mCTA-eACA, -eACT, -eAGC). The partitioning of AFLP variation within *Chiloglottis* is shown in Table 3. A UPGMA tree (Fig. 1) shows three main clusters, corresponding to the Reflexa, Formicifera and Valida clades recovered under DNA sequence data (Mant et al. 2002) and illustrated with representative species in Fig. 2.

Statistical tests by random permutation in the AMOVA confirmed significant differentiation among clades, among the species within clades, and among species with respect to the total. However, the magnitude of the variation partitioned amongst the three levels varied greatly, with 73% of the total genetic variance accounting for the differences among the clades, with the remainder apportioned more or less equally among and within the species in each clade (15% and 12% respectively, see Table 3). A similar disparity in uncorrected pairwise DNA sequence diver-

Table 3. Results of Analysis of Molecular Variance (AMOVA) showing percentage of AFLP variation apportioned among groups and Phi-statistics. ***$P < 0.001$, **$P < 0.002$

Source	% Var, ϕ value
Chiloglottis	
Among Clades	73
Among Species/Clades	15
Indiv./Within Species	12
ϕ_{CT}	0.726***
ϕ_{SC}	0.545***
ϕ_{ST}	0.875***
C. reflexa v *C. trilabra*	
Among Species	16
Individuals.Within Species	84
ϕ_{ST}	0.157***
C. grammata v *C. triceratops*	
Among Species	52
Individuals.Within Species	48
ϕ_{ST}	0.525**

gences among (28%) versus within (0.2% to 0.3%) each of the three clades was recorded in Mant et al. (2002). Fixed AFLP band differences among the three clades were high (43 to 57) relative to the number of shared polymorphisms (from 5 to 23). However, bands unique to individual species were low, ranging from 1-7 in the Reflexa clade, 0 to 1 in the Formicifera clade and 1-14 in the Valida clade.

Within the analyses by clade, more variation was detected within species than among species within the Formicifera clade and there were no significant pairwise P-values among species, reflecting the low sample sizes. Thus, we were unable to statistically separate any of the species in this clade (see also the UPGMA analysis, Fig. 1). For the remaining two clades there was more overall genetic variation detected among species than within species (Table 3). However, individual pairwise P-values were mostly not significant. The UPGMA showed clustering along species lines in the Valida and Reflexa clades with high bootstrap support. However, relationships among those species were either not well resolved or exhibited poor bootstrap support. Among the two species pairs examined in more detail, *C. grammata* and *C. triceratops* separated along species lines with high bootstrap support in the NJ tree (Fig. 3a) and with high significant genetic differentiation ($\phi_{ST} = 0.525$, $P < 0.002$). Among the *C. reflexa* and *C. trilabra* samples, there was low but significant genetic differentiation ($\phi_{ST} = 0.16$, $P < 0.001$), with poor separation of the species in the NJ tree (Fig. 3b).

Examining congruence between orchid and wasp topologies. Figure 4 shows a comparison of the phylogeny of *Chiloglottis* (as estimated by maximum parsimony analysis of AFLPs) with that of their specific pollinators. Only those samples are included for which pollinator molecular data are available (16 plant-pollinator pairs). For the orchids, 182 parsimony informative characters were analyzed, finding one tree of length 270. For the wasps, two MP trees of length 1191 were found on analysis of 334 informative characters from the three genes. Figure 4 shows the strict consensus of those two wasp trees. Relationships within the *Chiloglottis* Reflexa group differ between parsimony (Fig. 4) and UPGMA (Fig. 1) trees. However, maximum parsimony finds the same topology when all taxa are included (tree not shown) and when the two taxa without pollinator data are excluded.

A mixture of matching and non-matching pattern between orchid and wasp topologies can be observed (Fig. 4). The topology of the Reflexa group matches that of its autumn-emerging pollinators. Analyses using the Templeton test (Table 4) revealed significantly longer tree-lengths when the best topologies of each group were forced onto the alternative dataset. The test was run with and without the orchid-pollinator pair, *C. grammata* and *Eirone*, as *Eirone* has previously been shown to be distantly related to the remaining *Neozeleboria* pollinators of *Chiloglottis* (data not shown). However, in all comparisons, the differences were found to be significant, leading to an overall rejection of the null hypothesis of cocladogenesis. The partition homogeneity test similarly rejects the null

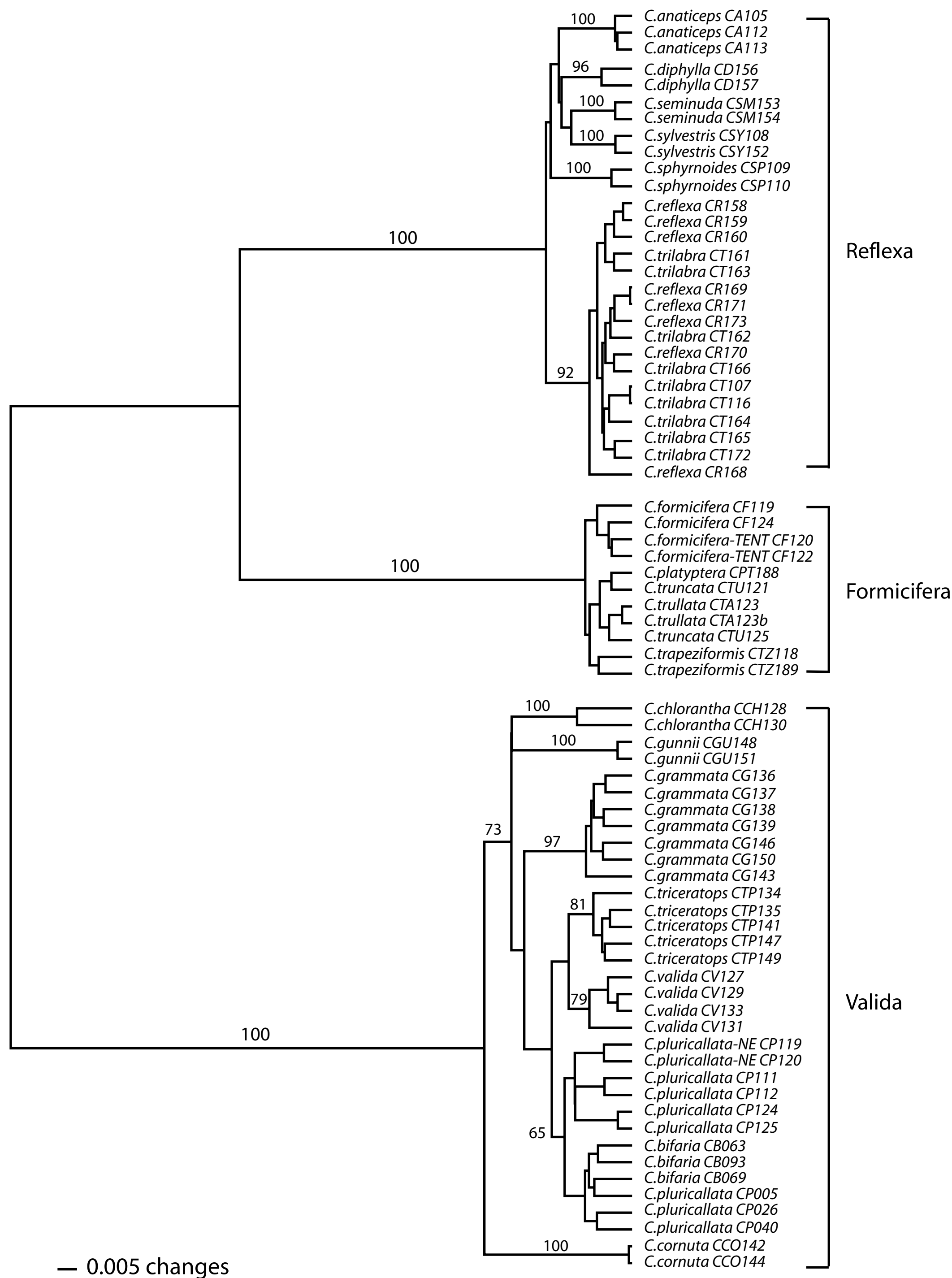

Fig. 1. UPGMA tree of *Chiloglottis* (72 samples, 251 AFLP bands, numbers on branches are bootstrap percentages). The genetic differentiation between the three major groups (Reflexa, Formicifera and Valida) is greater than that found among the species within each. The Reflexa group is autumn-flowering, whereas the other two groups flower in spring-summer. Multiple individuals and populations of each sampled species are generally supported by strong bootstrap percentages. However, relationships among species are not well supported

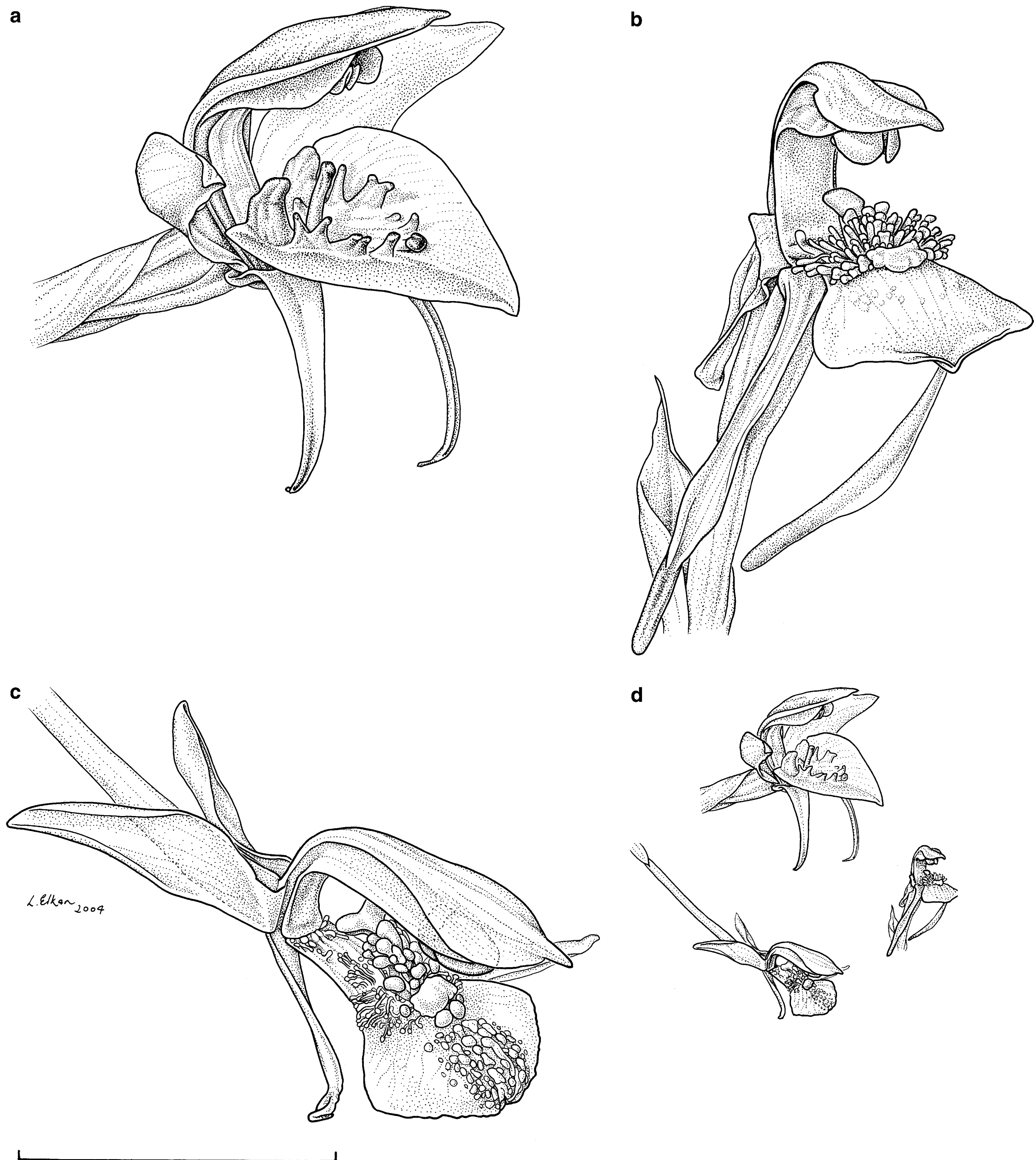

Fig. 2. Flowers of *Chiloglottis* species from the Valida, Reflexa and Formicifera clades. The labella are passively mobile, particularly in the Valida clade, allowing male wasps that are deceived by floral odours and the insectiform calli to be tipped towards the column during attempted copulation. Pollinia placement is to the wasp thorax. (**a**) *C. 'bifaria'* ms. D. L. Jones (*C.* affinity *pluricallata* D. L. Jones), scale = 1.5 cm (**b**) *C. sylvestris* D. L. Jones & M. A. Clements, scale = 0.75 cm (**c**) *C. formicifera* Fitzg. scale = 0.75 cm (**d**) Flowers drawn at same scale (scale = 4 cm)

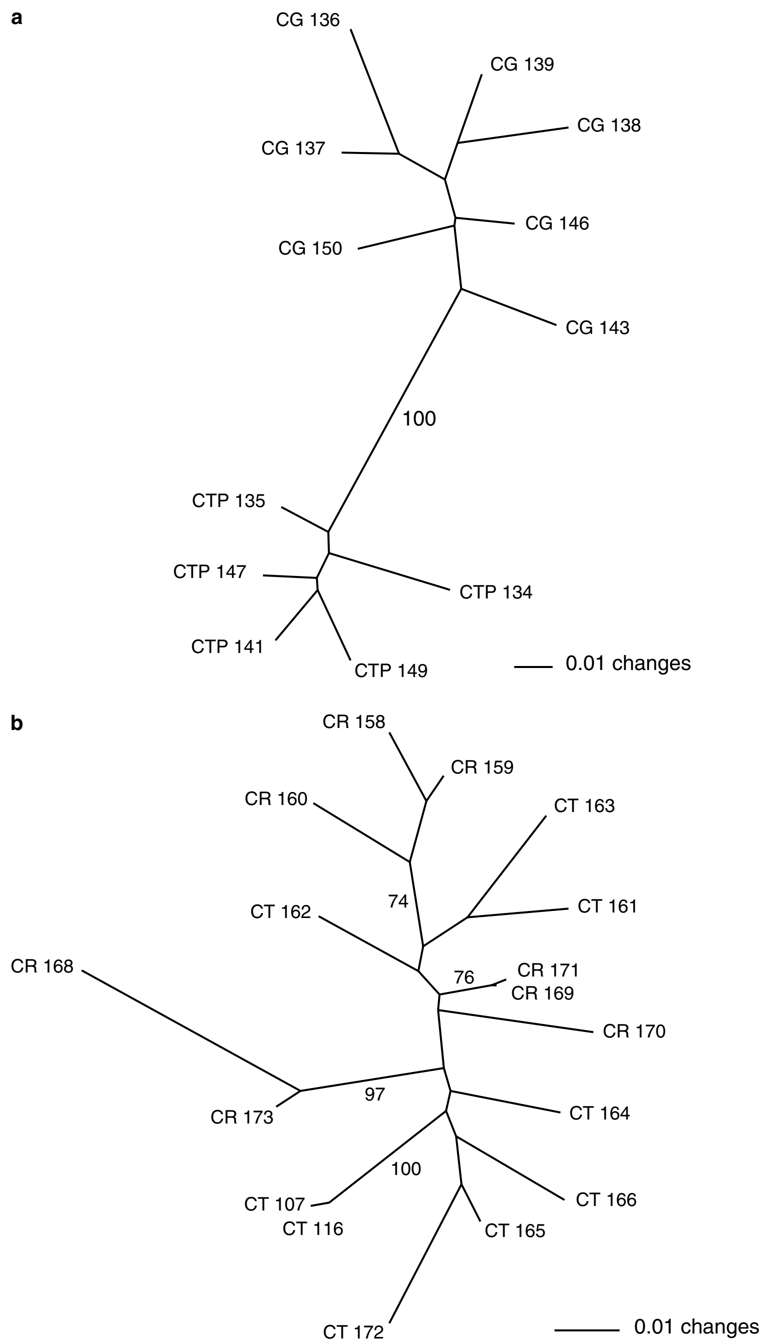

Fig. 3. (**a**) Neighbour joining (NJ) tree of co-flowering and sympatrically distributed *C. grammata* G. W. Carr (CG) and *C. triceratops* D. L. Jones (CTP) from Tasmania, (**b**) NJ of the co-flowering *C. reflexa* Labill. (CR) and *C. trilabra* Fitz. (CT). See Table 1 for localities

hypothesis of cocladogenesis by finding that the orchid and wasp datasets comprise a significant data partition ($P < 0.01$).

Discussion

The pattern of AFLP variation within *Chiloglottis* is largely congruent with DNA sequence data (Mant et al. 2002) in showing a marked disparity among three sub-generic clades compared to the variation found within each clade. The three clades are morphologically and phenologically divergent (Figs. 1 and 2). The Reflexa clade is entirely autumn-flowering, with a single flower held on a long peduncle (between 5 to 15 cm high) and recurved lateral

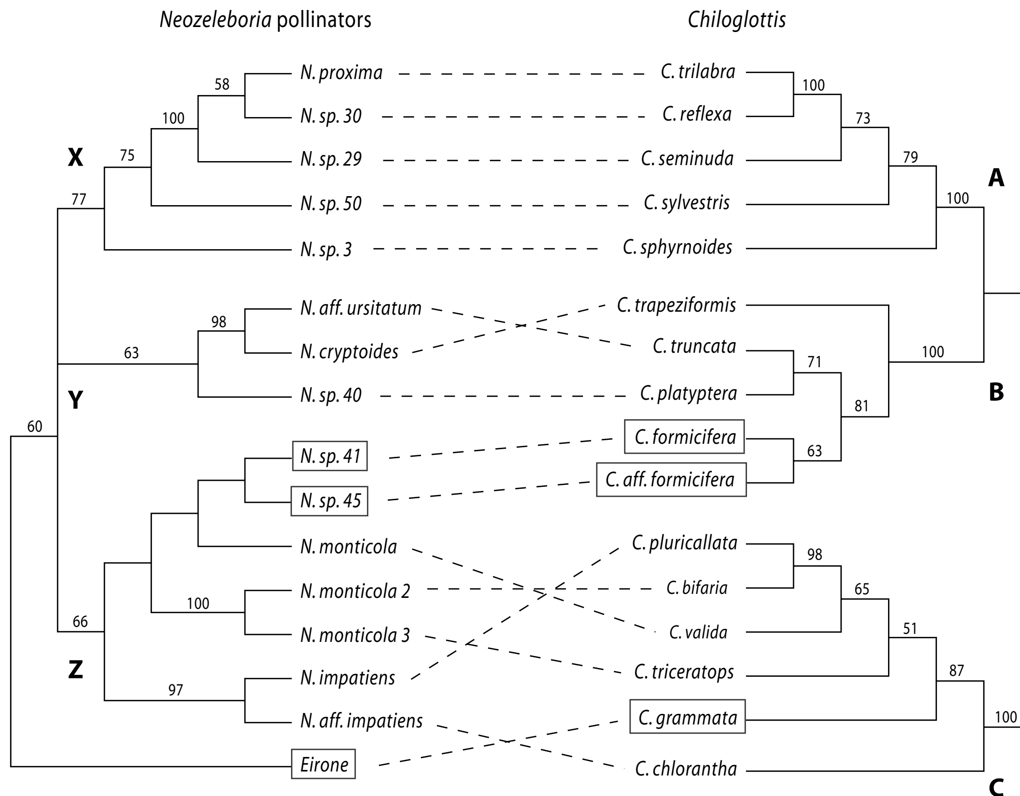

Fig. 4. A comparision of orchid and wasp phylogenies as estimated by AFLPs for *Chiloglottis* and DNA sequence data for the Thynnine wasp pollinators (*Neozeleboria* and *Eirone*). Orchids are aligned with their respective pollinators. The wasp phylogeny was based on a combined analysis of three genes (nuclear *wingless* and mitochondrial 16S and cyt*b*) presented in Mant et al. (2002). GenBank accessions can be found in that paper. The topology shown is the strict consensus of two MP trees of length 1191 (334 informative characters). The phylogeny of *Chiloglottis* (length 270) was based on 182 parsimony informative AFLP characters available for this reduced taxon set for which pollinator samples were present. The wasp tree is rooted using *Eirone* and the root in the orchid tree is the same as that found under DNA sequence data. The boxed taxa highlight cases of incongruence among the three main clades found in both orchid and wasp trees. Several other topological disagreements are evident within each of those three clades. Informal clade names: *Chiloglottis*, **A** Reflexa **B** Formicifera, **C** Valida; *Neozeleboria* **X** Proxima **Y** Cryptoides **Z** Monticola

petals. The labellum is narrow in the proximal first third, then expands into a broad triangular to spathulate apex. The *C. trapeziformis* clade is generally similar to the Reflexa clade in its peduncle length and labellum shape, but flowers in early spring. The late spring to

Table 4. Testing the null hypothesis of cocladogenesis between *Chiloglottis* and its thynnine wasp pollinators. The asterisk indicates significant difference from the shortest tree(s)

	Templeton Test Comparing Tree Lengths	
	Orchid	Wasp
Score based on orchid data set	270	424/426
P value	(best tree)	P < 0.001*
Score based on wasp data set	1320	1191

summer-flowering Valida clade differs by the presence of larger flowers held low to the ground, a cordate labellum, broad spreading lateral petals and generally less dense insectiform calli on the labellum (Jones 1991, Bower 1996).

Although differentiation among species in each main clade is usually higher than that found within species, those differences are not great. While AFLP patterns shown in the UPGMA tree indicate the distinct genetic clustering of many *Chiloglottis* species, the lack of extensive intraspecific sampling restricts the conclusions we can draw about the establishment of reproductive barriers among taxa. Where a higher number of individuals has been sampled (Fig. 3a,b), our results show evidence both for and against the establishment of genetic barriers. The Tasmanian species, *C. grammata* and *C. triceratops*, pollinated by an undescribed *Eirone sp.* and a form of *Neozeleboria monticola* respectively (Bower unpublished, Mant unpublished), separate strongly along species lines. The molecular pattern clearly supports the origin of reproductive barriers in these two co-flowering and sympatrically distributed species, despite the relatively low number of individuals that have been sampled. However, two geographically overlapping species from mainland Australia, *C. reflexa* and *C. trilabra*, pollinated by the closely related *N. sp. 30* and *N. proxima* respectively, are indistinguishable by AFLP data. Certain key taxa such as the *C. reflexa*-*C. trilabra* and *C. pluricallata* species complexes could be further investigated using more intensive population sampling to examine evidence for hybridisation, cross-attraction of pollinators and floral odour variation among taxa.

Although this AFLP evidence suggests *Chiloglottis* species attracting different pollinators have established reproductive barriers, the overall pattern of molecular variation across *Chiloglottis* makes it difficult to reject the possibility of interspecific gene flow over longer evolutionary timescales. In the case of *Chiloglottis*, both DNA sequences and AFLPs reveal a disparity in genetic variation among versus within its three major clades. AMOVA analyses of each of the major clades (Table 3) show that much of the AFLP variation is shared among closely related species. A similar pattern is found in *Ophrys* with low sequence differentiation among species (Soliva et al. 2001), a lack of any deep branching pattern leading to outgroup genera (Bateman et al. 2003), and a sharing of microsatellite variation among species (Soliva and Widmer 2003). Indeed, genetic patterns in *Ophrys* indicate interspecific gene flow is prevalent even among sympatric taxa that attract distinct pollinators and are strongly differentiated in floral odour (Mant et al. in press).

For *Chiloglottis*, two scenarios could account for a phylogenetic structure that lacks deep branching pattern while species at the tips of the trees share considerable variation. Firstly, a burst of species divergence occurred recently within each of the three clades leaving no trace of older lineages, which either did not exist or were lost to extinction. In this case, the sharing of molecular variation among species could be attributed to the retention of ancestral polymorphism rather than to interspecific gene flow (Avise 1994, Hilton and Hey 1997). While this scenario is possible, it seems unlikely that the process occurred repeatedly in three independent *Chiloglottis* lineages with divergent ecological and geographical distributions and different flowering phenologies. Under the second scenario, each of the three clades represents a single interbreeding evolutionary unit or ‘syngameon’ undergoing merge-and-diverge oscillations (Grant et al. 2004). Pollinator mediated selection may be strong enough to lead to divergence in certain traits, such as floral odour, that are important for pollinator attraction (Mant et al. 2002) and even to the disruption in gene flow among taxa following pollinator shifts (Fig. 3a). However, over the long term, any accumulated genetic differences are lost following the re-establishment of gene flow

across the group as a whole. The observed sharing of AFLP variation may thus be due to a long history of past introgression. The lack of deep branching pattern in the phylogeny of each *Chiloglottis* lineage may also reflect past introgression rather than a history of evolutionary stasis or extinction, although we are not able to arbitrate between these scenarios with current data. A similar evolutionary history could be envisaged for the sexually deceptive *Ophrys* and *Caladenia*.

Species divergence and cocladogenesis. The comparison of orchid and pollinator phylogenetic trees reveals a degree of matching topological pattern that suggests cospeciation should be considered as an explanation for the historical association. In particular, orchid species from the autumn-flowering Reflexa group show the same historical pattern as that of their pollinators from the autumn-emerging group of pollinators related to *N. proxima*. However, the occurrence of clear switches (non-matching pattern) between major clades, such as that seen in the distantly related *Eirone* (pollinator of *C. grammata*) and the pollination of *C. formicifera* and *C.* aff. *formicifera*-Tenterfield by *N.* sp. 41 and *N.* sp. 45, indicate that such switching events are common in the history of the interaction. Further, our tests of the overall cladistic congruence between orchid and wasp data favour a rejection of cocladogenesis, in accordance with the conclusions drawn in the DNA sequence study of Mant et al. (2002).

Unfortunately, topological comparison alone may not be able to arbitrate between these competing hypotheses (Sorenson, et al. 2004). It is noted that the tests employed examine congruence across the entire dataset while local patterns consistent with cospeciation may be overlooked. However, additional observations lead us to favour the hypothesis that orchid and wasp divergences have occurred independently even in cases of matching pattern as found in the Reflexa-Proxima association. Gas chromatography with electroantennographic detection studies from orchids and their pollinators demonstrate shared active floral scent components across thynnine-pollinated orchid taxa (Mant et al. 2002, Schiestl et al. 2003). This indicates that the tendency of related orchids to use related wasps as pollinators may reflect phylogenetic patterns in the sex pheromones of the wasps themselves. Secondly, a lack of temporal congruence in divergence times is suggestive of speciation occurring independently in both groups (Mant et al. 2002). Finally, the presence of non-pollinating *Neozeleboria* species that are close relatives of known pollinators suggests that if cospeciation was occurring, the association between plant and pollinator must have been repeatedly lost (Mant et al. unpubl. data). This argument also holds for the autumn-emerging Proxima group, which comprises both pollinators and non-pollinators (Mant et al. unpubl. data).

Bioassays have confirmed the chemical simplicity of the thynnine sex pheromone system and its mimicry by orchids. A single compound, chiloglottone, was shown to be solely responsible for pollinator attraction in *C. trapeziformis* (Schiestl et al. 2003), whereas two to three related compounds of minor structural variation to chiloglottone are active in other *Chiloglottis* species (Mant et al. 2002, Mant and Schiestl, unpublished). A single compound is also responsible for pollinator attraction in the ichneumonid wasp pollinated *Cryptostylis* (Schiestl, et al. 2004). To learn more about how chemical changes in the floral odour are associated with species divergence in *Chiloglottis,* future research should focus on identifying the active compounds of more orchid species and populations. Targeting sister-species identified from the phylogenetic reconstructions in this study provides a means of investigating adaptive shifts associated with fragrance divergence in this group.

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