Evolution, 56(5), 2002, pp. 888–898

A PHYLOGENETIC STUDY OF POLLINATOR CONSERVATISM AMONG SEXUALLY DECEPTIVE ORCHIDS

JIM G. MANT,[1,2,3] FLORIAN P. SCHIESTL,[1,4] ROD PEAKALL,[1] AND PETER H. WESTON[3]
[1]*School of Botany and Zoology, Australian National University, Canberra, Australian Capital Territory, 0200, Australia*
[2]*E-mail: Jim.Mant@anu.edu.au*
[3]*Royal Botanic Gardens Sydney, Mrs Macquaries Road, Sydney, 2000, Australia*
[4]*Geobotanical Institute ETH Zurich, Zollikerstrasse 107, Zurich, CH-8008, Switzerland*

Abstract.—Orchids of the genus *Chiloglottis* are pollinated through the sexual deception of male wasps mainly from the genus *Neozeleboria* (Tiphiidae: Thynninae). The orchids mimic both the appearance and sex pheromones of wingless female thynnines but provide no reward to the deceived males. Despite the asymmetry of this interaction, strong pollinator specificity is typical. Such plant-pollinator interactions would seem to be relatively flexible in the plant's adaptive response to variation in the local pollinator resource. However, we present DNA sequence data on both orchids and wasps that demonstrate a pattern of pollinator conservatism operating at a range of taxonomic levels. Sequence data from the wasps indicate 15 of 16 *Chiloglottis* pollinators are closely related members of one clade of Thynninae. A pattern of congruence between orchid and wasp phylogenies is also demonstrated below the generic level, such that related orchids tend to use related thynnine wasps as specific pollinators. Comparative physiological data on the wasp responses to the floral scents of two *Chiloglottis* species and one outgroup, *Arthrochilus*, indicate similar attractive volatile chemicals are used by related orchid taxa. By extension, we infer a similarity of sex pheromone signals among related thynnines. Thus, the conservative pattern of pollinator change in sexually deceptive orchids may reflect phylogenetic patterns in the sex pheromones of their pollinators.

Key words.—Deceptive pollination, evolutionary constraint, floral odor, gas chromatography with electroantennographic detection, pseudocopulation, specialization, thynnine wasp.

Received July 5, 2001. Accepted December 14, 2001.

The Orchidaceae possess an extraordinary variety of pollination systems and attract an equally impressive array of pollinators. Many of these systems are also highly specialized, attracting a single or very few pollinating species (Tremblay 1992). One of the more unusual modes of pollination involves the sexual deception of male Hymenoptera by chemical, visual, and tactile mimicry of the female insect, a syndrome often referred to as pseudocopulation. A combination of chemical and behavioral studies on the sexually deceptive *Ophrys* from Europe (Bergström 1978; Borg-Karlson 1990; Schiestl et al. 1999, 2000; Ayasse et al. 2000) has demonstrated that floral odors mimicking hymenopteran sex pheromones are of key importance to this plant-pollinator interaction. This form of sexual mimesis has arisen numerous times in the family yet finds its most diverse expression among the terrestrial Diurideae of Australia, with up to nine genera and more than 100 species pollinated by a range of sexually deceived Hymenoptera (Dafni and Bernhardt 1990). The evolution of floral diversity and, by extension, of reproductive isolation in angiosperms is often assumed to be mediated by pollinator selection (Grant 1994). Yet, because most plant species tend toward generalized pollination, claims of directional selection seem paradoxical (Ollerton 1996; Waser 1998). Although the extent of pollinator specialization in the angiosperms may indeed be exaggerated by pollination biologists (Waser et al. 1996), extreme specificity appears to be the rule in deceptive orchids that mimic the species-specific sexual traits of insects (Nilsson 1992). Recent studies on Australian sexually deceptive taxa have confirmed this view by documenting widespread specific pollination in both sympatric and allopatric species (Stoutamire 1975; Peakall 1989; Peakall and Handel 1993 ; Bower 1996; Peakall and Beattie 1996; Alcock 2000; Bower 2001). Thus, in sexually deceptive systems, the evolution of reproductive isolation or speciation may actually be closely associated with pollination, as changes in the chemistry of floral scents may permit the attraction of distinct pollinators (Paulus and Gack 1990; Grant 1994).

Given the link between specific pollinators and adaptive change, it is interesting that little attention has been paid to how specialization at the species level translates to specialization at higher taxonomic levels (Waser et al. 1996; Johnson and Steiner 2000). Are sexually deceptive orchids highly labile in the kind of pollinator used, switching readily among unrelated insects? Or are these deceptive orchids more often constrained to particular taxonomic groups of pollinators? One might expect shifts in specific pollinators to occur erratically, according to ecological advantage rather than pollinator phylogeny. Alternatively, pollinator conservatism among clades of sexually deceptive orchids may be favored by the nature of the chemical mimicry system employed. As it is, particular orchid groups vary greatly in the diversity of pollinators they use. In South Africa, diversification of the mainly food-deceptive *Disa* has been associated with major adaptive shifts in pollinators—from birds, hawkmoths, and honeybees—even among closely related species (Johnson et al. 1998). At the other extreme, specialized pollinator rewards restrict orchids in the Catasetinae solely to fragrance collecting male euglossine bees (Dressler 1981; Chase and Hills 1992). The European sexually deceptive *Ophrys* has pollinators deriving from six families across three superfamilies of Aculeate Hymenoptera (Borg-Karlson 1990) in some cases with intraspecific forms using different families (Paulus and Gack 1990). However, several solitary bee genera figure highly, including *Andrena* in the Andrenidae and *Eucera*, *Tetralonia*, and *Anthophora* in the Anthophoridae (Paulus and Gack 1990).

© 2002 The Society for the Study of Evolution. All rights reserved.

In the Diurideae, pollinators of sexually deceptive orchids derive from among five hymenopteran families, including Pergidae, Formicidae, and ichneumonid, scoliid, and tiphiid wasps (for pollinator accounts, see van der Cingel 2001 and references therein). As in *Ophrys*, the high pollinator diversity associated with sexually deceptive diurids might suggest a tendency in the group for shifts onto phylogenetically disparate pollinators. However, much of the pollinator diversity in the Diurideae may be accounted for by several independent origins of sexual deception. Recent molecular phylogenies (Kores et al. 2001) confirm this interpretation, indicating at least one origin in the thynnine wasp–pollinated *Caladenia* and between two and four separate origins in the remaining four orchid taxa: *Cryptostylis* (Ichneumonidae), *Leporella fimbriata* (Formicidae), *Calochilus* (Scoliidae), and subtribe Drakaeinae (Thynninae with one exception, *Caleana major* pollinated by a member of the Pergidae). These observations suggest that, although several pollinating taxa are used by sexually deceptive diurids, specialization is evident particularly at the generic level, with pollinator shifts among higher hymenopteran taxa being rare.

In this paper, we investigate the extent of pollinator conservatism and how it may be maintained by reconstructing both orchid and pollinator relationships among 16 species of one genus, *Chiloglottis* (Diurideae: Drakaeinae), pollinated by male thynnine wasps (Tiphiidae: Thynninae). By examining the responses of wasp olfactory receptors to orchid scents using gas chromatography with electroantennographic detection (GC-EAD), we assess the basic principles of pollinator attraction in two *Chiloglottis* species and one closely related genus, *Arthrochilus*. These phylogenetic and comparative physiological data are used to address the following questions: Are there patterns of phylogenetic congruence between *Chiloglottis* species and their wasp pollinators? Do specific pollinators show distinct physiological responses to *Chiloglottis* floral odors? Do the pollinators of different orchid species respond to similar compounds?

Materials and Methods

DNA Material

Sampling of orchids and their respective pollinators for this study follows pollinator determinations for the majority of the 23 described, and several undescribed species of *Chiloglottis* by C. C. Bower (1996; unpubl. data), following earlier reports by Stoutamire (1974, 1975). In total, 16 species of *Chiloglottis* and their pollinators have been sampled from throughout the eastern seaboard of Australia and Tasmania. Although not all *Chiloglottis* species whose pollinators have been determined could be sampled, the taxa collected represent a substantial survey of the genus (four described species are lacking pollinator accounts, one is self-pollinating, and two have wasp pollinators allied closely to species sampled in this study). Each of the sampled orchid species has distinct pollinators either in sympatry or allopatry and are distinguished by diagnostic, if in some cases minor, morphological characters (Jones 1991; Bower 1996). One orchid outgroup, *Arthrochilus huntianus*, and its pollinator, *Arthrothynnus huntianus* (Bower 2001), and three nonpollinating Thynninae outgroups are included.

The specific status of the majority of wasp pollinators sampled is not in doubt, although several await formal taxonomic treatment (G. Brown, pers. comm.). However, certain pollinators remain indistinguishable on morphological grounds and are referred to as *Neozeleboria monticola* (1), *N. monticola* (2), *N. monticola* (3), *N. impatiens* (1), and *N. impatiens* (2). Choice experiments among translocated orchid species suggests there is a complex geographical pattern of pheromonal variation among these morphologically similar forms of *N. monticola* and *N. impatiens* (Bower and Brown 1997; C. C. Bower, unpubl. data). The geographical forms of these two wasp species pollinate morphologically distinct species of *Chiloglottis* living in different geographical regions (see Table 1 for localities). Vouchers for orchid accessions are held at the National Herbarium of New South Wales (NSW) or Australian National Herbarium (CANB), and wasps at The Australian Museum, Sydney. Pollinating wasps were caught following procedures outlined in Peakall (1990) and wasp species identifications were confirmed by G. Brown (Dept. of Primary Industries and Fisheries, Northern Territory, Australia). Accession details of orchid and wasp samples are listed in Table 1.

Orchid Sequencing

DNA sequence data from one nuclear (ITS) and one chloroplast locus (*trn*T/L) were examined. Orchid DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Melbourne, Australia) and further purified using a modified version of the diatomite method of Gilmore et al. (1993). Primers ITS1F and ITS 2R from Baldwin et al. (1992) were used for amplification and sequencing of the entire ITS region. Two additional primers (A. Perkins, unpubl. data) anchored in the 5.8S gene (Cal-1R, 5′-CCAAGATATATCCATTGCCGAGAGTC-3′) and Cal-2F, 5′-CAGAATCCCGTGAACCATCGAG-3′) were used in ITS sequencing reactions. The chloroplast intergenic spacer between *trn*T (UGU) and the *trn*L (UAA) 5′ exon was sequenced using the published primers, B48557 and A49291 (Taberlet et al. 1991). Polymerase chain reactions (PCRs) (50 μl) contained 5 pmol of each primer, 10 mM Tris-Cl (pH 8.3), 3 mM MgCl, 50 mM KCl, 0.2 mM of each dNTP, 1 unit of *Taq* polymerase (Fisher Taq F1, Biotec Perth, Australia) and 4 μl of template. Amplification was carried out in a Hybaid (Middlesex, U.K.) OMN-E thermal sequencer using a 30-sec denaturation at 94°C, 30-sec extension at 72°C with a 2°C touchdown for annealing every second cycle, commencing at 65°C and reaching a low of 55°C. A final 4-min extension at 72°C completed the reaction. The *trn*T/L amplification followed the same procedure as ITS. PCR products were purified using Promega (Sydney, Australia) Wizard PCR Preps DNA Purification System. Sequencing reactions followed those for the wasps.

Wasp Sequencing

DNA was extracted from the thorax of fresh or ethanol-preserved specimens using the salting-out method of Sunnucks and Hales (1996). Two mitochondrial genes (16S and cytochrome *b*) and one nuclear gene (*wingless*) were sequenced. A fragment near the 5′ end of the mitochondrial

TABLE 1. Voucher numbers and locality information for taxa examined in the molecular study.

Taxon	Voucher	Latitude/Longitude	Locality[1]	GenBank accession number		
				*trn*T/L	ITS	
Chiloglottis chlorantha D. L. Jones	M180	33°51′16″S 150°01′52″E	NSW: Kanangra-Boyd	AY042125	AY042157	
C. formicifera Fitzg.	M161	33°24′01″S 151°02′53″E	NSW: Darug NP	AY042122	AY042154	
C. affinity formicifera D. L. Jones	M151	28°58′00″S 152°04′52″E	NSW: Tenterfield	AY042123	AY042155	
C. grammata G. W. Carr	M188	42°54′52″S 147°15′34″E	TAS: Mt Wellington	AY042130	AY042162	
C. platyptera D. L. Jones	M173	31°55′48″E 151°20′45″S	NSW: Barrington Tops	AY042124	AY042156	
C. pluricallata D. L. Jones	M220	31°56′43″S 151°23′06″E	NSW: Barrington Tops	AY042126	AY042158	
C. affinity pluricallata	M218	31°55′05″S 151°23′33″E	NSW: Barrington Tops	AY042127	AY042159	
C. sylvestris D. L. Jones &	M232	33°32′26″S 150°38′05″E	NSW: Kurrajong	AY042118	N/A	
M. A. Clements	M104	29°28′24″S 152°19′07″E	NSW: Washpool NP	N/A	AY042150	
C. seminuda D. L. Jones	M231	33°32′26″S 150°38′05″E	NSW: Kurrajong	AY042117	AY042149	
C. sphyrnoides D. L. Jones	M107	30°32′45″S 152°14′00″E	NSW: Styx River	AY042119	N/A	
	M103	31°39′36″S 151°48′44″E	NSW: Nowendoc	N/A	AY042151	
C. trapeziformis Fitzg.	M160	33°06′16″S 145°04′34″E	NSW: Orange	AY042121	AY042153	
C. triceratops D. L. Jones	M190	43°02′54″S 147°07′48″E	TAS: Mt Wellington	AY042128	AY042160	
C. trilabra Fitzg.	M100	31°56′13″S 151°22′23″E	NSW: Barrington Tops	AY042115	N/A	
	M106	30°27′07″S 152°18′47″E	NSW: Ebor	N/A	AY042147	
C. truncata D. L. Jones	M155	27°14′47″S 152°03′37″E	NSW: Toowoomba	AY042120	AY042152	
C. valida D. L. Jones	M181	33°51′16″S 150°01′52″E	NSW: Kanangra-Boyd	AY042129	AY042161	
Arthrochilus huntianus (R. Muell.) Blaxell	M266	35°21′26″S 148°40′02″E	ACT: Namadgi NP	AY042131	AY042163	
Pollinators				16S	Cytochrome *b*	*Wingless*
Neozeleboria cryptoides (Smith)	W73	35°16′38″S 149°05′15″E	ACT: Canberra	AY042101	AY02138	AY042170
N. impatiens (1) (Smith)	W78	33°51′16″S 150°01′52″E	NSW: Kanangra-Boyd	AY042106	AY042143	AY042175
N. impatiens (2) (Smith)	W102	31°57′23″S 151°26′38″E	NSW: Barrington Tops	AY042105	AY042142	AY042174
N. monticola (1) (Turner)	W83	35°21′26″S 148°40′02″E	ACT: Namadgi NP	AY042109	AY042146	AY042178
N. monticola (2) (Turner)	W101	31°57′23″S 151°26′38″E	NSW: Barrington Tops	AY042107	AY042144	AY042176
N. monticola (3) (Turner)	W91	41°16′23″S 145°36′58″E	TAS: Hellyer Gorge	AY042108	AY042145	AY042177
N. proxima (Turner)	W4	33°51′16″S 150°01′52″E	NSW: Kanangra-Boyd	AY042095	AY042132	AY042164
N. affinity ursitatum Brown	W52	27°09′50″S 152°10′24″E	QLD: Toowoomba	AY042100	AY042137	AY042169
N. sp. 3 Brown	W9	29°28′30″S 152°18′52″E	NSW: Washpool NP	AY042099	AY042136	AY042168
N. sp. 29 Brown	W115	33°30′09″S 150°25′11″E	NSW: Mt Wilson	AY042097	AY042134	AY042166
N. sp. 30 Brown	W7	33°30′09″S 150°25′11″E	NSW: Mt Wilson	AY042096	AY042133	AY042165
N. sp. 40 Brown	W70	31°55′48″S 151°20′45″E	NSW: Barrington Tops	AY042103	AY042140	AY042172
N. sp. 41 Brown	W64	33°08′04″S 151°14′24″E	NSW: Yarramalong	AY042102	AY042139	AY042171
N. sp. 45 Brown	W53	28°55′54″S 152°07′44″E	QLD: Tenterfield	AY042104	AY042141	AY042173
N. sp. 50 Brown	W10	29°28′30″S 152°18′52″E	NSW: Washpool NP	AY042098	AY042135	AY042167
Arthrothynnus huntianus Brown	W116	35°21′26″S 148°40′02″E	ACT: Namadgi NP	AY042114	N/A	AY042183
Eirone sp. nov.	W87	42°54′52″S 147°15′34″E	TAS: Mt Wellington	AY042110	N/A	AY042179
Lophocheilus anilitatus (Smith)	W140	35°21′26″S 148°40′02″E	ACT: Namadgi NP	AY0421111	N/A	AY042180
Thynnoturneria sp. nov.	W139	32°28′16″S 116°53′03″E	WA: Boyagin	AY042113	N/A	AY042182
Zaspilothynnus trilobatus Turner	W129	34°59′13″S 116°41′34″E	WA: Walpole	AY042112	N/A	AY042181

[1] NSW, New South Wales; TAS, Tasmania; ACT, Australian Capital Territory; QLD, Queensland; WA, Western Australia; NP, National Park.

LSU rDNA gene (16S), incorporating domains IV and V of the large subunit rRNA gene, was amplified using the primers LR-J-12887 and LR-N-13398 (Simon et al. 1994). A large fragment of the cytochrome *b* mtDNA gene was amplified using the CB1 and tRs primers (Jermiin and Crozier 1994) for *Neozeleboria* pollinators only. The published cytochrome *b* primer, tRs, was successful, despite the presence of a hairpin loop at the 3′ end. The nuclear gene, *wingless*, was amplified using the published primers LepWG1 and LepWG2 (Brower and DeSalle 1998) and two sequencing primers designed for this study HyWG1 (5′-ATGAGGCTTCCAAATTTCCG-3′) and HyWG2 (5′-CTACCGCAGCACATCAGTCG-3′). The three outgroups were sampled for 16S and *wingless* only, because cytochrome *b* was considered too variable for informative outgroup comparison. PCR reactions (25 μl) contained 5 pmol of each primer, 10 mM Tris-Cl (pH 8.3), 3 mM MgCl, 50 mM KCl, 0.2 mM of each dNTP, one unit of *Taq* polymerase (Fisher Taq F1, Biotech) and 2 μl of template. Amplification was carried out in a Corbett Research, (Sydney, Australia) FTS-960 thermal sequencer using a 45-sec denaturation at 94°C, 30-sec extension at 72°C and using a 5°C stepdown program for annealing with the first cycle at 65°C, and annealing in later cycles reduced by 5°C after every second cycle until reaching 45°C. An additional 35 cycles were run with annealing set at 45°C. Cytochrome *b* amplification followed the same conditions but the annealing temperature was reduced to 50°C. PCR products were precipitated and purified using ammonium acetate: ethanol (1:10) precipitation followed by a 70% ethanol wash.

A 10-μl sequencing reaction using the dideoxy chain termination method (Sanger et al. 1977) was carried out using ABI BigDye terminator chemistry (Perkin Elmer Boston, MA) following the manufacturer's instructions. The same primers used for amplification were used for sequencing reactions except in the case of *wingless.* Sequencing was performed using an ABI Prism 377 automated DNA sequencer. All DNA fragments were sequenced in both the forward and reverse directions.

Sequence Analysis

Sequence analysis was conducted using PAUP* version 4.08 (Swofford 2001). Sequences were aligned and edited using Sequencher version 3.1.1 (Gene Codes Corporation, Ann Arbor, MI). The 16S alignment was compared with published secondary structure reconstructions of *Bombus* (Buckley et al. 2000). Two regions corresponding to helices 68 and 75 in *Bombus* (Buckley et al. 2000) showed length variation that made alignment ambiguous and were excluded from further analyses. Informative indels were coded as binary characters in the cpDNA (three indels) and ITS (two indels) datasets. Indels in the wasp datasets were either lacking (cytochrome *b* and *wingless*) or ambiguous (16S), therefore none were coded for analysis. Sequences and their alignments have been submitted to GenBank (Table 1).

Parsimony analyses were implemented using heuristic searches with TBR branch swapping and 10 random addition sequence starting trees, with gaps treated as missing. Bootstrapping was performed with 100 replicates (Felsenstein 1985). An incongruence length difference (ILD) test (Farris et al. 1995) as implemented in PAUP* was used to assess the combinability of the three wasp datasets for *Neozeleboria* taxa only, after excluding uninformative characters. Transition/transversion ratios were calculated by averaging over all pairwise comparisons across all sites. To investigate the occurrence of cospeciation among organisms, two questions are usually addressed (Huelsenbeck et al. 1997). The first assesses the degree to which phylogenetic topologies are in agreement. The second examines whether speciation times are associated. Software is available for analyzing both of these questions (Page 1996; Huelsenbeck et al. 1997). However, these require fully resolved phylogenies. In the absence of a fully resolved orchid phylogeny, we chose to assess the congruence of orchid and wasp phylogenies with an ILD test. Orchid and wasp datasets were treated as two separate data partitions and the test was run with and without the two instances of topological incongruence (*C. formicifera* and *C. affinity formicifera*, see Fig. 1). To test whether divergence times in the orchid and wasp lineages were similar, we were again limited by the lack of a fully resolved orchid phylogeny and the absence of any external calibration dates for a molecular clock analysis. Therefore, divergence times were assessed by comparing the relative branch lengths of orchid and wasp trees.

Floral Odor

Sample collection, gas chromatography with electroantennographic detection

Orchid flowers were collected from the Blue Mountains, New South Wales (*Chiloglottis* spp.) and in the Brindabella Range, Australian Capital Territory (*Arthrochilus huntianus*). Individual labella were extracted in pentane for 24 h. Samples were concentrated by evaporation of solvent and stored in a freezer. Male pollinator wasps were collected by baiting with orchid flowers (Peakall 1990) in the area where the orchids grew. Gas chromatography with electroantennographic detection (GC-EAD) was performed according to Schiestl et al. (2000) and Schiestl and Ayasse (2000).

One microliter of each odor sample was injected splitless at 50°C (1 min) into a gas chromatograph (HP 6890, Hewlett Packard, Little Falls, DE) followed by opening the split valve and programming to 230°C at a rate of 10°C/min. The GC was equipped with a DB-FFAP column (30 m × 0.32 mm); helium was used as carrier gas. A GC effluent splitter (press-fit-connection; split ratio 1:1) was used and the outlet was placed in a purified and humidified airstream. This air was directed over a male wasp's antenna prepared as follows: The tip of the excised antenna was cut off and the antenna mounted between two glass electrodes filled with insect ringer solution. The electrode holding the base of the antenna was connected to grounded Ag-AgCl wire. The distal end of the antenna was connected in the same way via an interface box to a signal acquisition interface board (IDAC; Syntech, Hilversum, The Netherlands) for signal transfer to a PC. EAD signals and flame ionization detector (FID) responses were simultaneously recorded.

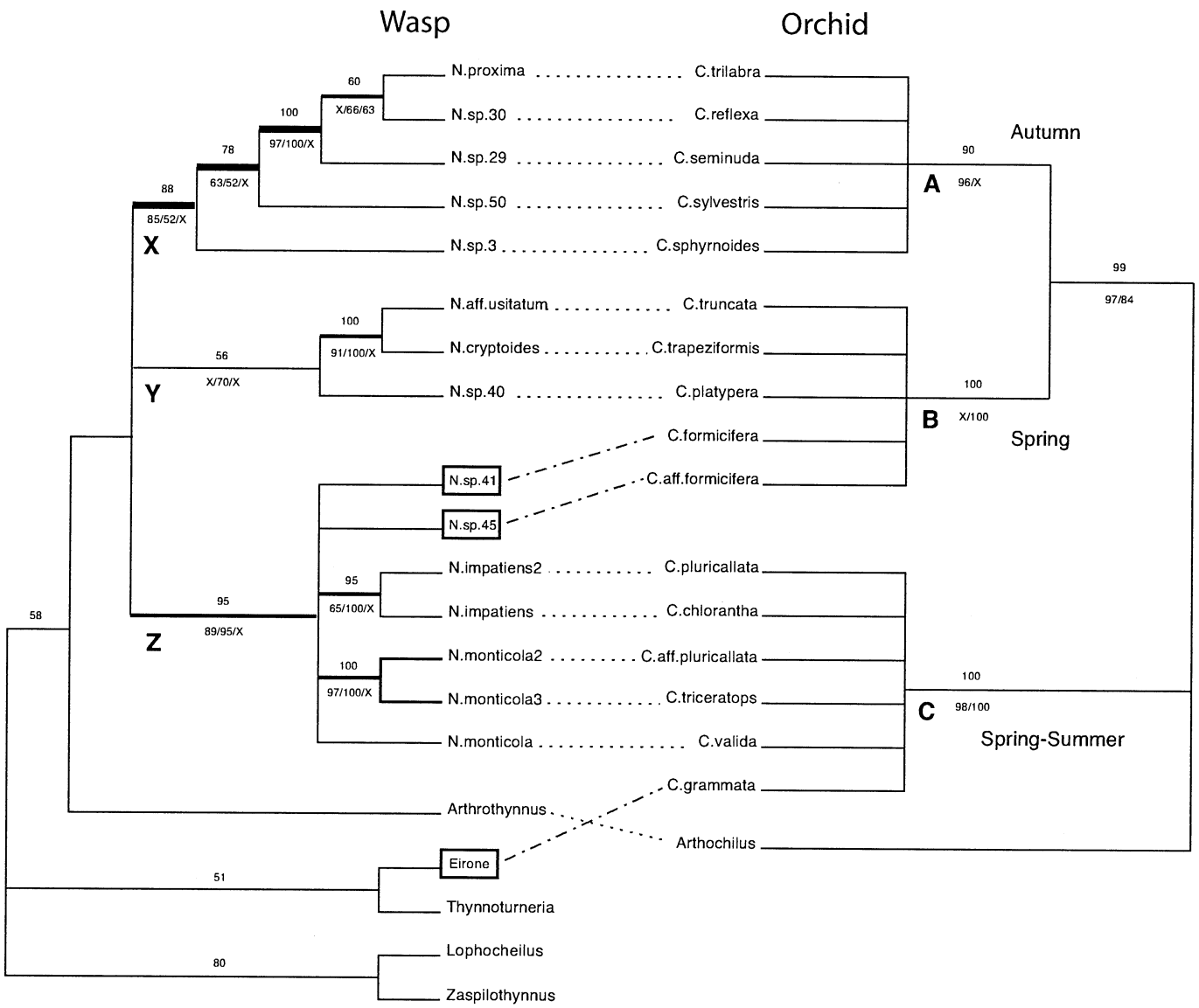

FIG. 1. Phylogenetic relationships among the orchid *Chiloglottis* and its thynnine wasp pollinators, estimated by maximum parsimony (MP). Orchid species are aligned with their specific pollinators. The three clades of *Chiloglottis* are pollinated by three groups of *Neozeleboria*, except where indicated by diagonal line and boxes. The wasp tree is a strict consensus of three MP trees, using the combined data of one nuclear (*wingless*) and two mitochondrial DNA (16S and cytochrome *b*) genes (tree length 1204, 366 parsimony informative characters, CI = 0.45, RI = 0.48). The orchid tree is a strict consensus of 21 MP trees using chloroplast (*trn*T/L) and nuclear (ITS) data (tree length 57, 44 characters, CI = 0.91, RI = 96). Bootstrap numbers for the wasps are for combined data above and separate 16S/cytochrome *b*/*wingless* below the line; for *Chiloglottis*, combined data are given above and separate *trn*T-*trn*L/ITS below. Bootstraps below 50% are represented as an X. Semi-bold lines indicate branches recovered also in separate analyses of 16S and cytochrome *b*. Bold lines indicate branches recovered in independent analyses of each of the three genes.

Treatment of data

For each sample type, approximately five GC-EAD runs were obtained. Peaks in the EAD recording were assumed to be responses from the antennal receptors if they were detectable in all recordings at the same recording time. For calculation of the relative retention indices (RRI), 1 μl of odor sample was injected in the GC together with 1 μl of a standard mixture of C9-C30 alkanes. For each coinjection, relative retention indices of biologically active peaks were calculated according to the formula (RT2 − RTX)/(RT2 − RT1), where RTX is retention time of active compound and RT1,2 are retention times of alkanes eluting before and after the active compound.

RESULTS

Orchids

Separate phylogenetic analyses of the nuclear and chloroplast data reveal a congruent pattern and pass the ILD test ($P = 0.51$). Three clades (A, B, C) are identified that correspond to three groups recognized by both morphology and flowering phenology (Figs. 1, 2). The nuclear and plastid

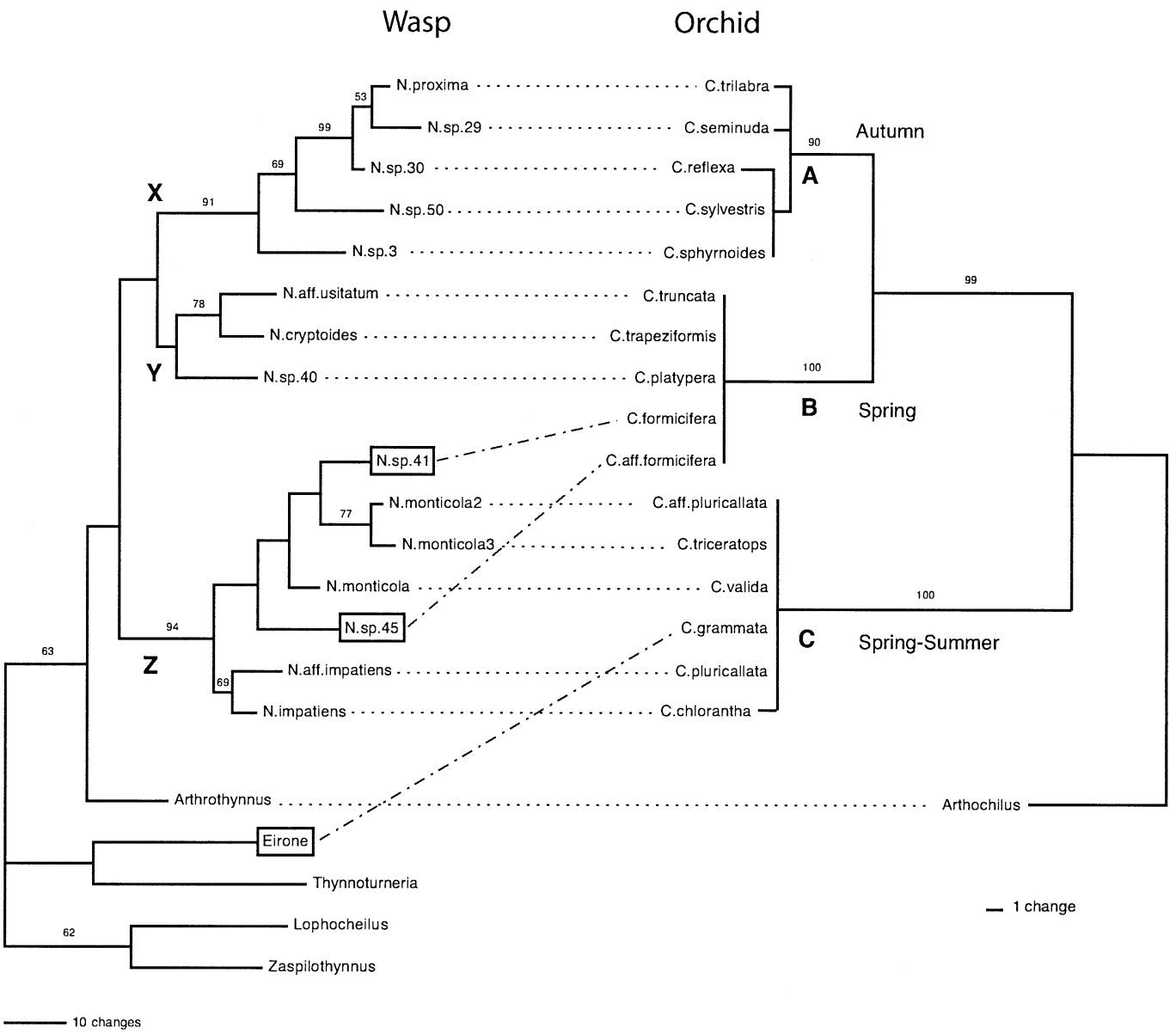

FIG. 2. Phylograms of the orchid *Chiloglottis* and its wasp pollinators estimated by maximum parsimony (MP). The wasp tree is based on combined 16S and *wingless* data (one of two trees at length 378, 121 parsimony informative characters, CI = 0.45, RI = 0.54). The orchid tree is one of 21 MP trees recovered from the analysis shown in Figure 1. Note the arrangement of taxa in the autumn flowering *Chiloglottis* clade A varies among the 21 MP trees and reduces to polytomy in the strict consensus (Fig. 1). Bootstraps are given above the line. Relative branch lengths are different in the orchid and wasp trees.

sequence data (Table 2) reveal considerable nucleotide difference between the three clades (average uncorrected divergence A:B = 1.0%, A:C = 2.3%, B:C = 2.6%) relative to the variation within each clade (average A = 0.2%, B = 0.09%, C = 0.08%). The monophyly of *Chiloglottis* is confirmed by outgroup comparison.

Wasps

Phylogenetic signal across the three genes satisfies the ILD test (P = 0.98), and separate analyses demonstrate considerable topological congruence. The same three main clades (X, Y, Z) are found when the two mitochondrial genes are analyzed separately, and when all datasets are combined (Fig. 1). However, the relationships between those clades are not well supported and are best represented as the soft polytomy shown in Figure 1. These three clades are not strongly supported by the nuclear DNA data, which exhibits low ingroup divergence. However, there are no nuclear DNA clades with high bootstrap support (BS) that are incongruent with the three main clades. The monophyly of the autumn emerging *Neozeleboria* clade X and the species relationships within it are congruent across all three genes, with the exception of the relationships among *N. proxima*, *N.* sp. 30, and *N.* sp. 29, which differ under 16S. However, the placement of *Neozeleboria* sp. 40 (pollinator of *C. platyptera*) with *N. cryptoides* and *N. ursitatum* is not strongly supported (BS = 56%, com-

TABLE 2. Molecular sequence results. Three loci were sequenced for the wasp pollinators (16S, cytochrome *b*, *wingless*) and two for the orchids (ITS, *trn*T/L). Wasp ingroup includes *Neozeleboria* taxa only. Cytochrome *b* is sequenced for *Neozeleboria* only. Cytochrome *b* first, second, and third codon positions are specified. 16S data excludes length variable regions.

Gene	Size of fragment (bp)	Variables sites (outgroup)	Parsimony informative (outgroup)	% Divergence uncorrected (outgroup)	A + T (%)	Ti/Tv
16S	484	95 (148)	66 (88)	1.7–11 (−15.1)	75.1	0.79
cytochrome *b* total	684	320	237	8.8–23.2	73.8	1.11
cytochrome *b* 1st	228	87	63	6.1–19.4	68.2	1.0
cytochrome *b* 2nd	228	36	19	0–9.6	69.7	1.19
cytochrome *b* 3rd	228	197	155	19.7–45.6	84	1.0
wingless	361	18 (42)	9 (33)	0–2.9 (−16.4)	50.5	n/a
ITS	736–740	36	30	0–4.4	47.2	1.32
*trn*T-*trn*L	508–623	10	8	0–1.9	69.3	0.92

bined data), reflecting conflicting signal also in morphological characters (J. G. Mant, unpubl. data). Most relationships within clade Z are not well supported in any of the three genes or in the combined dataset. Overall, the three *Neozeleboria* clades are largely corroborated by morphological characters associated with the male genitalia (J. G. Mant, unpubl. data) and correspond to differences in emergence time (see Fig. 1).

Orchid–Wasp Phylogenetic Congruence

Fifteen of the 16 sampled *Chiloglottis* species are pollinated by members of the *Neozeleboria* clade of Thynninae, as tested by outgroup comparison. The two datasets that include outgroups (16S and *wingless*) support the monophyly of the *Neozeleboria* pollinators, and position *Eirone* sp. (pollinator of *C. grammata*) as distantly related. *Eirone* is included in a distinct group sometimes recognized as the tribe Ragagastrini (Given 1954). However, *Arthrothynnus* is estimated as either sister to *Neozeleboria* (16S and combined data) or embedded within that genus (*wingless* only). The *wingless* data, in particular, strongly support the monophyly and close affinity of the sampled *Neozeleboria* taxa plus *Arthrothynnus* (maximum 3% ingroup uncorrected divergence; 4.5–6.9% between ingroup and three outgroups; 16.4% divergence between *Eirone* and ingroup).

Below the generic level, there is considerable phylogenetic congruence between the *Chiloglottis* and *Neozeleboria* topologies. One of the three *Chiloglottis* clades is exclusively pollinated by a clade of *Neozeleboria*, whereas the other two *Chiloglottis* clades are pollinated predominantly by species in two corresponding clades of *Neozeleboria* (Fig. 1). The autumn flowering clade A is restricted to autumn emerging wasps of clade X. Similarly, the spring-summer flowering clade C is pollinated by species from the spring-summer emerging clade Z, with the exception of *C. grammata*. However, this topological congruence is contradicted by the third orchid group, comprising *C. truncata*, *C. trapeziformis*, *C. platyptera*, *C. formicifera*, and *C. aff. formicifera*. The first three of these species use members of the poorly supported clade Y, whereas the latter two use members of clade Z (Fig. 1).

The ILD test supports the strong association of the *Chiloglottis* and *Neozeleboria* phylogenetic signals, with the exception of *C. formicifera* and *C. aff. formicifera*. The ILD test suggests the orchid and wasp datasets hold significantly different signals ($P = 0.01$). However, the source of this incongruence derives from the above two species. When both of these species (and their pollinators) are excluded, the orchid and wasp datasets are not significantly different ($P = 0.84$). When only one of these species is excluded, the difference remains significant ($P = 0.01$).

A statistical comparison of the relative branch lengths in orchid and wasp trees was not undertaken due to the lack of variation within the three clades of *Chiloglottis*. However, it is noted that the relative branch length pattern in the *Chiloglottis* and *Neozeleboria* gene trees differ markedly (Fig. 2). The patristic, or path-length, distances (Farris 1964) between the basal nodes of clades A, B, and C and the basal node for *Chiloglottis* are much longer than the patristic distances between the basal nodes of clades A, B, and C and the terminal nodes. By contrast, the patristic distances between the basal nodes of clades X, Y, and Z and the basal nodes for *Neozeleboria* are mostly shorter than the patristic distances between the basal nodes of clades X, Y, and Z and the terminal nodes. The degree of rate heterogeneity that would need to be postulated to account for these differences within a cospeciational model seems much greater than any observed heterogeneity in the phylograms. Moreover, an explanation invoking rate heterogeneity is implausible because it would require simultaneous deceleration of rates in three independent orchid lineages or simultaneous acceleration in three independent wasp lineages, or both. This suggests speciation events in orchid and wasp groups occurred independently, with *Chiloglottis* perhaps radiating onto an existing *Neozeleboria* lineage. Further phylogenetic resolution of the three *Chiloglottis* species groups will be needed for statistical tests of this hypothesis.

Biologically Active Floral Odor Compounds

In the three investigated orchid species, we found either one or two peaks eliciting electroantennographic responses in the respective pollinator antennae (Table 3, Fig. 3). Each orchid-pollinator pair produced a distinct pattern of EAD responses, although overlap in retention time and relative retention index suggests that partially similar or even identical compounds are produced by the orchids for pollinator attraction. Such overlap is evident in one active compound in the two *Chiloglottis* species (RT 16.7), and another one within *C. trilabra* and *Arthrochilus huntianus* (RT 16.8; Table 3). To test whether the different wasp pollinators are indeed

TABLE 3. Retention times (RT) and relative retention indices (RRI) of biologically active compounds in orchid-odor samples tested on pollinator and nonpollinator antennae.

Orchid sample	Insect antenna	Pollinator	RT (min) of active peaks (RRI)		
			Peak 1	Peak 2	Peak 3
Chiloglottis trilabra	*Neozeleboria proxima*	yes	16.72 (0.58)	16.82 (0.47)	
C. seminuda	*N.* sp. 29	yes	16.71 (0.58)		
Arthrochilus huntianus	*Arthrothynnus huntianus*	yes		16.81 (0.46)	18.16 (0.79)
C. trilabra	*A. huntianus*	no		16.82 (0.47)	

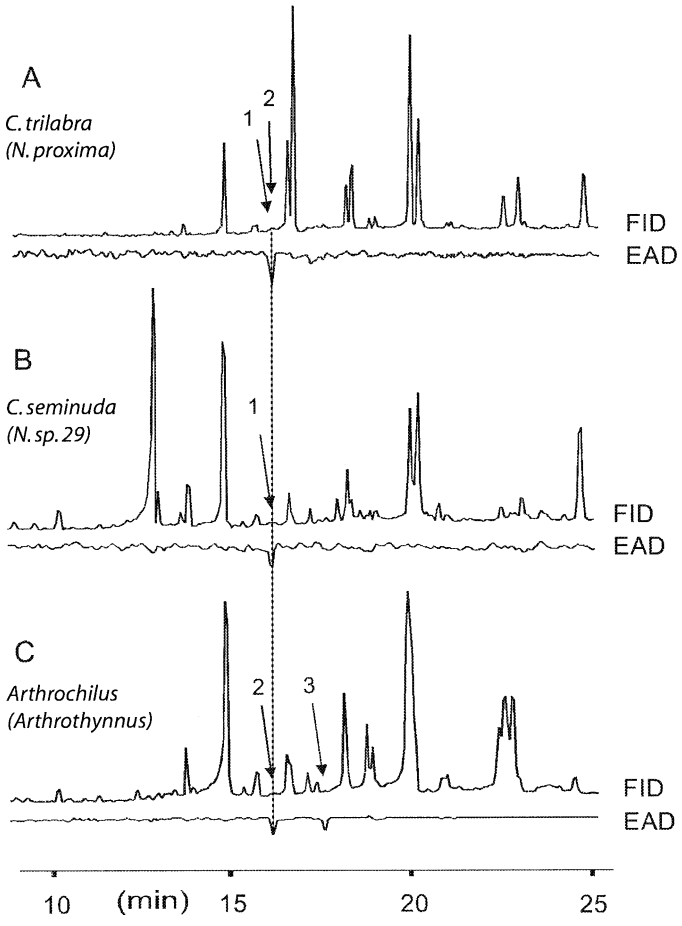

FIG. 3. Gas chromatography with electroantennographic detection recordings of (A) *Chiloglottis trilabra* and *Neozeleboria proxima*; (B) *C. seminuda* and *N.* sp. 29; (C) *Arthrochilus huntianus* and *Arthrothynnus huntianus*. The peaks in the flame-ionization-detector (FID) traces represent odor compounds present in the orchid flowers. The electroantennographic detector (EAD) traces display summed responses of olfactory neurones to particular odor compounds (Arn et al. 1975). Because FID and EAD responses are recorded simultaneously, EAD responses correspond to peaks at the same recording time. The numbered peaks refer to distinct antennal responses, the retention times of which are found in Table 3. Peak 1 and 2 in *C. trilabra* elute closely together, but are clearly resolved as two peaks in the EAD responses. The lack of clear FID peaks accompanying the EAD responses indicates the antennae are more sensitive to the presence of the odor compound in the samples than the gas chromatograph equipment.

responding to identical compounds in the different orchid species, we performed a reciprocal test of the *Arthrothynnus huntianus* EAD response to *C. trilabra*, which is normally pollinated by *N. proxima*. *Arthothynnus huntianus*, the pollinator of *A. huntianus*, responded to the identical peak in the *C. trilabra* samples as did *N. proxima*. This finding strongly suggests sharing of active compounds between orchid species.

DISCUSSION

Phylogenetic Congruence

Higher-level taxonomic patterns in the use of pollinators are usually identified simply by noting taxonomic congruence with a pollinating insect group (Anderson 1979; Chase and Hills 1992; Johnson and Steiner 2000). As much as current taxonomy reflects phylogeny, this procedure might allow the recognition of pollinator conservatism, yet only at the level of traditional Linnean hierarchies. In this study, the estimation of phylogenetic relationships in both orchids and their wasp pollinators has enabled the degree of pollinator specialization in a sexually deceptive genus to be examined at a range of hierarchical levels. Phylogenetic congruence between *Chiloglottis* and its thynnine pollinators is evident at three of those levels. First, the genera in the monophyletic Drakaeinae (containing *Chiloglottis*; Kores et al. 2001) are pollinated by wasps from subfamily Thynninae, with the exception of *C. major*, which is pollinated by the sawfly, *Pterygophorus* (Symphyta: Pergidae). Second, 15 of 16 sampled species of *Chiloglottis* are pollinated by *Neozeleboria* species, which are together resolved as a monophyletic group (including *Arthrothynnus*). Finally, two of the three clades that comprise *Chiloglottis* are pollinated by two clades within *Neozeleboria*. As in other plant-insect interactions, the degree of specialization may tend to be more diffuse at the level of closely related congenerics. In *Chiloglottis*, incongruence among the spring-flowering Formicifera clade and two spring-emerging groups within *Neozeleboria* (Fig. 1) indicates specialization is incomplete at the intrageneric group level.

Three exceptions to the *Neozeleboria* pollination of *Chiloglottis* are known, only one of which could be sampled in the present study. These are *Eirone* (pollinating *C. grammata*), *Arthrothynnus latus* (*C. diphylla*), and *Chilothynnus palachilus* (*C. palachila*; Bower 1996; unpubl. data). *Eirone* represents a substantial departure from the dominant pattern of specialization. In contrast, molecular data suggest that *Arthothynnus* is either sister to *Neozeleboria* or nests within that genus. We predict future molecular studies will also dem-

onstrate the close relationship of *Chilothynnus* given the morphological affinity it shows to *Neozeleboria* (Brown 1996a, 1996b). The sharing of pollinating wasp genera between *Chiloglottis* and *Arthrochilus* is consistent with the general pattern of pollinator conservatism found among the Drakaeinae. Overall, it is emphasized that the diversification of *Chiloglottis* has involved, with few exceptions, pollinator shifts among one clade of wasps within the subfamily Thynninae, a group comprising more than 50 genera and more than 500 described species in Australia (Given 1954).

The orchid sequence results reveal an interesting disparity in sequence divergence between, relative to within, the three *Chiloglottis* species groups (Fig. 2). This pattern is consistent with either a recent radiation of species within each of the groups or the erosion of molecular diversity via gene flow and selection among the recognized species. An explanation invoking heterogeneity of molecular evolutionary rates is implausible because it would require simultaneous deceleration of rates in three independent lineages. However, gene flow cannot account for the lack of variation in the nonrecombining chloroplast locus. Rather, a loss of ancestral chloroplast haplotypes needs to be invoked. To further distinguish between these processes, more variable multilocus markers are being investigated.

Pollinator Responses to Orchid Odor

Each of the three pollinator species sampled have a distinct GC-EAD response to the orchid odors. These species-specific responses are consistent with field experiments demonstrating specific pollinator attraction. Although electrophysiological activity of odor compounds does not, per se, prove a role for those compounds in the mating behavior of the insect, numerous previous investigations have demonstrated this link (e.g., Struble and Arn 1984). In the European sexually deceptive *Ophrys sphegodes*, electrophysiologically active odor compounds elicited mating behavior in the pollinator bee, *Andrena nigroaenea* (Schiestl et al. 1999, 2000). The responses of male thynnines to orchid odor compounds are likely to mirror the responses to sex pheromones documented in the *Andrena* pollination of *Ophrys*. However, thynnine wasps appear to respond to a relatively simple odor blend containing one or two active compounds, unlike the solitary bee, *A. nigroaenea*, which responds to a blend of 14 compounds (Schiestl et al. 2000).

Similarity of GC-EAD Responses across Species

The results of our GC-EAD experiments not only reveal orchid odors elicit distinct responses in each pollinator species, but that these responses center on similar active compounds. Theoretically, it is possible that different substances have the same retention times on a particular column. However, in our data, the similarity of these compounds is strongly suggested given that they originate from closely related orchids and are responded to by closely related wasps. Our GC-EAD setup uses sex pheromone receptors as a detector in the chemical analyses (Arn et al. 1975). Because one type of sex pheromone receptor typically responds to only one or a few similar compounds (Hansson 1995), it is possible to detect particular compounds in a sample. The response of *A. huntianus* to the same peak in *C. trilabra* as its pollinator, *Neozeleboria proxima*, therefore strongly supports a sharing of active odor compounds between orchid taxa.

Further support for these interpretations has recently emerged from two largely allopatric species, *C. trapeziformis* and *C. valida*. GC-EAD revealed that a single electrophysiologically active compound is shared by both orchids (F. P. Schiestl, unpubl. data). Subsequently, chemical identification and bioassays of the synthetic compound with *C. trapeziformis* have confirmed that this single compound elicits attraction and mating behavior in the pollinator males in equal intensity to the floral odor (F. P. Schiestl, unpubl. data).

Mating characters are often presumed to be highly homoplasious in relation to other suites of characters, yet there is little evidence to support such a generalization (see review by de Queiroz and Wimberger 1993). In thynnine wasps, pheromone differences between species may be constituted by variation in only a few compounds, with related taxa sharing broadly similar pheromone constituents. Thus, thynnine pheromones are likely to be strongly influenced by phylogeny. Unfortunately, given our small sample size, this suggestion must remain speculative. Comparative data on the sex pheromones of solitary Hymenoptera are lacking (Ayasse et al. 2001). However, among other sex pheromone systems, such as the well-characterized Lepidoptera, the evidence indicates that variation in pheromone chemistry may be congruent with phylogeny. Female lepidopteran sex pheromones achieve species-specific attraction with the use of few components that are conservative amongst taxonomic groups, such as the predominantly 14-carbon chain in subfamily Tortricinae and 12-carbon chain in Olethreutinae (Roelofs and Brown 1982; Löfsdedt and Kozlov 1996).

How Is Pollinator Conservatism Maintained?

Whereas pollinator shifts among *Chiloglottis* species have mostly been among wasps that are closely related, it would seem unlikely that this is a coevolutionary response to cladogenesis in *Neozeleboria*. The three instances of phylogenetic incongruence indicate that speciation among *Chiloglottis* can be independent from that of thynnine wasps. Furthermore, relative branch length differences between the plant and insect trees suggest speciation events in the two groups were not contemporaneous. Nonetheless, the level of phylogenetic conservatism in *Chiloglottis* is surprising for an orchid pollination system that would appear to favor both rapid and flexible shifts in pollinators (Paulus and Gack 1990; Nilsson 1992).

We suggest that several phylogenetically influenced factors have interacted to constrain the pattern of pollinator change in *Chiloglottis*. At the outset, any shift to a new pollinator will depend on the ecological availability of pollinator resources in a given geographical area and flowering time. For *Neozeleboria*, we have seen that variation in wasp emergence phenology can be predicted by phylogenetic relationship. In this case, all autumn-emerging pollinators form a clade, with the spring-summer *Neozeleboria* forming two groups (Fig. 1). Thus, for example, if pollinator shifts among autumn-flowering *Chiloglottis* occur mostly between *Neozeleboria* with the same autumn phenology, then those shifts are likely

to be among wasps from the same clade. Overall, however, the chemical mimicry of female wasp pheromones may operate to constrain the potential pollinator resource to the narrow taxonomic range of wasps seen in *Chiloglottis*. If the pheromonal differences among thynnine taxa are themselves phylogenetically constrained, such that related wasps have similar pheromone signals, then changes in floral odor sufficient to attract a distinct pollinator will tend also to attract a wasp species that is closely related. Our comparative GC-EAD data are at least consistent with the hypothesis of shared pheromone components among related wasp taxa. Conversely, it remains to be seen whether convergence in sex pheromone signals among more distantly related hymenopteran taxa can explain the phylogenetically disparate shifts seen within *Chiloglottis* and among other orchid taxa. In conclusion, we suggest that the hierarchical pattern of sex communication signals found among pollinators may largely frame the pattern of pollinator shifts in sexually deceptive orchids. In the case of *Chiloglottis*, the chemical mimicry of thynnine wasp pheromones that are themselves subject to phylogenetic constraints may lead to pollinator conservatism at a range of taxonomic levels.

Acknowledgments

The research for this study has been generously supported by the Hermon Slade Foundation and the Royal Botanic Gardens, Sydney. Funding for the investigation of the wasp responses to orchid odors was provided by the Australian National University, Australian Orchid Foundation, and the American Orchid Society. JGM was financially supported by an Australian Postgraduate Award and FPS by the Fonds fuer wissenschaftliche Forschung, Austria. Thanks in particular to L. Cook for laboratory assistance and advice, and to M. Ayasse for allowing us to work in his laboratory. Thanks also to M. Batley, C. Bower, G. Brown, G. Cassis, M. Clements, and D. Jones for discussion and advice. For laboratory or field assistance, thanks to J. Armstrong, C. Bower, R. Butcher, G. Goodes, P. Jobson, G. Leidreiter, C. Porter, A. Perkins, and H. Wapstra.

Literature Cited

Alcock, J. 2000. Interactions between the sexually deceptive orchid *Spiculaea ciliata* and its wasp pollinator *Thynnoturneria* sp. (Hymenoptera: Thynninae). J. Nat. Hist. 34:629–636.

Anderson, W. R. 1979. Floral conservatism in Neotropical Malpighaceae. Biotropica 11:219–223.

Arn, H., E. Städler, and S. Rauscher. 1975. The electroantennographic detector: a selective and sensitive tool in the gaschromatographic analysis of insect pheromones. Z. Naturforsch. Sect. C 30:722–725.

Ayasse, M., F. P. Schiestl, H. F. Paulus, C. Löfstedt, B. S. Hansson, F. Ibarra, and W. Francke. 2000. Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: How does flower-specific variation of odor signals influence reproductive success? Evolution 54:1995–2006.

Ayasse, M., R. J. Paxton, and R. Tengö. 2001. Mating behaviour and chemical communication in the order Hymenoptera. Annu. Rev. Entomol. 46:31–78.

Baldwin, B. G., M. J. Sanderson, J. M. Porter, M. F. Wojciechowski, C. S. Campbell, and M. J. Donoghue. 1992. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Mo. Bot. Gard. 82:247–277.

Bergström, G. 1978. Role of volatiole chemicals in *Ophrys*-pollinator interactions. Pp. 207–230 *in* G. Harbourne, ed. Biochemical aspects of plant and animal coevolution. Academic Press, New York.

Borg-Karlson, A.-K. 1990. Chemical and ethological studies of pollination in the genus *Ophrys* (Orchidaceae). Phytochemistry 29: 1359–1387.

Bower, C. C. 1996. Demonstration of pollinator-mediated reproductive isolation in sexually deceptive species of *Chiloglottis* (Orchidaceae: Caladeniinae). Aust. J. Bot. 44:15–33.

———. 2001. Pollination of the elbow orchid, *Arthrothynnus huntianus* (F. Muell.) Blaxell subsp. *huntianus*. Orchadian 13: 366–371.

Bower, C. C., and G. R. Brown. 1997. Hidden biodiversity: detection of cryptic thynnine wasp species using sexually deceptive, female mimicking orchids. Mem. Natl. Mus. Vic. 56:461–466.

Brower, A. V. Z., and R. DeSalle. 1998. Patterns of mitochondrial versus DNA sequence divergence among butterflies: the utility of *wingless* as a source of characters for phylogenetic inference. Insect Mol. Biol. 7:73–82.

Brown, G. R. 1996a. *Arthrothynnus*, a new genus of orchid-pollinating Thynninae (Hymenoptera: Tiphiidae). Beagle 13:73–82.

———. 1996b. *Chilothynnus*, a new genus of Australian Thynninae (Hymenoptera: Tiphiidae) associated with orchids. Beagle 13: 61–72.

Buckley, T. R., C. Simon, P. K. Flook, and B. Misof. 2000. Secondary structure and conserved motifs of the frequently sequenced domains IV and V of the insect mitochondrial large subunit rRNA gene. Insect Mol. Biol. 9:565–580.

Chase, M. W., and H. G. Hills. 1992. Orchid phylogeny, flower sexuality, and fragrance-seeking. BioScience 42:43–49.

Dafni, A., and P. Bernhardt. 1990. Pollination of terrestrial orchids of southern Australia and the Mediterranean region. Evol. Biol. 24:193–252.

de Queiroz, K., and P. H. Wimberger. 1993. The usefulness of behaviour for phylogeny estimation: levels of homoplasy in behavioural and morphological characters. Evolution 47:46–60.

Dressler, R. L. 1981. The orchids: natural history and classification. Harvard Univ. Press, Cambridge, MA.

Farris, J. S. 1964. The meaning of relationship and taxonomic procedure. Syst. Zool. 16:44–51.

Farris, J. S., M. Källersjö, A. G. Kluge, and C. Bult. 1995. Conducting a significance test for incongruence. Syst. Biol. 44: 570–572.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Gilmore, S., P. H. Weston, and J. A. Thompson. 1993. A simple, rapid, inexpensive and widely applicable technique for purifying plant DNA. Aust. Syst. Bot. 6:139–148.

Given, B. B. 1954. A catalogue of the Thynninae (Tiphiidae, Hymenoptera) of Australia and adjacent areas. N. Z. Dep. Sci. Ind. Res. Bull. 109:1–89.

Grant, V. 1994. Modes and origins of mechanical and ethological isolation in angiosperms. Proc. Natl. Acad. Sci. U.S.A 91:3–10.

Hansson, B. S. 1995. Olfaction in Lepidoptera. Experientia 51: 1003–1027.

Huelsenbeck, J. P., B. Rannala, and Z. Yang, 1997. Statistical tests of host-parasite cospeciation. Evolution 51:410–419.

Jermiin, L. S., and R. H. Crozier. 1994. The cytochrome *b* region in the mitochondrial DNA of the ant *Tetraponera rufoniger*: sequence divergence in Hymenoptera may be associated with nucleotide content. J. Mol. Evol. 38:282–294.

Johnson, S. D., and K. E. Steiner. 2000. Generalisation versus specialization in plant pollination systems. Trends Ecol. Evol. 15: 140–143.

Johnson, S. D., H. P. Linder, and K. E. Steiner. 1998. Phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). Am. J. Bot. 85:402–411.

Jones, D. L. 1991. New taxa of Australian Orchidaceae; *Chiloglottis* R. Br. Aust. Orchid Res. 2:36–44.

Kores, P., M. Molvray, S. Hopper, P. H. Weston, A. Brown, K. Cameron, and M. Chase. 2001. A phylogenetic analysis of Diurideae (Orchidaceae) based on plastid DNA sequence data. Am. J. Bot. 88:1903–1914.

Löfsdedt, C., and M. Kozlov. 1996. A phylogenetic analysis of pheromone communication in primitive moths. Pp. 473–489 *in* R. T. Cardé and W. J. Bell, eds. Insect pheromone research: new directions. Chapman and Hall, New York.

Nilsson, L. A. 1992. Orchid pollination biology. Trends Ecol. Evol. 7:255–259.

Ollerton, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. J. Ecol. 84:767–769.

Page, R. D. M. 1996. Temporal congruence revisited: comparison of mitochondrial DNA sequence divergence in cospeciating pocket gophers and their chewing lice. Syst. Biol. 45:151–167.

Paulus, H. F., and C. Gack. 1990. Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidaceae). Isr. J. Bot. 39:43–79.

Peakall, R. 1989. The unique pollination of *Leporella fimbriata* (Orchidaceae): pollination by pseudocopulating male ants (*Myrmecia urens*, Formicidae). Plant Syst. Evol. 167:137–148.

———. 1990. Responses of male *Zaspilothynnus trilobatus* Turner wasps to females and the sexually deceptive orchid it pollinates. Func. Ecol. 4:159–167.

Peakall, R., and A. J. Beattie. 1996. Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tenticulata*. Evolution 50:2207–2220.

Peakall, R., and S. N. Handel. 1993. Pollinators discriminate among floral heights of a sexually deceptive orchid: implications for selection. Evolution 47:1681–1687.

Roelofs, W. L., and R. L. Brown. 1982. Pheromones and evolutionary relationships of Tortricidae. Annu. Rev. Ecol. Syst. 13: 395–422.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.

Schiestl, F. P., and M. Ayasse. 2000. Post mating odor in females of the solitary bee, *Andrena nigroaenea* (Apoidea, Andrenidae) inhibits male mating behavior. Behav. Ecol. Sociobiol. 48: 303–307.

Schiestl, F. P., M. Ayasse, H. F. Paulus, C. Löfsdedt, B. S. Hansson, F. Ibarra, and F. Francke. 1999. Orchid pollination by sexual swindle. Nature 399:421–422.

Schiestl, F. P., M. Ayasse, H. F. Paulus, C. Löfsdedt, B. S. Hansson, F. Ibarra, and W. Francke. 2000. Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): patterns of hydrocarbons as the key mechanism for pollination by sexual deception. J. Comp. Physiol. A 186:567–574.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87:651–701.

Stoutamire, W. P. 1974. Australian terrestrial orchids, thynnid wasps and pseudocopulation. Am. Orchid Soc. Bull. 43:13–18.

———. 1975. Pseudocopulation in Australian terrestrial orchids. Am. Orchid Soc. Bull. 44:226–233.

Struble, D. L., and H. Arn. 1984. Combined gas chromatography and electroantennogram recording of insect olfactory responses. Pp. 161–178 *in* H. E. Hummel and T. A. Miller, eds. Techniques in pheromone research. Springer, New York.

Sunnucks, P., and D. F. Hales. 1996. Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera, Aphididae). Mol. Biol. Evol. 13: 510–524.

Swofford, D. L. 2001. PAUP*4.0b: phylogenetic analysis of parsimony (* and other methods). Sinauer, Sunderland, MA.

Taberlet, P., L. Gielly, G. Pautou, and J. Bouvet. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17:1105–1109.

Tremblay, R. L. 1992. Trends in the pollination ecology of the Orchidaceae. Can. J. Bot. 70:642–650.

van der Cingel, N. A. 2001. An atlas of orchid pollination: America, Africa, Asia and Australia. A. A. Balkema, Rotterdam, The Netherlands.

Waser, N. M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. Oikos 82:198–201.

Waser, N. M., L. Chittka, M. V. Price, N. M. Williams, and J. Ollerton. 1996. Generalisation in pollination systems, and why it matters. Ecology 77:1043–1060.

Corresponding Editor: J. Conner