

A phylogeny of the oil bee tribe Ctenoplectrini (Hymenoptera: Anthophila) based on mitochondrial and nuclear data: Evidence for Early Eocene divergence and repeated out-of-Africa dispersal

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Abstract

The bee tribe Ctenoplectrini, with two genera, comprises nine species in tropical Africa and ten in Asia and Australia. Most of them collect floral oil, pollen, and nectar from Cucurbitaceae, but three species are thought to be cleptoparasites. The unusual morphology of Ctenoplectrini has made it difficult to infer their closest relatives, in turn preventing an understanding of these bees' geographic and temporal origin. We used two mitochondrial and two nuclear markers (4741 nucleotides) generated for most of the species to test the monophyly of the tribe, its relationships to other Apidae, and its biogeographic history. Ctenoplectrini are strongly supported as monophyletic and closest to the Long-horned bees, Eucerini. The presumably cleptoparasitic species form a clade (*Ctenoplectrina*) that is sister to the remaining species (*Ctenoplectra*), confirming the independent evolution of cleptoparasitism in this tribe. Tree topology and molecular dating together suggest that Ctenoplectrini originated in Africa in the Early Eocene and that *Ctenoplectra* dispersed twice from Africa to Asia, sometime in the Late Eocene, 30–40 my ago, from where one species reached the Australian continent via Indonesia and New Guinea in the mid-Miocene, c. 13 my ago. Dry and cool mid-Miocene climates also coincide with the divergence between *Ctenoplectra bequaerti* from West Africa and *Ctenoplectra terminalis* from East and South Africa, perhaps related to fragmentation of the equatorial African rainforest belt.

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1. Introduction

Systematic relationships in bees (Anthophila), a group of more than 16,000 species, remain insufficiently resolved, although molecular phylogenetic data are rapidly improving the situation (Danforth et al., 2006a, 2006b; Cameron et al., 2007). The most diverse of the nine currently accepted families of Anthophila are the Apidae, which comprise Nomadinae, Xylocopinae, and Apinae (Engel, 2001, 2005; Michener, 2007). Each of these three subfamilies is fairly well circumscribed, with only a few taxa not yet clearly assigned as to subfamily. One such taxon is the tribe

Ctenoplectrini, with 19 species in Africa, Asia, and Australia (Eardley, 2003; Engel, 2007; H. Schaefer and M. Engel, manuscript; our Fig. 1). Depending on the interpretation of their relatively short glossa and labial palpi, Ctenoplectrini have variously been placed as a subfamily (Ctenoplectrinae) in the Melittidae, which are short-tongued (S-T) bees (Michener, 1944), as a distinct family (Ctenoplectridae) and sister group to all long-tongued (L-T) bees (Michener and Greenberg, 1980; Alexander and Michener, 1995), or as a tribe (Ctenoplectrini) within the Apidae of the L-T bees (Roig-Alsina and Michener, 1993; Silveira, 1993a). The most recent comprehensive classification of bees assigns Ctenoplectrini to the large L-T subfamily Apinae, which includes 19 tribes, including the commercially important Apini (honeybees), Bombini (bumblebees), and Meliponini (stingless bees) (Michener, 2007). This place-

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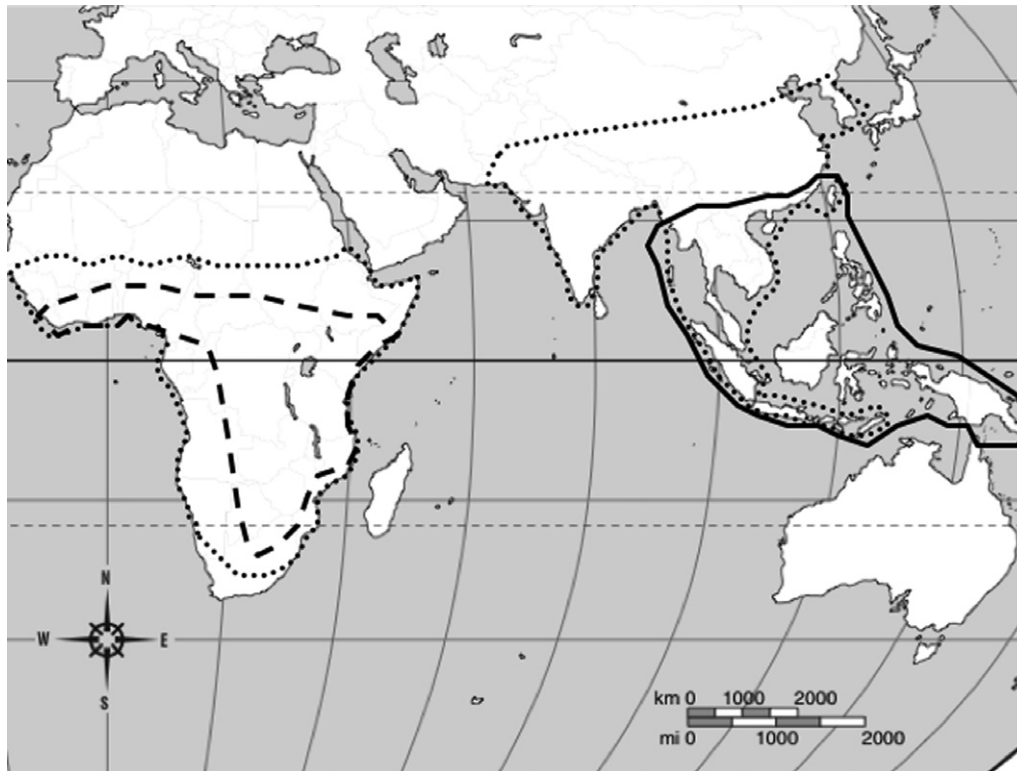


Fig. 1. Distribution map of the oil bee tribe Ctenoplectrini (Apidae): Australian/Asian *C. kelloggi* clade (bold line), *Ctenoplectrina* (broken line), *C. polita* clade in Africa and *C. davidi* clade in Asia (dotted lines). The Australian range of the genus is confined to a very small region of Northern Queensland's Cape York Peninsula.

ment is supported by the morphological study of Roig-Alsina and Michener (1993), who used 131 characters of adult bees. In a recent analysis, Danforth et al. (2006b) added DNA data from five markers to the morphological data set of Alexander and Michener (1995) and found *Ctenoplectra albolimbata* (the only included Ctenoplectrini) nested among the twelve representatives of Apidae sampled.

Ctenoplectrini comprise two genera, *Ctenoplectrina* and *Ctenoplectra*, with the former having three species endemic in Africa, the latter nine species in Asia, one in Australia, and six in Africa (H. Schaefer and M. Engel, manuscript). There are two morphological groups (Vogel, 1990), each spanning Africa and Asia, namely large, metallic bluish-green species and small, brown or black species. *Ctenoplectra* bees are oligolectic on Cucurbitaceae flowers from which they obtain pollen and floral oil as larval food, as well as nectar to cover the energy requirements of the adults (Vogel, 1990; H. Schaefer and S. Renner, unpublished data). *Ctenoplectrina* females have lost the morphological features associated with oil or pollen collection and are therefore thought to be cleptoparasitic, probably on small *Ctenoplectra* species (Rozen, 1978; Michener, 2007; field observations by HS).

As part of a study on the evolution of pollinator relationships in the Cucurbitaceae, we set out to test the monophyly and relationships of Ctenoplectrini and to infer their biogeographic history, focusing on major divergence events

in the tribe. The unclear relationships among the 19 tribes of Apinae required relatively broad sampling of potential outgroups to achieve proper rooting of Ctenoplectrini. We therefore focused on mitochondrial and nuclear markers widely used in bee phylogenetics (Schwarz et al., 2004; Fuller et al., 2005; Danforth et al., 2006a, 2006b; Cameron et al., 2007). A second reason for relatively broad outgroup sampling was the need to include taxa with fossil records to constrain a molecular clock for Ctenoplectrini. Specific questions we wanted to answer were, (i) where and when did Ctenoplectrini evolve and are they monophyletic; (ii) what is the relationship between the Asian and African species and how old are these disjunctions; (iii) what is the phylogenetic relationship of the (presumed) cleptoparasites to their hosts; and (iv) is *Ctenoplectra* diversification temporarily correlated with particular climates and the evolution of special biota, such as savannah or dry forest biomes.

2. Materials and methods

2.1. Taxon sampling

Ctenoplectrini are poorly represented in collections, and most museum specimens are too old or too valuable for destructive sampling. DNA sampling therefore relied mostly on specimens collected during the first author's fieldtrips to Africa and Australasia in 2005, 2006, and 2007. Our taxon sampling includes all six African species

of *Ctenoplectra*, four of the nine Asian species, and the sole Australian species. *Ctenoplectra* material from Laos, the Philippines, and New Guinea was available for morphological comparison only. *Ctenoplectrina* is represented by one of the two described species and a new species from Nigeria (H. Schaefer and M. Engel, manuscript). Based on the results of Roig-Alsina and Michener (1993) and Danforth et al. (2006b), we included the following outgroups: *Ancyla*, *Apis*, *Bombus*, *Diadasia* (GenBank No. AF300533, AY585110), *Eucera*, *Liotrigona*, *Lithurgus* (DQ067195, DQ141116), *Nomada*, *Trigona*, *Megachile* (DQ067196), *Melissodes* (AF181616), *Melitoma* (AF300516, AF300550), *Paratetrapedia* (DQ225337), *Ptilothrix* (AF300517, AF300562), *Tetrapedia* (DQ225332), *Toromelissa* (AF300518, AF300565) and *Xylocopa* (AY005224, AY005251). Several of these have a fossil record, required to constrain a molecular clock (see Section 2.5). Names and geographic origins of all extracted bee specimens are shown in Table 1. Voucher specimens have been deposited in the following public collections: Snow Entomological Museum, Lawrence (Kansas); Natural History Museum, London; and American Museum of Natural History, New York.

2.2. DNA extraction, PCR, cloning and sequencing

Total genomic DNA was isolated from bees preserved in concentrated Ethanol (96–99%) and from a few dry, pinned specimens. Tissue was taken from the thoracic musculature without destruction of the voucher specimen or by grinding entire legs with glass beads. The DNA was then isolated using commercial animal tissue extraction kits (NucleoSpin Tissue, Macherey–Nagel, Düren, Germany), following the manufacturer's manual, but with a prolonged incubation of up to 8 h in Lysis buffer. For older specimens with more degraded DNA an extraction kit for food samples (NucleoSpin, Macherey–Nagel) was used, again following the manufacturer's protocol.

Two mitochondrial and two nuclear gene regions were amplified, and all fragments were sequenced in both directions. The mitochondrial regions are from the protein-coding genes cytochrome *b* (mt *cytb*) and cytochrome oxidase 1 (*cox-1*). The nuclear regions are the F2 copy of elongation factor 1 α (*Ef-1 α*) and the carbamoylphosphate synthase domain of the fusion protein carbamoylphosphate synthetase, aspartate transcarbamylase and dihydroorotase (*CAD*). The primer sequences used for polymerase chain

Table 1
Species and newly sequenced loci for this study, their sources and geographic provenience, and GenBank accession numbers

Species	Geographic origin of the sequenced material	<i>Ef1-alpha</i> gene, (F2 copy)	<i>CAD</i> gene	<i>cox-1</i> gene	<i>cytb</i> gene
<i>Ctenoplectra albolimbata</i> Magretti	Tanzania, Lindi District	EU122133	EU122059	EU122082	EU122105
<i>Ctenoplectra albolimbata</i> Magretti	Tanzania, Pugu Hills	EU122134	EU122060	EU122083	EU122106
<i>Ctenoplectra antinorii</i> Gribodo	Tanzania, West Usambara Mountains	EU122136	EU122062	EU122085	EU122108
<i>Ctenoplectra armata</i> Magretti	Tanzania, East Usambara Mountains	EU122137	EU122063	EU122086	EU122109
<i>Ctenoplectra australica</i> Cockerell	Australia, Cape York Peninsula	EU122138	EU122064	EU122089	EU122110
<i>Ctenoplectra bequaerti</i> Cockerell	Nigeria, Cross River State	EU122139	EU122065	EU122087	EU122111
<i>Ctenoplectra davidi</i> Vachal	PR China, Yunnan Province	EU122140	EU122066	EU122088	EU122112
<i>Ctenoplectra elsei</i> Engel	Indonesia, Sulawesi	—	—	Pseudogene	EU122113
<i>Ctenoplectra florissomnis</i> van der Vecht	PR China, Guangdong Province	EU122141	EU122067	EU122090	EU122114
<i>Ctenoplectra florissomnis</i> van der Vecht	PR China, Yunnan Province	EU122142	EU122068	EU122091	EU122115
<i>Ctenoplectra kelloggi</i> Cockerell	PR China, Guangdong Province	EU122143	EU122069	Pseudogene	EU122116
<i>Ctenoplectra kelloggi</i> Cockerell	PR China, Guangxi Province	EU122144	EU122070	Pseudogene	EU122117
<i>Ctenoplectra polita</i> (Strand)	Tanzania, East Usambara Mountains	EU122146	EU122072	EU122093	EU122119
<i>Ctenoplectra polita</i> (Strand)	Tanzania, Dar-Es-Salaam, Pugu Hills	EU122147	EU122073	EU122094	EU122120
<i>Ctenoplectra polita</i> (Strand)	Tanzania	EU122148	EU122074	EU122095	EU122121
<i>Ctenoplectra polita</i> (Strand)	Nigeria, Cross River State	EU122145	EU122071	EU122092	EU122118
<i>Ctenoplectra terminalis</i> Smith	Tanzania, East Usambara Mountains	EU122150	EU122076	EU122097	EU122124
<i>Ctenoplectra terminalis</i> Smith	Tanzania, East Usambara Mountains	EU122151	EU122077	—	EU122125
<i>Ctenoplectrina alluaudi</i> (Cockerell)	Nigeria, Cross River State	EU122135	EU122061	EU122084	EU122107
<i>Ctenoplectrina</i> spec. nov.	Nigeria, Cross River State	EU122149	EU122075	EU122096	EU122122
<i>Ctenoplectrina</i> spec. nov.	Togo, leg. S. Vogel	—	—	—	EU122123
Outgroups					
<i>Ancyla asiatica</i> Friese	Greece, Rhodos, leg. A. Mueller	—	—	—	EU122102
<i>Apis mellifera</i> L.	Germany, Bavaria	EU122132	—	EU122080	EU122103
<i>Bombus pascuorum</i> (Scopoli)	Germany, Bavaria	—	EU122058	EU122081	EU122104
<i>Eucera nigrescens</i> Pérez	Germany, Bavaria	—	—	—	EU122126
<i>Liotrigona</i> spec.	Tanzania, East Usambara Mountains	EU122152	—	EU122098	EU122127
<i>Megachile willughbiella</i> (Kirby)	Germany, Bavaria	EU122153	—	—	EU122128
<i>Nomada fucata</i> Panz.	Germany, Bavaria	EU122154	EU122078	EU122099	EU122129
<i>Trigona collina</i> Smith (subgenus <i>Heterotrigona</i>)	PR China, Yunnan Province	EU122155	—	EU122100	EU122130
<i>Xylocopa violacea</i> L.	Germany, Bavaria	EU122156	EU122079	EU122101	EU122131

reaction (PCR) amplification are listed in Table 2. Primers for the *cytb* region were taken from Schwarz et al. (2004). For the *cox-1* gene region, we used two sets of primers that produce a fragment of c. 1300 nucleotides (nt) near the 3' end of *cox-1*: M414 and M399 of Schwarz et al. (2004) and a second set consisting of the universal primers UEA7 and UEA10 designed by Lunt et al. (1996). Because of pseudogene problems (Section 3) we used an additional more specific primer pair for the Australian/Asian taxa from the list in Simon et al. (2006): C1J2195 and C1N2776. Regarding the nuclear markers, *Ef-1 α* has two copies in bees, *Ef-1 α* F1 and *Ef-1 α* F2, which are expressed at different stages of development (Danforth and Ji, 1998). The primers used here for PCR amplification of the *Ef-1 α* F2 region included the F2-specific forward primer HaF2-For1 and the reverse primer F2-Rev1 designed by Danforth et al. (2004), plus two newly designed internal primers for degraded DNA samples: EF1int-F and EF1int-R. The primers for the *CAD* region are from Moulton and Wiegmann (2004, 581F and 680R) and Danforth et al. (2004, apCADfor1 and ap835rev1, ap787for2 and ap1098rev2, apCADfor3 and apCADrev3a).

The PCR protocols used were as follows: Initial denaturation at 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C for denaturation, 1 min for primer annealing at 48–55 °C (depending on DNA quality) and 1 min 40 s at 72 °C for DNA elongation, followed by a final elongation period of 7 min at 72 °C. Reactions were performed with 10 μ M of primers, 25 μ M MgCl₂, 1.25 μ M of each dNTP, 2.5 μ M of 10 \times PCR buffer, 0.5 U of Taq DNA polymerase, and 10–50 ng of template DNA per 25 μ l reaction volume. When amplification failed, the more reactive Phusion polymerase was used (Phusion TM High Fidelity PCR Kit, Finnzymes) according to the manufacturer's protocol. For

cloning we used the pGEM-T Vector Kit (Promega). Cloning of both nuclear markers was performed in two species to test for the presence of multiple copies. Mitochondrial sequences were cloned when amino acid translation of sequences revealed stop codons within the highly conserved proteins.

All reaction products were purified with Wizard SV gel and PCR clean-up kits (Promega), and cycle sequencing was performed with BigDye Terminator v3.0 cycle sequencing kits (Applied Biosystems), using 1/4-scale reaction mixtures. The dye terminators were removed by Sephadex G-50 Superfine gel filtration (Amersham Biosciences) on MultiScreen TM-HV membrane plates (Millipore) according to the manufacturer's protocol. Purified sequencing reactions were run on an ABI Prism 3100 Avant sequencer. The PCR primers were used for sequencing. Sequences were edited with Sequencher 4.6 (Gene Codes, Ann Arbor, MI, USA) and aligned by eye, using MacClade 4.06 (Maddison and Maddison, 2003).

2.3. Phylogenetic analyses

Equally weighted parsimony searches were conducted with version 4.0b10 of PAUP (Swofford, 2002). Gaps were treated as missing data. Parsimony searches used 10 random taxon-addition replicates, tree-bisection-reconnection (TBR) swapping and steepest descent, but multrees not in effect. Statistical support was measured by non-parametric bootstrapping as implemented in PAUP, using a random starting tree, 1000 replicate heuristic searches, each with 100 random taxon-addition replicates and otherwise the same settings as in the tree searches. Mitochondrial and nuclear data sets were analyzed separately. A partition homogeneity test (implemented in PAUP; equivalent to

Table 2
PCR primers used in this study

Primer name	Gene region	Primer sequence (5'–3')	Source
cb1 (forward)	<i>cytb</i>	TAT GTA CTA CCA TGA GGA CAA ATA TC	Schwarz et al. (2004)
cb2 (reverse)	<i>cytb</i>	ATT ACA CCT CCT AAT TTA TTA GGA AT	Schwarz et al. (2004)
M414 (forward)	<i>cox-1</i>	CCT TTT ATA ATT GGA GGA TTT GG	Schwarz et al. (2004)
M399 (reverse)	<i>cox-1</i>	TCA TCT AAA AAC TTT AAT TCC TG	Schwarz et al. (2004)
UEA7 (forward)	<i>cox-1</i>	TAC AGT TGG AAT AGA CGT TGA TAC	Lunt et al. (1996)
UEA10 (reverse)	<i>cox-1</i>	TCC AAT GCA CTA ATC TGC CAT ATT A	Lunt et al. (1996)
C1J2195 (forward)	<i>cox-1</i>	TGA TTC TTT GGW CAC CCW GAA GT	Simon et al. (2006)
C1N2776 (reverse)	<i>cox-1</i>	GGT AAT CAG AGT ATC GWC GNG G	Simon et al. (2006)
HaF2For1	<i>Ef-1α</i> F2	GGG YAA AGG WTC CTT CAA RTA TGC	Danforth et al. (2004)
F2-Rev1	<i>Ef-1α</i> F2	AAT CAG CAG CAC CTT TAG GTG G	Danforth et al. (2004)
EF1int-F	<i>Ef-1α</i> F2	TCY KSH AAR ATG CCY TGG TTY A	newly designed by HS
EF1int-R	<i>Ef-1α</i> F2	AGY GGA AGY CBG AGY GCR TT	newly designed by HS
581F	<i>CAD</i>	GGW GGW CAA ACW GCW YTM AAY TGY GG	Moulton and Wiegmann (2004)
680R	<i>CAD</i>	AAN GCR TCN CGN ACM ACY TCR TAY TC	Moulton and Wiegmann (2004)
apCAD for1	<i>CAD</i>	GGW TAT CCC GTD ATG GCB MGW GC	Danforth et al. (2004)
ap835 rev1	<i>CAD</i>	GCA THA CYT CHC CCA CRC TYT TC	Danforth et al. (2004)
ap787 for2	<i>CAD</i>	TGC TTY GAR CCD AGY CTH GAT TAY TG	Danforth et al. (2004)
ap1098 rev2	<i>CAD</i>	ATA TTR TTK GGC ARY TGD CCK CCC	Danforth et al. (2004)
apCAD for3	<i>CAD</i>	CTC HGT KGA RTT YGA TTG GTG YGG	Danforth et al. (2004)
apCAD rev3a	<i>CAD</i>	CAR GGR TAR CCR ACY TCY TCR CAA AAT TC	Danforth et al. (2004)

the ILD test of Farris et al., 1994) with 100 replicates, 10 random taxon-addition sequences, TBR swapping on best trees only and the multrees option on was performed to test whether the mitochondrial and the nuclear data sets could be combined (uninformative sites were excluded for this test).

We performed one parsimony analysis of the combined data and a second analysis with the third codon position of all mitochondrial sequences excluded because previous analyses of allodapine bees have found long-branch effects when mitochondrial third positions were included (Schwarz et al., 2004, 2006). A third analysis used six data partitions, namely *cytb*, *cox-1*, *Ef-1 α* coding, *Ef-1 α* non-coding, *CAD* coding, and *CAD* non-coding.

To find the best substitution models for our data, we used ModelTest 3.7 (Posada and Crandall, 1998) under the Akaike information criterion. The best-fitting model for each of the individual partitions as well as the combined data was the general time reversible (GTR) model plus a gamma shape parameter (G) and proportion of invariable sites (P-invar).

Maximum likelihood (ML) analyses were performed using GARLI 0.951 (Zwickl, 2006, available at www.bio.utexas.edu/faculty/antisense/garli/Garli.html), with model parameters estimated over the duration of specified runs. Several runs were performed, and of the resulting trees, the one with the best log likelihood value was chosen. Bootstrap support values were estimated in GARLI with 100 replicate heuristic searches under the same model as used in the searches. Additional ML analyses under the same model with the combined, unpartitioned and the six partitions data set were performed with RAxML-VI-HPC version 4.0.0 (A. Stamatakis, 2006, available at <http://phylobench.vital-it.ch/raxml-bb/index.php>).

Bayesian inference also used the GTR + G + P-invar model and relied on MrBayes (Huelsenbeck and Ronquist, 2001). We first analyzed the combined, unpartitioned dataset and then four partitions (mitochondrial first and second position, mitochondrial third position, *Ef-1 α* , CAD) and six partitions (see above), allowing partition models to vary by unlinking gamma shapes, transition matrices, and proportion of invariable sites. Bayesian runs started from independent random starting trees and were repeated twice. Markov chain Monte Carlo (MCMC) runs extended for 1 million generations, with trees sampled every 100th generation. We used the default priors in MrBayes, namely a flat Dirichlet prior for the relative nucleotide frequencies and rate parameters, a discrete uniform prior for topologies, and an exponential distribution (mean 1.0) for the gamma-shape parameter and branch lengths. Convergence was assessed by checking that the standard deviations of split frequencies were <0.01; that the log probabilities of the data given the parameter values fluctuated within narrow limits; that the convergence diagnostic (the potential scale reduction factor given by MrBayes) approached 1; and by examining the plot provided by MrBayes of the generation number versus the log probability of the data.

Trees saved prior to convergence were discarded as burn-in (1000 trees) and a consensus tree was constructed from the remaining trees.

2.4. Biogeographic analyses

For dispersal-vicariance analysis, we coded the distribution range of each species in a binary matrix, with the categories being presence/absence in Africa, Europe, Asia/Australia, and America. We then used DIVA 1.1 (Ronquist, 1996, 1997) to infer vicariance and dispersal events. The maximum number of areas that could be simultaneously occupied by hypothetical ancestral lineages was experimentally varied between two, three, and four; otherwise we used the DIVA default settings.

2.5. Dating methods

Bee fossils are uncommon and rarely show sufficient diagnostic characters to be reliably associated with particular nodes in a molecular tree. As there is no fossil of *Ctenoplectrini* (M. Engel, University of Kansas, personal communication, June 2006), trees could not be calibrated internally but only by using fossils from related clades (as specified below; the confusingly named fossil amber bee genus *Ctenoplectrella* belongs to the Megachilidae; Engel, 2001, 2006).

Divergence time estimation relied on a strict clock, a Bayesian relaxed clock (Thorne et al., 1998), and penalized likelihood (Sanderson, 2002). To estimate divergence times from a strict clock, *Ef-1 α* branch lengths were calculated under a GTR + G + P-invar + clock model on the preferred ML topology. This locus has relatively clock-like behaviour (as ascertained in a likelihood ratio test). The tree was imported into PAUP, rooted on *Megachile* and *Lithurgus* and branch lengths then calculated under the “enforce clock” option. The resulting branch length table was saved, the distance between a calibration node and the present divided by the age of the calibration node to obtain a substitution rate, and this rate then used to calculate the age of divergence events of interest. Calibration came from two sequentially used calibration points: (i) *Cretotrigona prisca* from the Maastrichtian (70–65 million years [my] ago), a bee conserved in amber that is a representative of the Meliponini and morphologically closest to the African *Dactylurina* of *Trigona* s.l. (Engel, 2000a, 2001). We included two specimens of *Trigona* s.l. in our analyses, *Liotrigona* spec. from Africa and *Trigona collina* of subgenus *Heterotrigona* from China, and constrained the *Trigona* stem lineage (the split Meliponini–Apini) to minimally 65 my. *Trigona* apparently is not monophyletic (Rasmussen and Cameron, 2007) but the Meliponini are. (ii) For an alternative calibration, we included *Xylocopa violacea* of subgenus *Xylocopa* and *Xylocopa tranquebarica* of subgenus *Nyctomelitta*. Based on the results of Leys et al. (2002), we constrained the split between these subgenera to minimally 43 my. Following Renner and Meyer

(2001), we used binomial probability theory to estimate the standard deviation (SD) of the distance from the fixed calibration node to the tips and then used this value to obtain the SDs of the estimated ages of interest.

Penalized likelihood dating was performed using *r8s* version 1.71 (Sanderson, 2002; available from <http://loco.biosci.arizona.edu/r8s/>). The input topology and branch lengths were those obtained from the combined data analyzed under ML, and the appropriate smoothing parameter was found via cross-validation.

Bayesian relaxed clock dating was performed using the approach developed by Thorne and Kishino (2002) and Thorne et al. (1998). These authors' *multidivtime* software was used in R, relying on LAGOPUS, an R wrapper script written by C. Heibl & N. Cusimano (Department of Biology, University of Munich, available at www.christophheibl.de/mdt/mdtlnr.html). With this macro, model parameters were estimated under the F84 + G model, this being the only model implemented in *multidivtime*. Based on Baltic amber fossils and morphological studies, Engel (2001) has suggested that the Apidae are c. 90 my old, and we therefore set the prior on the mean age of the root node to 90, with an equally large standard deviation. The prior distribution of the root rate was set to 0.0019, a value obtained by dividing the median of the distances between the root and the tips in the *estbranches* phylogram by the root age (branch length estimation in *estbranches* being the first step of *multidivtime* age estimation). A comparable root rate was obtained in an *Efl- α* plus *cox-1* data matrix for allodapine bee, constrained similarly to ours (Schwarz et al., 2006). The prior for the Brownian motion parameter, which controls the magnitude of autocorrelation per my along the descending branches of the tree and its standard deviation were set to 1.11. MCMC chains comprised 1 million cycles and were sampled every 100th generation, with a burn-in of 100,000 cycles. Confidence in node ages was assessed from the 95% credibility intervals calculated by the program.

For absolute ages we relied on the geologic time scale of Gradstein et al. (2004).

3. Results

3.1. Locus lengths, base frequencies, and rate heterogeneity

Mitochondrial and nuclear sequences were generated for 13 of the 19 (68%) species of Ctenoplectrini. For six species, we sequenced two individuals from different locations, and for the widespread species *Ctenoplectra polita*, we sequenced four individuals covering the geographic range of the species. The total matrix comprised 13 ingroup species (21 accessions) plus 18 outgroup species. Sequences of *cytb* were obtained for all 13 ingroup species, *cox-1* for 11, and *EF-1 α* and *CAD* for 12. All newly created sequences have been deposited in GenBank (Table 1).

We recovered 462, 1301, 985, and 2116 nt of *cytb*, *cox-1*, *EF-1 α* , and *CAD*, respectively, of which 209 (45%; 146% and 32% just for the ingroup), 429 (33%; 327%

and 25% just for the ingroup), 261 (26%; 124% and 13% just for the ingroup), and 553 (26%; 265% and 13% just for the ingroup) nucleotides were parsimony-informative. Alignment was straightforward for the mitochondrial sequences and for the exon regions of the nuclear sequences. Intron regions of the latter were more difficult to align, especially between the ingroup and the more distant outgroups. Two highly variable regions of 36 and 87 aligned nt of the *CAD* introns were excluded from the analyses because of alignment problems. All data matrices and trees have been deposited in TreeBASE (<http://www.treebase.org/>). The combined ingroup data matrix consisted of 4710 characters (862%, or 18%, of them parsimony-informative); a matrix that included the 18 outgroup taxa had a length of 4741 (1504%, or 32%, parsimony-informative). To assess the homogeneity of base frequencies across taxa, we ran χ^2 tests in PAUP for each of the individual data sets, excluding missing or ambiguous sites. Results for the four genes were: $\chi^2 = 51$, $df = 93$, $P = 1.0$ for the *cytb* region; $\chi^2 = 35$, $df = 57$, $P = 0.99$ for the *cox-1* region; $\chi^2 = 22$, $df = 78$, $P = 1.0$ for the *EF-1 α* region; $\chi^2 = 4$, $df = 42$, $P = 1.0$ for the *CAD* region. These tests revealed no nucleotide bias among taxa. Empirical base frequencies in the 39 taxon and 21 taxon files (with or without outgroups) were A = 0.31, C = 0.16, G = 0.19, T = 0.35.

The oldest specimens successfully used for DNA extraction were an undescribed species of *Ctenoplectrina* collected by S. Vogel in 1985 and a specimen of *Ctenoplectra elsei* collected by G. Else also in 1985. Two legs were used for each extraction, and reasonable *cytb* sequences were obtained.

3.2. Cloning and *cox-1* pseudogenes

Clones of the nuclear loci *EF-1 α* and *CAD* were all identical, confirming the characterization of these genes as low copy markers (Danforth et al., 2004). Mitochondrial *cytb* was not cloned because none of the sequences had double peaks, nor did amino acid translation result in any stop codons. The mt *cox-1* sequences of the three Australian/Asian species *Ctenoplectra australica*, *Ctenoplectra kelloggi*, and *C. elsei*, however, produced divergent and apparently non-functional copies. We therefore cloned *cox-1* from the first two and sequenced 20 clones of each. Cloning of *C. elsei* sequences was unsuccessful. Judging from stop-codons in the amino acid sequences, only one of the 20 copies from *C. australica* was functional (clone 1) and not a single of the 20 obtained from *C. kelloggi*. The number of different copies is too high to be explained entirely with PCR artifacts although it is possible that PCR artifacts are among them. The remaining species of Ctenoplectrini yielded unambiguous *cox-1* sequences, and only these were included in the final analyses (next section). Analysis of all *cox-1* sequences including the pseudogenes shows that they form a paraphyletic cluster in which the *C. australica/C. kelloggi/C. elsei* clade is embedded (Fig. 2).

3.3. Phylogenetic analyses

Parsimony trees resulting from mitochondrial and nuclear data (not shown) did not contradict each other in any statistically well-supported nodes, and the partition homogeneity test also revealed no significant incongruence between data sets ($P = 0.09$). The combined data yielded 867 most parsimonious trees that did not differ from each other in the relationships found for the ingroup. As in individual nuclear and mitochondrial trees, *Ctenoplectrina* was sister to *Ctenoplectra*. Exclusion of third codon positions in the mitochondrial data sets had no effect on tree topology. Maximum likelihood analyses of the combined data produced a topology (Fig. 3) similar to most parsimony topologies for the ingroup; and Bayesian tree searches also yielded a topology that was similar (not shown).

Ctenoplectrini always formed a monophyletic group (with 80% ML bootstrap support and 100% posterior probability [PP]; these values may be equivalent, see Suzuki et al., 2002), sister to the Eucerini *Eucera* and *Melissodes* (with 76% ML bootstrap support and 100% PP). The two sequenced species of *Ctenoplectrina* form a monophyletic group (100% supports under either optimality criterion) and are sister to *Ctenoplectra*. The latter is also monophyletic (100% PP, 64% ML bootstrap support) and consists of three well supported clades: (i) an African group with the

large green and blue species *Ctenoplectra bequaerti* and *Ctenoplectra terminalis*; (ii) an Asian group with the small brownish-black species *Ctenoplectra davidi* and *Ctenoplectra florisomnis*; and (iii) a clade consisting of an Australian/Asian group (*C. kelloggi* and its relatives) plus another African group with small brownish-black species (the *C. polita* clade).

3.4. Biogeographic analyses

All three runs (with different assumptions on the maximum number of areas occupied by a hypothetical ancestor) resulted in identical solutions that favored Africa as the most likely range of the most recent common ancestor of Ctenoplectrini. The DIVA results also support two independent dispersal events from Africa to Asia, one of them followed by extension into Australia.

3.5. Molecular dating

Estimates for the divergence times of major clades obtained with the three dating methods overlap (Table 3); we here focus on the results from the Bayesian relaxed clock and their 95% confidence intervals. The age of the Ctenoplectrini stem group (i.e., the split of Ctenoplectrini from Eucerini) is estimated as 56 (CI 67–44) my old (Early

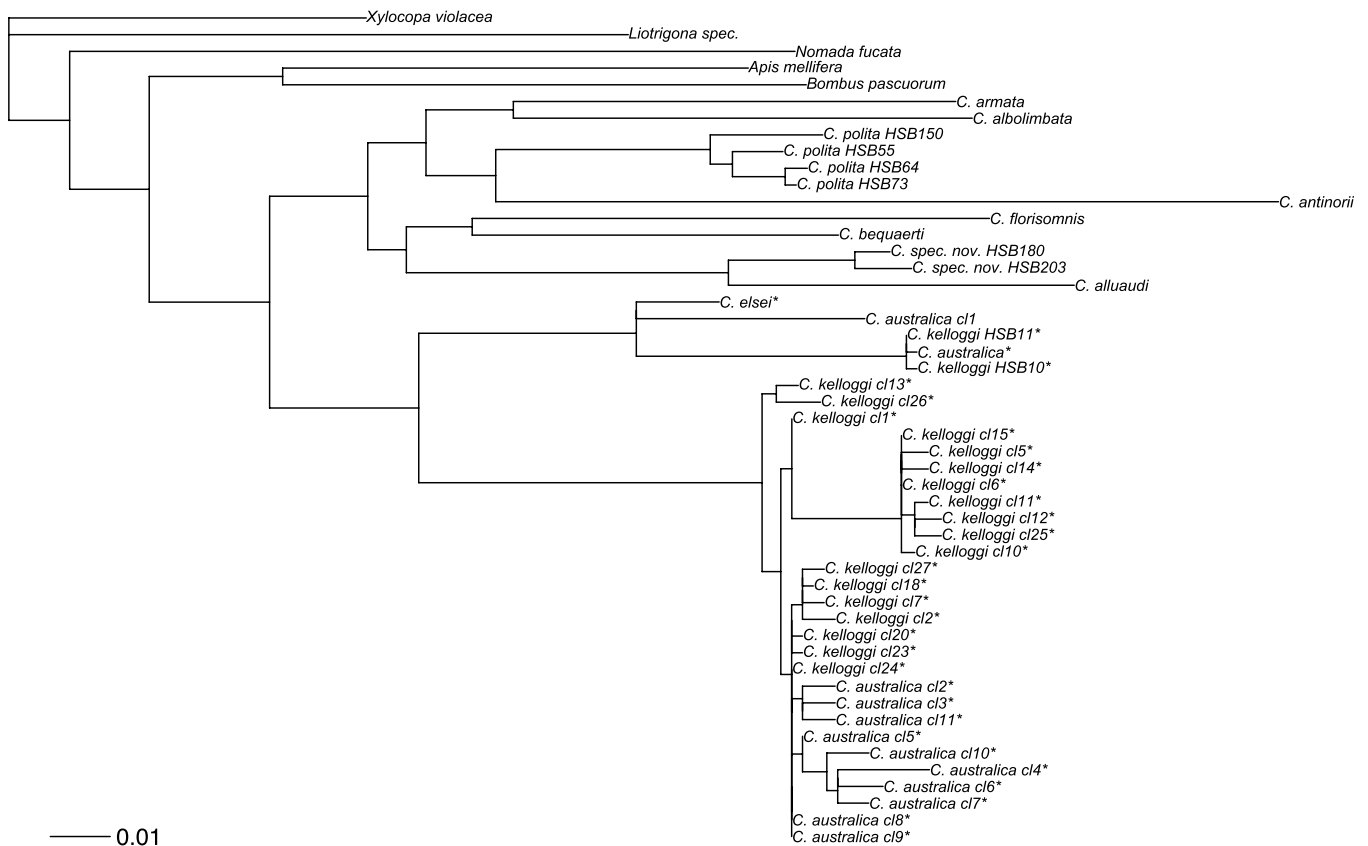


Fig. 2. Maximum likelihood phylogram for Ctenoplectrini (rooted on *Xylocopa*) obtained from directly sequenced and cloned mt *cox-1* sequences, the latter are marked with the prefix “cl”, followed by a number. Pseudogenes are marked with an asterisk.

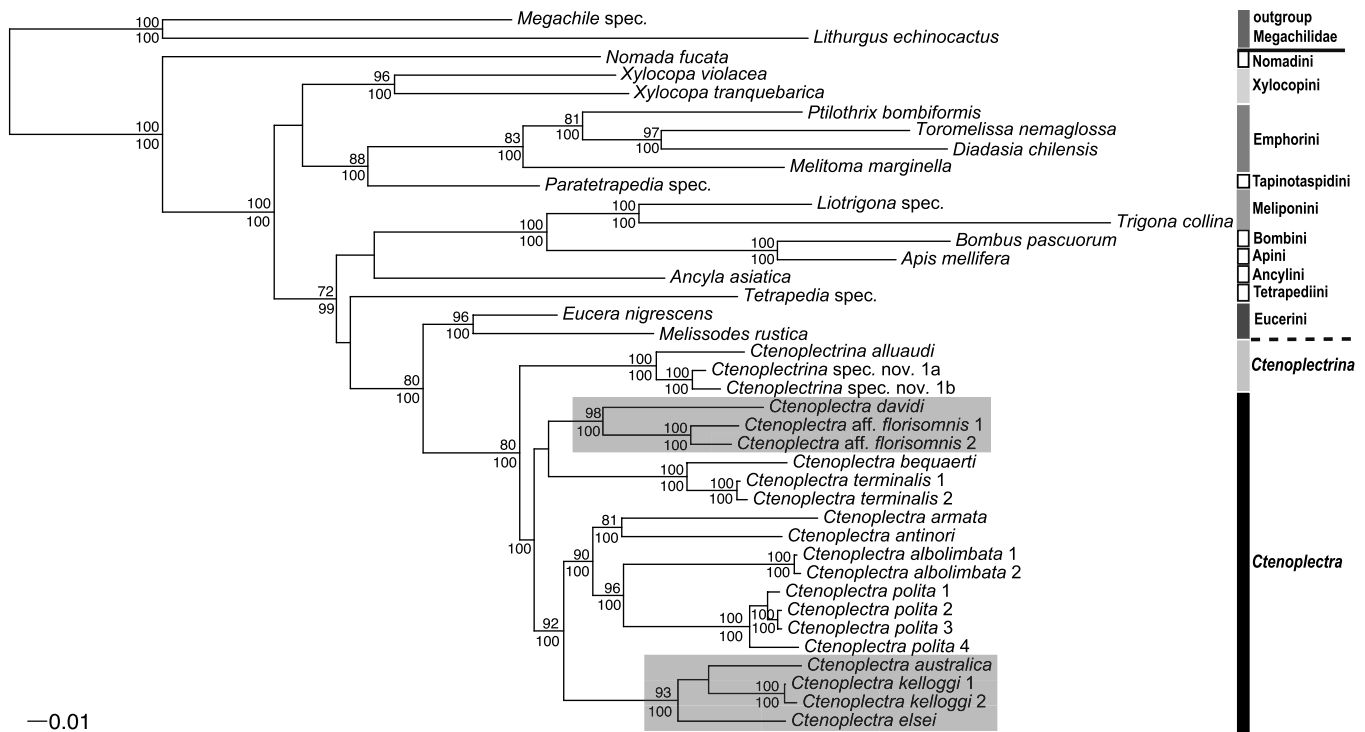


Fig. 3. Maximum likelihood phylogram for Ctenoplectrini and other Apidae (rooted on Megachilidae based on Danforth, 2006) obtained from mt *cytb*, mt *cox-1*, nc *EF-1 α* , and nc *CAD* (4741 nucleotides). Values above branches indicate likelihood bootstrap $\geq 80\%$, values below branches Bayesian posterior probabilities (PP) $> 95\%$. The grey boxes mark the two Asian (Australasian) clades of *Ctenoplectra*, viz. the *C. davidi* clade and the *C. kelloggi* clade in Fig. 1. The remaining species of Ctenoplectrini occur in Africa.

Eocene) and the split between *Ctenoplectrina* and *Ctenoplectra* as 42 (50–33) my old (mid-Eocene). Dispersal from Africa to Asia occurred 36 (44–28) and 34 (42–26) my ago. The *C. polita* group of non-parasitic small African species evolved some 30 my ago. The Australian/Asian clade of large blue species has a crown group age of 17 (24–12) my. Dispersal to Australia occurred 13 (18–9) my ago.

4. Discussion

4.1. Phylogeny and evolution of the Ctenoplectrini

This study confirms that Ctenoplectrini are a member of Apinae as found by Danforth et al. (2006b), whose study placed *C. albolimbata* as sister to *Apis mellifera*. A related analysis that included two Eucerini (Danforth et al., 2006a) placed them as sister to Apini and Centridini, but did not include any Ctenoplectrini. The sister group relation between Ctenoplectrini and Eucerini discovered here (Fig. 3) requires further testing with a denser sample of Apinae and Eucerini. A sister group relationship between Ctenoplectrini and Ancylini, which might have been suggested by the shared reduced labial palpi (at least in the type genus *Ancyla*; see Baker, 1998), is rejected by our data, implying that short tongues in these clades evolved independently. Short tongues in *Ancyla* could be an adaptation to shallow-flowered plant hosts, such as Apiaceae (Silveira, 1993b), and similarly in Ctenoplectrini, short tongues may

fit their cucurbitaceous hosts, which have easily accessible nectar (Schaefer and Renner, unpublished). Similar short labial palpi among L-T bees are only found in a few parasitic Allodapini that are unlikely to be the sister group to Ctenoplectrini (Michener, 2007).

Previous morphological studies of Apinae divided them into two groups, the Apine line (including among others the Centridini, Anthophorini, and Apini) and the Eucerine line (including for example the Eucerini, Emphorini, Tapinotaspidini, Ancylini, and Exomalopsini). Synapomorphies for Apine are a distinct stipital sclerite and for Eucerine an apically 2–4-lobed seventh sternum in the males and an anterior tentorial arm that is fused to the head wall, forming a large triangular subantennal area (Silveira, 1993a). Based on these characters, Silveira (1993a) placed the Ctenoplectrini in the Eucerine line, which is supported by our molecular data. The number of four ovarioles per ovary found in *C. albolimbata* from South Africa (Rozen, 2003) is the basic number in Apidae, but larval characters are insufficiently known to discern relationships of Ctenoplectrini within Apidae (Straka and Bogusch, 2007).

Given their distinct morphology, the monophyly of Ctenoplectrini is perhaps not surprising, but the sister group relation between *Ctenoplectra* and *Ctenoplectrina* is unexpected. (A taxonomic revision of Ctenoplectrini by H. Schaefer and M. Engel is in preparation and will include a morphological data matrix.) *Ctenoplectrina* and the small

Table 3
Divergence time estimates for major clades of Ctenoplectrini obtained from different clock approaches and alternative calibrations (see Section 2)

Dating method	Split Eucerini	Split Ctenoplectrina-Ctenoplectrina	1st dispersal to Asia	2nd dispersal to Asia	C. polita clade	C. davidi clade (crown group)	Australian/Asian clade (crown group)	Ctenoplectrina (crown group)	Dispersal to Australia	C. bequaerti clade
Strict clock (SD) Calibration <i>Creto-trigona</i>	66 (±3)	52 (±3)	44 (±3)	42 (±2)	39 (±2)	30 (±2)	26 (±2)	18 (±2)	18 (±2)	16 (±2)
Strict clock (SD) Calibration <i>Nyctomelitta</i>	48 (±2)	38 (±2)	32 (±2)	30 (±2)	28 (±2)	21 (±2)	18 (±1)	13 (±1)	13 (±1)	11 (±1)
Relaxed clock, likelihood	54	40	38	34	29	29	17	13	13	14
Relaxed clock, Bayes (CI)	56 (44–67)	42 (33–50)	36 (28–44)	34 (26–42)	29 (22–38)	28 (20–37)	17 (12–24)	16 (10–23)	13 (9–18)	13 (9–19)

SD, standard deviation; CI, 95% confidence interval.

African species of *Ctenoplectra* are morphologically similar, and it therefore seemed likely that the former might have evolved from the latter, which turned out not to be the case. The third species of *Ctenoplectrina*, *C. politula*, however, has not yet been sequenced, and the monophyly of *Ctenoplectrina* therefore needs further testing. Cleptoparasitism in *Ctenoplectrina* is so far only inferred from the absence of any pollen or oil collecting apparatuses in the females and the strictly sympatric occurrence with *Ctenoplectra*. Nests of *Ctenoplectra* are very difficult to find, and the hosts of the presumed parasites are therefore unknown (Rozen, 1978; Michener and Greenberg, 1980; Vogel, 1990; H.S., personal observation in Tanzania and PR China, 2005). Cleptoparasitism is more common in bees than is social parasitism, and there are thought to be over 3000 cleptoparasitic species belonging to at least 25 main evolutionary lineages (Rozen, 2000, 2001; Grimaldi and Engel, 2005). It is widespread in Apidae, Megachilidae, and Halictidae, rare in Colletidae, and unknown in the other families (Michener, 2007). In Apidae, cleptoparasitism may have evolved 11 times, based on adult morphology, or six times based on larval morphology (Straka and Bogusch, 2007). Ten of the 11 cleptoparasitic lineages are in the Apinae (as circumscribed by Michener, 2007), one of them being the *Ctenoplectrina* clade. The sister relationship between the cleptoparasite and its host found here (Fig. 3) fits with similar cases of parasite clades having radiated on clades of closely related hosts in *Bombus* (Kawakita et al., 2004), *Braunsapis* (Fuller et al., 2005), *Exaerete* (Engel, 1999; Anjos-Silva et al., 2007), and Augochlorini (Engel et al., 1997; Engel, 2000b), all providing examples of Emery's (1909) rule that parasitic aculeate Hymenoptera often have evolved from their hosts.

4.2. Biogeography of *Ctenoplectrini*

Eucerini, the apparent closest relatives of Ctenoplectrini, are distributed in temperate zones of Eurasia, Africa and the Americas; they are represented here by a European species (*Eucera nigrescens*) and a North American species (*Melissodes rustica*). Based on our molecular clock estimates, the split between Eucerini and Ctenoplectrini occurred in the Early Eocene, conceivably in Africa as shown in Fig. 4a. An initial diversification in tropical Africa would fit with the geographic ranges and evolutionary sequence of the oil-offering Cucurbitaceae clades on which Ctenoplectrini are oligolectic (Schaefer and Renner, unpublished data).

The evolution of the cleptoparasite lineage *Ctenoplectrina*, with three species, all in tropical and Southern Africa, apparently occurred soon after the initial diversification of their hosts (Table 3). The next-deepest divergence in Ctenoplectrini is that between the *C. bequaerti* and *C. davidi* clades on the one hand and the *C. polita*/*C. kelloggi* clade on the other. Of the four species in the latter clade, two (*C. albolimbata* and *C. armata*) are adapted to savannah habitat and two (*C. antinorii* and *C. polita*) are found in

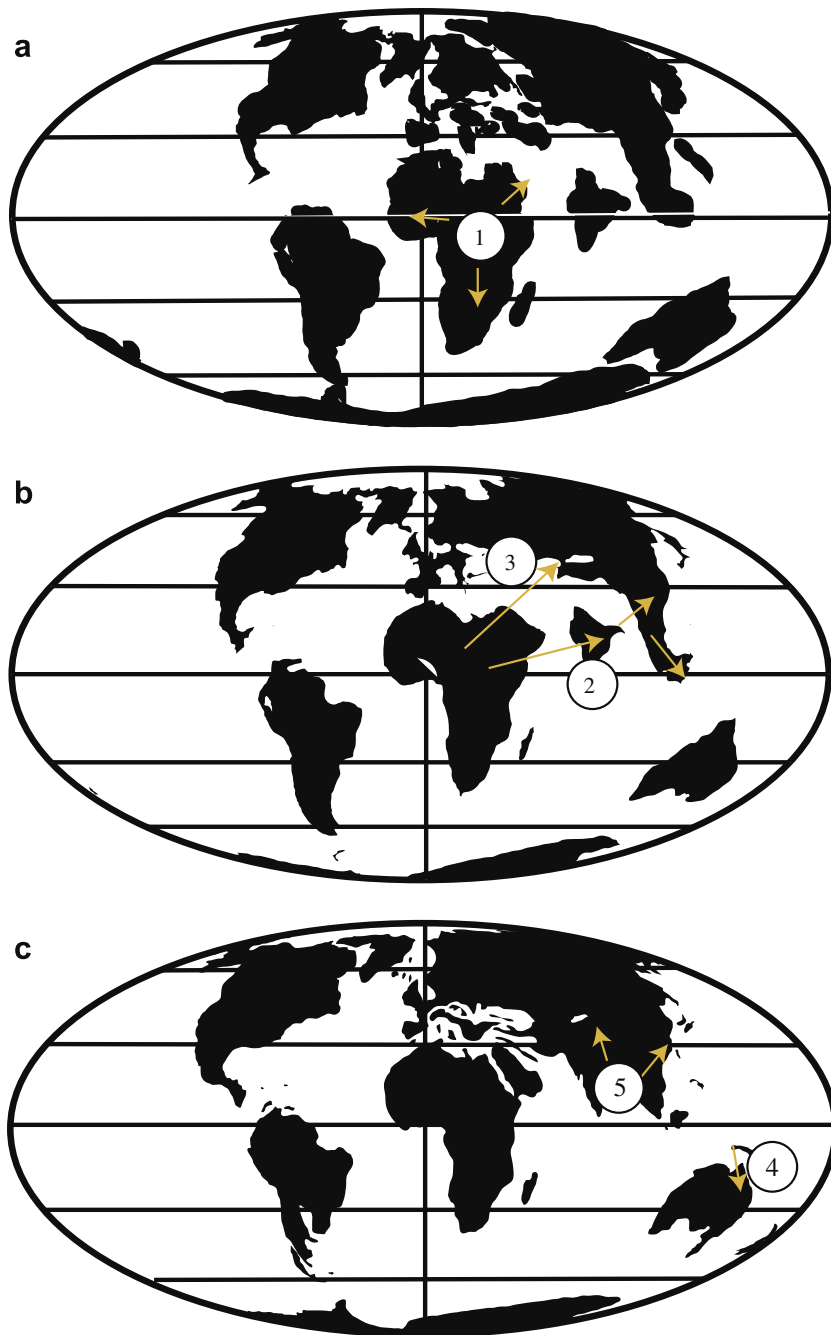


Fig. 4. Biogeographic scenario inferred for Ctenoplectrini from the tree topology in Fig. 3, and a Bayesian relaxed molecular clock (see text). (a) Early Eocene (c. 53 my ago), Ctenoplectrini diverge from their sister clade Eucerini and diversify in tropical Africa; (b) Early Oligocene (c. 30 my ago), the ancestors of the *C. davidi* and the *C. kelloggi* clades disperse into Asia, either via Proto-India (2) or via Europe (3); (c) mid-Miocene (c. 12 my ago), the ancestor of *C. australica* spreads into Australia via New Guinea (4) and the *C. davidi* clade diversifies in South/Central China and South Russia (5). Mesozoic and Cenozoic coastlines from Smith et al. (1994).

dry habitats as well as perhumid tropical forest. The divergence between *C. bequaerti* and *C. terminalis* apparently occurred during the Middle Miocene (c. 13 my ago). Both species depend on rainforest habitats, with *C. bequaerti* today restricted to West Africa, *C. terminalis* to East and Southern Africa. Judging from their divergence time, aridification of mid-latitude continental regions during the mid-Miocene (Jacobs, 2004) may have led to habitat fragmentation and thus contributed to geographic speciation.

Sometime during the Late Eocene (at around 36 and 34 my ago), each of two *Ctenoplectra* clades dispersed to Asia (Fig. 4b), giving rise to two morphologically divergent species groups (marked in grey in Fig. 3). One consists of *C. davidi* and *C. florisomnis* plus three other species not yet sequenced, viz. *Ctenoplectra cornuta*, *Ctenoplectra thladianthae*, and *Ctenoplectra yoshikawai* (based on morphological characters; H. Schaefer and M. Engel, manuscript), the other comprises *C. kelloggi*, *C. australica*,

C. elsei, and two further species, *Ctenoplectra vagans* and *Ctenoplectra chalybea* (based on morphological characters). The *C. davidi/C. florissomnis* group comprises small brown/black bees that mostly visit flowers of the oil-offering Cucurbitaceae genus *Thladiantha*. These bees occur in rainforest and drier habitats from Indonesia to Russia and Pakistan (compare Fig. 1). The *C. kelloggi/C. australica* group comprises large blue bees that are specialized on the large-flowered *Momordica cochinchinensis* and close relatives. This group occurs exclusively in tropical and subtropical forests from subtropical China to tropical Australia (Fig. 1; Vogel, 1990; H.S., personal observation, PR China, 2005, Australia, 2007).

Based on the molecular clock estimates, dispersal to Australia occurred in the Middle Miocene (Fig. 4c), when the ancestors of *C. australica* appear to have reached Australia from Indonesia via New Guinea. In this period, the north drifting Australian tectonic plate reached tropical latitudes and collided with the Sunda Island Arc of the Asian plate. Low sea levels resulted in additional stepping stone islands that permitted extensive faunal exchange (Braby et al., 2007, and references cited therein). Other bee genera, for example *Xylocopa* (Leys et al., 2002) and *Braunsapis* (Fuller et al., 2005), based on molecular clock estimates reached Australia during the same period.

4.3. *cox-1*—a region for standard bar-coding?

cox-1 has emerged as the most promising DNA region for zoological bar-coding projects (e.g., Hebert et al., 2004a). In lower level bee systematics, it is generally used without pseudogene problems (B.N. Danforth, Cornell University, personal communication to H.S., March 2007), and only a handful of publications point to possible problems with this marker. In insects, Jordal and Hewitt (2004) excluded putative *cox-1* pseudogenes in a beetle study, and Hebert et al. (2004b) reported cases of *cox-1* heterozygosity in butterflies. Other cases have been reported from sea urchins (Jacobs and Grimes, 1986) and copepods (Bucklin et al., 2000; Williams and Knowlton, 2001). The pseudogene sequences produced in this study probably result from mitochondrial DNA transferred to the nuclear genome, where mutation rates are considerably lower than in the mitochondria. This explains why sister species relations are no longer resolved in the pseudogene phylogram (Fig. 2). Extremely high levels of mitochondrial-nuclear transfers (NUMTs) have been reported from the honeybee (*A. mellifera*; Pamilo et al., 2007), which together with our discovery of NUMTs in Ctenoplectrini points to the importance of carefully screening for non-functional copies before relying on *cox-1* for bar-coding purposes.

5. Conclusions

In answer to the questions posed at the outset, our results from phylogenetic analysis of mitochondrial and

nuclear loci show that Ctenoplectrini are monophyletic and closest to Eucerini from which they appear to have diverged in the Early Eocene. *Ctenoplectra* dispersed twice from Africa to Asia, with both disjunctions occurring at a similar time in the Late Eocene. The (presumed) cleptoparasitic genus *Ctenoplectrina* is the sister clade of *Ctenoplectra* and evolved early during the diversification of its host. The estimated time of 13 my for the split between the sister species *C. bequaerti* from West Africa and *C. terminalis* from East and South Africa coincides with the fragmentation of the equatorial African rainforest belt during the dryer and cooler climate of the mid-Miocene. At the same time, low sea levels appear to have provided stepping stone islands that permitted dispersal from Indonesia to New Guinea and Australia.

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