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Evolution

Multiple origins of advanced eusociality in bees inferred from mitochondrial DNA sequences

(Apidae/molecular systematics/ribosomal RNA)

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ABSTRACT **The remarkably high level of colony organization found in the honey bees and stingless bees (family Apidae) is extremely rare among animals. Yet there is controversy over whether these two groups independently evolved advanced eusocial behavior or inherited it from a common ancestor. Phylogenetic analyses of DNA sequence information from the mitochondrial genome (large-subunit ribosomal RNA gene) of representative apid bees suggest that advanced eusocial behavior evolved twice independently within this assemblage. These results depart from previous hypotheses of apid relationships by indicating a close phylogenetic relationship between the primitively eusocial bumble bees and the stingless bees.**

Advanced eusocial behavior is extremely rare among the insects and has evolved in only two orders, the termites (Isoptera) and the ants, bees, and wasps (Hymenoptera). The mechanisms of evolution of social insect behavior continue to be debated (1) after more than two decades of intense examination following Hamilton's groundbreaking theory on the evolution of altruism (nonreproductive workers) in Hymenoptera (2). A wealth of recent data on hymenopteran behavior (3, 4), ecology (5), and population structure (6) has led to fresh insights and refined hypotheses of the social evolutionary process (7, 8). However, we still understand very little about the actual historical pattern of hymenopteran social evolution, even though it is becoming increasingly clear that knowledge of phylogenetic history is crucial for testing hypotheses of evolutionary processes (9). This conundrum is exemplified by the bees of the family Apidae, one of the most useful groups for investigating independent patterns of social evolution because they exhibit all gradations of social organization, from solitary to advanced eusocial (10). A review of previous phylogenetic investigations shows that despite serious effort to apply the best methods of comparative morphological and behavioral analyses (11, 12), there is still no consensus of relationships for the Apidae. Hypotheses of apid social evolution (5), therefore, are currently untestable because we lack a strongly supported phylogeny. Here I report a phylogenetic hypothesis of apid tribal relationships based on mitochondrial DNA (mtDNA) nucleotide sequences, representing a comprehensive phylogeny using on comparative DNA sequences for a social insect family. These results constitute a major departure from all previous hypotheses of apid relationships by indicating a close phylogenetic relationship between bumble bees and stingless bees. Furthermore, in contrast to recent views, these results offer fresh support for the hypothesis that advanced eusocial behavior in the apid bees evolved twice independently within two distantly related lineages, the honey bees and the stingless bees (13, 14).

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The Apidae traditionally have been divided into four subfamilies or tribes: the advanced eusocial honey bees (Apini) and stingless bees (Meliponini), the primitively eusocial bumble bees (Bombini), and the solitary (10) to primitively eusocial (5) orchid bees (Euglossini). Although honey bees and stingless bees share striking similarities in their complex social organization [e.g., large, perennial colonies with a morphologically and behaviorally distinct queen modified for egg laying, female offspring (workers) with a high degree of task specialization and complex communication] (5, 10), the mechanics of their social systems (e.g., recruitment, colony founding, nest architecture) are strikingly different (15). For example, honey bees recruit nest mates to food sources and nest sites via a symbolic dance–language and food odors, whereas stingless bees use a system of trail pheromones that leads recruits directly to the resource. When honey bees initiate a new colony, the old queen leaves her nest accompanied by a swarm of workers who search for an appropriate new nest site; in stingless bees a young queen leaves the old nest to take up residence in a new one previously constructed over several weeks by workers from the old colony. Whether these differences in advanced eusocial behavior are the result of alterations from a common advanced eusocial ancestor or reflect the independent evolution of advanced eusociality can be tested with an independently estimated phylogenetic hypothesis (16, 17).

There are currently four principal hypotheses of relationships among the apid tribes (Fig. 1), based on cladistic analyses of morphological characters (18). As stated by Michener (11), there are currently too few discrete, phylogenetically informative (synapomorphic) morphological characters to corroborate the tribal relationships strongly (Fig. 1).

For this study, mtDNA sequences were used to provide an independent set of characters for estimating phylogenies (19) and testing the above competing phylogenetic hypotheses.† This technique is becoming increasingly popular because aligned sequences of nucleotides can provide an enormous number of additional homologous characters (20, 21) to supplement those available from morphology and behavior.

MATERIALS AND METHODS

Specimens Analyzed. Sequences from the mitochondrial large subunit (16S) ribosomal RNA gene (rRNA) (22) were compared in 14 exemplars representing the four apid tribes Apini: *Apis mellifera, Apis cerana, Apis dorsata, Apis florea*, and *Apis koschevnikovi*; Bombini: *Bombus pennsylvanicus, Bombus avinoviellus*, and *Psithyrus variabilis*; Meliponini: *Melipona compressipes, Scaptotrigona luteipennis, Trigona hypogea*, and *Trigona pallens*; Euglossini: *Eulaema poly-*

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†The sequences discussed in this paper have been deposited in the GenBank data base (accession nos. L22891–L22906).

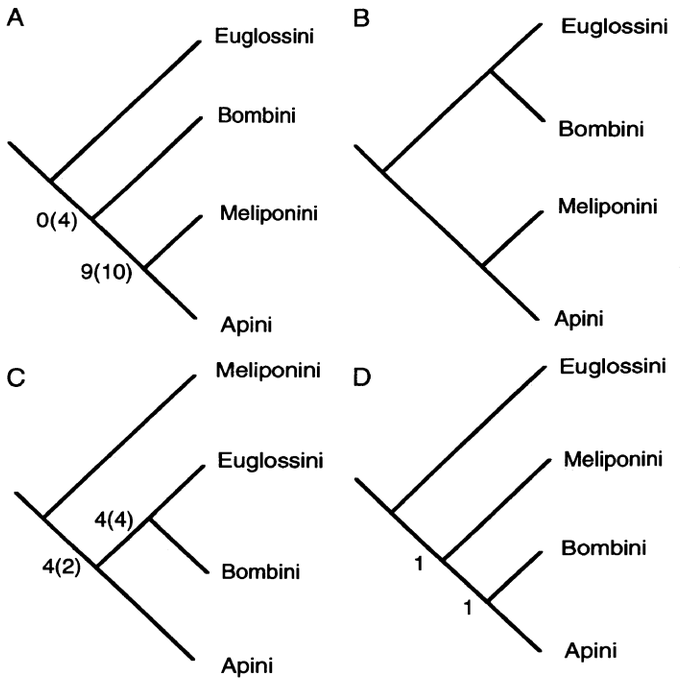


FIG. 1. Four different systematic hypotheses of the four tribes of Apidae based on morphological analyses. (*A–D*) Putative synapomorphies are indicated on the branches uniting tribes (number in parentheses refers to alternative analyses). Synapomorphies are not indicated in the topology of *B* because, as stated by the author, the lines of descent were subjectively determined (10).

chroma and *Eufriesia caerulescens*. Exemplars from the Xylocopini, *Xylocopa virginica*, and the Allodapini, *Exoneura bicolor*, were chosen as outgroups for the analyses.

The use of exemplars to represent a tribe was justified on the basis that each tribe has been recognized as a monophyletic group (11). The outgroups were selected from the subfamily Xylocopinae (family Anthophoridae), considered to be monophyletic and the closest relatives of Apidae (23). Voucher specimens of all taxa used in this investigation are deposited in the Entomology Museum at the University of Arkansas.

DNA Extraction. Sequences were obtained from fresh, frozen, and ethanol-preserved tissue. Cellular DNA was extracted by using modifications of standard procedures (24). Thoracic tissue was used for the larger specimens, but for small specimens the entire animal (minus wings and other appendages) was included. On the basis of OD readings, extracted DNA samples were adjusted to a concentration of 100–250 ng/μl and stored at 4°C.

PCR Amplification. A 536-bp fragment of the 16S ribosomal subunit from the mitochondrial genome was amplified by the PCR using primers described by Cameron *et al.* (21), developed from sequences of *A. mellifera*. The 16S rRNA gene was chosen for this study because it is known to contain regions of conserved nucleotides (25), appropriate for examining higher level relationships. PCR was done as described (26) with several modifications. The 50-μl PCR mixture contained from 5–100 ng of genomic DNA/50 mM KCl/10 mM Tris·HCl, pH 8.4/2.5 mM $MgCl_2$/200 μM each dATP, dTTP, dGTP, and dCTP/10 pmol (0.2 μM) of each oligonucleotide primer/1 unit of *Taq* DNA polymerase (Cetus). Each of 35 cycles entailed denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 70°C for 2 min and 30 sec (with an additional 3-sec extension per cycle). PCR products were visualized by agarose gel electrophoresis and precipitated overnight with 2 vol of ethanol and 1/10 vol of 2 M NaCl.

Purification of Amplified DNA. The amplified mtDNA samples were purified by 3.5% PAGE (20–25 cm vertical gel plates with 1.6-mm spacers and combs) run at 300–400 V for ≈3 hr. The gel was stained with ethidium bromide (10 mg/ml) for 10 min and rinsed in distilled water. The mtDNA bands were visualized on a long-wave UV transilluminator, and the

Table 1. Sequence identity values

	Am	Ad	Ac	Af	Ak	Mc	Tp	Th	Sl	Bp	Ba	Ps	Eu	Ef	Xy	Ex
Am		.861	.863	.861	.848	.747	.758	.752	.728	.740	.744	.752	.769	.779	.741	.749
Ad	398		.848	.855	.848	.784	.762	.769	.751	.722	.742	.773	.788	.783	.738	.764
Ac	403	392		.859	.904	.745	.764	.762	.743	.753	.690	.765	.759	.767	.754	.742
Af	402	395	403		.859	.778	.775	.789	.747	.753	.768	.769	.774	.797	.771	.778
Ak	396	392	425	403		.773	.786	.782	.786	.783	.761	.791	.791	.808	.775	.766
Mc	343	360	342	357	355		.886	.895	.886	.826	.810	.830	.778	.782	.762	.799
Tp	347	349	350	355	360	406		.915	.893	.819	.795	.810	.788	.797	.762	.792
Th	345	353	350	362	359	411	419		.884	.810	.795	.802	.782	.771	.758	.788
Sl	337	347	344	346	364	407	409	406		.820	.798	.823	.777	.786	.758	.804
Bp	341	333	347	347	361	379	375	372	378		.846	.887	.790	.804	.765	.802
Ba	343	342	318	354	351	372	364	365	368	390		.847	.792	.820	.766	.802
Ps	345	355	351	353	363	381	371	368	378	407	389		.786	.797	.769	.802
Eu	359	364	357	363	372	357	361	359	360	364	365	361		.882	.788	.795
Ef	364	362	359	373	378	359	365	354	364	371	378	366	413		.771	.790
Xy	343	341	349	357	359	350	349	348	351	353	353	353	365	357		.790
Ex	314	320	311	326	321	335	332	330	337	336	336	336	333	331	331	

Numbers of identical sites for all pairs of aligned sequences are given in the lower triangle. Percentages of sites identical between the paired sequences are given in the upper triangle. See legend for Fig. 2 for two-taxon name code. Boxed areas indicate principal comparisons among the social tribes.

appropriate fragments were cut out with sterile blades. The mtDNA was electroeluted as described (24).

Double-Stranded DNA Sequencing. Amplified double-stranded mtDNA was sequenced directly by dideoxynucleotide chain-termination (27), with the same primers used in the above PCR amplifications. A Sequenase polymerase sequencing kit (United States Biochemical) was used with the following modifications. To 100–500 ng of mtDNA dissolved in 6 μl of distilled water were added 2 μl of 5× Sequenase reaction buffer (United States Biochemical) and 2 μl of the appropriate primer (2 ng/μl). The mixture was heated to 90–95°C for 3 min to denature the DNA and plunged into ice (annealing of the primer occurs rapidly during cooling). After several minutes, the following was added to each DNA mixture: 2 μl of dGTP mix (United States Biochemical, 1:20 dilution), 1 μl of 0.1 M dithiothreitol, 1 μl of [α-^{32}P]ATP (3000 Ci/mM; 1 Ci = 37 GBq), and 2 μl of Sequenase polymerase (1:8 dilution in enzyme dilution buffer). Aliquots of 3.5 μl of the reaction mixture were added to each of four tubes containing 2.5 μl of the appropriate dideoxyribonucleotide chain-termination solution (ddTTP, ddCTP, ddATP, ddGTP) and incubated at 45°C for 10 min. After incubation, 4 μl of formamide dye was added to the reactions, which were then heated to >90°C for 3 min and cooled on ice. Sequencing reactions were electrophoresed in 5–6% polyacrylamide gels (0.4 mm thick) and visualized by autoradiography. The sequences for each taxon were compared, and the homologous regions were aligned by hand and checked by computer alignment (28).

Sequence Analysis. Aligned sequences for the 16 taxa under consideration were analyzed by using the optimality criterion of maximum parsimony (29). Only informative nucleotide positions were used as characters in the analyses. A character is considered informative when it exhibits at least two-nucleotide states, each shared by two or more taxa. Gaps were treated as a fifth character in the results reported below; alternative analyses excluding variable-length regions did not contradict the results reported here. Transitions and transversions were treated both with equal and differential weight. Differential weights of 1.1, 1.2, and 1.3 were applied to transversions (30) using a step matrix implemented in PAUP. Parsimony analyses were performed on a MacIntosh Quadra 700 computer, using the Branch and Bound option implemented in PAUP, version 3.0s (31), which guarantees finding all most parsimonious trees. Maximum-likelihood analysis (32) implemented in PHYLIP, version 3.41 (33), and bootstrap analysis of particular clades (34), implemented in PAUP (10,000 replicates), were applied as heuristic methods to test for the reliability of the results on the basis of maximum parsimony. PAUP was also used to obtain strict consensus trees for use in computing a decay index (35) to evaluate the relative reliability of particular clades and to generate a tree-length distribution of 100,000 trees drawn at random from the set of all possible trees (random-trees option). The coefficient of skewness of this distribution (g_1 statistic) was estimated as a measure of phylogenetic signal in the 16S sequences (36).

RESULTS

Characterization of 16S Sequences. Aligned sequences of the 16S rRNA fragment can be obtained from the GenBank data base or directly from the author. Of the possible 536 bp comprising the entire amplified fragment, sequences were obtained for 418–472 bp for all 16 taxa. Considerable length polyporphism was apparent among the taxa, the result of insertions/deletions in several A+T-rich hypervariable regions. A significant overall bias in adenines and thymines (80%) was also apparent for the entire fragment. A result of the strong A+T bias was a correspondingly large number of symmetrical A+T transversion-substitutions (61.1% of all substitutions). Nonetheless, there were more than twice as many transition substitutions as would be expected by chance under these base frequencies. Percentages of sequence identity between all pairs of sequences are given in Table 1 for the aligned sites.

Phylogeny Reconstruction. Omission of all invariant and autapomorphic sites left 171 informative characters. Parsimony analysis of the 171 informative sites resulted in two equally parsimonious tree topologies (Fig. 2 *A* and *B*), differing only by the placement of *Melipona* (*M. compressipes*) and *Scaptotrigona* (*S. luteipennis*) within the tribe Meliponini. The results are consistent with monophyly of the currently recognized tribes. In both trees, Meliponini is most

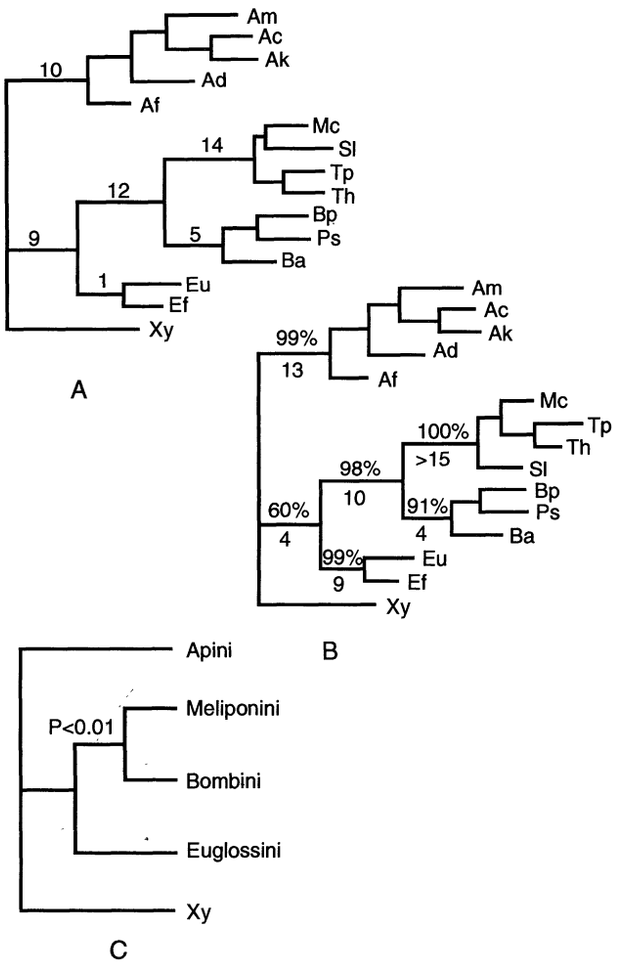


FIG. 2. The two maximum-parsimony trees (*A* and *B*), rooted by the outgroup (*X. virginica*) at an internal node with basal polytomy (PAUP option). (*C*) Simplified tree representing only the tribal topology. Am, *A. mellifera*; Ac, *A. cerana*; Ad, *A. dorsata*; Af, *A. florea*; Ak, *A. koschevnikovi*; Bp, *B. pennsylvanicus*; Ba, *B. avinoviellus*; Ps, *P. variabilus*; Mc, *M. compressipes*; Sl, *S. luteipennis*; Th, *T. hypogea*; Tp, *T. pallens*; Eu, *El. polychroma*; Ef, *Ef. caerulescens*; Xy, *X. virginica*; Ex, *Ex. bicolor*. Tree length for 171 informative sites with 15 taxa = 502 steps; consistency index = 0.536; retention index = 0.612. Numbers associated with the internal branches of tree *A* indicate the number of unambiguous nucleotide changes supporting the branch; percentages above branches (*B*) indicate percentage of times the branch was recovered in 10,000 bootstrap replicates; numbers below branches indicate the number of additional steps required to collapse a given clade (35) in both of the maximum-parsimony trees. A global maximum-likelihood analysis (33) for the same 15 taxa resulted in the same tribal topology (*C*), with a high level of confidence for the Meliponini plus Bombini clade ($P < 0.01$).

closely related to Bombini, and Euglossini is the sister group to this clade. The tribe Apini is inferred to be the sister group to the lineage containing Meliponini plus Bombini plus Euglossini. A bootstrap consensus tree (34) estimated by heuristic search from 10,000 replications resulted in >98% support for the Bombini plus Meliponini clade. Several other statistical analyses support the Bombini plus Meliponini clade (Fig. 2, Table 2). The simplified tribal tree topology (Fig. 2*C*) represents a significantly more parsimonious arrangement for the mtDNA data than each of those in Fig. 1 (Table 2). Furthermore, all trees ($n = 28$) from one to three steps longer than the most parsimonious trees have the same tribal topology as that of Fig. 2. There is strong phylogenetic signal in the 16S sequence data, as evident by the highly significantly skewed distribution of 100,000 randomly generated trees ($g_1 = -0.626$; $P < 0.01$) (36).

DISCUSSION

These molecular results are discordant with previous hypotheses of apid relationships based on morphology (Fig. 1). In particular, no morphological analysis has ever indicated that bumble bees and stingless bees form a monophyletic group. It is unclear at present why the mtDNA data conflict strongly with hypotheses derived from morphological data, although none of those hypotheses enjoys a strong consensus. A major problem for the morphological approach has been the inability to find sufficient synapomorphies (shared derived characters) among the apid tribes to strongly support any of the tribal relationships (11). This problem may stem from the ancient age of these tribes (39, 40), which has allowed the evolution of a large number of tribal autapomorphies to obscure shared features. In contrast, the molecular phylogeny presented here is corroborated by an independent (although preliminary) investigation (41) of sequences from the large subunit nuclear rRNA, representing data from an unlinked genome, resulting in the same tribal relationships as those shown in Fig. 2.

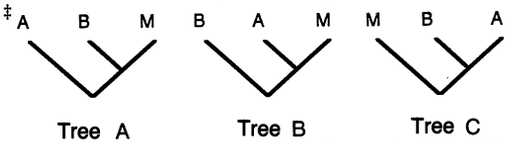
Possible sources of error in these analyses are here considered: (*i*) the occurrence of large insertions and deletions in the hypervariable regions, resulting in uncertain alignment, could have caused sufficient noise in the data to allow a misleading result; (*ii*) a high frequency of multiple substitutions at informative sites, hence loss of homology, can give a misleading picture of relationships (42); (*iii*) secondary structure, known to impose selective constraints on nucleotide substitutions (42), could lead to constraints on nucleotide substitutions in the 16S rRNA molecule, resulting in a lack of independence among different informative sites (but see ref. 43); (*iv*) sorting of ancestral polymorphism among lineages leading to a lack of congruence between gene trees and species trees (44); and (*v*) choice of outgroups (45). The first and second potential sources of error were addressed by excluding from a second analysis all hypervariable regions (likely to be high in multiple substitutions) that were difficult to align; this is a form of character weighting. This procedure resulted in the same tribal topology as that in Fig. 2. The third source of error (and the first) can be examined by considering other nucleotide sequences from single-copy nuclear genes to determine the degree of phylogenetic congruence among different genes and by examining the secondary structure of apid 16S rDNA. With respect to the fourth source of error, the times between sequential divergences among the extremely old apid tribes [meliponines may be 80 million years old (39)] are unlikely to be short enough for ancestral polymorphisms to significantly affect these phylogenetic results. Regarding the choice of outgroup, this investigation included another tribal exemplar from the Xylocopinae (Allodapini: *Exoneura*) as an additional outgroup in a separate analysis, resulting in three maximum parsimony trees, each with the same tribal topology as that depicted in Fig. 2, except that Euglossini formed a clade with Apini; the Bombini plus Meliponini relationship was retained. In light of new morphological information (C. D. Michener, personal communication) suggesting that Xylocopinae is no longer the closest possible outgroup, future work should include additional analyses of other outgroup taxa from the Anthophoridae.

Table 2. Results of Wilcoxon nonparametric paired-comparisons tests of alternative topologies (37) depicted in Figs. 1 and 2 and Wilcoxon (37) and Cavender (38) tests of alternative three-taxon topologies, inferred from parsimony analysis (31)

Maximum parsimony mtDNA tree vs. alternatives (Fig. 1 *A–D*)	Wilcoxon*† Tree 2A, *P*	Tree length 501	Cavender,† *P*
Tree 1*A*	≤0.01*	518	
Tree 1*B*	≤0.01*	522	
Tree 1*C*	<0.01*	518	
Tree 1*D*	<0.006*	520	
Three-taxon topologies‡			
Tree *A* vs. *B*	<0.01†		<0.05
Tree *A* vs. *C*	<0.01†		<0.05
Tree *B* vs. *C*	>0.05†		>0.05

*The Wilcoxon analysis tests the hypothesis that for the mtDNA data the maximum parsimony tree in Fig. 2*C* is shorter, with fewer substitutions, than each of the alternatives in Fig. 1 *A–D*. In all comparisons, the tribal topology in Fig. 2*C* is significantly shorter than the alternatives.

†In the three-taxon topologies, both Wilcoxon and Cavender tests were used to test the inference that Bombini and Meliponini form a sister group (topology *A*) relative to Apini. Both tests significantly support a Bombini plus Meliponini clade. The Wilcoxon test is based on Templeton's criteria for nucleotide sequence data (37). Cavender's test is based on Felsenstein's modification (38); it is limited to tests of three taxa plus an outgroup.

‡ 

Branches of the trees are as follows: A, Apini; B, Bombini; and M, Meliponini; the outgroup is *Xylocopa* (not shown).

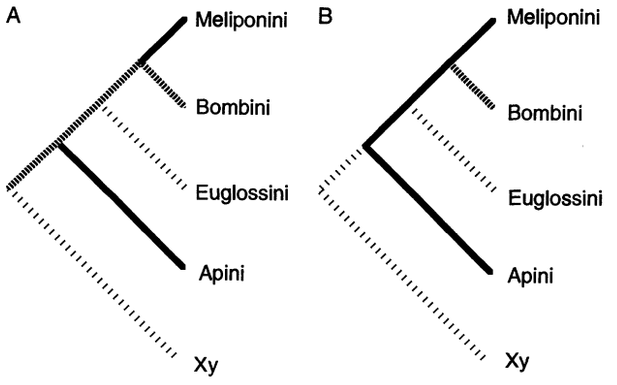


FIG. 3. The maximum-parsimony mtDNA tribal topology, indicating alternative hypothetical ancestral character states for sociality. (*A*) Topology assumes that highly eusocial behavior is derived from primitively eusocial behavior. (*B*) Topology does not assume that highly eusocial behavior is derived. On the basis of these two contrasting assumptions about ancestral character states, topology (*A*) requires four changes (gains or losses of sociality) from the solitary/parasocial ancestor (Xylocopinae); topology (*B*) requires three changes. See legend of Fig. 2 for two-letter taxon names. Social states are indicated by patterns on branches: ▨, solitary; ▩, primitively eusocial; □, highly eusocial.

If further corroborated, the apid relationships reported here will have a profound influence on our interpretation of advanced eusocial evolution in the bees. If advanced eusocial behavior is derived from less complex primitively eusocial behavior, as has often been assumed (13), it arose twice independently (Fig. 3*A*). Alternatively, if one relaxes the assumption that advanced eusociality evolved from primitive eusociality and allows a reversal of eusociality, a more parsimonious interpretation (Fig. 3*B*) is that advanced eusocial behavior arose only once and was lost entirely in Euglossini or modified into primitive eusociality in Bombini. Such a reversal of eusociality to less strongly social forms is generally considered unlikely (13). Support for either of these two contrasting interpretations may ultimately require an assessment of potentially homologous traits shared between bumble bees and stingless bees. For example, striking similarities in two traits, design of nest architecture (46) and the recycling of wax from pupal cocoons (10), have been reported but never directly compared between these two groups. In addition, the assumption that advanced eusociality is an irreversible stage in social evolution requires rigorous investigation of the plasticity of social behavior in the Hymenoptera.

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