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Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus* Blochmannia gen. nov.

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The systematic relationships of intracellular bacteria of 13 *Camponotus* species (carpenter ants) from America and Europe were compared to those of their hosts. Phylogenetic trees of the bacteria and the ants were based on 16S rDNA (*rrs*) gene sequences and mitochondrial cytochrome oxidase subunit I (COI) gene sequences, respectively. The bacterial endosymbionts of *Camponotus* spp. form a distinct lineage in the γ-subclass of the *Proteobacteria*. The taxa most closely related to these bacteria are endosymbionts of aphids and the tsetse fly. The bacterial and host phylogenies deduced from the sequence data show a high degree of congruence, providing significant evidence for cospeciation of the bacteria and the ants and a maternal transmission route of the symbionts. The cloned *rrs* genes of the endosymbionts contain putative intervening sequences (IVSs) with a much lower G+C content than the mean of the respective *rrs* genes. By *in situ* hybridization specific 16S rDNA oligonucleotide probes verified the presence of the bacteria within tissues of three of the eukaryotic hosts. It is proposed that the endosymbionts of these three carpenter ants be assigned to a new taxon '*Candidatus* Blochmannia gen. nov.' with the symbionts of the individual ants being species named according to their host, '*Candidatus* Blochmannia floridanus sp. nov.', '*Candidatus* Blochmannia herculeanus sp. nov.' and '*Candidatus* Blochmannia rufipes sp. nov.'.

Keywords: 16S rDNA, endosymbiosis, *Camponotus* spp., '*Candidatus* Blochmannia', cytochrome oxidase subunit I

INTRODUCTION

Symbioses between bacteria and insects are very widespread and frequently found in the orders *Coleoptera*, *Blattaria*, *Homoptera* and *Hymenoptera* (Buchner, 1965; Dasch *et al*., 1984; Douglas, 1989). In many cases these interspecies interactions are thought to be obligate and mutualistic. In fact, highly developed symbiotic systems are found in insects specializing on unbalanced diets such as plant sap or mammalian blood. The best characterized examples for such interactions are the intracellular endosymbionts of aphids (*Buchnera aphidicola*), tsetse flies (*Wigglesworthia glossinidia*), weevils and cockroaches (Aksoy, 1995; Baumann *et al*., 1995; Bandi *et al*., 1995; Heddi *et al*., 1999). In all of these symbiotic interactions the micro-organisms appear to be involved in metabolic processes, e.g. provision with essential amino acids and vitamins or recycling of uric acid nitrogen for their host organisms (Baumann *et al*., 1995; Cochran, 1985; Douglas, 1998; Heddi *et al*., 1999; Nogge, 1982). Inside the body of their host species such symbiotic bacteria are resident within specialized host cells, the mycetocytes, which can form

Abbreviations: COI, cytochrome oxidase subunit I; IVS, intervening sequence.

The GenBank accession numbers for the partial cytochrome oxidase subunit I sequences reported in this paper are AF187954–AF187962.

symbiotic organs, so-called mycetomes. Alternatively, the bacteria-containing mycetocytes can be intercalated between regular enterocytes of the mid-gut epithelium. In agreement with the intimate association of the bacteria and their host, the micro-organisms generally cannot be cultivated outside the host tissues and the host organism usually suffers when they lose their symbionts (Baumann & Moran, 1997).

Essential symbiotic bacteria appear to be transmitted maternally, although in most cases the actual transmission pathway is not known. The fossil records of aphids suggest a very ancient coexistence of aphids with *Buchnera*, probably dating back to 160–250 million years (Baumann *et al*., 1997; Lambert & Moran, 1998; Moran *et al*., 1993). In agreement with this long-lasting association and with a vertical transmission route, the comparison of the phylogeny of the symbiotic bacteria and their host animals revealed a strictly congruent evolution (Baumann *et al*., 1997; Moran *et al*., 1993). A similar evolutionary relationship was recently described for tsetse flies and cockroaches with their respective intracellular symbiotic bacteria (Bandi *et al*., 1995; Chen *et al*., 1999). Other intracellular bacteria, for instance those belonging to the parasitic *Wolbachia* group, do not show cospeciation with their hosts indicating that horizontal exchanges have occurred (O'Neill *et al*., 1992).

In 1887 Blochmann described a close association of 'bacteria-like structures' with the tissues of the mid-gut and the ovaries of the ant species *Camponotus ligniperdus* (formerly *Camponotus ligniperda*; Bolton 1996) and *Formica fusca* which both belong to the subfamily *Formicinae* (Blochmann, 1887). These bacteria are Gram-negative rods of variable length, large numbers of which reside in the cytoplasm of mycetocytes intercalated between normal epithelial cells (Dasch *et al*., 1984; Schröder *et al*., 1996). The same bacteria are also found in the cytoplasm of oocytes of queens and workers, which suggests a transovarial transmission route of the bacteria (Buchner, 1965; Kolb, 1959; Schröder *et al*., 1996).

The recent comparison of the 16S rDNA sequences of the endosymbionts of four different *Camponotus* species revealed that they were most closely related to each other forming a distinct taxonomic group within the γ-subclass of *Proteobacteria* (Schröder *et al*., 1996). The most closely related taxa are symbionts of the tsetse fly and of the aphids (Aksoy, 1995; Baumann *et al*., 1995; Schröder *et al*., 1996). The differences within the 16S rDNA sequences of the four *Camponotus* symbionts isolated from two European and two American ant species correlated with the geographical distribution of their host animals; this suggested the possibility of a concordant evolution of the ant symbionts and their host species (Schröder *et al*., 1996). In the present study we extended this analysis and investigated the systematic relationships and phylogeny of the symbionts of 13 *Camponotus* species isolated from Europe and America. In addition, partial mitochondrial cytochrome oxidase subunit I (COI) gene sequences of their host species were determined, allowing the comparison of the molecular phylogeny of both the symbionts and their host species. The phylogenetic trees obtained for the bacterial endosymbionts as well as for their host species suggests a highly synchronous cospeciation process of both partners.

METHODS

Ants, bacterial strains and growth conditions. The origin of the ant species used in this study is presented in Table 1. The ants were cultivated at 25 °C with 50 % humidity in plastic boxes providing artificial nests. They were fed with honey water and cockroach pieces (*Nauphoeta cinera*) twice a week. For cloning experiments the *Escherichia coli* strain DH5α (Pharmacia) was used and grown in Luria–Bertani (LB) broth (Gibco). If required, ampicillin was added at a final concentration of 50 μg ml^{-1}.

Characterization of the *rrs* genes of the endosymbionts. DNA cleavage with restriction enzymes, PCR, DNA ligation, preparation of plasmid DNA, gel electrophoresis on agarose gels and transformation of *E. coli* DH5α was performed according to standard procedures (Sambrook *et al*., 1989). DNA sequencing was carried out on an ABI PRISM 377 (PE Applied Biosystems) automatic sequencer according to the manufacturer's protocol. As templates for the PCR reactions, crushed mid-gut preparations of the ant species shown in Table 1 were used. The *rrs* genes of the endosymbionts were selectively amplified using the previously described primers SL (5′-TTGGGATCCAGAGTTTGATCATGGCTCAGAT-3′) and SR (5′-CACGAATTCTACCTTGTTACGACTTCACCCC-3′) (Schröder *et al*., 1996). The primer sequences contain restriction enzyme cleavage sites for cloning experiments and are complementary to conserved regions characteristic for eubacterial *rrs* genes (Amann *et al*., 1991, 1995). The PCR reactions were performed in a total volume of 100 μl containing 0·2 mM dNTPs, 1 mM $MgCl_2$, 70 pmol each oligonucleotide and 2·5 U *Taq* DNA polymerase (Promega). Amplification was performed in a Bio-Med 60 thermocycler with the following conditions: 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C and 3 min primer extension at 72 °C. Generally 40 PCR cycles were run. The amplified DNA fragments were purified using a QIAquick PCR Purification Kit (Qiagen) and ligated into pUC18 (Pharmacia).

Genetic linkage of the 16S and 23S genes in the rDNA operons of the endosymbiotic bacteria shown in Table 1 was investigated by PCR amplification of the intervening DNA segment between these two genes as described previously (Aksoy, 1995; Aksoy *et al*., 1997). Control PCR reactions were performed in parallel using primers specific for the *rrs* genes of the endosymbionts (camp.L, 5′-GAATTACTGGGCGTAAAGAGT-3′; camp.R, 5′-GGAACGTATTCACCGTGAC-3′) (Schröder *et al*., 1996).

Sequencing of partial mitochondrial COI of the ant species. DNA of individual ants was isolated using a CTAB-phenol extraction method (Hunt & Page, 1994). Part of the mitochondrial COI gene was amplified using the primers provided by Ted Schultz, Smithsonian Institution (unpublished data; for primer sequences see Gadau *et al*., 1999). PCR reactions were performed in a total volume of 25 μl containing 0·2 mM dNTPs, 1 mM $MgCl_2$, 70 pmol each

Table 1. Ant species, their geographical location and GenBank accession numbers for the 16S rDNA and partial COI sequences

Species (subgenus)	Geographical origin	Accession no. (16S rDNA)	Accession no. (COI)
Camponotus (*Myrmothrix*) *atriceps*	Trinidad	AJ245591	AF186361
Camponotus (*Tanaemyrmex*) *balzani*	Madre de Dios, Peru	AJ245596	AF187955
Camponotus (*Tanaemyrmex*) *castaneus*	Bayhead, Florida, USA	AJ245594	AF187959
Camponotus (*Myrmothrix*) *floridanus*	Ft Pierce, Florida, USA	X92549	AF186362
Camponotus (*Camponotus*) *herculeanus* (E)*	Bavaria, Germany	X92550	AF176687
Camponotus (*Camponotus*) *herculeanus* (A)*	Idaho, USA	AJ250715	Sequence identical to AF176687
Camponotus (*Camponotus*) *ligniperdus*	Leinach, Germany	X92551	AF176686
Camponotus (*Camponotus*) *pennsylvanicus*	USA	AJ245598	AF189360
Camponotus (*Myrmothrix*) *rufipes*	Rio, Brazil	X92552	AF187957
Camponotus (*Myrmothrix*) *rufipes* B	El Bagual, Argentina	AJ245597	AF187956
Camponotus (*Myrmepomis*) *sericeiventris*	Missiones, Argentina	AJ245593	AF187960
Camponotus (*Tanaemyrmex*) *silvicola*	Cuzco Amazonico, Peru	AJ245592	AF187954
Camponotus (*Tanaemyrmex*) *socius*	Florida, USA	AJ245595	AF187958

* *Camponotus herculeanus* was isolated in Europe (E) and in the USA (A).

oligonucleotide and 2·5 U *Taq* polymerase (Promega). The samples were amplified in a Bio-Med 60 Thermocycler and the conditions used for thermal cycling were as follows: denaturation of the target DNA at 94 °C for 3 min, followed by 35 cycles consisting of 1 min denaturation at 94 °C, 30 s primer annealing at 47 °C and 1 min 30 s extension at 72 °C. At the end of the cycles, the reaction mixture was kept at 72 °C for 5 min. PCR products were purified using microcon 100 microconcentrators (Amicon), followed by automated sequencing of double-stranded products (Big Dye-kit; Perkin Elmer). We sequenced from both directions and all sequences were unambiguously aligned by eye.

***In situ* hybridization.** The oligonucleotides used for *in situ* hybridization were described previously (Schröder *et al.*, 1996). They were enzymically labelled with digoxigenin-11-ddUTP by terminal transferase (Boehringer Mannheim) following the manufacturer's instructions. Mid-gut cryosections were fixed in 4 % (w/v) paraformaldehyde dissolved in PBS buffer, pH 7·2, for 30 min at 4 °C. Then they were rinsed in PBS, incubated for 15 min with 0·5 µg proteinase K ml^{-1}, rinsed and fixed once more as described above. Hybridization was carried out overnight with 60 ng labelled oligonucleotide in 20 µl 5 × SET hybridization buffer [5 × SET: 0·75 M NaCl, 5 mM EDTA, 0·1 M Tris, 0·2 % blocking reagent (Boehringer Mannheim), 0·025 % SDS] at 42 °C in the case of the hercu2 oligonucleotides and at 37 °C for the flori3 and rufi2 oligonucleotides. The slides were washed for 15 min in 0·2 × SET at room temperature: non-specific binding was blocked by covering the slides with a solution containing 100 mM maleic acid, 150 mM NaCl, 0·5 % blocking reagent for 30 min. The slides were then incubated with alkaline-phosphatase-conjugated Fab fragment specific for the digoxigenin moiety (Boehringer Mannheim). Unbound Fab fragments were removed by washing with a solution containing 100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5. Alkaline phosphatase activity was visualized by the formation of a darkly coloured insoluble precipitate. As a substrate, a 1:50 dilution of the nitro blue tetrazolium/bromo-4-chloro-3-indolylphosphate stock solution (Boehringer Mannheim) in a solution containing 100 mM Tris/HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5, was used.

Phylogenetic analysis. 16S rDNA sequences of the endosymbiotic bacteria were compared with the 16S DNA database of members of the γ-subclass of *Proteobacteria* which are deposited in the ribosomal database project (RDP) (Maidak *et al.*, 1997). Similarity values were transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The least square distance method of De Soete (1983), as well as the programs Neighbour-joining and Maximum-likelihood contained in the PHYLIP program package (Felsenstein, 1993; Saitou & Nei, 1987), were used for construction of phylogenetic dendrograms. The default settings of the program DNAML version 3.5c (transition/transversion rate 2·0; use of empirical base frequencies) were used for maximum-likelihood analysis (Felsenstein, 1993). For calculation of bootstrap values, 500 trees were analysed using the programs NJFIND and NJBOOT. The program PAUP 4.0 (Swofford, 1998) was used for the phylogenetic analysis of the mitochondrial COI DNA sequences.

RESULTS

Characterization of the 16S rDNA sequences of endosymbiotic bacteria of *Camponotus* species

The *rrs* genes encoding the 16S RNA of endosymbiotic bacteria from nine *Camponotus* species were amplified and analysed in addition to the four previously described *rrs* sequences (Schröder *et al.*, 1996) (Table 1). In all but three cases (*Camponotus atriceps*, *Camponotus sericeiventris* and *Camponotus pennsylvanicus*) almost the entire *rrs* gene DNA sequences were analysed corresponding to about 96 % of the *E. coli rrs* sequence. The shorter sequences contained between 1270 and 1320 nt. The comparison of the sequences with the 16S DNA sequences deposited in the RDP revealed that all of the endosymbionts are more closely

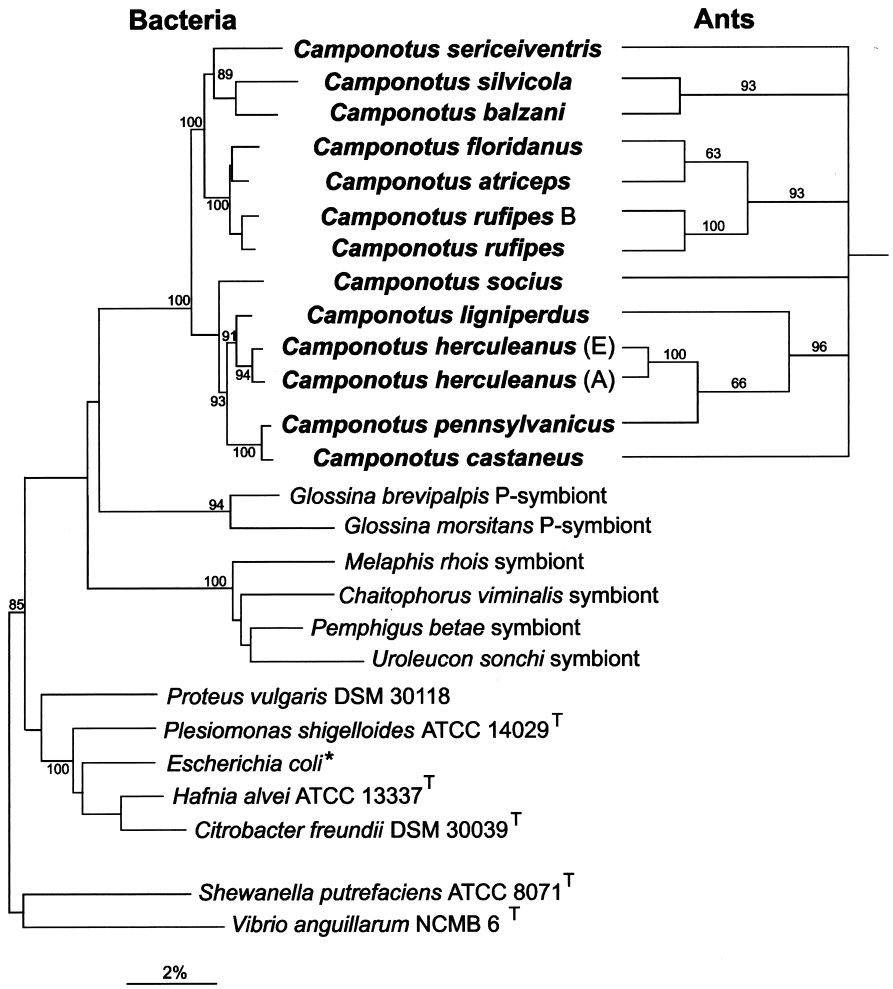

Fig. 1. Comparison of the phylogenetic trees of the endosymbionts of *Camponotus* species and their host organisms. The phylogenetic tree of the endosymbionts is based on their 16S rDNA sequences and was calculated according to De Soete (1983), whereas the tree of their host species is based on COI sequences calculated using the PAUP 4.0 program (Swofford, 1998). The bar represents 2% difference in the corrected sequences (Jukes & Cantor, 1969), as determined by measuring the length of the horizontal lines connecting two species. Numbers refer to bootstrap values of calculated 500 trees. For comparison, two primary endosymbionts of two tsetse fly species, four primary symbionts of aphids and several members of the *Enterobacteriaceae* are shown. The EMBL accession numbers of the 16S rDNA sequences used for this analysis are as follows: *Proteus vulgaris*, X07652; *Plesiomonas shigelloides*, M59159; *E. coli*, V00333; *Hafnia alvei*, M59155; *Citrobacter freundii*, AJ233408; *Shewanella putrefaciens*, X82133; *Vibrio anguillarum*, X16895; P-symbionts of *Glossina brevipalpis*, L37341; *Glossina morsitans*, L37339; *Melaphis rhois*, M63255; *Chaitophorus viminalis*, M63252; *Pemphigus betae*, M63254; *Uroleucon sonchi*, M63250.

related to each other than to any other bacterial species. In agreement with previous results, all of the symbionts are members of the γ-subclass of the *Proteobacteria*, where they form a new taxon which shows between about 10 and 14% divergence from the most closely related taxa, namely the endosymbionts of tsetse flies and those of aphids. Together these three taxonomic groups form a cluster of endosymbiotic bacteria, which is placed adjacent to members of the family *Enterobacteriaceae* (Fig. 1).

With the different tree algorithms used, identical phylogenetic branching patterns were obtained (Fig. 1). Within the *Camponotus* symbionts three subclusters are apparent, in which the strains are more related to each other than to the members of the other subclusters. The topology of this branching pattern is supported by highly significant bootstrap values (89–100%). The first subcluster consists of the symbionts of the South American ant species *Camponotus balzani* (Peru) and *Camponotus silvicola* (Peru), sharing a similarity value of 96·8%. The symbiont of *C. sericeiventris* (Argentina) also clusters with these two species, although it appears to be more distantly related (95·5–96·2% similarity). The second group contains the symbionts of four ant species with a similarity value of 97·9–98·9%, namely *Camponotus floridanus* (Florida, USA), *C. atriceps* (Trinidad),

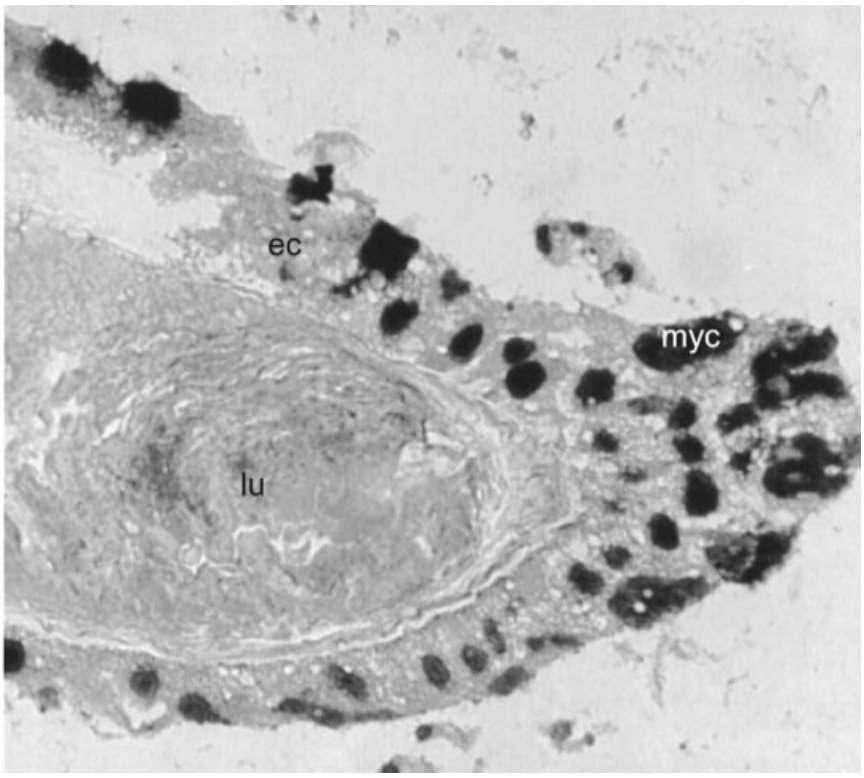

Fig. 2. *In situ* hybridization with oligonucleotides specific for the *C. floridanus* symbiont in a mid-gut preparation of *C. floridanus*. myc, Mycetocyte with hybridizing bacteria; ec, mid-gut epithelial cell; lu, lumen of the mid-gut with excrement.

Camponotus rufipes (Brazil) and the closely related *Camponotus rufipes* B (Argentina). The symbionts of the two ant species *C. ligniperdus* (Germany) and *Camponotus herculeanus* (Germany and USA) form a group together with the bacteria from two other species resident in the USA, *C. pennsylvanicus* and *Camponotus castaneus*, exhibiting a similarity of 98·1–99·7%. The symbiont of *Camponotus socius* (Florida, USA) does not fall in any of the clades, although it appears to be more closely related to the clade containing *C. ligniperdus*, *C. herculeanus*, *C. pennsylvanicus* and *C. castaneus* (similarity of 97·0–97·2%) than to the other clades (94·3–97·2%). Members of the first and second subcluster (no sequence information is available for the symbiont of *C. sericeiventris*) are characterized by 4–7 additional bases located at position 1550–1453 (*E. coli* sequence).

In situ detection of symbionts in mid-gut preparations of several *Camponotus* species

To confirm that the 16S rDNA sequences derived from the PCR reaction of mid-gut preparations of the ants indeed correspond to the symbiotic bacteria, we performed *in situ* hybridization experiments with three of the ant species, *C. rufipes*, *C. ligniperdus* and *C. floridanus*, using oligonucleotide primers specific for the respective symbionts. Fig. 2 shows a typical result obtained with a mid-gut preparation of *C. floridanus* and the oligonucleotide specific for its symbiont. The dark coloured cells represent the mycetocytes filled up with the symbiotic bacteria, which are intercalated between normal epithelial cells. No labelling was obtained using oligonucleotides specific for the other species (data not shown).

Intervening DNA sequences (IVSs) are present in the symbiont *rrs* genes

In several bacterial genera, including *Campylobacter*, *Helicobacter* and *Clostridium*, so-called IVSs were found representing DNA sequences present in the respective *rrs* genes, which are absent from the mature 16S rRNA sequences (see Rainey *et al*., 1996). Interestingly, the alignment of the endosymbiont 16S rDNA sequences with that of e.g. *E. coli* also revealed the presence of putative IVSs which are located between positions 82–88/83–87 (insert I) and 207–212/208–211 (insert II) of the *E. coli rrs* sequence. The length of the insert I sequences ranges between 7 and 21 nt, that of insert II between 6 and 31 bases. Only 2 bp constitute insert I in *C. ligniperdus* and *C. herculeanus* symbionts. Inserts are absent in the symbionts of tsetse fly and aphid symbionts as well as in *Enterobacteriaceae*. As in other cases, these sequences differ in their G+C content very much from the mean of the respective *rrs*

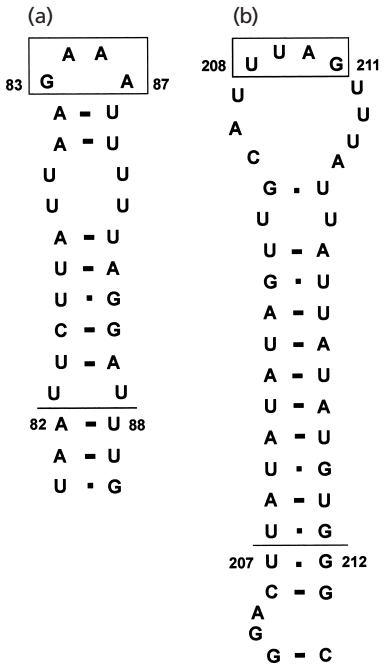

Fig. 3. Structure of the putative IVSs present in the 16S rDNA sequences of *C. rufipes* endosymbionts. Numbers refer to positions in the *E. coli* 16S rDNA sequence. (a) Insert I; (b) insert II.

genes. The G+C content of the 16S rDNA of the *Camponotus* endosymbionts ranges between 48 and 50 mol% which is in a similar range as that of tsetse fly and aphid symbionts, while that of *Enterobacteriaceae* is slightly higher (53–55%). The G+C content of the inserts ranges between 5 and 33%. Only the inserts of the *C. socius* symbiont exhibit a G+C content of 43 mol%. The inserts form a rather stable secondary structure. An example of the secondary structure of the inserts of the *C. rufipes* symbiont, which exhibits the longest inserts in both regions, are depicted in Fig. 3.

Structural organization of the rDNA operons of symbiotic bacteria

In the case of the free-living *Enterobacteriaceae* the rRNA genes are organized in the order 5′-16S–23S-5S-3′, forming a single transcription unit. The aphid symbionts are somewhat unusual, because their rRNA genes are organized into two transcription units (Baumann *et al.*, 1995). In fact, in *Buchnera* spp. the gene encoding the 16S RNA is not located upstream of the 23S RNA gene (Rouhbakhsh & Baumann, 1995). This is in contrast to the tsetse fly symbionts, where the 16S rDNA gene is located upstream of the 23S rDNA gene (Aksoy, 1995). Using a PCR strategy we attempted to analyse the relative position of the 16S rDNA in the case of the ant symbionts. As shown in Fig. 4, in the case of all 12 *Camponotus* symbionts tested so far, the 16S rDNA apparently is not located upstream of the 23S rDNA, and the 16S and 23S genes seem to be organized in different transcription units like in the aphid symbionts.

Characterization of the mitochondrial COI DNA sequence of several *Camponotus* species

DNA fragments (385 bp) containing part of the coding region of the mitochondrial COI from 13 *Camponotus* species were obtained and used for a phylogenetic analysis. These data were analysed using the branch-and-bound method implemented by PAUP 4.0 (Swofford, 1998). Two most parsimonious trees were found (tree length, 335; consistency index, 0·56; retention index, 0·55). Both trees differed only in the relative position of the three closely related taxa (*C. ligniperdus*, *C. herculeanus* and *C. pennsylvanicus*) and otherwise had the same topology. Bootstrap values based on 1000 replications are given in the strict consensus tree in Fig. 1. Clades with high bootstrap support are: (1) the *Camponotus sensu stricto* subgenus (*C. ligniperdus*, *C. herculeanus* (E), *C. herculeanus* (A) and *C. pennsylvanicus*); (2) the South American species of the subgenus *Tanaemyrmex* (*C. silvicola* and *C. balzani*); and (3) the North and South American species of the subgenus *Myrmothrix* (*C. floridanus*, *C. atriceps*, *C. rufipes* and *C. rufipes* B). *C. rufipes* B is not yet described as a valid species but sequence data and significant differences in certain physiological and life history features make it very likely that *C. rufipes* and *C. rufipes* B are in fact different species. The two North American species of the subgenus *Tanaemyrmex* (*C. socius* and *C. castaneus*) do not form a cluster, but *Tanaemyrmex* is a huge and very diverse subgenus and it is not surprising that these species do not cluster together. *C. sericeiventris* is, as the only member of the subgenus *Myrmepomis*, also part of the unresolved polytomy at the basis of the tree (Fig. 1).

Comparison of the phylogenetic trees of the bacterial endosymbionts and their host organisms

The phylogenetic tree of the endosymbionts of 13 different *Camponotus* species based on the 16S RNA sequences indicated the presence of at least three distinct groups, with the members of each group displaying greater similarity to each other than to the members of the other groups. Within these three groups, the symbionts of *C. sericeiventris* and *C. socius* are quite distantly related to their direct neighbours. The establishment of a phylogenetic tree of their host species based on COI DNA sequences allowed the comparison of the branching pattern of the symbiont and host animal trees. This analysis reveals a strikingly similar branching pattern in both trees (Fig. 1). The exception is *C. castaneus*, which cannot be related to any other species on the basis of the COI analysis, but its symbiont appears to be very closely related to those of *C. pennsylvanicus* and the other *sensu stricto Camponotus* species. Nevertheless, both trees show very significant taxonomic relationship with each other

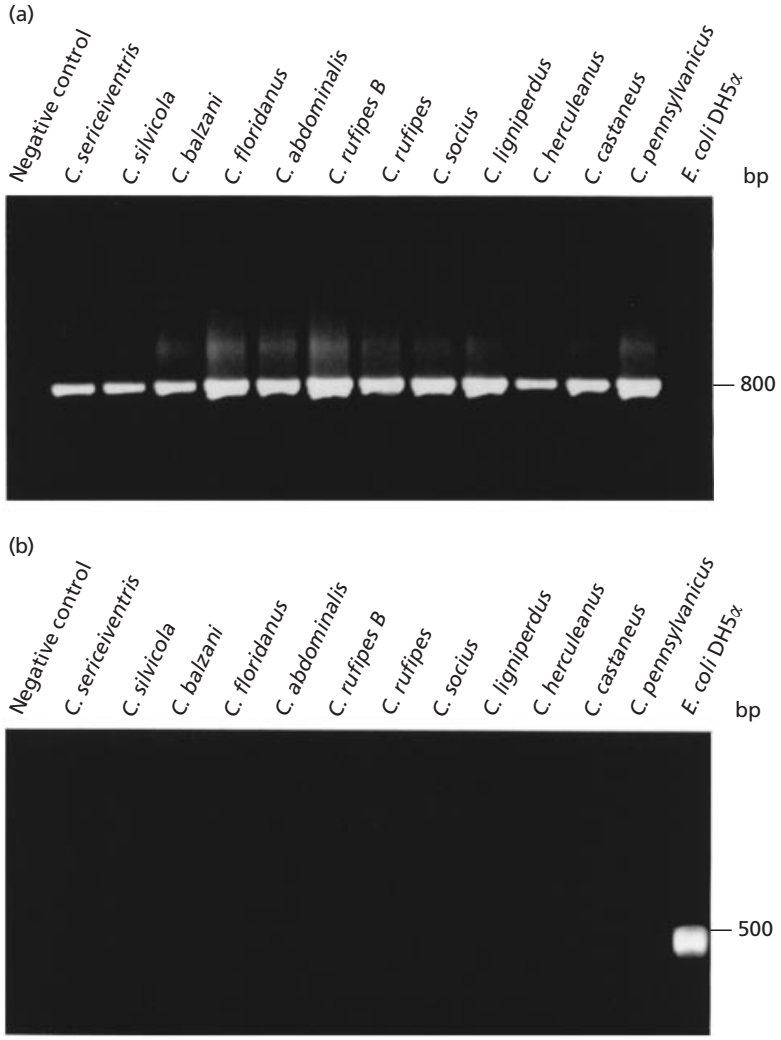

Fig. 4. (a) Agarose gel showing PCR products derived from primers specific for *Camponotus* symbionts binding within the respective 16S rDNA genes. (b) Agarose gel showing PCR products obtained with primers hybridizing with highly conserved regions of the 16S rDNA and 23S rDNA of eubacteria.

demonstrating a congruent evolution of the symbiotic bacteria and their host species.

DISCUSSION

It is generally believed that the transmission of intracellular endosymbionts residing in mycetocytes occurs vertically (Buchner, 1965). Accordingly, the evolution of the symbiotic bacteria and their host animals should have occurred in a highly congruent fashion. Such a cospeciation of symbiotic partners has in fact been documented for several bacteria/insect associations including those of aphids, tsetse flies and cockroaches with their respective symbionts (Baumann *et al*., 1995; Aksoy, 1995; Bandi *et al*., 1995). In the case of the ant/bacteria symbiosis, the phylogenetic trees of the bacteria and their host organisms are highly congruent for 12 out of the 13 ant/bacteria relationships analysed and generally fit very well with classical systematics based mainly on morphological criteria.

The North and South American species of the subgenus *Myrmothrix* (*C. floridanus*, *C. atriceps*, *C. rufipes* and *C. rufipes* B) form a distinct cluster within both phylogenetic trees derived from the 16S rDNA and COI sequences, respectively. The same holds true for the *Camponotus sensu stricto* group [*C. ligniperdus*, *C. herculeanus* (E), *C. herculeanus* (A) and *C. pennsylvanicus*]. On the basis of classical systematics *C. sericeiventris* belongs to the subgenus *Myrmepomis*. In agreement with this position, the analysis of the COI sequence did not reveal a significant relationship to the other ant species analysed here. Similarly, the 16S rDNA sequence of its symbionts also demonstrates a relatively isolated position of *C. sericeiventris*, but provides first evidence for a significant relation to several South American species (*C. silvicola*, *C. bal-*

zani) belonging to the subgenus *Tanaemyrmex*. The systematics of the subgenus *Tanaemyrmex* is complex. It is a huge and apparently quite heterogeneous subgenus which includes several species of uncertain classification. In fact, *C. silvicola* and *C. balzani* are the only species belonging to this subgenus for which a significant clustering is obvious on the basis of both the COI and the 16S rDNA data presented here. The other two *Tanaemyrmex* species analysed here, *C. socius* and *C. castaneus*, are found in different clusters of the symbiont dendrograms or cannot be resolved on the basis of the COI dendrograms. In the case of *C. socius*, the 16S rDNA sequence of its symbiotic bacteria indicates, however, a somewhat closer relationship of *C. socius* with the *Camponotus sensu stricto* subgenus (*C. ligniperdus*, *C. herculeanus* and *C. pennsylvanicus*) than with other species. We expect that further molecular analysis of other members of the subgenus *Tanaemyrmex* will lead to a major revision of their systematic positions.

The generally high degree of congruence in the phylogenetic trees of the endosymbiotic bacteria and their host ant species strongly indicates that they have diversified in parallel and supports the maternal transmission route. Despite the congruence of the phylogenetic trees of both the symbionts and their host animals, their geographical distribution is reflected only partially in the phylogenetic trees. For example, the species *C. floridanus* is living in Florida, but is more closely related to *C. rufipes*, isolated from geographically distant regions in South America, than to other species resident in Florida, such as *C. castaneus* and *C. socius*. This indicates that long distance migrations of ants have occurred in the past and that in general no horizontal transmission of the symbionts had occurred since then despite the geographical overlap or close neighbourhood of several distinct ant species.

The geographical distribution of *C. herculeanus* is of particular interest, because this species is found in Europe, Asia and in North America. In this study we have analysed the symbionts of a European *C. herculeanus* population which clusters together with the European and American species *C. ligniperdus* and *C. pennsylvanicus*, respectively. This fact is in agreement with classical systematics which classifies these species in the *Camponotus sensu stricto* subgenus. Moreover, the COI sequence of *C. herculeanus* derived from an American population was recently found to be identical to the COI sequence of the European counterpart (Gadau *et al*., 1999) and, as shown here, the 16S rDNA sequences of their symbionts show 99·2% similarity. This suggests that *C. herculeanus* populations have been separated only recently between the two continents, possibly after the disruption of the land bridge between Alaska and Asia at the end of the last ice age about 8000 years ago.

The only exception to this pattern of congruent evolution appears to be *C. castaneus*. Among the 13 species analysed it is the only one for which no congruence of the symbiont and COI based dendrograms is obvious. In fact, the close relationship of *C. castaneus* to *C. pennsylvanicus* detected on the basis of the symbiont dendrogram is neither supported by the phylogenetic analysis of the COI DNA sequences nor by morphological criteria (Creighton, 1950). Additionally, *C. herculeanus* and *C. ligniperdus* are clearly the closest relatives of *C. pennsylvanicus* and, in fact, they form a clade in the COI tree (bootstrap value 96%). Currently, we do not have an explanation for this discrepancy. *C. castaneus* and *C. pennsylvanicus* populations show extensive overlap in geographic range. Whether this indicates an exceptional event of a horizontal transmission of the symbionts is difficult to evaluate and must await further analysis in the future.

The phylogenetic depth of the cluster of the ant symbionts is as high as that of the symbionts of different tsetse fly species and different families of aphids. It is therefore possible that, as suggested also for the *Buchnera*/aphid association, symbiosis may have been an original trait present already in a common ancestor of the ants living more than 100 million years ago. On the other hand, so far the symbionts have been detected only in the ant subfamily *Formicinae*, but not in species of the ancestral subfamilies *Nothomyrmeciinae*, *Myrmeciinae* and *Ponerinae*. The careful analysis of ancestral ant lineages will provide insights in the age of this symbiosis in the future. Interestingly, the phylogenetic tree based on the 16S rDNA sequences also implies that the ant and aphid symbionts have a common ancestor. In many ecosystems ants and aphids are closely associated in beneficial symbiosis. In this context it is also noteworthy, that in contrast to free living *Enterobacteriaceae* and the symbionts of the tsetse flies, the ant and aphid symbionts both have unlinked 16S and 23S rRNA genes. The disruption of rRNA operons is a feature observed also in other intracellular bacteria of unrelated phylogenetic lineages such as the rickettsiae. Such a scrambled gene organization appears to be correlated with genome reduction, which is a typical feature of many obligate intracellular bacteria (Andersson & Kurland, 1998).

The extension of two helical regions by putative IVSs characterizes the 16S rDNA of all symbionts of *Camponotus* species. The shortest variations are detected in the *C. socius* symbiont which also exhibits a higher G+C content. These features correlate with the isolated position of this organism in the phylogenetic trees (Fig. 1). The length and G+C content differ among members of the individual 16S rDNA subclusters and do not allow affiliation of symbionts to these clusters on the basis of properties of the IVSs.

The phylogenetic distances between individual ant endosymbionts are in many cases larger than those found between various genera of *Enterobacteriaceae*, indicating that they may form different genera by themselves. However, as no sequence data of other genes from the ant symbionts are available, it is

possible that the substitution rate of the 16S rDNA sequences of the ant endosymbionts is higher than the normal isochronic substitution rate. In fact, recent data demonstrate that in endosymbiotic bacteria with a long history of maternal transmission destabilizing base substitutions accumulate in their 16S RNA genes (Lambert & Moran, 1998) and their coding genes show an excess of nucleotide substitutions causing amino acid replacements (Moran, 1996). In the case of the aphid symbionts the rate of 16S RNA evolutionary change was estimated to be about twice that of free-living bacteria and about 36-times greater than in homologous regions of the host 18S RNA (Moran, 1996; Moran *et al*., 1995).

The maternal transmission route of the symbiotic bacteria is in line with the fact that they are also found in the cytoplasm of the oocytes (Schröder *et al*., 1996), which distinguishes the ant symbionts from other intracellular endosymbionts such as those of the tsetse flies which could not be detected in the oocytes (Aksoy *et al*., 1997). In fact, there is increasing evidence that the bacterial invasion of the oocytes of the ants is the basis for the maternal transmission of the bacteria, because the symbionts can be detected in the endoderm forming the mid-gut epithelium very early during embryogenesis (C. Sauer, B. Hölldobler & R. Gross, unpublished results). In oviparous aphids, the bacteria are endocytosed by the ovum and occur as a 'symbiont ball' at the posterior pole of the egg. In viviparous aphids, the mycetocytes are found in close proximity to the aphid embryos and the bacteria are able to enter the blastocoel of young embryos. Finally, they are also endocytosed by the mycetocytes of the embryos (Baumann *et al*., 1995; Buchner, 1965; Hinde, 1971). In this respect it may be interesting to note, that in contrast to the ant symbionts, which are found free in the mycetocyte cytoplasm, *Buchnera* is located in vacuoles within the mycetocytes, but not free in the cytoplasm. The transmission routes of *Wigglesworthia* in the tsetse flies remains obscure, because they are neither found in the ovaries nor in milk glands of their host organisms (Aksoy *et al*., 1997). Therefore, despite several similar features of the various mycetocyte bacteria, there are substantial differences in their dissemination strategies which may reflect different origins or adaptation to their host's biology.

According to Murray & Schleifer (1994) the properties of uncultured organisms should be recorded by a '*Candidatus*' designation. Therefore, we propose for the *Camponotus* symbionts the designation '*Candidatus* Blochmannia' (Bloch.man′ni.a′ N.L. fem. n. referring to F. Blochmann, a German zoologist who provided the first description of the endosymbiotic bacteria in ants as early as 1887). '*Candidatus* Blochmannia' comprises Gram-negative rod-shaped bacteria which are found in the cytoplasm of specialized cells, so-called mycetocytes, intercalated between mid-gut epithelial cells or in oocytes of ants of the subfamily *Formicinae*. Bacterial cells are non-sporulating and divide by septation. They are 0·5–1·5 μm in width and have a quite variable length between 12 and 30 μm. On the basis of the 16S rDNA sequences, these bacteria belong to the γ-subclass of the *Proteobacteria*. The 16S RNA encoding *rrs* genes are not located upstream of the 23S RNA genes. Cospeciation with their host animals indicates that transmission of the bacteria generally occurs vertically. For the symbionts of the ant species *C. floridanus*, *C. rufipes* and *C. ligniperdus* the identification of unique regions within the 16S rDNA allowed their unequivocal identification by *in situ* hybridization in their respective host organisms. On the basis of these unique sequences we propose the symbionts of these three species to be designated as follows.

'*Candidatus* Blochmannia floridanus' (flo′ri.da.nus. N.L. masc. adj.) [(*Proteobacteria*) NC; NAS (GenBank no. X92549), oligonucleotide sequence complementary to a unique region of the 16S rRNA gene 5′-CTCTACTCAGTTCTTTGGG-3′; S (*Camponotus floridanus*, mycetocytes)] (Schröder *et al*., 1996).

'*Candidatus* Blochmannia rufipes' (ru.fi.pes. N.L. masc. adj.; *C. rufipes* is characterized by red legs) [(*Proteobacteria*) NC; NAS (GenBank no. X92552), oligonucleotide sequence complementary to a unique region of the 16S rRNA gene 5′-GTCTATGTAGT-TCTTTGG-3′; S (*Camponotus rufipes*, mycetocytes)] (Schröder *et al*., 1996).

'*Candidatus* Blochmannia herculeanus' (her′cu. lea.nus. N.L. masc. adj.) [(*Proteobacteria*) NC; NAS (GenBank no. X92550), oligonucleotide sequence complementary to a unique region of the 16S rRNA gene 5′-GTGGGCTATTACCCCG-3′; S (*Camponotus herculeanus*, mycetocytes)] (Schröder *et al*., 1996).

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