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Phylogeny of Fungus-Growing Ants (Tribe Attini) Based on mtDNA Sequence and Morphology

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We examined the phylogenetic relationships among taxa of attine or "fungus-growing" ants (Tribe Attini) using parsimony analyses of molecular and morphological data. We sequenced a region of mitochondrial DNA from 13 taxa of attines and from one closely related outgroup species, *Wasmannia auropunctata*. Our study sequence included the 3′ end of the cytochrome c oxidase subunit I (CO I) locus (183 to 198 total sites; 91 informative sites), an intergenic spacer region of variable size (0 to 152 sites), the tRNA leucine locus (65 to 74 sites), and the 5′ end of the cytochrome c oxidase subunit II (co II) locus (292 total sites; 140 informative sites). The inferred relationships among the attine taxa based on an unweighted analysis of the nucleotide sequence data closely matched the relationships inferred from an analysis of larval morphological characters from 11 of the taxa. In fact, the inferred relationships were completely congruent among the protein parsimony analysis of amino acid data, the morphology analysis, and "total evidence" analysis combining the amino acid and morphology data. The congruent conclusions we obtained from two independent data sets increases our confidence in the reliability of our analyses. © 1998 Academic Press

INTRODUCTION

Attine ants (Tribe Attini) are unique among ants in their habit of growing a symbiotic fungus for food. All attine ants are obligately dependent on the their fungus. Different species of attines, however, vary greatly in their ecologies (Weber, 1972; Wetterer, 1994). The 203 described species of attines are divided into 11 genera (Kempf, 1972; Weber, 1972; Hölldobler and Wilson, 1990). Ants of the 9 "lesser" genera (*Mycetarotes*, *Myrmicocrypta*, *Mycocepurus*, *Apterostigma*, *Cyphomyrmex*, *Mycetosoritis*, *Mycetophylax*, *Sericomyrmex*, and *Trachymyrmex*) tend to have small and inconspicuous colonies (fewer than 3000 workers) and generally collect insect excrement or small pieces of dead plant material to use as substrate for their fungal gardens. In contrast, ants belonging to the "advanced" genera of attines (*Acromyrmex* and *Atta*) have colonies that can grow to include many thousands to several million workers. Ants of these 2 genera generally depend on cutting fresh leaves and vegetation for their fungal gardens and consequently are commonly called "leaf-cutting" ants. Many species belonging to these 2 genera are major agricultural pests.

Fundamental evolutionary questions concerning attine ants have remained unanswered due to poor understanding of their phylogenetic relationships (see Wetterer, 1994; Schultz and Meier, 1995). The relationships among the attine genera proposed by earlier researchers show little agreement. For example, Wheeler (1910) proposed that *Myrmicocrypta* was the most "primitive" (i.e., basal) attine genus, but Weber (1972) and Hölldobler and Wilson (1990) both considered *Cyphomyrmex* the genus that retains the most primitive traits. Emery (1912) presented a phylogenetic tree of the attine ants, depicting a basal dichotomy with *Myrmicocrypta* and *Apterostigma* on one branch and all other attines (with *Cyphomyrmex* the most basal) on the other. In contrast, Kusnezov (1963) proposed that *Apterostigma*, *Myrmicocrypta*, and *Mycocepurus* were the most basal genera and considered *Cyphomyrmex* to be a fairly derived genus. The only agreement among all the proposed phylogenies was that *Trachymyrmex*, *Acromyrmex*, and *Atta* were closely related.

In the present study, we obtained mitochondrial DNA sequence data (the 3′ end of the CO I locus, an intergenic spacer region, the tRNA leucine locus, and the 5′ end of the CO II locus) from a variety of attine

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ants and performed parsimony analyses using both nucleotide data and translated amino acid data. We compared the results of these analyses with a similar analysis of data on the larval morphology of attine ants (Schultz and Meier, 1995). Finally, we combined the two data sets in a single "total evidence" analysis (Kluge, 1989).

MATERIALS AND METHODS

We sequenced mitochondrial DNA from attine ant samples representing 13 named taxa in seven genera: *Mycocepurus goeldii, Apterostigma collare, Apterostigma* cf. *pilosum, Cyphomyrmex rimosus, Sericomyrmex amabilis, Sericomyrmex* sp. nov., *Trachymyrmex* cf. *saussurei, Trachymyrmex zeteki, Acromyrmex octospinosus, Acromyrmex volcanus, Atta cephalotes, Atta sexdens sexdens,* and *Atta sexdens rubropilosa.* As a nonattine outgroup species, we used *Wasmannia auropunctata.* Parsimony analyses of larval characters indicated that this species is closely related to the attine ants (Schultz and Meier, 1995). We have deposited voucher ant specimens in the National Museum of Natural History at the Smithsonian Institution.

For most samples, we extracted DNA from whole live pupae. We used pupae because DNA yield was much higher than that for adults. Also, pupae have sterile digestive tracts and therefore have lower chance of contamination from gut bacteria. We had lesser success obtaining usable DNA from alcohol-preserved pupae and no success from alcohol-preserved adult workers.

Using the polymerase chain reaction (PCR), we amplified a segment of mitochondrial DNA using "universal" insect primers "George I" (sense strand) and "Marilyn" (anti-sense strand), devised by R. Harrison from sequence conserved between *Drosophila yakuba* and *Apis mellifera.* "George I" = 5′-ATACCTCGACGTTATTC-AGA-3′ with the 3′ end at position 2792 in the cytochrome c oxidase subunit I (CO I) locus (numbered according to the *D. yakuba* sequence from Clary and Wolstenholme, 1985). "Marilyn" = 5′-TCATAAGTTCAA/GTATCATTG-3′ with the 3′ end at 3383 in the cytochrome c oxidase subunit II (CO II) locus. For amplification we used 30 cycles of 45 s at 94°C, 45 s at 46°C, and 1 min at 72°C. Ant sequences are deposited in GenBank under Accession numbers AF016012 to AF016026.

For our morphological analyses, we used data on larval morphology presented in Schultz and Meier (1995). To compare our molecular analyses and the morphological analyses, we used a "pruned" version of the preferred tree of Schultz and Meier (1995) derived from a morphological data set consisting of 44 characters and 67 species, but "pruned" of all taxa except the 11 in the present molecular analyses. Two species in our molecular analyses, *A.* cf. *pilosum* and *T. zeteki,* were not represented in the morphological study.

RESULTS

We obtained a PCR product consisting of the 3′ section of the mitochondrial CO I region (183 to 198 total sites; 91 informative sites), an intervening highly variable intergenic spacer region (0 to 152 sites), the tRNA leucine region (65 to 74 sites), and the 5′ section of the mitochondrial CO II region (292 total sites; 140 informative sites). For four taxa of attine ants, we sequenced samples from two separate colonies. In *Acromyrmex octospinosus* and *Atta sexdens sexdens,* the coding regions of the replicate samples had identical sequences, and for *Sericomyrmex amabilis,* the coding regions of the replicates differed by only four bases, all silent substitutions. However, our two samples of *Cyphomyrmex rimosus,* one from Costa Rica and one from Brazil, differed by 66 nucleotide substitutions and 14 amino acid substitutions. We therefore treated *C. rimosus* and *C. rimosus* 2 as two separate taxa. *C. rimosus,* as currently defined, probably comprises a number of distinct species (J. Longino, pers. comm.).

The protein-coding CO I and CO II sequences were unambiguously aligned by eye for all species. CO I was the same length for *Wasmannia auropunctata, Mycocepurus goeldii, C. rimosus, Trachymyrmex zeteki T.,* cf. *saussurei, Sericomyrmex S. amabilis,* sp. nov., *Acromyrmex Ac. octospinosus,* and *volcanus.* However, *Atta cephalotes, Atta At. sexdens sexdens,* and *sexdens rubropilosa* each had 3 additional nucleotide bases (coding for one additional amino acid), *Apterostigma collare* had 9 more bases (three more amino acids), and *A.* cf. *pilosum* had 15 more bases (five more amino acids).

The intergenic spacer sequences could not be aligned with confidence, and for this reason we excluded them from parsimony analysis. The intergenic spacer region was considerably longer in leaf-cutting (*Atta* and *Acromyrmex*) species (from 134 bases in *Ac. octospinosus* to 152 bases in *At. sexdens sexdens*) than in the other species surveyed (from 0 bases in *A.* cf. *pilosum* to 42 bases in *T.* cf. *saussurei*). Sequence data from this region may ultimately prove to be phylogenetically informative, at least within genera.

For the tRNA leucine locus, only the sequences of the stems, the regions connecting stems, and the anticodon loop were alignable among species. The other loop regions varied in length among species and site homologies could not be assessed with confidence. We therefore included in our analysis only the 11 informative sites that were alignable among all the species surveyed.

We performed unweighted parsimony analysis of nucleotide bases for 242 informative characters (91 CO I sites, 140 CO II sites, and 11 tRNA sites) using the program PAUP 3.1 (Swofford, 1993) and obtained a single most parsimonious "nucleotide sequence" tree (Fig. 1). The ratio of unambiguous transitions to unam-

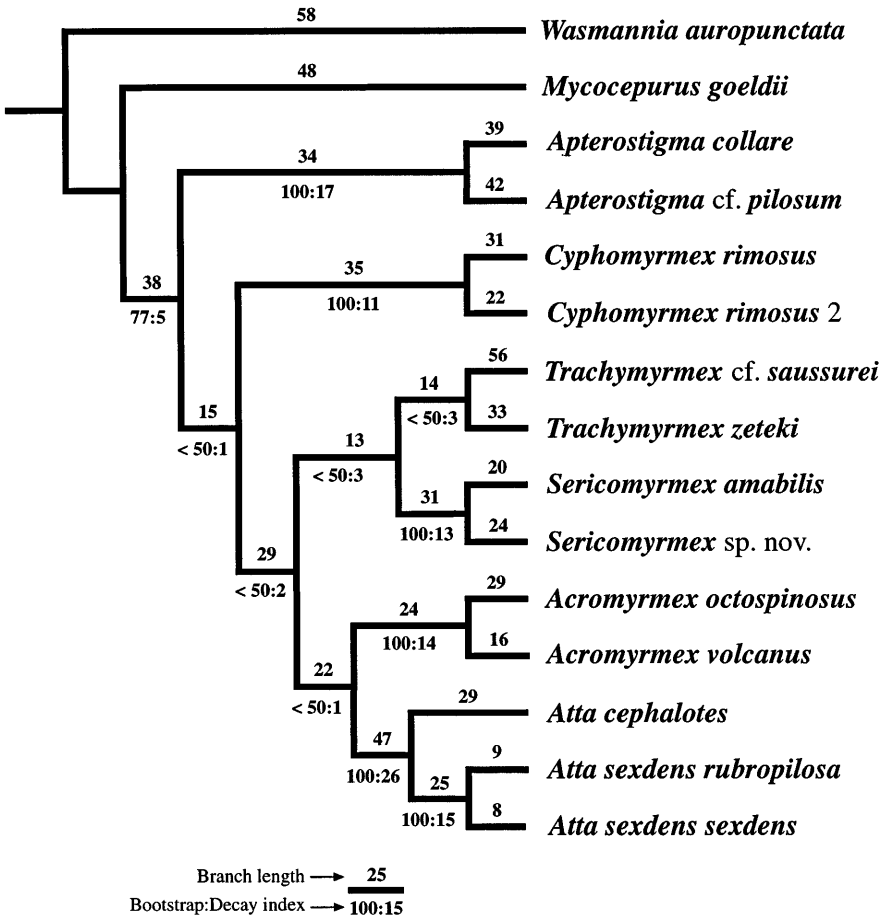

FIG. 1. The "nucleotide sequence" tree resulting from an unweighted parsimony analysis of 231 informative cytochrome oxidase and 11 informative tRNA nucleotide characters conducted in PAUP 3.1 (Swofford, 1993) using a heuristic search with 10 random-addition replicates. Length = 791, CI = 0.48, RI = 0.49. Numbers above each branch indicate branch length. The first number below each branch indicates bootstrap support from 1000 pseudoreplicates with 10 random addition heuristic searches per pseudoreplicate in PAUP 3.1 (Swofford, 1993); the second number below each branch is the decay index (a.k.a. Bremer support).

biguous transversions implied by the "nucleotide sequence" tree is 0.89 to 1 over all sites (0.76 for first + second codon positions; 0.99 for third positions). This predominance of transversions appears to relate to the high overall A/T content of the sequenced regions (0.74 for first + second positions; 0.85 for third positions). Under these conditions, the most rapidly evolving sites may have experienced multiple hits and the potential for nonhomologous parallel occurrences of As and Ts is high. This problem is most severe for third codon positions, 77% of which are variable across taxa (versus 39% for first + second positions).

To test for saturation at the third codon position, we partitioned the data into first and second positions only and third positions only and performed unweighted parsimony analyses. We obtained two most-parsimonious trees (MPTs) from the first + second position analysis (length = 282, CI = 0.557, RI = 0.619). The strict consensus of these two trees is well resolved and largely agrees with the morphology tree. We obtained five MPT's from the third position analysis (length = 477, CI = 0.442, RI = 0.408). The strict consensus of these five trees entirely lacks resolution for intergeneric relationships except for an almost certainly spurious basal position for the higher attine genus *Sericomyrmex*. This result is consistent with the pattern expected from rapidly evolving "silent" sites in which the ratio of noise to signal may be dangerously high. To determine whether significant incongruence exists between these data partitions, we conducted an Incongruence Length Difference (ILD) test (Farris *et al.*, 1995) using the "con-test" command in the program DADA (Nixon, 1994) with 100 iterations, five "autospin" random-additions searches per iteration, and the "mh*" command of Hennig86 (Farris, 1988). The result ($P = 0.52$) indicates a level of between-data-set incongruence in the center of the distribution of such measures obtained from random partitions drawn from the unpartitioned data, consistent with saturation at third positions or with some combination of partial satura-

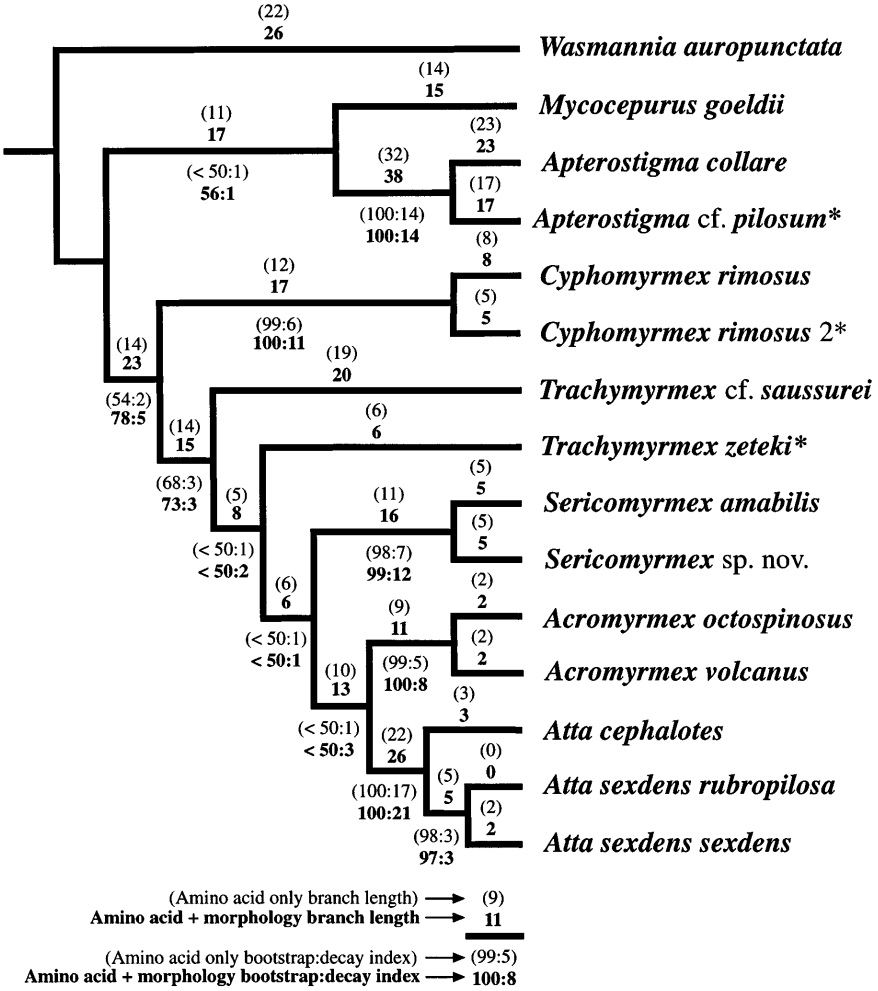

FIG. 2. The single most parsimonious tree for both the "amino acid sequence" and the "total evidence" (i.e., amino acid + morphology) analyses obtained from heuristic search with 10 random-addition replicates in PAUP 3.1 (Swofford, 1993). The topology is identical to that of the tree obtained when the preferred tree derived from larval morphological data (Schultz and Meier, 1995) is "pruned" of all taxa except those 11 taxa included in the molecular analyses. Asterisks (*) indicate taxa absent from the morphological data set; these were scored as missing values for the morphological characters in the "total evidence" analysis. Numbers above each branch indicate branch lengths for the amino acid sequence tree (in parentheses) and "total evidence" tree (boldface). Numbers below each branch indicate bootstrap support and decay indices for the amino acid sequence tree (in parentheses) and the "total evidence" tree (in boldface). Bootstrap values were obtained from 1000 pseudoreplicates, 10 random addition heuristic searches per pseudoreplicate, in PAUP 3.1 (Swofford, 1993).

tion and congruence, but inconsistent with strongly supported conflicting signal between the two data sets.

Under a scenario of strong phylogenetic signal at first and second positions and high levels of noise at third positions, we would expect only a small decrease in goodness-of-fit when third position character data are constrained to conform to the optimal tree topologies produced by analyzing the first+second position data relative to their fit on the most optimal trees for the third position data. Alternatively, we would expect a large decrease in goodness-of-fit when the first+second position data are constrained to conform to the topologies that are optimal for the third position data set relative to their fit on the most optimal trees for the first+second position data. We compared the length increase obtained when each data set is mapped onto the best-fitting tree produced by the alternative data set using the "compare 2 trees" command (1000 branch-and-bound iterations) in PAUP* (version 4.0d54; Swofford, 1997), evaluating significance using a permutation-based test (Archie, 1989; Faith, 1991) under the conservative two-tailed criterion (Engel and Schultz, 1997). This test avoids recent criticisms of permutation tests (Carpenter, 1992; Swofford *et al.*, 1996) because the null model of random behavior is appropriate for saturation due to multiple hits. The results indicate that the length difference of 9 obtained by constraining the third position data to the best-fitting first+second position tree is indistinguishable from that expected from a data set comprised of pure noise ($P = 0.102$ in

the most significant of five pairwise comparisons). Conversely, the length difference of 28 obtained by constraining the first+second position data to conform to the best-fitting third position tree is significantly different from that expected from noise ($P = 0.001$ in each of the two pairwise comparisons).

Given these results, and given that silent substitutions can also occur at first and second positions, we chose to dampen noise at rapidly evolving sites by converting the protein-coding nucleotide sequences to amino acid sequences and to analyze the data using protein parsimony (Felsenstein, 1991). We created the protein-parsimony step matrix in MacClade (Maddison and Maddison, 1992) and analyzed the data by heuristic search (10 random-taxon-addition iterations) in PAUP 3.1 (Swofford, 1993). From this analysis, we obtained a single most parsimonious "amino acid sequence" tree (Fig. 2). This tree is identical in topology to that of the "pruned morphology" tree for the 11 species in both analyses.

Finally, we subjected the combined morphological and molecular data to a "total evidence" analysis (Kluge, 1989; Eernisse and Kluge, 1993), simultaneously applying the parsimony criterion to the morphological characters and the protein parsimony criterion to the amino acid characters. In order to combine the data, we assigned missing values for morphological characters to *A.* cf. *pilosum* and *T. zeteki*. Because the 11 tRNA characters are not protein-coding, and because ILD tests indicate that the data partition consisting of these 11 characters may be significantly incongruent with the morphology data set ($P = 0.06$; see Table 1), we chose to exclude them from the combined analysis. From this "total evidence" analysis, we obtained a single most parsimonious tree that is identical to the "amino acid sequence" tree (Fig. 2), and increases support for the majority of branches (Table 2).

TABLE 1

Results of the ILD Partition Congruence Significance Test (Farris *et al.*, 1995)

Data partition	*P*	Result (90% confidence)
Morphology vs all nucleotides	0.06	Reject congruence
Morphology vs tRNA	0.06	Reject congruence
Morphology vs amino acids	0.80	Accept congruence

Note. Tests assume a null hypothesis of congruence. Three data partitions after eliminating two non overlapping taxa, *Apterostigma* cf. *pilosum* and *Trachymyrmex zeteki*. The first two tests were carried out using the "con-test" command in the computer program DADA (Nixon, 1994) with 100 iterations and 10 autospin random addition searches per iteration using the "ie-" command in Hennig86 (Farris, 1988). The third test, employing a protein parsimony step matrix, was carried out with the "partition heterogeneity test" in PAUP 4.0d54 (Swofford, 1997) with 100 replicates and 10 random-addition heuristic searches per replicate. Assumed confidence level is 90%.

TABLE 2

Decay Indices (a.k.a. Bremer Support) for the Same Branches in Three Trees, the Morphology Tree, the Amino Acid Tree, and the Tree Obtained When the Two Data Sets are Combined (Fig. 2)

Branch	Morph tree	A.A. tree	Combined tree	Increase over A.A. tree
1. *Apterostigma* (2 spp.)	n.a.	14	14	0
2. 1 + *Mycocepurus*	2	1	1	0
3. *Cyphomyrmex* (2 spp.)	3	6	11	5
4. *Sericomyrmex* (2 spp.)	4	7	12	5
5. *Atta sexdens* (2 subspp.)	0	3	3	0
6. *Atta* (3 spp.)	2	17	21	4
7. *Acromyrmex* (2 spp.)	2	5	8	3
8. 4 + 6 + 7	n.a.	1	1	0
9. *Trachymyrmex zeteki* + 8	n.a.	1	2	1
10. *Trachymyrmex* cf. *saussurei* + 9	n.a.	3	3	3
11. 3 + 10	1	2	5	3
Total increase				24

Note. The last column indicates the increase in support over that found in the amino acid tree when the morphological characters are added to the analysis. In no case does a decrease in support occur for the amino acid tree. A single decrease occurs on branch 2 of the morphology tree.

DISCUSSION

The phylogenetic relationships we infer from our parsimony analyses of mitochondrial DNA data are congruent with those obtained from parallel analyses of larval morphological characters (Schultz and Meier, 1995). These congruent conclusions, drawn from two independent data sets, increase the confidence we place in the broad phylogeny Schultz and Meier (1995) obtained for the Attini (see Littlewood and Smith, 1995). The mitochondrial cytochrome oxidase I and II genes appear to be excellent for phylogenetic comparisons of genera and species within the tribe Attini, at least at the amino acid level. This region has also been successfully employed in other lower level phylogenetic studies of Hymenoptera and other insects, but with similar problems of high A/T content and saturation, particularly at the third codon position (e.g., Willis *et al.*, 1992; Spicer, 1995; Emerson and Wallis, 1995; Pedersen, 1996; Ayala *et al.*, 1996), making this region less useful for higher level phylogenetic inference (Liu and Beckenbach, 1992).

Establishing a reliable phylogeny for the attine ant species helps answer many long-standing evolutionary questions about these ants and their fungi. For example, whereas most attines grow their fungus in the form of mycelium, one species group, *C. rimosus*, grows its fungus in yeast form. If the yeast-growing *Cyphomyrmex* species were the basal attine clade, then it would be possible that growing yeast was the primitive condi-

tion among attine ants, as suggested by Weber (1972). Our analyses, however, support Kusnezov's (1963) proposal that *Apterostigma* and *Mycocepurus* are more basal genera than *Cyphomyrmex* and thus that yeast cultivation by *C. rimosus* is almost certainly a derived trait.

Weber (1982) referred to the genera *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta* as the "higher" attine genera, and our analyses suggest that species in these four genera do, in fact, form a clade, though the relationships within this clade remain uncertain. Many other questions concerning the phylogeny of attine ants deserve further attention (see Wetterer, 1994; Schultz and Meier, 1995). The present analysis suggests that as analyses of molecular and morphological characters are carried out for additional attine species, the phylogenetic hypotheses for this interesting and important group of ants will continue to converge on a stable result.

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