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MOLECULAR EVIDENCE FOR THE ORIGIN OF WORKERLESS SOCIAL PARASITES IN THE ANT GENUS *POGONOMYRMEX*

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Abstract.—Speciation of two social parasites from their respective hosts is tested using a molecular phylogeny. Alignment of 711 DNA base pairs of mitochondrial cytochrome *b* gene was used to assess phylogenetic relationships of inquiline species to their hosts and to other members of the genus. We show that the inquiline social parasites of the North American seed harvester ants are monophyletic, descending from one of the known hosts (*Pogonomyrmex barbatus*) in the recent past and shifting hosts in a pattern similar to that observed in other Hymenopteran social parasites. In addition, the host populations unexpectedly were found to be polyphyletic. Populations of *Pogonomyrmex rugosus* from an area east of the Chiricahua Mountains in Southern Arizona belong to a mitochondrial clade separate from the more western clade of *P. rugosus* from the Sonoran and Chihuahuan Deserts. Evidence of mitochondrial DNA introgression between *P. rugosus* and *P. barbatus* was also observed. We conclude that Emery's rule does not strictly hold for this system, but that the hosts and parasites are very closely related, supporting a loose definition of Emery's rule.

Key words.—Cytochrome *b*, Emery's rule, phylogeny, *Pogonomyrmex*, social parasitism, speciation.

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An underappreciated phenomenon in hymenopteran social insects (ants, social bees, and social wasps) is the existence of taxa that have evolved to exploit the social living conditions of closely related species. Hymenopteran social parasites live in, and depend upon, the host nest of another social insect species. These relationships span a continuum from nest sharing to host queen execution with enslavement of host workers to the ultimate level of workerless parasitism, where the parasites coexist with the host queen while losing the ability to make workers themselves (Wilson 1971; Buschinger 1986). These parasitic species (termed inquilines) are rare, making them difficult to study and relatively unknown outside of the social insect field. However, the phenomenon has evolved more than 100 times in ants, bees, and wasps, revealing an inherent vulnerability of insect societies to exploitation (Hölldobler and Wilson 1990). It has long been noticed that host and parasite pairs share morphological similarity suggesting that they are closely related (Wheeler 1919). This hypothesized ancestral host-parasite relationship is known as Emery's Rule (Emery 1909). Carpenter et al. (1993) highlighted the two important questions raised by Emery's observation that help to understand the significance of the rule and how it might be tested: (1) whether the host and parasite have an immediate common ancestor; and (2) whether ecological and host deception requirements restrict social parasites to related hosts.

Underlying the first question is the larger question of whether parasites arise from their host through sympatric speciation. Focusing on the predicted pattern and putting aside the question of reproductive isolation (for possible scenarios see Buschinger 1986, 1990; West-Eberhard 1986; Bourke and Franks 1991; Nonacs and Tobin 1992), the sympatric speciation hypothesis has been tested against two types of possible relationships: the strict and loose definition of Emery's rule (Wilson 1971; Buschinger 1990; Ward 1996). In the strict case each parasite is a sister taxon to its host (Fig. 1A), and in the most recent version of the loose case, the most closely related free living clade should contain all of the hosts (Fig. 1B; Ward 1996). This loose case allows for diversification of hosts and parasites. The problem is that the pattern shown in Figure 1A would not rule out a free-living sister taxon evolving into a parasite on its sister taxon (Wilson 1971). On the other hand, with enough time, host shifting and extinction events could result in relationships outside of those presented in Figure 1, even with sympatric speciation of social parasites (Buschinger 1990). Thus, the question of sympatric speciation can not be definitively addressed by simply examining extant patterns of relationships among highly evolved social parasites and their hosts without assuming: (1) no speciation events; (2) no extinction events; and (3) no host shifting. Nevertheless, a compelling case for the possibility is beginning to emerge as more intermediate cases of social parasitism are discovered (e.g., Schultz et al. 1998) and added to those reviewed by Buschinger (1990). Indeed, only the youngest parasitic lineages would seem to offer any hope for determining the relationship of initial host to initial parasite.

The second question of ecological requirements and the need to circumvent nest-mate recognition is more approachable. This hypothesis predicts that the more restricted social parasites are to their relatives, the more closely parasite lineages should evolve in parallel with their hosts. This restriction makes possible other relationships outside of the strict and loose framework, where a social parasite could radiate across a genus and across related hosts (Fig. 1C). If this restriction is not important, then we should find social parasites distributed across diverse species of ants as a function of geographic distance. Whether the relationships shown in Figure 1C are interpreted as technically satisfying a loose definition of Emery's rule (they were not by Ward [1996]), this pattern is still consistent with the tendency of close host-parasite relationships underlying the original observation.

However, the generality of Emery's rule has been called into question, as there appears to be a continuum of cases.

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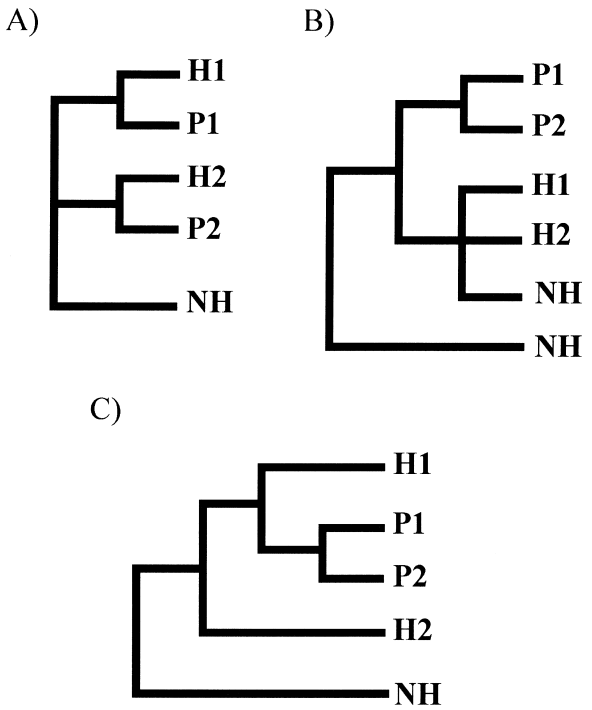

FIG. 1. Three different phylogenetic patterns predicted by various definitions of Emery's rule: (A) the strict definition that host and parasite are sister taxa; (B) one loose definition that the nearest nonparasitic outgroup to the social parasite clade contains the hosts; and (C) the most general, loose version that social parasites tend to parasitize closely related taxa. H and P indicate a host species or parasite species, respectively, and NH designates nonhosts.

Recent studies have found likely strict cases in ants (reviewed in Bourke and Franks 1991; Schultz et al. 1998), a parallel loose case similar to Figure 1B in the bees (Lowe and Crozier 1997), various ambiguous and complex patterns in wasps and ants (Carpenter et al. 1993; Baur et al. 1995, 1996; Sanetra and Buschinger 2000), as well as several cases contradicting the rule in ants (Agosti 1994; Ward 1989, 1996; Heinze 1998). Clearly, different processes appear to be at work in different cases (Agosti 1994), raising the question of exactly how relatedness and ecological factors, taken together or separately, interact in the evolution of social parasitism.

Several more practical difficulties arise when attempting to test Emery's rule based on morphological analysis in social insects. Many parasitic species have lost their own worker caste and rely entirely on host workers. However, much of social insect taxonomy, especially in ants, is based on worker characters. This requires parasitic taxa to be treated with a post facto method using different characters than for the rest of the analysis (Taber 1990). A second problem is morphological convergence among social parasites and among host-parasite pairs. Similarities arising from the parasitic lifestyle can lead to morphological convergence of social parasites, most notable a reduction in queen size (Nonacs and Tobin 1992; Aron et al. 1999). At the same time, convergence of parasite and host can occur after the relationship is established because the parasite and host share the same nest and ecological environment, biasing an analysis toward confirming Emery's rule. A good example of this can be found in bumble bees, where selection for Mullerian mimicry better explains the convergence of host and parasite coloration than common ancestry (Plowright and Owen 1980). Of course, this particular bias strengthens the conclusions of those morphological studies that falsify the rule. Modern techniques that measure presumably neutral genetic characters such as DNA sequence are especially suited for helping to resolve these species relationships. Indeed, the results of most molecular studies thus far have contradicted the strict interpretation of Emery's rule while supporting various degrees of the loose interpretations (Carpenter et al. 1993; Choudary et al. 1994; Baur et al. 1995, 1996; Lowe and Crozier 1997).

This study examines two questions related to Emery's rule and the evolution of the two social parasites in the North American seed harvester ant genus *Pogonomyrmex*. Does social parasitism in *Pogonomyrmex* have one or multiple origins? How closely related are the two parasites to their hosts? We addressed these questions by analyzing a sequence of the mitochondrial cytochrome *b* gene. This gene has been well studied (Crozier and Crozier 1993; Jermiin and Crozier 1994; Simon et al. 1994; Crozier et al. 1995) and used to address similar questions in the past (Carpenter et al. 1993; Choudary et al. 1994; Baur et al. 1995, 1996; Lowe and Crozier 1997).

MATERIALS AND METHODS

Social Parasites in the Ant Genus Pogonomyrmex

The Myrmicine ant genus *Pogonomyrmex* is limited to the New World and consists of 60 species in both North America (32 species) and South America (28 species; Taber 1998). Before the subgenus *Ephebomyrmex* was synonymized with *Pogonomyrmex* (Bolton 1995), the North American taxa were divided into three monophyletic species complexes (*P. californicus* complex, *P. occidentalis* complex, and *P. barbatus* complex; Cole 1968). These are in turn monophyletic with respect to the rest of *Pogonomyrmex* and the former *Ephebomyrmex*.

Pogonomyrmex anergismus and *P. colei* are workerless social parasites that live within a host nest and produce only sexual offspring. Based on morphology, these two species of social parasites and their hosts belong to the *P. barbatus* complex. *Pogonomyrmex barbatus* and *P. rugosus* are hosts for *P. anergismus*, whereas *P. rugosus* is the only known host for *P. colei* (Cole 1968; Snelling 1981; Rissing 1983; MacKay and Van Vactor 1985; Johnson 1994; Johnson et al. 1996). Taber (1990) has described the social parasites *P. anergismus* and *P. colei* as sister species arising from a common ancestor that was also the ancestor of both hosts. However, in the description of *P. colei*, Snelling (1981) indicated that it was unlikely that either species of social parasite is derived from the other. Snelling (1981) also stated that it was unlikely for the two social parasites evolved from their respective hosts, but that hosts and social parasites might share a common ancestor. More recently, Tabor revised his view, suggesting that the social parasites are sister taxa arising from *P. barbatus* (Taber 1998).

Colonies of the two social parasites are perennial, and host queens survive parasitism. Mating is followed by dispersal flights during 2–3 days following summer and fall rains in both social parasites. Parasitic females mate with nestmates (males do not fly), then fly from the nest to locate a potential host colony by following foraging trails, probably by respond-

ing to recruitment pheromones (Johnson et al. 1996). Based on current records, the two social parasites have a parapatric distribution pattern; *P. colei* lives in central Arizona, southern Nevada, and southeastern California, whereas *P. anergismus* lives to the east in southwestern New Mexico and northern Texas (Johnson 1994; Johnson et al. 1996). Throughout their ranges, these social parasites are extremely rare, with about 1% of potential colonies infected in host populations where social parasites exist (Johnson 1994; Johnson et al. 1996).

Field Collections

We collected 17 species of *Pogonomyrmex* from 56 populations across the south-central and western United States and northwestern Mexico. The species included in this study were based on availability, subject to the constraint that samples include representatives of the former subgenus *Ephebomyrmex* (two species) and all three species complexes in the North American taxon *Pogonomyrmex* (*P. barbatus* complex, eight species; *P. occidentalis* complex, three species; *P. californicus* complex, four species; Table 1, Fig. 2). Extensive sampling of species across the North American taxa was combined with intensive sampling of the two host species across their geographic ranges. The outgroups were necessary to address the hypothesis that the social parasites evolved from a different species complex, but are morphologically convergent with their current host species. Because of their rarity, collections for the two species of social parasites were limited to multiple colonies at one site for each. For all other species, we collected numerous workers and reproductive males and females (if available) from one to three colonies per site. All ants were collected live and placed in isopropanol or frozen at −70°C.

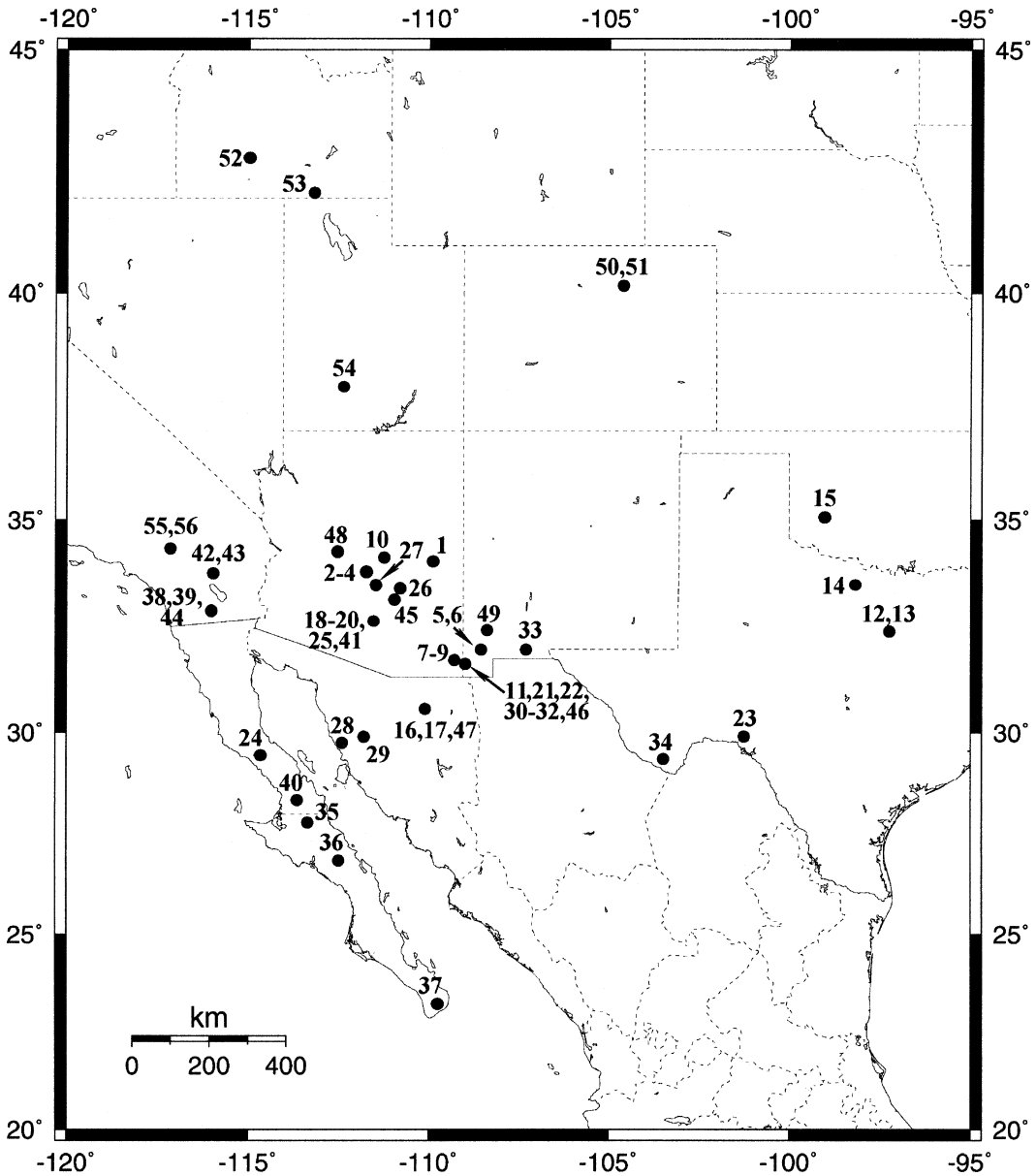

FIG. 2. Location of populations used to study the molecular phylogeny of social parasites in the ant genus *Pogonomyrmex*. Collection information is given in Table 1.

TABLE 1. Collection data for specimens of the ant genus *Pogonomyrmex* that were used in this study. All locales are in the United States, except as noted. All locales are mapped in Figure 2. For collector: SPC, Stefan P. Cover; RAJ, Robert A. Johnson; JDP, Joel D. Parker; SWR, Steven W. Rissing; GCS, Gordon C. Snelling. Voucher specimens are deposited at the Los Angeles County Museum of Natural History and the Robert A. Johnson collection, Tempe, Arizona.

Taxon and locality	Latitude	Longitude	Elevation (m)	Collector and accession number	Genbank accession number
Former *Ephebomyrmex*					
P. huachucanus Wheeler					
1. AZ: Apache Co., 2.4 km N Vernon	34°18′N	109°41′W	1760	RAJ AZ1686	AF202472
2. AZ: Maricopa Co., Mazatzal Mtns., 0.6 km S Pigeon Springs	33°37′N	111°15′W	1720	RAJ 300	AF202473
P. imberbiculus Wheeler					
3, 4. AZ: Maricopa Co., Mazatzal Mtns., at 17.3 km E Hwy 87	33°44′N	111°25′W	1245	JDP 300, 301	AF202474-5
P. barbatus complex					
P. anergismus Cole					
5, 6. NM: Grant Co., 6.1 km E Separ	32°10′N	108°22′W	1375	RAJ NM19, NM1546	AF202476-7
P. apache Wheeler					
7–9. AZ: Cochise Co., 3 km NW Portal	31°55′N	109°14′W	1640	JDP 106a, 106b, 106c	AF202480-2
P. barbatus (F. Smith)					
10. AZ: Yavapai Co., 1.6 km E Cornville	34°43′N	111°54′W	1040	RAJ AZ744	AF202483
11. NM: Hildago Co., 3 km NW Rodeo	31°52′N	109°02′W	1250	RAJ NM1933	AF202484
12. TX: Tarrant Co., Hwy 287 at 2 km S Alvord	32°58′N	97°25′W	265	JDP 233	AF202486
13. TX: Tarrant Co., Hwy 287 at 4 km S Alvord	32°57′N	97°23′W	255	JDP 231	AF202487
14. TX: Witchita Co., Hwy 44N at 5.5 km S Burkbenett	34°02′N	98°33′W	320	JDP 234	AF202486
15. OK: Beckham Co., Hwy 40 at 6 km W Sayre	35°15′N	99°45′W	590	JDP 237	AF202488
P. bicolor Cole					
16, 17. Mexico: Sonora, 31.0 km S Santa Ana at 8 km E Hwy 5	30°15′N	111°02′W	825	RAJ SON1025, SON1027	AF202489, AF202490
P. colei Snelling					
18–20. AZ: Pinal Co., 8 km NE Casa Grande	32°56′N	111°42′W	430	JDP 191, 303; RAJ AZ1001	AF202493–AF202495
P. desertorum Wheeler					
21, 22. NM: Hildago Co., 1.6 km S Jct. Hwys 9 and 80	31°54′N	109°02′W	1260	JDP 192, 193	AF202496, AF202497
23. TX: Terrel Co., Hwy 90 at 11 km W Dryden	30°00′N	101°59′W	640	JDP 222	AF202498
P. rugosus Emery					
24. Mexico: Baja California, Laguna Chapala	29°25′N	114°21′W	645	RAJ BC96-14A	AF202509
25. AZ: Pinal Co., 8 km NE Casa Grande	32°56′N	111°42′W	430	JDP 170	AF202510
26. AZ: Pinal Co., Kearny	33°03′N	110°55′W	560	RAJ AZ750	AF202511
27. AZ: Maricopa Co., 4.8 km W McDowell Peak	33°40′N	111°51′W	510	JDP 167	AF202512
28. Mexico: Sonora, Sierra Cirio	29°49′N	112°38′W	10	RAJ SON95-10	AF202513
29. Mexico: Sonora, Hwy 15 at 31 km S Santa Ana	30°16′N	111°06′W	660	RAJ SON1019	AF202514
30–32. NM: Hildago Co., Jct. Hwys 9 and 80	31°56′N	109°02′W	1260	JDP 189, 199-25, 199-43	AF202515–AF202517
33. NM: Luna Co., 1–10 at 15 km W Las Cruces	32°16′N	107°01′W	1350	JDP 206	AF202518
34. TX: Brewster Co., N entrance Big Bend Nat'l. Park	29°18′N	103°31′W	750	JDP 213	AF202519
P. tenuispina Forel					
35. Mexico: Baja California, 12.3 km N Rancho El Progresso	28°23′N	113°07′W	440	RAJ BC96-11	AF202525
36. Mexico: Baja California Sur, Hwy 1 at 20.0 km N Ejido Alfredo Bonfil	27°30′N	112°49′W	285	RAJ BC96-32	AF202526
37. Mexico: Baja California Sur, Sierra la Laguna	23°36′N	109°51′W	890	RAJ BC96-29B	AF202527
P. californicus complex					
P. anzensis Cole					
38, 39. CA: San Diego Co., Anza Borrego Desert State Park, Split Mtn	33°01′N	116°07′W	260	SPC 4807, 4808	AF202478, AF202479
P. californicus (Buckley)					
40. Mexico: Baja California, 8 km S Bahía de los Angeles	28°53′N	113°32′W	5	RAJ BC96-12	AF202491
41. AZ: Pinal Co., 8 km NE Casa Grande	32°56′N	111°42′W	430	SWR AZ 868	AF202492
P. magnacanthus Cole					
42, 43. CA: Riverside Co., Palm Desert	33°48′N	116°25′W	75	RAJ CAL1005, CAL1006	AF202499, AF202500
44. CA: San Diego Co., Anza Borrego Desert State Park, 8 km S Split Mtn	32°59′N	116°09′W	260	GS 98-052	AF202504
P. maricopa Wheeler					

TABLE 1. Continued.

Taxon and locality	Latitude	Longitude	Elevation (m)	Collector and accession number	Genbank accession number
45. AZ: Pinal Co., 4.8 km N Superior	33°19′N	111°07′W	940	RAJ AZ902	AF202501
46. NM: Hildago Co., Jct. Hwys 9 and 80	31°54′N	109°03′W	1260	JDP 107	AF202502
47. Mexico: Sonora, Hwy 15 at 31.0 km S Santa Ana	30°16′N	111°06′W	660	RAJ SON1022	AF202503
P. occidentalis complex					
P. occidentalis (Cresson)					
48. AZ: Yavapai Co., Chino Valley	34°45′N	112°27′W	1450	JDP 185	AF202505
49. NM: Hildago Co., Hwy 90 at 32 km N Lordsburg	32°30′N	108°35′W	1635	JDP 271	AF202506
50, 51. CO: Boulder Co., 0.8 km E Boulder	40°01′N	105°11′W	1630	JDP 195a, 195b	AF302507, AF202508
P. salinus Olsen					
52. ID: Elmore Co., Mountain Home	43°08′N	115°30′W	995	JDP 196	AF202520
53. ID: Oneida Co., I-89 at 15 km N state border	42°03′N	112°55′W	1430	JDP 197	AF202521
54. UT: Millard Co., Dog Valley Pass	38°38′N	112°37′W	1880	JDP 198	AF202522
P. subnitidus Emery					
55. CA: Los Angeles Co., San Gabriel Mtns., 3.2 km S Charlton Flat	34°16′N	118°01′W	1530	RAJ CAL1043	AF202523
56. CA: Los Angeles Co., San Gabriel Mtns., 1.6 km NW Oakwilde	34°16′N	118°12′W	910	RAJ CAL1044	AF202524

TABLE 2. Primer sequences used for polymerase chain reaction and sequencing of the mitochondrial DNA gene cytochrome *b*. Site number is the first binding site according to the honeybee (*Apis mellifera*) genome (Crozier and Crozier 1993). Primers followed by an asterisk are from Jermiin and Crozier (1994); others were designed for this study. L and H refer to the light and heavy strands, respectively. These primers worked for all of the taxa in this study.

Site number	Name and direction	Sequence
11400	CB1, L	5′-TATGTACTACCATGAGGACAAATATC-3′*
11661	CBm-1, L	5′-AATTCCATTCCATGTATATTTTAC-3′
11685	CBm-2, H	5′-GTAAAATATACATGGAATGGAATT-3′
11884	CB2, H	5′-ATTACACCTCCTAATTTATTAGGAAT-3′*
11910	CB2b, H	5′-ACAGTAAAGAATAATAAGATGA-3′
12096	ATR, H	5′-TGAATTAAAGGGTTAATAATGAA-3′
12251	tR^s, H	5′-TATTTCTTTATTATGTTTTCAAAAC-3′*

DNA Extraction and Sequencing

DNA was extracted using a method designed for minute wasps (Landry et al. 1993). Both sexes and workers were used for extractions depending on availability. In the case of alate females and workers, the gasters were removed to minimize contamination by endoparasites (Jermiin and Crozier 1994). Male gasters were not removed to take advantage of the high mitochondrial content of sperm. DNA was extracted from samples stored in isopropanol or at −70°C using the same method, except that those stored in isopropanol were first dried at 55°C in open tubes. Individuals were placed on dry ice, crushed, and 160 μl lytic buffer (200 mM Tris-HCl, pH 8.0, 70 mM EDTA, 2 M NaCl, 20 mM sodium metabisulfite) was added during grinding. An additional 40 μl of 5% sodium sarcosyl was added with mixing and the tubes were incubated at 55°C for 2 h with occasional mixing. Debris was removed by centrifuging samples for 20 min at 14,000 rpm. The supernatant was transferred to a tube containing 200 μl isopropanol and 90 μl of 10 M ammonium acetate, mixed, and then placed at −20°C for more than 2 h. DNA was pelleted, washed several times with 70% ethanol, dried at room temperature, and resuspended in 40 μl filter sterilized GC-HPLC grade water (Fisher Scientific, Pittsburgh, PA).

Sequencing templates were obtained for the 3′ end of the cytochrome *b* gene using the primers described in Table 2. Standard reaction conditions were 1/10 to 1/100 volume of the DNA preparation (1–5 μl), 0.5 μM final primer concentration, 1/10 final volume 10× buffer (Gibco-BRL, now Invitrogen, Carlsbad, CA), 3 mM Mg_2Cl final concentration, and 2.5 units TaQ DNA polymerase per 100-μl reaction volume. Sequencing templates were amplified using a Perkin-Elmer (now Applied Biosystems, Foster City, CA) 480 thermocycler with a preheating cycle of 2 min at 92°C, 30 cycles of 1 min denaturation at 92°C, 1 min annealing at 42–57°C (temperature empirically determined for each species and primer set), and 1–2.5 min extension at 72°C, with a final extension time of 7 min. The longer extension times were used for longer sequences. Polymerase chain reaction (PCR) products were purified with Millipore (Bedford, MA) spin columns using the manufacturer's specifications. The final product was resuspended in 12 μl PCR water with yields of approximately 1 μg. Both DNA strands were sequenced on an ABI 377 Automated DNA Sequencer (Applied Biosystems, Foster City,

CA) at the core sequencing facility at Arizona State University. All sequences were verified using at least two individuals from each collection site.

Analysis of DNA Sequences

Fifty-six sequences were translated (MacDNAsis, Hitachi Software Engineering, Yokohama, Japan) and aligned using ClustalW (Thompson et al. 1994). Sequences were truncated to a standard length of the shortest sequence (711 bp) to avoid any alignment ambiguities. Transition:transversion ratios, base composition, and codon usage were calculated using MEGA (Kumar et al. 1993). Consistency of base composition among taxa was tested with PAUP* 4.0b4a (Swofford 1998).

The two questions, whether the social parasites are monophyletic and how they are related to their hosts, were initially assessed by constructing phylogenetic trees using both maximum-parsimony and distance methods. Using the 46 unique haplotypes from the 56 samples, a maximum-parsimony tree using all sites was constructed using PAUP* 4.0b4a (Swofford 1998). The program settings included heuristic search, addition sequence set to random (10,000 replications), and the tree-bisection-reconnection algorithm. The gamma parameter (an estimate of the distribution of rates of evolution among sites) was estimated from the 60 most parsimonious trees ($\alpha = 0.40$) with the parsimony approximation function in PAUP. A gamma corrected Tamura-Nei distance-based neighbor-joining tree was then created with MEGA (Kumar et al. 1993). Both trees were tested with 500 bootstrap replicates.

The two hypotheses were more directly assessed with a maximum-parsimony-based test (Templeton test) and a distance-based test (four way cluster analysis). For these tests, we first calculated the most parsimonious trees for a truncated dataset using only samples from the *P. barbatus* complex (27 of the 56 samples). Reducing the number of taxa allowed us to conduct a branch-and-bound search rather than a heuristic search while eliminating noise from rearrangements within outgroups. We also gained three more codons for a total of 720 total bases when truncating to the shortest sequence under the same criteria as before.

For maximum-parsimony-based tests, we used PAUP* 4.04b to perform Templeton tests. This is a nonparametric, Wilcoxon sign-rank test that gives significance values for the increase in number of steps observed when comparing the best topology from the original analysis to the best topology under imposed constraints (groupings). We tested the social parasites grouped with their respective hosts as predicted by a strict interpretation of Emery's rule. We then pooled the social parasites as a monophyletic group and tested with each of the two nonhost clades from the *P. barbatus* complex (*P. apache*/*P. desertorum* and *P. tenuispina*/*P. bicolor*) in an attempt to falsify the loose interpretations of Emery's rule.

A series of distance-based interior branch length tests were done using PHYLTEST (Kumar 1995). This least-squares-based method compares the three possible topologies of four monophyletic groups of taxa. The best supported of the three possible topologies yields a positive length for the middle branch. This analysis is not biased by the variance within clusters and thus can be used when sample size varies across the four groups of taxa. We used the gamma distance with

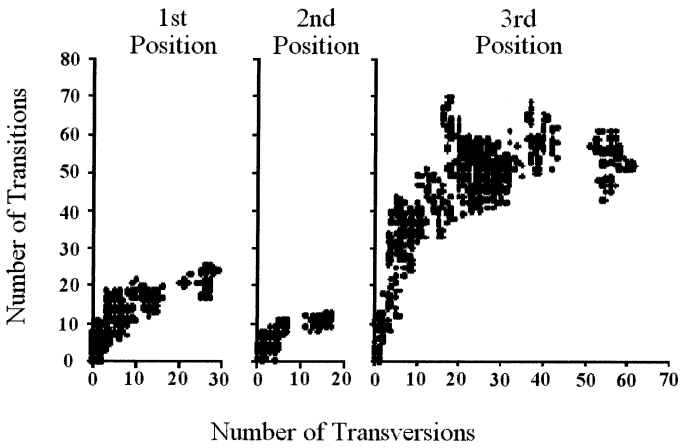

FIG. 3. The relative rates of evolution among the three codon positions as depicted by plotting the number of transitions versus the number of transversions for all pairwise comparisons of the 46 unique haplotypes in the study.

the gamma parameter ($\alpha = 0.28$) estimated from the two most parsimonious trees recovered for the *P. barbatus* complex dataset. Monophyletic groups were used in analyses (as per the above topologies) and included combinations of the two social parasites, their hosts, and nonhost species from the *P. barbatus* complex. Three lineages, *P. colei* (18–20), *P. anergismus* (5, 6), and the western *P. rugosus* clade (10, 24–29, 33) were included in the first three analyses. (The numbers in parentheses after the groupings refer to the sample identifications used in Figure 2 and Table 1.) The fourth group consisted of *P. anergismus* host clades, with the test run separately using the eastern (12–15) and western *P. barbatus* (11, 30–32, 34). A final test grouped the two social parasites with other groups including the eastern clade of *P. barbatus* (the putative ancestor), the *P. apache*/*P. desertorum* clade (7–9/21–23), and the *P. tenuispina*/*P. bicolor* clade (35–37/16, 17). All of these clusterings were repeated placing the two possible introgression samples (*P. barbatus*, 10 and 11) within their morphological species group.

RESULTS

Sequence Properties

The sequenced length of the cytochrome *b* reading frame from the CB1 primer to the termination codon varied from 237 codons in *P. imberbiculus* (taxa 3 and 4 in Table 1) to 253 codons in *P. rugosus* (taxon 33 in Table 1). Differences in the number of repeated asparagines at the 5′ end resulted in gaps that made alignment difficult. Thus, only the longest unambiguous alignment of 237 codons without gaps was used in the genuswide analyses. This left 46 unique haplotypes for the analysis, although all 56 complete sequences were deposited in Genbank (Table 1).

Base composition of the analyzed 711 base pairs is similar across taxa ($\chi^2 = 31.005$, df = 135, $P = 1.000$). However, the base composition shows a high AT content (72%). As expected, the rate of evolution varied across the three codon positions, with the most changes occurring in the first and third positions due to the redundancy of the genetic code (Fig. 3). The number of transitions versus the number of

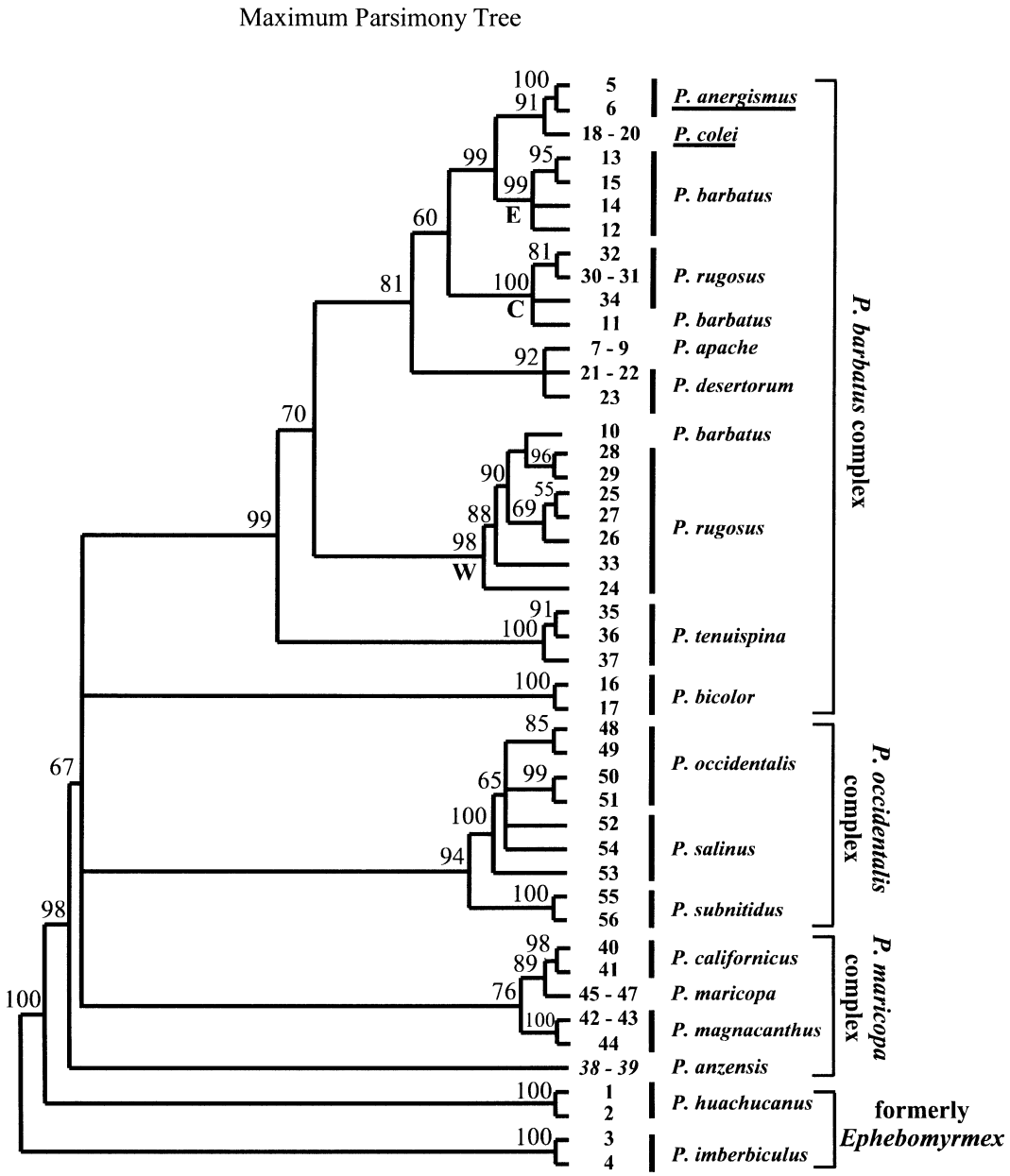

FIG. 4. The strict consensus tree of the 60 most parsimonious trees found by heuristic search. Numbers at the nodes indicate bootstrap values from 500 pseudoreplicates (values <50% not shown). Numbers to the left of the taxon names correspond to localities in Table 1 and Figure 2. The letters E, C, and W denote the eastern, central, and western clades of *Pogonomyrmex barbatus* and *P. rugosus* referred to in the text. The social parasites are underlined.

transversions increases linearly when comparing close relatives, but becomes asymptotic for comparisons with more distantly related outgroups. The transition:transversion ratio is 1.85. The codon usage pattern is typical of Hymenoptera with TAA used for all terminations (data not shown).

Phylogenetic Analysis

The strict consensus maximum-parsimony tree supports the hypothesis that the two social parasites are monophyletic and share a common ancestor with *P. barbatus* (Fig. 4). The heuristic search recovered one tree island of 60 most parsimonious trees (975 steps, RI = 0.78, CI = 0.47). The phylogeny of the outgroup species was consistent with current taxonomy, with the exception of placing *P. bicolor* and *P. anzensis* outside their accepted complexes of *P. barbatus* and *P. californicus*, respectively (Fig. 4).

The 17 samples for the two host species (*P. barbatus* and *P. rugosus*) form three mitochondrial clades, with occurrence in each clade corresponding to geographic location rather than to taxonomic identity (designated W, C, and E in Figs. 4–6). The eastern clade (E) consists of four samples of *P. barbatus* from northern Texas and southwestern Oklahoma (12–15). These individuals are more closely related to another host clade (C) that has a central geographic location relative to the total

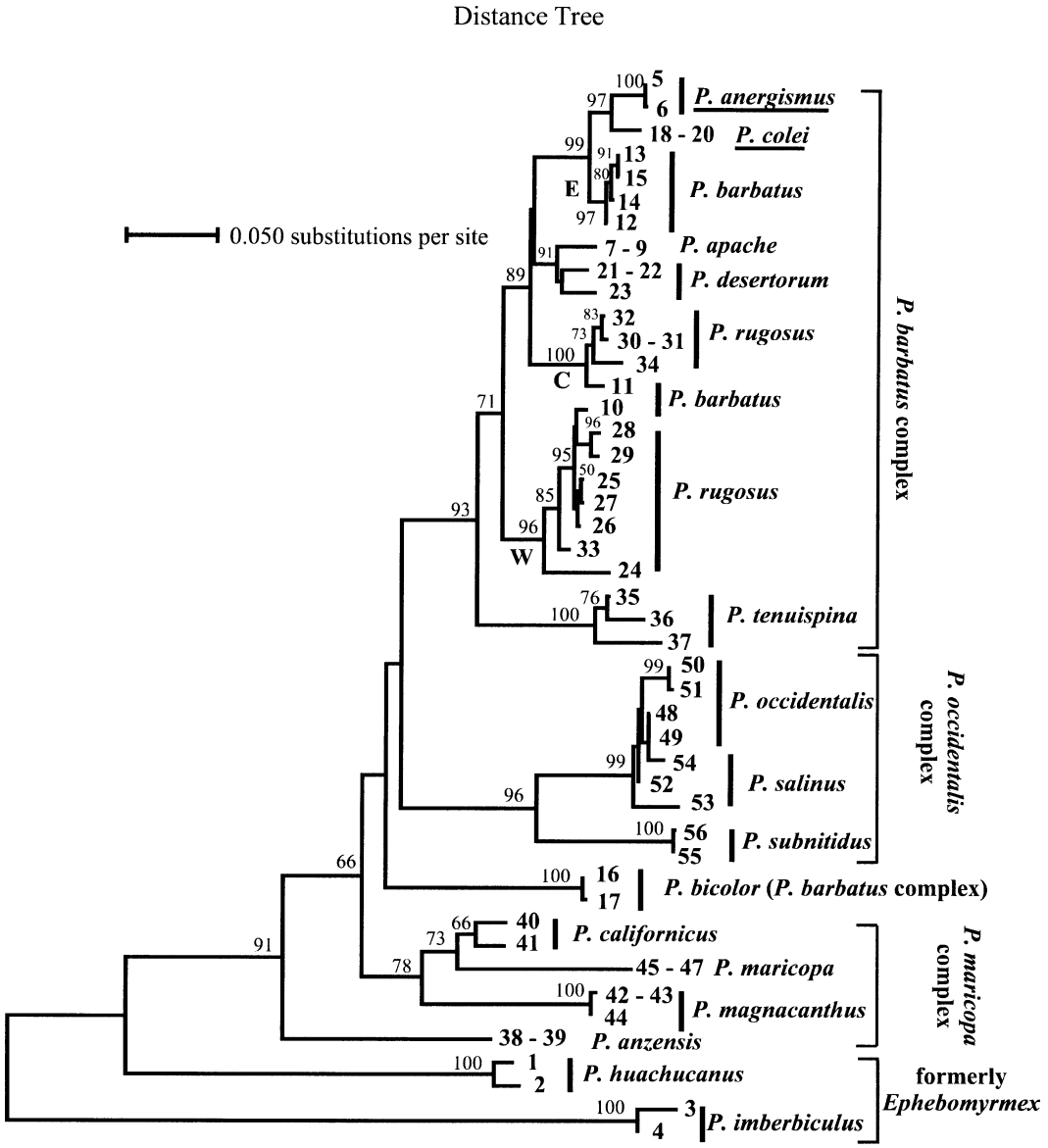

FIG. 5. Neighbor-joining tree with Tamura-Nei gamma distance and branch lengths. The tree was constructed by MEGA using an empirically determined gamma distance from PAUP. Numbers at the nodes indicate bootstrap values from 500 pseudoreplicates (values <50% not shown). Numbers to the left of the taxon names correspond to localities in Table 1 and Figure 2. The letters E, C, and W denote the eastern, central, and western clades of *Pogonomyrmex barbatus* and *P. rugosus* referred to in the text. The social parasites are underlined.

range of both species than to the most westerly clade of *P. rugosus* (W). The five samples in this central clade existed at locations from western Texas to southwestern New Mexico and included specimens of *P. rugosus* (30–32, 34) and *P. barbatus* (11; Figs. 2, 4, 5). The western clade consists of seven samples from Arizona and the Mexican states of Baja California and Sonora, and one sample from southern New Mexico; specimens of *P. rugosus* (24–29, 33) and *P. barbatus* (10) also exist in this clade. Unexpectedly, samples of *P. desertorum* and *P. apache* also fell within the host clades (Fig. 4).

The topology using the gamma-corrected Tamura-Nei distance shows no significant differences from the maximum-parsimony tree (Figs. 4, 5). Thus, the distance-based method confirms the monophyletic origin of the social parasites and their association with *P. barbatus*. The only topological difference from the maximum-parsimony analysis was the placement of the *P. desertorum*/*P. apache* clade as sister to the clade containing *P. barbatus* and the parasites. However, this relationship is not well supported.

Templeton and Four-Way Cluster Tests

Templeton tests support the monophyletic origin of the two social parasites (Table 3). The best four topologies resulting from placing each social parasite with their respective host(s) added 42 steps to the tree ($P < 0.0001$) relative to two most parsimonious topologies. Trees resulting from pooling the two social parasites along with the nearest nonhost

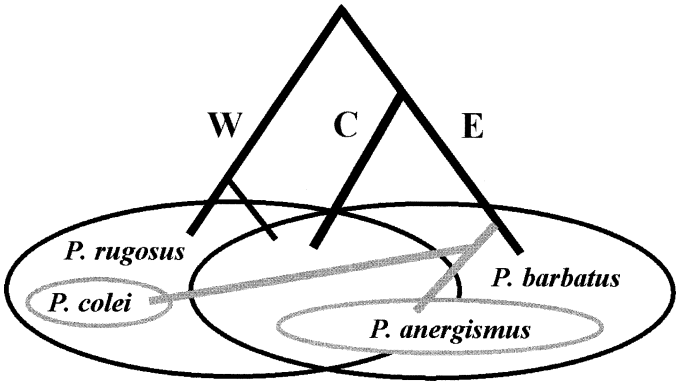

FIG. 6. Venn diagram of the geographic overlap of the hosts' and parasites' ranges (west to east, left to right), with the predominant mitochondrial lineages super imposed. E, C, and W represent the western, central, and eastern clades as described in Figures 4 and 5 and the text. The two large black ovals represent ranges of the hosts *Pogonomyrmex rugosus* and *P. barbatus*. The area of overlap includes both host species with hybrids and descendents of hybrids. Most of our samples from this region descend from a mitochondrial line that is most closely related to *P. barbatus*, though predating the origin of the social parasite line (gray lines). The social parasites (two gray ovals) came to occupy the ranges shown. *Pogonomyrmex colei* only occurs on western *P. rugosus* and *P. anergismus* on both *P. rugosus* and *P. barbatus* in the central area and on eastern *P. barbatus*. The border of the Sonoran (western) and Chihuahuan (eastern) Deserts falls at the far west side of the *P. barbatus* range.

species also added a significant number of steps (13 steps) to the trees (*P. apache*/*P. desertorum*, $P < 0.005$; *P. tenuispina*/*P. bicolor*, $P < 0.02$; Table 3). Thus, the branching patterns observed in the recovered trees with the parasites being monophyletic descending from the eastern clade of *P. barbatus* were verified.

Four-cluster tests also support the hypothesis that the two social parasites are monophyletic and most closely related to the eastern *P. barbatus* clade. The first three tests show that *P. colei* and *P. anergismus* are significantly more closely related to each other than to any host or nonhost clade (Table 4). The last test shows the social parasite lineage to be more closely related to a known host than to other members of the *P. barbatus* complex (Table 4).

TABLE 3. Results of the Templeton tests for determining the closest relative(s) of the two social parasites in the ant genus *Pogonomyrmex* using a sequence from the cytochrome *b* gene. Tests compared constraint trees to the two best trees found for *P. barbatus* complex taxa. Taxa included in analyses (corresponding collection locations as shown in Figures 2, 4, and 5 and Table 1) are the two social parasites (*P. colei*: 18–20; *P. anergismus*: 5, 6), their two hosts (*P. rugosus*: 10, 24–29, 33 and *P. barbatus* eastern: 12–15, and western: 11, 30–32, 34 clades), and nonhost species in the *P. barbatus* complex (*P. apache*: 7–9, *P. desertorum*: 21–23, *P. tenuispina*: 35–37, and *P. bicolor*: 16, 17).

Constraints	Number of trees found	Steps added	P[1]
(*colei, rugosus*)(*anergismus, barbatus*—E and W clades)(*apache*/*desertorum tenuispina*/*bicolor*)	4	42	<0.0001
(*coli, anergismus, apache*/*desertorum*)(*rugosus, barbatus*—E clade, *barbatus*—W clade, *tenuispina*/*bicolor*)	6	13	<0.005
(*colei, anergismus, tenuispina*/*bicolor*)(*rugosus, barbatus*—E clade, *barbatus*—W clade, *apache*/*desertorum*)	13	13	<0.02

[1] Each of the two original most parsimonious trees were compared to all of the most parsimonious trees found under each of the three constraints shown. Only the least significant comparison is given relative to each constraint.

DISCUSSION

Sequence Characteristics

The truncated cytochrome *b* sequences did not display any properties that would interfere with addressing hypotheses related to the origin of the two social parasites. The transition: transversion ratio is close to 2.0. Additionally, the base composition remains relatively constant across taxa, indicating similar evolutionary constraints, although the high AT content may lower the resolution of more distant relationships by limiting observable variability. Indeed, plots of transitions versus transversions asymptote in comparisons between the more distantly related taxa outgroups because of saturation (Fig. 3). This results in the poor resolution for the deep nodes evident in a number of observed polytomies (Figs. 4, 5). However, the linear portions of these curves contain the host

TABLE 4. Results of four-cluster analyses for determining the closest relative(s) of the two social parasites in the ant genus *Pogonomyrmex* using sequence from the cytochrome *b* gene. Patterns with positive interior branch lengths are presented. Taxa included in the groupings are as described in Table 3.

Taxa included in each analysis	Best supported branch pattern		Interior branch length	*P*
(*colei*)(*anergismus*)(*rugosus*) (*barbatus*—E and W clades)	(*colei*) (*anergismus*)	(*rugosus*) (*barbatus*—E and W clades)	0.036	<0.001
(*colei*)(*anergismus*)(*rugosus*) (*barbatus*—W clade)	(*colei*) (*anergismus*)	(*rugosus*) (*barbatus*—W clade)	0.052	<0.001
(*colei*)(*anergismus*)(*rugosus*) (*barbatus*—E clade)	(*colei*) (*anergismus*)	(*rugosus*) (*barbatus*—E clade)	0.021	<0.003
(*colei, anergismus*) (*barbatus*—E clade)(*apache, desertorum*)(*tenuispina, bicolor*)	(*colei, anergismus*) (*barbatus*—E clade)	(*apache, desertorum*) (*tenuispina, bicolor*)	0.026	<0.013

and parasite comparisons (Fig. 3) and the more recent evolutionary divergences that are important for this study.

Host Relationships

The mitochondrial phylogeny for the two host species, *P. rugosus* and *P. barbatus*, recovered here reflects to geographic location rather than established morphological taxonomic identity (Figs. 4–6). Interpreting this pattern necessitates understanding the biogeography of the two hosts, both of which are geographically widespread throughout the south-central and southwestern United States and Mexico (Fig. 2). Of the two species, *P. barbatus* has a more eastern distribution, as it is found from western Louisiana to central Arizona, whereas *P. rugosus* lives from central Texas to California. Thus, the two hosts have broadly overlapping geographic ranges from central and southeastern Arizona to western Texas, but both species also inhabit large areas lacking the other species (Johnson 2000a,b). Where the ranges overlap, the two species exist in distinct patches with the separation related to soil type and survivorship at colony founding stage (Johnson 2000a).

We interpret the three mitochondrial clades of host species to represent samples of *P. rugosus* (24–28 in Figs. 2, 4, 5), *P. barbatus* (12–15 in Figs. 2, 4, 5), and populations descended from hybrids of these two species (11, 30–32, 34 in Figs. 2, 4, 5). There was also one *P. barbatus* with a *P. rugosus* haplotype from the extreme western edge of the *P. barbatus* range (10 in Figs. 2, 4, 5). The eastern clade of *P. barbatus* (12–15), from northern Texas and southwestern Oklahoma, includes samples collected east of the geographic range of *P. rugosus*. We believe this clade represents *P. barbatus sensu stricto*, especially given that these samples are morphologically distinct from congeners collected in areas of geographic overlap with *P. rugosus* (R. Johnson, pers. obs.).

The host mitochondrial clade from southern New Mexico (central in Figs. 4, 5) is most closely related to the eastern clade of *P. barbatus sensu stricto* along with a nonhost clade (*P. desertorum*/*P. apache*). Four of the five samples in the central host species clade are of *P. rugosus* (Figs. 4, 5), suggesting genetic introgression between the two hosts. The westernmost clade consists of six samples of *P. rugosus* from the far western United States and one from the central area. This clade also includes one sample of *P. barbatus* (10), which again appears to result from genetic introgression (Figs. 4, 5). Thus, the host phylogeny suggests that *P. rugosus* and *P. barbatus* hybridize across widespread areas where their geographic ranges overlap. Indeed, many samples of *P. rugosus* × *P. barbatus* hybrids have been collected (Cole 1968; Hölldobler 1976). The pattern of introgression probably results from swamping of the least common haplotype, with *P. rugosus* displaying *P. barbatus* haplotypes in areas where *P. barbatus* is the more common species and *P. barbatus* displaying *P. rugosus* haplotypes in areas where *P. rugosus* is more common.

The unexpected and unresolved placements of *P. desertorum* and *P. apache* within the hosts clades and *P. bicolor* outside of the *P. barbatus* complex are problematic for the taxonomy of the genus and raise questions regarding the placement of unsampled species from the *P. barbatus* complex (four species). Reassuringly, this same unresolved polytomy with *P. rugosus* ancestral to the polytomy was also found in the most recent morphological analysis of this complex (Taber 1998). This makes an informative comparison, as hybridization and introgression of characters is likely responsible for the lack of resolution in this morphology-based tree as well. Alternatively, the mitochondrial ambiguities could be the result of incongruence of the mitochondrial and species trees due to an insufficient passage of time for lineage sorting to result in reciprocal monophyly of the haplotypes. In either case, the *P. barbatus* complex appears to have evolved too quickly and/or undergone too much hybridization for either this mitochondrial study or for classical morphological analysis to clearly resolve these relationships. This comparison highlights the need for comprehensive work on the systematics of this genus with multiple types of datasets.

Emery's Rule

The two phylogenetic analyses (distance, maximum parsimony) and two other tests (four-way cluster, Templeton tests) support the monophyly of *P. anergismus* and *P. colei* (Figs. 4, 5), with the eastern clade of *P. barbatus* as the most closely related extant taxon (Figs. 4, 5). This directly contradicts the strict definition of Emery's rule (Fig. 1A) as the parasites are most closely related to each other and not to their respective hosts. Indeed, *P. anergismus* contradicts the strictest definition by having more than one host. However, our data do provide support for the loose definitions, that hosts and parasites are closely related, because the parasites descend directly from one of the three closely related host lineages and infect related hosts. *Pogonomyrmex anergismus* fits the version of the loose rule depicted in Figure 1B because it is more closely related to its hosts, *P. barbatus* and *P. rugosus*, than to any other free-living species (noting that the grouping with *P. desertorum* and *P. apache* is not significant in the distance analysis, Fig. 4). In contrast, *P. colei* technically fails one loose definition because it has not been observed in eastern *P. barbatus* colonies and may have skipped over a nonhost clade (*P. desertorum*/*P. apache*) to infect the western *P. rugosus*. The advantage of defining specific testable branching patterns for the rule as we did here must be tempered with the reality that ''closely related to'' can never be an objective criterion. Clearly in this case, our findings show a relationship between hosts and parasites with parasites shifting across hosts that have a close relationship to each other. This is not the one-to-one host-parasite correspondence of the strict rule, nor does it satisfy the requirement of the closest clade to the parasites containing all of the hosts in the looser interpretations of Wilson (1971) and Ward (1996). However, Emery's rule can be still be said to hold for this genus, remembering that parasite divergence, host divergence, host shifting, and loss of parasite infection can all contribute to eroding host-parasite relationships over time (Buschinger 1990).

Origin of Social Parasitism in Pogonomyrmex

Two earlier cladistic analyses using morphology also supported monophyly of the two social parasites. The first differed in suggesting these two species form a sister group to the *P. rugosus*/*P. barbatus* clade, supporting the possibility of cospeciation (Taber 1990). In this case, the discrepancy with our dataset is likely due to inadequate sampling across the taxa.

Had we sampled only host taxa from the central range we likely would have discovered the same relationship. A later morphological analysis by the same author (Taber 1998) again found the social parasites to be monophyletic, but this time it could not be determined whether the parasite clade was more closely related to *P. barbatus*, *P. wheeleri* (not included in our study), or a clade including *P. desertorum*, *P. apache*, *P. bicolor*, and *P. texanus* (not included in our study). Taber could not have obtained the same trees recovered in our study because his methodology required taking each established species as a taxonomic unit by definition, and he thus could not resolve the central host lineage. However, Taber's approach would give a better picture of species designations if this central lineage is the result of failure of the mitochondrial genes to achieve reciprocal monophyly between *P. rugosus* and *P. barbatus*. In this case, the occurrence of only *P. anergismus* in the central clade of *P. rugosus* could be attributed to *P. anergismus* being limited to the Chihauhaun Desert and *P. colei* to the Sonoran Desert.

The findings regarding the host relationships, taken with biogeographic information are summarized in Figure 6. The resulting patterns, taken with behavioral observations of the parasites, offer an interpretation of the origin of the *Pogonomyrmex* inquilines and an explanation of why they loosely support Emery's observation, if not his rule. Bourke and Franks (1991) suggested that *P. colei* queens join pleometrotic associations of *P. rugosus* queens at the colony founding stage. However, we now know that *P. colei* foundresses use host-worker recruitment chemicals to locate and enter mature colonies (Johnson et al. 1996) and that both host species use identical recruitment pheromones (Regnier and Hölldobler 1973; Hölldobler et al. 2001). If the ancestor of *P. barbatus* and the social parasite included a variant that followed trail pheromones and entered into a polygynous or parasitic relationship during the colony-founding stage, then it would be free from the soil type restriction separating the two host species at the colony-founding stage. The parasite would be free to expand far into the range of *P. rugosus*, where it would have become isolated from its ancestral population. This scenario shares a similarity to the hypothesized speciation path of the apple maggot fly, *Rhagoletis*, where a novel host range became available, allowing range expansion with simultaneous restriction of herbivore gene flow (Feder et al. 1988; McPheron et al. 1988; Smith 1988). The observation of trail following also provides three alternative explanations for the pattern of host shifting and subsequent divergence of the parasites: (1) the ancestral inquiline speciated allopatrically from *P. barbatus* to parasitize the western clade of *P. rugosus*, then shifted back onto *P. barbatus* (West-Eberhard 1986) with divergence to *P. colei* and *P. anergismus*; (2) the parasite speciated allopatrically from a subspecies of *P. barbatus*, evolving into a parasite (Wilson 1971) that split into two species as it expanded onto *P. rugosus*; or (3) the parasite speciated sympatrically from *P. barbatus* (Buschinger 1986, 1990; Bourke and Franks 1991; Nonacs and Tobin 1992) and expanded onto *P. rugosus*, splitting into two species. Unfortunately, the pattern discovered here cannot resolve between these potential scenarios. However, in all cases, expansion across new and large host ranges would have facilitated the divergence of the two social parasites, with isolation by distance, rarity, small effective sizes, and inbreeding contributing to divergence.

In conclusion, Emery's rule in the strict sense has not held for molecular DNA sequence tests in the case of social insects. However, the original observation guiding Emery appears solidly based on social insect systems being prone to exploitation by closely related species. Thus, most social parasites tend to exploit related lineages for hosts. Our study, taken with others, indicates that social parasites tend to arise from other species of social parasites at least as readily as they arise from nonparasitic ancestors. This additional speciation within parasitic lineages accompanied by host shifting obscures tests of Emery's rule. The key factors for *Pogonomyrmex* social parasite speciation appear to be partially overlapping ranges of otherwise well separated host sister taxa, host sister taxa ranges restricted at the colony-founding stage, and a mechanism for entering the host nest. It will be interesting to see whether similar factors can be identified in other instances of social parasitism and speciation among clades of social parasites.

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