Phylogenies and pheromones

Defensive symbionts, phylogenetic affiliations and olfactory communication in beewolves (Philanthini, Hymenoptera, Crabronidae)

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If you find yourself in a hole, you must stop digging.

Will Rogers

Für den kleinen Moritz

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LIST OF PUBLICATIONS

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Kaltenpoth M, Goettler W, Dale C, Stubblefield JW, Herzner G., Roeser-Mueller K, Strohm, E (2006) *'Candidatus* Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. International Journal of Systematic and Evolutionary Microbiology 56 (6): 1403-1411 (chapter 2).

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Roeser-Mueller K, Strohm E, Kaltenpoth M (2010) Larval rearing temperature influences amount and composition of the marking pheromone of the male beewolf, *Philanthus triangulum*. Journal of Insect Science 10:74 available online: insectscience.org/10.74 (chapter 4).

CHAPTER 1

GENERAL INTRODUCTION

The fitness of an individual and its maximisation are of central importance for evolutionary processes. There are different ways how organisms can increase their fitness and pass on their genes to the next generation. This thesis deals with two such aspects that might contribute to the fitness of an organism: an association with symbionts and sexual selection in courtship pheromones. First, the defensive symbiosis between beewolf digger wasps (Hymenoptera, Crabronidae) and *Streptomyces* bacteria is addressed. Another part of this thesis deals with the sex pheromone of male beewolves and how it is influenced by the temperature an individual is exposed to during development. In addition to these two fitness relevant traits, a reconstruction of the phylogenetic relationships among beewolves and closely related species is presented, that provides a basis for understanding the evolution of both the symbiosis and the composition of the sex pheromones in beewolves. The present chapter summarizes the current knowledge on symbioses and pheromones, in particular their relevance for mate choice, and it ends with an overview of the biology of beewolves.

1.1 Symbioses

Symbioses have played an important role in the evolution of life on earth, and examples can be found in all kingdoms of microorganisms, plants and animals (Margulis 1999). According to the original definition of de Bary (1879), the term "symbiosis" encompasses the living together of unlike organisms over significant parts of their live span. This definition includes the whole range of biological interactions from mutualistic relationships via commensalism to parasitism, without specifying costs and benefits for the partners involved in the association (Douglas 2010). However, according to the most common definition of "symbiosis" in the current literature (Douglas 2010), in the present thesis the term "symbiosis" is used in its stricter sense, including only mutualistic associations.

The variety of symbiotic interactions is characterized by different traits and definitions. Regarding the degree of interdependence between the symbiotic partners symbioses are either classified as facultative or obligate: Associations with a low dependency on the partner are defined as facultative, symbioses with a strong interdependence, meaning that a symbiont cannot survive without its partner, are defined as obligate (Douglas 2010; Ishikawa 2003). Concerning the location of the symbionts two main types are distinguished. When an organism lives on another, this association is called ectosymbiosis (Ahmadjian & Paracer 2000). When the symbiont is located inside another organism, this is defined as endosymbiosis (Buchner 1965; Kikuchi 2009). Furthermore, endosymbionts can live either extracellularly (e.g. inside the intestinal tract) or intracellularly, where they often live in specialized cells (Houk & Griffiths 1980; Ishikawa 2003).

Insect-bacteria symbioses

The mutualistic associations between insects and microorganisms encompass an astonishing diversity (Buchner 1921), and the bacterial symbionts might have played a significant role for the evolutionary success of insects (Margulis & Fester 1991; Maynard-Smith 1989). About half of all insects are estimated to be associated with endosymbiotic bacteria (Buchner 1965; Ishikawa 2003), and some of these endosymbioses have evolved as early as several hundred million years ago (Harris et al. 2010).

The benefits for insect hosts and bacterial symbionts are multifaceted. Insects often provide nutrients for their bacterial inhabitants, an ecological niche with stable conditions and the safety of being passed on to their progeny (Currie 2001; Douglas 1994; Margulis & Fester 1991). In return, bacteria provide benefits for their host insects nutritionally by supplying them with essential nutrients, especially for blood-sucking, wood-feeding and phloem-sucking insects and by improving the host's digestive functions (Akman et al. 2002; Dettner 1999; Dillon & Dillon 2004; Douglas 2006; Gaudermann et al. 2006; Moran & Baumann 2000; Shigenobu et al. 2000; Zientz et al. 2004). Furthermore, the bacterial symbionts may influence the hosts' reproduction (Zchori-Fein et al. 2004) or confer heat tolerance (Russell & Moran 2006). Additionally, bacterial symbionts are known for providing their hosts with substances needed as pheromone components (Dillon et al. 2000; Dillon et al. 2002; Matsuura 2003). Finally, bacteria are known to support the host's defence against parasitoids (Oliver et al. 2003) and pathogens (Currie et al. 1999; Dillon et al. 2005; Hu & Webster 2000; Kaltenpoth et al. 2005; Piel 2004; Scarborough et al. 2005; Takatsuka & Kunimi 2000).

Defensive insect-bacteria symbioses

The role of defensive characteristics in symbiotic associations between insects and bacteria has recently attracted increased scientific interest (e.g. Brownlie & Johnson 2009; Crotti et al. 2010; Ferrari & Vavre 2011; Kaltenpoth 2009; Oliver & Moran 2009). Symbiotic gut bacteria e.g. are known to improve the host's defence against pathogenic attacks in numerous insect species either by efficiently exploiting limiting nutrients and thereby outcompeting pathogens (Dillon & Dillon 2004; Godfray et al. 1999) or by releasing antimicrobial substances (Currie et al. 1999; Dillon & Charnley 1995; Poulsen et al. 2011; Takatsuka & Kunimi 2000). *Wolbachia* bacteria reduce the susceptibility of *Drosophila melanogaster* against RNA-viruses (Hedges et al. 2008; Teixeira et al. 2008). Members of the beetle genus *Paederus* harbour endosymbiotic *Pseudomonas* bacteria which produce the anti-predator toxin pederin (Kellner 2002; Piel 2002). Aphids do not only live in a nutritional symbiosis with *Buchnera* bacteria (Douglas 1998), they also show several fascinating defensive associations with vertically (and horizontally) transmitted secondary symbionts, which e.g. improve the aphids' resistance against hymenopteran parasitoids (partly by the involvement of a bacteriophage) or provide protection against fungi (Ferrari et al. 2004; Moran et al. 2005; Oliver et al. 2010; Oliver & Moran 2009; Oliver et al. 2006; Oliver et al. 2003; Scarborough et al. 2005).

Actinobacteria seem to be predisposed for defensive associations because of their potential to produce diverse antibiotic substances. Surprisingly, there are only few examples for defensive symbioses between insects and members of this clade of bacteria. Two of them concern fungus-farming insects: The symbiosis between pine beetles and *Streptomyces* bacteria and the association of attine ants and symbiotic actinobacteria of different genera. Southern pine beetles (*Dendroctonus frontalis*; Coleoptera, Curculionidae) excavate galleries between the inner bark and the phloem of pine trees. These galleries are inoculated with their symbiotic fungus, providing food for the larvae. The growth of this fungus and thus the development of the larvae are endangered by antagonistic fungi. *Streptomyces* symbionts may protect the beetles' symbiotic fungus against these competitors by producing an antifungal molecule termed mycangimycin (Scott et al. 2008). A similar defensive symbiosis with diverse genera of bacterial symbionts contained in oral secretions has also been described for another *Dendroctonus* species (Cardoza et al. 2006).

The symbiotic association between leaf-cutter ants (Hymenoptera, Formicidae) and bacteria has recently received considerable attention. More than 200 species of attine ants are known for cultivating and harvesting fungus gardens on plant material in their subterranean nests, serving as food for larvae and adult ants. The ants live in an obligate symbiosis with their specific fungus from the family Lepiotaceae, and the association evolved about 50 million years ago (Mueller 2005; Mueller et al. 1998; Mueller et al. 2001). Despite intense maintenance and hygienic behaviour, the ants' fungus gardens are endangered by the parasitic fungus *Escovopsis* (Currie 2001). As defence against the fungal threat, the ants are associated with symbiotic bacteria that belong to the genera *Pseudonocardia*, *Streptomyces* and, possibly, *Amycolatopsis*. Several of these actinobacteria have been shown to produce antiobiotics *in vitro* (Barke et al. 2010; Currie et al. 1999; Haeder et al. 2009; Oh et al. 2009a; Schoenian et al. 2011; Sen et al. 2009), and in one case the presence of antibiotics has been demonstrated *in vivo* by mass-spectrometric imaging (Schoenian et al. 2011). Some of the bacteria are cultivated on the ants' cuticle (Currie et al. 1999), and their antibiotics have been shown to inhibit the growth of *Escovopsis* but do not affect the symbiotic fungus (Cafaro et al. 2011; Currie et al. 1999; Haeder et al. 2009; Oh et al. 2009a).

1.2 Sexual selection, pheromones and temperature

Sexual selection and mate choice

When Charles Darwin (1859) published his theory of evolution by means of natural selection he already encountered the evolution of ostentatious male traits like colorful ornaments, bird song and weaponry. These conspicuous features in behaviour and outer appearance are expected to reduce the males' chance of survival rather than enhance it, and thus should be eliminated by natural selection (Andersson 1994). Darwin explains his paradoxical findings by proposing 'sexual selection' as an additional selective force besides natural selection (Darwin 1859, 1871). Thus,

sexual selection "depends on the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction" and "not on a struggle for existence, but on a struggle […] for possession of the females; the results is not death to the unsuccessful competitior, but few or no offspring" (Darwin 1859).

Sexual selection results from a basal conflict between the sexes concerning reproduction (Darwin 1871; Thornhill & Alcock 1983): Generally, females are limited by resources like nesting sites or food, have low reproduction rates and produce only few and costly germ cells. In contrast, reproductive success in males is mainly limited by the number of available mating partners; they have high reproduction rates and produce countless small sperm cells (Trivers 1972). Additionally, males - in contrast to females - often do not invest any resources in rearing the offspring. These asymmetric costs of reproduction in the sexes lead to a conflict of interests: Males can usually increase their fitness by maximizing the number of matings (Andersson 1994; Trivers 1972), but females should be choosy and select the most suitable genitor for their few and costly offspring (Gould & Gould 1997; Trivers 1972).

These circumstances lead to a rivalry between males for the access to females and to an evolution of traits which increase the males' mating success, e.g. signals for attracting females and enhancing their willingness for mating (Halliday 1980). Hence, males have evolved numerous advertisement signals that might also contain information on mate quality and species affiliation, thus providing a basis for an adaptive female mate choice (Droney & Hock 1998; Jones & Hamilton 1998; López et al. 2003). These signals are often costly and thus constitute a sort of "handicap", thereby representing a good indicator for the male's fitness, because only a superior, healthy male is able to develop and maintain such costly signals (Ahtiainen et al. 2005; McGraw et al. 2002; Rantala et al. 2002; Rantala & Kortet 2004; Zahavi 1975). When females choose a male of high quality as mating partner on the basis of an honest signal, they can either benefit directly from this choice, e.g. by male nuptial gifts or reduced risk of infection (Engqvist & Sauer 2003; Sakaluk 2000; Stalhandske 2002), or they gain indirect benefits, when the offspring quality depends on the mate's genetic background. Different theories have been proposed to explain the evolution of female mate choice (Krebs & Davies 1993): Well known is the "good-genes" model, which predicts the existence of some males in a population with an especially high-quality genetic background, which constitute the best choice for all females (Andersson 1994; Hine et al. 2002; Møller & Alatalo 1999). By contrast, the model of the "best compatibility/complementary" predicts that each female has an individual male as optimal mating partner (Colegrave et al. 2002; Halliday 1983; Neff & Pitcher 2005; Reinhold 2002), thus there is no single best male.

Female mate choice decisions may be based on a variety of morphological, physiological, or immunological traits (e.g. size, age or immunocompetence) of the mating partner (Adamo & Spiteri 2005; Hasegawa et al. 2011; López et al. 2003). Beyond that, the degree of kinship between male and female also can play an important role for mate choice (Charlesworth & Charlesworth 1987; Charpentier et al. 2005; Keller & Waller 2002; Lihoreau & Rivault 2010). Many

studies demonstrate that individuals of different taxa are able to recognize kin and thus can avoid inbreeding (Ehman & Scott 2001; Garner & Schmidt 2003). Especially in aculeate Hymenoptera, kin discrimination is of great importance due to their sex determination system, the single-locus complementary sex-determination (sl-CSD) (Beye et al. 2003; Cook 1993). Usually, unfertilized, haploid eggs develop into males, and fertilized, diploid eggs become females. If diploid eggs are homozygous at the sex-determination locus, however, they develop into diploid males that are mostly effectively sterile (Cook 1993; Cook & Crozier 1995). Because the probability of homozygosity at this locus dramatically increases in cases of matings among close relatives, the fitness costs of inbreeding are paticularly high in species with sl-CSD. Hence, selection should favour females avoiding to mate with close kin.

Pheromones and mate choice

To avoid cheating in species with female choice, the males' advertising signals have to be costly, resulting in a correlation between male quality and signal development (Zahavi 1975). Numerous studies have focused on mate choice decisions on the basis of acoustic and visual signals (Andersson 1994; Klappert & Reinhold 2003; Møller & Alatalo 1999). By contrast, the role of chemical signals in mate choice has attracted relatively little attention (Ali & Tallamy 2010; Brodt et al. 2006; Droney & Hock 1998; Eisner & Meinwald 1995; Kortet & Hedrick 2005; Sappington & Taylor 1990a, 1990b; Vainikka et al. 2006). This might be due to the fact that the human sense of olfaction is less developed in comparison with insects (Angioy et al. 2003; Kaissling 1971), and that olfactory signals are less accessible and require sophisticated chemical-analytical equipment for adequate analysis. Due to their complexity and high variability in quantity and quality, however, chemical signals often communicate a wealth of information that can be used by receivers for an adaptive mate choice (Ayasse et al. 2001; Hölldobler 1995).

Pheromones are chemical signals produced by an organism that can elicit behavioural or physiological responses in conspecifics (Karlson & Luescher 1959). In insects, pheromones are the predominant modality of communication. Although most insect sex pheromones are released by females (Alexander et al. 1997), the existence of male pheromones has been shown for several taxa (Jutsum & Gordon 1989; Landolt & Phillips 1997; Shelly & Whittier 1997). Some studies could show that male pheromones can reveal mate qualities to females (Droney & Hock 1998; Moore 1997; Thornhill 1992) and that females choose adaptively on the basis of the male pheromones (Hine et al. 2002; Jones & Hamilton 1998; Jones et al. 1998).

Temperature and pheromones

For many biological processes, temperature is the most important environmental factor. It directly affects the kinetics of biochemical reactions (Johnston & Wilson 2006) and is known to play a role for a variety of morphological, physiological, and life-history traits, e.g. development time (Ratte 1984), size (Atkinson 1994; Blanckenhorn 1997) or fecundity (Nabeta et al. 2005).

The impact of temperature on the quantity and quality of pheromones has up to now received little attention. The studies in this field mainly focus on the effect of ambient temperature in adult Lepidoptera (with predominantly female pheromones) and changes in amount or composition are ascribed to the immediate influence of temperature on biochemical pathways (Ono 1993; Raina 2003). Even less is known about the impact of temperature during early larval development on the pheromone of imagos (Ono 1993). In theory, environmental conditions should affect the composition of sex pheromones only to a limited degree, because the signal would otherwise loose important basic information like species affiliation and could not be recognized by potential mating partners any more. Thus, a certain level of developmental stability can be expected (Møller & Swaddle 1997; Paterson 1985). Nonetheless, environmental conditions during larval development may affect pheromone composition and/or amount and, thus, reveal important information for potential mating partners relevant for mate choice decisions.

In summary, the relevance of male sex pheromones for female mate choice decisions in insects is still largely unknown, despite their importance for mate attraction and their potential as honest indicators of male qualities. Beyond that, the factors shaping these chemical signals have hitherto received little attention, and especially the effects of environmental conditions during larval development on the composition of the adult pheromone are still virtually unknown.

1.3 The genus *Philanthus*

The solitary digger wasp genus *Philanthus* (Hymenoptera, Crabronidae) is a member of the subfamily Philanthinae, one of the largest groups in the family Crabronidae. In the past decades, several studies have revealed astonishing behavioural and physiological features of this genus with regard to resource allocation, interaction with parasites, mate attraction, chemical communication and the defence against pathogenic microorganisms.

Geographical distribution and systematic background of *Philanthus*

The genus *Philanthus* (Hymenoptera, Crabronidae) currently comprises 137 described species that are distributed almost over the whole world with the exception of Australia, South America and the Antarctic. The largest number of species (~70%) occurs in Africa and Eurasia (Pulawski 2010). Despite the high diversity of about 50 species on the African continent, they have unfortunately received little attention from entomologists (Pulawski 2010). By contrast, the 34 New World

Philanthus species have been studied much more extensively, and especially the studies of Evans and O'Neill have advanced our knowledge on the biology of this genus (e.g. Evans 1964; Evans & O'Neill 1988, 1991; O'Neill 1983; O'Neill & Evans 1983). However, most studies have undoubtedly been published on the European beewolf, *P. triangulum* (e.g. Herzner et al. 2007; Herzner et al. 2005; Kaltenpoth et al. 2005; Kroiss et al. 2006; Schmitt et al. 2003; Strohm 2000; Strohm et al. 2008; Strohm & Linsenmair 1995, 1997, 1998). This species has a wide distribution, reaching from Scandinavia to South Africa and from Portugal to the Near and Middle East (Blösch 2000; Bohart & Menke 1976; Ebrahimi 2005; Pulawski 2010). Together with the genera *Philanthinus* and *Trachypus*, *Philanthus* represents the tribe Philanthini, building the sister group to the clade consisting of the Cercerini and Aphilanthopini. Eight genera are included in the three tribes, which altogether constitute the subfamily Philanthinae (Alexander 1992).

Male behaviour

Male *Philanthus* defend small territories which are mostly located in the vicinity of female nest aggregations (Kroiss et al. 2010; Simon-Thomas & Poorter 1972, Figure 1). These areas do not contain any resources that might be relevant for females and are defended against conspecific males (Evans & O'Neill 1988; Strohm 1995). With a clypeal brush the male applies a cephalic gland secretion on plant materials within its territory (Evans & O'Neill 1988, Figure 2). Very probably this secretion is produced in a mandibular gland (McDaniel et al. 1992; O'Neill & Evans 1983; Schmidt et al. 1990; Schmidt et al. 1985), and there is strong evidence that it functions as sex pheromone to attract receptive females for mating (Evans & O'Neill 1988, 1991; Schmitt et al. 2003; Simon-Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000). Chemical analyses of the pheromone composition in *P. triangulum* revealed a complex blend with (Z)-11-eicosen-1-ol as the main compound (Kroiss et al. 2006; Schmidt et al. 1985; Schmitt et al. 2003).

Figure 1. Male beewolf watching over its territory

The pheromone of male *P. triangulum* possibly conveys a wealth of information about male qualities to the female: Herzner et al. (2006) showed that the male pheromone reflects relatedness, finding that the pheromone blends of brothers were more similar than those of non-related individuals. The study of Kaltenpoth et al. (2007) revealed that the pheromone also mirrors population affiliation and geographical distances. Additionally, the pheromone composition contains information on the male's age, and Kaltenpoth & Strohm (2006) hypothesized that physiological constraints could explain the reduction in costly substances in the pheromones of older males. All these studies show that the males' pheromone has the potential to provide a useful basis for females to choose the optimal mating partner (Herzner et al. 2006; Kaltenpoth et al. 2007; Kaltenpoth & Strohm 2006), especially with regard to the lek mating system in *Philanthus* where females visit male clusters for mating (Höglund & Alatalo 1995; Kroiss et al. 2010), and the associated multitude of males among which females can choose when searching for a mating partner (Evans & O'Neill 1988; Kroiss et al. 2010; Simon-Thomas & Poorter 1972).

Figure 2. Male beewolf scent-marking its territory

Female behaviour

Philanthus females establish subterranean nests by digging nest burrows in sandy soil. They hunt bees or other Hymenoptera and paralyze the prey by stinging it into the thorax (Evans & O'Neill 1988; Strohm 1995, Figure 3). Then, the prey is carried into the female's nest in flight (Olberg 1953). One or several paralyzed prey items are consecutively brought to the nest, and a brood cell is provisioned with the prey (Strohm 1995). Subsequently, the female lays an egg on one of the prey items and closes the brood cell. After hatching, the *Philanthus* larva feeds on the paralyzed prey and subsequently starts spinning a cocoon which is attached to one narrow side of the elliptical brood cell (Strohm 1995; Strohm & Linsenmair 1995). The imago either ecloses later in the same year after the completion of the holometabolous development, or the larva overwinters in the cocoon and hatches in the following summer.

Figure 3. Female European beewolf paralyzing a honey bee

Due to warm and humid conditions in the subterranean brood cells, the prey items and the *Philanthus* offspring are threatened by fungus infestation. Several lines of defence have evolved in this genus to maximize the offspring's survival during its subterranean development period: It was shown that female *Philanthus triangulum* preserve their prey items by embalming them with a secretion from a postpharyngeal gland (Herzner et al. 2007; Herzner & Strohm 2007). This treatment reduces the risk of fungal infestation of the prey items due to a physical effect: The embalming procedure results in a hydrophobic layer on the surface of the prey items. This treatment prevents water condensation and thus constrains spore germination (Herzner & Strohm 2007). The next line of defence is produced by the egg: It has been shown to significantly delay the growth of fungi in the brood cell (Strohm 1995) by releasing the gas nitric oxide with its strong antimicrobial effects (Engl 2011).

During the long-lasting phase of hibernation in the cocoon, the beewolf larva is protected by a highly specialized association with symbiotic bacteria. Adult females harbour and grow these endosymbionts that belong to the genus *Streptomyces* in specialized antennal glands (Goettler et al. 2007; Kaltenpoth et al. 2005). Before a female oviposits it applies a white secretion from the antennal glands containing the symbiotic bacteria to the ceiling of the brood cell (Kaltenpoth et al. 2005; Strohm 1995; Strohm & Linsenmair 1995). When the larva starts spinning the cocoon, it locates this secretion, takes it up and incorporates the symbionts into the silk threads of the cocoon wall (Kaltenpoth et al. 2005; Strohm & Linsenmair 1995). Kaltenpoth et al. (2005) demonstrated that the absence of the symbionts dramatically reduces the survival probability of the larvae in *P. triangulum* by preventing fungal infestation. This is mediated by the production of at least nine different antibiotics on the beewolf cocoon, which provide protection against a broad range of potential fungal pathogens (Kroiss et al. 2010)

1.4 Outline of the thesis

Symbiosis between beewolves and *Streptomyces* bacteria

Chapters 2-3 deal with the unique symbiotic alliance between beewolves of the digger wasp tribe Philanthini (Hymenoptera, Crabronidae, including the genera *Philanthus*, *Trachypus* and *Philanthinus*) and *Streptomyces* bacteria:

In chapter 2, the presence of symbiotic *Streptomyces* bacteria is described for 28 different *Philanthus* species and subspecies. Based on genetic, ultrastructural and morphological data the new monophyletic taxon '*Candidatus* Streptomyces *philanthi*' is proposed for the bacterial symbionts.

In Chapter 3, we reconstructed the phylogenetic relationships among the genera of the subfamily Philanthinae to date the origin of the beewolf-*Streptomyces* symbiosis and to enable future investigations on coevolutionary processes in this association. We compiled and analyzed a large data set including about 5 kb of sequence data based on six molecular markers. We dated the origin of the symbiosis by using a molecular clock approach considering the relevant Philanthinae fossil record. Beyond that, we investigated the relationships within the genus *Philanthus*, because up to now no systematic analyses had been conducted within the subfamily Philanthinae. The resulting species groups were compared to the current morphological classification, and the relation of the genus *Philanthus* to its sister genus *Trachypus*, whose systematic position had not yet been fully understood, is addressed.

Male sex pheromone and temperature during development

Several studies had already demonstrated the variability of the pheromone in European beewolf males: The pheromone blend is known to be shaped by a variety of influencing factors, i.e. relatedness (Herzner et al. 2006), age (Kaltenpoth & Strohm 2006), and geographical distance (Kaltenpoth et al. 2007). Thus, the male pheromone contains information on the male's characteristics and hence might play an important role for female mate choice decisions. Because little is known about the influence of environmental conditions during larval development on the adult pheromone, we investigated the impact of different rearing temperatures on the composition and amount of the sex pheromone of adult male *P. triangulum* (Chapter 4). We also discuss the relevance of the results with regard to female mate choice decisions.

CHAPTER 2

'*CANDIDATUS* STREPTOMYCES PHILANTHI', AN ENDOSYMBIOTIC STREPTOMYCETE IN THE ANTENNAE OF *PHILANTHUS* DIGGER WASPS

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2.1 Summary

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 the 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, the new taxon '*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.

2.2 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 (Douglas 1998; Bourtzis & Miller 2003). 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 (Moran et al. 1993; Bandi et al. 1995; Baumann et al. 1997; Chen et al. 1999; Sauer et al. 2000; Lo et al. 2003).

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*), paralyze them by stinging and provision one to five honeybees as larval food in each brood cell (Strohm 1995; Strohm and 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 the 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 &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 (Strohm &Linsenmair 1995; Kaltenpoth et al. 2005). 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, unpubl. 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 (Currie et al. 1999; Cafaro &Currie 2005).

In the present study, we investigated 28 different *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.

2.3 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 in Cape Town, South Africa. The US species were identified according to Bohart and 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, unpubl. 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 objects were then critical point dried (CPD 030; BALTEC), 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 postfixation 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 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 fD1 and rP2 can be used to amplify a wide range of eubacterial 16S rRNA gene, 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 of template, 1x 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 the *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'-TACCGATCGCATGGTTGGTG-3',
Strep_phil_fwd2: 5'-TATGACTACYGAYCGCATGG-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 to 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, *S. aureofaciens* DSM 40631, and *S. 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 40230T*, S. rimosus* DSM 40260T and *Bacillus subtilis* DSM 402 served as negative controls for the specific probe. The SPT177 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 labeled probe, and samples were incubated for 90 min. at 45°C for hybridization. For hybridization within the antennae, fresh female antennae were cut into sections with a razor 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 labeled 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 ≥ 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.

2.4 Results

Localization of endosymbionts

Scanning electron micrographs of the antennal surface of *Philanthus triangulum*, *P. loefflingi*, and *P. 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; Figure 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 in the brood cell.

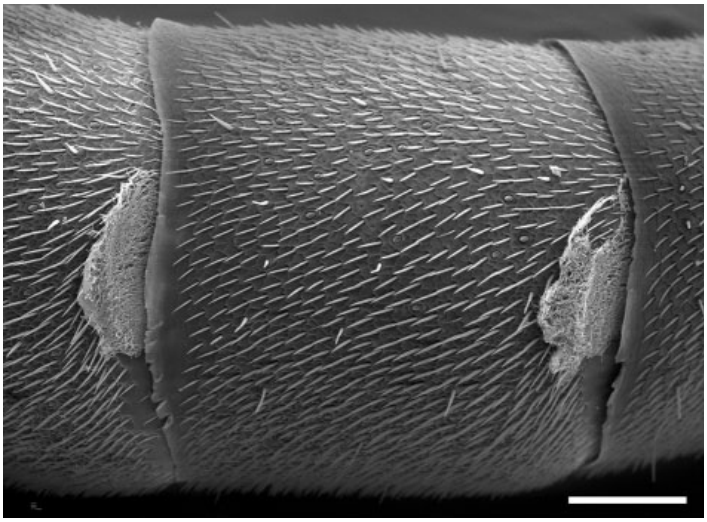

Figure 1. SEM image of an antenna of a female European beewolf (*P. triangulum*) with symbiotic *Streptomyces* bacteria being secreted from the antennal glands. Scale bar = 100 µm.

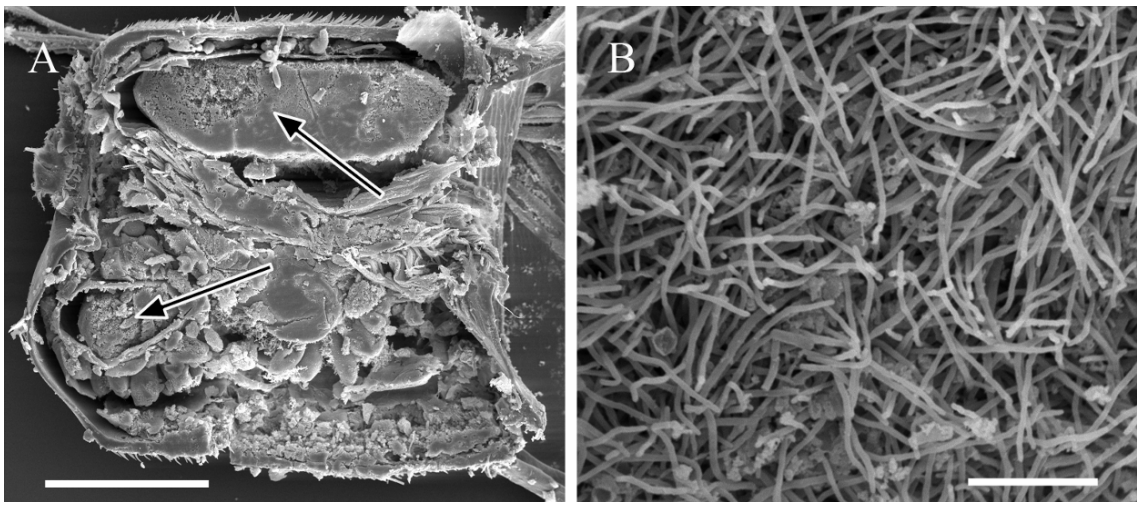

Figure 2. SEM image of the interior of an antennal segment of a female *P. loefflingi*. (A) Longitudinal section of a flagellomer. The reservoir of the antennal gland is indicated by arrows. (B) Symbiotic *Streptomyces* bacteria forming a dense cluster within the antennal gland. Scale bars = 200 µm (A) and 10 µm (B).

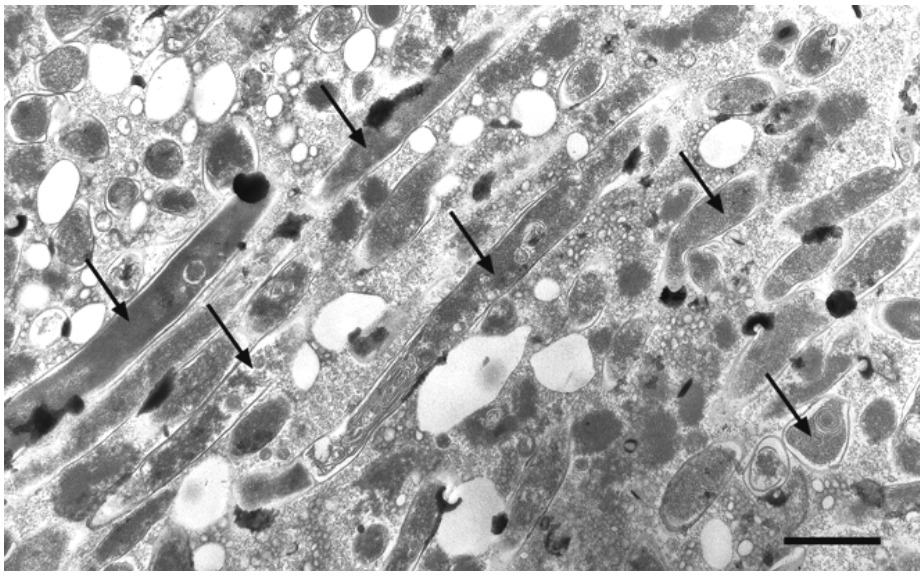

Figure 3. TEM image of a cross-section through the antennal gland of a female *P. triangulum*. Some endosymbiotic *Streptomyces* are indicated by arrows. Scale bar = 1 µm.

When a flagellomer was cut open, filamentous bacteria were clearly visible in large numbers within the gland reservoir (Figure 2A), where they formed a dense cluster of cells (Figure 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 (Figure 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). 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 (Figure 4).

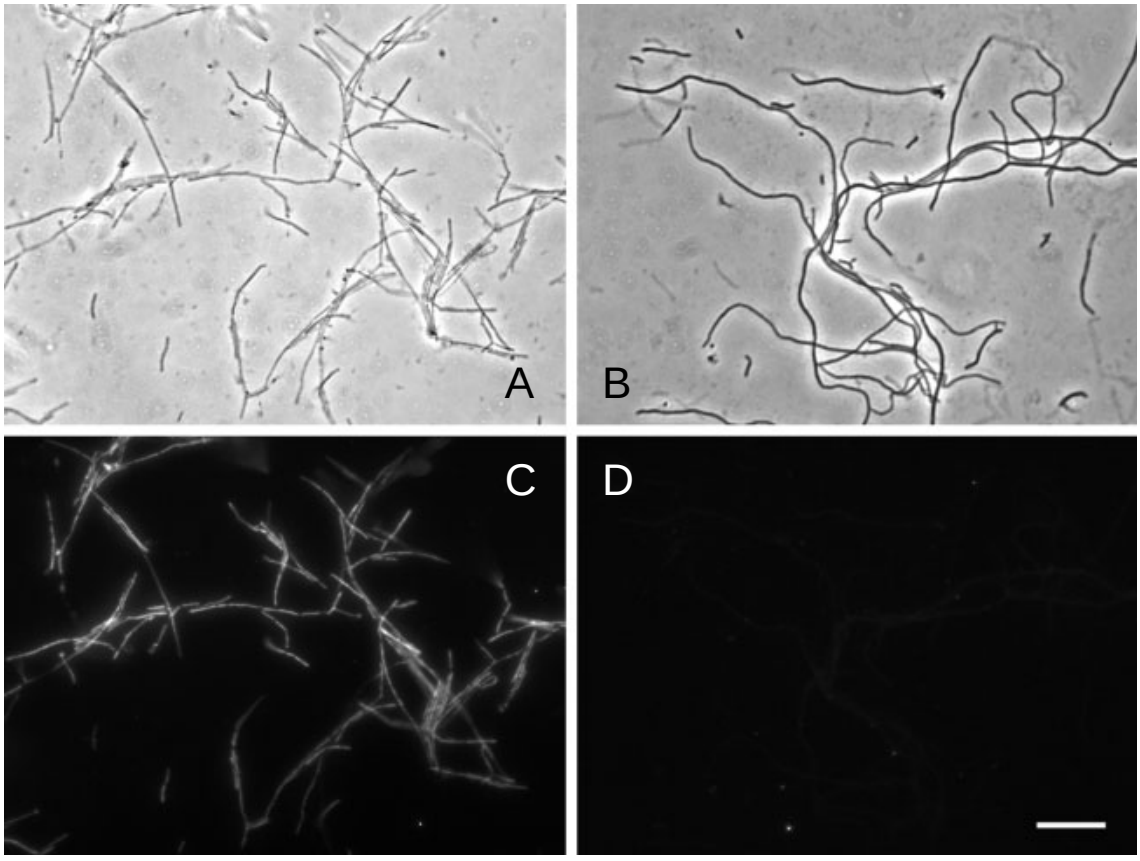

Figure 4. FISH of antennal *Streptomyces* endosymbionts. Phase-contrast micrograph of symbiotic bacteria in the antennal gland secretion of a female beewolf (A) and of a negative control strain of *Streptomyces rimosus* DSM 40260^T (B). (C, D) Epifluorescence micrographs of the same areas after staining with the specific Cy3-labeled probe SPT177. Scale bar = 10 µm.

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. 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 (Figure 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 PCR reactions with the specific 16S rRNA 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* also yielded no amplicons in one of the PCR reactions, but gave strong amplicons in all other PCRs.

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

Specimens of the other wasp genera of the subfamily Philantinae (*Aphilanthops, Clypeadon* and *Cerceris*) yielded no amplicons in any of the specific PCR reactions. 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 surrounding soil during the life of the digger wasps within subterranean nests (data not shown). Thus, the 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.

The *Streptomyces* control strains yielded no amplicons in most of the PCRs, demonstrating 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 (Figure 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.

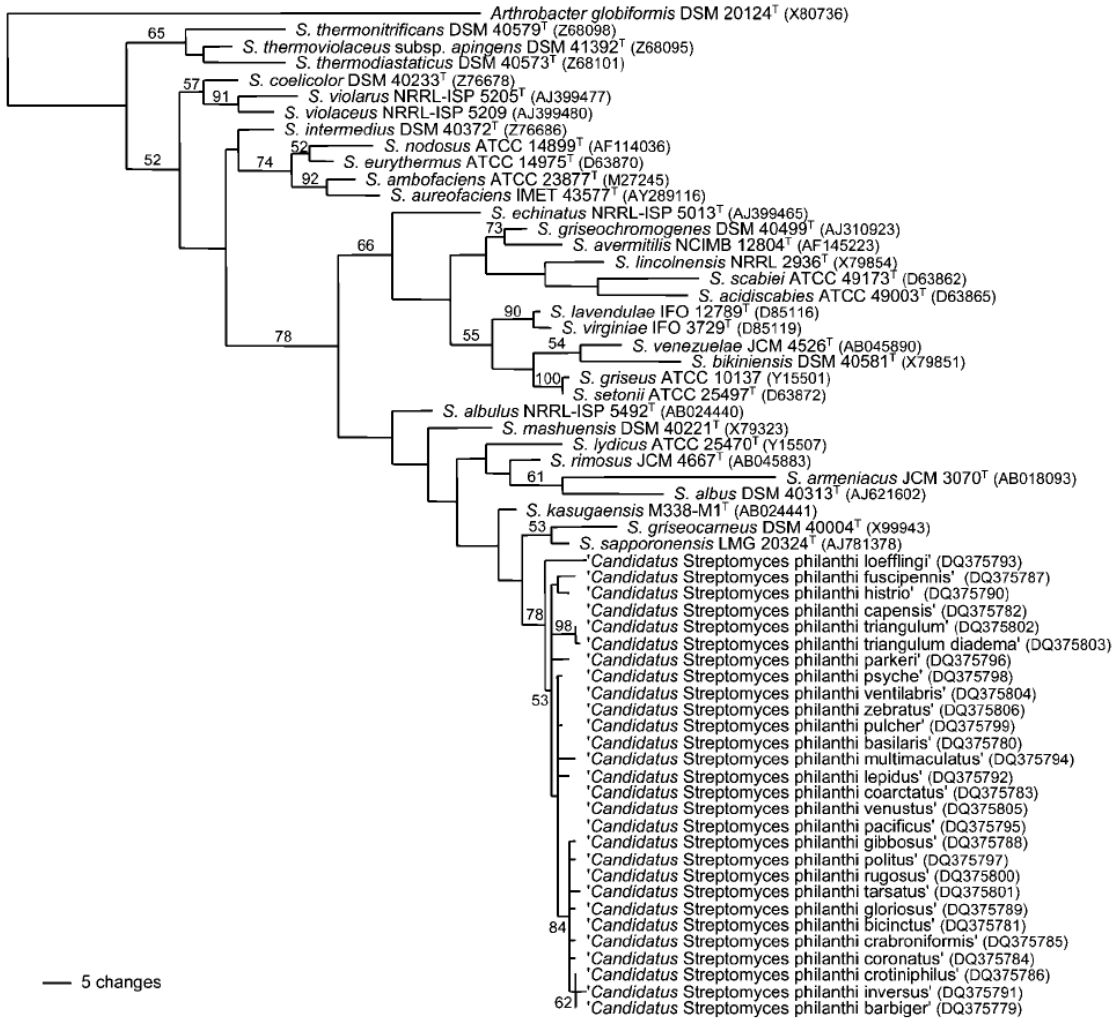

Figure 5. Phylogenetic position of *Philanthus* endosymbionts within the genus *Streptomyces* based on 1324 bp of 16S rRNA gene sequence (104 parsimony-informative characters). First of 600 equally parsimonious trees from a full heuristic search with random addition sequence (10 replicates) and TBR branch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

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* (Figure 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 deposited in the GenBank database with accession numbers DQ375779-DQ375806. The accession numbers for specific ecotypes are shown in Figure 5 and Table 1.

2.5 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 (Buchner 1921; Baumann & Moran 1997; Moran and Telang 1998; Ishikawa 2003). 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 (Strohm, unpubl. 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 online supplementary data, chapter 2.7).

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 on average 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 (Sembiring et al. 2000; Manfio et al. 2003; Liu et al. 2005). Therefore, it is desirable to include ecological characteristics in the description of new species (Cohan 2002; Konstantinidis & Tiedje 2005). Among *Philanthus* endosymbionts, the 16S rRNA gene sequence similarity is relatively high (98.9% to 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 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; Moran & Baumann 2000; Sauer et al. 2000; Clark et al. 2001; Ishikawa 2003). 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; Nyholm et al. 2000; Nishiguchi 2002; 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 to 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 to 0.02 substitutions per site per 50 million years (Ochman and Wilson 1987; Moran et al. 1993; Bandi et al. 1994), 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 and 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 cocoonspinning 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 groundnesting digger wasps cope with the threat of pathogenic soil microorganisms infecting their progeny.

Acknowledgements

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2.6 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'-TACCGATCGCATGGTTGGTG-3'; S (*Philanthus*, antennal glands); M]. Kaltenpoth et al., this study.

2.7 Online supplementary data

Attempts to cultivate '*Candidatus* Streptomyces philanthi'

In a first attempt to cultivate the *Philanthus* antennal symbionts, secretions from *Philanthus triangulum* female antennal glands were harvested from the brood cells and suspended in 100 µl of sterile water. 10-100 µl of the suspensions were spread onto a range of different solid media. Additionally, whole antennae of freshly killed female *P. triangulum* were plated out on the same media.

The following media were tested: LB agar (DSM Medium 381), Streptomyces Medium (DSM Medium 65), Streptomyces Medium supplemented with streptomycin (100 µg/ml) and kanamycin (50 µg/ml), Streptomyces Medium supplemented with homogenized bees from beewolf brood cells (12 bees per 500 ml medium), Streptomyces Medium supplemented with homogenized *P. triangulum* females (eight females per 20 ml medium), Powdered Chitin Agar (Hsu and Lockwood, 1975), Powdered Chitin Agar supplemented with cycloheximide (100 µg/ml), and beewolf cocoon agar (a medium containing 30 empty *P. triangulum* cocoons per 250 ml agar medium). Plates were incubated at 25°C and 30°C under aerobic conditions for six to eight weeks.

Bacteria from culture plates were spread onto six-field microscope slides for fluorescence in-situ hybridization (FISH). The specific probe SPT 177 (Kaltenpoth et al. 2005) was used to screen for '*Candidatus* Streptomyces philanthi', and the general eubacterial probe EUB 338 (Amann et al. 1990) served as a positive control. Although bacterial colonies grew on all media tested and several of the colonies showed actinomycete morphology, none of the colonies was stained by the specific probe SPT 177. Amplification and sequencing of partial 16s rDNA sequences from some of the colonies with general eubacterial primers fD1 and rP2 (Weisburg et al. 1991) revealed the presence of *Acinetobacter* sp. and *Streptomyces* sp.

In a second cultivation attempt, female beewolf antennae were surface sterilized before cultivation. Therefore, four antennae were removed from live adult wasps and rinsed for 5 minutes in 1 ml of a sterile solution of 0.5% Triton X-100 to remove surface debris. The antennae were then surface sterilized by immersion in 1 ml of a freshly made sodium hypochlorite solution with 0.6 % available chlorine for 2 minutes. The antennae were then rinsed five times in 1 ml sterile water and transferred aseptically to a Dounce ground glass subcellular homogenizer (Kontes Scientific Glassware, Vineland, NJ) along with 1 ml sterile Mitsuhashi-Maramorosch (MM) basal medium (ICN Biomedicals). The antennae were then homogenized for 2 min to release bacteria and the homogenate was used as inoculum in a range of culture attempts.

Culture attempts were made using a range of solid media formulations under aerobic, anaerobic and microaerobic conditions. The media formulations tested included Streptomyces Medium (Sigma), supplemented with 0.2% (w/v) casamino acids (Difco), Potato Dextrose agar (Difco), MM

agar (Dale et al., 2005), and Medium 199 (Gibco), solidified by addition of molten low-melt agarose (1% w/v final concentration) at 55 °C.

Cultures were initiated on solid phase media by streaking 20 µl of the antennal homogenate onto plates. Plates were incubated at 25 °C under an air atmosphere (to provide aerobic conditions) or in sealed gas jars flushed with at least 20 volumes of either nitrogen (for anaerobic conditions) or a mixture of 5% oxygen, 10% carbon dioxide and 85% nitrogen (for microaerophilic conditions). Plates were maintained for 7 days and then removed and inspected under a stereo microscope. Bacterial colonies were removed and inoculated directly into PCR tubes. PCR was performed using universal bacterial 16S rDNA primers (Hugenholtz et al. 1998). The 16S rDNA amplicons were cloned into TOPO vectors, and sequenced using vector specific primers. The resulting sequences were then submitted to BLAST at the NCBI database. Unfortunately, no *Philanthus* endosymbiont 16S rDNA sequences were detected; the 16S rDNA sequences obtained were all closely related to the genus *Serratia*.

Media Formulations

LB Agar

Trypone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g
Distilled water	1000.0 ml

pH adjusted to 7.0 with KOH before addition of agar and autoclaving.

Powdered Chitin Agar

Colloidal Chitin	4.0 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$MgSO_4 \bullet 5\ H_2O$	0.5 g
$FeSO_4 \bullet 7\ H_2O$	0.01 g
$ZnSO_4$	0.001 g
$MnCl_2$	0.001 g
Agar	20.0 g
Distilled water	1000.0 ml

Streptomyces medium

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
$CaCO_3$	2.0 g
Agar	12.0 g
Distilled water	1000.0 ml

pH adjusted to 7.2 with KOH before addition of agar and autoclaving.

MM agar

Sodium Chloride	7.0 g
Lactalbumin hydrolysate	6.5 g
Yeast extract	5.0 g
Glucose	4.0 g
$CaCl_2$	0.15 g
$MgCl_2$	0.05 g
KCl	0.2 g
$NaHPO_4$	0.17 g

Make up in 800 ml of water, add 0.12 g sodium bicarbonate, adjust pH to 6.9 and filter sterilize. Equilibrate the sterile media in a 55 °C water bath and then add 200 ml of autoclaved (and still molten) 5% agarose. Pour plates and pre-equilibrate in a gas jar, if necessary.

CHAPTER 3

65 MILLION YEARS OF DEFENSIVE ALLIANCE: MOLECULAR PHYLOGENY OF BEEWOLVES REVEALS THE AGE OF A PROTECTIVE SYMBIOSIS WITH *STREPTOMYCES* BACTERIA

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Abbreviations:

cds: coding sequence
ncs: non-coding sequence
bp: base pairs
wnt-1: Wingless
LWRh: long-wavelength rhodopsin
ArgK: arginine kinase
EF1α: elongation factor 1α
COI: cytochrome oxidase subunit 1
ML: maximum likelihood
BI: Bayesian inference
MP: Maximum parsimony

3.1 Abstract

Beewolf digger wasps of the genera *Philanthus*, *Trachypus,* and *Philanthinus* (Hymenoptera, Crabronidae) engage in a unique defensive symbiosis with *Streptomyces* bacteria. The symbionts are located inside antennal glands and transferred to the larval cocoon by the larvae, where they provide protection against detrimental fungi by producing a complex cocktail of at least nine different antibiotic substances. In order to date the origin of the symbiotic association with *Streptomyces* we set up a data set of more than 5 kb DNA sequences per taxon, including six molecular markers (wingless, long-wavelength rhodopsin, arginine kinase, cytochrome oxidase subunit 1, 28S rRNA, and elongation factor 1α) for a reconstruction of the phylogenetic relationships within the Crabronid subfamily Philanthinae. Maximum likelihood, Bayesian inference, and maximum parsimony analyses revealed congruent phylogenetic relationships with high support values in all reconstructions among 43 species and subspecies of *Philanthus*, six species of *Trachypus*, one *Philanthinus* species*,* and several outgroup taxa. *Philanthinus* was found to be the

most basal genus that is associated with protective symbionts and the genus *Philanthus* was found to be paraphyletic with respect to the morphologically well-defined South American genus *Trachypus*. Molecular clock analyses placed the origin of the symbiosis between beewolves and *Streptomyces* to 65-97 million years. This study is the first estimate of the evolutionary age for any protective insect-bacteria symbiosis and provides the basis for understanding the evolution of this association as well as of other traits of beewolf digger wasps.

3.2 Introduction

Beewolves of the digger wasp tribe Philanthini (Hymenoptera, Crabronidae, including the genera *Philanthus*, *Trachypus* and *Philanthinus*) represent a small group of solitary digger wasps comprising about 173 species worldwide. Several characteristics of this group are unusual or even unique among insects and render the Philanthini especially interesting model organisms for ecological and evolutionary research. Male beewolves establish small territories, defend them against intruding males and scent mark them with a sex pheromone from cephalic glands to attract receptive females (Evans & O'Neill 1988; Kroiss et al. 2010; Simon-Thomas & Poorter 1972; Strohm 1995). The pheromone has been shown to contain information about relatedness, population affiliation, and age, which may be used by females for adaptive mate choice (Herzner et al. 2006; Kaltenpoth et al. 2007; Kaltenpoth & Strohm 2006).

As in many other solitary wasps, female beewolves dig nest burrows and mass-provision their larvae with Hymenopteran prey (Bohart & Menke 1976; Evans 1964; Koedam et al. 2009; Polidori et al. 2009; Strohm 1995; Strohm & Linsenmair 1995). Due to the warm and humid conditions in the beewolf brood cells, both the offspring and the provisions are endangered by fungal or bacterial infections from the surrounding soil (Strohm & Linsenmair 2001). To reduce the risk of microbial attack, beewolves have evolved a number of efficient defence mechanisms: Females of the genus *Philanthus* have been shown to preserve their prey items by embalming them with a secretion from a postpharyngeal gland (Herzner et al. 2007; Herzner & Strohm 2007). Interestingly, the secretion reduces fungal infestation by a physical rather than a chemical effect. The treatment of the prey strongly reduces water condensation on the bees and thereby hinders spore germination and growth (Herzner & Strohm 2007). The second line of defence is mediated by a specialized association with endosymbiotic bacteria of the genus *Streptomyces* that beewolf females cultivate in unique antennal gland reservoirs and secrete into the brood cell prior to oviposition (Goettler et al. 2007; Kaltenpoth et al. 2006; Kaltenpoth et al. 2005). The symbionts are later taken up by the larva and incorporated into the cocoon silk (Kaltenpoth et al. 2010a), where they provide protection against a broad range of potentially harmful microorganisms by producing a complex cocktail of at least nine different antibiotic substances (Kaltenpoth et al. 2005; Kroiss et al. 2010). The symbiotic *Streptomyces* have been detected in the antennae of all *Philanthus*, *Trachypus,* and *Philanthinus* species investigated so far, but not in any other genera of closely related wasps (i.e. *Cerceris*, *Aphilanthops*, *Clypeadon*, Kaltenpoth et al. 2006, 2010b; Kaltenpoth et al. submitted), suggesting that the symbiosis evolved somewhere along the branch leading to the tribe Philanthini (Kaltenpoth et al. submitted). Although

the symbionts of different species form a monophyletic group within the genus *Streptomyces*, horizontal transmission among host species also seems to occur (Kaltenpoth et al. 2010a; Kaltenpoth et al. submitted).

At present, the phylogenetic relationships within and among genera of the Crabronid subfamily Philanthinae are poorly understood. The most detailed phylogenetic study thus far was based on 37 morphological characters and yielded insights into genus-level relationships (Alexander 1992). The results revealed three monophyletic tribes within the Philanthinae: the Aphilanthopini (*Aphilanthops* + *Clypeadon*), the Cercerini (*Pseudoscolia* + (*Cerceris* + *Eucerceris*)), and the Philanthini (*Philanthinus* + (*Philanthus* + *Trachypus*)) (Alexander 1992). However, no systematic analyses have so far been published on the intrageneric level, although several authors classified species from the same geographical regions into subgroups based on morphological traits (Arnold 1925; Bohart & Grissell 1975; de Beaumont 1961; Evans & O'Neill 1988; Ferguson 1983b). Additionally, the systematic classification of the genera *Trachypus* and *Philanthus* is still unclear: Currently, they are treated as sister genera. However, several authors suggested that *Philanthus* is paraphyletic with respect to *Trachypus*, but did not formally synonymize the two genera (Alexander 1992; de Beaumont 1961).

Here we used molecular markers to reconstruct the phylogenetic relationships among and within genera of the Philanthini. On the basis of 5040 bp of sequence data from six different nuclear and mitochondrial genes, we reconstructed a well-supported phylogenetic tree of 43 species and subspecies of *Philanthus*, six *Trachypus* species, and one species each from the genera *Philanthinus, Aphilanthops, Clypeadon, Cerceris*, respectively, as well as several outgroup genera. The phylogeny allowed us to date the origin of the symbiotic association between Philanthini and their defensive *Streptomyces* symbionts by using a molecular clock approach, and it lays the foundation for comparative phylogenetic analyses of hosts and symbionts to test for co-diversification. Beyond that, the phylogeny provides a basis for reconstructing the evolution of the male pheromone, the evolution of the other defence mechanisms and of different ecological characteristics of beewolves.

3.3 Materials and methods

Insect samples

Specimens of 43 *Philanthus* species and subspecies from North America, Europe, India, and South Africa, six *Trachypus* species from South America, and one *Philanthinus* species from Turkey were collected (Suppl. Table 1). As outgroup taxa, Crabronid species of the closely related genera *Aphilanthops, Clypeadon, Cerceris, Bembix*, and *Bicyrtes*, as well as the more distantly related *Apis mellifera* (Apidae) were used (Suppl. Table 1).

Molecular methods

DNA was extracted either from insect thoraces or, to allow for later morphological determination of single specimens, from three legs. The MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) was used for DNA isolation according to the manufacturer's instructions. PCR amplifications were performed on a TGradient Thermocycler (Biometra, Göttingen, Germany). The PCR master mix with a final reaction volume of 12.5 µl was composed of 1 µl genomic DNA extract, 1 µl of each primer (10 µM), 1.5 µl dNTP-Mix (2 mM; Fermentas, St. Leon-Rot, Germany), 1.25 µl Peqlab reaction buffer (200 mM Tris-HCl (pH 8.55 at 25 °C), 160 mM $(NH_4)_2SO_4$, 0.1% Tween 20 and 20 mM $MgCl_2$) and 0.5 units SAWADY Taq DNA polymerase (Peqlab, Erlangen, Germany). Cycle parameters were as follows: 3 min initial denaturation at 94°C, followed by 35 cycles of 94°C for 40 sec, the primer-specific annealing temperature for 40 sec, and 72°C for 40 sec (or 90 sec for longer fragments), and a final extension of 4 min at 72°C. Primer sequences and references are listed in Table 1, details on primer combinations, annealing temperatures and the corresponding fragment lengths are summarised in Table 2. Prior to sequencing, PCR products were purified with the peqGOLD MicroSpin Cycle-Pure Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) following the manufacturer's protocol. Sequencing was done commercially at Seqlab Sequence Laboratories (Göttingen, Germany).

Table 1. Sequences and references of the PCR primers used in this study

Gene	Primer	Sequence	Reference
Wingless	beewgfor	TGCACNGTSAAGACCTGYTGGATGAG	Danforth et al. 2004
	Lepwg2a	ACTICGCARCACCARTGGAATGTRCA	Brower & DeSalle 1998, Danforth et al. 2004
Long	LWRH_Rev1744	GCDGCTCGRTAYTTHGGATG	this study
Wavelength	LWRhFor4_N	GAGAARAAYATGCGNGARCAAGC	this study (modified from Danforth et al. 2004)
Rhodopsin	LWRhFor1	AATTGCTATTAYGARACNTGGGT	Mardulyn & Cameron 1999, Danforth et al. 2004
	LWRhRev1	ATATGGAGTCCANGCCATRAACCA	Mardulyn & Cameron 1999, Danforth et al. 2004
EF1a	For1deg	GYATCGACAARCGTACSATYG	Danforth et al. 2003
	F2Rev1	AATCAGCAGCACCTTTAGGTGG	Danforth et al. 2003
	HaF2for	GGGYAAAGGWTCCTTCAARTATGC	Danforth et al. 1999
	Cho10	ACRGCVACKGTYTGHCKCATGTC	Danforth et al. 2003
Arginine	ArgK_Loretta	TGATCGATGATCACTTCCTTTTCAA	this study
kinase	ArgK_fwd2	GACAGCAARTCTCTGCTGAAGAA	Kawakita et al. 2003
	ArgK_KLTrev2	GATKCCATCRTDCATYTCCTTSACRGC	www.danforthlab.entomology.cornell.edu/resources.html
CO	fwd1	TGGAGCHTCWTTYAGATTAATAATYCG	this study
	rev2	TCCWCCAATWGTRAATAATAARAYA	this study
	LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
	Ben	GCWACWACRTAATAKGTATCATG	Kronauer et al. 2004
28s	3665F	AGAGAGAGTTCAAGAGTACGTG	Cameron & Mardulyn 2001
	4749R	GTTACACACTCCTTAGCGGA	Danforth et al. 2006

Partial sequences of six different genes were obtained, all of which have previously been shown to be useful for phylogenetic analyses in Hymenoptera (Cameron & Mardulyn 2001; Danforth et al. 2004; Kawakita et al. 2003; Ramirez et al. 2010): A fragment of the subunit 1 of the mitochondrial cytochrome oxidase gene (COI; 841 bp) was amplified and sequenced, as well as a fragment of the ribosomal 28S gene (28S; 865 bp). Additionally, the following four single-copy nuclear genes were used: Wingless (wnt-1, comprising of 378 bp cds), long-wavelength rhodopsin (LWRh, comprising of

608 bp of cds and 156 bp ncs), arginine kinase (ArgK, with 825 bp cds and 111 bp ncs) and elongation factor 1α (EF1a, including 1041 bp cds and 696 bp ncs). The listed fragment lengths are those of the processed sequences used for the phylogenetic analyses. Some outgroup sequences for *Apis*, *Bembix,* and *Bicyrtes* could be obtained from the NCBI database.

Table 2. Primer pairs with corresponding fragment lengths and PCR annealing temperatures

Primer pair	Gene	Annealing temperature in ℃	approx. f ragment lentgh
beewgfor / Lepwg2a	Wingless	65.6	450 bp
LWRhFor1 / LWRhRev1	Opsin	58.5	650 bp
LWRhFor1 / LWRH_Rev1744	Opsin	53.8	1200 bp
LWRhFor4_N / LWRH_Rev1744	Opsin	53.8	800 bp
ArgK_fwd2 / ArgK_KLTrev2	ArgK	50.5	1200 bp
ArgK_Loretta / ArgK_KLTrev2	ArgK	53.0	700 bp
For1deg / F2Rev1	EF1α	56.8	1300 bp
HaF2for / Cho10	EF1α	58.0	1700 bp
28s_3665F / 28s_4749R	28s	62.9	1000 bp
LCO / Ben	CO	49.0	1100 bp
fwd1 / rev2	CO	52.8	1000 bp

Phylogenetic analyses

Sequences were aligned using the programs BioEdit 7.0.5.3 (Hall 1999) and SeaView 4.2.6 (Gouy et al. 2010). All alignments were checked and improved manually. Open reading frames and intron/exon boundaries were identified by comparison with published coding sequences for *Apis mellifera* (LWRh: BK005514.1; ArgK AF023619.1; EF1a: NM_001014993.1) or via a blast search against non-redundant sequences in the Genbank database. Coding sequences and introns should be treated as separate data sets due to a differing sequence evolution. Thus, we differentiated between coding (cds) and non-coding (n-cds) sequence parts within a single locus. We determined nine individual partitions: 28S, COI, wnt-1, LWRh-cds, LWRh-ncs, ArgK-cds, ArgK-ncs, EF1a-cds, and EF1a-ncs. Unfortunately, all non-coding sequences could only be reliably aligned among the Philanthini species. Therefore, we recoded the intron sequences of all outgroup taxa to missing data and thus excluded them from the analyses.

In a first step we built nine gene trees using fast likelihood inferences with the software RAxML v7.0.4 (Stamatakis 2006; Stamatakis et al. 2008; Stamatakis et al. 2005) corresponding to the nine partitions determined above. Maximum likelihood (ML) searches were conducted with the rapid hill-climbing algorithm (Stamatakis et al. 2005) under the General Time-Reversible model with four gamma parameters GTR+G (Tavaré 1986; Yang 1993, 1994). Support values (100 bootstrap steps) were calculated for each node and topologies were manually compared among the gene trees. Because none of the strongly supported nodes were different, we combined all loci in one supermatrix.

Additionally, searches for a saturation effect within one of the three codon positions were conducted for the genes wnt-1, COI, LWRh, ArgK, and EF1a by calculating homoplasy indices (HI) for each codon position and gene separately. The software PAUP* 4.0 beta (Swofford 2003) was used for these analyses. The homoplasy index of the third codon position of the genes COI and LWRh (HI(COI)=0.66, HI(LWRh)=0.46) were higher compared to the first and second positions (HI: CO-1^{st}=0.52, COI-2^{nd}=0.25, LWRh-1^{st}=0.34, LWRh-2^{nd}=0.18). Therefore, we excluded the third codon positions of the genes COI and LWRh for further analyses, or we used the translated amino acid sequences (stated for each analysis).

In a next step, multiple independent analyses with different data partitioning strategies (1-4) were performed to test for the robustness of the phylogenetic reconstructions: (1) unpartitioned, (2) four partitions with combined nuclear introns, exons and mitochondrial sequences separately, plus 28S sequences, (3) nine partitions with single genes separately and splitting coding and non-coding sequence parts, (4) complete random partitioning in 9 partitions; all analyses were conducted with excluded third codon positions of the genes COI and LWRh and also with base sequences translated into amino acid sequences. The best fitting evolutionary model for the amino acid-translated sequences (COI, LWRh) was inferred with ProtTest v1.4 (Abascal et al. 2005). The CPREV model showed the highest fit for LWRh, and the MTREV for COI; both are empirical models of amino acid substitution that indicate relative rates of amino acid replacement. Out of these different runs we chose the tree with the highest likelihood for presentation. Bootstrap support values were obtained through a full non-parametric bootstrap inference with 10000 replicates, carried out separately with RAxML.
Bayesian inferences were run with the program MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). The searches were also conducted under the GTR+G model with four rate categories. We ran each analysis for 10,000,000 generations and sampled trees every 1000 generations. A "Burnin" of 20% was used, i.e. the first 20% of the sampled trees are discarded. We checked if the standard deviation of split frequencies consistently was less than 0.01 and discarded all runs not meeting this criterion. We computed 50% majority rule consensus trees for each analysis with posterior probability values for every node. Different partition schemes (1-4) were analyzed as well (see above). However, combined data sets of DNA and protein sequences cannot be used in MrBayes, so only the nucleotide sequences of the first and second codon positions were taken for COI and LWRh.

Equal weighted maximum-parsimony (MP) analyses were performed using the program PAUP* 4.0 beta (Swofford 2003). We used a heuristic search and TBR (tree-bisection-reconnection) for branch swapping. Bootstrap supports were obtained from 1000 independent replicates. The third codon positions of COI and LWRh were excluded for all MP analysis as well. Further, MP analyses were only conducted for the partition schemes (1) and (3).

Dating of the symbiosis

Divergence time estimations were inferred using BEAST v1.6.1 (Drummond & Rambaut 2007). Bayesian analyses were conducted under a strict clock (using a single rate of sequence evolution across the phylogeny) and two relaxed clock (allowing variable substitution rates) models (uncorrelated lognormal, random local clock model; Drummond et al. 2006). In each analysis 25 million steps were performed. To estimate the influence of partitioning, analyses were conducted with a partitioned (9 partitions) as well as with the unpartitioned dataset.

Four calibrations points were included in the dating analysis: (A) The age of the Bembicinae with oldest fossils known from lower Oligocene (~37 Mya) Florissant beds in Florida (Cockerell 1906; Pulawski & Rasnitsyn 1980), (B) the age of the oldest *Cerceris* fossil from Late Stampian (~30 Mya) shales in France (Timon-David 1944), and (C) the age of the oldest *Philanthus* fossil (*P. saxigenus*) from Florissant shales (~37 Mya) in Florida (Rohwer 1909), and (D) the root age was calibrated with the bee fossil *Cretotrigona prisca* from Maastrichtian amber (~65 Mya) in New Jersey/USA (Engel 2000; Michener & Grimaldi 1988). Because of their unclear systematic position, the fossils of *Philanthus annulatus* (~30 Mya, Theobald 1937) and *Prophilanthus destructus* (~35 Mya, Cockerell 1906) were not considered in the analysis. Minimum age constraints were modelled with lognormal distributions. Maximum age constraints were set to 125 Mya (A-C), the estimate of the crabronid-bee divergence based on the Apoidea fossil record (Ohl & Engel 2007). A maximum constraint for the root age was set to 140 Mya, the estimated rise of the angiosperms. Due to their dependence on and tight association with angiosperms, bees and the closely related crabronid wasps have very likely evolved after the origin of angiosperms (Brady et al. 2009).

Further, one analysis was conducted without sequence data and fixed topology (ML topology) to account for a possible bias imposed through the calibrations alone without considering the DNA information. Additional analyses were conducted to examine the influence of the root age calibration. Here, two analyses were performed, one without constraining the root age as well as another with only the root age as calibration point. Evaluation and comparison of the results were performed using Tracer v 1.5, TreeAnnotator, LogCombiner (Drummond & Rambaut 2007), and FigTree v1.3.1 (Rambaut 2010). Confidence intervals were estimated as 95% highest posterior density intervals (HPD). The clock model producing the smallest confidence intervals summed over all analyzed parameters was considered as most appropriate for the data (Drummond et al. 2006). The uncorrelated lognormal model outperformed the random local clock model in this respect. Therefore, we used the uncorrelated lognormal model with 25 million replicates and a sample frequency of 2500 steps for the final divergence time estimation. Three independent runs were performed. We used the ML tree (see Figure 2) as starting tree for these analyses. The first 1000 trees (10%) were discarded as burnin for each sample and then the three runs were combined into one single file. The maximum clade credibility tree was inferred with TreeAnnotator and visualized with FigTree. The combined log file was analyzed with the software Tracer.

3.4 Results

Phylogenetic analysis

The final concatenated alignment consisted of 5521 aligned nucleotides, comprising 1378 parsimony informative sites (excluding third position of COI/LWRh: 5040 bp, 1077 parsimony informative sites). Maximum likelihood analyses, Bayesian inference analyses and maximum parsimony analyses based on the full and partitioned dataset showed highly congruent topologies (Figure 1; for MP tree see Suppl. Figure 1). Likewise, most of the different analytical scenarios and partitions yielded similar results. Some discrepancies were observed for the analyses including the third codon position of LWRh and COI, which were likely caused by the saturation of the third positions.

In all analyses, the genus *Philanthinus* was positioned at the base of the Philanthini with high statistical support. However, the genus *Trachypus* grouped deeply within the genus *Philanthus* and represented the sister group to all North American *Philanthus* species. This was supported by the combined dataset as well as all single-gene analyses with the exception of the LWRh intron and the – probably misleading – analyses including the 3rd positions of LWRh or COI. However, in the LWRh intron analysis based on only 156 bp of aligned sequence data, there was only weak statistical support for the genera *Philanthus* and *Trachypus* being monophyletic sister clades. The strong support for the paraphyly of *Philanthus* with respect to *Trachypus* in all other single-gene trees and, most notably, the analyses based on the combined dataset strongly suggest that the genera *Philanthus* and *Trachypus* should be taxonomically treated as one genus.

The reconstruction of the phylogenetic affiliations within the genera *Philanthus* and *Trachypus* were highly congruent across the different ML, BI and MP analyses. Separate analyses based on single-gene coding sequences and on introns yielded similar intrageneric relationships, respectively, indicating that the results were not biased by selective pressures acting on the coding sequences. The species groups recovered in this study as well as the present morphological classification are listed in Table 3. Due to the extensive studies of Evans and O'Neill (1988), the best investigated *Philanthus* species are those of the North American continent. Based on morphological and behavioural characters, they were classified into four species groups (Table 3): the *zebratus* group, *gibbosus* group, *pacificus* group and *politus* group (Evans & O'Neill 1988). The species *P. ventilabris*, *P. lepidus*, *P. bilunatus*, *P. solivagus* and *P. albopilosus* were considered as separate species, not clearly fitting the traits of one of the four groups. In the molecular phylogenies, the *zebratus* group agreed with the composition proposed by Evans & O'Neill, and *P. solivagus* could be added to this group. Based on the molecular data, the *gibbosus* group proposed by Evans & O'Neill (1988) was split into two separate groups, the second one of which is here called "*inversus* group". Additionally, the two species *P. barbiger* and *P. bilunatus* could be assigned to the *gibbosus* group in our analyses. There were some discrepancies between molecular and morphological data with regard to the placements of *P. psyche, P. serrulatae,* and *P. pulcher* within the *pacificus* and the *politus* group, which is unsurprising as these groups comprise the species that are both the smallest and the morphologically most difficult to identify among North American *Philanthus*.

As suggested by Evans & O'Neill (1988), *P. ventilabris* is quite distinct in the phylogenetic analyses, but with close affinity to the *zebratus* group. *P. albopilosus* and *P. lepidus* could also not clearly be assigned to one of the four groups, but in our analyses the two species were positioned at the base of the *pacificus/politus* species complex.

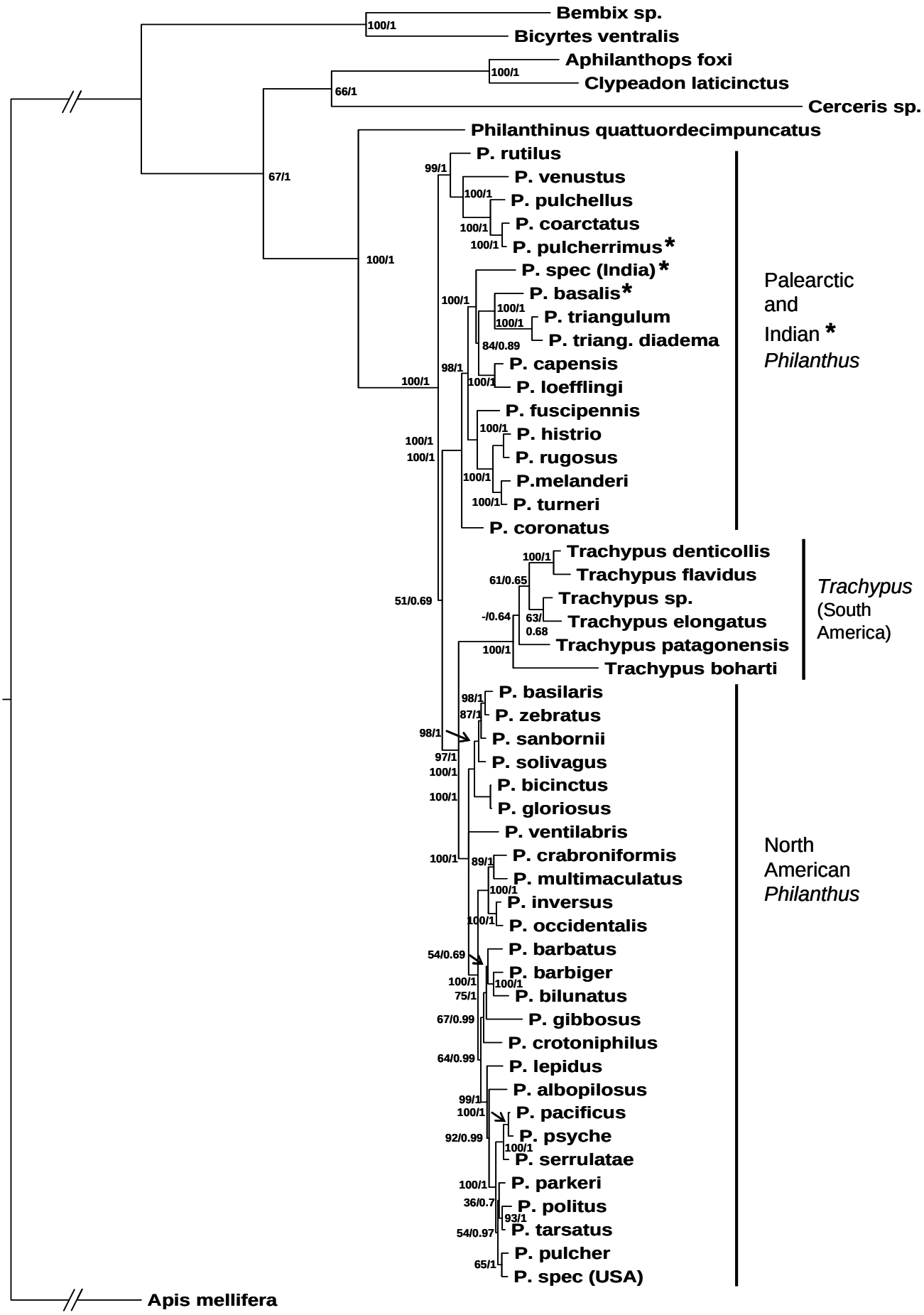
Bembix sp.
Bicyrtes ventralis
Aphilanthops foxi
Clypeadon laticinctus
Cerceris sp.
Philanthinus quattuordecimpunctatus
P. rutilus
P. venustus
P. pulchellus
P. coarctatus
P. pulcherrimus*
P. spec (India)*
P. basalis*
P. triangulum
P. triang. diadema
P. capensis
P. loefflingi
P. fuscipennis
P. histrio
P. rugosus
P.melanderi
P. turneri
P. coronatus
Palearctic and Indian * Philanthus
Trachypus denticollis
Trachypus flavidus
Trachypus sp.
Trachypus elongatus
Trachypus patagonensis
Trachypus boharti
Trachypus (South America)
P. basilaris
P. zebratus
P. sanbornii
P. solivagus
P. bicinctus
P. gloriosus
P. ventilabris
P. crabroniformis
P. multimaculatus
P. inversus
P. occidentalis
P. barbatus
P. barbiger
P. bilunatus
P. gibbosus
P. crotoniphilus
P. lepidus
P. albopilosus
P. pacificus
P. psyche
P. serrulatae
P. parkeri
P. politus
P. tarsatus
P. pulcher
P. spec (USA)
North American Philanthus
Apis mellifera
0.06

Figure 1. Maximum-likelihood phylogeny of Philanthinae. Best maximum-likelihood tree obtained with RAxML v7.04, including 43 species of *Philanthus*, six species of the genus *Trachypus,* one species of each of the genera *Philanthinus*, *Cerceris*, *Aphilanthops*, and *Clypeadon*, two other Crabronid genera (*Bembix* and *Bicyrtes*) as well as *Apis mellifera* as outgroups. Values at the nodes represent ML bootstrap support values (obtained by a full non-parametric bootstrap search with 10000 iterations) and Bayesian posterior probabilities of the Bayesian analysis. The data set consisted of a partitioned 5040bp alignment including nucleotide sequences of six genes (wnt-1, 28s, ArgK, EF1a, LWRh and COI [excluding 3rd positions in the cds]).

Earlier studies on Palearctic and African *Philanthus* species are scarce and less detailed (Arnold 1925; de Beaumont 1949, 1951, 1961). Our phylogenetic analyses indicate that the *rutilus* and *coarctatus* groups proposed earlier (de Beaumont 1961; Gayubo 1991) should be combined. Furthermore, the *triangulum* group was extended by five more members, and the *fuscipennis* species group by four other species.

Age of the symbiosis between beewolves and *Streptomyces* bacteria

Model comparison in Tracer with pairwise Bayes factor analyses on the tree likelihood clearly revealed the relaxed uncorrelated lognormal model as most appropriate in comparison to the strict clock and the random local clock model. Comparing the analyses regarding partitioning, the 9-partition data set was preferred over the unpartitioned data set in the Bayes factor analyses, but due to a trade off between computational power and time and comparable results of the analyses of both partitioning data sets regarding topology and branch lengths the unpartitioned data set was used for the final analysis. In the analyses where the root date was the only calibration point, the results for the estimated age of the symbiosis were similar to the analyses without root calibration. The same was true for the analyses with and without consideration of the alignment, indicating that the analysis was robust. The relaxed clock assumption that branches differ in their substitution rates was confirmed: with a value of 0.508 the coefficient of variation indicated a moderate rate variation (Drummond et al. 2007). The common ancestor of the Philanthinae that is supposed to live in symbiosis with *Streptomyces* bacteria was dated to 65 mya, with a 95% confidence interval of 48-88 mya.

THIS STUDY	EVANS & O'NEILL 1988
zebratus group:	
P. basilaris	P. basilaris
P. zebratus	P. zebratus
P. sanbornii	P. sanbornii
P. bicinctus	P. bicinctus
P. gloriosus	P. gloriosus
P. solivagus	
gibbosus group:	**gibbosus group:**
P. barbatus	P. barbatus
P. gibbosus	P. gibbosus
P. crotoniphilus	P. crotoniphilus
P. barbiger	
P. bilunatus	
inversus group:	
P. crabroniformis	P. crabroniformis
P. multimaculatus	P. multimaculatus
P. inversus	P. inversus
P. occidentalis	P. occidentalis
pacificus group:	
P. pacificus	P. pacificus
P. psyche	P.pulcher
P. serrulatae	P. barbiger
politus group:	
P. politus	P. politus
P. parkeri	P. parkeri
P. tarsatus	P. tarsatus
P. pulcher	P. psyche
	P. serrulatae
Other species:	
P. ventilabris	P. ventilabris
P. albopilosus	P. albopilosus
P. lepidus	P. lepidus
	P. solivagus
	P. bilunatus

THIS STUDY	DE BEAUMONT 1961
rutilus group:	**rutilus group:**
P. rutilus	P. rutilus
P. pulcherrimus	
	coarctatus group:
P. coarctatus	P. coarctatus
P. venustus	P. venustus
P. pulchellus	P. pulchellus (Gayubo 1991)
triangulum group:	
P. triangulum	P. triangulum
P. tr. diadema	
P. spec (India)	
P. basalis	
P. capensis	
P. loefflingi	
fuscipennis group:	
P. fuscipennis	P. fuscipennis (Arnold 1925)
P. histrio	
P. rugosus	
P. melanderi	
P. turneri	

Table 3. Species groups according to this and earlier studies (only species investigated in this study are listed). Species differing from earlier species group classifications are highlighted in bold letters.

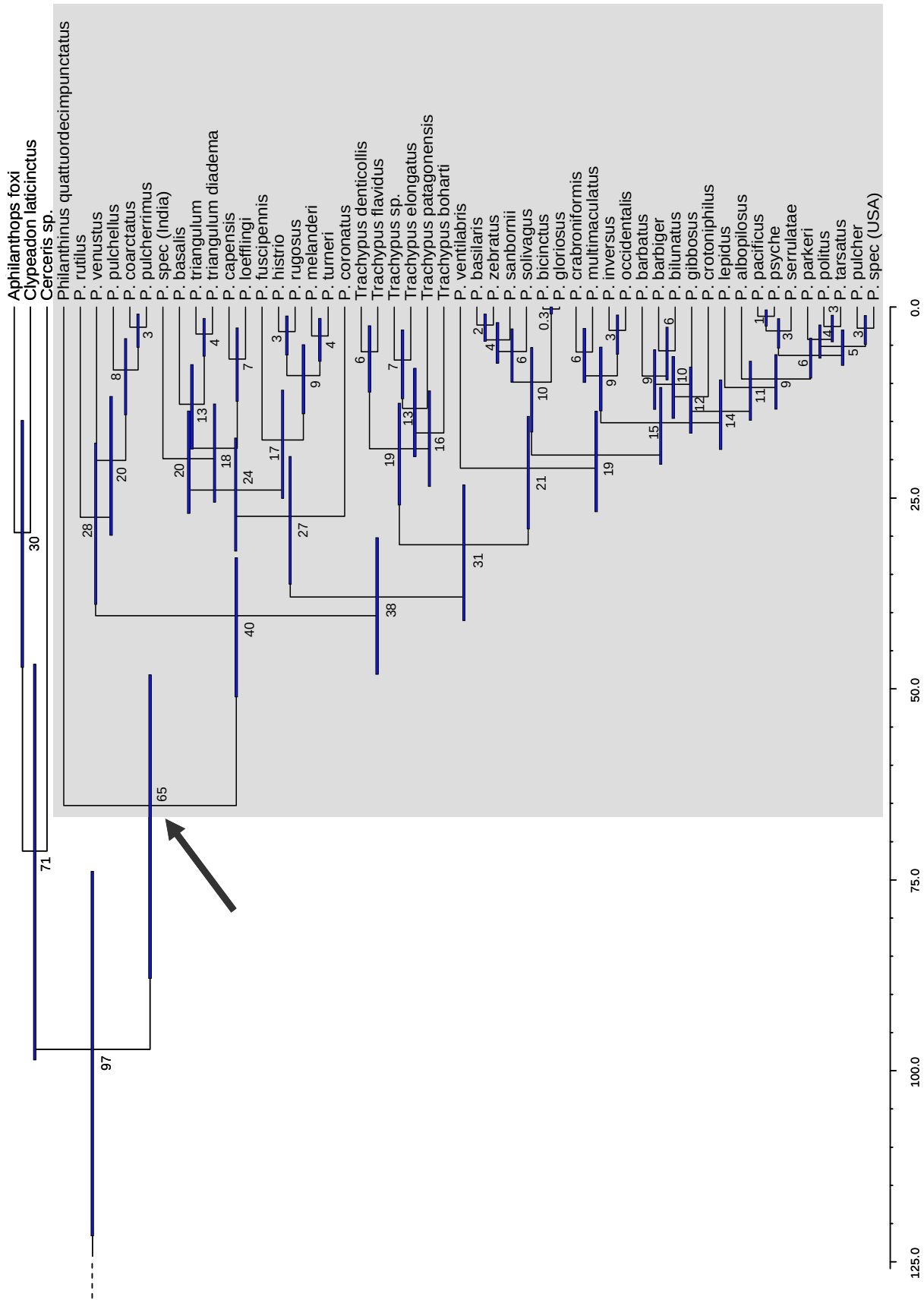

Figure 2. Dated phylogeny of the Philanthinae. Phylogenetic tree with the highest clade credibility resulting from BEAST analyses under the uncorrelated lognormal model, basing on the combined, unpartitioned 6-gene-data set. Node ages are shown in million years ago (mya) with their 95% HPD interval bars (equivalent to 95%

confidence intervals). Taxa with symbiotic *Streptomyces* bacteria in the antennae are highlighted with grey background, and an arrow indicates the reconstructed origin of the symbiosis.

3.5 Discussion

Phylogenetic relationships within Philanthinae

In the present study, we provide the first molecular phylogeny of the Crabronid wasp subfamily Philanthinae based on specimens of 43 species of *Philanthus*, six species of *Trachypus*, and one species each from the genera *Philanthinus*, *Cerceris*, *Aphilanthus*, and *Clypeadon,* respectively. Various phylogenetic analyses with the concatenated dataset of about 5 kb from two mitochondrial and four nuclear genes as well as subsets of single genes consistently yielded the same phylogenetic relationships within and among genera. Our results confirm earlier findings of a monophyletic tribe Philanthini comprising the genera *Philanthus*, *Trachypus*, and *Philanthinus* (Alexander 1992), which is inconsistent with an earlier placement of the genus *Philanthinus* within the tribe Aphilanthopini (Bohart & Menke 1976).

Interestingly, the results of this study also correspond with the suggestion of Alexander (1992) to combine the genera *Trachypus* and *Philanthus* into a single genus. Up to now, the two genera had been treated as separate genera (e.g. see Bohart & Menke 1976; Evans & O'Neill 1988; Pulawski 2010), which was mainly based on two distinct morphological characters of *Trachypus*: the truncate distal antennal segment and the petiolate gaster. However, there are some described Southeast Asian *Philanthus* species with a petiolate gaster (Bohart & Menke 1976), so this character has evolved independently at least twice in the tribe Philanthini and therefore likely represents a comparatively simple transition from a non-petiolate state. Still, according to Bohart and Menke (1976), the antennal difference between *Philanthus* and *Trachypus* alone "seems to be adequate for generic separation". However, the phylogenetic analyses of the present study agree with other authors who describe the monophyletic genus *Trachypus* as a group derived from the paraphyletic *Philanthus* (Alexander 1992; de Beaumont 1961).

The division of the North American *Philanthus* species groups overall corresponded reasonably well with the classification of Evans & O'Neill (1988). There were some discrepancies between molecular and morphological data, but the main groups were recovered by both methods (Table 3). However, the molecular phylogeny allowed for more detailed classifications and for the assignment of species with ambiguous morphological characters to distinct species groups.

The molecular phylogeny of the Philanthinae allows for some interesting speculations on the geographic origin of this group. According to Ohl & Engel (2007) and Engel (2001), bees and spheciform wasps most probably originated in the Southern hemisphere, as an analysis of the preferred habitats of basal taxa suggested semi-arid regions of Gondwana (Engel 2001). Additionally, the majority of very ancient bee lineages can be found in the southern hemisphere (Danforth 2007), and Danforth et al. (2006) trace the origin of the bees to Africa. Likewise, the rise

of angiosperms most probably occurred in Gondwana (Raven & Axelrod 1974; Taylor & Hickey 1992), and their adaptive radiation is tightly interwoven with the evolution of their probably most important pollinators, the bees. The results of the present study suggest that the Philanthini – as predators of bees – have their origin in the Palearctic or Paleotropics, possibly in Africa, with the earliest branches in the trees consisting of the Palearctic genus *Philanthinus* and the African and Palearctic *Philanthus* species. All derived species, including those of *Trachypus*, are from the Americas.

Following the molecular data contained in this study, the most probable scenario is that South America has most probably been colonized from Africa and then split into the North American *Philanthus* and the South American *Trachypus* clade. The exchange between Africa and South America has already been described for many plant (e.g. Morley 2000, 2003) and animal taxa (e.g. de Queiroz 2005; Rowe et al. 2010). These exchanges either were long-distance dispersal events or enabled by land bridges between Africa and South America. The Thulean bridge existed until about 50 million years ago and is considered an important dispersal route for temperate taxa (Kuhlmann 2009; Sanmartin et al. 2001; Tiffney 1985; Tiffney & Manchester 2001). The northern land bridge Beringia was usable for warm-adapted taxa during a later period in the Eocene with higher global temperatures (about 55–35 mya, Wolfe 1975) and might be another possibility for the colonization of the Americas. The colonization of South America also might have happenend by transoceanic dispersal events, which are known for a broad variety of different taxa (de Queiroz 2005), even for vertebrates (e.g. transatlantic dispersal in worm lizards, ca. 40 mya; Vidal et al. 2008). The exchange between South and North America probably occurred via land bridges, because already before the formation of the Panamanian Isthmus ca. 3 Mio years ago there had been land bridges between the Americas: A fragmented bridge including the proto Antilles existed during the Middle Eocene (Graham 2003; Pennington & Dick 2004) and a later, brief connection involving the submerged Aves ridge – a part of an extinct volcanic arc – in the Eocene-Oligocene boundary, fitting the time of the estimated splitt-off between *Trachypus* and the north American *Philanthus* clade (ca. 31 mya, see Figure 2; Iturralde-Vincent & MacPhee 1999). Interestingly, the South Indian *Philanthus* species were interspersed among Asian and African taxa in our phylogeny, suggesting that the Indian subcontinent was colonized repeatedly from Africa (*P. basalis* and *P. spec.*) and from Asia (*P. pulcherrimus*).

Age of the symbiosis between beewolves and *Streptomyces* bacteria

Our analyses allow estimating the origin of the symbiosis between beewolves and protective *Streptomyces* bacteria. Earlier studies indicated that the symbionts probably occur in all species of *Philanthus, Trachypus*, and *Philanthinus* (the tribe Philanthini), but are likely absent from all other genera of Crabronid wasps (Kaltenpoth et al. 2006; Kaltenpoth et al. submitted). Based on our molecular phylogeny, the split of the Philanthini from the other tribes in the subfamily happened around 97 mya (95% confidence limits 74 and 122 mya), and the most recent common ancestor of the three genera in the Philanthini probably lived about 65 million years ago (95% confidence limits 48 and 88 mya, respectively). Thus, the protective symbiosis between beewolves and *Streptomyces*

bacteria likely evolved 65-97 mya. Incidentally, this estimate for the age of the beewolf-*Streptomyces* symbiosis overlaps with an earlier analysis by Kaltenpoth et al. (2006), who used DNA sequences of the *Streptomyces* symbionts and estimated substitution rates to date the symbiosis back to about 26-67 mya. However, the latter estimate was based on the erroneous assumptions that the symbiosis is limited to the genus *Philanthus* and that hosts and symbionts have co-diversified (but see Kaltenpoth et al. 2010a; Kaltenpoth et al. submitted). In comparison with other insect-bacteria symbioses for which the evolutionary origin has been dated so far, the beewolf-*Streptomyces* association is of relatively recent origin: the symbiosis of aphids and their endosymbiotic *Buchnera* bacteria has been estimated to be 160-280 million years old (Moran et al. 1993) and the origin of the symbiotic alliance between cockroaches or termites and *Flavobacterium-Bacteroides* bacteria dates back 135-280 million years (Bandi et al. 1995). The associations between cicadas and their endosymbiotic bacteria probably evolved in the period from 170 to more than 270 million years ago (Gosalbes et al. 2010). However, all of these symbioses represent intimate and obligate associations between insects and intracellular symbionts that are strictly vertically transmitted. By contrast, the evolutionary histories are likely to be different for extracellular symbionts that are transmitted outside of the host's body and can also experience horizontal transfer (Kaltenpoth et al. 2010a; Kaltenpoth et al. submitted).

To date, only two protective symbioses that are similar to the beewolf-*Streptomyces* association have been described, and both involve fungus-farming insects: the symbiosis of leaf-cutter ants with *Pseudonocardia* bacteria (Currie et al. 1999), and the association between bark beetles and *Streptomyces* (Scott et al. 2008). While little is known about the intimacy and evolutionary stability of the association between bark beetles and *Streptomyces*, the leaf-cutter ants and their protective bacterial symbionts have recently received considerable attention. The ants' fungus gardens are defended against the parasitic fungus *Escovopsis* by antibiotic secretions of bacteria belonging to the genera *Pseudonocardia, Streptomyces,* and, possibly, *Amycolatopsis* (Barke et al. 2010; Currie et al. 1999; Haeder et al. 2009; Oh et al. 2009a; Schoenian et al. 2011; Sen et al. 2009). Although at least the *Pseudonocardia* symbionts are transmitted vertically and there is some degree of specificity between hosts and symbionts, horizontal transmission and/or *de novo* uptake of symbionts from the environment appears to happen frequently (Cafaro et al. 2011). The presence of symbiotic bacteria on a leaf-cutter ant from Dominican amber provides a minimum age estimate of 15-20 mya for the age of the ant-Actinobacteria symbiosis (mentioned in Cafaro et al. 2011). Since the original form of ant agriculture by growing fungi for nutrition evolved about 50 million years ago (Schultz & Brady 2008), the association with bacteria is probably of similar or more recent evolutionary origin. Thus, this symbiosis appears to be slightly younger than the association between Philanthini and *Streptomyces* bacteria.

Evolution of the symbiosis

The early evolution of the beewolf-*Streptomyces* symbiosis remains enigmatic. Since the bacteria-containing antennal gland secretion also provides a directional information to the beewolf larva that is later necessary for successful emergence (Strohm & Linsenmair 1995), previous studies suggested that the antennal gland reservoirs evolved for this purpose (Strohm & Linsenmair 1995) and that the glands were secondarily invaded by the symbiotic bacteria (Goettler et al. 2007). However, the lack of basal Philanthini with morphologically simple bacteria-free gland reservoirs is in favour of a tight link between the evolution of the gland reservoirs and the origin of the symbiosis (Kaltenpoth et al. submitted). Interestingly, the symbiont cultivation organs in beewolf antennae are more complex than the cuticular crypts of attine ants (Currie et al. 2006). Possibly, the availability of alternative defence mechanisms in the social leaf-cutter ants by means of metapleural gland secretions (Yek & Mueller 2010) or hygienic behaviour (Currie & Stuart 2001) reduced the selection pressure to increase the investments in the symbiotic association with *Pseudonocardia*. Because the solitary Philanthini have no further contact with the developing brood after oviposition, the protective mechanisms must be long-lasting and more efficient than in attine ants. Thus, symbiotic bacteria are ideal allies to combat potential pathogens of the beewolf offspring during the long phase of hibernation in the soil, and increased investment in cultivation of such defensive symbionts is likely to provide a selective advantage. As these traits should be adaptive for other ground-nesting Hymenoptera as well, it seems unlikely that symbiotic interactions with Actinobacteria are restricted to beewolves, leaf-cutter ants and pine beetles. Future research on the microbial communities associated with ground-dwelling insects will undoubtedly yield fascinating insights into possible defensive alliances with bacteria.

3.6 Acknowledgements

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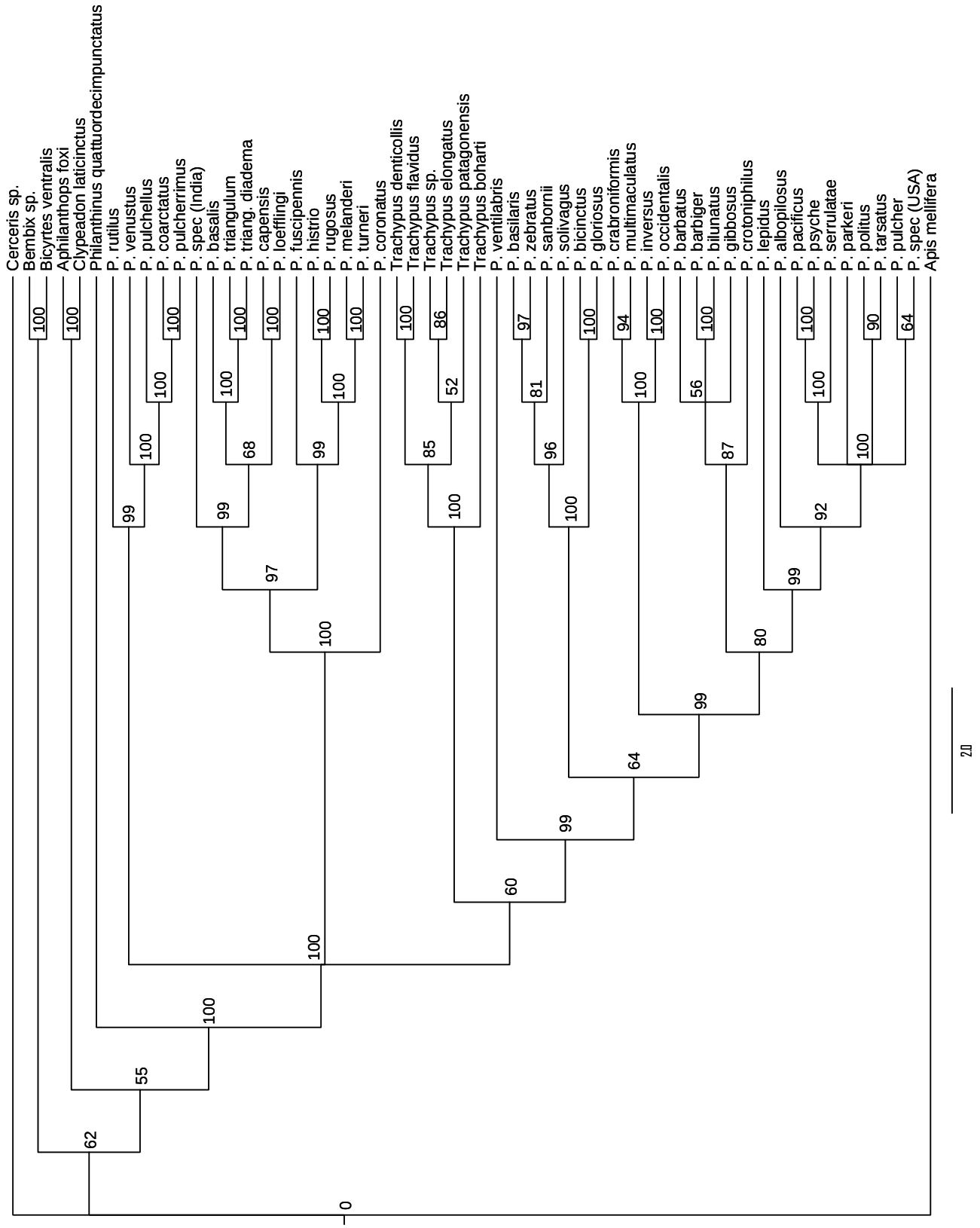
3.7 Supplementary material



Suppl. Figure 1. Phylogenetic relationships in *Philanthus* inferred from maximum parsimony analysis (PAUP v4.0 beta) as indicated by a 50% majority rule consensus tree of 2 trees. Numbers represent bootsrap values (1000 replicates). The data set consisted of a partitioned 5040 bp alignment, including DNA sequences of six genes (wnt-1, 28s, ArgK, EF1α , LWRh and COI (excluding 3d position in the cds).

Suppl. Table 1: Information on the specimens used in this study, including collection localities and GenBank accession numbers for each of the genes (wnt-1, LWRh, ArgK, EF1a, 28s, COI; - : no sequence data available due to problems with PCR amplification, NYK = not yet known)

		Collection	Gen Bank Nr					
Species	**Sample no.**	**locality**	**Wingless**	**Opsin**	**EF1a**	**28s**	**ArgK**	**COI**
P. albopilosus	USA-E56	USA	JN674198	NYK	NYK	JN674251	NYK	NYK
P. barbatus	USA-E18	USA	JN674199	NYK	NYK	JN674252	NYK	NYK
P. barbiger	UT-E15	USA	JN674200	NYK	NYK	JN674253	NYK	NYK
P. basalis	IN-E035	India	JN674201	NYK	NYK	JN674254	NYK	NYK
P. basilaris	UT-E6	USA	JN74202	NYK	NYK	JN674255	NYK	NYK
P. bicinctus	USA-E29	USA	JN74203	NYK	NYK	JN674256	NYK	-
P. bilunatus	USA-BS34	USA	JN74204	NYK	NYK	JN674257	NYK	-
P. capensis	SA-E62	South Africa	JN74205	NYK	NYK	JN674258	NYK	NYK
P. coarctatus	MO-1	Oman	JN74206	NYK	NYK	JN674259	NYK	-
P. coronatus	m1	Germany	JN74207	NYK	NYK	JN674260	NYK	NYK
P. crabroniformis	USA-E10	USA	JN74208	NYK	NYK	JN674261	NYK	NYK
P. crotoniphilus	USA-E39	USA	JN74209	NYK	NYK	JN674262	NYK	-
P. fuscipennis	SA-E69	South Africa	JN74210	NYK	NYK	JN674263	NYK	NYK
P. gibbosus	USA-E188	USA	JN74211	NYK	NYK	JN674264	NYK	-
P. gloriosus	USA-E60f	USA	JN74212	NYK	NYK	JN674265	NYK	NYK
P. histrio	SA-E58	South Africa	JN74213	NYK	NYK	JN674266	NYK	NYK
P. inversus	USA-E53b	USA	JN74214	NYK	NYK	JN674267	NYK	-
P. lepidus	CAN-E1	Canada	JN74215	NYK	NYK	JN674268	NYK	-
P. loefflingi	SA-E13	South Africa	JN74216	NYK	NYK	JN674269	NYK	NYK
P. melanderi	SA-E79	South Africa	JN74217	NYK	NYK	JN674270	NYK	NYK
P. multimaculatus	UT-E76	USA	JN74218	NYK	NYK	JN674271	NYK	NYK
P. occidentalis	CAL-Eth4	USA	JN74219	NYK	NYK	JN674272	NYK	NYK
P. pacificus	USA-E19	USA	JN74220	NYK	NYK	JN674273	NYK	NYK
P. parkeri	UT-E45	USA	JN74221	NYK	NYK	JN674274	NYK	NYK
P. politus	JS-32a	USA	JN74222	NYK	NYK	JN674275	NYK	-
P. psyche	UT-E154/ *JS-A	USA	JN74223	NYK	NYK *	JN674276	NYK	NYK
P. pulchellus	SP-001	Spain	JN74224	NYK	NYK	JN674277	NYK	NYK
P. pulcher	USA-E8b	USA	JN74225	NYK	NYK	JN674278	NYK	NYK
P. pulcherrimus	IN-E064	India	JN74226	NYK	NYK	JN674279	NYK	NYK
P. rugosus	SA-E23	South Africa	JN74227	NYK	NYK	JN674280	NYK	NYK
P. rutilus	JS-32	-	JN74228	NYK	NYK	JN674281	NYK	-
P. sanbornii	m28	USA	JN74229	NYK	NYK	JN674282	NYK	NYK
P. serrulatae	JS-63	USA	JN74230	NYK	NYK	JN674283	-	-
P. solivagus	USA-BS36	USA	JN74231	NYK	NYK	JN674284	NYK	-
P. spec	IN-E010	India	JN74232	NYK	NYK	JN674285	NYK	NYK
P. spec	CAL-Eth14	USA	JN74233	NYK	NYK	JN674286	NYK	-
P. tarsatus	JS-44	USA	JN74234	NYK	NYK	JN674287	NYK	-
P. triangulum	N14/ *JS-B	Germany	JN74235	NYK	NYK *	JN674288	NYK	NYK
P. triangulum diadema	SA-E8	South Africa	JN74236	NYK	NYK	JN674289	NYK	NYK
P. turneri	SA-E116	South Africa	JN74237	NYK	NYK	JN674290	NYK	NYK
P. ventilabris	USA-E50	USA	JN74238	NYK	NYK	JN674291	NYK	NYK
P. venustus	Ph02	Greece	JN74239	NYK	NYK	JN674292	NYK	-
P. zebratus	USA-E25	USA	JN74240	NYK	NYK	JN674293	NYK	NYK
Trachypus boharti	BR-002	Brasil	JN74250	NYK	NYK	JN674294	NYK	NYK
Trachypus denticollis	JS-11	Chile	JN74241	NYK	NYK	JN674295	NYK	-
Trachypus elongatus	BR-E032	Brasil	JN74242	NYK	NYK	JN674296	NYK	NYK
Trachypus flavidus	BR-E067	Brasil	JN74243	NYK	NYK	JN674297	NYK	NYK
Trachypus patagonensis	BR-E092	Brasil	JN74244	NYK	NYK	JN674298	NYK	NYK
Trachypus spec	JS-52	Chile	JN74245	NYK	NYK	JN674299	NYK	-
Philanthinus quattuordecimp.	TU-EY-E027	Turkey	JN74246	NYK	NYK	JN674300	NYK	NYK
Aphilanthops foxi	CAL-Eth10	USA	JN74247	NYK	NYK	JN674301	NYK	NYK
Bembix amoena/ *B. troglodytes	-	-	EU367331.1	-	EU367212.1	EU367154.1	-	EF203767.1 *
Bicyrtes ventralis	-	-	-	DQ116701.1	AY585161	AY654458.1	-	-
Cerceris rybiensis/Eucerceris	*Cerc1/**Cerc2/***JS-C	USA	* JN74248	NYK **	NYK ***	AY654460.1	NYK **	-
Clypeadon laticinctus	UT-E177/ *BS32a/ **JS-D	USA	JN74249	NYK *	NYK **	JN674302	NYK	NYK
Apis mellifera	-	-	AY703618.1	U26026.1	NM_001014993.1	AY703551.1	NM_001011603.1	AF214668.1

CHAPTER 4

LARVAL REARING TEMPERATURE INFLUENCES AMOUNT AND COMPOSITION OF THE MARKING PHEROMONE OF MALE EUROPEAN BEEWOLVES (PHILANTHUS TRIANGULUM)

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4.1 Abstract

Pheromones play an important role for courtship and mating in many insect species, and they are shaped by a complex interaction of genetic and environmental factors. Developmental temperature is known to have a strong influence on adult life history, morphology, and physiology, but little is known about its effect on pheromone characteristics. In the present study, the influence of temperature during larval development on the amount and composition of the complex marking pheromone from the cephalic glands of adult male European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) was investigated. Additionally, we examined temperature effects on several life-history traits. European beewolf larvae were reared at three different constant temperatures (20°C, 25°C, and 30°C). Males reared at 20°C showed longer development times and higher mortality, suggesting that low temperatures constitute stressful conditions for developing larvae. After eclosion, the amount and composition of the scent marking secretion of the adult males was analyzed by coupled gas chromatography – mass spectrometry. Males that had been reared at 20°C had significantly less secretion than individuals reared under warmer conditions (25°C and 30°C). Furthermore, larval rearing temperature had a significant effect on the composition of the adult males' pheromone gland content, with warmer rearing conditions leading to higher relative amounts of compounds with high molecular weight. Our results show that the temperature during larval development significantly affects the amount and composition of the content of the male beewolf pheromone glands, probably due to physiological constraints and competing processes for limited energetic resources. Thus, the pheromone gland content may contain information on developmental conditions of male beewolves, which may have consequences for female mate choice decisions and male reproductive success.

4.2 Introduction

In many organisms, communication between mating partners is mediated by pheromones (e.g. Johansson & Jones 2007; e.g. Jones & Hamilton 1998). These chemical signals do not only facilitate the localization and identification of conspecific individuals (Johansson & Jones 2007), they may also contain information on the morphological and/or physiological condition of the potential mating partner, e.g. size, age, symmetry, fertility, or immunocompetence (Jones et al. 1998; Marco et al. 1998; Martín & López 2000; Reusch et al. 2001). This information may provide a potential partner with cues for an adaptive mate choice. Mating with a high-quality partner might provide direct (e.g. reduced risk of parasite infection, higher chances of receiving fertile gametes, defence, brood care) and/or indirect benefits (e.g. "good genes" for the offspring) to choosy individuals (Andersson & Iwasa 1996; Eisner & Meinwald 1995; Penn & Potts 1998; Wagner & Harper 2003).

For a number of species, a genetic basis for the variation in pheromone characteristics has been shown (Collins & Cardé 1985; Herzner et al. 2006; Roelofs & Rooney 2003; Sappington & Taylor 1990a; Sheck et al. 2006). Some authors also found that the amount and/or composition of sex pheromones is influenced by environmental factors (Clark et al. 1997; Moore 1997; Sappington & Taylor 1990b). However, most of these studies investigated the effect of environmental conditions on pheromone characteristics during the adult stage. The impact of conditions during larval development has as yet received little attention (but see e.g. Conner et al. 1990; Ono 1993; Tillman et al. 1999).

Temperature is one of the most important environmental factors since it influences many morphological, physiological, and life-history traits, e.g. size, fecundity, and development time (Atkinson 1994; Blanckenhorn 1997; Nabeta et al. 2005; Ratte 1984). In some adult Lepidoptera, ambient temperature has an immediate impact on pheromone amount and/or composition probably by directly affecting biochemical pathways (Ono 1994; Raina 2003). In potato tuberworm moths, different rearing temperatures during larval development resulted in changes in the amount and composition of the females' sex pheromone (Ono 1993).

The effect of environmental conditions on pheromone composition may reduce the efficiency of communication processes or even result in the loss of signal function. Thus, a certain degree of developmental stability has to be expected to retain the signal's information content for conspecifics (Møller & Swaddle 1997; Paterson 1985). On the other hand, variation in pheromone composition due to environmental effects might also increase the information content of a signal. Assuming that a male pheromone varies in response to different environmental conditions in a defined way and that these changes have some fitness implications, a female might be able to assess some indicator for quality from pheromone composition. For example, if the temperature during development depends on decisions and/ or competitive abilities of a male's mother (e.g. by choosing and defending suitable oviposition or nesting sites), a male that developed at optimal temperatures might be more attractive to females. If these maternal characteristics are heritable,

choosing a son of such a female as mating partner should be beneficial for a female (Andersson 1994; Møller & Alatalo 1999). Here we investigated one precondition for this scenario and asked whether developmental temperatures affect quantity and composition of the male pheromone of a hymenopteran species, the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae).

European beewolves are solitary digger wasps that live in warm and sandy areas (Strohm & Linsenmair 1995). Females excavate a nest burrow with several separate brood cells, each of which is provisioned with one to five paralysed honeybees (*Apis mellifera*) as food for the developing larva (Strohm & Linsenmair 1995, 1997, 2000, 2001). To prevent fungal infestation in the warm and humid brood cell, the prey is preserved by the females by embalmment with a gland secretion (Herzner & Strohm 2007). Moreover, beewolf females provide the brood cell with a whitish substance that originates from specialized antennal glands (Göttler et al. 2007) and that contains symbiotic bacteria which protect the cocoon from fungal infestation (Kaltenpoth et al. 2005, 2006).

Male beewolves establish and defend small territories (about 0.25 m^2) in the vicinity of female nest aggregations (Simon-Thomas & Poorter 1972; Strohm 1995). They scent-mark these territories with a pheromone from cephalic glands to attract receptive females (Evans & O'Neill 1988; Schmitt et al. 2003; Simon-Thomas & Poorter 1972; Strohm 1995; Strohm & Lechner 2000). Territories of different males are often aggregated, thereby forming a lek that might facilitate female choice on the basis of pheromone quality and quantity of the competing males (Evans & O'Neill 1988; Strohm and Kroiss, submitted; Simon-Thomas & Poorter 1972). Females approach these territories from the downwind side in a zig-zagging flight pattern, probably orienting towards the windborne pheromone (Evans & O'Neill 1988). Since the copulation is not preceded by any kind of visual display, female choice appears to be, at least predominately, based on information obtained from the male's secretion (E. Strohm & M. Kaltenpoth, unpubl. obs.). Although the components are rather long chained and, thus, intuitively seem not to be volatile, we have ample evidence that at least most of the compounds are volatile enough to be detected by olfaction (Herzner et al. 2005; Schmitt et al. 2007), i.e. without contact. There is no conspicuous antennation of the territory by females, and there is no visual courtship by males. Females simply alight in a territory, the male approaches her, sits on her back and inserts its genitalia. The scent marking secretion consists of at least 55 components, including long-chain aliphatic hydrocarbons and some compounds with functional groups (Kroiss et al. 2006; Schmitt et al. 2003). These components are also found in extracts from male territories (E. Strohm, T. Schmitt, G. Herzner, J. Kroiss and M. Kaltenpoth, unpubl. data). It is already known, that the amount and composition of the male marking pheromone is influenced by family affiliation (Herzner et al. 2006; Kaltenpoth et al. 2007), age (Kaltenpoth & Strohm 2006), and geographical origin (Kaltenpoth et al. 2007).

We reared male European beewolves at different temperatures and, using coupled gas chromatography and mass spectrometry, analysed pheromone quantity and composition of the mature males as well as several life-history traits (survival probability of the larva, development

time, and adult weight). Moreover, it was assessed whether there were predictable trends in the temperature dependent variation of the pheromone composition regarding the chain length of the individual pheromone substances. The results are discussed with regard to possible physiological and ecological constraints and their relevance for mate choice.

4.3 Materials and methods

Specimens and rearing conditions

Female European beewolves were taken from a laboratory population that represented the F1 generation of females caught from populations in the vicinity of Würzburg (Germany). They were kept individually in observation cages which consisted of a flight compartment and an attached nesting area where the females could establish their nests (see Strohm & Linsenmair 1995). The cages were kept in a greenhouse at an average temperature of 25°C (with a range from 20°C to 30°C caused by external conditions), and an average humidity of 45% (range: 30-80 %), with additional illumination of the flight cage by neon lamps for 14 hours a day. The females were provided with honey and live honeybees *ad libitum*, and each cage was checked several times each day for new brood cells. The content of brood cells with two bees was transferred to artificial brood cells in Petri dishes: Each Petri dish was filled with a fixed amount (130 g) of autoclaved sand, and an artificial, cylindrical brood cell with a standardized diameter of about 2.6 cm and a depth of 1 cm was formed. The paralyzed bees with the beewolf egg as well as the white antennal gland secretion which contains the symbiotic bacteria (Kaltenpoth et al. 2005, 2006) were transferred to the artificial brood cell. The humidity in the brood cell was kept constant at 4% H_2O (w/w) by weighing brood cells every other day and replacing evaporated water through four small holes in the lid of the Petri dish.

Each brood cell was randomly assigned to one temperature treatment. Experimental brood cells were stored in three conditioning cabinets (ATS1373 So, Ehret GmbH) at 20°C, 25°C, and 30°C, respectively. The eggs and larvae respectively were examined every day, and life-history parameters were recorded (hatching date, cocoon spinning, eclosion from the cocoon, occurrence of mould infestations, and death of the larva).

Each emerged adult male was weighed (Mettler AE 160; +/- 0.1g), individually marked on the thorax with dots of acrylic paint and released into a climate chamber (2.4 m x 1.8 m x 2.1 m, 25/20°C day/night and 12h/12h light/dark cycles) containing sand-filled buckets (for digging sleeping burrows) and provided with honey *ad libitum*. Since *P. triangulum* males need about 5-9 days to develop the complete pheromone blend (Kaltenpoth & Strohm 2006), they were left in the climate chamber until the age of 12 days. Then they were caught and transferred into small polystyrol vials (height: 80 mm, diameter: 35 mm) provided with moist sand and honey for another two days. During this time, males that had depleted their pheromone in the climate

chamber by scent marking could replenish their pheromone glands. Finally, the males were anesthetized with CO_2, killed by freezing and kept at -30°C until chemical analysis.

Chemical analysis

The frozen males were thawed, decapitated and their heads were incised on both sides below the eyes to open up the postpharyngeal gland, which is the storage organ of the male sex pheromone (Herzner et al. 2007; Kroiss et al. 2006). Heads were placed individually in glass vials (1.5 ml), and 20 µl of a solution of 1 µg/µl octadecane in hexane (equivalent to a final amount of 20 µg of octadecane) was added as an internal standard to each vial to allow absolute quantification of the pheromone. The heads were then submerged in approximately 1 ml of distilled hexane, and the gland contents were extracted for four hours. The extracts were immediately analyzed by coupled capillary gas chromatography-mass spectrometry (GC-MS) with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 mass selective detector. The GC was equipped with a DB-5ms+ fused silica capillary column (J&W, 30 m x 0.25 mm ID; df = 0.25 µm; temperature program: from 60°C to 300°C at 5°C/min, held constant for 1 min at 60°C and for 10 min at 300°C). Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector (250°C) was used with the purge valve opened after 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. Since preliminary analyses had revealed that the total amount of chemicals in the sample has an effect on the detection and quantification of certain components, samples in which the concentration of the gland extract was either too high (overlapping peaks) or too low (with compounds below detection threshold) were rerun after adjusting the pheromone concentration by addition or evaporation of hexane.

In the pheromone gland extracts, 21 components could be reliably detected in all samples, and their peaks were manually integrated with MSD ChemStation software (Agilent Technologies). The substances were identified by comparison of mass spectra and retention times with earlier analyses (Kroiss et al. 2006; Schmitt et al. 2003). Not all substances described as components of the pheromone by Kroiss et al. (2006) could be detected due to the low concentrations of the gland content extracted from single males. The peaks of (*Z*)-11-eicosen-1-ol and (*Z*)-9-tricosene were not fully separated in all chromatograms and were therefore pooled and treated as one peak for the statistical analyses. Because of the by far higher eicosenol fraction (Schmitt et al. 2003) the peak is labelled as "eicosenol" in the following. This procedure is conservative with regard to the hypotheses tested. For each sample, the total peak area was standardized to 100%, and the relative amount of each peak was calculated. Since the relative peak areas represent compositional data, they were transformed to logcontrasts prior to statistical analyses (Aitchison 1986). Using the octadecane peak as an internal standard, the total amount of the gland content was estimated.

Statistical analysis

The influence of the rearing temperature on development time and adult weight was analyzed by Kruskal-Wallis tests. χ^2-tests were used to evaluate the survival rate, and analyses of variance and covariance (with adult weight as the covariate) were conducted to assess the influence of temperature on the quantity of the pheromone gland content (including post hoc tests with Scheffe's multiple comparisons). Tests were calculated using BIAS 8.1 and SPSS 11.0.

Multivariate analyses were conducted to test for differences in the chemical profiles of males reared at different temperatures: The peaks were subjected to a principal component analysis (PCA with varimax rotation, principal components with eigenvalues > 1 were included in the subsequent analyses) to reduce the number of describing variables. The extracted PCA factors were then subjected to a discriminant analysis (DA) to assess whether males confronted with different rearing temperatures exhibit differences in their pheromone profiles. To investigate the influence of the rearing temperature on individual components of the pheromone gland, a multivariate analysis of variance (MANOVA) was conducted. Additionally, a correlation analysis was used to test for an influence of temperature on the relative amounts of the substances depending on molecule size (chain lengths or molecular weights respectively). SPSS 11.0 was used for the calculation of all tests.

Earlier studies revealed a distinct chemical dimorphism in the pheromone blend of *P. triangulum* males (Kroiss et al. 2006), with either *Z*-(9)-pentacosene (C_{25}-type) or *Z*-(9)-heptacosene (C_{25}/C_{27}-type) as the major component. Therefore, all analyses on the composition of the pheromone gland extracts were conducted with the data of the C25-chemotype only, in addition to the analysis including both chemotypes. The sample size of C_{27}-type individuals was too small to allow separate analyses. Since the analyses including both types and those including only C_{25}-type males yielded qualitatively identical results, only the data based on the complete dataset are presented here.

4.4 Results

Life history traits

Development time of males differed significantly between temperature groups (Kruskal-Wallis test, H = 39.7, df = 2, p < 0.001; Figure 1). On average, males reared at 30°C needed 20 days from the egg stage until eclosion from the cocoon, whereas males reared at 20°C had just started cocoon spinning at that point of time (Q_{10} value of average development times = 3.33). Male adult weight was not significantly affected by rearing temperature (Kruskal-Wallis test, H = 0.441, df = 2, p = 0.802). Survival probability of developing males, however, was strongly influenced by rearing temperature (contingency test, χ^2 = 9.24, p = 0.01). Males reared at 20°C had a significantly higher mortality rate (74%) than males in any of the other two temperature groups

(25°C: 39% mortality, 30°C: 43% mortality; pairwise comparisons: 20°C versus 25°C: $\chi^2 = 7.435$, p = 0.006; 20°C versus 30°C: $\chi^2 = 4.795$, p = 0.029, 25°C versus 30°C: $\chi^2 = 0.015$, p = 0.904).

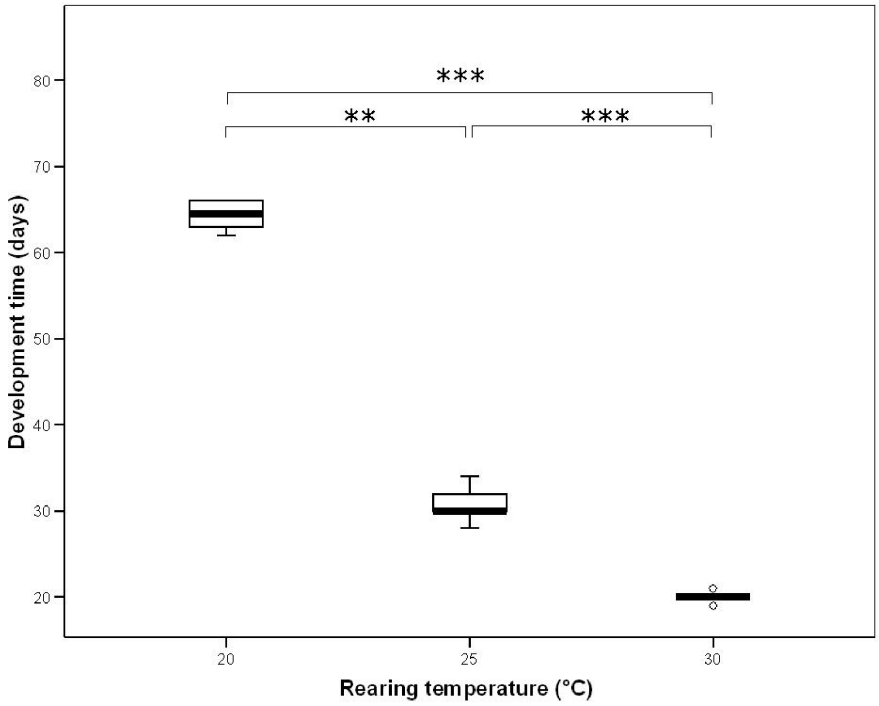

Figure1. Median development time (oviposition to eclosion of imago) of beewolf males at different rearing temperatures ($N_{20°C}$ = 7, $N_{25°C}$ = 24, $N_{30°C}$ = 17; ** significant at p < 0.01, *** significant at p < 0.001). Bold lines represent medians, boxes comprise the interquartile range, and whiskers indicate minimum and maximum values, except outliers, these are represented by circles.

Quantity of the pheromone gland content

The total amount of the content of the pheromone gland from male heads ranged from 165 to 659 µg (mean ± SD = 387 ± 125 µg). The temperature during larval development had a significant influence on the quantity of marking pheromone of adult males (ANOVA, $F_{2,40} = 4.86$, p = 0.013, N = 43). Since body weight might be a confounding factor for the amount of gland content (Pearson's regression, regression coefficient r = 0.354; p = 0.020), we also conducted an analysis of covariance with body weight as covariate, but the temperature effect persisted (ANCOVA; $F_{2,39} = 4.189$, p = 0.022, N = 43): Adult males reared at 20°C had significantly less marking pheromone than males reared at either 25°C or 30°C (Scheffe's multiple comparisons, p < 0.05; Figure 2).

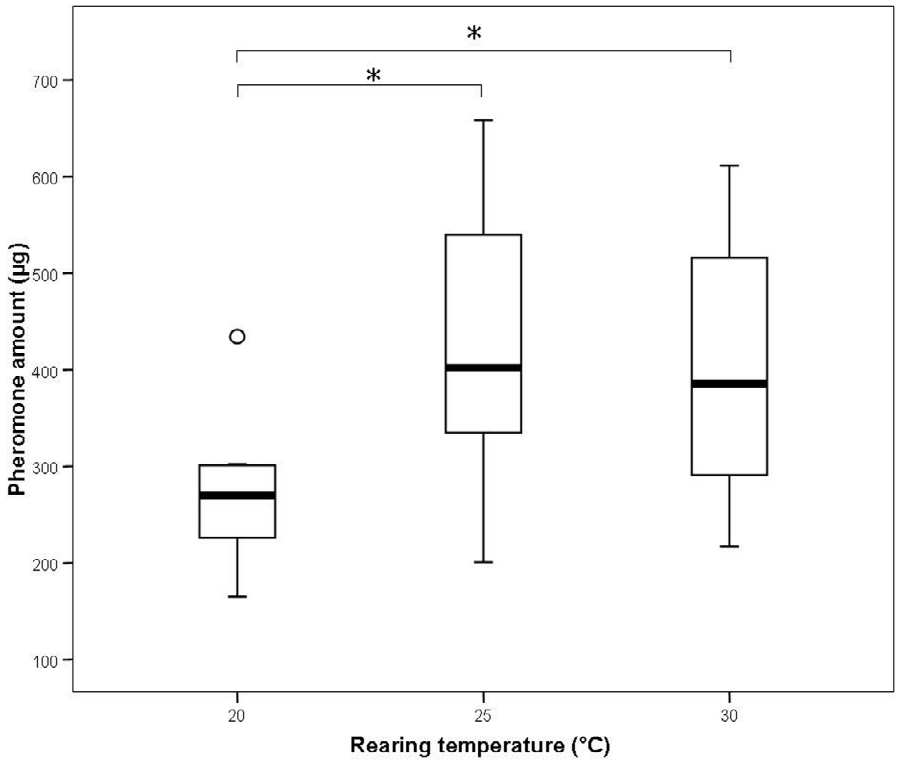

Figure2. Absolute marking pheromone amount (in µg) of adult beewolf males reared at different temperatures ($N_{20°C}$ = 7, $N_{25°C}$ = 22, $N_{30°C}$ = 14; * significant at $p < 0.05$). Bold lines represent medians, boxes comprise the interquartile range, and whiskers indicate minimum and maximum values, except outliers, these are represented by circles.

Composition of the pheromone gland content

In the extracts of male beewolf heads, 21 compounds were found in all samples and were, thus, included in the analysis (Table 1), with (*Z*)-11-eicosen-1-ol (including minor amounts of (*Z*)-9-tricosene) constituting the component with by far the highest relative amount (mean ± SD = 61.21 ± 3.83 %). The PCA produced five principal components with eigenvalues larger than 1 (eigenvalues: 5.8, 3.8, 3.5, 2.1, 1.5), explaining 79.9% of the total variance. The DA on these principal components significantly separated the three temperature groups (Wilks $\lambda = 0.541$, $\chi^2 = 24.3$, df = 4, $p < 0.001$, N = 43; Figure 3). Between 57 and 64% (on average 60.5%) of the males were correctly assigned to the temperature treatment by the DA - only 33% correct classifications would be expected by chance.

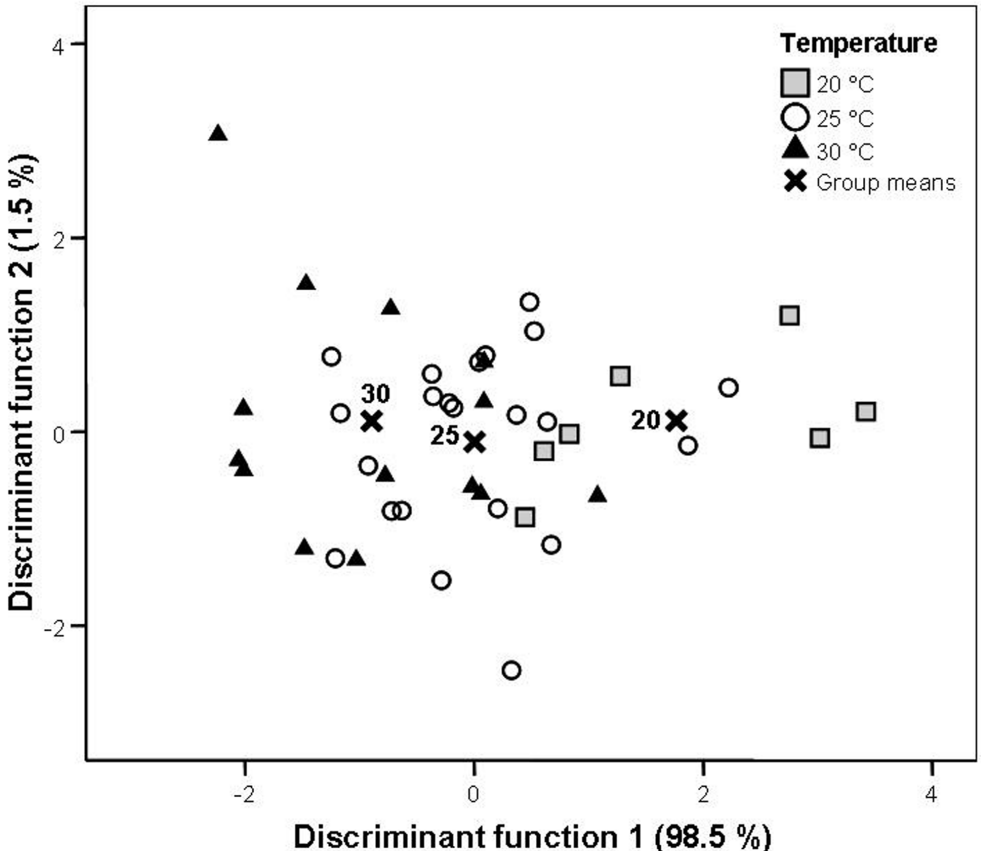

Figure 3. Discriminant analysis of the marking pheromone composition of males with different rearing temperatures (squares: 20°C, N = 7; circles: 25°C, N = 22; triangles: 30°C, N = 14).

A multivariate analysis of variance (MANOVA) was conducted with the 21 components of the pheromone gland extracts to elucidate the contribution of the individual components of the pheromone blend to the differences between rearing temperatures (Table 1). Since several components differ significantly in their relative amount between the two chemotypes (Kroiss et al. 2006), only males of the C_{25}-type (N = 82) were included in this analysis. The MANOVA revealed a significant temperature effect on the composition of the marking pheromone ($p = 0.035$, Pillai's trace = 1.453, $F = 1.896$). Ten of the 21 components (five with and five without functional groups) differed significantly in their relative amounts among temperature groups (Table 1).

To test whether the temperature dependent changes in pheromone composition show a trend with regard to the size of the molecules, a correlation analysis of the molecular weight of each component and the ratio of its relative amounts at 20°C and 30°C was conducted (Figure 4). The analysis showed a significant negative correlation (Pearson correlation, correlation coefficient $r = -0.67$; $p = 0.001$). Thus, males reared at a low temperature (20°C) exhibited significantly higher relative amounts of components with low molecular weights, while the blend of males reared at a high temperature (30°C) contained higher amounts of the hydrocarbons with high molecular

weights. The same effect was found if the hydrocarbon chain length (number of C atoms) was used instead of the molecular weights (Pearson correlation, correlation coefficient $r = -0.72$; $p < 0.001$).

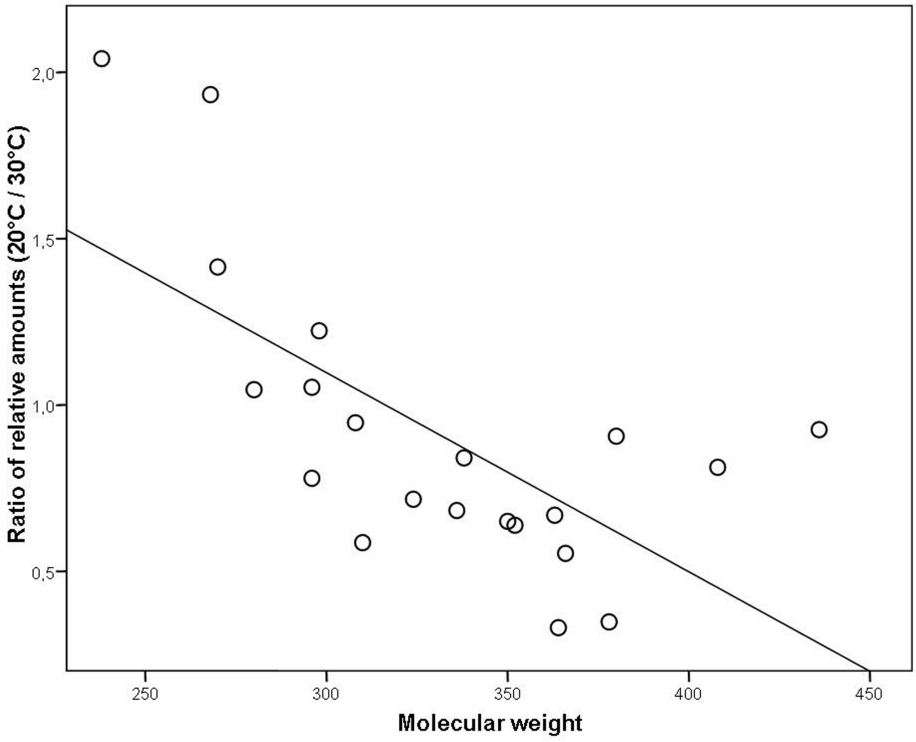

Figure 4: Correlation between the molecular weight of each component and the ratio of its mean relative amounts at 20°C and 30°C.

Table 1: Compounds of the males' scent marking secretion. Given are retention indices, length of C-backbone, molecular weight (MW), and the relative amount (mean values +/- SD) of each component. The results of the MANOVA of the effect of larval rearing temperature are shown for all blend components (C25-type males only, N = 82). Significant differences among temperature groups are highlighted in bold.

	Retention	Length of	MW	Relative amount (%)		MANOVA	
Compound name	index	C-backbone	(g/mol)	Mean	SD	F	p
(*S*)-2,3-Dihydrofarnesoic acid	1767	18	238	4.92	3.31	2.249	0.121
(*Z*)-9-Octadecen-1-ol	2061	18	268	0.35	0.18	8.691	**0.001**
(*Z*)-10-Nonadecen-2-one	2079	19	280	6.37	0.93	4.615	**0.017**
1-Octadecanol	2085	18	270	2.43	0.70	10.722	**<0.001**
Heneicosane	2100	21	296	0.17	0.05	0.210	0.811
Unidentified substance	2168	22	308	0.15	0.06	1.322	0.280
Docosane	2200	22	310	0.12	0.05	4.238	**0.023**
(*Z*)-11-Eicosen-1-ol+(*Z*)-9-Tricosene	2271	20	296	61.21	3.83	4.014	**0.027**
1-Eicosanol	2287	20	298	2.69	0.39	15.639	**<0.001**
Tricosane	2300	23	324	4.34	0.72	1.252	0.299
(*Z*)-9-Tetracosene	2372	24	336	0.67	0.22	3.361	**0.047**
Tetracosane	2400	24	338	0.78	0.25	0.830	0.444
(*Z*)-9-Pentacosene	2477	25	350	12.06	3.21	2.192	0.127
Pentacosane	2500	25	352	1.49	0.29	3.713	**0.035**
(*Z*)-9-Hexacosene	2573	26	364	0.10	0.09	15.217	**<0.001**
Hexacosane	2600	26	366	0.04	0.02	2.713	0.081
Δ-16-Pentacosen-8-one	2656	25	363	0.10	0.05	0.452	0.640
(*Z*)-9-Heptacosene	2674	27	378	0.91	1.51	13.082	**<0.001**
Heptacosane	2700	27	380	0.70	0.15	0.951	0.396
Nonacosane	2900	29	408	0.35	0.08	0.570	0.571
Hentriacontane	3100	31	436	0.06	0.02	0.318	0.730

4.5 Discussion

Life history traits

Larvae reared under higher ambient temperatures exhibited significantly shorter development times and lower mortalities than those kept under low-temperature conditions. Such negative correlations between temperature and development time have been described for numerous insect species (Ratte 1984) and have also been found in earlier studies on European beewolves (Strohm 2000). This effect is generally ascribed to the temperature-dependence of basal biochemical processes (Schmidt-Nielsen 1999). The temperature tolerance of an organism follows an optimum curve (Ratte 1984; Schmidt-Nielsen 1999) and deviations from the ideal temperature range lead to developmental stress or can even be lethal. Generally, a short development time is advantageous, because it might reduce the mortality risk in the vulnerable larval stage (Sibly & Calow 1986). Selection for a fast development is likely to be particularly strong in beewolves, because the larvae are exposed to a high density of pathogenic microorganisms in their subterranean brood cells and, thus, face a high risk of bacterial or fungal infestation (Herzner & Strohm 2007; Kaltenpoth et al. 2005; Strohm & Linsenmair 1998, 2001). To reduce larval development times and enhance the survival probability of the offspring, *P. triangulum* females in Central Europe strongly prefer areas

with favourable climatic conditions, i.e. warm places with high solar irradiation, for nesting (Olberg 1953; Rathmayer 1962). Additionally, short larval development times may allow the emergence of a second generation in one flight season.

Amount of pheromone gland content

Our results show that low larval rearing temperatures cause a reduction in the quantity of pheromone gland contents of adult European beewolf males. Males reared at 20°C possessed significantly less marking pheromone than those reared at 25°C and 30°C, and this effect was not caused by differences in body size. Suboptimal developmental conditions probably lead to competing processes for limited energetic resources causing a higher investment into traits important for current survival (like e.g. immunocompetence) at the expense of future reproductive traits (Buchanan et al. 2003; Kemp & Rutowski 2007; Landete-Castillejos et al. 2002; Ratte 1984; Zwaan et al. 1992). For example, the number of pheromone gland cells (Göttler & Strohm 2008) could be reduced when males are confronted with developmental stress, resulting in a reduced pheromone production.

In European beewolves, the characteristics of the male sex pheromone and the gland morphology suggest that pheromone quantity probably is a crucial factor for male reproductive success: First, regarding the huge amount of gland content applied to the territory the pheromone constitutes an exaggerated signal targeted at the females' sensory sensitivity (Herzner et al. 2005). The conspicuousness of a male territory for females is most probably positively correlated with the amount of secretion applied to the territory. Thus, sexual selection may promote a high rate of pheromone production. Second, the gland tissues involved in the production and the storage of the pheromone are greatly enlarged, and details of their morphology suggest a high level of metabolic activity (Göttler & Strohm 2008). There is evidence that territory owners deplete most of their marking pheromone over their daily activity period and that they replenish the stores over night (E. Strohm unpubl. data). Thus, the amount of gland content available to a male probably limits its ability to scent mark its territory and to attract receptive females. Most substances applied to the substrate are quite long-lasting (J. Kroiss, unpubl. data), but independent of the degree of volatilization of different components males that are able to produce larger amounts of pheromone within a given time probably have a selective advantage because the more pheromone is applied the more conspicuous or attractive will it be for females. Correlations between pheromone production and male reproductive success have been shown for several other insect taxa (e.g. Droney & Hock 1998; e.g. Löfstedt et al. 1990).

Composition of the pheromone gland content

There was a significant influence of the larval rearing temperature on the composition of the pheromone gland content of adult male European beewolves. These changes in composition were gradual and there was no loss or addition of components. Although there was a broad overlap between the blends of males reared at 20°C, 25°C, and 30°C, the three groups were significantly separated by a discriminant analysis (Figure 3). The relative amounts of about half of the

components of the marking pheromone differed significantly among groups (Table 1). Interestingly, changes in the relative amounts of the components with the rearing temperature were correlated with the molecule size of the substances with higher temperatures leading to an increase in the proportions of long chained components (Figure 4). Thus, there is a predictable trend in the temperature dependent variation of the composition of the marking pheromone.

Changes in the ratio of pheromone components due to different larval rearing temperatures have been described for the moth *Phtorimaea operculella* (Lepidoptera: Gelechiidae; Ono 1993). This effect has been ascribed to physiological limitations and different temperature dependencies of enzymes involved. In contrast to the European beewolf, however, in *P. operculella* the females are the pheromone-producing sex, as it is the case in most moth species (Svensson 1996).

Since sexual signals produced by males and females are subjected to considerably different selection pressures (Phelan 1992, 1997), the fitness consequences of temperature-induced changes in pheromone composition are expected to differ between the sexes as well. Due to asymmetric parental investment (Andersson 1994) females should be more discriminating than males when choosing a mating partner. The influence of larval conditions on the composition of the male sex pheromone potentially provides information for female choice. There are several possibilities why it might be beneficial for a female to choose a male with regard to its rearing temperatures. First, a male that developed at an optimal temperature might be healthier and more fertile than others, thereby potentially providing direct benefits (like more viable sperm) to females. Second, assuming that ideal nesting sites are limited (Strohm et al. 2001) only competitively superior females might be able to defend such sites. Thus, choosing a male whose pheromone indicates optimal temperatures during development might be beneficial for a female since this male may carry high quality alleles that it inherited from its mother.

The results of the present study show that the temperature during larval development has a significant effect on the amount and composition of the marking pheromone of adult male European beewolves. These changes may reflect physiological constraints and competing processes for limited energetic resources during early development. To our knowledge, this is the first study on the effects of developmental temperature on amount and composition of the pheromone gland content in a species with a male sex pheromone. The enormous importance and widespread distribution of chemical signals as well as the potentially high information content of male sex pheromones and its implications for female mate choice decisions call for further studies on the impact of developmental conditions on pheromone characteristics.

4.6 Acknowledgements

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CHAPTER 5

GENERAL DISCUSSION

5.1 Symbiosis between beewolves and *Streptomyces* bacteria

Chapters 2-3 deal with the unique symbiotic interaction between members of the digger wasps tribe Philanthini (Hymenoptera, Crabronidae) and *Streptomyces* bacteria located in special reservoirs in female antennae. In these chapters, a new monophyletic taxon was proposed for the symbiotic bacteria, and phylogenetic studies on hosts and symbionts were presented. Additionally, the age of the symbiotic association has been estimated. Because the analyses of the systematic relations within the genus *Philanthus* in comparison to the current morphological classification and its relation to the genus *Trachypus* as well as the dating of the symbiosis have already been discussed in detail in chapter three, these points will be omitted here. In the following, different facets of the evolution of this fascinating association are discussed in the context of similar symbioses found in insects.

Systematic position of beewolf symbionts

In general, insects are associated with an enormous variety of symbiotic microorganisms (Buchner 1965). The systematic diversity of endosymbiotic bacteria found in insects ranges from Proteobacteria (Chen et al. 1996; Clark et al. 1992; Moran et al. 2005a), Mollicutes (Hurst et al. 1999; Williamson et al. 1999), Spirochetes (Breznak 2002; Hongoh et al. 2003) to the Flavobacteria/Bacteroidetes group (Bandi et al. 1995; Moran et al. 2005b). Members of the Actinobacteria have up to now been comparatively rarely described as symbiotic partners (see Kaltenpoth 2009 for review), but they were e.g. found in pine beetles (Scott et al. 2008), leaf-cutter ants (Currie et al. 1999; Haeder et al. 2009; Oh et al. 2009a; Schoenian et al. 2011), termite guts (Bignell et al. 1991) or in true bugs (Hill et al. 1976). Especially regarding defensive associations Actinobacteria are predestined as symbiotic partners, because this group is famous for producing a large number of antibiotics. Members of the genus *Streptomyces* alone produce 75% of all antibiotics used in human and veterinary medicine (Miyadoh 1993).

As humans, beewolves also use antibiotics produced by their symbiotic Actinobacteria for defence against pathogens: Nine antibiotics are used as combination prophylaxis to defend the offspring in the brood cells (Kroiss et al. 2010), and thus represent an example for the effectiveness of such a symbiosis with Actinobacteria. The actinobacterial *Philanthus* symbionts were shown to form a monophyletic clade and could clearly be ascribed to the genus *Streptomyces* by genetic methods (diagnostic PCR and fluorescence in-situ hybridization; chapter 2). This genus is also known to be

involved in symbioses with attine ants (Barke et al. 2010; Haeder et al. 2009; Schoenian et al. 2011), pine beetles (Scott et al. 2008) and, possibly, other arthropods (Gebhardt et al. 2002). Thus, actinomycetes as bacterial symbionts in insects seem to be more common than previously thought (Kaltenpoth 2009).

Distribution of symbiotic *Streptomyces* among Philanthinae (Hymenoptera, Crabronidae)

In the second chapter we could show that all investigated species of the genus *Philanthus* harbour the symbiotic bacteria. The paraphyly of the genus *Philanthus* with respect to *Trachypus* had already been proposed by Alexander (1992) and could be confirmed by genetic data (chapter 3). Taken together these findings suggest that the genus *Trachypus* should harbour the endosymbiotic bacteria as well, which was confirmed by Kaltenpoth et al. (2010) for two South American *Trachypus* species. The genus *Philanthinus* was proposed as closest relative to the *Philanthus*/*Trachypus* complex (chapter 3), following the morphologically based phylogeny of Alexander (1992), but hitherto it was not yet known if this rare genus is cosely related enough to harbour the endosymbiotic bacteria as well. However, Kaltenpoth et al. (submitted) recently demonstrated the existence of the *Streptomyces* symbionts in the species *Philanthinus quattuordecimpunctatus*.

All others members of Philanthinae except the genera *Eucerceris* and *Pseudoscolia* have been investigated with regard to *Streptomyces* symbionts, but they all lack the bacteria (chapter 2; Kaltenpoth et al. submitted; Figure 1a). The two missing genera *Eucerceris* and *Pseudoscolia* are not expected to harbour the symbionts due to the close relationship to *Cerceris* (Alexander 1992), which already have been shown to lack the endosymbionts (chapter 2) and then must have lost the symbionts secondarily. Thus, the symbiosis must have evolved somewhere along the branch leading to the tribe Philanthini (chapter 3, Kaltenpoth et al. submitted). Assuming the most parsimonious scenario that the emergence of the symbiosis was a unique event, the placement of *Philanthinus* as a sister group of *Philanthus* and *Trachypus* (Alexander 1992) is preferred over Bohart & Menke's (1976) placement of the genus within the tribe Aphilanthopini (see Figure 1b). Additionally, the former scenario is strongly supported by the molecular phylogeny (chapter 3).

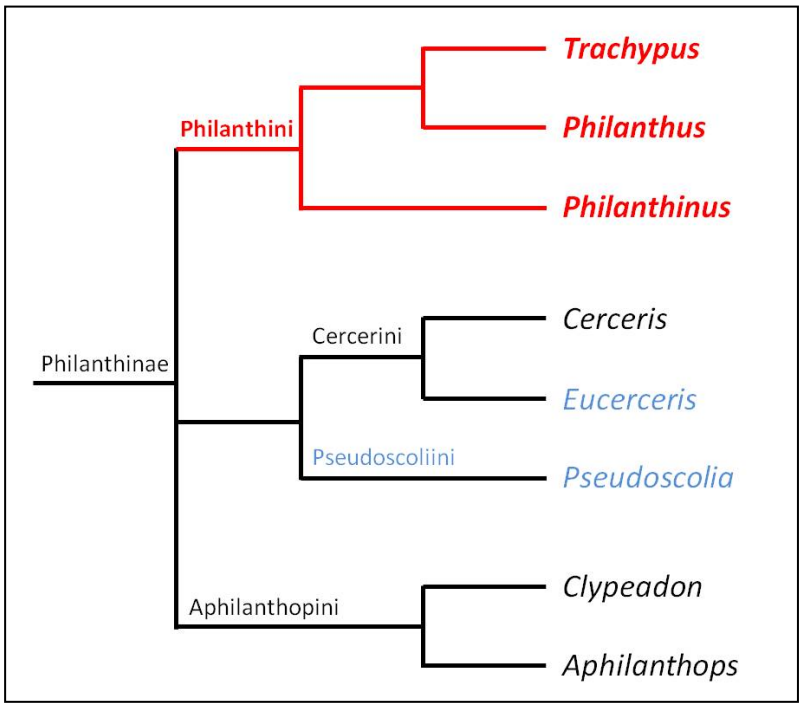

Figure 1a: Distribution of antennal Streptomyces bacteria in wasps of the Crabronid subfamily Philanthinae. The schematic phylogeny was reconstructed by Alexander (1992) based on morphological data. Taxa with symbiont-containing antennal gland reservoirs are highlighted in bold red, those that have not been investigated for the presence of antennal symbionts yet are given in light blue. *(from Kaltenpoth et al, subm.)*

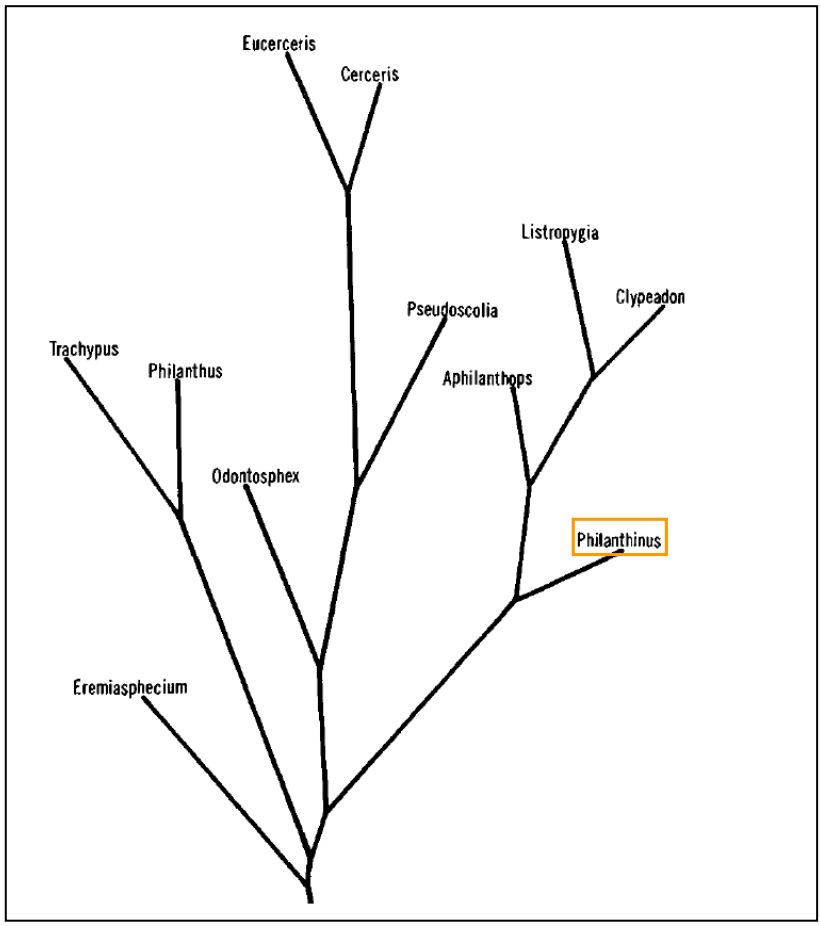

Figure 1b: Dendrogramm suggesting relationships in the Philanthinae *(from Bohart & Menke 1976)* The position of the genus *Philanthinus* is highlighted in orange.

Evolution and co-evolution

During the past years, the knowledge on defensive mutualistic interactions for saving host or food resources from pathogens, parasitoids or predators has been growing steadily (Brownlie & Johnson 2009; Douglas 2010; Jaenike et al. 2010; Kaltenpoth 2009; Kroiss et al. 2010; Schoenian et al. 2011; Vorburger & Gouskov 2011). Especially insects with underground nesting sites experience a high selection pressure for developing defensive mechanisms against pathogen attack. The existence of this high pressure is reflected in the three lines of defence that evolved in the genus *Philanthus*: First, the provisioned prey items are embalmed with a hydrocarbon-containing secretion from a postpharyngeal gland to reduce the risk of spore germination (Herzner & Strohm 2007, 2008), the second line of defence consists of an antimicrobial gas released by the beewolf egg (Engl 2011). Finally, the cocoon stage of the larva is protected by the highly specialized symbiosis with *Streptomyces* bacteria that produce at least nine antibiotic substances for defence against pathogenic fungi (Kaltenpoth et al. 2005; Kroiss et al. 2010).

As already mentioned above, the symbiosis between beewolves and *Streptomyces* bacteria must have evolved somewhere along the branch to the tribe Philanthini, but one only can speculate about the initial steps leading to this highly specialized association. Originally, the glands might have served for providing directional information for the offspring on where to exit the brood cell, locate the open main tunnel, and successfully leave the nest, a function that is still relevant today (Strohm & Linsenmair 1995). *Streptomyces* bacteria, which are known to be very common in the soil (Dari et al. 1995; Kutzner 1981), then could have secondarily colonized the glands as commensals or even parasites, with the gland secretions serving as potential nutrients. Alternatively, the symbiotic bacteria could first have been cultivated on the cuticle of the beewolf, like it is the case in the similar and also highly specialized symbiotic association between attine ants and their actinobacterial symbionts (Currie et al. 2006). The recently described association of *Streptomyces* bacteria and two species of solitary sphecid mud dauber wasps (Poulsen et al. 2011) comprises another stage with regard to the evolution of such a symbiosis: This potentially might constitute the primary stage of a symbiotic association, comparable to an early stage of the evolution of the beewolf-*Streptomyces* association. In the beginning, a high diversity of different *Streptomyces* strains might have been located on the beewolf cuticle as it is the case in mud dauber wasps (Poulsen et al. 2011). Then this loose association could have developed from a commensalism or an unspecific ectosymbiosis to a highly specialized endosymbiosis by the colonization of cuticular cavities in the antennae, which then could have developed into the present complex symbiont cultivation organs. In this coevolutionary process the bacteria gained safe and increasing space to grow and a secured transmission to the next wasp generation. The wasps profited by the antibiotics produced by the bacteria to save their offspring during development. That symbiotic associations can develop very quickly when a strong selection pressure is present was shown recently in *Drosophila*, where the pressure is carried out by a parasitic nematode that destroys an individual's fitness by a sterilizing effect (Jaenike et al. 2010). Jaenike et al. could show a major adaptive change to a symbiont-based mode of defence, which rapidly spreads its profound defensive effects in the *Drosophila* population to fend off the parasitic nematode.

Regarding the investigation of coevolutionary processes in the evolutionary history of the beewolf-*Streptomyces* association this thesis has provided a molecular phylogeny of the host species. Regarding the mode of transmission, both vertical and horizontal transmission have been described to occur in this symbiosis: After the exclusion of a possible transovarial transfer of the symbionts (Kaltenpoth 2006), vertical transmission from mother to daughter via the cocoon of the larva was shown to be likely (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010a). Vertical transmission from mother to offspring seems to be the prevalent mode of symbiont transfer in insects (e.g. Baumann & Moran 1997; Bourtzis & Miller 2003; Buchner 1965). During subsequent molecular symbiont screening studies, the exchange of symbiont strains between species most probably via predator-prey relations within the genus *Philanthus* was suggested (M. Kaltenpoth, pers. comm.). Other possibilities could be symbiont transmissions by chrysidid wasp parsitoids and by the reuse or usurpation of other females' nests. In addition, it was shown that *Philanthinus* symbionts cluster deep within the monophyletic clade of beewolf symbionts, also suggesting transmission events via horizontal transfer (Kaltenpoth et al. submitted), what also had been proposed for *Trachypus* symbionts, which in contrast to their hosts do not form a monophyletic clade (Kaltenpoth et al. 2010b). Thus, in addition to the predominant vertical mode of symbiont transmission there also seems to occur horizontal transfer or even *de novo* uptake from the environment occasionally (Kaltenpoth et al. 2010b), which is also known from the bacterial symbiosis in leaf-cutter ants (Cafaro & Currie 2005; Cafaro et al. 2011; Kost et al. 2007; Mueller et al. 2008; Poulsen et al. 2005).

Most endosymbiotic bacteria in insects are located in the gut or in specialized cells in abdominal organs, e.g. in specialized bacteriomes in the digestive tract, in the fat body or in the ovaries (Blochmann 1892; Buchner 1921; Dillon & Dillon 2004; Ishikawa 2003; McLean & Houk 1973). In beewolves, the symbionts are harbored in unique antennal gland reservoirs constituting invaginations of the outer cuticle (chapter 2; Goettler et al. 2007). The association between beewolves and *Streptomyces* bacteria is the first known symbiosis located in insect antennae. Despite the unique localization, the symbiosis shows a high similarity to the leaf-cutter ant association, a defensive symbiosis with elaborate cuticular crypts supported by specialized exocrine glands (Currie et al. 2006; Currie et al. 1999). As in the ant-*Pseudonocarida* symbiosis (Currie et al. 2006), there is some evidence for differences in the structure and complexity of the antennal symbiont cultivation organs involved in the Philanthini symbiosis: Kaltenpoth et al (submitted) showed that the investigated species of the genus *Philanthinus* possesses antennal gland reservoirs in six antennal segments, as opposed to only five in the genera *Philanthus* and *Trachypus* (Goettler et al. 2007; Kaltenpoth et al. 2010b). Additionally, the *Philanthinus* glands are structurally slightly less complex than in most *Trachypus* and *Philanthus* species (Kaltenpoth et al. submitted). Thus, the complexity of the antennal gland reservoirs may have increased during the evolution of the association (Kaltenpoth et al. submitted). A reason for that could be that with more complex structure the gland reservoirs provide more space. Thus, there are more bacteria in the reservoirs and a stable defence can be warranted for the offspring (Kaltenpoth et al. submitted). The question, why the more derived Philanthini species have glands in fewer antennal segments than

Philanthinus has, is more difficult to answer: The fact that the symbionts are released by an increased hemolymph pressure caused by the female by pressing the antennae against the wall of the brood cell might be relevant in this context. Maybe the sixth reservoir could not be fully exploited because the female is not able to apply enough pressure to all reservoirs. If the contribution made by the sixth reservoir is not significant for the offspring's fitness, this reservoir could have got lost secondarily during evolution. Even though this hypothesis still has to be tested by analysing the amount of symbionts released from the different antennal segments, the recent findings on the evolution of this symbiosis suggest an increasing complexity of the symbiont-cultivation organs during its past, and show that the association between hosts and symbionts is characterised by predominantly vertical and occasional horizontal transmission events or *de novo* uptake from the environment.

5.2 The male pheromone and the impact of rearing temperature

Chapter three deals with the pheromone of European Beewolves (*P. triangulum*, Hymenoptera, Crabronidae) which males apply to their territories to attract females for mating. In the following, the role of the sex pheromone as an indicator of male quality for female mate choice decisions and the relevance of larval rearing temperature as an environmental factor influencing the adult male pheromone are discussed.

Relevance of the male marking pheromone for female mate choice decisions

Due to the asymmetry regarding the costs of reproduction in the sexes, females in general should be choosier than males (Andersson 1994; Gould & Gould 1997; Trivers 1972). Through their choice, females either can profit directly e.g. by receiving resources from the male (Bateson 1983; Halliday 1983; Hamilton & Zuk 1982) or indirectly e.g. by ensuring that their offspring inherits "good genes" from the high-quality mating partner (Andersson 1994; Møller & Alatalo 1999). As *Philanthus* females do not seem to gain any direct benefits from the mating partner (Kroiss et al. 2010; Strohm 1995), only indirect benefits are considered in the discussion below.

Following Fisher's (1930) 'runaway process' (also known as 'sexy-son-hypothesis', Weatherhead & Robertson 1979), there is a correlation between female choice traits and male attractiveness traits (Bakker 1993; Brooks & Couldridge 1999; Gwinner & Schwabl 2005; Hine et al. 2002; Houde 1994). If a female mates with an attractive male, the sons will be attractive as well and their daughters will inherit the choosiness. The trait underlying the male's attractiveness can be linked to the male's fitness, but that is not a prerequisite for the process to work. In beewolves, males do not have sons because unfertilized eggs develop into males. But here another mechanism, so to speak a 'fit-daughter' effect, could play a role: Females of this species prefer sun-exposed areas to construct their nests. Because these sites are limited, females compete for the best nesting sites (Evans & O'Neill 1988; Simon-Thomas & Simon-Thomas 1972; Strohm 1995). Females of low

competitiveness have to nest in suboptimal areas, where the offspring has to develop at lower temperatures, which affects the sons' sex pheromone composition (chapter 3). Thus, the pheromone of these males reflects the low-quality conditions during larval development and thus the poor competitiveness of the male's mother. Hence, females may gain fitness benefits by choosing the son of a 'competitive mother-in-law', because this might possibly lead to daughters with similar qualities.

The 'good genes' model hypothesizes that a male's signal conveys honest information on its genetic quality and thus makes it possible for females to choose a mate of high quality and hence increases the fitness of their progeny (Andersson 1994; Hamilton & Zuk 1982). It has been considered an alternative to Fisher's hypothesis in the past, but meanwhile the two models are seen as two points on a continuum of sexual selection (Kokko et al. 2002; Mead & Arnold 2004). As for Fisher's model there also are several studies supporting the 'good genes' model, showing positive fitness consequences of mate choice for the offspring (Barber et al. 2001; Doty & Welch 2001; Partridge 1983; Tallamy et al. 2002). Studies investigating adaptive female choice based on olfactory signals are rare (Jones et al. 2000; Jones & Hamilton 1998; Jones et al. 1998; Vainikka et al. 2006), but there are several publications showing the transmission of mate quality aspects to females in insects (Droney & Hock 1998; Kortet & Hedrick 2005; Moore 1997; Thornhill 1992), salamanders (Marco et al. 1998), fish (Milinski 2003), reptiles (Martín & López 2000) and mammals (Kavaliers et al. 2003). In beewolf males, the pheromone might have resulted from selection pressures caused by female selection for an honest signal of male quality: Males with lower rearing temperature had less pheromone, reflecting a developmental trade-off between basal physiological processes for development and the formation of a trait relevant for reproduction, thus representing an honest signal due to the fact that it causes costs (Zahavi 1975). This form of trade-off caused by developmental conditions is known from numerous other taxa (Buchanan et al. 2003; Gimenez & Anger 2005; Landete-Castillejos et al. 2002; Ratte 1984; Soma et al. 2006; Woodgate et al. 2010; Zwaan et al. 1992). A higher pheromone amount is supposed to be of advantage for a male, because it can apply more pheromone to its territory, thus attract more females and gain more matings than other males (Droney & Hock 1998; Lloyd 1979; Otte 1974). This is supported by the observation that in *P. triangulum* females seem to prefer mating with bigger males (E. Strohm, J. Kroiss, M, Kaltenpoth, G. Herzner, unpubl. data). The effect of different factors influencing the pheromone amount have been investigated in several species (Abernathy et al. 1994; Jones & Hamilton 1998; Subchev & Jurenka 2001), but only little is known about the impact of rearing temperature on pheromone quantity (Ono 1993). The effect of rearing temperature on the composition of a male's pheromone as it is the case in male *Philanthus*, hitherto has only been investigated in moth (Ono 1994). Just as the relevance of pheromone amount discussed above the composition as well constitutes an honest signal and can be used by females for mate choice. Thus, the pheromone in *Philanthus* with its high information content might be a good indicator for a male's fitness and provides optimal preconditions for an adaptive female choice.

5.3 Final conclusions and future prospects

The beewolf-*Streptomyces* association is the first example of a symbiosis with microorganisms located inside specialized antennal glands. To complement previous studies on the ecology of the symbiosis and the morphology of the symbiont-cultivation organs, genetic and systematic background information was collected to yield insights into the taxonomic distribution and the evolutionary history of this extraordinary association between insects and bacteria. With these data it was also possibly to date the origin of this extraordinary symbiosis. Thus, the studies presented in this thesis provide a basis for future investigations on co-evolutionary processes in this insect-bacteria symbiosis. Additionally, it is very likely that other defensive symbioses with actinomycetes will be discovered in the future, since other Hymenoptera and ground-nesting insects are confronted with similar problems to cope with pathogen infestations in underground brood cells. Moreover, the growing knowledge on the variety of antibiotic substances involved in this symbiosis might also become relevant in the field of human medicine.

In addition to several factors that were already known to influence the pheromone of male beewolves, we could show that conditions during larval development also play an important role in shaping the pheromone of adult males. Pheromone quantity and quality can convey information on the quality of a male's mother to receptive females. These findings show that not only the genetic background or immediate environmental factors, but also conditions during early development can influence the properties of an insect's sex pheromone and thereby may play an important role in female mate choice decisions.

The reconstruction of the phylogenetic relationships within the Philanthinae provides the basis for future comparative studies on different aspects of beewolf biology. The prey spectrum of different *Philanthus* species (groups) and other Philanthini could be mapped onto the beewolf phylogeny and thus deliver insights into the evolution of prey specialization in this tribe. Another fascinating aspect in the biology of *P. triangulum* is the defence of the beewolf egg against fungal growth in the brood cell, which is mediated by the release of the gas nitric oxide and its strong antimicrobial effects. Comparative studies on this protective trait could reveal the range of beewolf species that employ this defence, and in combination with the beewolf phylogeny the evolutionary history of the nitric oxide defence could be investigated. Regarding the pheromone of male *Philanthus* and the knowledge on the diverse factors influencing this chemical signal, a next step could be to reconstruct the evolutionary history of this sexually selected trait by mapping a chemotaxonomy of the male pheromone onto the phylogenetic tree. This could also be done with the chemical profiles of the prey-embalming secretion of beewolf females, which increases the offspring's survival chances and thus is subject to natural selection. Thereby, the effects of the different selection pressures of natural vs. sexual selection could be investigated and yield insights into the evolution of chemical traits. Thus, the findings presented in this thesis provide the basis for understanding the evolutionary history of different ecological and behavioural aspects in beewolves, most importantly the defensive symbiosis with *Streptomyces* bacteria and the male beewolf sex pheromone.

CHAPTER 6

SUMMARY

6.1 Symbiosis between beewolves and *Streptomyces* bacteria

Symbiotic associations basically contributed to the evolution of life on earth. In insects, most symbionts provide their hosts with important nutrients. However, during the past years an increasing number of defensive symbioses has been discovered, where symbionts protect their hosts against predators, parasitoids or pathogens. Beewolf digger wasps (Hymenoptera, Crabronidae, Philanthini) engage in a highly specialized defensive symbiosis with *Streptomyces* bacteria that are cultivated in glands within the females' antennae, a location which has not yet been described for any other symbiosis. Females apply these symbionts to their subterranean brood cells, and when the larva starts spinning the cocoon, the symbionts are incorporated into the cocoon wall. There the symbionts protect the cocoon against fungal infestation by producing an antimicrobial cocktail.

Here, the presence of symbiotic bacteria was detected in 28 different *Philanthus* species and subspecies by using different genetic, ultrastructural and morphological methods. Systematic analyses showed that the investigated symbiont is an as yet undescribed species belonging to the genus *Streptomyces*, and the new monophyletic taxon '*Candidatus* Streptomyces philanthi' is proposed for the bacterial symbionts.

Besides phylogenetic analyses of the symbionts a large data set of six molecular markers of the hosts was analyzed. A molecular phylogeny of the subfamily Philanthinae was reconstructed, which provided the basis to date the origin of the symbiotic association between *Streptomyces* and Philanthini digger wasps. The origin of the beewolf-*Streptomyces* association must most probably have evolved somewhere along the branch leading to the tribe Philanthini, and molecular clock analyses placed the origin of the symbiosis between beewolves and Streptomyces to 65-97 million years, thus constituting the oldest dated defensive symbiosis known at present. Additionally, the phylogenetic relationships within the genus *Philanthus* were investigated. The results roughly corresponded with a previous morphological classification into species groups. Beyond that, the controversial relationship between the sister genera *Philanthus* and *Trachypus* was examined closer, with results clearly indicating paraphyly of *Philanthus* with respect to *Trachypus*, thus contradicting the current division in two discrete genera. The reconstruction of the phylogenetic relationships within the Philanthinae additionally provides the basis for future comparative studies on different aspects of beewolf biology like the pheromone blend of males or the prey spectrum of different beewolf species.

The new insights into the phylogenetic relationships within hosts and symbionts involved in the beewolf-*Streptomyces* association presented in this thesis provide a profound basis for future investigations on coevolutionary processes in the history of this symbiosis. Additionally, further studies on this exceptional symbiotic association may yield valuable knowledge on the relevance of actinomycete bacteria for pathogen defence in other insects and might lead to the discovery of unknown antibiotic substances that could be useful for human medicine.

6.2 Male pheromone and temperature effects during larval development

The role of sexual selection and female mate choice decisions based on male visual ornaments and acoustic signals has been studied extensively. However, little is known on the role of pheromones for adaptive female choice, despite their large potential for communicating information on mate qualities by variations in quantity and quality. Only few studies focus on the factors that influence the shape of insect pheromones, like the effect of environmental conditions on the amount and composition of adult sex pheromones and the conditions during larval development in particular.

In this thesis we focused on the male marking pheromone of the digger wasps species *Philanthus triangulum* (Hymenoptera, Crabronidae). The males defend small territories and mark them with a pheromone blend from cephalic glands to attract receptive females for mating. Because these territories are concentrated in the vicinity of female nest aggregations, females can choose the mating partner from numerous males. Previous studies had shown that the pheromone varies with kinship, geographical distance, size and age, but the effect of developmental conditions on the male pheromone had not yet been studied. We could show that the temperature during a male's larval development strongly affects pheromone quantity and quality of the imago. Thus, the information comprised in a male's pheromone blend may provide a solid basis for female mate choice decisions, not only reflecting the condition of the adult but also early developmental conditions of a potential mating partner.

CHAPTER 7

ZUSAMMENFASSUNG

7.1 Symbiose zwischen Bienenwölfen und Streptomyceten

Symbiontische Interaktionen haben einen essentiellen Beitrag zur Evolution des Lebens auf der Erde geleistet. Symbionten von Insekten versorgen ihre Wirte in demeisten Fällen mit essentiellen Nährstoffen, aber in den letzten Jahren wuchs auch die Zahl an beschriebenen Verteidigungssymbiosen, bei denen die Symbionten ihre Wirte vor Beutegreifern, Parasitoiden oder Pathogenen schützen. Bienenwölfe (Hymenoptera, Crabronidae, Philanthini), die zu den Grabwespen gehören, leben in einer hochspezialisierten Verteidigungs-Symbiose mit Bakterien der Gattung *Streptomyces*: Die Symbionten werden von den Weibchen in speziellen Drüsen in ihren Antennen kultiviert, ein Ort, für den bisher keine anderen Symbiosen beschrieben sind. Die Weibchen geben diese Bakterien bei der Verproviantierung mit in ihre unterirdischen Brutzellen. Wenn die Larve später beginnt, einen Kokon zu spinnen, werden die Symbionten mit in die Kokonwand eingearbeitet, wo sie den Kokon durch Abgabe eines antimikrobiellen Cocktails vor Pilzbefall schützen.

Die Anwesenheit von symbiontischen Bakterien bei 28 verschiedenen *Philanthus*-Arten und Unterarten konnte hier mit verschiedenen genetischen, ultrastrukturellen und morphologischen Methoden festgestellt werden. Anschließende systematische Analysen zeigten, dass der bakterielle Symbiont zu einer bisher unbeschriebenen Art der Gattung *Streptomyces* gehört und für die Symbionten wird das neue, monophyletische Taxon ‚*Candidatus* Strepotmyces philanthi' vorgeschlagen.

Neben einer phylogenetischen Analyse der Symbionten wurde auch ein umfassender Datensatz basierend auf sechs genetischen Markern der Wirte untersucht. Eine molekulare Phylogenie der Unterfamilie Philanthinae wurde rekonstruiert, welche die Grundlage für eine Datierung der Symbiose zwischen *Streptomyces* und den Philanthini darstellte. Die Enstehung der Bienenwolf-*Streptomyces* Assoziation hat sich höchstwahrscheinlich an irgendeinem Punkt des Astes entwickelt, der zu der Unterfamilie der Philanthini führt. Analysen auf der Basis molekularer Uhren haben den Ursprung der Symbiose auf einen Zeitraum von vor 65 bis 97 Millionen Jahren datiert, womit diese Assoziation die älteste aller bisher datierten Verteidigungssymbiosen darstellt. Zusätzlich wurden die phylogenetischen Beziehungen innerhalb der Gattung *Philanthus* untersucht. Die Ergebnisse decken sich weitgehend mit einer früheren morphologischen Einteilung in verschiedene Artengruppen. Des Weiteren wurde die kontroverse Beziehung zwischen den beiden Schwestergattungen *Philanthus* und *Trachypus* genauer untersucht. Die Ergebnisse sprechen für eine deutliche Paraphylie von *Philanthus* bezüglich *Trachypus*, was der derzeitigen Einteilung in zwei getrennte Gattungen widerspricht. Neben ihrer Relevanz für Symbiose und Systematik ist die molekulare Phylogenie der Philanthinae auch eine Grundlage für vergleichende Studien in anderen

Bereichen der Biologie der Bienenwölfe, wie z.B. des Pheromons der Männchen oder der Beutespektren verschiedener Bienenwolfarten.

Die neuen Einsichten der vorliegenden Arbeit in die phylogenetischen Beziehungen innerhalb der Wirte und der Symbionten, die an der Bienenwolf-*Streptomyces* Assoziation beteiligt sind, bilden eine solide Grundlage für die weitere Erforschung koevolutionärer Prozesse in der Geschichte dieser Symbiose. Darüber hinaus können weitere Studien dieser außergewöhnlichen symbiontischen Assoziation wertvolle Einsichten in die Relevanz der bakteriellen Actinomyceten für die Pathogenabwehr bei anderen Insekten liefern und zur Entdeckung unbekannter antibiotischer Substanzen beitragen, die vielleicht auch in der Humanmedizin Anwendung finden könnten.

7.2 Männchenpheromon und Temperatureinfluss während der Larvalentwicklung

Die Rolle der sexuellen Selektion und der Weibchenwahl anhand optischer Ornamente und akustischer Signale von Männchen wurde in der Vergangenheit in zahlreichen Studien untersucht. Über die Bedeutung von Pheromonen für eine adaptive Weibchenwahl dagegen ist sehr wenig bekannt, obwohl Pheromone durch ihre hohe Variabilität in Qualität und Quantität über ein hohes Potential zur Vermittlung von Männchen-Qualitäten verfügen. Es gibt nur wenige Studien, die sich mit den Einflussfaktoren auf Pheromone beschäftigen, wie beispielsweise die Bedeutung von Umweltbedingungen für die Menge und Zusammensetzung des Sexualpheromons adulter Insekten, im speziellen während der Larvalentwicklung.

Der Schwerpunkt dieser Arbeit war das Männchen-Pheromon der Grabwespenart *Philanthus triangulum* (Hymenoptera, Crabronidae): Männchen dieser Art verteidigen kleine Territorien und markieren diese mit einem Pheromongemisch aus Kopfdrüsen, um damit paarungsbereite Weibchen anzulocken. Da diese Territorien geballt in der Nähe von Nestaggregationen der Weibchen auftreten, haben die Weibchen die Möglichkeit, ihren Paarungspartner aus einer Vielzahl verschiedener Männchen auszuwählen. Frühere Studien haben gezeigt, dass das Pheromon von verschiedenen Faktoren wie dem Verwandtschaftsgrad, geographischer Distanz, von Größe und Alter beeinflusst wird, aber die Bedeutung der Entwicklungsbedingungen für das Männchenpheromon wurde bisher nicht untersucht. Hier konnten wir zeigen, dass die Temperatur während der Larvalentwicklung eines Männchens sowohl die Pheromon-Qualität als auch die Pheromonmenge des Adulttiers erheblich beeinflusst. Daher könnten die Informationen, die in dem Männchenpheromon enthalten sind, eine solide Basis für die Weibchenwahl darstellen, da sie nicht nur die augenblickliche Verfassung eines Adulttieres, sondern auch die larvalen Entwicklungsbedingungen eines potentiellen Paarungspartners widerspiegeln.

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