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Phylogeny of ants (Formicidae) based on morphology and DNA sequence data

C. Astruc,[a,b] J.F. Julien,[b] C. Errard,[a] and A. Lenoir[a,*]

[a] *IRBI Institut de Recherche sur la Biologie de l'Insecte, CNRS UMR 6035, Faculté des Sciences et Techniques, 37200 Tours, France*
[b] *Centre de Génétique Moléculaire—C.N.R.S, 1 Avenue de la Terrasse. 91198 Gif-sur-Yvette, France*

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Abstract

In order to reconstruct ants' phylogeny, we analysed DNA sequences for two nuclear genes, *abdominal-A* and *Ultrabithorax*, from 49 species of ants and two outgroups. As these genes control the development of the first segments of the abdomen in insects, which are very variable in ants (petiole, postpetiole, and gaster constriction), we hypothesized that the morphological variations between the subfamilies may be correlated with mutations of some *abd-A* or *Ubx* regions. Contrarily to our hypothesis, these sequences are highly conserved. The differences observed concern mainly third codon positions and present some saturation. Phylogenetic reconstructions were carried out using the genetic raw sequence data and by combining them with a set of morphological data (Total Evidence). Relations among subfamilies of ants remains poorly resolved with molecular data only, but adding these data to morphological characters confirms and reinforce the topology of Baroni Urbani et al. (1992): a Poneroid complex [Ponerinae, Cerapachyinae, Leptanillinae and army ants], a Formicoid complex [Dolichoderinae, Formicinae] and a Myrmecoid complex [Myrmicinae, Myrmeciinae, Pseudomyrmecinae, Nothomyrmeciinae]. Our molecular results allow resolution near the branch tips and three subfamilies (Dolichoderinae, Formicinae and Pseudomyrmecinae) always appear as monophyletic. The Formicinae and the Dolichoderinae have close relationships. The Camponotini appear as a strong clade inside the Formicinae. The Ponerinae are separated in two parts: the Ectatommini and all other tribes. The Cerapachyinae, Dorylinae, and Ecitoninae belong to the same clade, the Cerapachyinae being confirmed in their subfamily status. The Myrmicinae appears to be very heterogeneous, with the Attini forming a very stable and well-separated group.

Keywords: Ant phylogeny; Formicidae; Hox genes; Abdominal-A; Ultrabithorax

1. Introduction

The higher phylogeny and classification of ants remain controversial in many respects. These social insects are characterised by complex and sometimes very specialised behaviours, the evolutionary origin of many of them remains unclear. Whereas fungus growing has been demonstrated to be characteristic of only one monophyletic tribe, the Attini (North et al., 1997), the evolutionary origin of other behavioural traits, such as sewing or legionary foraging, is not ascertained. Conversely, some morphological features, such as the monstrous modified queens, that is clearly correlated with nomadism in legionary ants, can be interpreted either as a landmark for a common origin of the species concerned (Brady, 2003), or as the result of convergent evolution.

All ant species are grouped into a single family, the Formicidae, which is subdivided into anywhere from 10 to 20 subfamilies, according to various authors. Both the number and the relationships of these subfamilies have been repeatedly modified since Brown worked out the first modern classification of ants (Brown, 1954 and see Wilson, 1971). Major improvements were achieved with the proposal to create a Formicoid complex, regrouping the Formicinae, Dolichoderinae, and Aneuretinae, put forward by Taylor (1978) and the gathering of the Dorylinae, Aenictinae, Cerapachyinae,

* Corresponding author. Fax +33-247367285.
E-mail address: alain.lenoir@univ-tours.fr (A. Lenoir).

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and Ecitoninae in a clade as proposed by Bolton (1990a,b). However, Hölldobler and Wilson (1990) presented an alternate vision of ant relationships, in which Taylor's Formicoid complex was disbanded, and the Formicinae emerged as a sister group to all the other ants. Last but not least, Baroni Urbani et al. (1992) proposed the most exhaustive review to date, which recognised 17 subfamilies on the basis of a cladistic analysis of a set of 68 morphological and behavioural characters. In their paper, both the Formicoid and the Poneroid complexes, *sensu* Taylor, were re-established. However, the authors themselves concluded that their data set was insufficient to support a fully resolved ant phylogeny. More recently, Grimaldi et al. (1997) added the fossil Sphecomyrminae, and Perrault the Probolomyrmicinae (Perrault, 2000). The main object of the present work, therefore, was to increase the available character set by providing molecular data.

Mitochondrial or ribosomal DNA sequences have frequently been used, in the family Formicidae to establish phylogenetic relationships at the generic or tribal or subfamily level (Ayala et al., 1996; Baur et al., 1993; Baur et al., 1995; Baur et al., 1996; Brandão et al., 1999; Chenuil and McKey, 1996; Chiotis et al., 2000; Crozier et al., 1995; Feldhaar et al., 2003; Johnson et al., 2003; Savolainen and Vepsalainen, 2003; Ward and Brady, 2003; Wetterer et al., 1998). Preliminary complete trees of the present Formicidae family based on ribosomal RNA have been presented on the web by (Sullender, 1998; see also Sullender and Johnson, 1998) and Maddison (2002). However, the large excess of AT nucleotides in the hymenopterans mitochondrial genome makes its use difficult for long-range phylogenetic studies, unless it is accounted by a mutability of G and C nucleotides sufficiently high to account for the AT-richness as in the honeybee (Koulianos and Crozier, 1999). We thus focused our efforts on relatively stable nuclear genes: *abdominal-A* (*abd-A*) and *Ultrabithorax* (*Ubx*), which are part of an important set of developmental genes, structurally linked within the homeotic complex (Martin et al., 1995), *Ubx* being also involved in wing development (Abouheif and Wray, 2002). In all apocrite hymenopterans (including ants), the first abdominal segment is constricted, separating the mesosome from the gaster and fusing with the first abdominal segment to the thorax. In the ants, the second abdominal segment is also constricted and constitutes the petiole. In some subfamilies such as the Myrmicinae and the Pseudomyrmecinae the third abdominal segment becomes also constricted, constituting the post-petiole. As this region is developing under homeotic genes control, we hypothesized that morphology may be correlated with mutations of some of the *abdominal-A* and *Ultrabithorax* regions (Niculita et al., 2001).

Preliminary comparisons of the *abd-A* homeodomain coding sequences between a few Formicidae species had revealed that saturation is low for this gene within the Formicidae (from 6 to 11%) while it is clearly observed in inter-order comparisons between insects (from 22 to 26% among the Hymenoptera–Formicidae vs. honeybee) and reaches 35% between different insect orders (Table 1).

In order to increase the phylogenetic signal we chose to analyse both the *Ubx* homeobox and a 300 nucleotides fragment from the *abd-A* gene that includes the homeobox coding region and 173 nucleotides downstream. In *Drosophila melanogaster* this fragment encompasses a stretch of Glutamine codons, constituting a microsatellite that presents an important intraspecific polymorphism (Michalakis and Veuille, 1996). Our results show that this is not the case in the Formicidae neither in the Vespidae nor in the Mutillidae studied here. The protein sequences encoded by the fragments analysed here are highly conserved. Most of the observed variations lead to synonymous codons and show some saturation. Thus, the resolution of some of the deeper branches of the Formicidae phylogeny was made possible only by the combination of our molecular data with some of the morphological characters defined by Baroni Urbani et al. (1992).

2. Methods

2.1. Specimens

The species sampled, their taxonomic affinities, and their geographic origins are listed in Table 2. Forty-nine species of Formicidae encompassing 11 subfamilies and two hymenopteran outgroups (Vespidae and Mutillidae) have been used. We did not include ants of the rare Aneuretinae and Aenictinae subfamilies as well as the extinct subfamilies.

Collected specimens were frozen at −80 °C or placed in 100% ethanol at −20 °C. One species *Nothomyrmecia macrops* was only represented by a dried specimen from the Muséum National d'Histoire Naturelle de Paris collection, and only *adb-A* 137 bp were analysed. This species was recently included in the fossil genus *Prionomyrmex* and the subfamily Nothomyrmeciinae renamed Prionomyrmecinae (Baroni Urbani, 2000), but it was contradicted by Ward (Ward and Brady, 2003), so we used the name *Nothomyrmecia*.

2.2. DNA amplification and sequencing

The equivalent of approximately 10 mg of fresh tissue, corresponding to one or more individuals, was crushed for 5 min in 100 μl of extraction buffer (0.1 M EDTA; 1% SDS, 0.1 M Tris–HCl, pH 9.0) in a Eppendorf tube using a rotating pestle. Twenty-five microliters of 5 M potassium acetate were added and the tube placed at 4 °C for 5 min. After 15 min of centrifugation

Table 1
Distance matrix (uncorrected) expressed in percent of differences in a 173 nucleotides long fragment coding for the abd-A homeobox

	Formicidae	Formicidae	Formicidae	Formicidae	Formicidae	Hymenoptera	Diptera	Lepidoptera	Orthoptera	Coleoptera
	Formica selysi	*Lasius alienus*	*Myrmica rubra*	*Myrmecia piliventris*	*Ectatomma ruidum*	*Apis mellifera*	*Drosophila melanogaster*	*Manduca sexta*	*Schistocerca gregaria*	*Tribolium castaneum*
Formica selysi		6	9	10	11	26	29	23	25	26
Lasius alienus			7	8	7	23	26	25	24	25
Myrmica rubra				7	6	24	26	25	26	24
Myrmecia piliventris					7	23	26	25	24	25
Ectatomma ruidum						22	23	28	24	27
Apis mellifera							22	22	14	35
Drosophila melanogaster								28	22	27
Manduca sexta									23	25
Schistocerca gregaria										35
Tribolium castaneum										

Niculita et al. (2001) *for* Myrmica rubra, Balavoine and Petrochilo, unpublished data other ant species, Walldorf et al. (1989) for honeybee and *Drosophila*, Tear et al. (1990) for *Schistocerca gregaria*, Nagy et al. (1991) for *Manduca sexta*, Shippy et al. (1998) for *Tribolium castaneum*. In dashed areas distances between ants.

at 10,000*g* the supernatant was collected and precipitated with 0.5 volume of isopropanol. The DNA precipitate was dissolved in 100 µl of 1 mM EDTA, 10 mM Tris–HCl, pH 7.5. The yield was approximately 10 µg of genomic DNA per sample. Ten to 20 ng were used in each PCR.

The initial set of primers used for amplifying the *abd-A* homeodomain coding region was derived from the sequence obtained by Walldorf et al. (1989) for the *abd-A* gene of *Apis mellifera*. Sequences flanking the *abd-A* homeobox were obtained by Ligation Mediated PCR in the formicine ant *Formica selysi* (J.-F. Julien, unpublished results). This enabled us to generate a new set of primers capable of amplifying a 300 nucleotide fragment including the entire homeobox and 169 nucleotides downstream (Table 3). The primers used to amplify the *Ubx* homeodomain coding region were derived from the sequence of a genomic clone of the *Ubx* gene of *Myrmica rubra* (J.F. Julien unpublished results). They were chosen in regions highly conserved between *Drosophila melanogaster* and *Myrmica rubra*. The nucleotide sequences analysed in this work span 129 nucleotides encoding the homeodomain of the *Ubx* gene (33 ant species) and the equivalent region of the *abd-A* gene plus the 169 nucleotides downstream encoding most of the C-terminal part of the protein (297 nt for 49 ant species).

PCR amplifications were carried out in a volume of 25 µl under the following conditions: 94 °C for 40 s, annealing for 40 s, 72 °C for 40 s. This cycle was repeated 35 times. The annealing temperature varied from 52 to 64 °C for *abd-A* and 58–72 °C for *Ubx*, according to the species. The determination of the optimal annealing temperature and amplification conditions, were carried out on a Stratagene Gradient Robocycler. PCR products were purified by gel filtration using S-400 Micro-Spin columns (Pharmacia). Sequencing reactions were performed on both strands with the 33P-terminator Thermosequenase kit from Amersham.

2.3. Phylogenetics analysis

The Pile-up program was used to align sequences (GCG, Washington University Genetic Computing Group). For some species whose *Ubx* sequence was not available, analysis was limited to the sequence of *abd-A*.

In a first step, we used the raw sequence data to make reconstructions that generally exhibit a good resolution at the terminal level, among species, genera or even tribes while the deeper branches, at the subfamily level, are poorly supported as estimated by bootstrap percentage (Felsenstein, 1985). Two weighting methods were used in order to get rid of this homoplasic noise. First a rough successive weighting scheme was applied to the sequence data: all polymorphic sites within a group supported by a bootstrap percentage higher than 0.9 were eliminated, i.e., zero weighted, from the overall

Table 2
Species names, subfamily, their geographic localisation, and GenBank Accession code

Subfamily	Genus	Species	Abbr.	Geographic area	GenBank Accession	
					abd-A	Ubx
Cerapachyinae	*Cerapachys*	*sp.*	sp.	Cameroon	AY185213	AY185265
Dolichoderinae	*Azteca*	*sp.*	sp.	French Guyana	AY185214	AY185266
	Dolichoderus	*bidens*	b.	French Guyana	AY185215	AY185267
	Dolichoderus	*sp.*	sp.	Cameroon	AY185216	AY185268
	Tapinoma	*melanocephalum*	a.	French Guyana	AY185218	
Dorylinae	*Anomma*	*nigricans*	n.	Cameroon	AY185219	AY185270
Ecitoninae	*Eciton*	*burchelli*	b.	French Guyana	AY185220	AY185271
	Neivamyrmex	*pilosus*	p.	French Guyana	AY185221	
Formicinae	*Camponotus*	*femoratus*	f.	French Guyana	AY185222	AY185272
	Camponotus	*sp.*	sp.	French Guyana	AY185223	AY185273
	Camponotus	*vagus*	v.	France	AY185224	AY185274
	Dendromyrmex	*sp.*	sp.	French Guyana	AY185225	AY185275
	Formica	*selysi*	s.	France	AY185226	AY185276
	Gigantiops	*destructor*	d.	French Guyana	AY185227	
	Lasius	*alienus*	a.	France	AY185228	AY185277
	Oecophylla	*longinoda*	l.	Cameroon	AY185229	AY185278
	Polyrhachis	*laboriosa*	l.	Cameroon	AY185230	AY185279
Leptanillinae	*Leptanilla*	*sp.*	sp.	Spain	AY185231	
Myrmeciinae	*Myrmecia*	*nigriceps*	n.	Australia	AY185262	
	Myrmecia	*tarsata*	t.	Australia	AY185263	AY185299
Myrmicinae	*Acromyrmex*	*subterraneus*	s.	Brazil	AY185232	AY185280
	Atta	*sexdens*	s.	French Guyana	AY185233	
	Crematogaster	*brasiliensis*	b.	Brazil	AY185234	AY185281
	Crematogaster	*limata parabiotica*	lp.	French Guyana	AY185236	AY185283
	Cyphomyrmex	*salirini*	s.	French Guyana	AY185235	AY185282
	Manica	*rubida*	r.	France	AY185237	AY185284
	Messor	*capitatus*	c.	Spain	AY185238	AY185285
	Myrmica	*rubra*	r.	France	AY185239	AY185286
	Tetramorium	*bicarinatum*	b.	Japan	AY185240	AY185287
Nothomyrmeciinae	*Nothomyrmecia*	*macrops*	m.	Australia	AY185264	
Ponerinae	*Amblyopone*	*australis*	a.	Australia	AY185241	AY185288
	Ectatomma	*quadridens*	q.	French Guyana	AY185242	
	Ectatomma	*ruidum*	r.	Mexico	AY185243	AY185289
	Gnamptogenys	*striatula*	sp.	Brazil	AY185244	AY185290
	Leptogenys	*sp.*	sp.	French Guyana	AY185245	AY185291
	Odontomachus	*haematodus*	h.	Brazil	AY185246	AY185292
	Odontomachus	*magi*	m.	French Guyana	AY185247	
	Pachycondyla	*apicalis*	a.	Mexico	AY185248	
	Pachycondyla	*goeldi*	g.	French Guyana	AY185249	
	Pachycondyla	*obscuricornis*	o.	Brazil	AY185251	AY185293
	Platythyrea	*sinuatae*	s.	French Guyana	AY185252	AY185294
	Ponera	*coarctata*	c.	France	AY185253	AY185295
	Prionopelta	*sp.*	sp.	French Guyana	AY185254	
	Typhlomyrmex	*sp.*	sp.	French Guyana	AY185255	
	Paraponera	*clavata*	c.	French Guyana	AY185250	
Pseudomyrmecinae	*Myrcidris*	*epicharis*	e.	Brazil	AY185256	
	Pseudomyrmex	*gracilis*	g.	French Guyana	AY185257	
	Pseudomyrmex	*termitarius*	t.	French Guyana	AY185258	AY185296
	Tetraponera	*aethiops*	a.	Cameroon	AY185259	AY185297
Outgroups						
FAMILY: Mutillidae *Mutilla*		*sp*	i.	France	AY185260	AY185298
FAMILY: Vespidae *Polistes*		*sp.*	sp.	France	AY185261	

Abbr, abbreviation on figures.

set. In a second attempt "Normal Parsimony" was used as a weighting method (De Laet and Albert, 1999). In this method, all variable positions are weighted by a factor equivalent to the retention index minus the minimum number of steps necessary to produce the variation observed for that position. None of these methods significantly improved the resolution or the statistical support of the trees obtained.

Phylogenetic reconstruction was performed by Maximum Parsimony (MP) for species where both *abd-A*

Table 3
Primer localisation on the *M. rubra abd-A* (Niculita et al., 2001) and *Ubx* sequences

M. rubra Ubx sequence

```
                                          T TGTTCCAGGA GCGAACGGAA TGC  7240
5' TATGCATGGA AGACAAGACA GCCGCGCAAC TCTGGTTCGT TGTTCCAGGA GCGAACGGAA TGCGCAGGCG CGGCCGACAG ACCTACACGC GCTACCAGAC TCTGGAGCTG GAAAAAGAAT
3' ATACGTACCT TCTGTTCTGT CGGCGCGTTG AGACCAAGCA ACAAGGTCCT CGCTTGCCTT ACGCGTCCGC GCCGGCTGTC TGGATGTGCG CGATGGTCTG AGACCTCGAC CTTTTTCTTA
          10         20         30         40         50         60         70         80         90        100        110        120

         130        140        150        160        170        180        190        200        210        220        230        240
   TCCACACGAA CCACTATCTC ACCAGGCGGA GGCGGATCGA GATGGCACAC GCACTCTGCC TGACGGAACG GCAAATCAAG ATCTGGTTCC AGAATCGGCG AATGAAATTG AAGAAAGAGA
   AGGTGTGCTT GGTGATAGAG TGGTCCGCCT CCGCCTAGCT CTACCGTGTG CGTGAGACGG ACTGCCTTGC CGTTTAGTTC TAGACCAAGG TCTTAGCCGC TTACTTTAAC TTCTTTCTCT

         250        260        270        280        290        300        310        320        330        340        350        360
   TTCAGGCGAT CAAGGAGCTA AACGAGCAGG AAAACGAAGC GCAGGCGCAA AAGGCGGCGG TAGCGGCAGC GCGGTTGCGC ATCAGCAGCA AGGGGGCGGC GGGGGACCGG AAGGGGGCAA 3'
   AAGTCCGCTA GTTCCTCGAT TTGCTCGTCC TTTTGCTTCG CGTCCGCGTT TTCCGCCGCC ATCGCCGTCG CGCCAACGCG TAGTCGTCGT TCCCCCGCCG CCCCCTGGCC TTCCCCCGTT 5'
7241 GTCCGCTA GTTCCTCGAT TTGCTCGTCC TTTTGCTTCG CG
                                          7242 GTCCGCGTT TTCCGCCGCC ATCGCCGTCG CGCCA
```

M. rubra abd-A sequence

```
   GACTAGT-CC -GAGGCGCAG GGGGCGGCAG ACGTACA  1650
   GACTAGTGCC CGAGGCGCAG AGGTCGACAA ACA  7858
5' GACTAGTGCC CGAGGCGCAG AGGTCGACAA ACACACACGC GGTTCCAAAC ACTGGAATTA GAAAAAGAGT TTCACTTCAA TCACTATCTG ACGCGACGAC GACGAATAGA GATTGCGCAT
3' CTGATCACGG GCTCCGCGTC TCCAGCTGTT TGTGTGTGCG CCAAGGTTTG TGACCTTAAT CTTTTTCTCA AAGTGAAGTT AGTGATAGAC TGCGCTGCTG CTGCTTATCT CTAACGCGTA
          10         20         30         40         50         60         70         80         90        100        110        120

         130        140        150        160        170        180        190        200        210        220        230        240
   GCACTCTGCC TGACGGAACG ACAGATCAAA ATCTGGTTCC AGAATCGGCG AATGAAACTG AAAAAGGAAT TAAGGGCTGT CAAAGAGATC AACGAGCAAG CCAGACGCGA GCGCGAGGAA
   CGTGAGACGG ACTGCCTTGC TGTCTAGTTT TAGACCAAGG TCTTAGCCGC TTACTTTGAC TTTTTCCTTA ATTCCCGACA GTTTCTCTAG TTGCTCGTTC GGTCTGCGCT CGCGCTCCTT
                                          1651 GCCGC CTACTTTAAC TTCTTCCTCA ACTCCCGCCA CTTTC

       250        260        270        280        290        300        310        320        330        340        350        360
   CAAGACATGA TGAAGAAACA GCAGGCGGAA AAACAGGCCA AATTACAACA GGAGCAGCAG AGCGCCGCCC TTCAGCATCA GCAGCAACAT CACGTAAGCG GCCTGGAAAA AACGCAAAGC
   GTTCTGTACT ACTTCTTTGT CGTCCGCCTT TTTGTCCGGT TTAATGTTGT CCTCGTCGTC TCGCGGCGGG AAGTCGTAGT CGTCGTTGTA GTGCATTCGC CGGACCTTTT TTGCGTTTCG
                                                                                                  8112 CGC CGGACCTTTT TTGCGTTTCG
                                                                                                                        8113  CG
         370        380        390        400        410        420        430
   GATCTTCTGA AGGCAGTCAG TAAGGTCCCG CGAGCGACGC CACGAACAGG CTTTTGGCTC GCAGATGACG AA 3'
   CTAGAAGACT TCCGTCAGTC ATTCCAGGGC GCTCGCTGCG GTGCTTGTCC GAAAACCGAG CGTCTACTGC TT 5'
   CTAGA
   CTAGAAGACT TCCGTCAGTC ATTCCA
```

In bold italics primers used.

and *Ubx* were known (Felsenstein, 1985) and Neighbour Joining (NJ) for all the species (Saitou and Naei, 1987), using Phylowin software (Galtier et al., 1996). The software did not allow MP analyses when data are missing.

Finally the "Total Evidence" (TE) approach was used, combining the published morphological characters with sequence data (Eernisse and Kluge, 1993), which revealed useful to the phylogeny of strepsiptera among the holometabolous insects (Whiting et al., 1997) or of fungus-growing ants (Wetterer et al., 1998) and to assess the evolution of social bees (Chavarria and Carpenter, 1994), army ants (Brady, 2003) or the "primitive" Australian ants (Ward and Brady, 2003). In all these examples, molecular analysis were not sufficient. For that analysis, our nucleotide data set was combined with a subset of the morphological characters (Baroni Urbani et al., 1992). Amongst these, we retained those characters that were present and non-polymorphic inside the subfamilies analysed here. We rejected polymorphic characters, as our samples generally did not cover all the different status of the character, inducing a bias in the construction. This subset represents more than 40% of the characters used by Baroni Urbani et al. They were encoded in Table 4 as an artificial DNA sequence in order to simplify and homogenise the data treatment, where "G" means presence and "A" means absence. Such a set, added to our molecular data, will thus strengthen the cohesion of the subfamilies, with only minor changes in their internal structure. We verified that, when used alone for phylogeny reconstruction, this morphological subset yields essentially the same tree as the consensus tree published by these authors.

3. Results

The deduced amino-acid sequences of *abd-A*, within as well as outside the homeodomain, are very highly conserved, including the outgroups (data not shown). Among the 49 species studied, *Gigantiops destructor* and *Prionopelta sp.* are the only ones to show a different number of residues: a deletion of one amino acid in the C-terminal region of *abd-A* (respectively, 89 and 94) for the two species. This particularity is accompanied by the substitution of Serine 87 by an Asparagine. This latter feature is shared by the three species of Dolichoderinae analysed here (and also by *Tapinoma erraticum*, GenBank codes AY185217 for *abd-A*, AY185269 for *Ubx*, data not analysed here). Unique amino-acid substitutions also affect the C-terminal region of *abd-A* in the two species of *Crematogaster*, *Ponera coarctata*, *Cerapachys sp.* and in *Myrmecia nigriceps*. The remaining ant species present a perfectly identical peptidic sequence.

The *Ubx* sequences are completely conserved in length and in amino acid sequence. The strong peptidic identity observed among taxa separated for several scores of MYR implies naturally a certain amount of saturation in the nucleotide changes that, in turn, affect mainly third codon positions. The amount of silent position substitutions (Ks) observed is actually 30% when sequences originating from different Formicidae subfamilies are compared (data not shown).

The phylogenetic trees are presented on Figs. 1 and 2 for the sequences and Figs. 3 and 4 for the "Total Evidence" (TE) dataset (NJ and MP, respectively, in both cases). At the highest levels of the phylogeny of the Formicidae, the sequence data do not support clear phylogeny (Figs. 1 and 2). On the contrary, the Total Evidence data set, analysed by NJ, highlights two groups: a first with Ponerinae, Cerapachyinae, Leptanillinae, and army ants (Poneroid complex), a second with the other sub-families (Fig. 3, bootstrap 1). This second group is separated in a Formicoid complex [Dolichoderinae + Formicinae] supported by a bootstrap percentage of 0.59 and a Myrmecoid complex [Myrmicinae + Myrmeciinae + Pseudomyrmecinae + Nothomyrmeciinae]. Inside the Myrmecoid complex, Myrmeciinae (bulldog ants) and Nothomyrmeciinae (*Nothomyrmecia*) form one clade, separated from Pseudomyrmecinae (acacia ants and relatives) plus Myrmicinae. However, when the same data set is analysed by MP (Fig. 4), this clade is disrupted with the Dolichoderinae separated from all other ants, probably due to the rooting.

The Dolichoderinae form a stable group at the sequence level with bootstrap supports of 0.9 by NJ (Fig. 1) and 0.73 by MP (Fig. 2), confirmed by TE (1 by NJ, Fig. 3 and 0.87 by MP, Fig. 4). Within this subfamily, the tribe Tapinomini, represented here by *Tapinoma* and *Azteca*, presents a stronger support than the grouping of the two *Dolichoderus* species.

The Formicinae subfamily behaves as a stable group, reconstituted by all the methods with a bootstrap percentage varying from 0.38 (NJ, Fig. 1) to 0.46 (MP, Fig. 2) using the nucleotide data set and 0.95–0.96 in TE analysis (Figs. 3 and 4). Inside this group *Camponotus*, *Dendromyrmex*, and *Polyrhachis* constitute a robust clade (bootstrap percentage from 0.87 to 0.96) that confirms their grouping into the tribe Camponotini. *Formica* (Formicini) and *Lasius* (Lasiini) seem related, being always part of the same clade, while *Gigantiops* (Gigantiopini) may (NJ–TE) or may not (NJ-genes only) be part of the group. *Oecophylla* (Oecophyllini) seems more closely related to the Camponotini than to *Formica* and *Lasius* in all the trees obtained (Figs. 1–4).

It is also clear that the Cerapachyinae are located well outside the Ponerinae and form a monophyletic group with the "Army Ants," Dorylinae (*Anomma)* and Ecitoninae (*Eciton*, *Neivamyrmex*). These results are

Table 4
Characters defined in Baroni Urbani et al. (1992) and used for the Total Evidence analysis

Taxa	No. of character in Baroni Urbani et al. (1992)																							
	12	18	22	25	27	28	29	30	31	33	35	36	38	39	40	41	43	46	53	54	55	57	60	65
Cerapachyinae	G	A	G	G	G	A	A	G	A	A	A	A	A	A	A	A	A	A	G	G	G	A	A	A
Dolichoderinae	A	G	A	A	A	A	A	A	A	A	A	A	G	A	G	A	A	A	G	A	A	A	A	A
Dorylinae	G	A	G	G	A	A	A	G	G	A	A	A	A	G	A	A	G	G	A	G	A	G	A	A
Ecitoninae	G	A	G	G	A	A	A	G	A	G	A	A	A	A	A	A	G	A	A	A	G	A	G	A
Formicinae	A	G	A	A	A	A	A	A	A	A	G	G	G	A	A	A	A	G	G	A	A	A	A	A
Leptanillinae	G	G	G	G	A	A	A	A	A	A	A	A	A	A	A	A	G	G	G	G	G	A	A	A
Myrmeciinae	G	G	A	G	G	A	A	A	A	A	A	A	A	A	A	G	A	A	A	A	G	A	A	A
Myrmicinae	A	G	A	G	A	G	A	A	A	A	A	A	A	A	A	A	A	A	G	A	G	A	A	A
Ponerinae	A	G	G	G	G	A	G	A	A	A	A	A	A	A	A	A	A	A	G	G	G	A	A	A
Pseudomyrmecinae	G	G	A	G	A	G	A	A	A	A	A	A	A	A	A	A	A	A	G	A	G	A	A	G
Mutillidae	A	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Vespidae	A	A	G	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

Character number in Baroni Urbani et al. (1992)	Description of the characters
12	Opening of the metapleural gland covered from above by cuticular flange (G), or absent (A)
18	Presternite of abdominal segment III protruding ventrally (A), or not (G)
22	Abdominal segment III without complete fusion of tergum and sternum (G), or absent (A)
25	Abdominal segment IV without differentiated presclerites (A), or without differentiated presternite and pretergite (G)
27	Presclerites of abdominal segment IV longer than one half of A III and with the sides running parallel (G) or not (A)
28	Presternite of abdominal segment IV, when present, subequal to or longer than the prestergite (A), or notably shorter than the pretergite (G)
29	Tergum and sternum of abdominal segment IV completely fused (G) or not (A)
30	Spiracles of abdominal segment 5–7 visible without dissection, (G), or not (A)
31	Pygidium bidentate (G), or not (A)
33	Pygidium reduced to a narrow U-shaped sclerite impressed or concave dorsally (G), or not (A)
35	Acidopore present (G), or not (A)
36	Sting and lancets articulated (G), or disarticulated (A)
38	Proventriculus sclerotised (G), or not (A)
39	Epithelium of the Dufour gland crenellate (G), or not (A)
40	Pavan's gland present (G), or absent (A)
41	Sting bulb gland present (G), or absent (A)
43	Gyne dichthadiiform (G), or not (A)
46	Gyne Bursa copulatrix exposed (G), or not (A)
53	Male Propodeal spiracle slit-shaped (A), or round to elliptical (G)
54	Male With tergosternal fusion of abdominal segment III (G), or not (A)
55	Male Abdominal segment IV whithout differentiated presclerites (A), or with differentiated presclerites (G)
57	Male Abdominal sternite VIII with (G) or without (A) long anterior apodemes
60	Male Genitalia with normally thin lamina annularis (A), or with very large lamina annularis, almost egg-shaped (G)
65	Larve with (G) or without (A) conspicuous food pocket

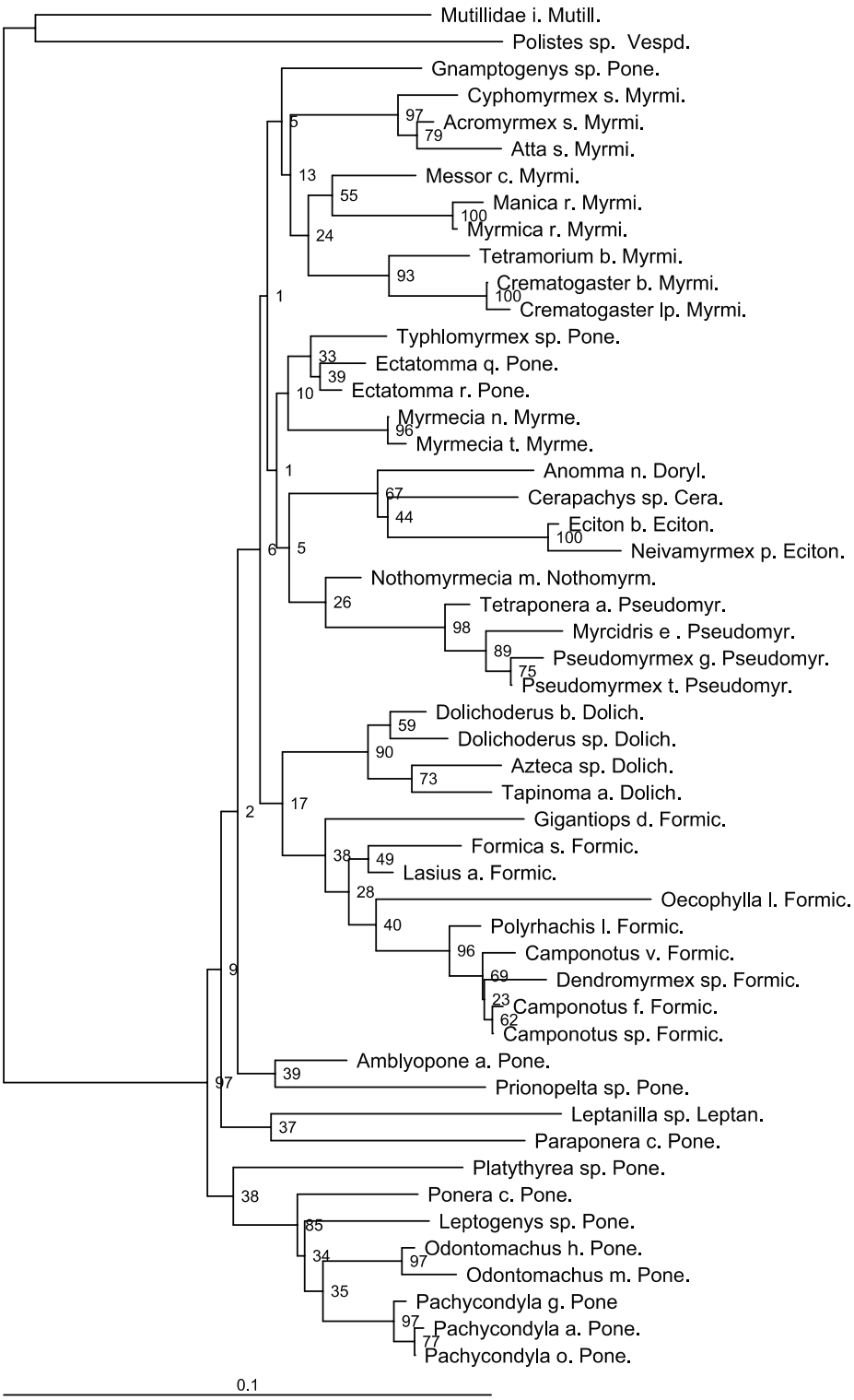

Fig. 1. Consensus phylogenetic tree from 1000 bootstrap replicates, obtained from the sequences alone, by the Neighbour Joining method. Distances were calculated according to Tajima and Nei. For the 51 species where the Ubx sequence were determined (49 ants, a Vespidae and a Mutilid), *abd-A* and *Ubx* sequences were concatenated. Percentage of bootstrap support are indicated.

supported with the sequences alone with a bootstrap varying, respectively, from 0.67 to 0.80 in NJ and MP (Figs. 1 and 2) and confirmed by bootstrap percentages from 0.96 to 0.99 in TE analysis (Figs. 3 and 4).

The results produced by the TE data set, with the two methods, splits up the subfamily Ponerinae in two distinct groups: the Ponerini, Amblyoponini, and Platythyreini, on the one hand, and *Ectatomma*,

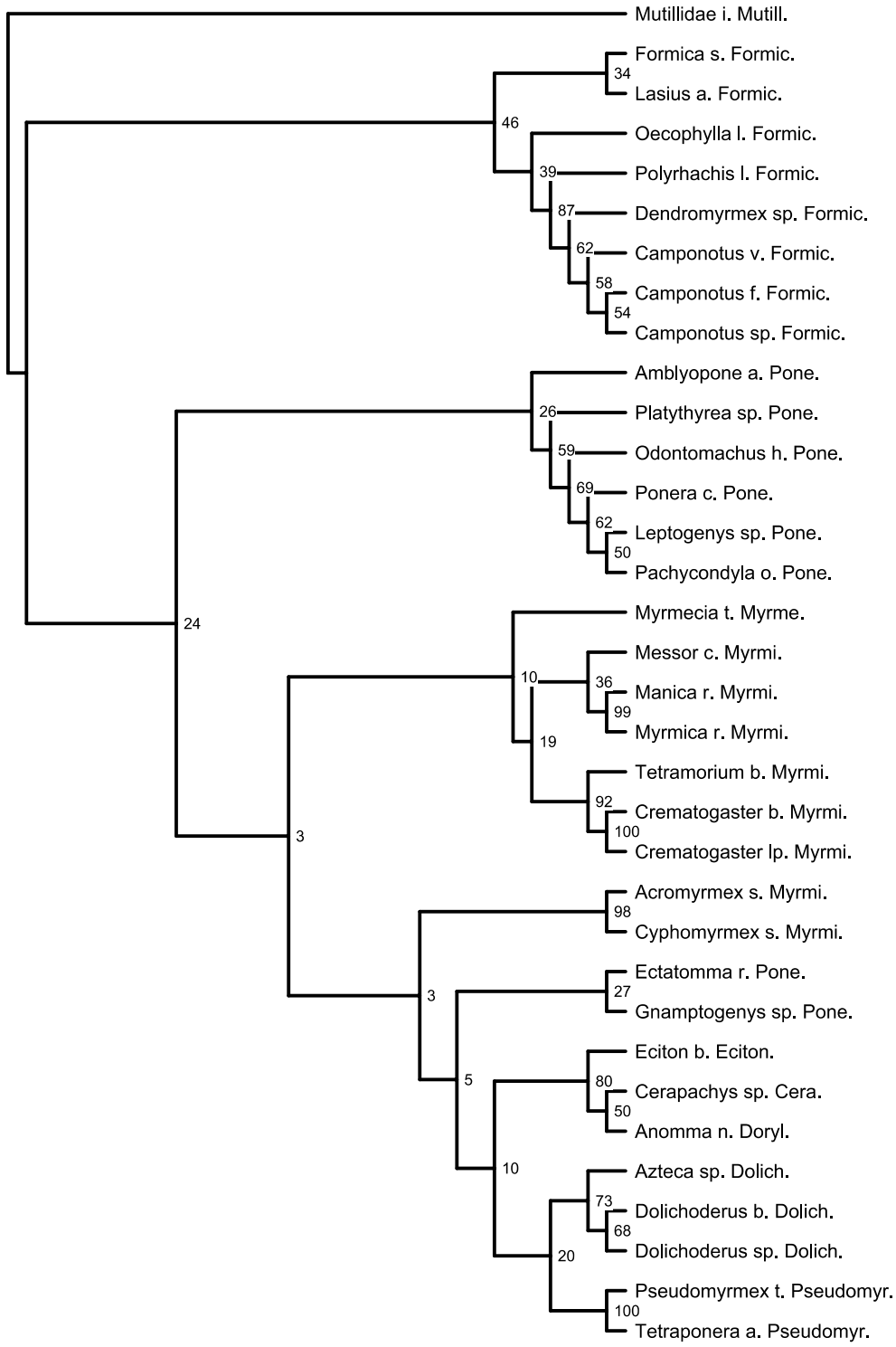

Fig. 2. Maximum-parsimony consensus tree from 1000 bootstrap replicates, obtained from the concatenated *abd-A* and *Ubx* sequences in the 35 species where both sequences are available.

Gnamptogenys, and *Typhlomyrmex* on the other. Although the two groups thus formed are not supported by strong bootstrap values, they are a constant feature of all the reconstructions we performed. In the absence of the *Ubx* sequence for the corresponding species, only *abd-A* sequences were taken into account in the following conclusions: *Ectatomma ruidum* seems paradoxically closer to *Typhlomyrmex sp.* than to *Ectatomma quadridens*. The position of *Paraponera clavata* is ambiguous; this species was once included in the Ectatommini subfamily, an arrangement which is never recovered in the tree reconstructions reported here

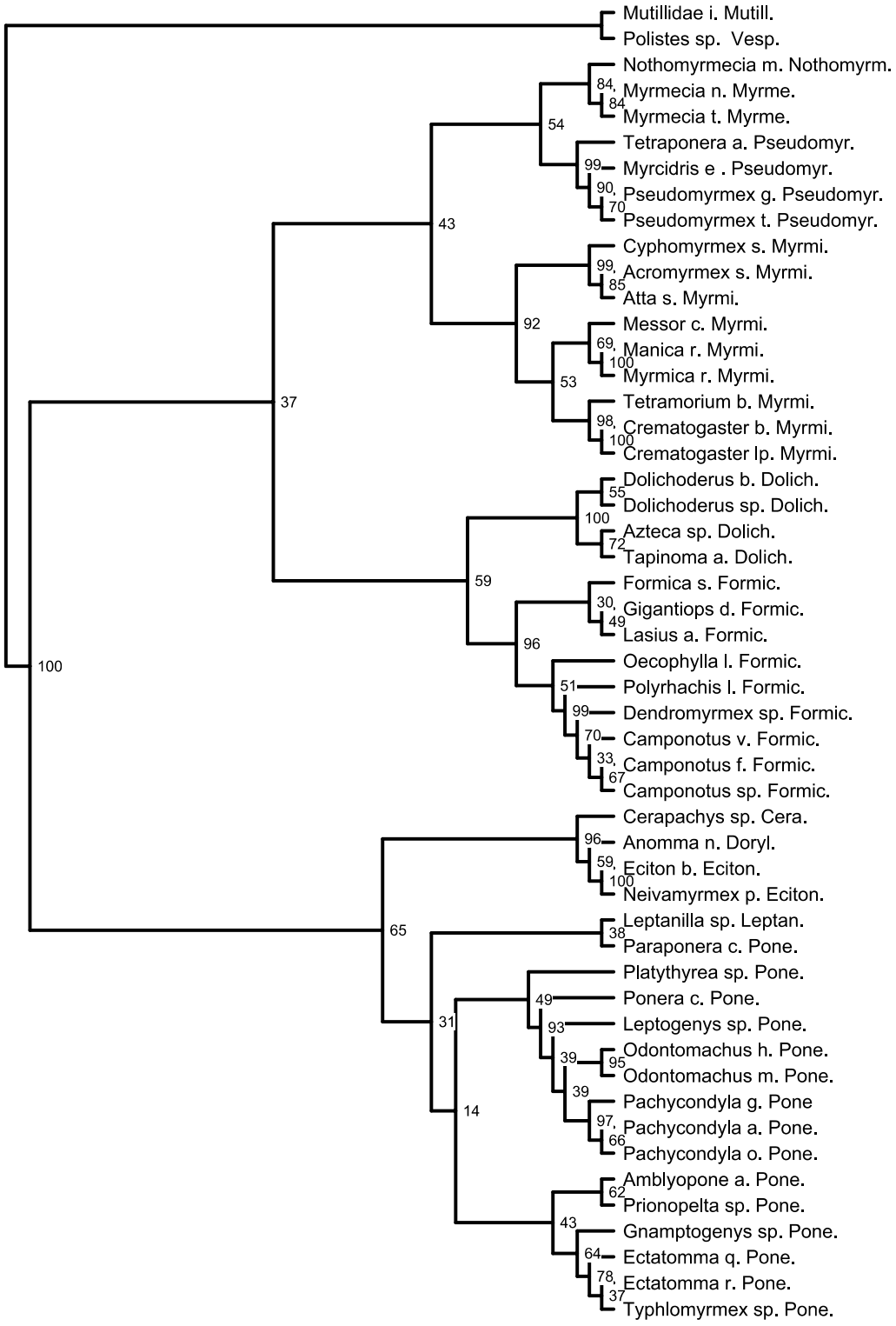

Fig. 3. Neighbour-joining consensus tree constructed from the "Total Evidence" dataset, including the *Ubx* sequence when available. Percentage of support from 1000 bootstrap replicates are indicated.

where this species segregates with *Leptanilla* (Leptanillinae), well apart from the Ectatommini, as well as from the clade formed by the Ponerini and *Odontomachus*. Along this latter branch, that regroups *Ponera*, *Leptogenys*, *Odontomachus* and *Pachycondyla*, *Ponera* emerges first, followed by *Leptogenys*, *Odontomachus*, and *Pachycondyla*. The Ponerinae subfamily appears to be deeply divided into at least two groups; comprising

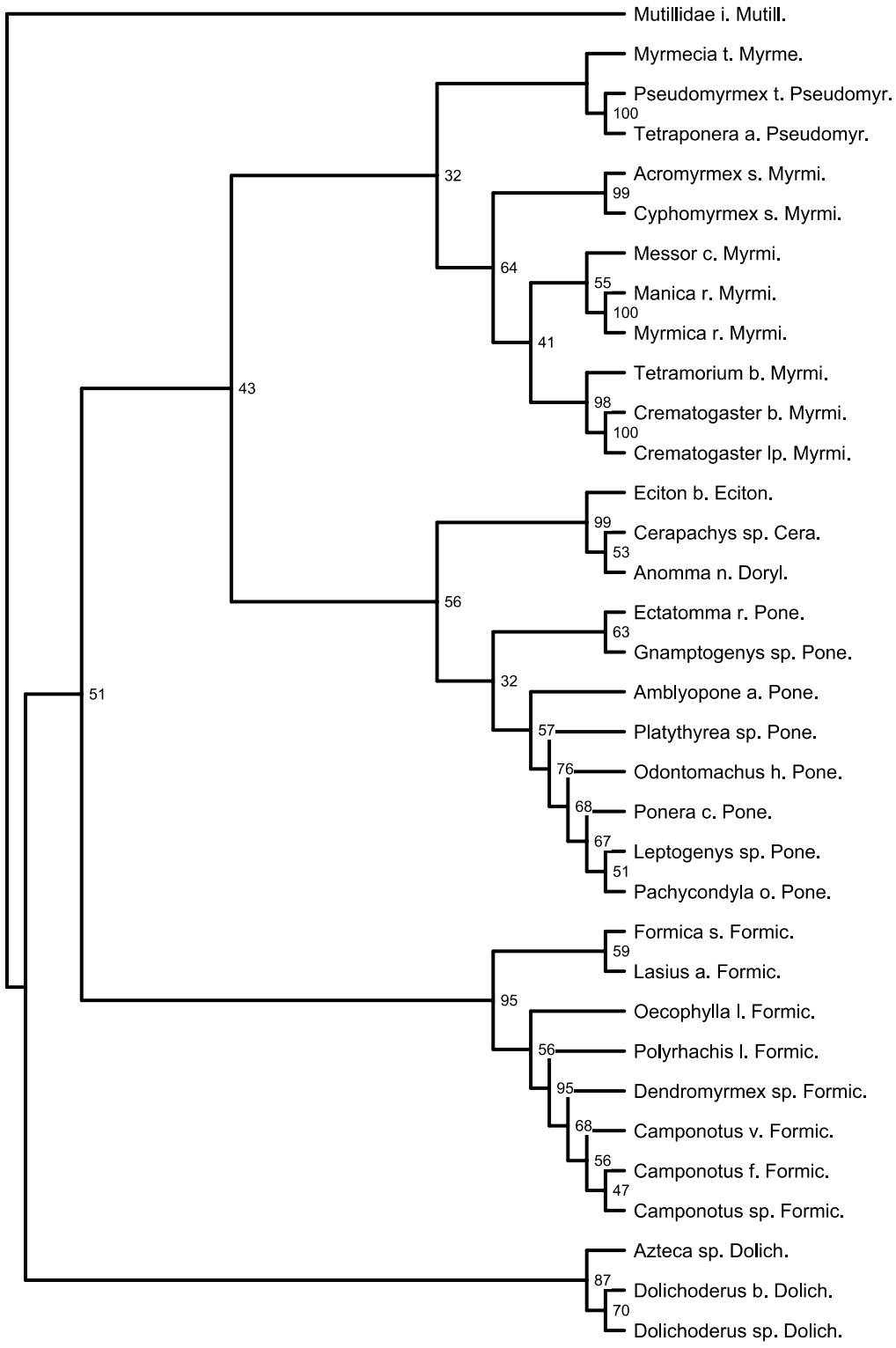

Fig. 4. Maximum-parsimony consensus tree from 1000 bootstrap replicates, obtained from the "Total Evidence" dataset, in the 35 species where both *abd-A* and *Ubx* sequences are available. Percentage of 1000 bootstrap trees are indicated.

on one hand the Ectatommini and, on the other, an assemblage composed of the Amblyoponini and the Ponerini, leaving the Leptanillinae and *Paraponera* in an undefined position.

While the Ponerinae subfamily is generally considered as a problematic one owing to its morphological and behavioural heterogeneity, the Myrmicinae are traditionally viewed as a well-supported clade.

This simplistic view is not confirmed by the reconstructions made from the sequences. The bootstrap support in the NJ tree is very low (0.13) and the Myrmicinae subfamily is split up in the MP tree. When the TE dataset is used, three groups emerge, one made up by *Tetramorium* (Tetramoriini) and *Crematogaster* (Crematogastrini), the second by *Myrmica*, *Manica* (Myrmicini) and *Messor* (Pheidolini) and the third by the tribe Attini that seems to have emerged earlier than the other two. Within the Attini, *Cyphomyrmex* emerges in a basal position.

The Pseudomyrmecinae, represented in our analysis by four species, form a solid monophyletic group supported by a bootstrap percentage ranging between 0.98 and 1 in all the analyses carried out and thus represents the most homogeneous subfamily met with. Within the Pseudomyrmecinae, in all the reconstructions, the same order of emergence is always observed: *Tetraponera* appears first, followed by *Myrcidris*, and *Pseudomyrmex*. This pattern is supported by high bootstrap indices from 0.98 to 1. Therefore, on the basis of DNA sequences analysis, *Myrcidris* cannot be considered as a basal genus in this family. The Pseudomyrmecinae subfamily is grouped with the Myrmicinae and the [Myrmeciinae + Nothomyrmeciinae] in the reconstruction operated from the TE dataset, confirming the existence of a Myrmecoid complex.

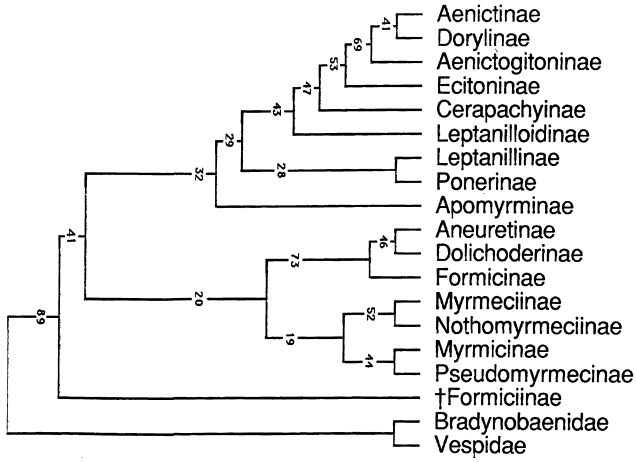

Fig. 5. Ant phylogeny derived from bootstrap analysis by Baroni Urbani et al. (1992)—Fig. 7 of Baroni Urbani.

4. Discussion

At the highest levels of phylogeny, our molecular based trees have many nonsensical deep relationships that result from the high degree of saturation at the third codon position and too conserved at the amino acid level. Longer sequences should be necessary. The Total Evidence trees therefore appear to come mainly from the morphological data and logically confirm the preferred tree presented by Baroni Urbani et al. (1992) with a Poneroid complex, and another branch grouping a Formicoid complex and a Myrmecoid one (including the Myrmicinae, Pseudomyrmecinae, Myrmeciinae, and Nothomyrmeciinae) (Fig. 5). It is noticeable that many bootstrap values are enhanced with our molecular data, compared to the Baroni Urbani preferred tree, and therefore reinforce it. These data are congruent also with the trees presented by Grimaldi et al. (1997) and Maddison (2002), the principal difference being that the Myrmecoid complex is dismantled, [Myrmicinae + Pseudomyrmecinae] being separated early from [Nothomyrmeciinae + Myrmeciinae]. These trees are very different from Sullender's one who considers all subfamilies of early emergence without any of the three complexes found here (Sullender, 1998).

In spite of the strong conservation and fairly high saturation level of the nuclear sequences used here, molecular phylogeny offers a reasonable reconstruction of the internal phylogeny of ants at a lower level within tribes. It should be stressed that, when considering only those bootstrap values higher than 50%, the two techniques of reconstruction tested, MP and NJ and the two data sets, molecular and Total Evidence used in this analysis, provide congruent results.

Although our molecular data alone bring little support to relationships among the subfamilies of ants, we have useful resolutions near the branch tips. Leaving aside the groupings supported by lower bootstrap values, on the basis of the sole molecular data set, three subfamilies, the Dolichoderinae, Formicinae, and Pseudomyrmecinae, always appear as monophyletic. Shattuck (1992) also demonstrated the monophyly of Dolichoderinae and Formicinae (Aneuretinae being probably included in this group). The subfamily Formicinae appears heterogeneous at the molecular level, formed at least by two clades. Inside this subfamily, the tribe Camponotini (including *Camponotus*, *Dendromyrmex*, and *Polyrhachis)* appears the only one to be very strongly based as indicated by Johnson et al. (2003).

Three other subfamilies, the Dorylinae, Ecitoninae, and Cerapachyinae always constitute a strong cluster. These data confirm the proposal of Bolton to raise the *Cerapachys* to a subfamily status and their grouping with army ants (Bolton, 1990a,b), but they are not congruent with Brady (2003) who put Cerapachyinae outside the army ant cluster.

The paraphyly of the Ponerinae is confirmed. This subfamily is clearly and deeply divided between a small Ectatommini section and a larger one, regrouping the Ponerini and all the other Ponerinae analysed here, including some apparently distant taxa, such as the Amblyoponini and the Platythyreini. Previous authors also observed this split between Ectatommini and all the other Ponerinae (Sullender, 1998; Sullender and

Johnson, 1998; Ward and Brady, 2003). It is difficult to choose between a radical and old splitting of this group, leaving alone the Ectatommini, or a sudden explosion, blurring the relationships of the two sections, but it should be necessary to consider the Ectatommini as a valid subfamily.

Whereas the monophyly of Ponerinae has been often questioned, the Myrmicinae has always been considered as very homogeneous. Molecular data give a very different view. The Attini are a robust group where *Cyphomyrmex* emerges in basal position as indicated by Wetterer et al. (1998).

Lower bootstrap indices mostly affect the more diverse and populous subfamilies, the Ponerinae and the Myrmicinae, with the Ponerinae constituting an extreme case. According to Philippe et al. (1994), this feature can be viewed as the landmark of an extremely rapid internal diversification, rather than that of a very ancient origin for these two subfamilies. Indeed the sparse information gathered from fossil remains suggests that the Dolichoderinae and the Myrmicinae subfamilies are equally ancient. Yet, on the basis of the DNA sequence data the bootstrap support is quite strong for the Dolichoderinae and insignificant at the best for the Myrmicinae. In the absence of any reasonable alternative, we are reluctant to question the monophyly of the Myrmicinae. The difference in the behaviour of the DNA sequences between these two groups is better explained in terms of effective population sizes. Our proposal is that the Myrmicinae have diverged more because their populations have been consistently larger than those of the Dolichoderinae. Because negative selection has limited the divergence of the DNA sequences, more divergence simply generates more noise, which in turn lowers the support for the group.

This use of nuclear DNA sequences has mainly confirmed our understanding of the evolution of the Ants, however, it has brought strong support to some of the alternative proposals put forward by others over the years. Features such as the Formicoid complex and the monophyly of the army ants will be necessary components of any future Ant phylogeny. Progress will come with a better knowledge of the internal structure of the Ponerinae and the Myrmicinae and obviously with a better understanding of the relationships between these families. In the face of our results, we are confident that the use of longer and much less conserved nuclear genes, in terms of amino-acid sequences, will allow the construction of a fully resolved ant phylogeny.

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