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Mitochondrial-DNA Sequence Evidence on the Phylogeny of Australian Jack-Jumper Ants of the *Myrmecia pilosula* Complex

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Australian ants of the *Myrmecia pilosula* species complex include some individuals (in *M. croslandi*) with the lowest possible metazoan chromosome number of $2n = 2$. Others in this cluster of sibling species have much higher numbers, the known maximum being $2n = 32$. Two species (*M. pilosula* and *M. 'banksi'*) are believed on cytogenetic and morphological grounds to have hybridized over a long period. To investigate the phylogeny and age of this group relative to the congeneric outgroup species *M. gulosa*, we sequenced part of the cytochrome *b* gene and the intergenic sequence between it and a primer anchored on the nearby $tRNA^{Ser}_{UCN}$ gene and analyzed the coding region using bootstrapped parsimony and neighbor-joining trees using the numbers of synonymous and nonsynonymous codons per site. The intergenic space demonstrated a profusion of repeated sequences, and only very closely related sequences (as judged by that for cytochrome *b*) showed detectable similarity at this almost 100% A+T region. In agreement with predictions from karyotype studies, the phylogenetic analyses showed that *M. croslandi* is the sister group to the other siblings; the time of separation of *M. croslandi* from the rest of the *pilosula* group is unexpectedly ancient. Other relationships were poorly resolved, but the results suggest that *M. 'banski'* and *M. pilosula* cluster together, as expected on cytogenetic grounds, and the tentative suggestion of close affinity of the *M. pilosula* samples and two "PB" samples supports derivation of PB from female *M. pilosula* and male *M. 'banksi.'*

INTRODUCTION

Ants of the genus *Myrmecia* have long been known to be among the most karyotypically variable animals in terms of chromosome number (Imai *et al.*, 1977; Crozier, 1981). That reputation was sealed with the discovery that ants of the *Myrmecia pilosula* group include some with a single pair of chromosomes (Crosland and Crozier, 1986). With chromosome numbers ranging from $n = 1$ to $n = 42$, the genus *Myrmecia* is the most diverse among animals in this respect (Imai *et al.*, 1990).

Further studies showed that there are several cryptic species in the *Myrmecia pilosula* group, differing minimally in morphology but considerably in karyotype (Crosland *et al.*, 1988; Imai *et al.*, 1988a,b; Imai and Taylor, 1989). The known range of chromosome numbers in the group is now $2n = 2–32$ (Imai *et al.*, 1994). The chromosomal rearrangements seen in the group include centric fissions or fusions ("Robertsonian rearrangements"), translocations, and centromere shifts (including pericentric inversions). The array of karyotype changes has been too complex to permit construction of a network linking all of the known karyotypes, although the pathways within each species are reasonably well understood.

Imai *et al.* (1994) summarized our knowledge of the *Myrmecia pilosula* complex, but some background should be given here.

The species with the lowest (possible) animal chromosome number is *Myrmecia croslandi* (Taylor, 1991), which has $2n = 2–4$. *Myrmecia 'imaii'* (provisional name) has $2n = 6–8$. A further species, with the provisional name *M. 'banksi,'* is distinguished by the greenish tinge of its head, and has a uniform karyotype with $2n = 10$, except for one colony apparently carrying a rare fusion and having $2n = 9$. *Myrmecia 'haskinsorum'* has $2n = 12–24$. *Myrmecia pilosula* s str. itself has $2n = 18–32$.

Along the southern edge of the range of *M. 'banksi'* several colonies were found that were interpreted as F1 hybrids of this form with *M. pilosula*. These are termed by Imai *et al.* (1994) PBF1-1 and PBF1-2, being differentiated by slight color differences. Other ants have standard *M. pilosula* s. str. karyotypes but are

Sequences reported in this paper have been deposited in the GenBank/EMBL database under Accession Nos. U15668–U15682.

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judged by Imai *et al.* (1994) to show phenotypic evidence of the introgression of genes from *M. 'banksi'* into *M. pilosula*. Following HTI's formalization (Imai *et al.*, 1994), we denote these colonies "PB-1" and "PB-2," being again differentiated by slight color differences.

A map showing the origins of the ants studied is given in Fig. 1.

The luxuriant diversity of karyotypes in a group of animals told apart only with great difficulty on morphological criteria raises a number of interesting questions, which can be addressed using phylogenetic information.

Several general questions arise. For example, what are the relationships of the forms with one another? How do the relationships from molecular information accord with those derived by Imai *et al.* (1994) from considerations of karyotypes and models of karyotype evolution (Imai *et al.*, 1986, 1988a)? In particular, the cytogenetic data suggest that *M. croslandi* is the sister group to the rest of the pilosula group, and that *M. pilosula* and *M. 'banksi'* should associate more closely than either does with *M. 'imaii.'* The F1 hybrids between *M. 'banksi'* and *M. pilosula* might also reveal which species, if either, predominantly supplies the maternal genome.

What is the approximate time scale over which this extensive karyotypic repatterning has occurred? The conclusion from studies of *Rhytidoponera* ants has been that karyotype evolution can occur rapidly in ants (Crozier *et al.*, 1986), but the karyotypic diversity of the *M. pilosula* complex is much greater than that known for the entire genus *Rhytidoponera*, and the possibility remains that the species of the *M. pilosula* complex are relatively ancient.

In this paper we examine the relationships between the various species in the *M. pilosula* complex, with the exception of *M. 'haskinsorum,'* for which material suitable for the study was not available. We use mitochondrial DNA (mtDNA) sequences from the cytochrome *b* gene. mtDNA in insects tends to be AT-rich, and that of another hymenopteran, the honeybee *Apis mellifera*, is the most AT-rich known for animals (Crozier and Crozier, 1993). But such high AT contents are not invariable, even in the Hymenoptera, with the ant *Tetraponera rufonigra*[3] having a lower AT content for its cytochrome *b* gene than does *Drosophila* (Jermiin and Crozier, 1994).

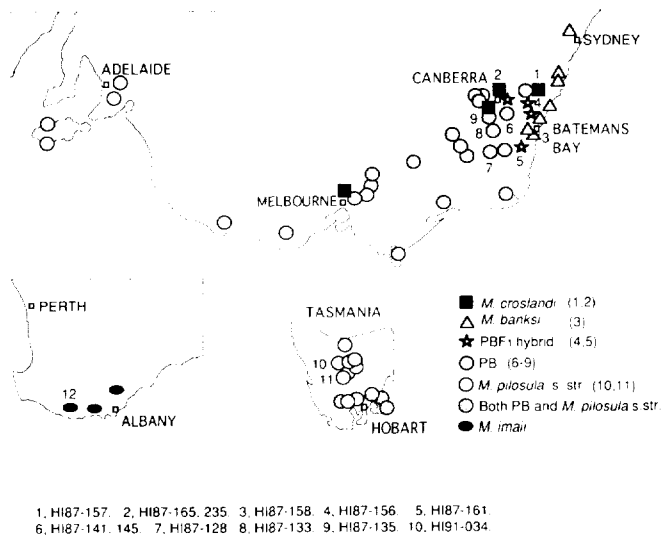

FIG. 1. Map of southeastern Australia and (inset) Western Australia showing cytologically verified occurrences of the species studied here in the *Myrmecia pilosula* complex (Imai *et al.*, 1994), with the collections used in this study indicated. Further explanation in text.

MATERIALS AND METHODS

Sources of Materials

The sources of the ants used in this study are given in Table 1.

Extraction of DNA, Amplification, and Sequencing

The molecular methods used were essentially those of Jermiin and Crozier (1994).

The primers used were derived from considerations of known insect mtDNA sequences (Clary and Wolstenholme, 1985; Crozier and Crozier, 1992, 1993; Jermiin and Crozier, 1994). These primers, their designations, and the coordinates to their equivalents in the honeybee sequence (in the direction of amplification) are as follows.

CB1	5'- TATGTACTACCATGAGGACAAATATC -3'	11400–11425
CB2	5'- ATTACACCTCCTAATTTATTAGGAAT -3'	11884–11859
CB3	5'- CCTATTCATATTCAACC -3'	11802–11818
tR^s	5'- TATTTCTTTATTATGTTTTCAAAAC -3'	12250–12226

Sequence Analysis and Phylogeny Inference

Basic sequence analyses were carried out using the MacVector (IBI) and DNA-Strider (C. Marck, SBGM) packages and programs written by RHC (Crozier and Crozier, 1993).

Maximum parsimony analyses were performed without constraints using PAUP (Swofford, 1989) and appropriate programs in the PHYLIP package (Felsenstein, 1990). We also used the bootstrap method (Felsenstein, 1985) to analyze the stability of the results using 1000 replicates. MacClade (Maddison and Maddison, 1992) was also used to determine the length penalty of suboptimal trees.

The four-taxon test of Steel *et al.* (1993), which corrects for base composition differences between sequences, was also used to test the significance of

[3] Jermiin and Crozier (1994) erroneously referred to this species as *Tetraponera rufoniger*.

TABLE 1

Collections of *Myrmecia* Ants Used in This Study

Species/code	Locality	Chromosome number, 2n (n)
M. gulosa		
ABNH-1	Helensburgh, NSW	
M. 'banksi'		
HI87-158	Sheep Station Creek, NSW	10
M. croslandi		
HI87-157	nr. Shoalhaven River, NSW	2
HI87-165	Canberra, ACT	4
HI87-235	Canberra, ACT	(2/3/4)
M. 'imaii'		
HT91-8	31 km W of Denmark, WA	7
M. pilosula		
HT91-34	Murderer's Hill, TAS	22/23
HT91-35	Derwent Bridge, TAS	(11/12/13)
M. pilosula (PB-1)		
HI87-128	Wambrook Creek, NSW	29
HI87-133	Shannon's Flat, NSW	24
HI87-135	Gudgenby nr. Naas, NSW	23/24
HI87-141	nr. Captain's Flat, NSW	32
HI87-145	nr. Captain's Flat, NSW	32
Putative F1 hybrids between *M. pilosula* and *M. 'banksi'*		
PBF1-1		
HI87-156	near Charleyong, NSW	19
PBF1-2		
HI87-161	Yowrie, NSW	15

Note. Codes with HI denote ants collected by H. T. Imai, and the next two digits indicate the year of collection. Colony ABNH-1, the colony used of the outgroup *Myrmecia gulosa,* was collected by Y. C. Crozier and R. Holiday in 1986; this colony was not karyotyped but the karyotype of this species has been considered elsewhere (Imai *et al.,* 1977). ACT, Australian Capital Territory; NSW, New South Wales; TAS, Tasmania; WA, Western Australia.

branch points identified in the parsimony analyses. This test was implemented using the program WAG4-SUM6 written by R.H.C.

The numbers of synonymous and nonsynonymous substitutions under the insect mitochondrial genetic code (Clary and Wolstenholme, 1985; see also Crozier and Crozier, 1993) were determined by Li's (1993) method. The method was implemented using a program listing kindly supplied by Dr Li and incorporated in a PASCAL program by J.-M.C. This program also counted the numbers of synonymous and nonsynonymous substitutions between sequences and used these to produce neighbor-joining (NJ) trees (Saitou and Nei, 1987) with bootstrap analysis (1000 replicates) to test their stability. In addition, a similar analysis was performed using the total number of substitutions as a distance measure for the NJ method.

Phylogenetic analysis was restricted to the coding region of the cytochrome *b* sequence because the other sequence obtained showed too high a level of indel variation to permit alignment with confidence.

Sequence was also obtained from the congener *Myrmecia gulosa* to allow this species to be used as an outgroup.

RESULTS AND DISCUSSION

Characteristics of the Sequences

The sequences obtained are given in Fig. 2. There is considerable variation in the length of sequence between the end of the cytochrome *b* gene and the primer based on the $tRNA^{Ser}_{UCN}$. This intergenic space is highly AT-rich (e.g., Fig. 3) and is highly variable between the various sequences obtained.

Some of the sequences display repeats in this space. For example HI87-156 has six copies, tandemly repeated, of 5′-TTTATTTATTAACTACCATTA-3′ beginning at position 813, HI87-135 has 11 copies, tandemly repeated, of 5′-TTTTTAAATATTA-3′ beginning at position 856, as does HI87-133 starting at position 831, and HI87-128 has four tandem copies of 5′-AATTAATTTTATTAA-3′ beginning at position 734. Dot matrix plots show that similarity for this region is very low for all but very closely related sequences. The common sequence for HI87-133 and HI-135 has been noted above; the *M. croslandi* sequences show more extensive TA runs than any of the others.

The general findings from this region may be summarized:

(1) Two *M. pilosula* colonies, HI91-034 and HI91-035, have essentially the same sequence.

(2) Two PB colonies (HI87-141 and HI87-145) have essentially the same sequence.

(3) Two PB colonies (HI87-133 and HI87-135) have essentially the same sequence.

(4) The PB colony HI87-128 differs from the pairs under (2) and (3), but has some small stretches of similarity with them.

(5) The three *M. croslandi* have nonidentical but very similar sequences.

The coding sequences are also highly AT-rich (see Table 2). As seen for honeybee mtDNA (Crozier and Crozier, 1993), *Myrmecia* base composition bias is strongest at the third position and is expressed as very low proportions of cytosine and, especially, guanine. The sequences, while very similar in composition, do differ ($P < 0.001$, χ^2), with *M. gulosa* having an unusually low proportion of cytosine compared to the rest.

FIG. 2. The sequences used in this study, which fall between the end of the CB-1 and the start of the tR^S primer sequences. The placement of the sequences into species is given in Table 1. The putative end of the cytochrome *b* gene is indicated by underlining of the termination codon TAA. A gap has been inserted to suggest homology of the termination codon for all species; no other gaps have been inserted because homologies could not be established across all of the sequences for the variable region between the end of the cytochrome *b* gene and the tR^S primer sequence.

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                .         .         .         .         .         .         .        80
HI87-157 ATTCTGAGGGGCAACTGTCATTACTAACCTTATTTCCACAGTCCCTTTCTTTGGGACTGAAATTGTCCAATGACTTTGAG
HI87-165 .........A.....................................................A.....................
HI87-235 .........A.....................................................A.....................
HI91-008 ...T.....T.................T...G....A...A.......TA.A..AT.A..T...ACT......T.A..G.
HI87-158 .........A........T........T...G....T..TA.T.....T..A...T.A..C...AC.........A....
HI91-034 .........T........T........T...G....T...A....C..T..A..AT.A..C...AC.........C..G.
HI91-035 .........T........T........T...G....T...A....C..T..A..AT.A..C...AC.........C..G.
HI87-128 .........T........T........T...G....T...A....C..T..A..AT.A..C...AC..............
HI87-133 .........T........T........T...G....T...A.......T..A..AT.A..T...ACT........C....
HI87-135 .........T........T........T...G....T...A.......T..A..AT.A..T...ACT........C....
HI87-141 .........T........T........T...G....T...A.......T..A..AT.A..C...AC.........A....
HI87-145 .........T........T........T...G....T...A.......T..A..AT.A..C...AC.........A....
HI87-156 ...T.....A........T........T...G........A.T.....T..A..AT.A..C...AC.........A....
HI87-161 .........T........T........T...G....T...A.......T..A..AT.A..C...AC.........C....
ABNH-1   N..T.....T..T.....A.....A..TT.AG.C..A...A.T..A.ATA....ATTAA.T..A..T......T.A....

                .         .         .         .         .         .         .       160
HI87-157 GAGGATATTCTGTAAGAAACGCAACACTAAACCGATTTTACTCTCTTCATTTCATCTTACCATTTGTAATTCTAATTATA
HI87-165 ..........................................................................
HI87-235 ..........................................................................
HI91-008 .......C...........T......T....T...........A..C..................A.T...T........
HI87-158 ..................T..C...T...GA...........AA.C..................A.T.......C....
HI91-034 ....G..C...........T..T...T................AA.C.........C........A..............
HI91-035 ....G..C...........T..T...T................AA.C.........C........A..............
HI87-128 .T...........T.....T..C...T...GA...........AA.C.........C........A....C.........
HI87-133 .G.....C..A...........T...T....T...........A..C.........C.T......A....C..T......
HI87-135 .G.....C..A...........T...T................A..C.........C.T......A....C..T......
HI87-141 .G..G..C...........T..T...T................AA.C.........C.G......A..............
HI87-145 .G..G..C...........T..T...T................AA.C.........C.G......A..............
HI87-156 .T...........T......T.T...T................AA.A.........TC....T...A..............
HI87-161 .......C...........T..C...T....T........T..AA.A.........C.T......A..............
ABNH-1   .G....TC...A.T.AT..T.....TT....T........T..AA.......T..T.....T...A.....T........

                .         .         .         .         .         .         .       240
HI87-157 GTAATTCTTCATTTATATTTCTTACACTTAACCGGATCATCTAACCCTTTAGGAATTAACAGAAATTTAAATAAAATCCC
HI87-165 ..........................................................................
HI87-235 ..........................................................................
HI91-008 ......A.....C.......T.....TA....T...........T..............T.....C...........T..
HI87-158 ......A..............C.T........T.....................T...G.T.....C.....C.....T..
HI91-034 ......A.G............C....T.....A........A..T..............T..................T..
HI91-035 ......A.G............C....T.....A........A..T..............T..................T..
HI87-128 ......A.A............C....T.....A........A..T..C...........T..................T..
HI87-133 ......A.A............C....T.....A........A..T..............................T..
HI87-135 ......A.A............C....T.....A........A..T..............................T..
HI87-141 ......A.G...........TC....T.....A........A..T..............T..................T..
HI87-145 ......A.G...........TC....T.....A........A..T..............T..................T..
HI87-156 ......A..............C....T.....A........A..T..............T..................T..
HI87-161 ......A.A............C....T.....A........A..T..............T..................T..
ABNH-1   .....AT.A...........T.....T..TT.A...........T..............T..................T..

                .         .         .         .         .         .         .       320
HI87-157 TTTTAATACTTATTATTCTTTAAAAGATTTATTAGGATTTGTAATCTCACTACTTTTATTTACATTATTAATTTTAATAA
HI87-165 ..........................................................................
HI87-235 ..........................................................................
HI91-008 ........T...C......ACG......C............A..G..A.TT..A.A..C...........G..........
HI87-158 ........T...C......GC..........C........A..G.AG.CT....A..C..C.....................
HI91-034 ........T...C......AC.......C..C....G..CAC....A.TT....A..C..C........G..........
HI91-035 ........T...C......AC.......C..C....G..CAC....A.TT....A..C..C........G..........
HI87-128 ........T..........AC.......C..C.........AC....A.TT.GT.A..C..C.....................
HI87-133 ........T..........AC.......A..C.......CAC......TT.G..A..C..C.....................
HI87-135 ........T..........AC.......A..C.......CAC......TT.G..A..C..C.....................
HI87-141 ........T...C......AC.......C.TC.......CAC....A.TT....A..C..C.....................
HI87-145 ........T...C......AC.......C.TC.......CAC....A.TT....A..C..C.....................
HI87-156 ........T...C.T....A........C.TC.........AC....A.TT....A.....C.....................
HI87-161 ........T...C..C...AC..........C.......CAC....A.TT....A..C..C.....................
ABNH-1   A.......T.....T...AA.T......A............A....TATTT..T.AA.C...GT.C.T.....C...T...
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                                                                                                                    400
HI87-157 CTCCATACTTATTCAGAGACCCTGATAATTTCTTACCAGCAAATCCTCTAGTAACCCCAATTCACATTCAACCAGAATGA
HI87-165 ....G...........................................................................
HI87-235 ....G...........................................................................
HI91-008 ............T..T..T.................G........AT....C.....T.....T..C.............
HI87-158 ....T.............T.....................AT....C..T..T..C..T..C.............
HI91-034 ....G........T.....T.....................AT....C.....T.....T.....T.............
HI91-035 ....G........T.....T.....................AT....C.....T.....T.....T.............
HI87-128 ............T.....T..C..................AT....C.....T.....T.....T.............
HI87-133 ............T.....T.....................AT....C.....T.....T.....T.............
HI87-135 ............T.....T.....................AT....C.....T.....T.....T.............
HI87-141 ............T.....T.....................AT....C.....T.....T.....T.............
HI87-145 ............T.....T.....................AT....C.....T.....T.....T.............
HI87-156 ..................T.....C......C.T.....AT..A.C..T..T.....T.....T.............
HI87-161 ........C....T.....T.....................AT....C..A..T.....T.....T.............
ABNH-1   AA..T..T.....T.....T...........TACC..T......T.AA..A.T..A........T.............

                                                                                                                    480
HI87-157 TATTTCCTATTTGCCTATAGAATCCTTCGAGCTATCCCAAATAAATTAGGTGGAGTTATTGCCTTATTCAGATCAATTTG
HI87-165 ............T.....................T.....................................................
HI87-235 ............T.....................T.....................................................
HI91-008 ..C..T..T..C..G.............TT....T.............A..T.....C..TC.....................
HI87-158 ..C..T..T.....T.............TT..................A..T........T.....T.....................
HI91-034 ........T..C..T........T.....TT....T.............A...........TC.....................
HI91-035 ........T..C..T........T.....TT....T.............A...........TC.....................
HI87-128 .....T..T..C..A.............TT.............C....A..C........T.....................
HI87-133 .....T..C..C..T.............TT....T.............A...........TC.....................
HI87-135 .....T..C..C..T.............TT....T.............A...........TC.....................
HI87-141 ........T..C..T........T.....TT....T.............A...........TC.................A
HI87-145 ........T..C..T........T.....TT....T.............A...........TC.....................
HI87-156 ..C.....T.....T..C.....T.....TT....T.............A...........TC......T.............
HI87-161 ..C..T..T..C..T.............CT....T.............A.....C.....TC.....................
ABNH-1   .....T........A........TT.A...T.A..T..T......C...A.......T.A..AC....A..........T

                                                                                                                    560
HI87-157 CGTTTTATATTTTCTCCCCATAACAACATCCAAAATAAACTCATCCTCATTTTATCCTTTAAGCCAATTAATAATCTGAT
HI87-165 ...........................................................................................
HI87-235 ...........................................................................................
HI91-008 TA.C........C..A..A...TT......A.............G.................A.....TT.........
HI87-158 TA.C............A..A...TT...C.TT.............T.....C...........A.....TT....A....
HI91-034 TA.C............G..T...TT...C..T.................................G.A.....TT........C
HI91-035 TA.C............G..T...TT...C..T.................................A.....TT........C
HI87-128 TA.C............A..A...TT..AC..T..C............A.................A......T.........
HI87-133 TA.C............G..A...TT...T..T.....................C.........A.....TT.........
HI87-135 TA..............G..A...TT...T..T.....................C.........A.....TT.........
HI87-141 TA.C........C..A......TT...C..T.....................C.........A.....TT.........
HI87-145 TA.C........C..A......TT...C..T.....................C.........A.....TT.........
HI87-156 TA............A.A..A...TT...C..T.................T.................A.....T.....A....
HI87-161 TA.C.........A.A......CT...C..T.................T.....C.........GCA.....TT.........
ABNH-1   AA..C.T......T.AT.TT..TTT.AT..TT.T.....T.....T..T........AA.T.AT......T....T...A

                                                                                                                    640
HI87-157 TATTTTTCATAACTTTCATAATTTTAACATGACTAGGATCACAACCAATTGAATACCCTTATATTATTTCAGCCCAATTT
HI87-165 .................C.....................................................G.....................
HI87-235 .................C.....................................................G.....................
HI91-008 .......T........T.............................GTG......A...........TC.A....T...A..
HI87-158 C......T........T.............................GT.......C.T..G..C.....CA....T...A..
HI91-034 .......T.....C..T..............T....G......GT.......C.T.....C......A...........
HI91-035 .......T.....C..T..............T....G......GT.......C.T.....C......A...........
HI87-128 .......T.....C..T..............T...........GT.......C.T..A..C......AT..........
HI87-133 .......T.....C..T..............T....G......GT.......C.T.....C......A...........
HI87-135 .......T.....C..T..............T....G......GT.......C.T.....C......A...........
HI87-141 .......T.....C..T..............T...........GT.......C.T.....C.....CA...........
HI87-145 .......T.....C..T..............T...........GT.......C.T.....C.....CA...........
HI87-156 .......T........T..............T...........GT.......C.T.....C......A....T.......
HI87-161 .......T........T...........G...T...........GT.......C.T.....C......A....A.....C
ABNH-1   .......TTC.TTA..TTAT........T...T......TTG..T.........CT.T...C.C.TC..TT..T...C..
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FIG. 2—*Continued*

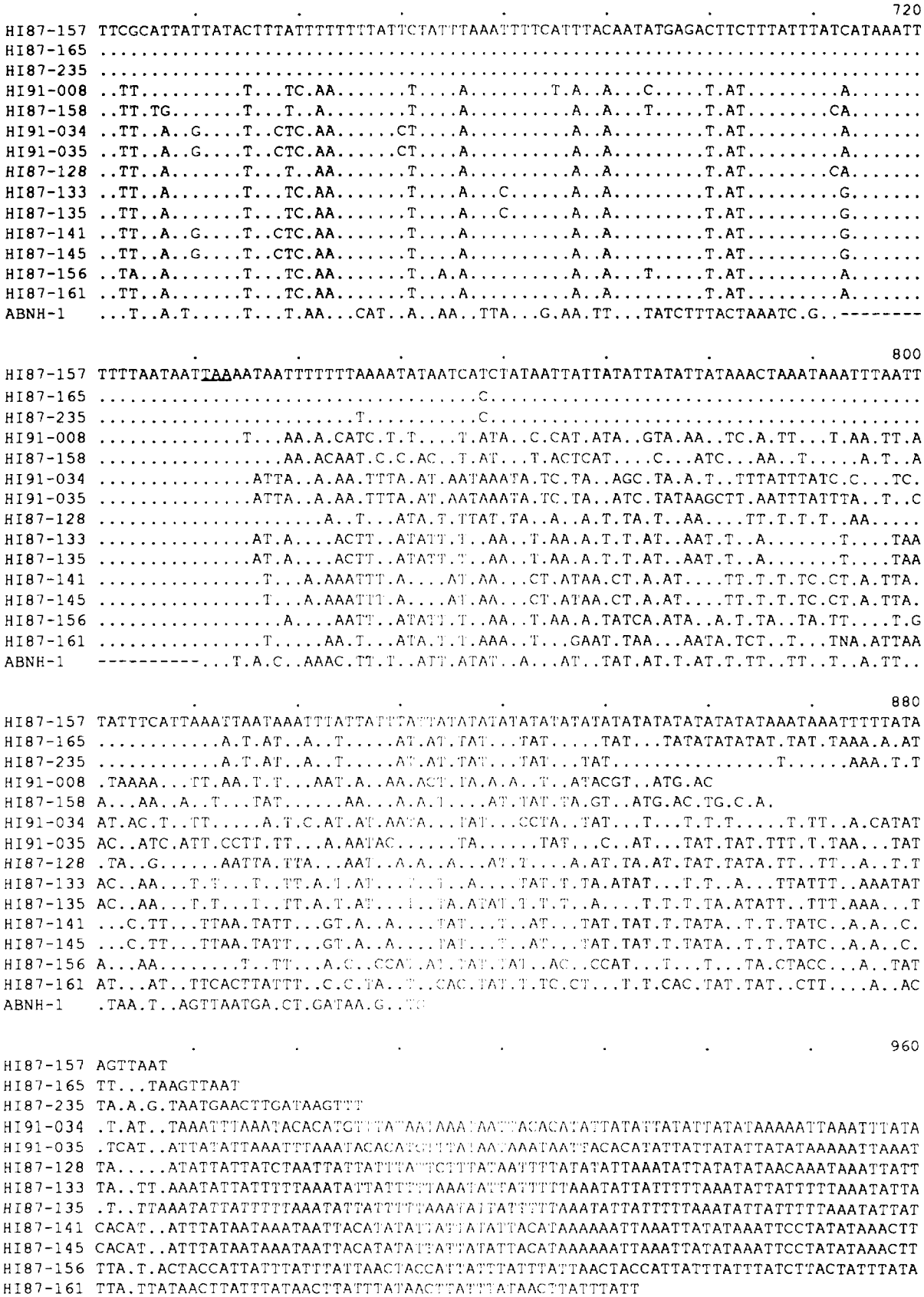

FIG. 2—*Continued*

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                                                                    1040
HI91-034 ATAAATTAAATTCCTATATAAACTTAAACTTTTAATAAATTAAAAATATTCTAAGTTAATGAGCTT
HI91-035 TTATAATAAATTAAATTCCTATATAAACTTAAACTTTTAATAAATTAAAAATATTCTAAGTTAATGA
HI87-128 ATATTATATAAAAATTAAATTATATTAAATTCTATATCTAACTAACTAAACTTAAATTTTTTTTTAATAAATAATTCAAA
HI87-133 TTTTTAAATATTATTATTTATTTTTTATAATTTTATATATTAAACATACCAAACAAGATATATTTATAATAAATAAATTT
HI87-135 TTTTAAATATTATTTTTAAATATTATTTTTAAATATTATTATTTATTTTTTATAATTTTATATATTAAACATACCAAACA
HI87-141 AAATTTTTATTAAATTAAAAATATCTAAGTTAATGA
HI87-145 AAATTTTTATTAAATTAAAAATATCTAAGTTAA
HI87-156 AATATTATTAATTAATATTATATTTATAATTATCAATTATCCATTAAAATTTAATATATTAAATTCAAACTTTATATAAT

                                                                    1120
HI87-128 AATATTTAAGTTAATGAA
HI87-133 ATTATTATTATATAAAAAATTAAATTATATAAATTTTATTTAAAATTTTAAATTTTTAATAAATAATTTTAAAAATATAT
HI87-135 AGATATATTTATAATAAATAAATTTATTATTATTATATAAAAAATTAAATTATATAAATTTTATTTAAAATTTTAAATTT
HI87-156 TTATAAATATAAGTTAATGAA

                                                                    1200
HI87-133 AAGTTAAT
HI87-135 TTAATAAATAATTTTAAAAAATATATAAGTTAATGA
```

FIG. 2—*Continued*

Phylogenetic Analyses

The tree obtained using the cytochrome *b* sequences analyzed under bootstrapped parsimony is shown in Fig. 4.

Using the totals of all substitutions per site as a distance and using the resulting distance matrices to obtained bootstrapped NJ trees yields a tree identical in topology to that of Fig. 4, except that colony HI87-128 does not fall within the PB + *pilosula* cluster but joins this cluster at its base.

Using either the synonymous or the nonsynonymous substitutions yields the results presented in Figs. 5 and 6.

All trees agree in placing the *M. croslandi* sequences distant to those of the other *M. pilosula* complex species. All trees except that based only on synonymous changes agree that the *M. gulosa* sequence joins the rest so as to indicate that *M. croslandi* is the sister group to the rest of the species in the complex. There are reasons to discount the synonymous-changes tree with respect to the root estimated for the *M. pilosula* complex. The estimated number of synonymous substitutions per site for the comparisons involving *M. gulosa* is greater than one (Fig. 7), indicating that saturation is likely to have occurred. For the comparisons involving *M. gulosa*, there is also a considerably greater spread of the estimates of synonymous than of nonsynonymous changes, whether or not the outlier point involving HI87-158 is included (CV synonymous = 25.42, CV nonsynonymous = 4.41) or not (CV synonymous = 11.12, CV nonsynonymous = 4.15), although the *M. gulosa* sequence would be expected to fall equally distant from all those of the *M. pilosula* complex.

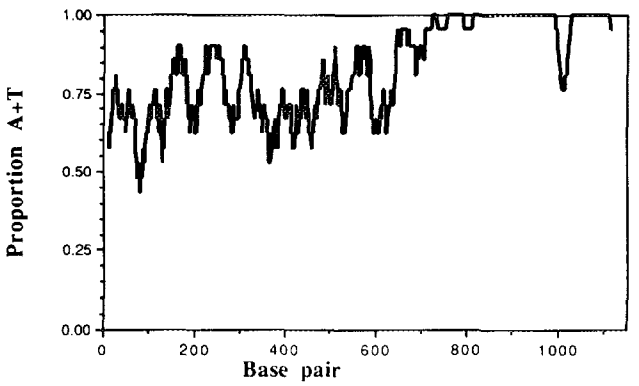

FIG. 3. Plot of the proportion of A+T for sequence HI87-135, smoothed using a 21-position window. See also Table 2 for the base composition of the coding portion of the sequence.

The root was also investigated using the Steel *et al.* (1993) frequency-dependent test, in terms of the grouping of the *M. gulosa* and the HI87-157 sequences to the exclusion of that of HI91-8 plus a variety of other sequences, using the cytochrome *b* sequence data. All seven such tests performed gave more support to the placement of the root shown in Figs. 4 and 5 than to the two alternatives in each case, and in three cases the rejection of alternatives was significant ($P < 0.05$).

The bootstrap values for the remainder of the tree of Fig. 4 do not strongly support associations between groups, although there are three pairs of sequences grouped in 100% of the replicates. These groups also appear in the other trees, except that the two PB sequences HI91-34 and HI91-35 are separated by HI87-161 in the tree derived from nonsynonymous changes. We suggest that, while nonsynonymous changes give a more reliable signal for distant relationships, they occur too sporadically to provide reliable clustering of closely related sequences, at least in this data set. Evidence for this conclusion comes from consideration of

TABLE 2

Base Composition Percentages of the Coding Region, by Codon Position

	First				Second				Third			
Species	A	T	G	C	A	T	G	C	A	T	G	C
M. gulosa	33.19	42.86	12.18	11.76	22.27	48.32	9.24	20.17	44.54	51.68	0.42	3.36
M. croslandi												
HI87-157	30.33	38.52	14.34	16.80	21.31	46.31	10.66	21.72	43.67	36.33	0.82	18.37
HI87-165	30.33	38.52	14.34	16.80	21.31	45.90	10.66	22.13	44.08	36.73	0.82	18.37
HI87-235	30.33	38.52	14.34	16.80	21.31	45.90	10.66	22.13	44.08	36.73	0.82	18.37
M. 'imaii'												
HI91-008	34.02	39.34	12.70	13.93	22.95	44.67	10.66	21.72	42.04	44.08	2.86	11.02
M. 'banksi'												
HI87-158	31.97	38.93	14.34	14.75	22.13	45.49	11.07	21.31	42.45	43.27	0.82	13.47
M. pilosula												
HI91-034	33.20	37.70	13.11	15.98	22.95	44.67	10.66	21.72	41.63	40.82	3.27	14.29
HI91-035	33.61	37.70	12.70	15.98	22.95	44.67	10.66	21.72	41.63	40.82	3.27	14.29
M. pilosula (PB-1)												
HI87-128	34.02	38.52	12.29	15.16	22.95	45.49	11.07	20.49	44.90	40.00	0.41	14.69
HI87-133	32.79	38.52	12.70	15.98	22.95	44.67	10.66	21.72	43.27	42.04	1.63	13.06
HI87-135	32.79	38.52	12.70	15.98	22.95	44.67	10.66	21.72	43.27	42.04	1.63	13.06
HI87-141	33.20	38.11	13.11	15.57	23.36	44.67	10.25	21.72	43.27	40.82	1.63	14.29
HI87-145	33.20	38.11	13.11	15.57	22.95	44.67	10.66	21.72	43.27	40.82	1.63	14.29
PBF1-1												
HI87-156	35.66	37.29	11.48	15.57	22.54	45.90	10.66	20.90	44.49	46.12	0.0	9.39
PBF1-2												
HI87-161	33.61	38.11	12.70	15.57	22.95	44.67	10.25	22.13	45.31	40.82	0.41	13.47
Mean	32.82	38.62	13.08	15.48	22.52	45.38	10.57	21.53	43.46	41.54	1.36	13.64
S.D.	1.51	1.28	0.89	1.29	0.70	1.01	0.43	0.58	1.15	3.90	1.05	3.87
Bias		0.29				0.27				0.47		

Note. Bias was calculated according to the formula $(2/3)\ \Sigma_{i=1}^{4}\ (|b_i - 0.25|)$, where b_i is the proportion of the ith base.

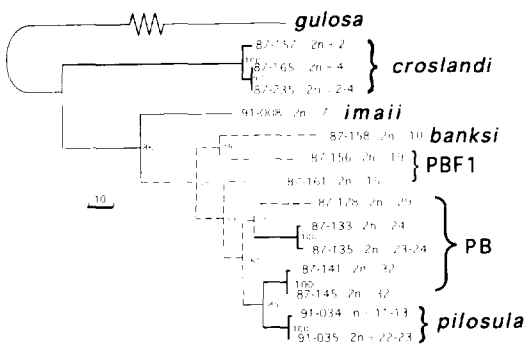

FIG. 4. Maximum-parsimony tree determined using the cytochrome *b* coding sequences. Bootstrap values (percentages of 1000 replicates) were determined using PAUP, and the branch lengths were estimated using MacClade. Groups obtained in more than 95% of the bootstrap replicates are connected by boldface lines, those obtained in 85 to 94% of the replicates by solid lines, and the rest by dashed lines. The "PBF1" sequences were obtained from ants with karyotypes indicating that they are F1 hybrids between *Myrmecia 'banksi'* and *M. pilosula*. The "PB" sequences come from ants with *M. pilosula* karyotypes but with morphological evidence of introgression from *M. 'banksi.'* The scale bar denotes 10 changes; the length of the branch going to *M. gulosa* is 155 units.

the intergenic region: HI91-34 and HI91-35 show high similarity along their lengths as determined by the dot matrix plots, but each shows much less similarity with HI87-161 for the intergenic space (Fig. 8). These considerations, plus other aspects of the characteristics of the intergenic space noted above, tentatively lend more support to the details of the PB–*pilosula* cluster shown in Fig. 4 and 5, as against those of Fig. 6.

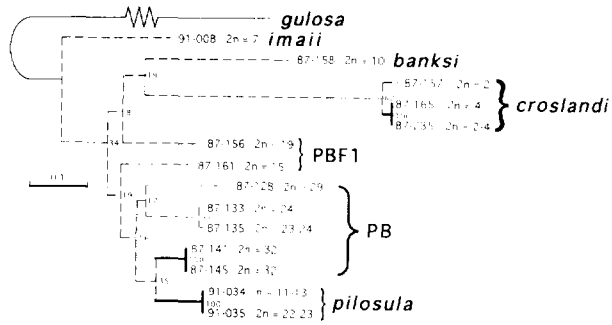

FIG. 5. Neighbor-joining tree determined using the number of synonymous substitutions/site as the metric. Bootstrap values from 1000 replicates are shown. The scale bar denotes 0.1 substitutions; the length of the branch going to *M. gulosa* is 0.95 substitutions. Other conventions are as for Fig. 4.

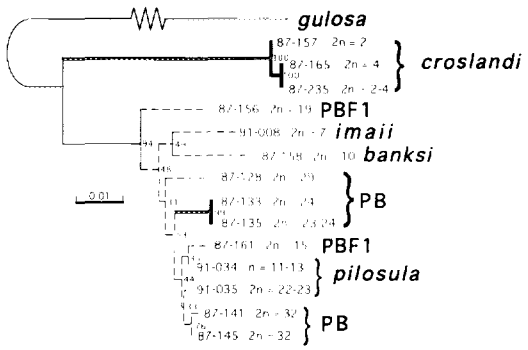

FIG. 6. Neighbor-joining tree determined using the number of non-synonymous substitutions/site. Bootstrap values from 1000 replicates are shown. The length of the scale bar is 0.01 substitutions; the length of the branch going to *M. gulosa* is 0.159 substitutions. Other conventions are as for Fig. 4.

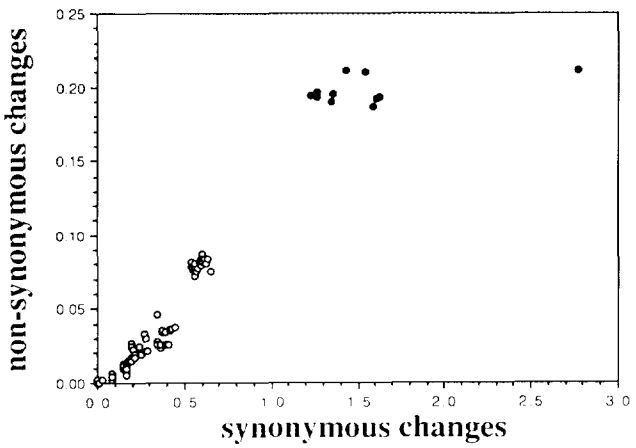

FIG. 7. Plot of estimated synonymous versus nonsynonymous changes/site for the sequences in this study. Comparisons involving the outgroup sequence, from *Myrmecia gulosa*, are shown as solid dots, and the others by open symbols.

Degree of Divergence

The synonymous and nonsynonymous substitutions estimated between the various colonies are given in Table 3. As a comparison, similar analyses between subspecies of the honeybee *Apis mellifera* (data from Garnery *et al.*, 1992) yield estimates of 0.04–0.09 synonymous substitutions per site and around 0.005 nonsynonymous estimates per site for the COXII gene, which evolves at a rate similar to that of the cytochrome *b* gene (Crozier and Crozier, 1993). The same data yield estimates between *A. mellifera* and the close relative *A. cerana* of 0.4 for synonymous and 0.01 for nonsynonymous substitutions per site. The *Myrmecia* cryptic species of the *M. pilosula* group are thus at least as different as well-recognized species of honeybee, and the difference between *M. gulosa* and the *pilosula* group is very large by comparison with the difference between two *Apis* species.

Age of the Species

The rate of evolution of the cytochrome *b* region of *Myrmecia* ants has not been calibrated. However, about 8% of potentially nonsynonymous sites differ between the *M. croslandi* sequences and the rest of the complex. Given that about 560 of the 714 sites used for this analysis are open to nonsynonymous changes, this value corresponds to about 6% of the sequences having substitutions "that would produce amino acid replacements" (DeSalle *et al.*, 1987), which would correspond for the ND1 gene of Hawaiian drosophilids to a time since divergence of about 150 My. Hymenopteran mtDNA appears to have evolved more than fly mtDNA since they diverged from a common ancestor (Crozier and Crozier, 1992, 1993; Jermiin and Crozier, 1994), which would reduce the estimated time since divergence, but the insect cytochrome *b* gene appears to evolve more slowly than that for ND1 (Crozier and Crozier, 1993).

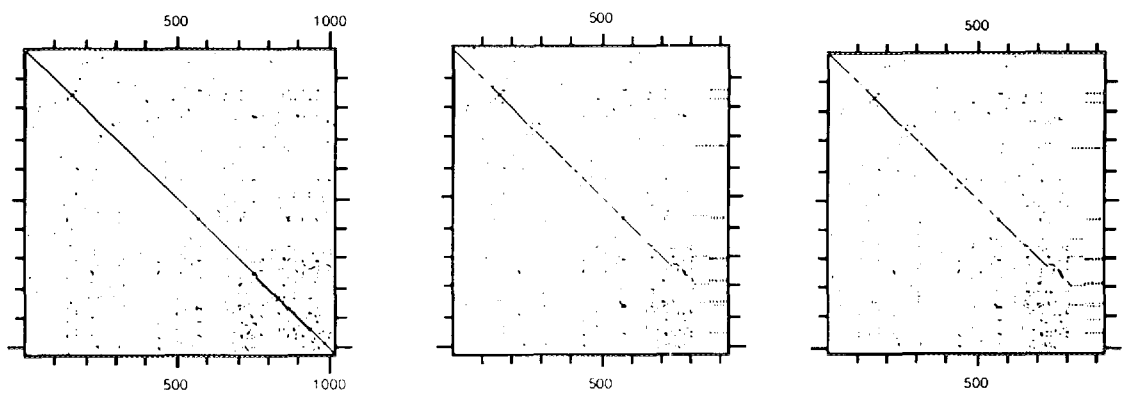

FIG. 8. Dot-matrix plots (window = stringency = 7) between (left) HI91-34 and HI91-35, (middle) HI91-34 and HI87-161, and (right) HI91-35 and HI87-161, showing the much greater similarity between HI91-34 and HI91-35 than between either and HI87-161, especially for the intergenic space.

TABLE 3

Numbers of Substitutions at Synonymous (Left) and Nonsynonymous (Right) Sites in the Coding Region, Estimated Using Li's (1993) Method

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 *M. gulosa*		0.20507	0.20315	0.20315	0.18974	0.20265	0.18750	0.18862	0.18213	0.18938	0.18938	0.18889	0.19150	0.18862	0.19086
2 HI87-157	1.42548		0.00179	0.00179	0.07913	0.07902	0.07767	0.07140	0.07788	0.07535	0.07336	0.07341	0.07539	0.07140	0.07833
3 HI87-235	1.43408	0.04068		0.00000	0.08118	0.08109	0.07972	0.07343	0.07993	0.07739	0.07539	0.07544	0.07744	0.07343	0.08038
4 HI87-165	1.43408	0.04068	0.00000		0.08118	0.08109	0.07972	0.07343	0.07993	0.07739	0.07539	0.07544	0.07744	0.07343	0.08038
5 HI91-008	1.14208	0.69711	0.69414	0.69414		0.03198	0.03855	0.02520	0.03319	0.02705	0.02522	0.02340	0.02523	0.02520	0.02570
6 HI87-158	1.77145	0.69385	0.67789	0.67789	0.47361		0.04216	0.03119	0.02899	0.03121	0.02932	0.02793	0.02982	0.03119	0.02934
7 HI87-156	1.03934	0.66556	0.63955	0.63955	0.49938	0.37041		0.02985	0.03267	0.02702	0.02520	0.02339	0.02521	0.02985	0.02152
8 HI87-135	1.20734	0.61821	0.60458	0.60458	0.47036	0.52032	0.33899		0.02174	0.01122	0.00942	0.01072	0.01253	0.00000	0.01481
9 HI87-128	1.18948	0.65337	0.66340	0.66340	0.48396	0.40036	0.34648	0.23731		0.01813	0.01631	0.01451	0.01632	0.02174	0.01810
10 HI87-141	1.11390	0.66894	0.65383	0.65383	0.40939	0.43344	0.26292	0.18818	0.24951		0.00178	0.00357	0.00536	0.01122	0.01072
11 HI87-145	1.11390	0.66894	0.65383	0.65383	0.40939	0.43344	0.26292	0.18818	0.24951	0.00000		0.00178	0.00357	0.00942	0.00892
12 HI91-035	1.17690	0.69344	0.65193	0.65193	0.44181	0.47259	0.31649	0.20714	0.25877	0.12895	0.12895		0.00178	0.01072	0.00713
13 HI91-034	1.17339	0.69344	0.65193	0.65193	0.44181	0.47259	0.31649	0.20714	0.25877	0.12895	0.12895	0.00000		0.01253	0.00534
14 HI87-133	1.20734	0.64286	0.62867	0.62867	0.44970	0.50986	0.35775	0.01352	0.22944	0.18818	0.18818	0.20714	0.20714		0.01481
15 HI87-161	1.11816	0.68908	0.67396	0.67396	0.45755	0.40299	0.32406	0.25915	0.29580	0.23248	0.23248	0.26078	0.26078	0.24279	

The age of the *M. pilosula* complex remains uncertain but would appear from these considerations to be on the order of 100 My. This conclusion is unexpected for two reasons: first, the close morphological similarity between these ants (but this may reflect selection for similarity of these ants in a mimetic complex), and second, the subfamily to which *Myrmecia* belongs, the Myrmeciinae, have a fossil record only to about 110 Mya (Brandão *et al.*, 1989; Brandão, 1990). If the same calculations are made using the nonsynonymous substitutions between *M. gulosa* and the *pilosula* complex (see Fig. 8), an age for the genus of > 150 My is obtained. The age of the ants as a whole is unlikely to be that great (Hölldobler and Wilson, 1990, p.23). It thus appears not only that the separation of *M. croslandi* from other species of the M. *pilosula* complex is ancient, more than half the age of the genus, but also that the evolution of ant mtDNA is more rapid than that of *Drosophila,* precluding ready calibration.

Cytogenetic Implications

Karyotypic comparisons suggested that *M. croslandi* is the most divergent species of the group, and the phylogenetic analyses strongly corroborate this. Species expected to be phylogenetically close on karyotypic grounds include various groups recognized as strongly assorted not only from the phylogenetic analysis but also from analysis of the noncoding region. As noted above, it also appears on cytogenetic grounds that *M. 'banksi'* would cluster with *M. pilosula,* and this is supported, although weakly, by the phylogenetic results. The very close association of the *M. pilosula* sequences with some of the PB sequences would, if confirmed, demonstrate derivation of these PB sequences from *M. pilosula* rather than *M. 'banksi'* and support the indications from the cytogenetic work (Imai, unpublished) that the PB populations stemmed originally from hybridization between *M. pilosula* females and *M. 'banksi'* males.

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