International Journal of Systematic and Evolutionary Microbiology (2006), **56**, 1403–1411 DOI 10.1099/ijs.0.64117-0

'*Candidatus* Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps

Martin Kaltenpoth,[1] Wolfgang Goettler,[1,2] Colin Dale,[3] J. William Stubblefield,[4] Gudrun Herzner,[2] Kerstin Roeser-Mueller[1] and Erhard Strohm[2]

Correspondence
Martin Kaltenpoth
martin.kaltenpoth@biozentrum.uni-wuerzburg.de

[1]University of Würzburg, Department for Animal Ecology and Tropical Biology, Am Hubland, D-97074 Würzburg, Germany

[2]University of Regensburg, Department of Zoology, D-93040 Regensburg, Germany

[3]University of Utah, Department of Biology, 257 South 1400 East, Salt Lake City, UT 84112, USA

[4]Fresh Pond Research Institute, 173 Harvey Street, Cambridge, MA 02140, USA

Symbiotic interactions with bacteria are essential for the survival and reproduction of many insects. The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) engages in a highly specific association with bacteria of the genus *Streptomyces* that appears to protect beewolf offspring against infection by pathogens. Using transmission and scanning electron microscopy, the bacteria were located in the antennal glands of female wasps, where they form dense cell clusters. Using genetic methods, closely related streptomycetes were found in the antennae of 27 *Philanthus* species (including two subspecies of *P. triangulum* from distant localities). In contrast, no endosymbionts could be detected in the antennae of other genera within the subfamily Philanthinae (*Aphilanthops*, *Clypeadon* and *Cerceris*). On the basis of morphological, genetic and ecological data, '*Candidatus* Streptomyces philanthi' is proposed. 16S rRNA gene sequence data are provided for 28 ecotypes of '*Candidatus* Streptomyces philanthi' that reside in different host species and subspecies of the genus *Philanthus*. Primers for the selective amplification of '*Candidatus* Streptomyces philanthi' and an oligonucleotide probe for specific detection by fluorescence *in situ* hybridization (FISH) are described.

INTRODUCTION

Many insects have evolved associations with endosymbiotic bacteria that are essential for reproduction or survival of the host (Moran & Baumann, 1994). Most of these bacteria are intracellular symbionts in specialist feeders, e.g. phloem-feeding, blood-sucking or wood-feeding insects (Baumann & Moran, 1997; Priest & Dewar, 2000). Since the diets of these insects lack essential nutrients, they depend on bacteria that are able to synthesize the necessary compounds (Bourtzis & Miller, 2003; Douglas, 1998). In many cases, symbiotic bacteria are transmitted vertically from one generation to the next, resulting in coevolution and cospeciation of hosts and symbionts which is reflected in congruent phylogenies (Bandi *et al.*, 1995; Baumann *et al.*, 1997; Chen *et al.*, 1999; Lo *et al.*, 2003; Moran *et al.*, 1993; Sauer *et al.*, 2000).

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) engages in a unique and highly specific symbiosis with bacteria of the genus *Streptomyces* (Kaltenpoth *et al.*, 2005). Female beewolves construct nest burrows in sandy soil, hunt honeybees (*Apis mellifera*), paralyse them by stinging and provision one to five honeybees as larval food in each brood cell (Strohm, 1995; Strohm & Linsenmair, 1995). After feeding on the provisioned prey, larvae spin a cocoon in which they usually overwinter and emerge the following summer (Strohm & Linsenmair, 1995). Since conditions in the brood cells are humid and warm, there is a continuous threat that the female's investment could be destroyed due to fungal or bacterial infection of the provisions or the immature wasp (Strohm

Abbreviations: FISH, fluorescence *in situ* hybridization; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the 28 ecotypes of '*Candidatus* Streptomyces philanthi' are DQ375779–DQ375806.

A description of the attempts made to cultivate '*Candidatus* Streptomyces philanthi' is available as supplementary material in IJSEM Online.

& Linsenmair, 2001). Recent studies have shown that symbiotic bacteria protect beewolf offspring against fungal infection at the cocoon stage (Kaltenpoth *et al.*, 2005).

The symbionts are cultivated in specialized antennal glands of the beewolf female and are secreted into the brood cell prior to oviposition (Kaltenpoth *et al.*, 2005; Strohm & Linsenmair, 1995). Later, they are taken up by the larva and applied to the outside of the cocoon, where they seem to serve as a protection against fungal infection, presumably by producing antifungal secondary metabolites (Kaltenpoth *et al.*, 2005). A second function of the secretion is to direct the cocoon-spinning of the larva which facilitates its eventual emergence (Strohm & Linsenmair, 1995). The bacteria certainly benefit from the association by obtaining an unoccupied and competition-free ecological niche and a reliable route of transmission into the next generation. They may also receive nutrients from the beewolf (M. Kaltenpoth and E. Strohm, unpublished data). A similar symbiotic relationship for pathogen defence between insects and actinomycetes has been described for leaf-cutter ants (Currie *et al.*, 1999). A species of the family *Pseudonocardiaceae* protects the ants' fungus gardens against a parasitic fungus by producing antibiotic substances (Cafaro & Currie, 2005; Currie *et al.*, 1999).

In the present study, we investigated 28 *Philanthus* species and subspecies and several closely related genera for the presence of endosymbiotic *Streptomyces* bacteria in their antennae. Ultrastructural and genetic data (16S rRNA gene sequences) are presented that support the description of '*Candidatus* Streptomyces philanthi', including 28 ecotypes in different host species and subspecies.

METHODS

Specimens. Specimens of 27 *Philanthus* species, including two subspecies of *P. triangulum*, two *Cerceris* species, *Aphilanthops frigidus* and two *Clypeadon* species, were collected in Germany, Greece, Oman, South Africa, Ukraine and the USA (Table 1). The South African specimens were identified by comparison with voucher specimens in the collection of the Albany Museum in Grahamstown, South Africa, and the South African Museum, Cape Town, South Africa. The US species were identified according to Bohart & Grissell (1975) and Ferguson (1983a, b). Because males lack the relevant glands (Strohm & Linsenmair, 1995) and the endosymbiotic bacteria have so far only been found in females (M. Kaltenpoth, unpublished data), only antennae from female specimens were used for electron microscopy and genetic analyses.

Electron microscopy. For scanning electron microscopy (SEM), specimens were fixed in alcoholic Bouin's fixative for 3 h and dehydrated in a graded acetone series. The specimens were then critical-point dried (CPD 030; BAL-TEC), sputtered with Pt/Pd (SCD 005; BAL-TEC) and examined with a digital scanning electron microscope (DSM 962; Zeiss). To investigate their interior ultrastructure, preserved antennae were cut with a razor blade before sputtering.

Specimens for transmission electron microscopy (TEM) were fixed for 2 h in a cold solution of 2 % glutaraldehyde, 2·5 % formaldehyde and 5 % sucrose buffered in 50 mM sodium cacodylate, pH 7·2. After post-fixation in 2 % OsO_4 and dehydration in an ethanol series, the specimens were embedded in Epon 812. Ultrathin sections of about 70 nm thickness (MT-7000 microtome; RMC; 45° diamond knife) were stained with 2 % uranyl acetate and Reynolds' lead citrate. Electron micrographs were taken with a transmission electron microscope (EM10; Zeiss) at 80 kV.

DNA extraction, PCR and sequencing. DNA was extracted from whole beewolf antennae according to a standard phenol/chloroform extraction protocol (Sambrook *et al.*, 1989). The following primer pairs were used for the amplification of *Streptomyces* 16S rRNA gene: fD1 (forward) (Weisburg *et al.*, 1991) and StrepF (reverse) (Rintala *et al.*, 2001), Act-S20 (forward) (Stach *et al.*, 2003) and rP2 (reverse) (Weisburg *et al.*, 1991). While primers fD1 and rP2 can be used to amplify a wide range of eubacterial 16S rRNA, the combination with StrepF and Act-S20 ensured that the PCR was specific for actinomycete 16S rRNA. PCR amplification was performed on Eppendorf Mastercyclers in a total reaction volume of 25 µl [containing 2 µl template, 1× PCR buffer (10 mM Tris/HCl, pH 8·8; 50 mM KCl; 0·08 % NP-40), 2·5 mM $MgCl_2$, 240 µM dNTPs, 20 pmol each primer and 1 U *Taq* DNA polymerase (MBI Fermentas)]. Cycle parameters were as follows: 3 min at 94 °C, followed by 32 cycles of 94 °C for 40 s, 65 °C for 1 min and 72 °C for 1 min, and a final extension time of 4 min at 72 °C. For sequencing, the following primers were used: fD1 (forward), Act-S20 (forward), Act-A19 (reverse) (Stach *et al.*, 2003), StrepF (reverse), rP2 (reverse).

For the selective amplification of *Philanthus* endosymbionts, the following forward primers were designed on the basis of the 16S rRNA gene sequences of the endosymbiotic *Streptomyces* and reference strains from the GenBank database: Strep_phil_fwd1, 5′-TACCGATCGC-ATGGTTGGTG-3′; Strep_phil_fwd2, 5′-TATGACTACYGAYCGCA-TGG-3′; Strep_phil_fwd3, 5′-CATGGTTRGTGGTGGAAAGC-3′; Strep_phil_fwd4, 5′-GTGGTGGAAAGCTCCGGC-3′ [binding to nucleotide positions 177–196, 170–188, 184–203 and 192–209, respectively, following the *Streptomyces ambofaciens* nomenclature (Pernodet *et al.*, 1989)]. The forward primers Strep_phil_fwd1–4 were used in combination with the general actinomycete reverse primer Act-A19. Temperature gradient PCRs were performed for all primer combinations and two Mg^{2+} concentrations were used to adjust the stringency of the reaction (1·5 and 2·5 mM). Final PCR conditions were the same as described above, except that 1·5 mM $MgCl_2$ was used for Strep_phil_fwd4/Act-A19. The annealing temperature was set at 65 °C for Strept_phil_fwd2/Act-A19 and to 68 °C for the three other primer combinations. DNA extracts from the antennae of 27 *Philanthus* species and one subspecies, two *Cerceris* species, *Aphilanthops frigidus* and two *Clypeadon* species (Table 1) were used as templates. Extracted DNA from cultures of *Streptomyces rimosus* DSM 40260^T, *Streptomyces aureofaciens* DSM 40631 and *Streptomyces venezuelae* DSM 40230^T was included to assess the specificity of the primers for *Philanthus* endosymbiont DNA.

Fluorescence *in situ* hybridization (FISH). The general eubacterial probe EUB 338 (Amann *et al.*, 1990) and the specific oligonucleotide probe SPT 177 (5′-Cy3-CACCAACCATGCGATCGGTA-3′) (Kaltenpoth *et al.*, 2005) were used for FISH. *S. aureofaciens* DSM 40631, *S. venezuelae* DSM 40230^T, *S. rimosus* DSM 40260^T and *Bacillus subtilis* DSM 402 served as negative controls for the specific probe. The SPT 177 probe is complementary to positions 177–196 of the *P. triangulum* endosymbiont 16S rRNA gene sequence (*S. ambofaciens* nomenclature; Pernodet *et al.*, 1989). Secretions of the white substance from beewolf females were harvested from brood cells and spread onto six-field microscope slides. Fixation and hybridization were carried out as described previously (Grimm *et al.*, 1998), with minor modifications: the hybridization buffer contained only 50 ng labelled probe and samples were incubated for 90 min at 45 °C for hybridization. For hybridization within the antennae, fresh antennae from female beewolves were cut into sections with a razor

Table 1. Occurrence of endosymbiotic *Streptomyces* bacteria in antennae of philanthine wasps (Hymenoptera, Crabronidae, Philanthinae) and amplification with the specific primers Strep_phil_fwd1–4 in combination with the general actinomycete primer Act-A19

To assess the specificity of the primers, the DNA of three cultivated *Streptomyces* species was included in the PCRs. ++, Strong amplification; +, weak amplification; −, no amplification; Y, symbionts present; N, symbionts not present; NA, not applicable; SA, South Africa; KZN, KwaZulu Natal; WCP, Western Cape Province; ECP, Eastern Cape Province. Standard two-letter abbreviations are used for US States.

Species	Specimens (*n*)	Geographical origin	Symbionts	Strep_phil amplicons				16S rRNA gene GenBank accession no.
				fwd1	fwd2	fwd3	fwd4	
***Philanthus* species**								
Philanthus barbiger	5	UT (USA)	Y	++	++	++	++	DQ375779
Philanthus basilaris	4	UT (USA)	Y	++	++	++	++	DQ375780
Philanthus bicinctus	3	WY (USA)	Y	++	++	++	++	DQ375781
Philanthus capensis	1	WCP (SA)	Y	++	++	++	++	DQ375782
Philanthus coarctatus	1	Oman	Y	++	++	++	++	DQ375783
Philanthus coronatus	1	Germany	Y	+	++	++	++	DQ375784
Philanthus crabroniformis	1	WY (USA)	Y	−	++	++	++	DQ375785
Philanthus crotoniphilus	2	UT (USA)	Y	++	++	++	++	DQ375786
Philanthus fuscipennis	4	ECP, WCP (SA)	Y	++	++	++	++	DQ375787
Philanthus gibbosus	4	UT (USA)	Y	+	++	++	++	DQ375788
Philanthus gloriosus	5	UT (USA)	Y	++	++	++	++	DQ375789
Philanthus histrio	1	WCP (SA)	Y	++	++	++	++	DQ375790
Philanthus inversus	2	UT (USA)	Y	+	++	++	++	DQ375791
Philanthus lepidus	3	MA (USA)	Y	++	++	++	−	DQ375792
Philanthus loefflingi	4	ECP, WCP (SA)	Y	++	++	++	++	DQ375793
Philanthus multimaculatus	7	UT (USA)	Y	++	++	++	++	DQ375794
Philanthus pacificus	4	UT (USA)	Y	++	++	++	++	DQ375795
Philanthus parkeri	6	UT (USA)	Y	++	++	++	++	DQ375796
Philanthus politus	1	MA (USA)	Y	+	++	++	+	DQ375797
Philanthus psyche	1	UT (USA)	Y	+	+	+	−	DQ375798
Philanthus pulcher	4	WY (USA)	Y	++	++	++	++	DQ375799
Philanthus rugosus	1	ECP (SA)	Y	+	++	++	++	DQ375800
Philanthus tarsatus	1	NE (USA)	Y	+	++	++	++	DQ375801
Philanthus triangulum triangulum	38	Germany, Greece, Ukraine	Y	++	++	++	++	DQ375802
Philanthus triangulum diadema	7	KZN, ECP, WCP (SA)	Y	++	++	++	++	DQ375803
Philanthus ventilabris	1	UT (USA)	Y	++	++	++	++	DQ375804
Philanthus venustus	3	Greece	Y	++	++	++	++	DQ375805
Philanthus zebratus	3	WY, CA (USA)	Y	++	++	++	++	DQ375806
Other wasp species								
Aphilanthops frigidus	1	MA (USA)	N	−	−	−	−	NA
Cerceris arenaria	1	Germany	N	−	−	−	−	NA
Cerceris rybyensis	3	Germany	N	−	−	−	−	NA
Clypeadon haigi	1	Utah (USA)	N	−	−	−	−	NA
Clypeadon laticinctus	5	Utah (USA)	N	−	−	−	−	NA
Control bacterial species								
Streptomyces aureofaciens	NA	NA	NA	−	−	−	−	NA
Streptomyces rimosus	NA	NA	NA	−	+	−	−	NA
Streptomyces venezuelae	NA	NA	NA	−	−	−	−	NA

blade and glued onto microscope slides. Fixation and pre-treatment of the samples was performed following a previously described protocol (Sauer *et al.*, 2002). Hybridization was carried out as for the bacterial samples, but with 3 h incubation with the labelled probe.

Phylogenetic analysis. BioEdit 7.0.4.1 software was used to assemble and align sequences and to calculate DNA distances with the DNADIST 3.5c algorithm by Joseph Felsenstein. The alignment was checked by eye and arbitrary alignment regions were excluded

from further analysis. The aligned sequences were imported into PAUP 4.0. Phylogenetic trees were constructed based on 1324 bp of 16S rRNA gene sequences in a full heuristic search with tree bisection and reconnection (TBR) branch swapping and 10 random addition sequence replicates, saving no more than 100 trees with a score $\geqslant$ 100 per replicate. Gaps were treated as a fifth character state and *Arthrobacter globiformis* DSM 20124^T was defined as the outgroup. Using the same settings, bootstrap values were obtained from a search with 1000 replicates.

RESULTS

Localization of endosymbionts

Scanning electron micrographs of the antennal surface of *P. triangulum*, *Philanthus loefflingi* and *Philanthus fuscipennis* females revealed that the bacteria are present at the openings of the antennal glands from which they are secreted into the brood cell (Kaltenpoth *et al.*, 2005) (Fig. 1). The appearance of symbiotic bacteria on the outer surface of the antennae is probably due to accidental compressions of the antennae prior to or during preservation; under natural conditions they are unlikely to be found on the antennal surface, except during the secretion process into the brood cell.

When a flagellomere was cut open, filamentous bacteria were clearly visible in large numbers within the gland reservoir (Fig. 2a), where they formed a dense cluster of cells (Fig. 2b). Transmission electron micrographs confirmed the presence of endosymbiotic bacteria within the antennal gland reservoir and suggest that the endosymbionts constitute the main component of the antennal gland content in female beewolves (Fig. 3). The bacteria showed a filamentous morphology with long and sometimes branched cells and were embedded in a matrix containing numerous vesicles in the gland reservoir. Bacterial cells were 0·38–0·62 μm wide and highly variable in length (5–20 μm).

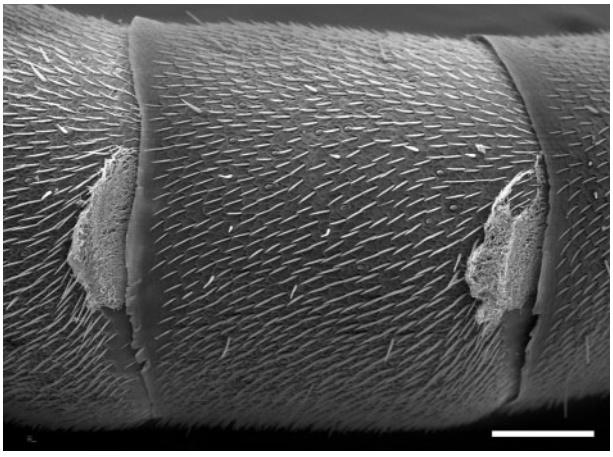

Fig. 1. SEM image of an antenna of a female European beewolf (*P. triangulum*) with symbiotic *Streptomyces* bacteria being secreted from the antennal glands. Bar, 100 μm.

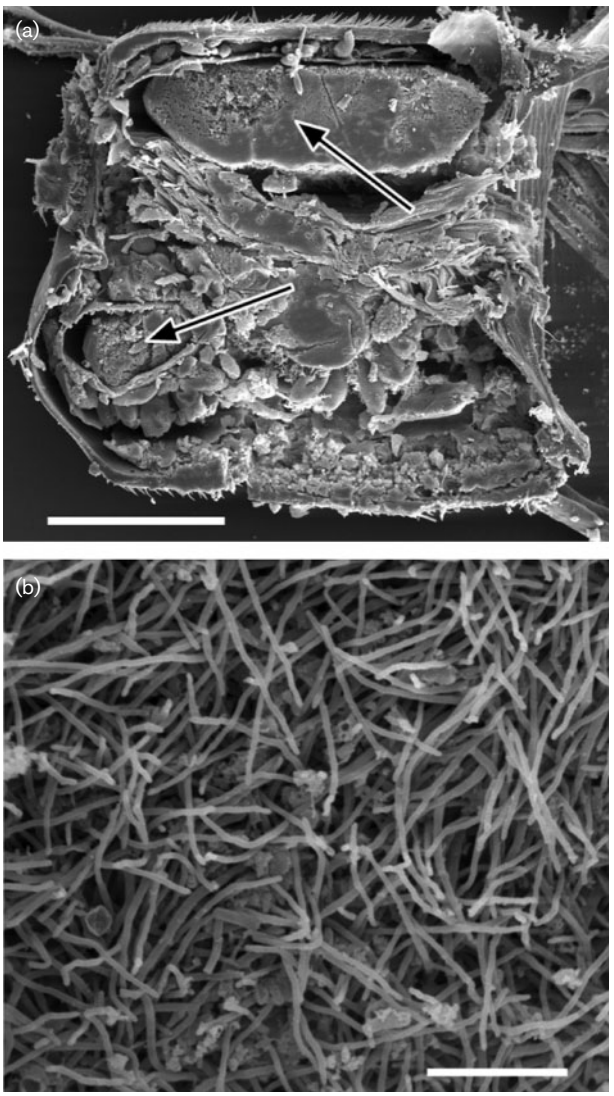

Fig. 2. SEM image of the interior of an antennal segment of a female *P. loefflingi*. (a) Longitudinal section of a flagellomere. The reservoir of the antennal gland is indicated by arrows. (b) Symbiotic *Streptomyces* bacteria forming a dense cluster within the antennal gland. Bars, 200 μm (a) and 10 μm (b).

The bacteria were clearly stained by the specific fluorescent probe SPT 177 both within female beewolf antennae and in the antennal gland secretion after it had been applied to the brood cell (Fig. 4). Reference strains of *S. aureofaciens*, *S. venezuelae*, *S. rimosus* and *B. subtilis* were not stained by the probe. The general eubacterial probe EUB 338 gave positive results in all cases.

Distribution of symbionts among philanthine wasps

All 28 *Philanthus* species, including the two subspecies of *P. triangulum*, yielded amplicons of the expected length in at least three of the four PCRs with the specific 16S rRNA

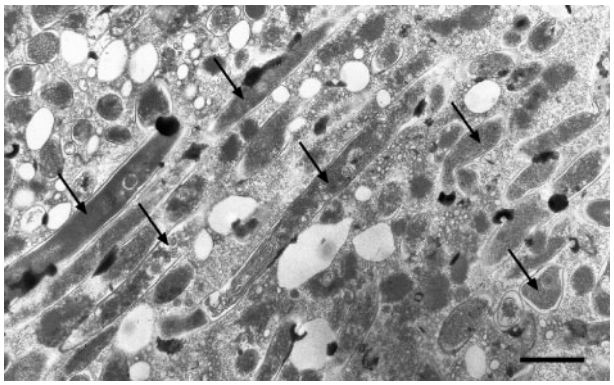

Fig. 3. TEM image of a cross-section through the antennal gland of a female *P. triangulum*. Some endosymbiotic *Streptomyces* are indicated by arrows. Bar, 1 μm.

primers Strep_phil_fwd1–4 in combination with the general actinomycete primer Act-A19 (Stach *et al.*, 2003) (Table 1). One species, *Philanthus psyche*, generally yielded only weak amplicons and failed to amplify altogether in one of the four specific PCRs. *Philanthus crabroniformis* and *Philanthus lepidus* each also yielded no amplicons in one of the PCRs, but gave strong amplicons in all other PCRs.

Specimens of the other wasp genera of the subfamily Philantinae (*Aphilanthops*, *Clypeadon* and *Cerceris*) yielded no amplicons in any of the specific PCRs. In PCRs with general actinomycete primers (Act-S20 and Act-A19), antennal DNA from *Aphilanthops*, *Clypeadon* and *Cerceris* yielded no, or very weak, amplicons. The sequences obtained from the weak amplicons were not closely related to the *Philanthus* endosymbionts and were probably due to contamination of the antennae from the surrounding soil during the life of the digger wasps within subterranean nests (data not shown). Thus, symbiosis with bacteria of the genus *Streptomyces* seems to be widespread among wasps of the genus *Philanthus*, but appears to be absent in other genera of the subfamily.

Streptomyces control strains yielded no amplicons in most of the PCRs, demonstrating the specificity of the primers for the *Philanthus* endosymbionts. However, Strep_phil_fwd2/Act-A19 did amplify the 16S rRNA gene of *S. rimosus* DSM 40260^T, a close relative of the *Philanthus* symbionts (Fig. 5) which shares around 98·0 to 98·5 % of its 16S rRNA gene sequence. Control PCRs with general actinomycete 16S rRNA primers (Act-S20/Act-A19) resulted in strong amplicons for all of the *Streptomyces* strains, showing that the lack of amplification in the specific PCRs was not due to general problems with the template DNA.

Phylogenetic position of '*Candidatus* Streptomyces philanthi'

The partial 16S rRNA gene sequences from the endosymbionts of 27 *Philanthus* species and one subspecies grouped together in a monophyletic clade within the genus *Streptomyces* (Fig. 5). The phylogenetic analysis indicates that the symbionts belong to the *Streptomyces armeniacus* group, the closest relatives being *Streptomyces kasugaensis* and *Streptomyces sapporonensis*, with a mean sequence divergence of about 1·1 and 1·2 %, respectively. The similarity among the endosymbionts of the 28 different *Philanthus* taxa was relatively high, ranging from 98·9 to 100·0 % 16S rRNA gene sequence similarity.

Almost complete 16S rRNA gene sequences for the 28 ecotypes of '*Candidatus* Streptomyces philanthi' have been

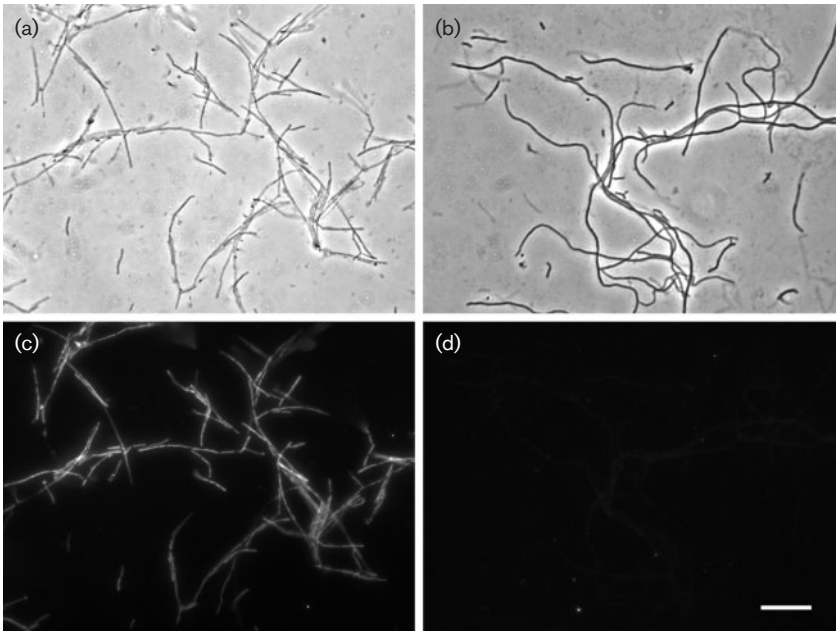

Fig. 4. FISH of antennal *Streptomyces* endosymbionts. Phase-contrast micrograph of symbiotic bacteria in the antennal gland secretion of a female beewolf (a) and of a negative control strain of *Streptomyces rimosus* DSM 40260^T (b). (c, d) Epifluorescence micrographs of the same areas after staining with the specific Cy3-labelled probe SPT 177. Bar, 10 μm.

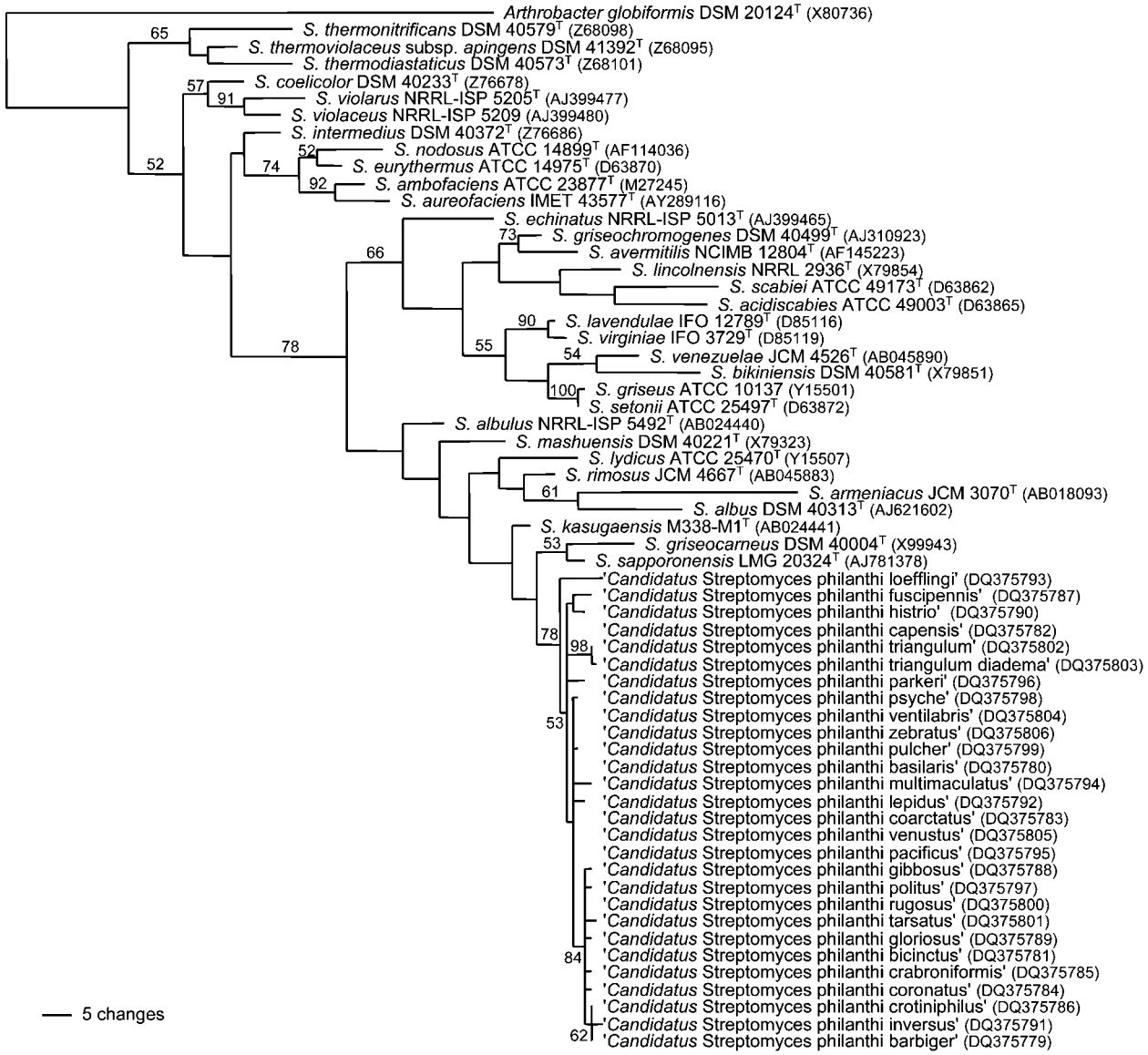

Fig. 5. Phylogenetic position of *Philanthus* endosymbionts within the genus *Streptomyces* based on 1324 bp of 16S rRNA gene sequence (104 parsimony-informative characters). First of 600 equally parsimonious trees from a full heuristic search with random addition sequence (10 replicates) and TBR branch swapping. *Arthrobacter globiformis* was defined as the outgroup. Bootstrap values at nodes are percentages of 1000 replicates. GenBank accession numbers are given in parentheses. Bar, 5 changes.

deposited in the GenBank database with accession numbers DQ375779–DQ375806. The accession numbers for specific ecotypes are shown in Fig. 5 and Table 1.

DISCUSSION

Endosymbiotic bacteria of insects are usually localized in the gut or reside within specialized host cells, so-called mycetocytes or bacteriocytes, which often form dedicated organ-like structures or are associated with the mid-gut epithelium (Baumann & Moran, 1997; Buchner, 1921; Ishikawa, 2003; Moran & Telang, 1998). The *Philanthus–Streptomyces* association represents the first case of endosymbiotic bacteria being localized in insect antennae. Correspondingly, the specialized antennal glands harbouring the symbionts have so far only been found in species of the genus *Philanthus* and appear to be absent even in closely related genera of philanthine wasps (E. Strohm, unpublished data). As is the case with many other endosymbiotic bacteria, attempts to cultivate the *Philanthus* symbionts using standard cultivation techniques and media were not successful (see Supplementary material in IJSEM Online).

The endosymbionts are present in the antennal gland reservoir of *Philanthus* females in large numbers and they can be detected by SEM, TEM, FISH (with a specific oligonucleotide probe) and by PCRs with specific primers. Genetic analyses of the 16S rRNA gene sequences of endosymbionts from the antennae of different beewolf species revealed that all species investigated so far harbour *Streptomyces* bacteria and that the *Philanthus* endosymbionts appear to represent a monophyletic clade within the genus *Streptomyces*. The antennal endosymbionts share an average of 98·8–98·9 % 16S rRNA gene sequence with their closest relatives, *S. kasugaensis* and *S. sapporonensis*. Despite this high sequence similarity, we propose the name '*Candidatus* Streptomyces philanthi' for the endosymbionts of *Philanthus* species because they are clearly separated from other species by their unique ecological niche. Several studies have shown that 16S rRNA gene sequence similarity alone is often inappropriate for the distinction of two species and the general rule of 3 % 16S rRNA gene sequence divergence between species tends to greatly underestimate the number of species (Cohan, 2002; Konstantinidis & Tiedje, 2005), as has been recently demonstrated for a number of *Streptomyces* groups (Liu *et al.*, 2005; Manfio *et al.*, 2003; Sembiring *et al.*, 2000). Therefore, it is desirable to include ecological characteristics in the description of novel species (Cohan, 2002; Konstantinidis & Tiedje, 2005). Among *Philanthus* endosymbionts, 16S rRNA gene sequence similarity is relatively high (98·9–100·0 %). We propose that the endosymbionts represent a single species with different ecotypes that are separated by their ecological niches (i.e. their host species).

The high degree of 16S rRNA gene sequence similarity among *Philanthus* endosymbionts suggests that they are transmitted vertically from mother to offspring, as has been described for many other endosymbiotic bacteria (Aksoy *et al.*, 1997; Clark *et al.*, 2000, 2001; Ishikawa, 2003; Moran & Baumann, 2000; Sauer *et al.*, 2000). Alternatively, the bacteria may be taken up from the environment with certain mechanisms preventing the uptake of non-symbiotic bacteria, a transmission route that has been demonstrated for the symbionts of the squid *Euprymna scolopes* (McFall-Ngai & Ruby, 1991; Nishiguchi, 2002; Nyholm *et al.*, 2000; Nyholm & McFall-Ngai, 2004). The following evidence points to vertical transmission of the bacteria from mother to offspring in *Philanthus*: (i) the bacteria are secreted into the brood cell and later taken up by the larva and (ii) a female larva that was reared in the absence of the white substance in its brood cell apparently lacked the symbiotic bacteria as an adult (Kaltenpoth *et al.*, 2005). However, further studies on the phylogenetic relationships of beewolves and their endosymbionts are needed to confirm vertical transmission and to determine whether horizontal transfer of symbionts between *Philanthus* species (e.g. via chrysidid parasitoids, interspecific nest usurpation or nest reuse) may have played a role in the evolution of the symbiosis.

Moran *et al.* (1993) estimated an evolutionary age of 160–280 million years for the symbiosis between aphids and their endosymbiont *Buchnera aphidicola* and Bandi *et al.* (1995) dated the origin of the association of cockroaches and termites with bacteria of the *Flavobacterium–Bacteroides* group to about 135–250 million years ago. Under the assumption of strictly vertical transmission of the symbionts, the low 16S rRNA gene sequence divergence among the endosymbionts of *Philanthus* wasps suggests that the symbiosis is of relatively recent origin. Assuming a mean rate of 0·008–0·02 substitutions per site per 50 million years (Bandi *et al.*, 1994; Moran *et al.*, 1993; Ochman & Wilson, 1987), the maximum sequence divergence of 1·07 % indicates that the origin of the symbiosis between beewolves and streptomycetes dates back about 26–67 million years. Taking into account that all *Philanthus* species investigated so far harbour the symbiotic bacteria, the association with bacteria probably evolved at around the time of origin of the genus *Philanthus*.

The evolution of specialized antennal glands in *Philanthus* females may have represented a key invention and evolutionary preadaptation for a symbiosis with *Streptomyces* bacteria. Strohm & Linsenmair (1995) demonstrated that the antennal gland secretion serves a second function by providing directional information to the beewolf larva that is necessary later for successful emergence. Thus, we hypothesize that the antennal glands originally evolved in the context of directing cocoon-spinning and emergence and that they might have been secondarily invaded by *Streptomyces* bacteria from the surrounding soil. In the beginning, the bacteria may have been commensals, or even parasites, in the antennal glands. In a sequence of evolutionary steps, including the uptake of the bacteria by the larva and their application to the cocoon, the antimicrobial activity of the streptomycetes might have been subsequently exploited by the beewolf hosts to protect their offspring against pathogen infection. Further studies are needed to investigate how related genera of ground-nesting digger wasps cope with the threat of pathogenic soil micro-organisms infecting their progeny.

Description of '*Candidatus* Streptomyces philanthi'

'*Candidatus* Streptomyces philanthi' [phi.lan′thi. N.L. n. *Philanthus* (Hymenoptera, Crabronidae) the generic name of the host organism; N.L. gen. n. *philanthi* of *Philanthus*, referring to the association with digger wasps of the genus *Philanthus*].

The reference strain is '*Candidatus* Streptomyces philanthi triangulum'.

Uncultured, Gram-positive, non-motile, possibly sporulating, filamentous bacteria with sometimes branched cells that can be assigned to the genus *Streptomyces* on the basis of their 16S rRNA gene sequence. A detailed description of the methods used in an attempt to cultivate the endosymbionts can be found as supplementary material in IJSEM Online. Cells are 0·38–0·62 μm wide and of highly variable length

(5–20 μm). The bacteria live as symbionts within specialized antennal glands of female digger wasps of the genus *Philanthus*. They are secreted into the brood cells, taken up by the larva and applied to the cocoon, where they appear to protect the beewolf offspring against fungal infection (Kaltenpoth *et al.*, 2005). Bacteria of different *Philanthus* species differ in their 16S rRNA gene sequence, but sequence divergence is relatively low (0–1·1 %). We propose that endosymbionts of different *Philanthus* species should be treated as ecotypes of '*Candidatus* Streptomyces philanthi' and named according to the host species. The 16S rRNA gene sequences of all ecotypes found so far can be amplified selectively by the specific forward primer Strep_phil_fwd3 (5′-CATGGTTRGTGGTGGAAAGC-3′) in combination with the general actinomycete reverse primer Act-A19 (Stach *et al.*, 2003). The ecotype '*Candidatus* Streptomyces philanthi triangulum' can be stained with the fluorescent probe SPT 177 (5′-Cy3-CACCAACCATGCGATCGGTA-3′) (Kaltenpoth *et al.*, 2005).

[(*Streptomyces*) NC; G+; F; NAS (GenBank accession number DQ375802), oligonucleotide sequence of unique region of the 16S rRNA gene is 5′-TACCGATCGCATG-GTTGGTG-3′; S (*Philanthus*, antennal glands); M]. Kaltenpoth *et al.*, this study.

ACKNOWLEDGEMENTS

We would like to thank Jon Seger, Michael Ohl, Protsenko Yura, Johannes Kroiss and Thomas Schmitt for providing *Philanthus* specimens. We are grateful to Fred and Sarah Gess and Simon van Noort for their help in collecting *Philanthus* in South Africa. The required collecting permits for South Africa were kindly issued by the nature conservation boards of KwaZulu Natal (permit no. 4362/2004), the Eastern Cape Province (permit no. 001-202-00026) and the Western Cape Province (permit no. 001-506-00001). We want to thank the Department of Microbiology at the University of Würzburg for providing *Streptomyces* reference strains and we thank Roy Gross for valuable comments on the manuscript. We gratefully acknowledge financial support from the German National Academic Foundation, the Arthur-von-Gwinner Foundation, the Unibund Würzburg and the Deutsche Forschungsgemeinschaft (DFG STR 532/2-1 and DFG STR 532/1-2).

REFERENCES

Aksoy, S., Chen, X. & Hypsa, V. (1997). Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). *Insect Mol Biol* **6**, 183–190.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. & Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.

Bandi, C., Damiani, G., Magrassi, L., Grigolo, A., Fani, R. & Sacchi, L. (1994). Flavobacteria as intracellular symbionts in cockroaches. *Proc Biol Sci* **257**, 43–48.

Bandi, C., Sironi, M., Damiani, G., Magrassi, L., Nalepa, C. A., Laudani, U. & Sacchi, L. (1995). The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. *Proc Biol Sci* **259**, 293–299.

Baumann, P. & Moran, N. A. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie van Leeuwenhoek* **72**, 39–48.

Baumann, P., Moran, N. A. & Baumann, L. (1997). The evolution and genetics of aphid endosymbionts. *Bioscience* **47**, 12–20.

Bohart, R. M. & Grissell, E. E. (1975). California wasps of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Bull Calif Insect Surv* **19**, 1–57.

Bourtzis, K. & Miller, T. A. (editors) (2003). *Insect Symbiosis*. Boca Raton, FL: CRC Press.

Buchner, P. (1921). *Tier und Pflanze in Intrazellulärer Symbiose*. Berlin: Verlag Gebrüder Borntraeger (in German).

Cafaro, M. J. & Currie, C. R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Can J Microbiol* **51**, 441–446.

Chen, X. O., Li, C. B., Zhao, S. Y. & Aksoy, S. (1999). Phylogeny of the symbionts of tsetse and the evolutionary relationships with their hosts. *Prog Nat Sci* **9**, 922–928.

Clark, M. A., Moran, N. A., Baumann, P. & Wernegreen, J. J. (2000). Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence. *Evolution Int J Org Evolution* **54**, 517–525.

Clark, J. W., Hossain, S., Burnside, C. A. & Kambhampati, S. (2001). Coevolution between a cockroach and its bacterial endosymbiont: a biogeographical perspective. *Proc Biol Sci* **268**, 393–398.

Cohan, F. M. (2002). What are bacterial species? *Annu Rev Microbiol* **56**, 457–487.

Currie, C. R., Scott, J. A., Summerbell, R. C. & Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**, 701–704.

Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu Rev Entomol* **43**, 17–37.

Ferguson, G. R. (1983a). Two new species in the genus *Philanthus* and a key to the *politus* group (Hymenoptera, Philanthidae). *Pan-Pacific Entomol* **59**, 55–63.

Ferguson, G. R. (1983b). Revision of the *Philanthus zebratus* group (Hymenoptera, Philanthidae). *J N Y Entomol Soc* **91**, 289–303.

Grimm, D., Merkert, H., Ludwig, W., Schleifer, K. H., Hacker, J. & Brand, B. C. (1998). Specific detection of *Legionella pneumophila*: construction of a new 16S rRNA-targeted oligonucleotide probe. *Appl Environ Microbiol* **64**, 2686–2690.

Ishikawa, H. (2003). Insect symbiosis: an introduction. In *Insect Symbiosis*, pp. 1–21. Edited by K. Bourtzis & T. A. Miller. Boca Raton, FL: CRC Press.

Kaltenpoth, M., Göttler, W., Herzner, G. & Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Curr Biol* **15**, 475–479.

Konstantinidis, K. T. & Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci U S A* **102**, 2567–2572.

Liu, Z. H., Shi, Y. L., Zhang, Y. M., Zhou, Z. H., Lu, Z. T., Li, W., Huang, Y., Rodriguez, C. & Goodfellow, M. (2005). Classification of *Streptomyces griseus* (Krainsky 1914) Waksman and Henrici 1948 and related species and the transfer of '*Microstreptospora cinerea*' to the genus *Streptomyces* as *Streptomyces yanii* sp. nov. *Int J Syst Evol Microbiol* **55**, 1605–1610.

Lo, N., Bandi, C., Watanabe, H., Nalepa, C. & Beninati, T. (2003). Evidence for cocladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. *Mol Biol Evol* **20**, 907–913.

Manfio, G. P., Atalan, E., Zakrzewska-Czerwinska, J., Mordarski, M., Rodriguez, C., Collins, M. D. & Goodfellow, M. (2003). Classification of novel soil streptomycetes as *Streptomyces aureus* sp. nov., *Streptomyces laceyi* sp. nov. and *Streptomyces sanglieri* sp. nov. *Antonie van Leeuwenhoek* **83**, 245–255.

McFall-Ngai, M. J. & Ruby, E. G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* **254**, 1491–1493.

Moran, N. & Baumann, P. (1994). Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *Trends Ecol Evol* **9**, 15–20.

Moran, N. A. & Baumann, P. (2000). Bacterial endosymbionts in animals. *Curr Opin Microbiol* **3**, 270–275.

Moran, N. A. & Telang, A. (1998). Bacteriocyte-associated symbionts of insects. *Bioscience* **48**, 295–304.

Moran, N. A., Munson, M. A., Baumann, P. & Ishikawa, H. (1993). A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc Biol Sci* **253**, 167–171.

Nishiguchi, M. K. (2002). Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. *Microb Ecol* **44**, 10–18.

Nyholm, S. V. & McFall-Ngai, M. J. (2004). The winnowing: establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* **2**, 632–642.

Nyholm, S. V., Stabb, E. V., Ruby, E. G. & McFall-Ngai, M. J. (2000). Establishment of an animal-bacterial association: recruiting symbiotic vibrios from the environment. *Proc Natl Acad Sci U S A* **97**, 10231–10235.

Ochman, H. & Wilson, A. C. (1987). Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J Mol Evol* **26**, 74–86.

Pernodet, J. L., Boccard, F., Alegre, M. T., Gagnat, J. & Guerineau, M. (1989). Organization and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* **79**, 33–46.

Priest, F. G. & Dewar, S. J. (2000). Bacteria and insects. In *Applied Microbial Systematics*, pp. 165–202. Edited by F. G. Priest & M. Goodfellow. Dordrecht: Kluwer Academic.

Rintala, H., Nevalainen, A., Ronka, E. & Suutari, M. (2001). PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. *Mol Cell Probes* **15**, 337–347.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sauer, C., Stackebrandt, E., Gadau, J., Holldobler, B. & Gross, R. (2000). Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus* Blochmannia gen. nov. *Int J Syst Evol Microbiol* **50**, 1877–1886.

Sauer, C., Dudaczek, D., Holldobler, B. & Gross, R. (2002). Tissue localization of the endosymbiotic bacterium '*Candidatus* Blochmannia floridanus' in adults and larvae of the carpenter ant *Camponotus floridanus*. *Appl Environ Microbiol* **68**, 4187–4193.

Sembiring, L., Ward, A. C. & Goodfellow, M. (2000). Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*. *Antonie van Leeuwenhoek* **78**, 353–366.

Stach, J. E. M., Maldonado, L. A., Ward, A. C., Goodfellow, M. & Bull, A. T. (2003). New primers for the class *Actinobacteria*: application to marine and terrestrial environments. *Environ Microbiol* **5**, 828–841.

Strohm, E. (1995). *Allokation elterlicher Investitionen beim Europäischen Bienenwolf Philanthus triangulum Fabricius (Hymenoptera: Sphecidae)*. Berlin: Verlag Dr. Köster (in German).

Strohm, E. & Linsenmair, K. E. (1995). Leaving the cradle: how beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology (Jena)* **98**, 137–146.

Strohm, E. & Linsenmair, K. E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecol Entomol* **26**, 198–203.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.