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Single-Copy Nuclear Genes Recover Cretaceous-Age Divergences in Bees

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Abstract.—We analyzed the higher level phylogeny of the bee family Halictidae based on the coding regions of three single-copy nuclear genes (long-wavelength [LW] opsin, *wingless*, and elongation factor 1-α [*EF-1*α]). Our combined data set consisted of 2,234 aligned nucleotide sites (702 base pairs [bp] for LW opsin, 405 bp for *wingless*, and 1,127 bp for *EF-1*α) and 779 parsimony-informative sites. We included 58 species of halictid bees from 33 genera, representing all subfamilies and tribes, and rooted the trees using seven outgroups from other bee families: Colletidae, Andrenidae, Melittidae, and Apidae. We analyzed the separate and combined data sets by a variety of methods, including equal weights parsimony, maximum likelihood, and Bayesian methods. Analysis of the combined data set produced a strong phylogenetic signal with high bootstrap and Bremer support and high posterior probability well into the base of the tree. The phylogeny recovered the monophyly of the Halictidae and of all four subfamilies and both tribes, recovered relationships among the subfamilies and tribes congruent with morphology, and provided robust support for the relationships among the numerous genera in the tribe Halictini, sensu Michener (2000). Using our combined nucleotide data set, several recently described halictid fossils from the Oligocene and Eocene, and recently developed Bayesian methods, we estimated the antiquity of major clades within the family. Our results indicate that each of the four subfamilies arose well before the Cretaceous–Tertiary boundary and suggest that the early radiation of halictid bees involved substantial African–South American interchange roughly coincident with the separation of these two continents in the late Cretaceous. This combination of single-copy nuclear genes is capable of recovering Cretaceous-age divergences in bees with high levels of support. We propose that LW opsin, *wingless*, and *EF-1*α (F2 copy) may be useful in resolving relationships among bee families and other Cretaceous-age insect lineages. [Bayesian methods; biogeography; molecular evolution; phylogeny.]

Bees are a monophyletic group of >16,000 species of pollen-feeding Apoidea (Melo, 1999; Michener, 2000). Bees and the angiosperms they pollinate have had an ancient and intimate association that most likely extends back well into the Cretaceous (Grimaldi, 1999; Engel, 2001). Currently, bees are divided into seven families: the highly derived long-tongued (LT) bees in the families Megachilidae and Apidae and the basal short-tongued (ST) bees in the families Colletidae, Stenotritidae, Andrenidae, Halictidae, and Melittidae (Michener, 2000). Previous morphological studies at the family, subfamily, and tribal levels (Roig-Alsina and Michener, 1993; Alexander and Michener, 1995) have demonstrated convincingly the monophyly of the LT bees and of most bee families and subfamilies, but many higher level relationships remain elusive. Alexander and Michener (1995) provided the most recent analysis of relationships among the ST bee families. They analyzed 109–114 morphological characters for 48 taxa of ST bees, 9 LT bees, and 8 spheciform wasp outgroups (from 2 of the 4 spheciform families recognized by Melo, 1999: Sphecidae and Crabronidae). Although their study provided important insights into relationships within families and helped establish family and subfamily monophyly for many groups, their results did not firmly establish relationships among the ST bee families. Alexander and Michener (1995:419) concluded that "the major clades (families) are distinct enough, but their relationships to one another remain uncertain . . . . New sets of characters (e.g., larval, molecular) should be tried." Understanding the phylogeny, antiquity, and early radiation of the bees is important because these families provide the most direct insights into the early evolution of the bees and the timing of bee/angiosperm coevolution.

Molecular data have shown promise for elucidating bee phylogeny, but to date most molecular studies have addressed questions at lower taxonomic levels, within genera (e.g., Danforth, 1999; Danforth et al., 1999; Sipes and Wolf, 2001; Pedersen, 2002; Kawakita et al., 2003), among genera within a tribe (e.g., Leys et al., 2000, 2002; Bull et al., 2003; Costa et al., 2003; Schwarz et al., 2003), or among closely related tribes within a subfamily (e.g., the corbiculate tribes; Cameron, 1993; Mardulyn and Cameron, 1999; Ascher et al., 2001; Cameron and Mardulyn, 2001). Not all these studies have been conclusive (e.g., compare the results of Ascher et al., 2001, with those of Mardulyn and Cameron, 2003), which suggests that we have not yet identified a suite of nucleotide data sets capable of recovering deep divergences in bees with high levels of support. Mitochondrial sequences (which may be useful at lower taxonomic levels) show little promise for resolving higher level (family, subfamily, and tribal) relationships in bees.

Single-copy nuclear genes provide a potential source of new characters for higher level insect phylogeny (Friedlander et al., 1992, 1994, 1996, 2000; Brower and DeSalle, 1994; Wiegmann et al., 2000), yet the utility of single-copy nuclear gene sequences has not been evaluated at the highest levels (e.g., among tribes, subfamilies, and families) in bees. We present here an examination of the utility of three single-copy nuclear genes, long-wavelength (LW) opsin, *wingless*, and elongation factor 1α (*EF-1*α), for elucidating higher level bee phylogeny.

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Our study focused on the Halictidae, one of the five ST bee families. Given that the halictids are among the more primitive bees, if single-copy nuclear genes can successfully recover phylogenetic relationships within this family, these same genes may be promising candidates for resolving relationships among families. Our results indicate that LW opsin, *wingless*, and *EF-1α*, when combined, are capable of resolving higher level Cretaceous-age phylogenetic relationships in bees. Our results were robust by all measures of tree stability, suggesting that these three genes in combination will be able to resolve long-standing questions concerning the family-level phylogeny of bees, such as whether colletids are sister to all other bees and whether Melittidae is monophyletic (Alexander and Michener, 1995).

Our study allowed us to firmly establish subfamily, tribal, and generic relationships in the family Halictidae. No previous morphological or molecular study has simultaneously resolved relationships among the halictid subfamilies, tribes, and genera. We combined our phylogenetic results with recent fossil data on the halictid bees to estimate the antiquity of key nodes within the phylogeny of halictid bees and used the resulting tree topology and divergence dates to develop a biogeographic hypothesis for the family. Based on our phylogenetic results, we have developed a new tribal classification for the family (Table 1).

TABLE 1. Alternative classifications of the halictid subfamily Halictinae. Tribal names used by us (This study) are based on family group names listed by Michener (1986). Numbers of species (in parentheses) are derived from Michener (2000) and other recent publications (e.g., Janjic and Packer 2003).

Michener (2000)	This study
Tribe Augochlorini[a] (25 genera, ~250 species)	Tribe Augochlorini (~250) 5 genera included[b]
Tribe Halictini (*sensu lato*)	Tribe Thrinchostomini (58)
Agapostemon Guérin-Méneville (41)	*Thrinchostoma*[b]
Caenohalictus Cameron (45)	*Parathrincostoma*[c]
Dinagapostemon Moure and Hurd (8)	Tribe Caenohalictini (148)
Echthralictus Perkins and Cheesman (2)	*Agapostemon*[b]
Eupetersia Blüthgen (29)[c]	*Agapostemonoides*[d]
Glossodialictus Pauly (1)	*Caenohalictus*[b]
Habralictus Moure (22)	*Dinagapostemon*[b]
Halictus Latreille (217)[c,e]	*Habralictus*[b]
Homalictus Cockerell (101)	*Paragapostemon*
Lasioglossum Curtis (>1200)[c,e]	*Pseudagapostemon*[b]
Mexalictus Eickwort (6)	*Rhinetula*[b]
Microsphecodes Eickwort and Stage (7)[c]	*Ruizantheda*[b]
Paragapostemon Vachal (1)	Tribe Sphecodini (288)
Parathrincostoma Blüthgen (2)[c]	*Eupetersia*[c]
Patellapis sensu lato Friese (161)	*Microsphecodes*[c]
Pseudagapostemon Schrottky (25)	*Ptilocleptis*[c]
Ptilocleptis Michener (3)[c]	*Sphecodes*[c,b]
Rhinetula Friese (1)	Tribe Halictini (>1,690)
Ruizantheda Moure (4)	*Glossodialictus*
Sphecodes Latreille (249)[c]	*Halictus*[b,c,e]
Thrincohalictus Blüthgen (1)	*Lasioglossum*[b,c,e]
Thrinchostoma Saussure (>56)	*Mexalictus*[b]
Urohalictus Michener (1)	*Patellapis* sensu lato[b]
	Thrincohalictus[b]

[a]See Eickwort (1969a) and Engel (2000) for generic treatments of the Augochlorini.
[b]Genera sampled for this study.
[c]Genera including some or all parasitic species.
[d]*Agapostemonoides* was recognized as a subgenus of *Agapostemon* by Michener. Recent cladistic studies (Janjic and Packer 2003) demonstrated that this is a valid genus.
[e]Genera with both solitary and eusocial species.
[f]*Lasioglossum* in our sense includes *Homalictus*, *Echthralictus*, and *Urohalictus*.

MATERIALS AND METHODS

Genes Analyzed

We generated a data set based on three single-copy nuclear genes that have previously shown promise for resolving deep divergences in insects. DNA extraction, PCR, and sequencing protocols followed standard methods previously detailed (Danforth, 1999; Ascher et al., 2001; Danforth and Ji, 2001).

Wingless.—The wnt family of protein coding genes is involved in early embryogenesis in insects and vertebrates (e.g., Rijsewijk et al., 1987; Uzvölgyi et al., 1988). At least a dozen subfamilies have been identified in this large and variable gene family (Sidow, 1992; Schubert et al., 2000). Of these subfamilies, wnt-1 has been phylogenetically informative at a variety of levels, from among phyla of metazoan animals (Schubert et al., 2000) to among and within Lepidopteran families (Brower and Egan, 1997; Brower and DeSalle, 1998; Brower, 2000; Campbell et al., 2000; Hsu et al., 2001). A recent study of phylogenetic relationships within the stalk-eyed flies (family Diopsidae; Baker et al., 2001) concluded that of the six genes analyzed *wingless* was the most useful in terms of congruence, data decisiveness, and bootstrap support.

In a preliminary evaluation of the *wingless* gene for relationships among the ST bee families, subfamilies, and tribes, we used primers developed for Lepidoptera (wg1a and wg2a; Brower and DeSalle, 1998). These primers nonspecifically amplified two or three *wingless* paralogs in most bee and spheciform taxa. Two paralogs differed in size and could be separated on low-melting-point agarose gels. When paralogs were similar in size we used PCR product cloning (T/A cloning) to isolate and sequence alternative paralogs. We characterized in bees a total of three *wingless* paralogs that could be separated unambiguously based on small or large indel mutations. The three paralogous copies in bees clustered unambiguously into three different *wingless* gene families (results not shown). Our most common paralog was in the wnt-1 family. Based on these wnt-1 sequences, we designed a new forward PCR primer (beewgFor; Table 2) that specifically amplifies 450 bp of the wnt-1 paralog in a variety of ST bee families and spheciform outgroups.

LW rhodopsin.—Bee LW rhodopsin is one of a family of three rhodopsin genes in bees: LW (green), blue, and ultraviolet rhodopsin (Chang et al., 1996; Towson et al., 1998). Rhodopsins are G-protein–coupled receptor proteins that perform the first steps in visual transduction in most organisms. LW rhodopsins have been characterized and sequenced in ants (Popp et al., 1996), mantids (Towner and Gärtner, 1994), butterflies (Briscoe, 1999), and flies (*Drosophila*; Huber et al., 1997). The LW rhodopsin (LW opsin) has been used for phylogenetic

TABLE 2. Primer sequences used for PCR assay of bees.

Primer	Sequence (5′ → 3′)
Opsin[a]	
Opsin For (=LWRhFor)	AAT TGC TAT TAY GAR ACN TGG GT
Opsin For3 (=LWRhFor3)	AGA TAC AAC GTR ATC GTS AAR GGT
Opsin For4	GAG AAR AAY ATG CGB GAR CAA GC
Opsin Rev (=LWRhRev)	ATA TGG AGT CCA NGC CAT RAA CCA
Opsin Rev4	GGT GGT GGT RCC GGA RAC GGT G
Opsin Rev4a	GGT GGT RCC GGA RAC GGT GGA DGT
wingless[b]	
Lep wg1a	GAR TGY AAR TGY CAY GGY ATG TCT GG
beewgFor	TGC ACN GTS AAG ACC TGY TGG ATG AG
Lep wg2a	ACT ICG CAR CAC CAR TGG AAT GTR CA

[a]PCR conditions. Opsin For/Opsin Rev: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min (35 cycles); Opsin For3/Opsin Rev: 94°C for 1 min, 54°C for 1 min, 72°C for 1 min (35 cycles); Opsin For4/Opsin Rev4: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min (35 cycles); Opsin For4/Opsin Rev4a: 94°C for 1 min, 50–60°C for 1 min, 72°C for 1 min (35 cycles).

[b]PCR conditions. Lep wg1a/Lep wg2a: 94°C for 45 sec, 54°C for 45 sec, 72°C for 45 sec (35 cycles); beewgFor/Lep wg2a: 94°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec (35 cycles).

analysis of bees (Mardulyn and Cameron, 1999; Ascher et al., 2001; Cameron and Mardulyn, 2001; Cameron and Williams, 2003), *Heliconius* butterflies (Hsu et al., 2001), cynipid wasps (Rokas et al., 2002), and aphids (Ortiz-Rivas et al., 2004). Previous phylogenetic analyses of bees (Mardulyn and Cameron, 1999; Ascher et al., 2001; Cameron and Mardulyn, 2001) involved opsin primers LWRhFor and LWRhRev, which produce an approximately 700-bp PCR product consisting of three exons and two introns.

We expanded the opsin data set beyond the range of previous studies using the 3′-RACE technique. Poly(A) mRNAs were isolated from heads of recently collected frozen bees (*Agapostemon virescens*, *Andrena carlini*, *Augochlorella striata*, *Lasioglossum leucozonium*, *Colletes inaequalis*, *Halictus ligatus*, and *Lasioglossum* (*Dialictus*) *zephyrum*; all collected in Ithaca, NY) using the Oligotex Direct mRNA Micro Kit (Qiagen, Chatsworth, CA). The mRNA was then reverse transcribed to generate complementery DNA (cDNA). PCR of cDNA fragments using opsin-specific primers and poly(T) reverse primers were used to generate PCR products, which were then ligated into a pGEM vector (Promega, Madison, WI) and transformed into DH5-α library efficiency competent cells (Gibco, Grand Island, NY). The transformed cells were plated out on Luria broth medium. Plasmids containing opsin inserts were isolated and sequenced using an ABI 373A automated sequencer. We aligned the sequences obtained with the three different opsin copies in *Apis* (Chang et al., 1996) to confirm that the cloned fragments matched the LW gene family. All sequences spanned the 3′ end of the gene and all included the stop codon plus the 3′ noncoding region. Based on these sequences we developed two reverse primers (Opsin Rev4, Opsin Rev4a; Table 2) to amplify the 3′ end of the gene (including one intron).

Our opsin data set spans positions 434–1,146 of the coding region in the *Apis mellifera* LW opsin paralog (Chang et al., 1996) and includes four exons and three introns (Fig. 1a). The region we sequenced spans transmembrane regions III–VII plus the intracellular loop downstream of transmembrane region VII (Fig. 1b). The locations of introns coincide with the junction of transmembrane and extramembrane regions, suggesting that the introns flank functional domains of the protein.

EF-1α.—*EF-1α* is a nuclear gene that encodes a protein involved in the GTP-dependent binding of charged tRNAs to the acceptor site of the ribosome during translation (Maroni, 1993; 126–134). *EF-1α* is a useful gene for studies of higher level phylogenetic relationships, especially in insects (Cho et al., 1995; Mitchell et al., 1997, 2000; Friedlander et al., 1998; Caterino et al., 2000; Regier et al., 2000; Yang et al., 2000). *EF-1α* occurs as two copies in bees (Danforth and Ji, 1998), beetles (Jordal, 2002), and flies (Hovemann et al., 1988). We have developed primers that specifically amplify the F1 or F2 copy in bees (Danforth et al., 1999; Danforth and Ji, 2001; Sipes and Wolf, 2001). The data set we used in this study spans positions 145–1,271 in the coding region of the *Apis* F2 copy (Danforth and Ji, 1998). Most sequences we used were obtained from previous publications on halictid phylogeny (Danforth, 2002), but several new outgroup and ingroup sequences were added for improved taxon sampling. Primers were described by Danforth et al., (2003).

Phylogenetic Methods

Alignments for all genes were generated with the Lasergene DNA Star software package using Clustal W. Reading frames and intron/exon boundaries were determined by comparison with published coding sequences for the honey bee, *Apis mellifera* (opsin [U26026]: Chang et al., 1996; *EF-1α* [AF015267]: Danforth and Ji, 1998) and for *Drosophila* (*wingless* [J03650]: Uzvölgyi et al., 1988).

Parsimony.—We included a total of 58 ingroup species from 33 genera of halictid bees representing all four halictid subfamilies, all tribes of the subfamily Halictinae (Table 1), and seven outgroups from the bee families Colletidae, Andrenidae, Melittidae, and Apidae (Table 3). Voucher specimens are deposited in the Cornell University Insect Collection. GenBank accession numbers and specimen voucher codes are listed in Table 3. Our combined data set is available from the *Systematic Biology* web site (http://systematicbiology.org/).

We performed maximum parsimony analyses using PAUP* 4.0b10 (Swofford, 2002). Initially, we performed equal weights parsimony analyses on each of the three data sets and then combined the data sets into a single analysis. We tested for data set congruence using the incongruence length difference (ILD) test (Farris et al., 1995) implemented in PAUP*. In spite of the limitations that this test may have (Barker and Lutzoni, 2002; Darlu and Lecointre, 2002; Dowton and Austin, 2002), it provides a useful heuristic measure of incongruence when data partitions are of similar size and patterns of nucleotide substitution are similar across partitions. Branch support for the individual data sets and for the combined data set was estimated using bootstrap analysis

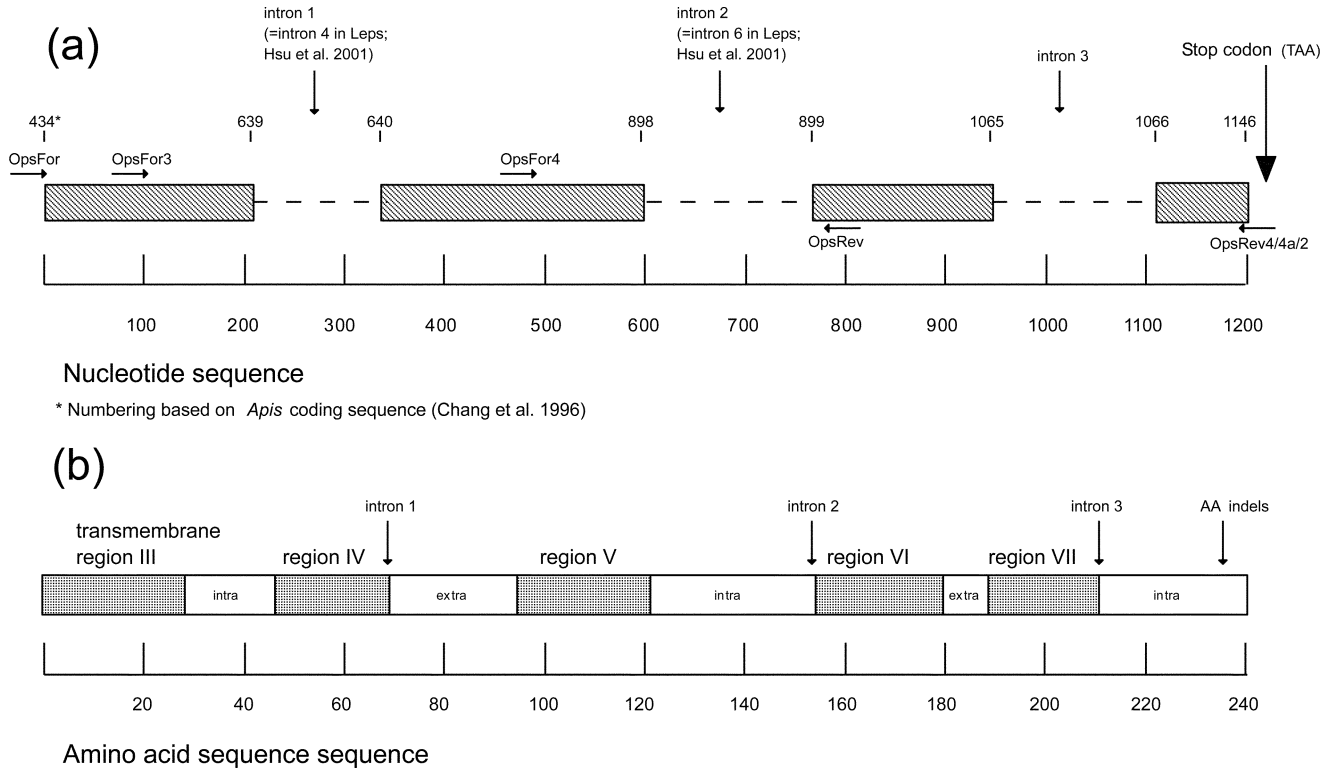

FIGURE 1. Map of the LW opsin gene region in bees used in this study. (a) Nucleotide sequence. Primer sites (see Table 2 for a complete list of primer sequences), introns, and exons are indicated. In the region analyzed there were three introns. (b) Amino acid sequence. Transmembrane, intracellular, and extracellular regions are shown (from Chang et al., 1996). Intron positions coincide with the junction between transmembrane domains and intra- or extracellular loops, suggesting that intron positions are functionally constrained.

(Felsenstein, 1985). For parsimony searches, we performed 100 random sequence additions. For calculating bootstrap proportions, we performed 500 replicates with 10 random sequence additions per replicate. Bremer support (Bremer, 1988) and partitioned Bremer support (Baker and DeSalle, 1997; Baker et al., 1998, 2001) were also calculated for the total data set using TreeRot 2 (Sorensen, 1999). See DeBry (2001) for problems associated with interpreting Bremer support values.

Maximum likelihood.—To evaluate the performance of different maximum likelihood (ML) models, we first calculated the likelihood scores of 20 possible models based on the parsimony trees obtained. Our models included four standard substitution models: Jukes–Cantor, Kimura two-parameter (K2P), Hasegawa–Kishino–Yano (HKY), and general time reversible (GTR) with five methods of accounting for rate variation among sites: no rate variation, proportion of invariant sites (I), a gamma distribution (G, with four rate catagories), invariant sites plus gamma distribution (I+G), and site-specific rates (SSR) with nine rate catagories associated with the three nucleotide (nt) positions (opsin nt1, opsin nt2, opsin nt3, *wingless* nt1, *wingless* nt2, *wingless* nt3, *EF-1α* nt1, *EF-1α* nt2, *EF-1α* nt3). As expected, the likelihood scores of the more complex models were significantly better than those for the less complicated models based on likelihood ratio (LR) tests (Felsenstein, 1988) (SSR models are not nested within the other models and therefore were not evaluated with the LR tests). For tree searching, we selected six models: K2P+I+G, HKY+I+G, GTR+I+G, K2P+SSR, HKY+SSR, and GTR+SSR. Starting trees for the ML analyses were based on the parsimony analysis, and we used a variety of branch swapping algorithms, including nearest neighbor interchange, supertree pruning and regrafting, and tree bisection–reconnection (in that order). We did not calculate bootstrap values on the ML tree because the size of the data set precluded thorough tree searches and the Bayesian analysis provided estimates of branch support based on the same suite of substitution models. SSR models have been criticized by Buckley et al., (2001) and Buckley and Cunningham (2002).

Bayesian methods.—For the Bayesian analyses, we used MrBayes 3.0 (Huelsenbeck and Ronquist, 2001; http://morphbank.ebc.uu.se/mrbayes3/). We analyzed the combined data set using the same six models: K2P+I+G, HKY+I+G, GTR+I+G, K2P+SSR, HKY+SSR, and GTR+SSR. For the invariant sites plus gamma models, we treated the separate genes as unlinked so that separate parameter estimates were obtained for each gene for all runs. For the SSR models, we allowed nine discrete rate catagories corresponding to the three codon positions within each gene. Analyses consisted of running four simultaneous chains for 10^6 generations. Analyses were run repeatedly to ensure that different starting points did not bias the resulting tree topologies and parameter

TABLE 3. Species included, locality data, and GenBank accession numbers.

Taxon	Abbrev.	Sample no.	Subfamily[a]	Locality data	GenBank nos.		
					Opsin (~1,200 bp)[b]	Wingless (450 bp)	EF-1α (~1,600 bp)
Apis mellifera	Apme		Xx	USA: New York, Tompkins Co.	U26026	AY222546	AF015267
Andrena (*Callandrena*) *brooksi*	Ansp	643	Xx	USA: New Mexico, Hidalgo Co.	AF344618	AY222551	AY230129
Andrena (*Simandrena*) *nasonii*	Anna	232	Xx	USA: New York, Tompkins Co.	AY227915	AY222550	na.[c]
Colletes skinneri	Cosk	632	Xx	USA: Arizona, Cochise Co.	AY227912	AY222547	AY230130
Hesperapis larreae	Hela	488	Xx	USA: California, Los Angeles Co. (J. Ascher)	AF344597	AY222552	AY230131
Hylaeus modestus	Hymo	279	Xx	USA: New York, Tompkins Co.	AY227913	AY222548	na.
Chilicolletes delahozii	Lesp	568	Xx	Chile: Elqui Prov. (J. G. Rozen, Jr.)	AY227914	AY222549	AF435392
Conanthalictus wilmattae	Cowi	351	Ro	USA: California (J. Neff)	AY227934	AY222553	AF435378
Dufourea malacothricis	Duma	235	Ro	USA: Michigan (P. Lincoln)	AY227917	AY222554	AF435382
Dufourea mulleri	Dumu	233	Ro	USA: Michigan (P. Lincoln)	AY227918	AY222555	AF435383
Dufourea novaeangliae	Duno	354	Ro	USA: New York, Cayuga Co.	AY227919	AY222556	AF435384
Goeletapis peruensis	Gope	569	Ro	Peru: Lima Dept. (J. G. Rozen, Jr.)	AY227923[d]	AY222560	AF435386
Penapis moldenkei	Pnpe	572	Ro	Chile: Huasco Prov. (J. G. Rozen, Jr.)	AY227921	AY222558	AF435401
Penapis toroi	Pnto	586	Ro	Chile: Region III (L. Packer)	AY227922	AY222559	AF435402
Protodufourea parca	Pfsp	237	Ro	USA: Arizona, Pima Co. (L. Packer)	AY227920	AY222557	AF435399
Sphecodosoma pratti	Sspr	355	Ro	USA: Texas, Mason Co. (J. Neff)	AY227924	AY222561	AF435410
Systropha curvicornis	Sycu	350	Ro	Austria: Vienna (M. Ayasse)	AY227925	AY222562	AF435411
Systropha planidens	Sypl	349	Ro	Austria: Vienna (M. Ayasse)	AY227926	AY222563	AF435412
Xeralictus bicuspidariae	Xrbi	566	Ro	USA: California, San Diego Co. (R. Snelling)	AY227927	AY222564	AF435413
Xeralictus timberlakei	Xrti	564	Ro	USA: California, Riverside Co. (R. Snelling)	AY227928	AY222565	AF435414
Dieunomia nevadensis	None	207	No	USA: Arizona, Cochise Co.	AY227931	AY222568	AF435396
Dieunomia triangulifera	Notr	286	No	USA: Kansas, Douglas Co.	AY227932	AY222569	AF435397
Lipotriches (*Austronomia*) *australica*	Noau	754	No	Australia: S. Australia, Cowell	AY227930	AY222567	AF435395
Pseudapis unidentata	Psun	241	No	Spain: Almeria Prov.	AY227933	AY222570	AF435404
Nomioides facilis	Nmsp	243	Nm	Spain: Granada Prov.	AY227929	AY222566	AF435394
Agapostemon leunculus	Agle	336	Hah	Republic of Panama: Panama Prov. (W. Wcislo)	AY227939	AY222576	AF435371
Agapostemon tyleri	Agty	230	Hah	USA: Arizona, Cochise Co.	AY227940	AY222577	AF140320
Caenohalictus sp. 2	Cnsp	578	Hah	Chile: Region I, Socoroma (L. Packer)	AY227941	AY222578	AF435376
Caenohalictus sp. 3	Cnsp	788	Hah	Brazil: Minas Gerais (D. Yanega)	AY227942	AY222579	AF435377
Dinagapostemon sp. 1	Dnsp	573	Hah	Republic of Panama: Chiriqui Prov. (L. Packer)	AY227943	AY222580	AF435380
Dinagapostemon sp. 2	Dnsp	790	Hah	Republic of Panama: Cocle Prov. (L. Packer)	AY227944	AY222581	AF435381
H. (*Halictus*) *quadricinctus*	Haqu	89	Hah	France: Dordogne (C. Plateaux-Quenu)	AY227956	AY222592	AF140334
H. (*Seladonia*) *tripartitus*	Hatr	93	Hah	USA: Arizona, Cochise Co.	AY227957	AY222593	AF140310
H. (*Vestitohalictus*) *vestitus*	Veve	373	Hah	Spain: Granada Prov.	AY227958[d]	AY222594	AF140313
Habralictus sp.	Hasp	786	Hah	Brazil: Minas Gerais (D. Yanega)	AY227945	AY222582	AF435387
L. (*Australictus*) *lithuscum*	Auli	711	Hah	Australia: Victoria	AY227962	AY222598	AF435372
L. (*Chilalictus*) *florale*	Chfl	320	Hah	Australia: S. Australia (S. Reyes)	AY227966	AY222602	AF264792
L. (*Chilalictus*) *lanarium*	Chla	316	Hah	Australia: Victoria (S. Reyes)	AY227967	AY222603	AF264793
L. (*Dialictus*) *zephyrum*	Dizp	74	Hah	USA: New York, Seneca Co.	AF448918[d]	AY222607	AF435379
L. (*Evylaeus*) *calceatum*	Evca	105	Hah	France: Dordogne (C. Plateaux-Quenu)	AF448877[d]	AY222608	AF435385
L. (*Hemihalictus*) *lustrans*	Helu	186	Hah	USA: Texas, Bastrop Co. (J. Neff)	AF448904[d]	AY222609	AF435388
L. (*Homalictus*) *megastigmus*	Homg	360	Hah	Australia: W. Australia, Bluff Knoll (S. Reyes)	AY227964	AY222600	AF264839
L. (*Homalictus*) *punctatus*	Hopu	245	Hah	Australia: S. Australia, Adelaide (S. Reyes)	AY227965	AY222601	AF435389
L. (*Lasioglossum*) *athabascense*	Laat	556	Hah	USA: New York, Tompkins Co.	AF448867	AY222604	AF435390
L. (*Lasioglossum*) *scitulum*	Lasc	550	Hah	Japan: Shimane Pref. (R. Miyanaga)	AY227968	AY222605	AF435391
L. (*Lasioglossum*) *zonulum*	Lazo	284	Hah	USA: New York, Tompkins Co.	AY227969	AY222606	AF264855
L. (*Parasphecodes*) *hybodinum*	Pahy	299	Hah	Australia: S. Australia (S. Reyes)	AY227963	AY222599	AF264857
Mexalictus arizonensis	Mxaz	98	Hah	USA: Arizona, Santa Cruz Co. (L. Packer)	AY227959	AY222595	AF140322
Patellapis (*Chaetalictus*) sp. 2	Chsp	603	Hah	South Africa: Kwazulu-Natal (L. Packer)	AY227952	AY222588	AF435374
Patellapis (*Pachyhalictus*) sp.	Phsp	357	Hah	Vietnam: Ban Don (L. Packer)	AY227954	AY222590	AF435400
Patellapis (*Lomatalictus*) sp.	Losp	604	Hah	South Africa: Gauteng (C. Eardley)	AY227953	AY222589	AF435393
Pseudagapostemon brasiliensis	Psbr	347	Hah	Brazil: Minas Gerais (D. Yanega)	AY227946	AY222583	AF140323
Pseudagapostemon pissisi	Psps	576	Hah	Chile: Region VIII, near Temuco (L. Packer)	AY227947	AY222584	AF435403
Rhinetula denticrus	Rhdn	799	Hah	Republic of Panama: Panama Prov. (D. Roubik)	AY227948	AY222585	AF435405
Ruizantheda mutabilis	Rumt	574	Hah	Chile: Region VIII, near Temuco (L. Packer)	AY227949	AY222586	AF435406
Ruizantheda proxima	Rupr	575	Hah	Chile: Region VIII, near Temuco (L. Packer)	AY227950	AY222587	AF435407
Sphecodes minor	Spmi	21	Hah	USA: New York, Tompkins Co.	AY227960	AY222596	AF140324
Sphecodes ranunculi	Spra	337	Hah	Canada: Nova Scotia (L. Packer)	AY227961	AY222597	AF140325
Thrinchostoma (*Diagozonas*) sp.	Trsp	464	Hah	Borneo: Sarawak (S. Heydon)	AY227951	na	na
Thrincohalictus prognathus	Thpr	434	Hah	Israel: Golan Heights (L. Packer)	AY227955	AY222591	AF140326
Augochlorella pomoniella	Aupo	592	Haa	USA: California, Inyo Co. (J. Ascher)	AY227935	AY222572	AF435373
Augochloropsis metallica	Aume	334/5	Haa	USA: New York, Tompkins Co.	AY227934	AY222571	AF140315
Corynura (*Cory*) *patagonica*	Copt	581	Haa	Chile: Region IX, near Temuco (L. Packer)	AY227936	AY222573	na
Megalopta genalis	Mgge	247	Haa	Republic of Panama: Panama Prov. (M. Engel)	AY227937	AY222574	AF140316
Neocorynura discolor	Ncdi	249	Haa	Colombia (M. Engel)	AY227938	AY222575	AF140317

[a] Xx = outgroup; Ro = Rophitinae; No = Nomiinae; Nm = Nomioidinae; Hah = Halictinae; Halictini (sensu Michener, 2000); Haa = Halictinae; Augochlorini.
[b] All sequences generated with Opsin For/Rev4 except where noted.
[c] na = not available.
[d] Sequences generated with Opsin For/Rev only.

estimates. Trees were sampled at intervals of 100 generations for a total of 10,000 trees. We plotted the likelihood scores against generation time to identify the region of the analysis in which the parameter estimates were stable. We discarded the burn-in region (trees and parameter estimates obtained before equilibrium; generally the first 1,000–2,000 trees) and calculated the mean, variance, and 95% credibility intervals of the parameter estimates using MrBayes. Trees were represented either as phylograms (with branch lengths averaged over many trees) or as 50% majority rule consensus trees using PAUP*.

Dating methods.—Before conducting the divergence dating analysis, we tested for constant rates among lineages using the LR test by comparing the likelihood score of a constant rate model with that of a variable rate model over the topology generated from the Bayesian GTR+SSR analysis. The LR test indicated violation of rate consistency among lineages, i.e., the lack of a molecular clock (-2 ln LR $= 513.37$; df $= 60$; $P \ll 0.001$). We then estimated branch lengths and divergence dates on this topology using computer programs (ESTBRANCHES and MULTIDIVTIME) that implement the Bayesian divergence dating method developed by Thorne and coworkers (Thorne et al., 1998; Kishino et al., 2001; Thorne and Kishino, 2002). This method allows for rate variation both among lineages and among genes. Branch lengths were estimated separately for each gene under an F84+G model (model complexity is limited by the dating program) with parameter values obtained from the program PAML 3.13a (Yang, 1997).

To infer posterior values for divergence times, we established 125 ± 30 million years before present (MYBP) as the mean and variance on the prior date for the root node (after Engel, 2001, pers. comm.). These dates reflect the consensus that bees originated sometime during the period of rapid angiosperm diversification in the early to middle Cretaceous (Lomholdt, 1982; Michener, 1979, 2000; Crepet, 1996; Grimaldi, 1999; Engel, 2001). Because this date is speculative, we tested the robustness of our results to the prior date on the root node by conducting additional analyses with these alternative values: 125 ± 60 MYBP (increased variance), 140 ± 30 MYBP (older prior), and 100 ± 30 MYBP (younger prior). We generated the prior for the initial rate at the root node using the penalized likelihood approach provided by the program r8s 1.50 (Sanderson, 2002).

We additionally constrained three internal nodes with minimum ages based upon the bee fossil record. We calibrated the root node of Augochlorina (a subtribe of Augochlorini; Engel, 2000) with a minimum age of 23 MYBP based on numerous fossils from this group in Dominican amber (Engel, 1995, 1996, 2000; Engel and Rightmyer, 2000). We similarly constrained the root node of Caenohalictini (=agapostemonine bees; Roberts and Brooks, 1987) with the same minimum date using the Dominican amber fossil *Eickwortapis dominicana* from this tribe (Michener and Poinar, 1996). We established a minimum age of 42 MYBP for the Halictini (Table 1) based on *Electrolictus antiquus* from Baltic amber (Engel, 2001).

A recent report of a "*Halictus?*" from the early Eocene (52–54.5 MYBP; Engel and Archibald, 2003) was not included because the specimen cannot be confidently placed to subfamily, much less to genus. Furthermore, we did not include supposed fossil halictid bee nests from the late Cretaceous (e.g., Cenomanian [Elliot and Nations, 1998; Genise, 2000; Genise and Verde, 2000] and Maastrichtian [Genise et al., 2002]) as calibration points because these nests are not attributable to particular groups within the halictid bees and may not be bee nests at all (J. G. Rozen, Jr., pers. comm.).

In conducting the Bayesian divergence dating analysis with the MULTIDIVTIME program, we performed multiple searches starting from different random states to ensure convergence of Markov chain Monte Carlo runs, each consisting of 100,000 cycles sampled every 100 times after an initial burn-in of 100,000 cycles. We also tested for correlated changes in rates between genes using this program, which appoximates the distribution of the rank correlation coefficient under a null model of no correlated rate change over time between two genes (Thorne and Kishino, 2002).

Biogeographic Analysis

To reconstruct the likely biogeographic history of the halictid bees, we used dispersal vicariance analysis (Ronquist, 1997) with the program DIVA 1.1 (Ronquist, 1996). We coded the taxa as being present or absent from the following areas: North America, Central America, South America, Eurasia, subsaharan Africa, and Indoaustralia. We simplified the analysis slightly by coding the distributions at the level of genus and subgenus (i.e., when a genus was widespread over two or more areas but the species we included in our analysis was restricted to one of those areas, we coded the species as present in both areas). We also coded some widespread taxa (e.g., Nomiinae) as being limited to their presumed area of origin, as recommended by Ronquist (1996). We coded Nomiinae as being primitively African because Africa includes the vast majority of genera and subgenera, and the Indoaustralian and North American genera/subgenera are considered to be derivatives (Michener, 1979). We also coded the Nomioidinae as primitively African, following the results of Pesenko's (2000) analysis of generic relationships. The data set is available from one of us (B.N.D.) as a Nexus file.

RESULTS

The combined three-gene data set consisted of a total of 2,234 aligned nucleotide sites, 779 of which were parsimony informative (Table 4). Although the overall data set showed an unbiased base composition, some partitions of the data set showed substantial base compositional bias (Table 4). *Wingless* third positions are strongly G/C rich, whereas *EF-1α* third positions show A/T bias. For all three genes combined, there was signficant base compositional heterogeneity among taxa only at third position sites (Table 4). The data set is complete for all genes except for four missing *EF-1α* sequences and one

TABLE 4. Descriptive results for each gene and codon position.

Gene	No. sites	No. parsimony-informative sites	% A/T	P^a	Relative rate
LW opsin					
nt1	234	65	57.4	1.00	0.800
nt2	234	35	56.6	1.00	0.306
nt3	234	192	33.2	<0.001	2.442
wingless					
nt1	135	20	41.6	1.00	0.293
nt2	135	6	54.2	1.00	0.063
nt3	135	108	15.6	<0.001	3.143
EF-1α					
nt1	376	36	44.3	1.00	0.273
nt2	376	17	58.3	1.00	0.109
nt3	375	300	57.2	<0.001	2.095
Overall	2234	779	49.0	<0.001	

[a]Probability of rejecting the null hypothesis of homogeneity among taxa in base composition.

missing *wingless* sequence (Table 3). This represents 3.4% of the total aligned data set in terms of base pairs.

Parsimony Analyses

Equal weights parsimony analysis of each of the three genes alone yielded trees with various degrees of resolution, with *wingless* alone providing the least resolved tree topology (presumably because of the small size of the data set; 405 total sites, 134 parsimony-informative sites). There was no conspicuous topological incongruence among the three data sets, except that the two species of *Homalictus* came out in the *wingless* data set far removed from their presumed close relatives in the genus *Lasioglossum*. This result is not due to having sequenced the wrong paralog of *wingless*; we resequenced several species of *Homalictus* with a wnt-1–specific forward primer and confirmed that these sequences clustered with the other bee sequences from the wnt-1 gene family. We suspect that the small size of the *wingless* data set combined with a possible long-branch artifact may explain this result. Bootstrap analyses of each data set alone revealed that all three data sets provide topologically congruent bootstrap consensus trees. Among data sets, we observed no incongruence that was supported by >50% bootstrap support. In fact, combining just *wingless* plus opsin yielded a tree virtually identical to tree based on *EF-1α* alone.

Analysis of the data incongruence using the ILD test indicated significant incongruence among the three genes when the two species of *Homalictus* were included in the data set ($P = 0.010$) and nonsignificant incongruence when *Homalictus* was removed from the data set ($P = 0.220$). This finding indicates that the incongruence among the data sets is due to the topological incongruence observed. Overall, we see no reason not to combine the three genes, given that significant incongruence is limited to just two sequences in the smallest of the three data sets.

A combined equal-weights parsimony analysis of the three genes yielded just two trees (length = 4,618; consistency index = 0.2983). To identify well-supported aspects of the tree, we performed a bootstrap analysis (PAUP* 4.0b10) and calculated Bremer support (TreeRot 2). Figure 2 shows the 50% bootstrap consensus tree (with bootstrap values above the nodes and Bremer support values below the nodes). Overall, the tree topology is extremely well supported, with bootstrap values >70% and Bremer support >4 for the majority of nodes. Furthermore, the tree recovers major aspects of halictid higher level phylogeny. Ingroup monophyly is supported by 79% bootstrap support, and Bremer support is 6. All four subfamilies (Rophitinae, Nomiinae, Nomioidinae, and Halictinae) are strongly supported (with bootstrap values ≥97% and Bremer support ≥14), and we recovered exactly the same relationships among the subfamilies as did Pesenko (1999) based on morphology. Analysis of the data set by LogDet distance methods yielded trees largely congruent with the parsimony results.

Our results also support the monophyletic groups traditionally recognized as tribes within the subfamily Halictinae by Michener (2000) and others (Pesenko, 1999): Augochlorini had 99% bootstrap support and Bremer support of 11, and Halictini sensu Michener had 77% bootstrap support and Bremer support of 3. Our results within the subfamily Halictinae strongly support generic relationships and are congruent with previous generic and tribal treatments of the halictine bees based on morphology (Eickwort, 1969a, 1969b; Michener, 1978a, 1978b; Roberts and Brooks, 1987; Danforth and Eickwort, 1997; Engel, 2000; Janjic and Packer, 2003). Given the huge number of species and the morphological diversity within the tribe Halictini (sensu Michener, 2000), we developed a revised tribal classification that more clearly reflects the morphological diversity and biogeographic distributions of this group (Table 1). Among the more interesting results of this study is the resolution of the relationships among the tribes Augochlorini, Thrinchostomini, Caenohalictini, Sphecodini, and Halictini.

Relationships among the genera of Halictini (sensu our revised classification; Table 1) were not resolved in the 50% bootstrap consensus. However, in the two shortest trees from the equal weights analysis relationships were recovered and were congruent with the generic relationships reported by Danforth (2002).

Overall, the parsimony results indicate strong phylogenetic signal in the combined data set extending well into the basal portions of the tree.

ML and Bayesian Analyses

ML trees are not shown but are identical in topology to those of the Bayesian analyses. We analyzed our combined data set based on six models: K2P+I+G, HKY+I+G, GTR+I+G, K2P+SSR, HKY+SSR, and GTR+SSR. Results of these six models were largely congruent. Trees based on the I+G models (Fig. 3) were identical in topology to the parsimony results (Fig. 2). Trees based on the SSR models, however, provided a slightly different hypothesis of relationships within the subfamily Rophitinae. In the SSR models, the South American

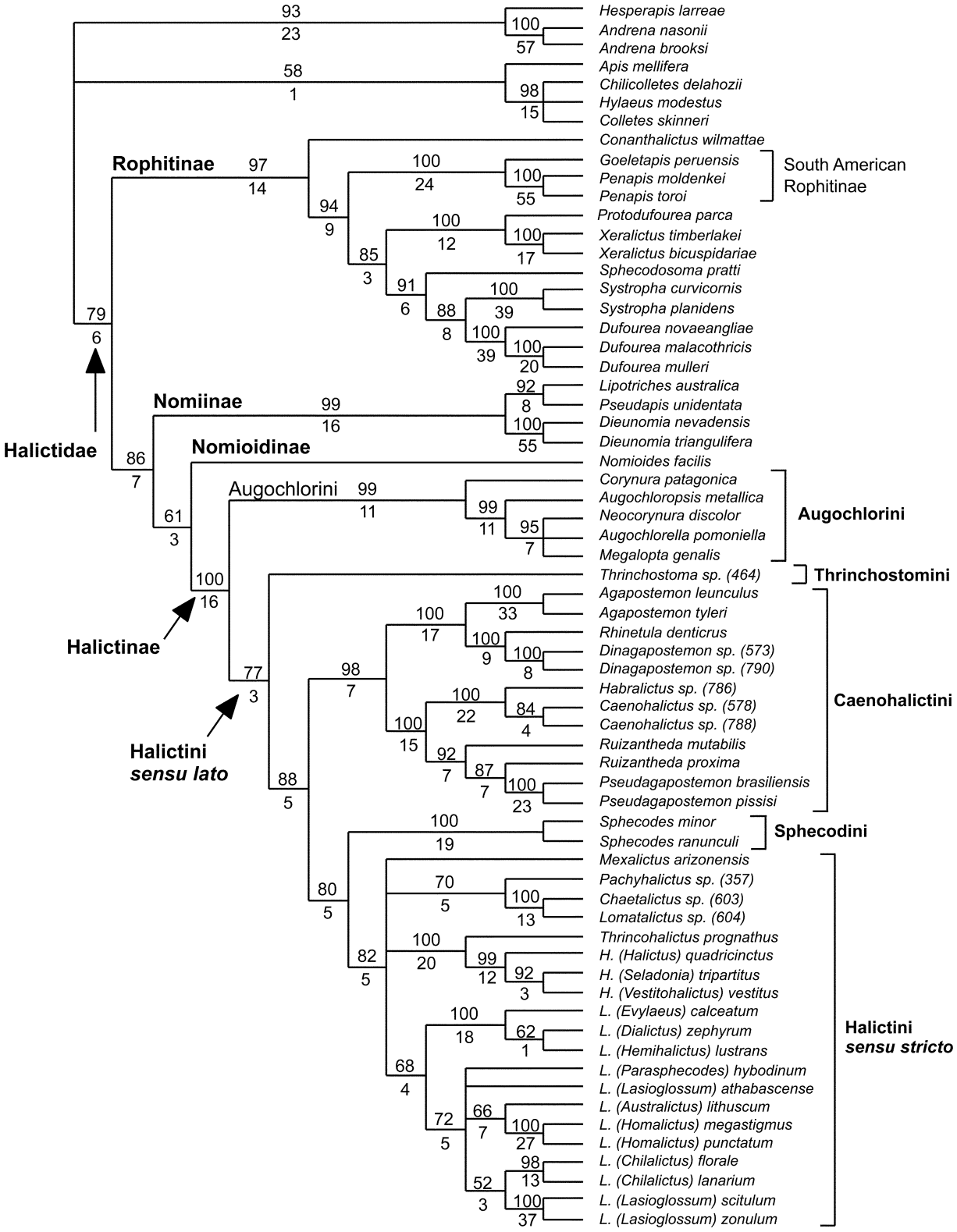

FIGURE 2. Parsimony bootstrap consensus tree of the combined data set. We performed 500 bootstrap replicates with 10 random sequence additions per replicate. Bootstrap values are shown above the nodes; Bremer support values (TreeRot 2; Sorensen, 1999) are shown below the nodes.

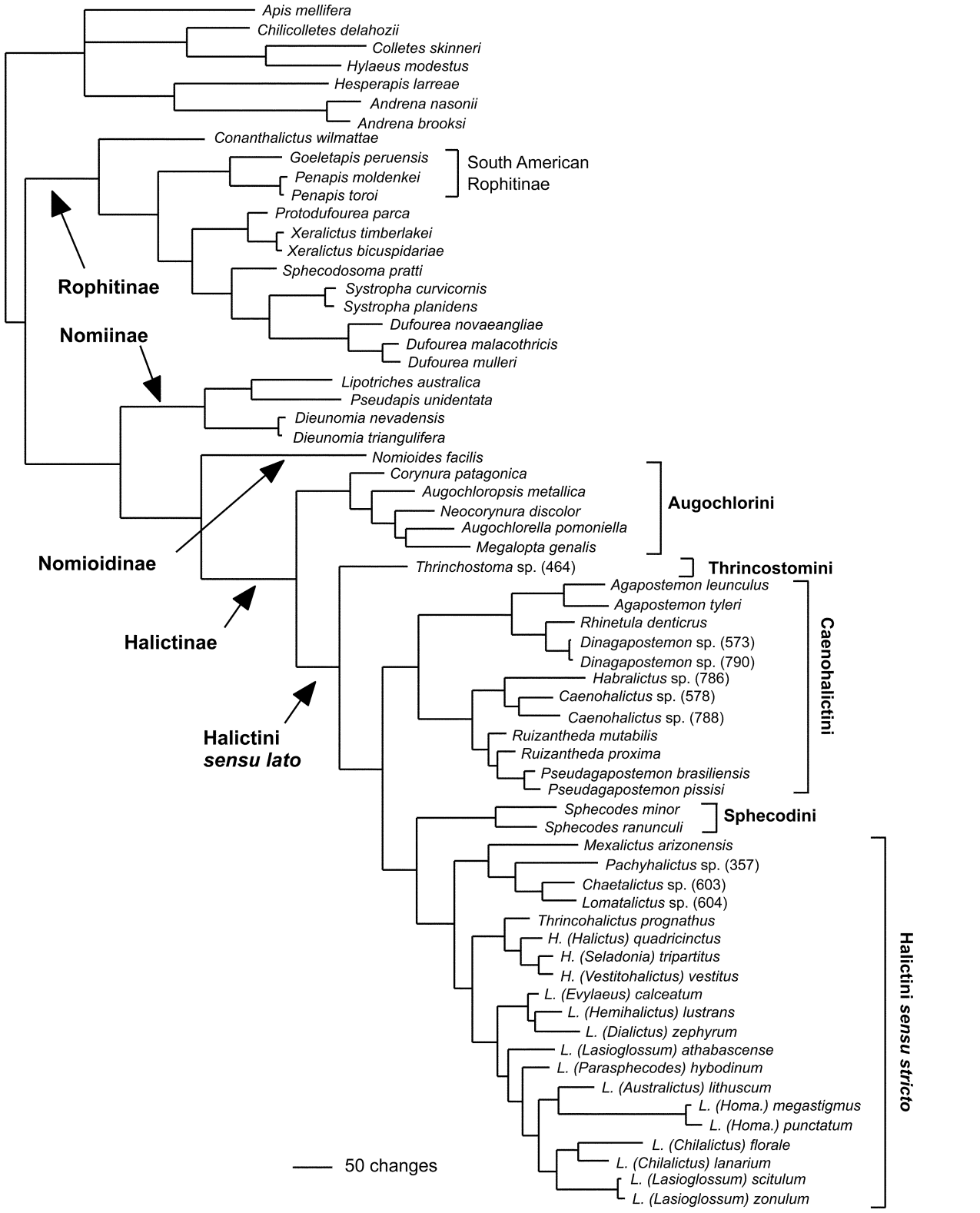

FIGURE 3. Bayesian tree based on the combined data set using a separate GTR+I+G model for each gene. Negative log likelihood = 24901.4 (averaged over last 1,000 tree topologies). Branch lengths are average branch lengths over last 1,000 tree topologies. Parameter estimates for each gene (averaged over last 1,000 tree topologies): opsin, pinv = 0.46 ± 0.0005, alpha = 1.32 ± 0.03; *wingless*, pinv = 0.58 ± 0.0007, alpha = 0.93 ± 0.02; *EF-1α*, pinv = 0.53 ± 0.0003, alpha = 1.32 ± 0.03.

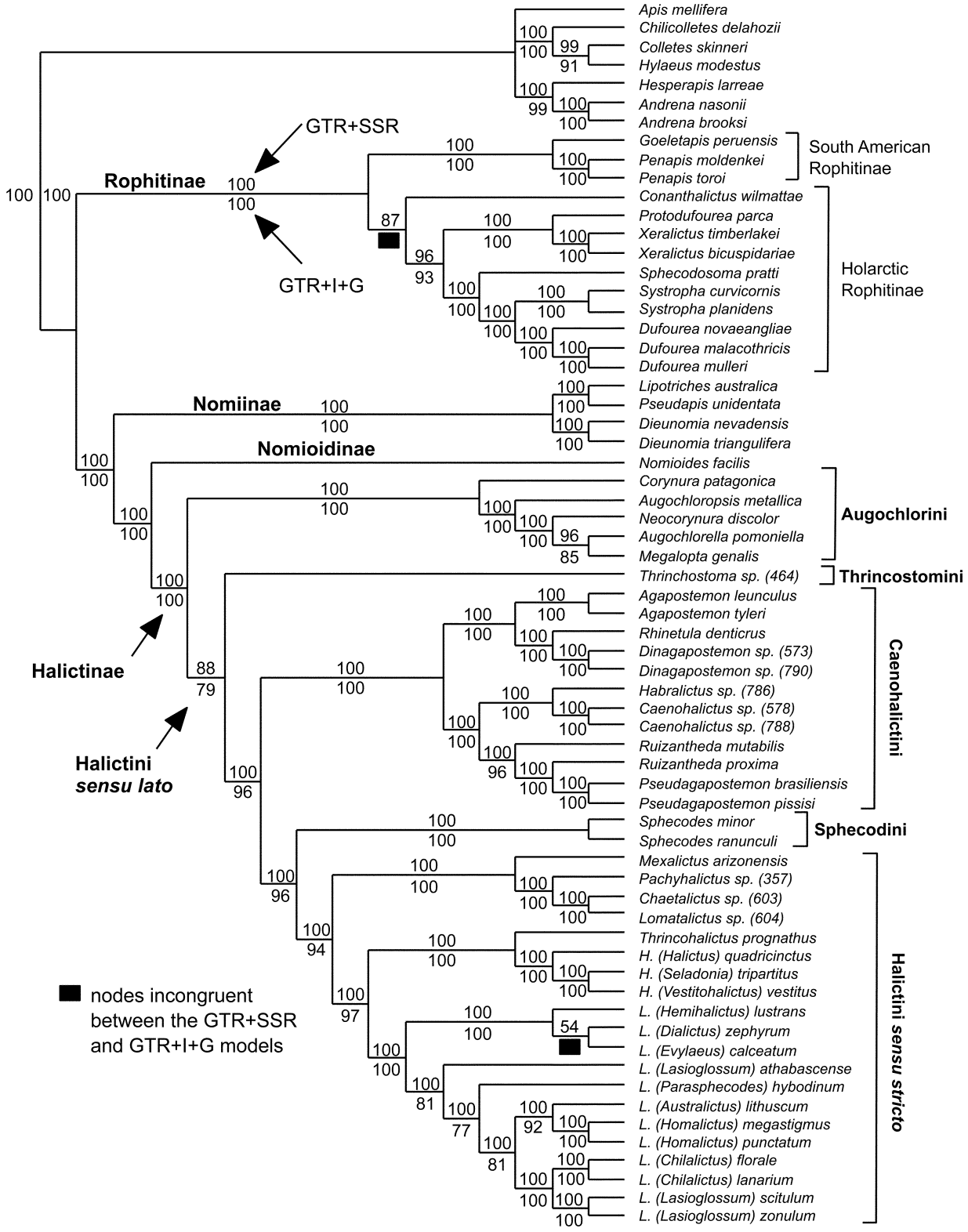

FIGURE 4. The 50% majority rule Bayesian tree based on the GTR+SSR model with nine rate categories: opsin nt1, opsin nt2, opsin nt3, *wingless* nt1, *wingless* nt2, *wingless* nt3, *EF-1α* nt1, *EF-1α* nt2, *EF-1α* nt3 (negative log likelihood = 25667.2; averaged over last 1,000 tree topologies). We discarded the first 1,000 trees and calculated the 50% majority rule consensus based on the remaining 9,000 tree topologies. Values above the branches indicate posterior probabilties based on the GTR+SSR model; values below the branches indicate posterior probabilities based on the GTR+I+G model (with parameters estimated separately for each gene; see Fig. 3). Nodes incongruent between the two analyses are indicated with solid squares.

Rophitinae (*Goeletapis*, *Penapis*, and *Ceblurgus* [not included in our analysis]; Rozen, 1997) is the sister group to the Holarctic Rophitinae (all remaining genera), whereas in the parsimony and Bayesian I+G models, *Conanthalictus* (a genus restricted to the arid regions of western North America) is the sister to all other rophitine genera. We summarize our Bayesian and ML results in Figure 4. The Bayesian posterior probabilities for the alternative among-site rate variation models are shown on the tree. The high posterior probabilities deep in the tree support the view that the combination of three single-copy nuclear genes provides a robust signal, even for the basal nodes.

Comparison among Genes

Comparing rates of substitution among the genes indicates that the three nuclear genes are evolving at roughly similar rates (Fig. 5), with opsin showing a slightly elevated rate at first and second positions, relative to the other genes. Relative to data sets involving combined nuclear and mitochondrial genes (e.g., Danforth et al., 2003), in which rate variation can range over two orders of magnitude, the variation in rates among the data sets here is small. Examination of the Bayesian parameter estimates (under a GTR+G model with parameters estimated separately for each gene) indicates that *wingless* shows the greatest heterogeneity in among-site rate variation and opsin shows the least. Parameter estimates for each gene (averaged over last 1,000 tree topologies) are as follows: opsin, alpha $= 0.30 \pm 0.0004$; *wingless*, alpha $= 0.18 \pm 0.0002$; *EF-1α*, alpha $= 0.23 \pm 0.0002$.

The rank correlations of rate change obtained from MULTIDIVTIME are positive for all pairwise comparisons between genes: opsin versus *wingless* (0.3376); opsin versus *EF-1α* (0.4326); *wingless* vs. *EF-1α* (0.4582). A positive correlation suggests the presence of lineage effects between genes, where sections of fast (or slow) evolutionary rate on the tree for one gene tend to be the same as those for the second gene. None of these comparisons are significant at the conventional 0.05 level when tested against the null hypothesis of independent rate changes between the two genes: opsin versus *wingless* ($P = 0.196$); opsin versus *EF-1α* ($P = 0.086$); *wingless* versus *EF-1α* ($P = 0.056$). The power of this test to detect these correlations, however, is still somewhat uncertain (Thorne and Kishino, 2002).

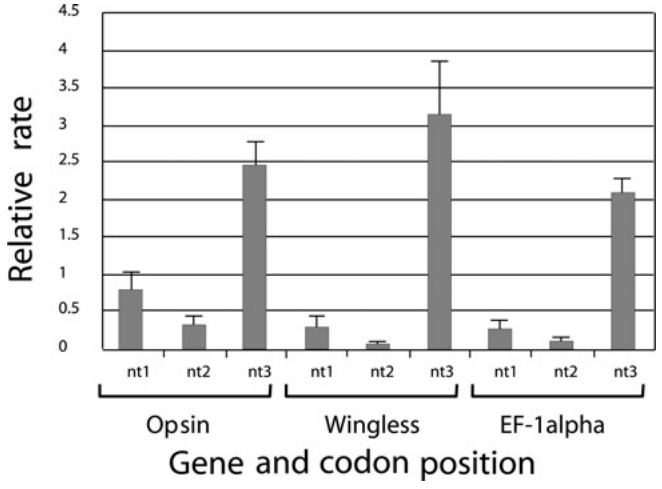

FIGURE 5. Relative rates obtained from the Bayesian GTR+SSR model. Bars indicate means, and error bars indicate 95% credibility intervals.

TABLE 5. Results of partitioned Bremer support (PBS) analysis.

Gene	PBS	Minimum steps	PBS/minimum steps
Opsin	426	601	0.71
wingless	182	268	0.68
EF-1α	262	643	0.41

To compare the relative contribution of each data set to the overall topology, we calculated partitioned Bremer support. Surprisingly, opsin contributes the most in terms of partitioned Bremer support (Table 5), with *EF-1α* providing the least (when total Bremer support is divided by the minimum number of steps; Baker et al., 2001). This finding is unexpected because equal weights parsimony analysis of each gene separately showed that *EF-1α* recovers the most fully resolved tree of the three data sets. Partitioned Bremer support may therefore not be a good indicator of the resolution one can expect from a particular data partition but rather the support that a particular data set provides to the overall tree topology in the combined analysis.

Dating Analysis

Combining our Bayesian GTR+SSR tree topology with three fossil calibration points and a prior root node date of 125 ± 30 MYBP, we converted our tree topology into a chronogram (Fig. 6). The dating analysis indicates that all four halictid subfamilies were present well before the end of the Cretaceous, the tribe Augochlorini diverged from the rest of the halictine bees near the end of the Cretaceous, and that the divergence among the remaining tribes (Thrichostomini, Caenohalictini, Sphecodini, and Halictini) and genera took place primarily during the Tertiary. Changing the prior value for the root node does not alter the basic result that the initial radiation of Halictidae occurred in the Cretaceous (Table 6). Changing the mean of the prior from 125 MYBP to 100 MYBP alters the posterior values by at most 10 MY, and even then the deepest nodes in the halictid tree remain Cretaceous, with 95% credibility intervals. Caution should be observed when interpreting any dating estimates derived by these methods (Benton and Ayala, 2003). However, we have taken a conservative approach, and our sensitivity analysis indicates that the prior on the root node has only a slight influence on the estimated dates (Table 6).

We combined our dating estimates (Fig. 6) with the bootstrap and Bremer support values for all ingroup nodes (Fig. 2) to assess how the level of support in the

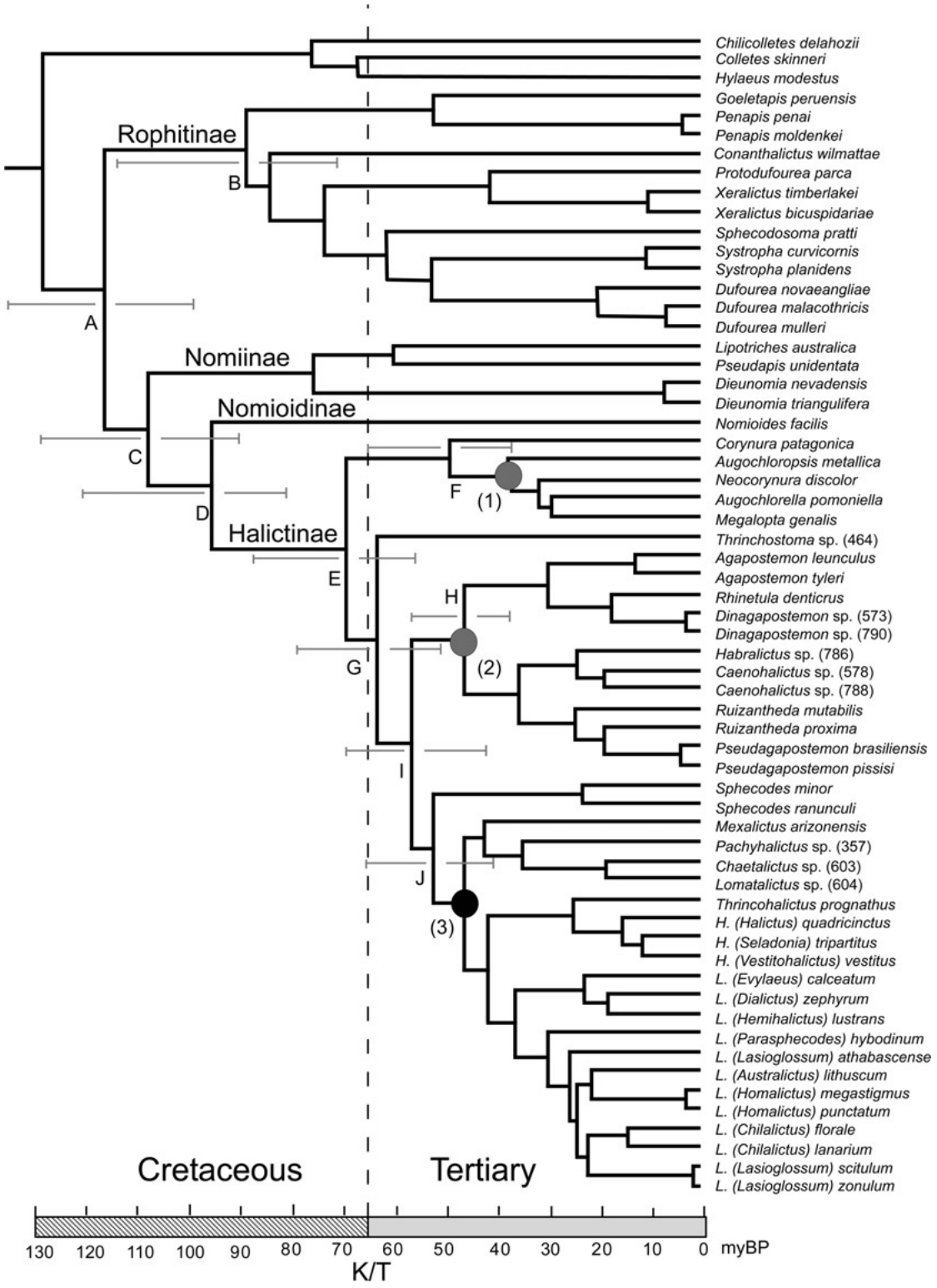

FIGURE 6. Chronogram derived from the Bayesian tree combined with three fossil calibration points: 1 = Dominican amber Augochlorini (minimum age = 23 MYBP; Engel, 1995, 1996, 2000; Engel and Rightmyer, 2000); 2 = Dominican amber Caenohalictini (*Eickwortapis dominicana*, minimum age = 23 MYBP; Michener and Poinar, 1996); 3 = Baltic amber *Electrolictus antiquus* (minimum age = 42 MYBP; Engel, 2001). Shaded circles indicate Dominican amber calibration points; solid circle indicates the Baltic amber calibration point. Error bars indicate 95% credibility intervals on selected nodes. Letters refer to nodes in Table 6.

TABLE 6. Mean and 95% credibility intervals on the antiquity of lettered nodes in Figure 6 under different prior values for root node. Nodes A–E remain Cretaceous even with different priors on the root node. Ages in millions of years.

	Prior values on root node			
Node	125 (95,155)	125 (65,185)	100 (70,130)	140 (110,170)
A	119 (97,145)	125 (97,164)	109 (89,132)	127 (104,154)
B	91 (70,114)	95 (70,129)	82 (64,104)	97 (75,122)
C	110 (90,134)	115 (89,151)	101 (83,123)	117 (95,143)
D	97 (79,120)	102 (79,134)	90 (73,110)	103 (83,128)
E	71 (58,88)	73 (58,97)	66 (56,81)	74 (60,93)
F	50 (35,68)	52 (36,73)	47 (34,63)	52 (37,72)
G	65 (52,81)	67 (53,89)	61 (51,75)	68 (54,86)
H	47 (37,61)	48 (37,66)	45 (36,56)	49 (38,64)
I	57 (48,72)	59 (48,79)	55 (47,66)	60 (49,76)
J	53 (49,66)	55 (45,72)	51 (44,61)	55 (46,70)

combined data set varies according to the age of clades. Our results (Fig. 7) indicate that both bootstrap and Bremer support levels remain high well into the Cretaceous. Bootstrap values are well above 80% (on average) until well over 100 MYBP, and Bremer support values remain >5 (on average) up to approximately 80 MYBP.

Biogeographic Analysis

The results of our biogeographic analysis are shown in Fig. 8. According to the DIVA analysis, the common ancestor of the Halictidae was widespread on (1) South America, Africa, and North America, (2) South America and Africa, or (3) North America and Africa. The last hypothesis seems highly unlikely given that North America and Africa are widely separated (although see below for an alternative scenario). Following the ambiguous root node, the program optimized the clade including Nomiinae, Nomioidinae, and Halictinae as primitively African, with two rounds of South American/African vicariance or two dispersal events to South America (numbers 2, 3). For the Rophitinae, the root node is ambiguous but most likely involved South American/North American vicariance dispersal from South America to North America (number 1), followed thereafter by dispersal to Eurasia. According to our chronogram (Fig. 6), the South American/African vicariance (or dispersal; numbers 2, 3) would have occurred roughly between 70 and 55 MYBP, long after the final separation of Africa and South America (100 MYBP; Smith et al., 1994) but perhaps before the continents became so widely separated that dispersal could no longer occur. In summary, our biogeographic analysis suggests an initial radiation in the southern continents of Africa and South America, with two subsequent dispersals to South America (giving rise to the Augochlorini and Caenohalictini). Much of early halictid evolution appears to have occurred in Africa or in Africa and South America.

DISCUSSION

Utility of Single-Copy Nuclear Genes

Our results provide the first conclusive demonstration that single-copy nuclear genes (in combination) are

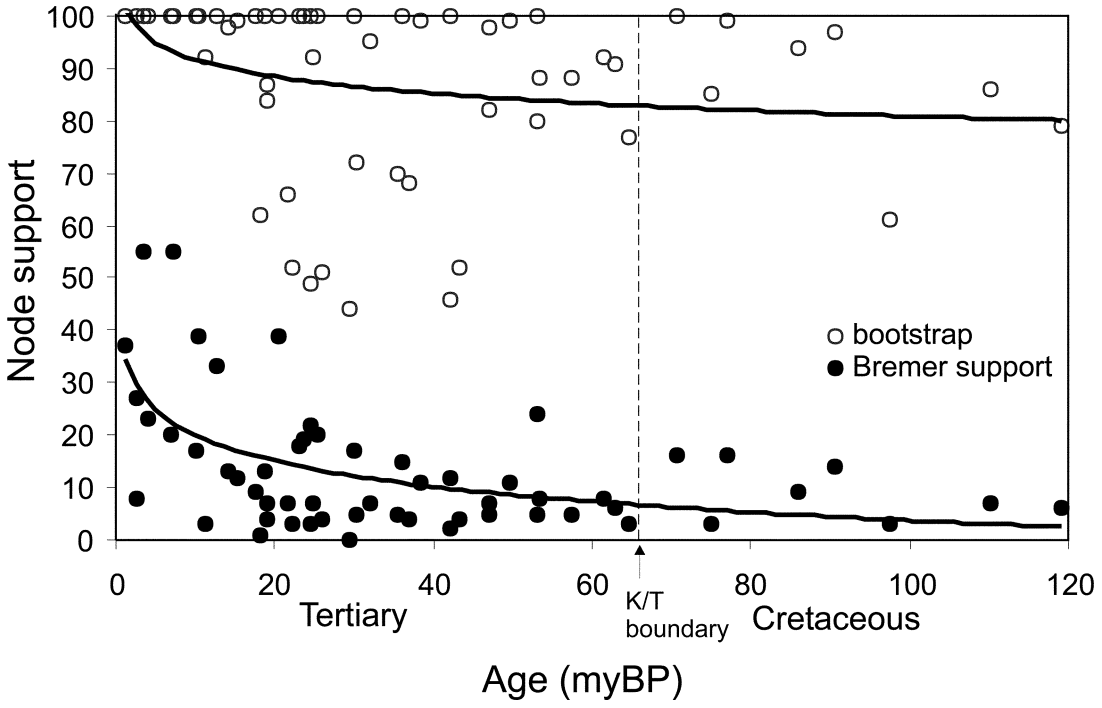

FIGURE 7. Relationship between bootstrap values (above, open circles) and Bremer support values (below, solid circles) for individual nodes within the tree and divergence time obtained from the Bayesian dating methods. The trendlines shown are logarithmic regression lines. On average, bootstrap values exceed 80% to at least 100 MYBP and Bremer support values exceed 5 to approximately 80 MYBP.

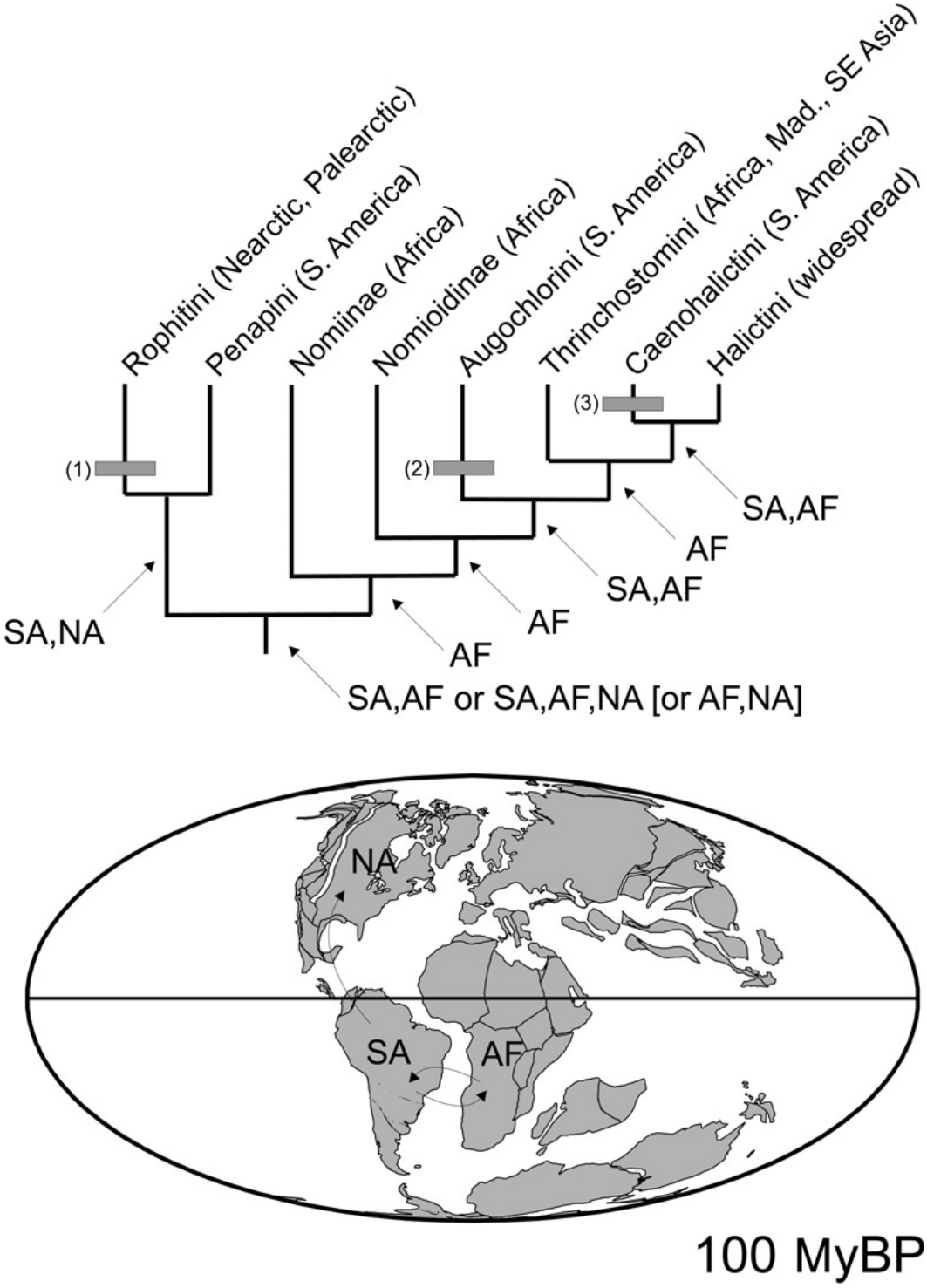

FIGURE 8. Results of the DIVA analysis showing ancestral character state reconstructions for the major branches in the phylogeny of halictid bees. AF = Africa; SA = South America; NA = North America. Hypothesized dispersal events are indicated as shaded boxes: 1 = dispersal of Rophitinae to northern hemisphere; 2 = dispersal of Augochlorini to South America; 3 = dispersal of Caenohalictini to South America. Ancestral distribution of the family would likely have been Africa and South America.

capable of resolving Cretaceous-age divergences in bees with high parsimony bootstrap, Bremer, and posterior probability values. No previous study has demonstrated such levels of support at or above the subfamily level in bees and no previous study has demonstrated robust results extending well into the Cretaceous (Fig. 7). Investigators have often noted difficulty in obtaining robust molecular reconstructions of approximately

Cretaceous-age relationships in various insect taxa (e.g., Mitchell et al., 1997, 2000; Fang et al., 2000; Yang et al., 2000; Caterino and Vogler, 2002; Johnson and Whiting, 2002; Ward and Brady, 2003). Although lineage-specific effects such as short deep internodes, rate variation among branches, and taxon sampling undoubtedly play a key role in some cases, the choice of molecular data presumably also has a major influence. Our results show that, with respect to our particular data set, support for deeper relationships only slightly declines and remains relatively high overall even into the Cretaceous (Fig. 7).We are optimistic that the combination of these three single copy nuclear genes (LW opsin, *wingless*, and *EF-1α*) will be capable of resolving the deepest and most problematic phylogenetic questions in bees, namely the family-level relationships among the basal or ST bee families (Colletidae, Stenotritidae, Andrenidae, Halictidae, and Melittidae).

We propose that the three genes we have utilized here become standard molecular markers for most higher level phylogenetic studies of bees. By focusing on the same set of molecular markers, bee systematists could accumulate substantial, mutually complementary data sets.

Historical Biogeography

Our results do not completely support Michener's (1979:307) hypothesis that halictid bees had a Northern Hemisphere origin with repeated colonization of the Southern Hemisphere:

> In view of the largely northern distribution of the subfamily Dufoureinae (=Rophitinae) and the rich representation of the other subfamilies in the Holarctic region, it seems likely that the origin or at least the initial radiation of the Halictidae was in the Laurasian continents. There were early major invasions of the southern continents, however, followed by extensive radiations, especially of Nomiinae in Africa, of Augochlorini in South America, and of Halictini in all three southern continents.

Our results suggest an African origin for the common ancestor of the Nomiinae, Nomioidinae, and Halictinae, with subsequent colonization of South America and (later) the Northern Hemisphere, and a South American origin for the Rophitinae, with subsequent dispersal and diversification in the Northern Hemisphere (Fig. 8). An alternative scenario could be invoked to explain the current seemingly primitive Southern Hemisphere distribution of the the halictid subfamilies. It is possible, but nearly impossible to verify without better fossil data, that these groups were widespread in the Northern Hemisphere and then later went extinct. The presence of one halictid bee fossil in Baltic amber (*Electrolictus antiquus*; Engel, 2001) could be viewed as supporting this hypothesis. Our results are also contingent on the Nomiinae and Nomioidinae having African origins. Future work will be needed to test this hypothesis.

There are some fascinating patterns that emerge from the biogeographic analysis. First, the parallel dispersals of halictine bees to South America lead to a striking morphological parallel. The two South American tribes of halictine bees (Augochlorini and Caenohalictini) include species that are for the most part brightly colored, metallic greenish bees. These groups are so similar in overall appearance that they were not recognized as distinct groups until Eickwort (1969b) described the numerous morphological characters that distinguish them. Why bright metallic greenish coloration would arise in parallel in South American halictines is not clear. Second, the phylogenetic placement of Thrichostomini (*Thrinchostoma* and its presumed cleptoparasitic derivative *Parathrincostoma*) raises fascinating biogeographic questions. *Thrinchostoma* occurs in southern and tropical Africa and Madagascar, with small numbers of species occuring in southern Asia (including India, Vietnam, Java, and Sumatra). The placement of *Thrichostoma* as a clade between Augochlorini and Caenohalictini (both South American groups) indicates the presence of halictine bees in Africa near the origin and early diversification of the subfamily Halictinae.

The dating analysis and the biogeographic scenario are largely congruent. The presumed dispersals from Africa to South America (nodes 2 and 3, Fig. 8) are estimated based on the dating analysis to have occurred between 70 and 55 MYBP (Fig. 6), considerably after the African/South American separation but during a period when the two continents were separated by a narrow water barrier (Smith et al., 1994). Furthermore, the presumed colonization of the Northern Hemisphere by the tribes Halictini (*sensu stricto*) and Sphecodini that is estimated to have occurred between 50 and 55 MYBP (Fig. 6, node J) is approximately coincident with first overland connections between North America and South America in the early Eocene (Maury et al., 1995).

The origin of all four halictid subfamilies before the Cretaceous/Tertiary (K/T) boundary provides important insights into patterns of bee diversity today. Each of the four halictid subfamilies (Rophitinae, Nomiinae, Nomioidinae, Halictinae) is morphologically distinct. Extinction of intermediate forms could help explain the morphological distinctiveness of these four subfamilies. The impact of the K/T extinction event on bees is difficult to gauge, but we speculate that it may have been substantial. Bee taxa could have experienced significant extinction at the K/T boundary for a number of reasons. First, the fossil record indicates that up to 80% of angiosperm plants in the Northern Hemisphere went extinct at the K/T boundary event (Johnson, 1992), and the most heavily impacted species were those with insect pollinators (Sweet, 2001). Recent evidence also suggests that insect herbivores with narrow host-plant preferences (specialists) suffered higher rates of extinction than did generalist herbivores (Labandeira et al., 2002). Given that many basal subfamilies and tribes of bees are primarily host-plant specialists (including Rophitinae, Nomiinae, and Nomioidinae in the Halictidae), the impact of angiosperm extinctions may have been most severe on those specialist lineages. The largest and most diverse halictid tribe is the Halictini (*sensu stricto*), a group of predominantly generalist species that

diversified after the K/T boundary (Fig. 6). Diversification of the Halictini (which includes >65% of all the species of halictid bees, Michener, 2000) after the K/T event may have been facilitated by extinction of other Cretaceous-age Northern Hemisphere bee groups.

Halictid Higher Level Classification

Our results are robust enough and our taxon sampling detailed enough to allow a revised tribal level-classification of the halictid subfamily Halictinae. Table 1 lists this classification, reflecting our phylogenetic results for this subfamily. A revised classification for the Nomiinae awaits a more thorough study of the generic and subgeneric relationships within this large, diverse, and widespread subfamily. We are currently developing a comparable single-copy nuclear gene data set for this subfamily. The subfamilies Rophitinae and Nomioidinae were recently treated by Michener (2000) and Pesenko (2000), respectively.

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