Friedrich-Schiller-University Jena

Faculty of Biological Sciences

Institute for Microbiology

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Specificity and regulation of symbiont transmission in beewolves (Hymenoptera, genus *Philanthus*)

Thesis

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Enes Baran Demiray

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Supervisors:

1. Prof. Dr. Martin Kaltenpoth

Departmant of Insect Symbiosis, Max Planck Institute for Chemical Ecology

2. Prof. Dr. Erika Kothe

Professorship for Microbial Communication, Friedrich Schiller University Jena

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1. Introduction

Evolution, the process of change in living organisms over generations, has shaped the dynamic of life on the earth. Within these multiple variable biological challenges, microorganisms and insects play crucial roles in various ecosystems. In particular, soil microorganisms are a diverse group of organisms that inhabit the soil, including bacteria, fungi, and protozoa. They form complex interactions with plants, insects, and other microorganisms, contributing significantly to nutrient cycling, decomposition, and soil structure maintenance (Grunseich *et al*. 2019).

Insects represent the most diverse and abundant group of animals over the million years of continuous evolutionary history on the planet. They inhabit almost every terrestrial and aquatic habitat, fulfilling essential ecological roles as pollinators, decomposers, predators, and prey. Moreover, the survival chance of many insect species is often affected by their symbiotic relationships with various of microorganisms (Engel and Moran 2013). Although the existence of predators, parasites, and pathogenic organisms in nature may crucially decrease the survival success of the insects, they evolved quite perceptive strategies such as chemical and behavioral defences to protect them against fungal and bacterial infections (Koehler *et al*. 2013). While behavioral defence systems are mostly mechanical protection, chemical defenses include the production of various types of antibiotics, antimicrobial peptides, or other chemical components of the host's immune system (Strohm and Linsenmair 2001). In certain cases, insects form defensive symbiotic relationships with specialized microorganisms (Douglas 2014). This collaboration can lead to the production of chemical defenses for the insects, while the microorganisms may find a safer habitat to obtain valuable nutrients in harsh environments. The investigation of these types of unique interactions between insects and microorganisms may reveal novel biological solutions for antibiotic production or pest control, which can be utilized in agriculture, ecology, and medicine.

1.1 Symbiosis

Symbiosis, with its dynamic structure persisting throughout evolutionary history, serves as a powerful survival mechanism, which leads to enhanced fitness for organisms. The term symbiosis itself was first described by the German scientist, Anton de Bary around 130 years ago to indicate an interaction between different organisms (de Bary 1887). Mutual interactions exist along a broad spectrum, where organisms have developed beneficial

survival strategies to help each other. Meanwhile, organisms can influence each other's fitness through various ecological interactions. In some cases, one organism may benefit at the expense of the other, causing harm (parasitism), while in other cases, an organism may benefit without causing harm (commensalism) to the other (Douglas 1994). In other words, this prolonged continuum can be imagined as a bridge between collaboration and antagonism (Dimijian, 2000).

Many multicellular animals, as well as fungi and plants, engage in mutualistic symbiotic relationships with microorganisms. These symbioses are often essential for the host's survival and reproductive success, particularly in habitats where the host coexists with an abundance of bacteria that could otherwise have detrimental effects on the host's chances of survival (Moran, 2006). In particular, many insects utilize this strategy to establish endosymbiotic associations with bacteria, which act as intracellular symbionts within specialized feeders, such as blood-sucking, phloem-feeding, or wood-feeding insects (Moran and Baumann, 1994). The primary purpose of this association is to supplement the insect's diet with essential nutrients that are insufficiently available. Symbionts play a crucial role in synthesizing important chemical compounds to compensate for these dietary deficiencies (Douglas, 1998). Most of the nutritional aid for insect-bacteria symbiosis is provided by *Proteobacteria*, *Bacteriodetes*, and *Firmicutes* (Bourtzis and Miller 2003). However, survival in nature cannot be achieved only by having access to food; it should be supported by defensive mechanisms to protect organisms against predators, environmental harshness, and pathogenic microorganisms. In this sense, insects have developed various strategies to protect themselves from the potentials dangers in their environment such as intricate immune system, chemical defenses, and behavioral tactics like brood care and nesting in habitats poor in predators/parasites (Batra 1968; Strohm *et al*. 2001).

Social insects demonstrate a collective behavioral defensive strategy by removing parasites, fungal spores, mites, nematodes, and other potential threats from the colony, thus preventing the emergence of hazardous infections (Reber *et al*. 2011). However, solitary species can not perform these types of behavioral defenses as social insects do, therefore, the survival chance of the offspring is mostly dependent on chemical protection, which is either provided by the insect itself or symbiotic microorganisms (Cremer *et al*. 2007; Koehler *et al*. 2013). The chemical protection provided by the symbiont can be mediated through three main methods: the elimination of pathogenic microorganisms through competitive exclusion (Koch and Schmid-Hempel 2011), boosting the host's immune system against bacterial or fungal

infestations (Evans and Lopez 2004), and releasing chemical compounds to directly kill the invaders (Mattoso *et al*. 2012). Interestingly, a significant number of insect species collaborate with the bacterial phylum Actinobacteria to form symbiotic associations (Kaltenpoth 2009). Because of their well-adapted biological features in the soil, such as the formation of mycelium to access nutrients and the production of various enzymes and secondary metabolites with antimicrobial properties, Actinobacteria, mainly *Streptomyces* (Kaltenpoth, 2009; Seipke *et al*. 2012; Koehler *et al*. 2013). One of the interesting defensive symbioses has also evolved between fungus-farming ants and their symbiont, which ants cultivate to protect their mutualistic food source, a basidiomycete fungi, from the pathogenic fungus *Escovopsis* (Currie *et al*. 1999; Goldstein *et al*. 2020). The growth of *Escovopsis* is heavily restricted with the help of the protective symbiotic *Pseudonocardia* (Currie *et al*. 1999; Goldstein *et al*. 2020). Moreover, the evolution of a symbiotic connection between ants and their symbiotic bacteria resulted in the formation of specialized glands in the insect host, which the bacteria can colonize (Goettler *et al*. 2007). For example, the antibiotic-producing bacteria of fungus-farming ants reside within cuticular crypts associated with ectodermal glands (Currie *et al.* 2006). The digestive tracts of *Tetraponera* ants also evolved bacterial sacs for their symbiosis (Billen and Buschinger, 2000). Likewise, *Dendroctonus frontalis* forms a symbiotic association with a bacteria belonging to the genus *Streptomyces*, which is present in both mycangia of the beetles and fungal galleries in the bark, resulting in the production of an antifungal substance, mycangimycin to protect nutritional resources against *Ophiostoma minus*, an antagonistic fungi (Scott *et al*. 2008; Oh *et al*. 2009).

1.2 Symbiotic relationship between *Philanthus triangulum* and *Streptomyces*

The early life stages of insects tend to be more vulnerable to pathogen attacks compared to their adult counterparts, which would be even worse if the organism evolved as solitary (Kaltenpoth and Engl, 2014). Due to the relatively vulnerable position of larvae in the larval stage, insect offspring should be protected by unique mechanisms, either communal or solitary defensive strategies (Kaltenpoth and Engl, 2014). There is a dynamic defensive symbiosis between *Philanthus triangulum* and its symbiont, *Candidatus* Streptomyces philanthi, within the specialized antenna gland reservoirs of female beewolf (Kaltenpoth *et al*. 2005, 2006, 2010).

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) is a solitary wasp that constructs nests under sandy soil, where the brood cells are located (Strohm and Linsenmair 1995). The life cycle of the European beewolf begins with the hunting of honeybees *(Apis mellifera*), during which the beewolf injects venom into the bee's thorax (Rathmayer 1962). The paralyzed honeybee prey is carried by the beewolf and placed in brood cells, with the number of honeybees in each cell varying from one to five (Seipke *et al*. 2012). However, because of the humid and warm environment inside the brood cells, the eggs could be exposed to significant threats from pathogenic fungi or bacteria. This could lead to serious infections that might hinder the successful development of mature wasps (Strohm and Linsenmair 2001). The beewolf has evolved several mechanisms to minimize the risk of infections in its brood cells (Ingham *et al*. 2023). First of all, the eggs of the beewolf sanitize the brood cell by introducing a high concentration of nitric oxide, which inhibits the growth of pathogenic soil-dwelling microorganisms (Strohm *et al*. 2019). The second strategy involves female beewolves applying a secretion containing high levels of long-chain saturated and unsaturated hydrocarbons from their postpharyngeal glands to the larval provisions, thereby minimizing water condensation and limiting the germination of pathogenic mold fungi (Herzner *et al*. 2007; Herzner *et al*. 2008). Furthermore, the specialized bacteria are cultivated within antennal gland reservoirs of the female beewolf and secreted into the brood cell before the oviposition (Strohm *et al*. 1995; Kaltenpoth *et al*. 2005). After the larvae are fed with provisions for about a week, they uptake the symbionts and transfer them to the cocoon while spinning it (Kaltenpoth *et al*. 2005). One of the reasons for the secretion of symbionts to the brood cell is to protect the larvae against microbial infestations, by pathogenic fungi and bacteria (Kaltenpoth *et al*. 2005). After the transfer of symbionts to the larval cocoon surface, the bacteria produce at least nine different antimicrobial compounds, mostly two different groups of antibiotics: streptochlorin and various piericidin derivatives, which results in defense against a broad range of pathogenic fungi (Kaltenpoth *et al*. 2005; Kroiss *et al*. 2010).

As a result of this symbiotic association, the larvae can be protected during the vulnerable phases of hibernation and metamorphosis (Kaltenpoth *et al*. 2005). Consequently, the survival fitness of beewolves is greatly enhanced through this symbiotic collaboration. The secretion of the white substance also functions as an orientational cue for the cocoon-spinning larvae, aiding in their emergence from the brood cell (Strohm and Linsenmair 1995). Furthermore, the evolution of the symbiotic relationship between *Streptomyces* bacteria and beewolves dates back at least 68 million years (Kaltenpoth *et al*. 2014). Over time, this interaction has

become tightly stabilized through the vertical transmission of the symbiont, leading to an association with a single clade of *Streptomyces* across Philanthine wasps throughout the evolutionary timeline (Kaltenpoth *et al*. 2014). It has also been revealed by phylogenetic analyses that there is a horizontal symbiont exchange between the beewolves and other Actinobacteria that can colonize the antennal glands of the beewolves (Kaltenpoth *et al*. 2014). However, despite the colonization of other free-living bacteria in gland reservoirs, they are not transmitted to the offspring due to strong partner fidelity between *Philanthus triangulum* and *Streptomyces philanthi* (Kaltenpoth *et al*. 2014).

1.3 Purpose of the research project

In this research project, we investigated the efficacy of symbiont transmission in beewolves by analyzing the distribution of bacteria within the antenna. In addition to the presence of the symbiont *Streptomyces philanthi*, previous findings show that other *Actinomycetes* can infect the beewolf antennae gland reservoirs in vitro. The core questions of the project were: Firstly, if other free-living bacteria such as *Streptomyces* sp. or *Actinomycetes* were to occupy the gland reservoirs of a beewolf, how frequently free-living bacteria can colonize the antennal gland reservoirs? Secondly, how is the distribution of bacterial composition in each antennal segment in terms of the prevalence of colonization of gland reservoirs by symbionts or other competitors?

2. Material and methods

2.1 Field collection and beewolf rearing

Female European beewolves, *Philanthus triangulum*, were collected from natural populations in Berlin, Halle, and Hamburg, Germany. In total, 39 female beewolves were kept in observation cages under a controlled environment in a greenhouse (14 h day, 10 h night; 23°C (+/− 3°C) at the Max Planck Institute for Chemical Ecology in Jena, Germany. Female beewolves were provided with honey bee workers (*Apis mellifera*) and honey *ad libitum*. They were tracked daily and collected right after they died for antennal processing.

2.2 Preparation of bacterial growth media

For 200 ml of working solution, 9.08 g of Grace's insect medium (Powder, Sigma-Aldrich) was weighed by using a scale (Scale XS105-Mettler Toledo). 80 µl of phenol red, 100 ml of Milli-Q water, 2 mL of tryptose phosphate broth (tryptose (20 g/L), dextrose (2 g/L), NaCl (5 g/L), disodium phosphate ((2.5 g/L), pH adjusted 7.3), cycloheximide (100 µg/ml), 10 µl of fetal bovine serum (5% of total volume) were added to the 200 ml of glass bottle. The solution was filled with Milli-Q water up to 200 ml. The pH of the solution was adjusted with a pH meter (Mettler Toledo-SevenExcellence™) by adding drops of 2M of NaOH slowly while the solution was stirred with a magnetic stirrer (Heidolph MR-1000-220V) to avoid precipitation of media components until the pH reached 6.5. After mixing and adjusting the pH, the solution was filtered through a vacuum filter (Corning® 500 ml Bottle Top Vacuum Filter, 0.22 µm Pore 33.2cm² PES Membrane) into the 250 ml storage bottle (Corning® 250 mL Square Polycarbonate Storage Bottle with 45 mm Cap). The filtrated solution was transferred into the storage bottle within the clean bench (Thermo Heraeus HERAsafe HS18) under sterile conditions. The solution was stored at + 4°C. In addition to the preparation of Grace's insect medium specialized for symbiont growth, ISP-2 medium, commonly used for the cultivation of Actinobacteria, particularly *Streptomyces*, was utilized to detect the growth of free-living bacteria. ISP-2 medium was prepared by mixing 1 liter of distilled water with 4 grams of yeast extract powder, 10 grams of malt extract powder, 4 grams of dextrose, and 20 grams of agar. After the sterilization procedure, the medium was poured into petri plates under sterile conditions, and the plates were stored at room temperature. This medium was used to cultivate bacteria that had already shown growth in liquid media.

2.3 Cultivation of *Streptomyces philanthi* and free-living bacteria from beewolf antennae

Beewolves were collected as soon as they died and stored in 1.5 ml reaction tubes (Eppendorf). One of the antennae of a collected beewolf was cut off and stored in a 1.5 ml reaction tube. The other antenna was stored in a 0.5 ml reaction tube filled with 4% of PFA (Paraformaldehyde) in PBS for FISH experiments at + 4°C. To begin with the processing of antennae for bacterial isolation, the surface of the whole antenna was disinfected by briefly rinsing it in 70% of ethanol for 15 seconds. Each of the segments of an antenna from four to eight was cut off carefully without crushing the segments with a tiny scissor suitable for insect studies under a stereo microscope (Leica M80 Stereomicroscope). Each of the segments was placed in five different 1.5 ml reaction tubes. The scissors was always disinfected with 70% ethanol in between the segment cuttings. 50 µl of Grace's insect medium was added into each of the 1.5 ml reaction tubes with segments and segments were crushed with pellet pestles until the turbidity became visible. 450 µl of Grace's insect medium was added into each well of the first five columns until the fourth row in the 24-well plate (Corning™ Costar™ 24-Well). 50 µl of medium-crushed segment mix was added to the first five wells in the first row of the 24-well plate for separate segments, from 4 to 8, respectively and a serial dilution was carried out by transferring 50 µl to the adjacent wells (1:10, 1:100, 1:1000). The 24-well plate was sealed with parafilm and packed into a plastic bag to prevent evaporation. The plate was incubated at 28 °C within an incubator (Memmert IPP110eco) for 2-3 weeks to visualize both free-living and symbiont growth. After the selection of candidate bacterial isolates, most of which were free-living *Streptomyces* on Grace's insect media, they were streaked onto ISP-2 agar media to visualize and detect bacterial morphology or other potential signs of contamination.

2.4 gDNA extraction with Epicentre MasterPure™ DNA kit

The purpose of extracting genomic DNA (gDNA) from bacterial isolates obtained from each of the antennal gland reservoirs (A4 to A8) in female beewolf antennae was to investigate the bacterial composition of the antennae in female individuals. 250 µl of bacterial samples in Grace's insect media was transferred to 1.5 ml of reaction tube for gDNA extraction. 300 µl of Tissue and Cell Lysis Solution (Epicentre MTC096H) was added into each of 1.5 ml of reaction tubes and mixed gently. The reaction tubes were incubated at 37 °C for 30 min with 600 rpm in a thermomixer (Eppendorf ThermoMixer® C). After 30 min of incubation, 5 µl of ProK (10 mg/ml) was added. The samples were incubated in a thermomixer (Eppendorf

ThermoMixer® C) at 65 °C for 15 min one more time. After the completion of the second incubation, the samples were placed on ice for 5 min. Then, 150 µl of MPC Protein Precipitation Reagent (Epicentre MMP095H) was added to each sample and vortexed vigorously for 10 sec. The samples were centrifuged at 10000 rpm for 10 min using a microcentrifuge (Eppendorf Centrifuge 5417 R). After the centrifugation, the supernatants of each sample were transferred to other clean 1.5 ml of reaction tubes, and the pellet was discarded. 500 µl of isopropanol was added into each reaction tube filled with supernatant and the tubes were inverted 30-40 times to mix the solution. The samples were stored at -20 °C for 60 min and then centrifuged at 14000 rpm for 10 min using microcentrifuge (Eppendorf Centrifuge 5417 R). The supernatant was carefully removed without disrupting the DNA pellet and discarded from each sample. 200 µl of 70% ice-cold ethanol was added into each reaction tube and the samples were centrifuged at 14000 for 5 min. The supernatant was removed and discarded. To ensure the complete evaporation of ethanol, the samples were placed in a vacuum dryer for 3-4 min at 45 °C (Eppendorf Concentrator plus™). The samples were resuspended with 50 µl of Low TE buffer (10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA solution). At the end of the procedure, the samples were stored at -20 °C for the downstream applications. Moreover, DNA concentrations of the samples were measured with a Nanodrop (Implen- NanoPhotometer® N60)

2.5 PCR of 16 S rRNA

PCR was performed to amplify the target region, 16S rRNA, for bacterial characterization. To amplify the target region, two universal bacterial primers, fd1(5'-AGAGTTTGATCCTGGCTGAG-3') and rp2(5'-ACGGCTACCTTGTTACGACTT-3') were used. The protocol was performed according to the PCR kit (Quick-Start Protocol *Taq* PCR Master Mix Kit-QIAGEN). The reaction mix per sample was prepared by adding of 25 µl of *Taq* PCR Master Mix, 2x, 1.25 µl of each primer, and 21.5 µl of RNAse-free water. Depending on the volume of samples that will be used for PCR, the volume of the reaction mix was adjusted. After the preparation of the reaction mix, the solution was pipetted gently and 49 µl of reaction mix was dispensed into each PCR tube (Biozym). Pre-isolated template DNA was added to each PCR tube containing the reaction mix. PCR tubes were transferred from ice to the thermal cycler (Eppendorf-Mastercycler EP Gradient S) with the block preheated to 94 °C.

The thermocycling routine of PCR was programmed as: initial denaturation at 94°C for 3 min, denaturation at 94°C for 40 sec, primer annealing at 55 °C for 1 min, extension at 72 °C for 1 min, final extension at 72 °C for 10 min, and holding at 4 °C. Denaturation, primer annealing, and extension was repeated 35 times. During the preparation of the reaction mix and placement of samples to PCR tubes were carried out under the PCR hood (VWR-PCR Workstation Pro) by keeping all components on ice. After amplification, the DNA concentrations of amplified samples were measured with a Nanodrop (Implen-NanoPhotometer® N60) and samples were stored at -20 °C for downstream applications.

2.6 Gel electrophoresis and purification of amplicons

After the PCR of the target region, gel electrophoresis of amplicons was performed to confirm the amplification of the target DNA region. To prepare 1% agarose gel, 100 ml of 1X buffer was mixed with 1 g of agarose (SeaKem LE agarose) and 6 µl of DNA stain (SYBR™ Safe DNA Gel Stain-Thermo Scientific). The PCR samples were mixed 2:1 with loading dye (DNA gel loading dye (6x)-Thermo Scientific) and loaded into the wells of the gel. GeneRuler 1 kb DNA Ladder-Thermo Scientific was used as a marker. The gel was run at 80 V for 40 min with a power supply (BIO-RAD). When the run was over, the gel was placed into a UV transilluminator (Syngene- GeneGenius Bio Imaging System), and the amplified fragments were digitally documented. After visualizing the target DNA amplification, which is approximately 1.5 Kbp in gel electrophoresis, the samples were purified using a kit (DNA Clean & Concentrator-5- Zymo Research) to remove any other impurities within the sample following the manufacturer's protocol. At the final step of the protocol, the purified amplicons were eluted in 25 µl of elution buffer. The DNA concentrations of the samples were measured using Nanodrop, and they were stored at -20 °C for longer storage and sequencing.

2.7 Sequencing

The sequencing was performed bi-directionally using fd1(5'-AGAGTTTGATCCTGGCTGAG-3') and rp2(5'-ACGGCTACCTTGTTACGACTT-3'). 6 µl of a reaction mix for each sample was prepared to start by adding 35 ng of purified DNA. Additionally, 0.5 µl of each primer, fd1, and rp2, was added separately to two different 0.5 µl of reaction tubes containing the DNA. Each tube was then filled up to 6 µl with dH_2O. Sequencing was done in the Department of Insect Symbiosis at the Max Planck Institute for

Chemical Ecology (Jena, Germany). The overlapping fragments of 16S rRNA were assembled from the same isolate to obtain an almost full-length 16S. The results were later analyzed using the Geneious software version 2023.0.4 (Dotmatics) and Basic Local Alignment Search Tool (BLAST) by the National Center of Biotechnology Information (NCBI) to assign taxonomy to the isolates.

2.8 Antennae processing by fluorescence in situ hybridization (FISH)

The antenna samples of female beewolves preserved in 4% of PFA in PBS were used to carry out fluorescence in situ hybridization experiment to detect the symbiotic *Streptomyces philanthi* and general Actinobacteria in the antennae gland reservoirs. The fluorescence in situ hybridization was started by the dehydration and infiltration of the antennal tissues. Dehydration of the samples was performed in the Histology Service of MPICE by following the protocol (Weiss and Kaltenpoth 2016) using an automated tissue processor (LYNX II). The dehydrated samples were then processed for embedding, which will be utilized for preparing sample sections for FISH. The infiltration was performed by placing the samples into 2 ml reaction tubes filled with 200 ml of LR-White infiltration solution. The samples were shaken on a shaker at 400 rpm for 2 hours. This procedure was repeated three times by changing the previous solution. After the infiltration, a fresh LR-white polymerization solution was prepared by combining 995 µl of the LR-White infiltration solution with 5 µl of the accelerator. The samples containing only the infiltration solution were replaced with 200 µl of the freshly prepared polymerization mix, and then the samples were shaken at 400 rpm for 30 minutes. This procedure was repeated twice by removing the old solution with a freshly prepared polymerization mix. Embedding and polymerization step was conducted according to the protocol (Weiss and Kaltenpoth 2016) and blocks were stored at +4 °C until the preparation of sections. After the preparation of polymerized blocks, the antennal samples were cut to prepare sections with a thickness of 1 µm for the fluorescence in situ hybdrization using a microtome (Leica-HistoCore AUTOCUT R). The FISH protocol on semi-thin sections was performed following the protocol (Weiss and Kaltenpoth 2016) . The hybridization buffer was initially heated to approximately 50 °C. To prepare the hybridization mix, three probes, Cy3-SPT177 (CACCAACCATGCGATCGGTA), Cy5-Act-A19 (CCGTACTCCCCAGGCGGGG), and Eub338-Cy7 (GCTGCCTCCCGTAGGAGT) were used. FISH probes were diluted 1:10 with distilled water before preparing the hybridization mix. For one slide, 100 µl of the hybridization mix was prepared by combining 85 µl of

hybridization buffer, 5 µl of Cy3- SPT177, 5 µl of Cy5-Act-A19, 5 µl of Eub338-Cy7, and 1 µl of DAPI (4',6-diamidino-2-phenylindole). Then, 100 µl of the hybridization mix was applied to each slide, and the slides were covered with cover glass. The samples were placed with hybridization buffer in a moist reaction chamber at 50 °C overnight. After hybridization, the cover glass was removed from the slide, and the slides were washed with wash buffer for 20 minutes in a 50 °C FISH cabinet. Then, the samples were washed twice with distilled water for 20 minutes each at room temperature. Once the samples were thoroughly washed, the slides were removed from the cuvette and placed onto tissue paper. 30 µl of Vectashield was applied to a cover glass, and the slide was covered with it. The samples were then stored at 4 °C for long-term storage. For visualization of hybridized antennal samples, they were analyzed under an inverted fluorescence microscope (Leica) by using thunder imaging systems with software (LAS X Thunder). The samples were then stored at 4 °C for long-term storage.

3. Results

3.1 Cultivation of Isolates in Liquid and Solid Media

Antennae from 39 beewolves were collected, and the antennomeres were subsequently used for bacterial isolation, as detailed in Table 1. BR, BZ, and BRMK1 samples were collected from Berlin, while HS samples were collected from Halle, Germany. There are five antennomeres (A4 to A8) where specialized gland reservoirs are located, housing the symbiont of the beewolf (Goettler *et al*. 2007). We tried to isolate the bacteria that colonized within the antennomeres by cutting the segments separately.

After 2-3 weeks of growth of bacteria in Grace's insect medium (GCIM) liquid media, the samples were selected for downstream application mostly based on their morphological differences such as pigmentation, colony shape, or mycelium structure to compare/analyze the symbiont and free-living bacteria. The isolates obtained from female beewolves, specifically BR2-29, BR2-31, HS-03, HS-04, and BZ-03, were selected due to their distinctive morphology compared to other isolates from antennal samples. These selected isolates were cultured in GCIM liquid media for further molecular applications. In addition to selecting morphologically different-looking bacteria compared to symbiont, several isolates from female beewolves, BR2-13 and BR2-24, which showed similar morphology to *Streptomyces philanthi*, were also used for cultivation and sequencing, as shown in Table 1. In addition to BR2-13 and BR2-24, BR2-09, BR2-19, and BR2-25 also displayed similar morphology to *Streptomyces philanthi* in the GCIM liquid media. The symbiont showed filamentous morphology as shown in Figure 1. in GCIM liquid media.

When the isolates with symbiont-like morphotype were streaked into the ISP-2 media, there was no growth in the ISP-2 agar medium even after waiting more than 3-4 weeks. The main purpose of visualizing the isolates on a petri dish was to distinguish and detect non-symbiont isolates based on their morphological structures before conducting molecular experiments. When the growth of the isolates was observed in GCIM liquid media, they were streaked onto ISP-2 solid media to visualize the growth of morphologically distinct free-living bacteria and symbionts. The free-living bacteria showed relatively fast growth, with colonies being visible after 2-3 days of incubation at 28 °C. As shown in Figure 2., isolates of BZ-03 antennomeres showed morphological similarity to *Streptomyces* species, showing filamentous growth of the colonies. Generally, the isolates of BZ-03 showed tough and sometimes leathery physical structures with sporulated and pigmented colony morphology such as pinkish pigmentation in

BZ-03 A7 and BZ-03 $A7_{(2)}$, or spore formation in BZ-03 A6 and BZ-03 $A6_{(2)}$. In three isolates, BZ-03 $A8_{(3)}$, BZ-03 $A8_{(4)}$, and BZ-03 $A8_{(5)}$, different bacterial morphologies were observed in the petri plate. As demonstrated in Figure 3., BR2-31 isolates also showed reddish pigmentation and sporulation, especially in three isolates, BR2-31 A8, BR2-31 $A8_{(3)}$, and BR2-31 $A8_{(2)}$, respectively. For the other isolates of BR2 and HS, the samples showed filamentous and irregular morphology along with circular forms. Furthermore, the other isolates of HS-03, HS-04, and BR2-29 also showed relatively similar morphology of filamentous bacteria like BR2-31 and BZ-03 isolates with pigmentation and sporulation. BR2-24 and BR2-13 isolates also showed similar morphotypes to the symbiont in GCIM liquid media after 2-3 weeks of incubation.

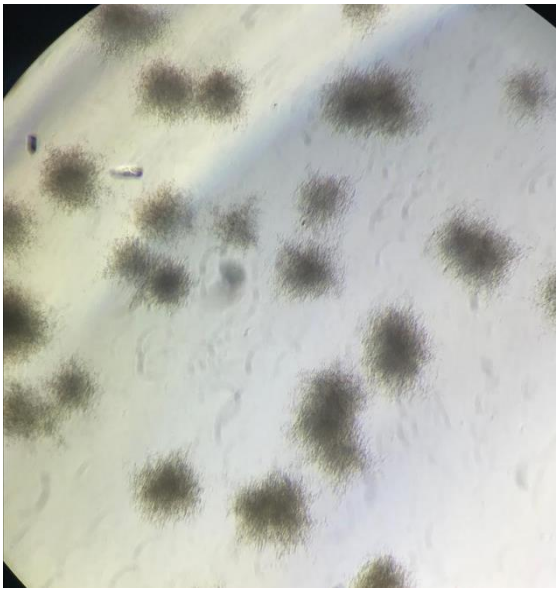

Figure 1. Microscopical image of filamentous bacteria sharing a similar morphology to the symbiont *Streptomyces philanthi* in a liquid Grace's insect media under an epifluorescence microscope at 10x magnification.

Table 1. Overview of collected European female beewolves and cultivated antennomeres, including sequencing success for cultivated segments (-, not cultivated and sequenced, +, cultivated and showed results for sequencing)

Female Beewolves	Growth on cultivation (Antennal Segments)	Sequencing
BR2-01	–	–
BR2-02	–	–
BR2-03	–	–
BR2-04	–	–
BR2-05	–	–
BR2-06	–	–
BR2-07	–	–
BR2-08	–	–
BR2-09	+	–
BR2-10	–	–
BR2-11	–	–
BR2-12	–	–
BR2-13	+ (A4, A5, A6, A7, A8)	+
BR2-14	–	–
BR2-15	–	–
BR2-16	–	–
BR2-17	–	–
BR2-18	–	–
BR2-19	+	–
BR2-20	–	–
BR2-21	–	–
BR2-22	–	–
BR2-23	–	–
BR2-24	+ (A4, A6, A8, A9)	+ (Except A4)
BR2-25	+	–
BR2-26	–	–
BR2-27	–	–
BR2-28	–	–

Table 1. (Continued)

Female Beewolves	Growth on cultivation (Antennal Segments)	Sequencing
BR2-29	+ (A12-8)	+
BR2-30	–	–
BR2-31	+ (A6, $A6_{(2)}$, A7, A8, $A8_{(2)}$,	+
HS01	$A8_{(3)}$)	–
HS02	–	–
HS03	+ (A12-8, A7, A5-4)	+
HS04	+ (A5, A7, $A7_{(2)}$, A8)	+
BZ01	–	–
BZ02	–	–
BZ03	+ (A4, A6, $A6_{(2)}$, A7, $A7_{(2)}$, A8, $A8_{(2)}$, $A8_{(3)}$, $A8_{(4)}$, $A8_{(5)}$	+ (Except A4, A8, $A8_{(2)}$, $A8_{(3)}$)
BRMK1	–	–

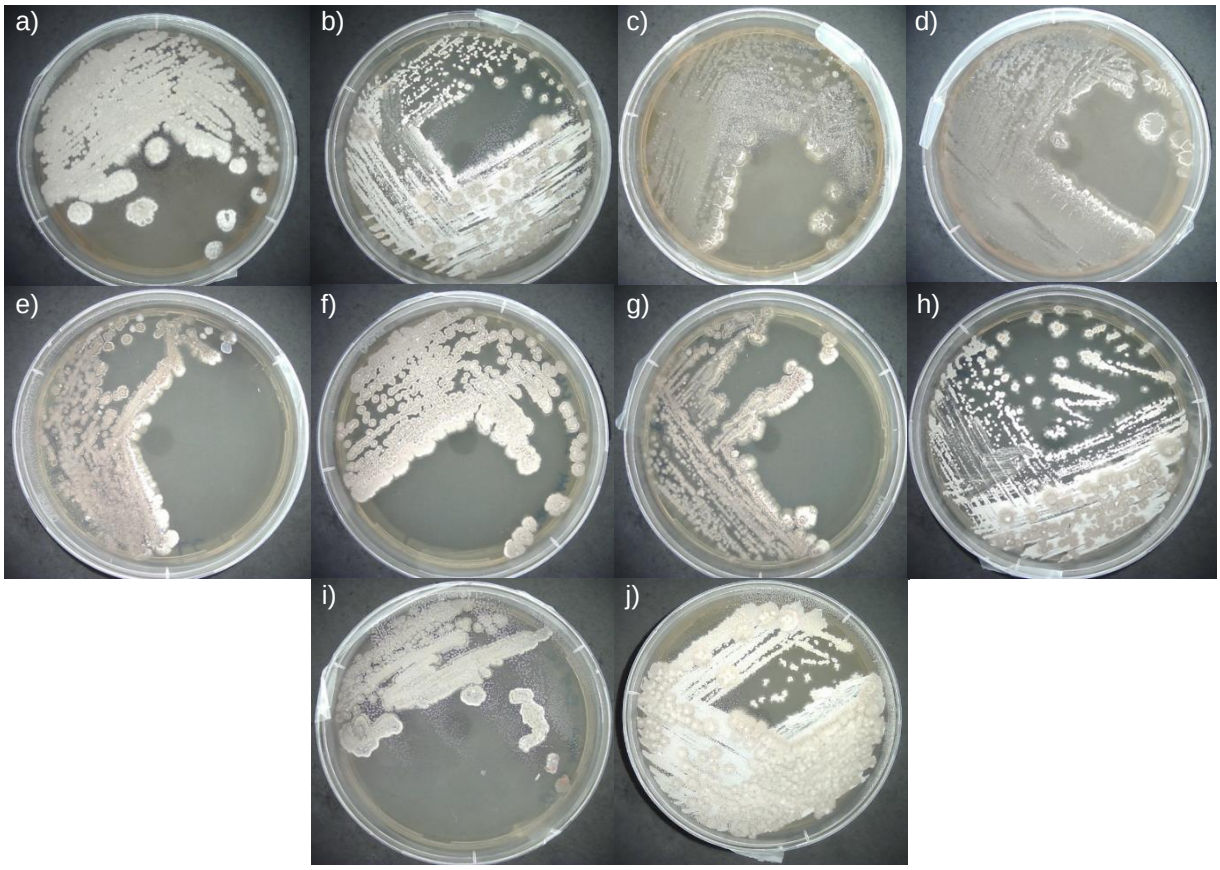

Figure 2. Bacterial isolates obtained from the antenna of female beewolf, BZ-03 **a)** BZ-03 A6 **b)** BZ-03 $A8_{(3)}$ **c)** BZ-03 A8 **d)** BZ-03 $A8_{(2)}$ **e)** BZ-03 A7 **f)** BZ-03 A4 **g)** BZ-03 $A7_{(2)}$ **h)** BZ-03 $A8_{(4)}$ **i)** BZ-03 $A6_{(2)}$ **j)** BZ-03 $A8_{(5)}$

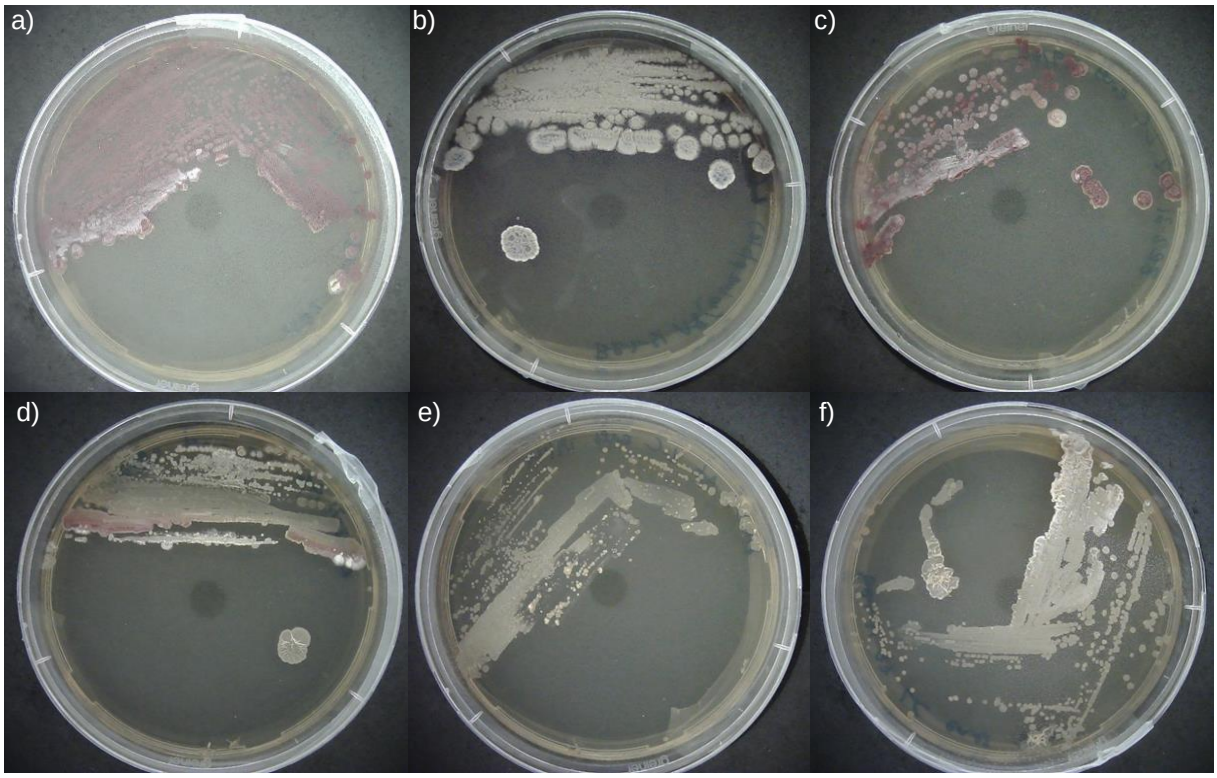

Figure 3. Bacterial isolates obtained from the antenna of female beewolf, BR2-31 a) BR2-31 A8 b) BR2-31 $A8_{(2)}$ c) BR2-31 $A8_{(3)}$ d) BR2-31 A6 e) BR2-31 $A6_{(2)}$ f) BR2-31 A7

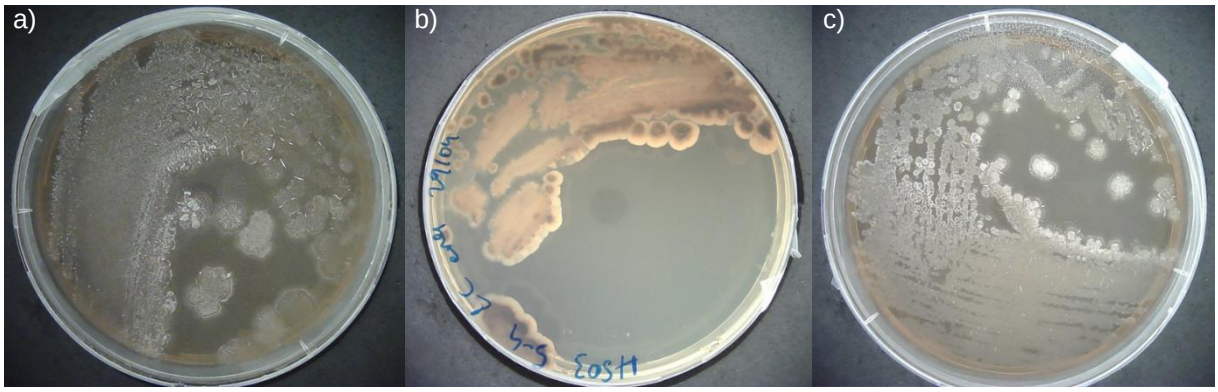

Figure 4. Bacterial isolates obtained from the antenna of female beewolf, HS-03 a) HS-03 12-8 b) HS-03 5-4 c) HS-03 A7

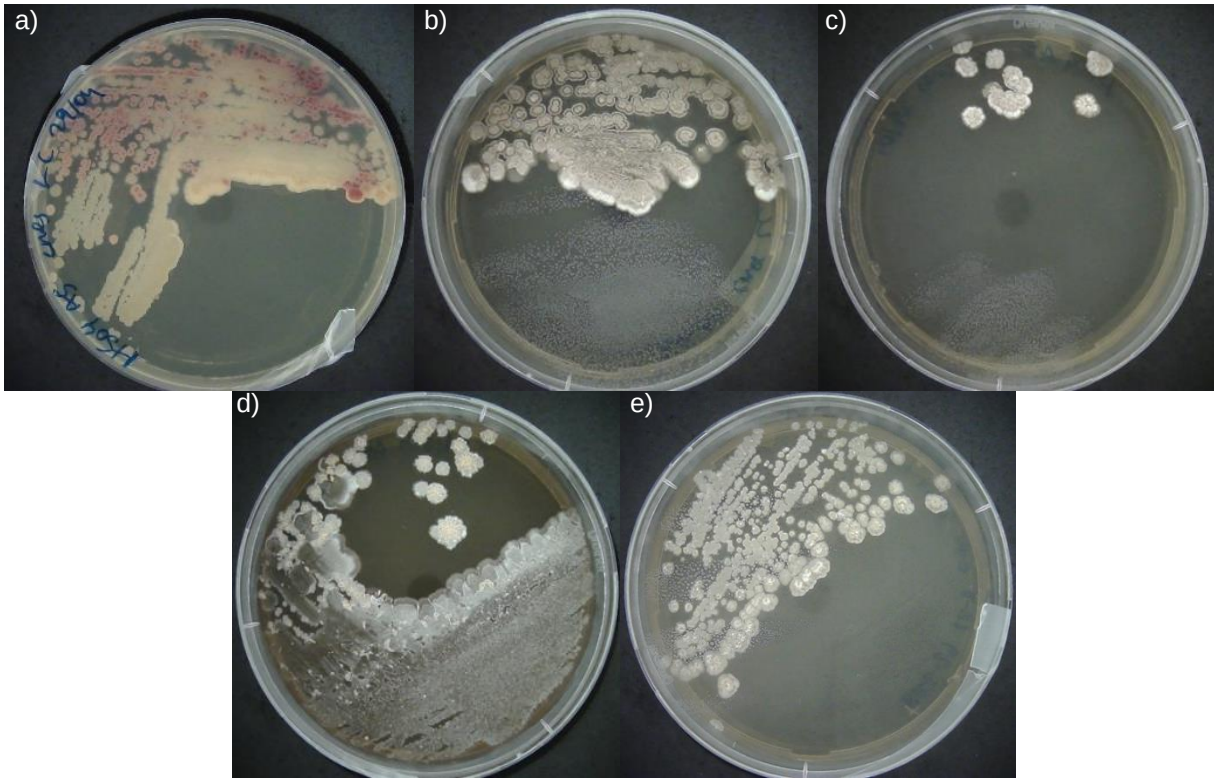

Figure 5. Bacterial isolates obtained from the antenna of female beewolf, HS-04 a) HS-04 A5 b) HS-04 A7 c) HS-07 $A7_{(2)}$ d) HS-04 A8 and BR2-29 e) BR2-29 12-8

3.2 Characterization of Isolates: DNA Extraction, PCR, and Sequencing

The bacterial isolates were investigated in both liquid and solid media, resulting in the selection of 33 isolates for molecular downstream applications. gDNA extractions of 33 samples were successfully carried out by using the Epicentre MasterPure™ DNA kit. The samples were successfully purified using the DNA Clean & Concentrator-5 kit from Zymo Research. Quantification and quality of DNA extracts of isolates, both prior and post-purification, were measured with Nanodrop. However, despite a high amount of amplicons being generated during PCR (approximately 300-400 ng/µl of DNA with remaining nucleotides and primers) for 33 samples, the samples exhibited a relatively low amount of purified amplicons after utilizing the purification kit, which was expected. The required volumes of purified amplicons were calculated to prepare the solution mix for sequencing. Figure 6 illustrates the sizes of amplified amplicons observed in agarose gel electrophoresis.

All samples, except BR2-31 $A8_{(2)}$, exhibited strong amplification of 16S rRNA. According to the sequencing results of the 33 samples by BLAST, 5 samples, BZ-03 $A8_{(3)}$, BZ-03 A4, BZ-03 $A8_{(4)}$, BR2-24 A4, BZ-03 $A8_{(5)}$ did not yield any data for confident taxonomic assignments by BLAST due to their short sequences. The sequence lengths of these five samples varied between 5 to 116, preventing further analysis.

The sequences of the isolates matched a complementary part of the 16S rRNA region of the culture collection strains and mainly resulted in various *Streptomyces* spp. such as *Streptomyces* sp., *Streptomyces venezuela*, *Streptomyces ardesiacus*, *Streptomyces tanashiensis*, *Streptomyces coelicolor*, *Streptomyces tendae*, and *Streptomyces rubrogriseus* for free-living strains as illustrated in Table 2. The majority of the samples approximately share 650-700 base pair length, which is less than the length of 16S rRNA. The four samples (BR2-13 A8 (11), BR2-13 A7 (12), BR2-24 A7 (25), HS-03 5-4 (33)) in which the sequences were assembled using both forward and reverse primers showed longer sequence lengths with 100% and 99.8% identity.

As expected from solid media observations for BR2-13 and BR2-24 isolates except BR2-13 A7 (12), BR2-13 A5 (8), BR2-13 A8 (11), BR2-24 A7 (25), BR2-24 A6 (27), BR2-13 A4 (28), BR2-13 A4 (29), and BR2-24 A8 (30) resulted in *Candidatus* Streptomyces philanthi. Interestingly, BR2-13 A7 (12) scored 99.8% identity with 1331 sequence length, indicating potential isolation of *Actinomycete Saccharothrix hoggarensis* sp., from the antennal gland.

Isolates matching a region of their 16S rRNAs to *Streptomyces venezuelae*, *Streptomyces ardesiacus*, *Streptomyces coelicolor*, *Streptomyces tendae*, and *Streptomyces tanashiensis*

were detected from various isolates of BZ-03 with 100% match scores and approximately 600-700 bp in length. The high sequence identity and low e-value of top hits of those isolates suggest a strong homology, indicating confidence in assigning those isolates to a particular species.

Notably, some of the isolates of BR2-31 (BR2-31 A6, BR2-31 $A6_{(2)}$, BR2-31 $A8_{(2)}$, BR2-31 A7) macthed to *Streptomyces* sp., while others (BR2-31 A8 BR2-31 $A8_{(3)}$) matched to *Streptomyces ardesiacus*. On the other hand, isolates obtained from the antenna of a female beewolf, HS-03, also showed homology to the reference strains, *Streptomyces tanashiensis* for two isolates and *Streptomyces rubrogriseus* for HS-03 5-4 (33), having a strong potential for characterization with 100% match score and 1237 bp in length. In HS-04 isolates, HS-04 A5 (14) comparatively resulted in low sequence length, which would not be enough to characterize the isolate, while HS-04 A8 (22) displayed homology to *Streptomyces venezuelae* strain with 100% identity with relatively acceptable length, 438 bp. Intriguingly, two other isolates of HS-04 were characterized with *Mesobacillus* sp. strain by the software analysis.

Table 2. BLAST results of 16S rRNA sequences of the isolates from antennae of *Philanthus triangulum*

Isolates	BLAST hit (16S rRNA partial sequence)	E-value	Identity	Query Covarage	Sequence Length
1- BR2-31 A6	*Streptomyces* sp. strain RFL*#10	0	100%	100%	650
2- BZ-03 A8	*Streptomyces venezuelae* strain ATCC 10595	0	100%	100%	610
3- BZ-03 A8(3)	No data	-	-	-	-
4- BZ-03 A7	*Streptomyces ardesiacus* strain YTAI3-1	0	100%	100%	651
5- BR2-31 A6(2)	*Streptomyces* sp. strain RFL*#10	6.93. e^{-92}	100%	100%	189
6- BR2-31 A8	*Streptomyces ardesiacus* strain YTAI3-1	0	100%	100%	643
7- HS-04 A7	*Mesobacillus* sp. strain 81E08	0	100%	100%	689
8- BR2-13 A5	*Candidatus* Streptomyces philanthi biovar triangulum	0	100%	100%	651
9- HS-03 12-8	*Streptomyces tanashiensis* strain CEMTC_5169	0	100%	100%	705
10- HS-03 A7	*Streptomyces tanashiensis* strain CEMTC_5169	0	100%	100%	692
11- BR2-13 A8	*Candidatus* Streptomyces philanthi biovar triangulum	0	100%	100%	1338

Table 2 (Continued)

Isolates	BLAST hit (16S rRNA partial sequence)	E-value	Identity	Query Covarage	Sequence Length
12- BR2-13 A7	*Saccharothrix hoggarensis* strain SA181	0	99.8%	100%	1331
13-HS-04 A7(2)	*Mesobacillus* sp. strain 81E08	0	100%	100%	601
14- HS-04 A5	*Streptomyces coelicolor* A3(2) strain CFB_NBC_0001	9.63. e^{-37}	100%	100%	89
15- BZ-03 A4	No data	-	-	-	-
16- BZ-03 A6	*Streptomyces coelicolor* JCM 4020	0	100%	100%	650
17- BZ-03 A7(2)	*Streptomyces ardesiacus* strain YTAI3-1	0	100%	100%	706
18- BR2-31 A8(2)	*Streptomyces* sp. CB09001	0	100%	100%	626
19- BR2-29 12-8	*Streptomyces* sp. strain XG184	0	100%	100%	671
20- BR2-31 A8(3)	*Streptomyces ardesiacus* strain YTAI3-1	0	100%	100%	643
21- HS-04 A8	*Streptomyces venezuelae* strain ATCC 10595	0	100%	100%	438
22- BR2-31 A7	*Streptomyces* sp. strain RFL*#10	5.07. e^{-114}	100%	100%	229
23- BZ-03 A6(2)	*Streptomyces tendae* strain LI03	1.83. e^{-154}	100%	100%	302

Table 2 (Continued)

Isolates	BLAST hit (16S rRNA partial sequence)	E-value	Identity	Query Covarage	Sequence Length
24- BZ-03 A8(3)	*Streptomyces tanashiensis* strain CEMTC_5169	0	100%	100%	649
25- BR2-24 A7	*Candidatus* Streptomyces philanthi biovar triangulum	0	100%	100%	1339
26- BZ-03 A8(4)	No Data	-	-	-	-
27- BR2-24 A6	*Candidatus* Streptomyces philanthi biovar triangulum	0	100%	100%	483
28- BR2-13 A4	*Streptomyces* sp. strain Je 1-426	0	100%	100%	672
29- BR2-13 A6	*Candidatus* Streptomyces philanthi biovar triangulum	6.75. e^{-118}	100%	100%	236
30- BR2-24 A8	*Candidatus* Streptomyces philanthi biovar triangulum	6.75. e^{-118}	100%	100%	236
31- BR2-24 A4	No data	-	-	-	-
32- BZ-03 A8(5)	No data	-	-	-	-
33- HS-03 5-4	*Streptomyces rubrogriseus* strain GPB8	0	100%	100%	1237

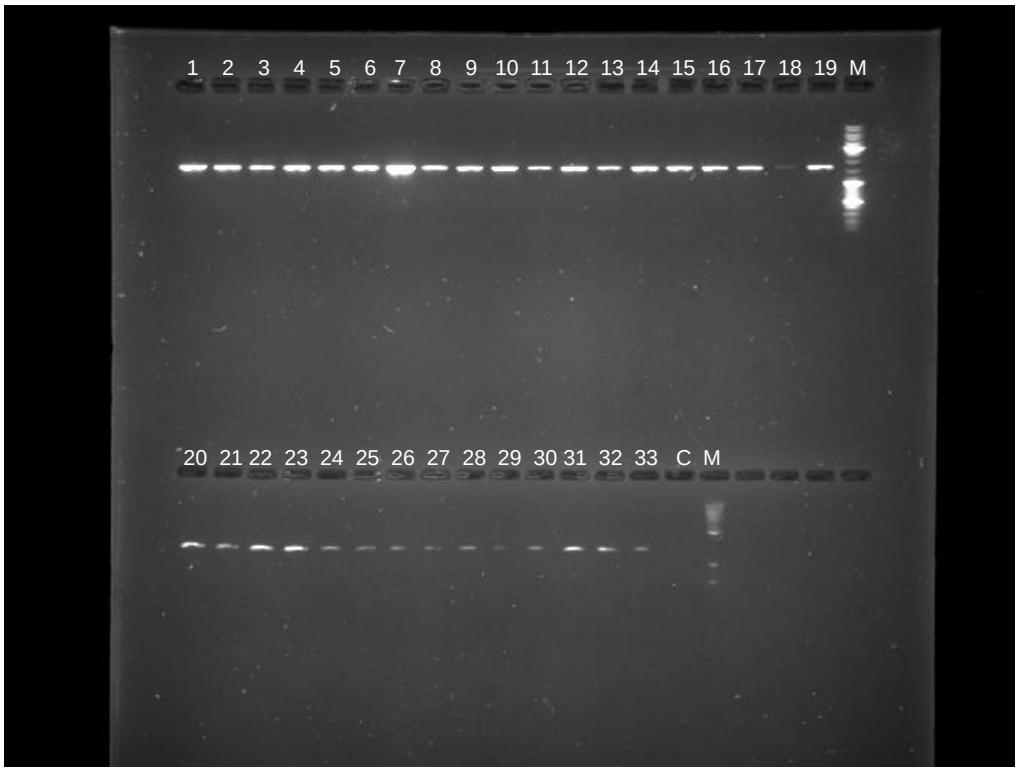

Figure 6. Gel electrophoresis result of 16S rRNA amplicons of 33 bacterial isolates after PCR (C: negative control M: marker)

3.3 Visualization of bacteria in beewolf antennal glands fluorescence in situ hybridization (FISH)

In antenna processing for FISH, 16 antennae from various female beewolves, 14 from *Philanthus triangulum*, and 2 from *Philanthus ventilabris*. Longitudinal semi-thin sections of antennae were prepared and used for FISH with three different probes to analyze the presence of the bacteria in gland reservoirs.

Out of 16 antennae from female beewolves, 14 samples showed positive results for the presence of bacteria in the gland reservoirs using FISH. Among the 16 antennae, which included 80 gland reservoirs expected to be filled with bacteria, 64 of the antennal gland reservoirs showed bacterial presence (80%). Signals created by all three probes or at least the two specific probes in FISH detected bacterial content in 11 out of 16 antennae. Moreover, two antennae showed no signals, while three antennal samples exhibited faint FISH signals with only one of the probes.

In BZ-03, the FISH signals were detected in each of the reservoirs except A4. Additionally, the signal detection for A6 was relatively low compared to other antennomeres. Figure 7 demonstrates that bacteria in the gland reservoirs were detected by each probe (Cy3-SPT177, Cy5-Act-A19, and Eub338-Cy7) at the same density. Additionally, gland cells were marked with DAPI, resulting in a blue color across the antenna. In the case of BR2-29, the reservoirs exhibited strong FISH signaling, suggesting that the bacteria in these antennal reservoirs were targeted by all of the probes as illustrated in Figure 8. In the results of BR2-31 and BR2-19 as seen in Figure 9. and Figure 10., the whole structure of the antennomeres and gland reservoirs were successfully observed, showing strong signal detection by each of the probes from A4 to A8 except Eub338-Cy7 in BR2-31. Moreover, some of the antennomeres in those samples were torn, likely during the process of sectioning the antennae.

The beewolves collected from Halle, HS-03, and HS-04 were also effectively processed, displaying that the bacteria within the gland reservoirs were intensely targeted by all the probes. However, due to the cutting issues in sectioning procedures, some parts of the antennomeres from A9 to A12 were not observed for both of these samples. As illustrated in Figure 17, although the gland reservoirs and the entire structure of antennomeres of BR2-25 were clearly observed under the fluorescence microscope, Eub338-Cy7 signals for eubacteria were not detected, whereas the signals of Cy3-SPT177 and Cy5-Act-A19 probes were visualized from A4 to A8, except for the 7th antennomere, in which no signal was detected. Furthermore, the quality of signal detection from the probes was not as bright as expected for

BR2-25. Another antenna of a female beewolf was utilized for FISH to investigate the bacterial accumulation in antennomeres, showing the perfect distribution of bacteria along the antenna. In Figure 19, BR2-14 showed the gland reservoirs filled with bacteria, targeted by Cy3-SPT177 and Cy5-Act-A19 probes brightly. The Eub338-Cy7 probe also did not show any signals for BR2-14. Intriguingly, other beewolves collected from Berlin, BR2-04, and BR2-05, were also processed for FISH, revealing empty gland reservoirs. Consequently, bacteria could not be targeted by the probes, as seen in Figure 18 and Figure 22, respectively.

In addition to the samples from Berlin and Halle, four more antennae of European beewolves collected from Hamburg were also investigated by FISH. In general, except H2 sample as seen in Figure 14., the other samples from Hamburg could not be targeted by Eub338-Cy7 probe. In Figure 13., H1 showed relatively bright signals for Cy3-SPT177 and Cy5-Act-A19, however, the structures of antennomeres could not be visualized as they should be. In Figure 14, the H2 sample exhibited strong signals for each of the probes in the A4, A5, and A6 antennomeres. The H3 sample also displayed that bacteria from A4 to A6 antennomeres are targeted with Cy3-SPT177 and Cy5-Act-A19 probes. However, the Cy3-SPT177 signals are more powerful than Cy5-Act-A19. In contrast to H3, the bacteria in the antennomeres of H4 were targeted intensely by Cy5-Act-A19 instead of Cy3-SPT177. Furthermore, only four antennomeres were visible and filled with bacteria in the H4 sample as shown in Figure 16.

To have more data about the bacterial existence in the antennae of beewolves, the antennae of two *Philanthus ventilabris* individuals were investigated by FISH as seen in Figure 20. and Figure 21. The bacterial content within the antennomeres from A4 to A8 was strongly targeted by Cy3-SPT177 and Cy5-Act-A19 probes, but not with Eub338-Cy7. Moreover, UT-E54 showed a lack of some antennomeres compared to UT-E61, indicating these parts got lost or missed during the sectioning procedure. In summary, all antennae samples including from European and US-based beewolves showed the signal detection for Cy3-SPT177 and Cy5-Act-A19, indicating the presence of the symbiont, *Streptomyces philanthi*, which belongs to the *Actinomycetes*. Interestingly, in some samples, Eub338-Cy7 signals were not detected, but the bacteria were targeted by other probes, Cy3-SPT177 and Cy5-Act-A19.

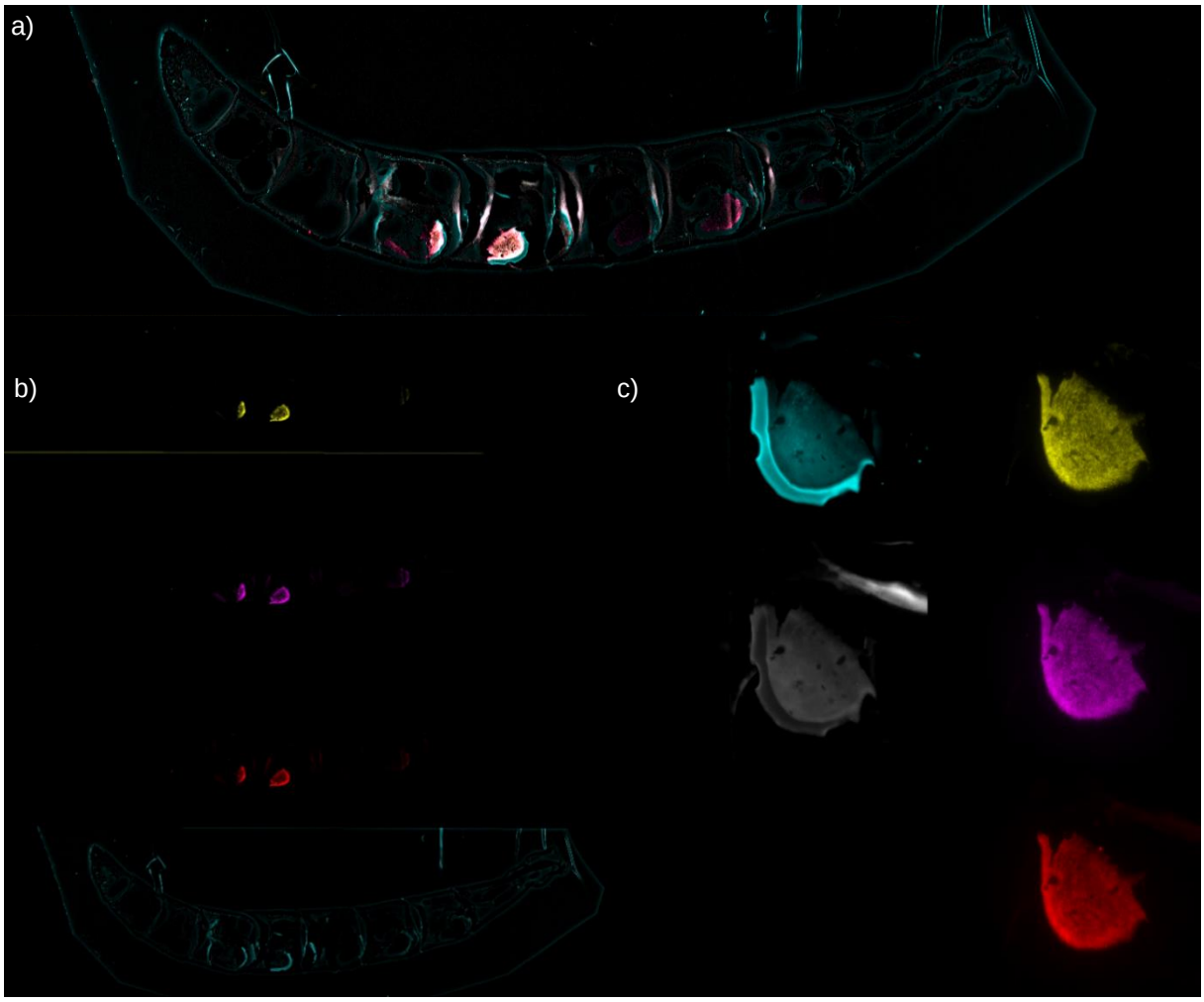

Figure 7. Whole structure of female beewolf antenna of BZ-03, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Yellow: Cy3- SPT177 Magenta: Cy5-Act-A19 Red: Eub338-Cy7 Blue: DAPI) a) Merged image of antenna targeted by all probes including DAPI b) Overview labeled gland reservoirs c) Bacteria in gland reservoir labeled by each probe with DAPI

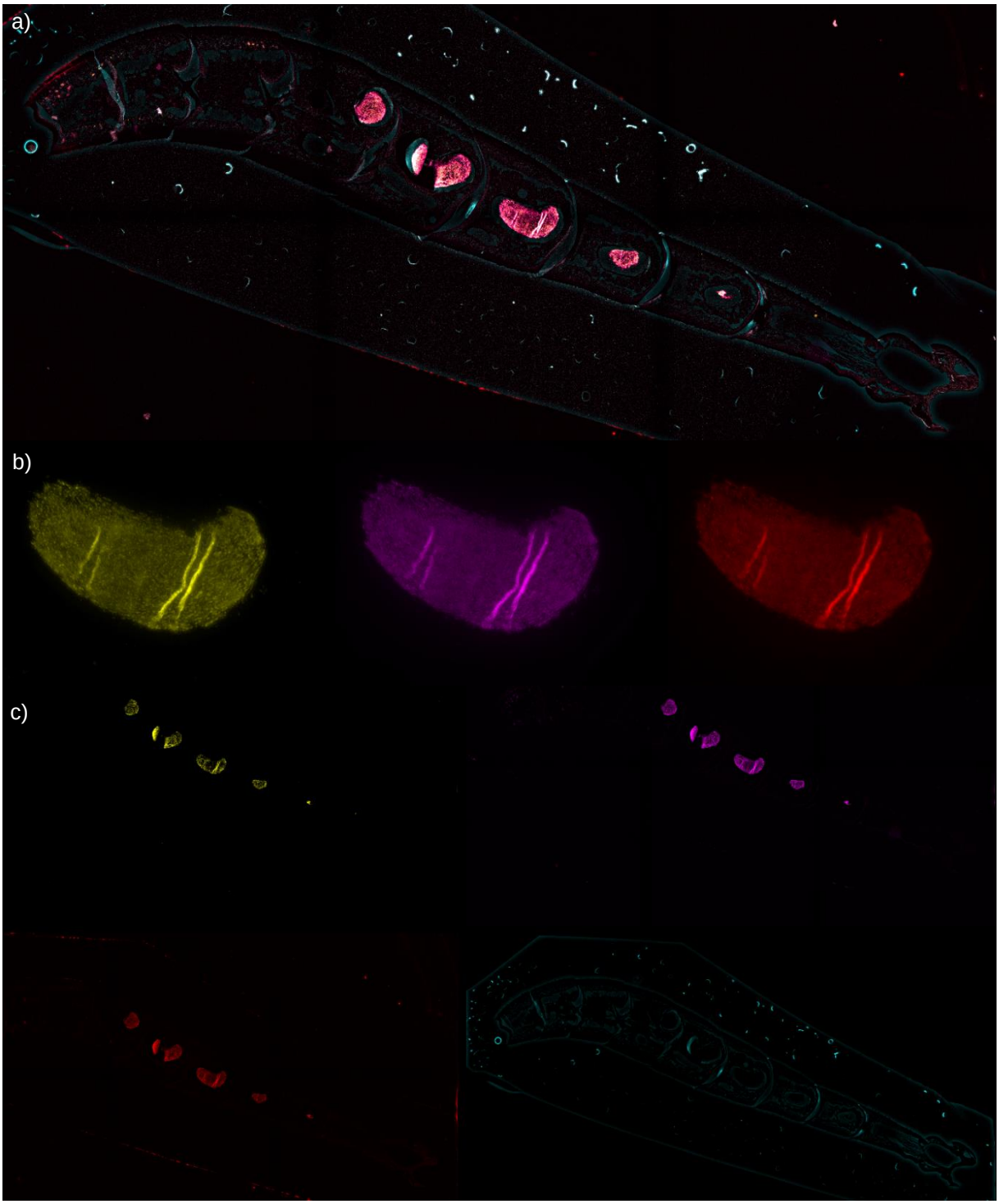

Figure 8. Whole structure of female beewolf antenna of BR2-29, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Yellow: Cy3- SPT177 Magenta: Cy5-Act-A19 Red: Eub338-Cy7 Blue: DAPI) a) Merged image of antenna targeted by all probes including DAPI b) Bacteria in gland reservoir labeled by each probe with DAPI c) Overview labeled gland reservoirs

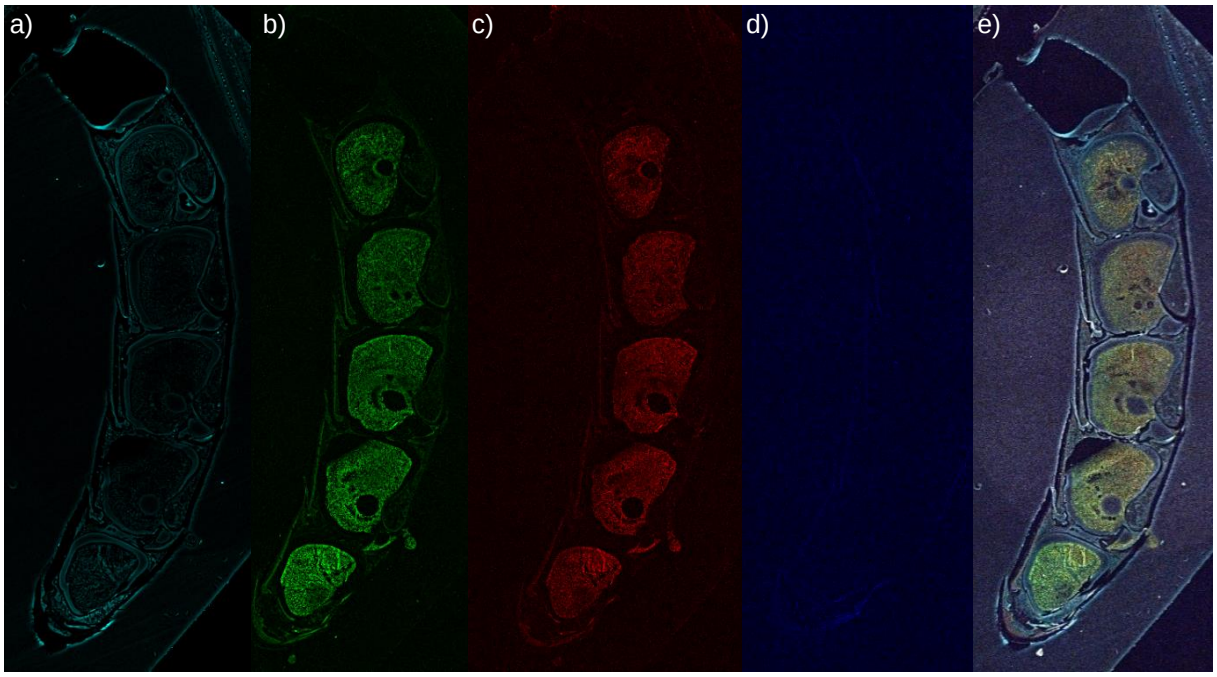

Figure 9. Whole structure of female beewolf antenna of BR2-31, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Bacteria in gland reservoirs labeled by Eub338-Cy7 e) Merged image of antenna targeted by all probes including DAPI

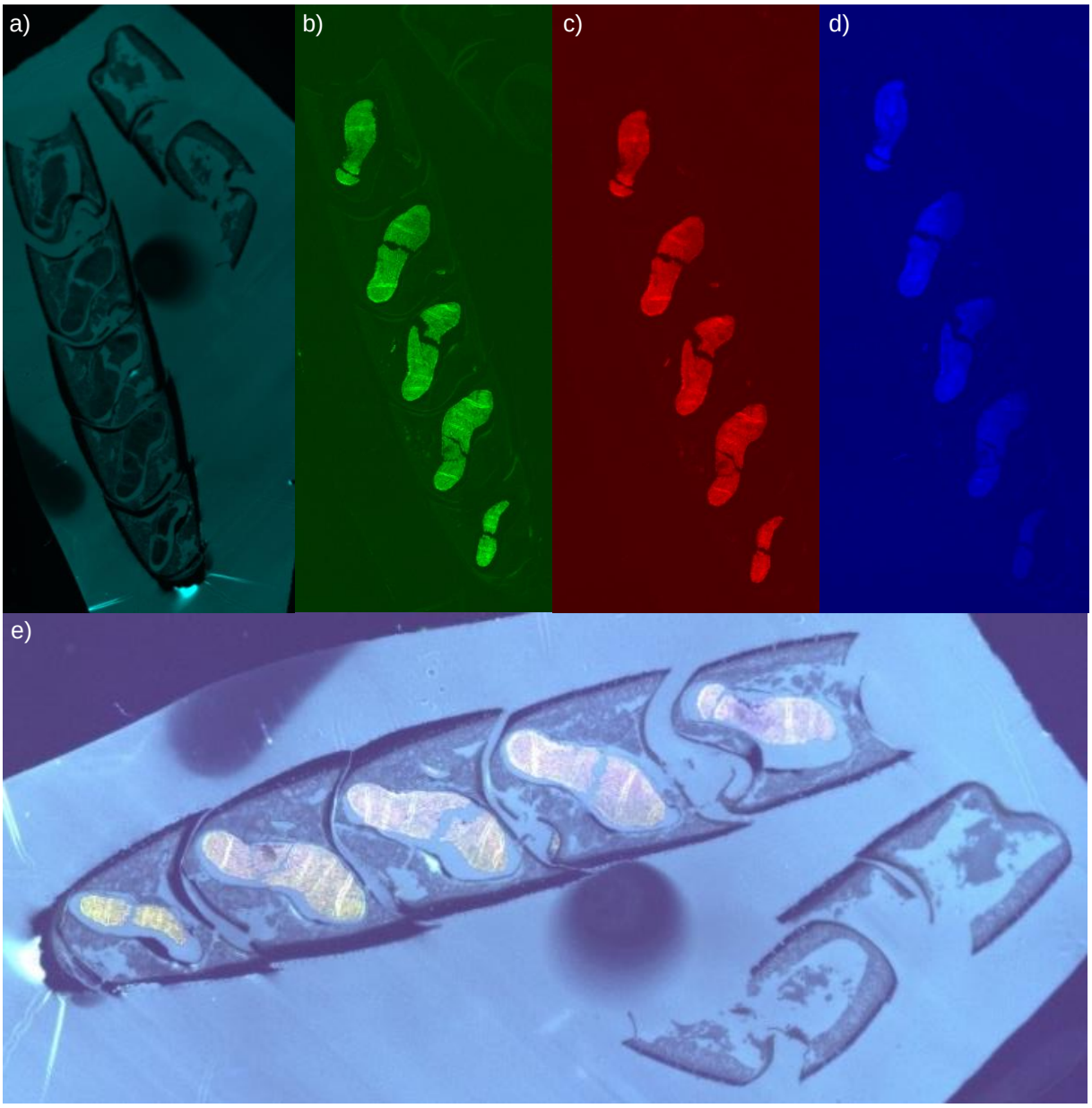

Figure 10. Whole structure of female beewolf antenna of BR2-19, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Bacteria in gland reservoirs labeled by Eub338-Cy7 e) Merged image of antenna targeted by all probes including DAPI

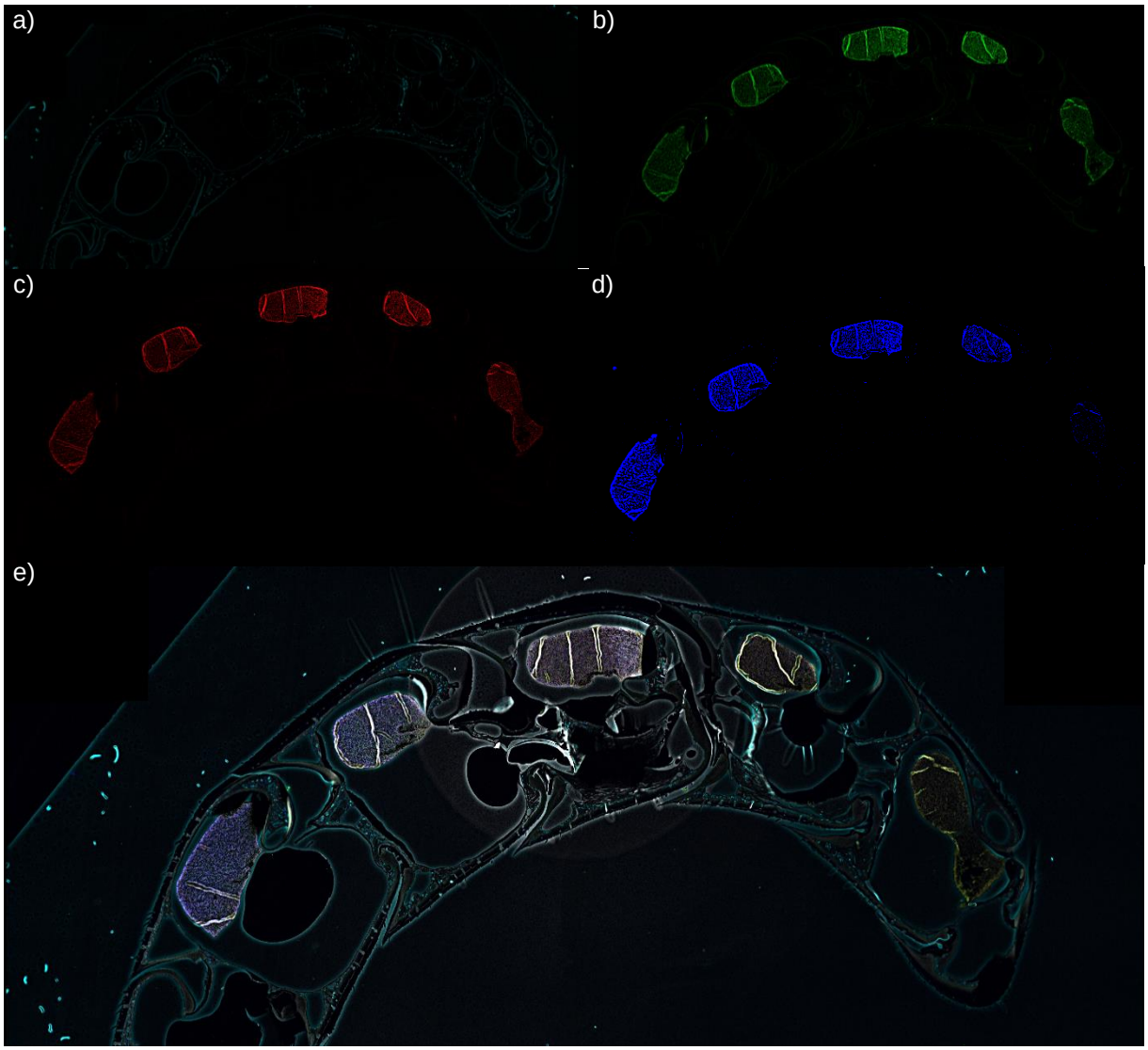

Figure 11. Whole structure of female beewolf antenna of HS-03, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Bacteria in gland reservoirs labeled by Eub338-Cy7 e) Merged image of antenna targeted by all probes including DAPI

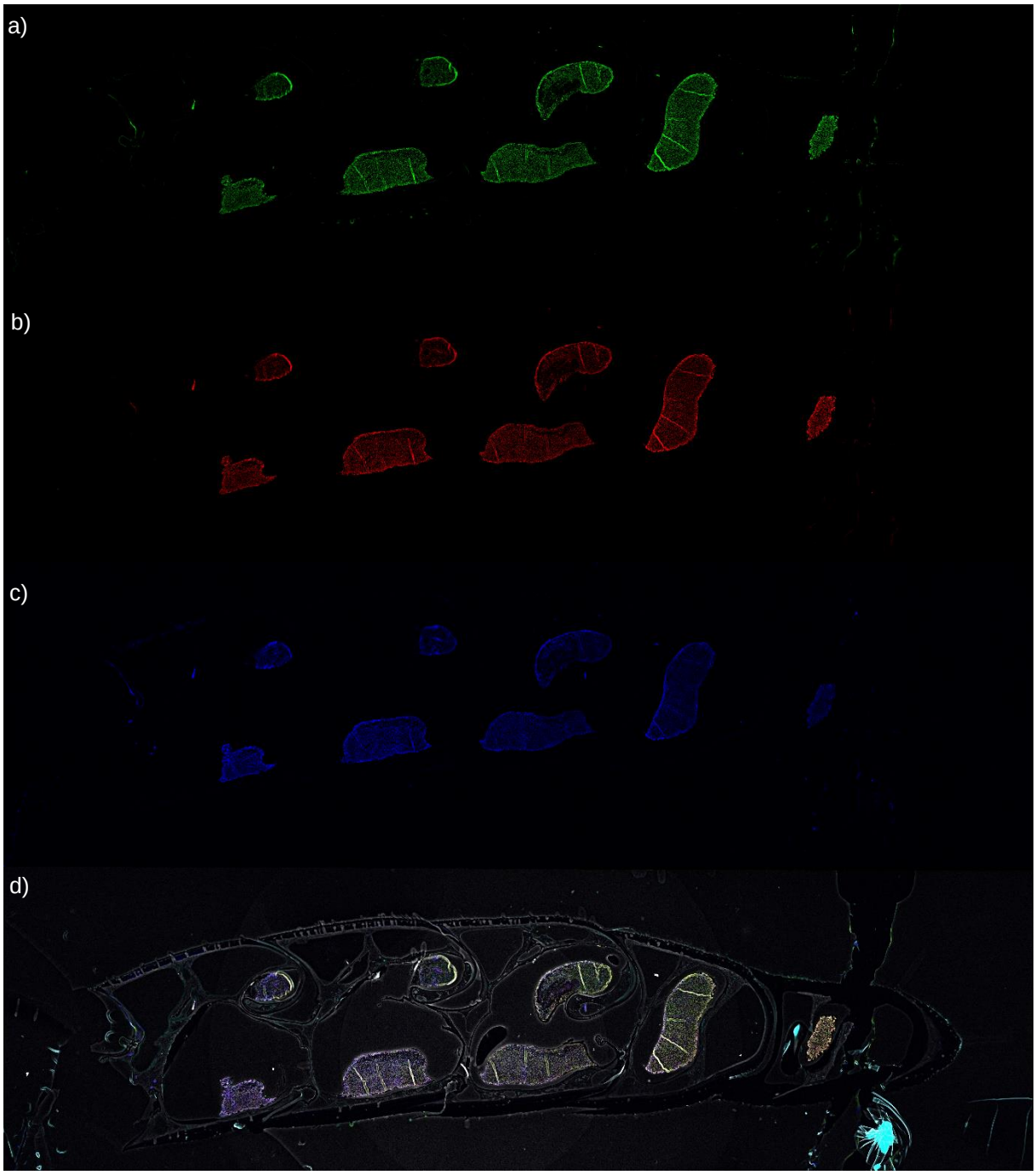

Figure 12. Whole structure of female beewolf antenna of HS-04, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Bacteria in gland reservoirs labeled by Cy3- SPT177 b) Bacteria in gland reservoirs labeled by Cy5-Act-A19 c) Bacteria in gland reservoirs labeled by Eub338-Cy7 d) Merged image of antenna targeted by all probes including DAPI

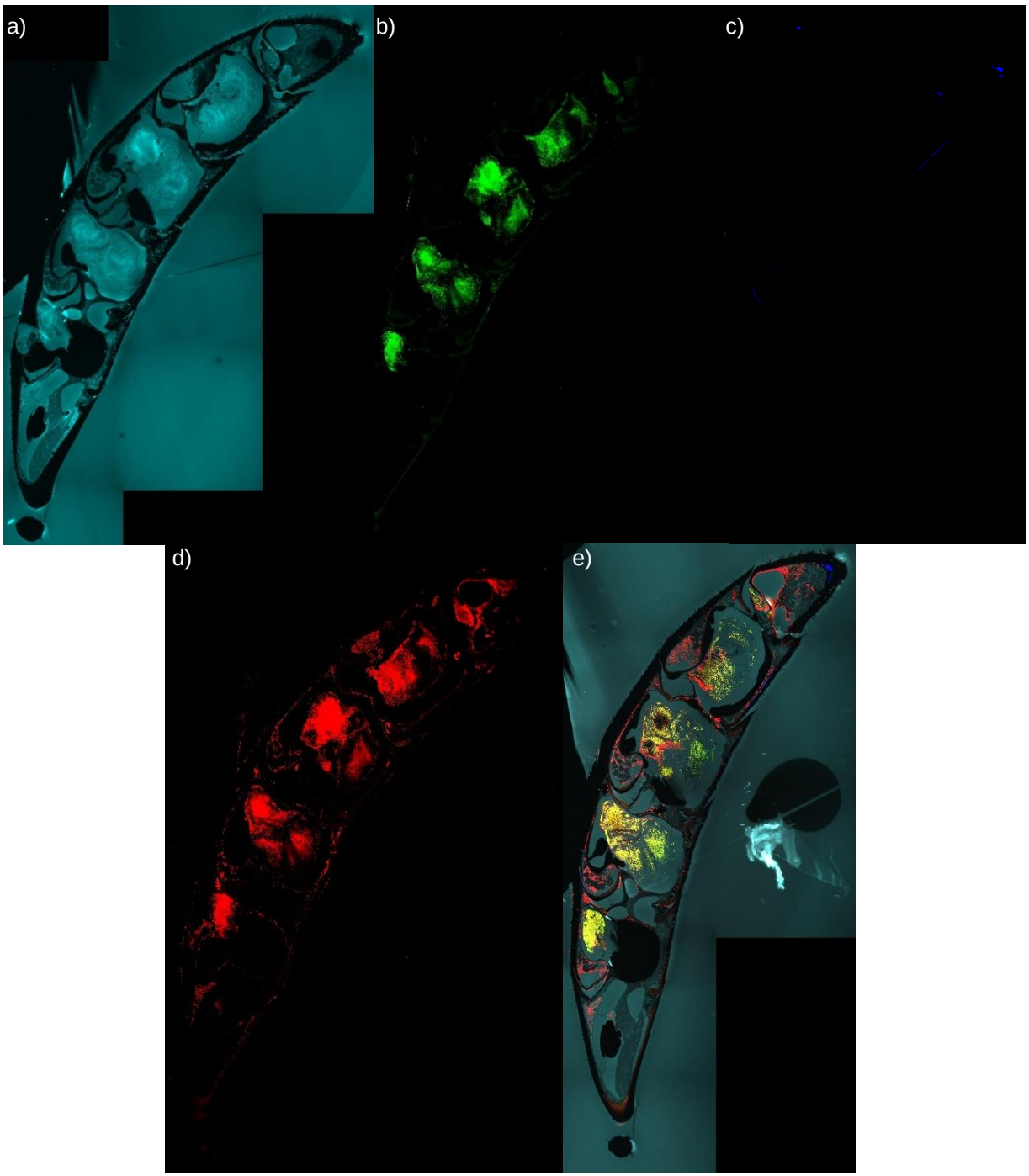

Figure 13. Whole structure of female beewolf antenna of H1, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Eub338-Cy7 d) Bacteria in gland reservoirs labeled by Cy5-Act-A19 e) Merged image of antenna targeted by all probes including DAPI

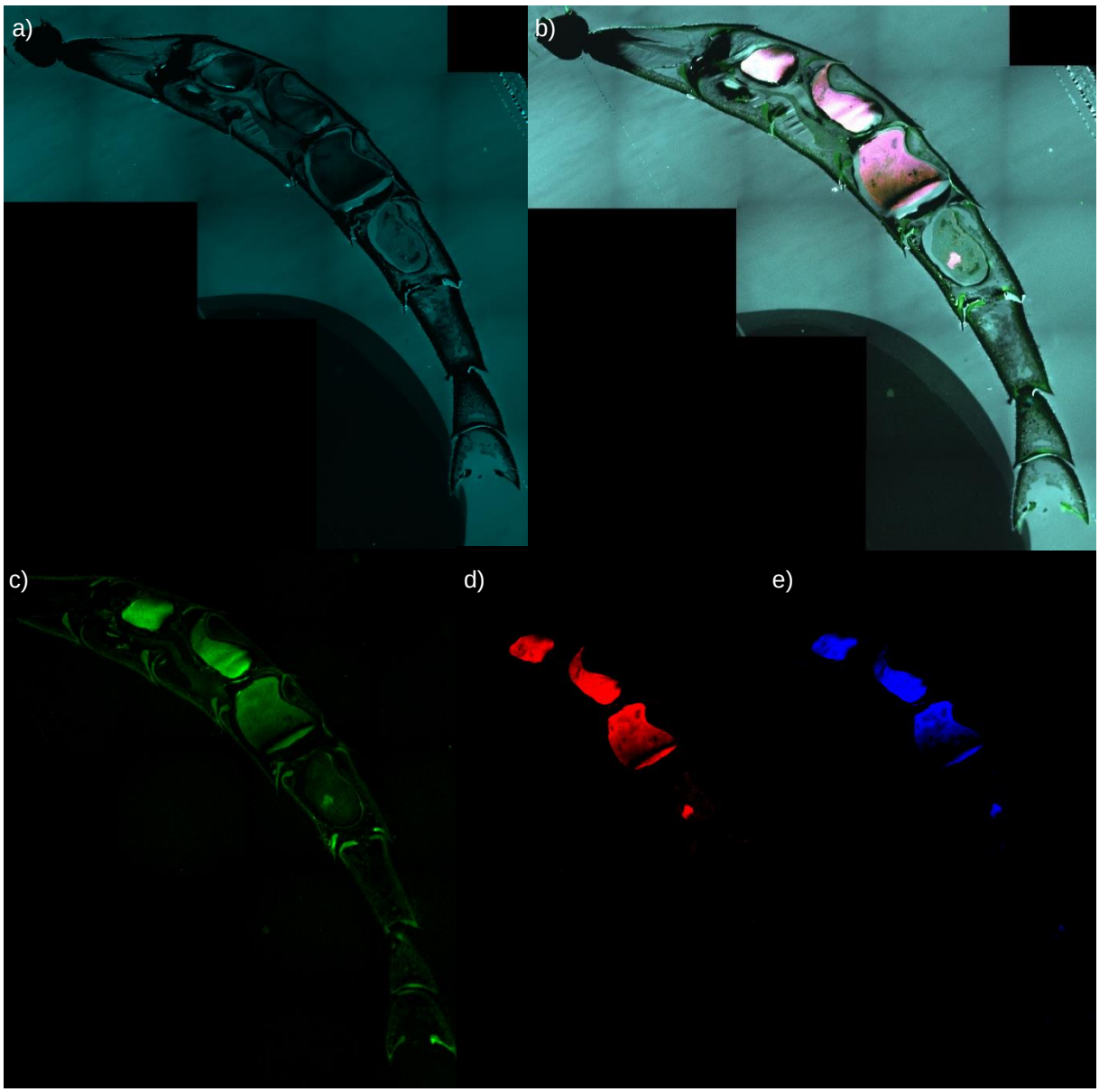

Figure 14. Whole structure of female beewolf antenna of H2, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Merged image of antenna targeted by all probes including DAPI c) Bacteria in gland reservoirs labeled by Cy3- SPT177 d) Bacteria in gland reservoirs labeled by Cy5-Act-A19 e) Bacteria in gland reservoirs labeled by Eub338-Cy7

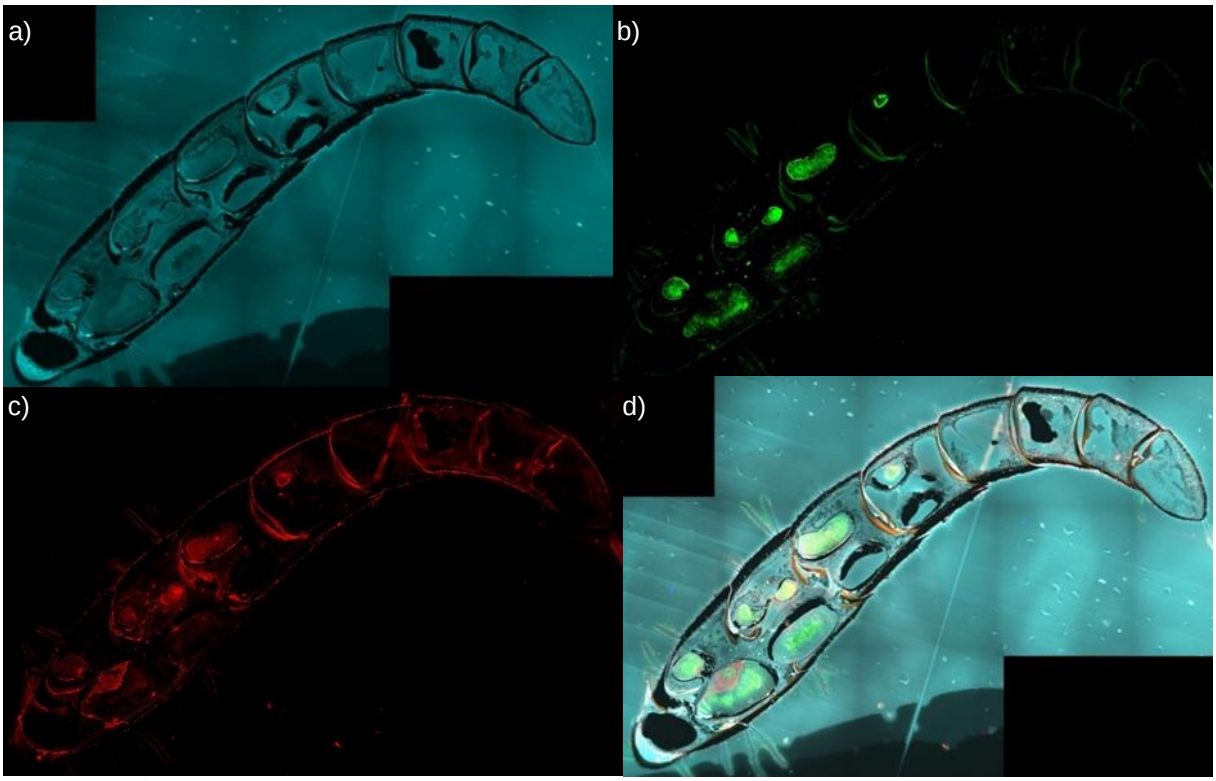

Figure 15. Whole structure of female beewolf antenna of H3, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Merged image of antenna targeted by all probes including DAPI

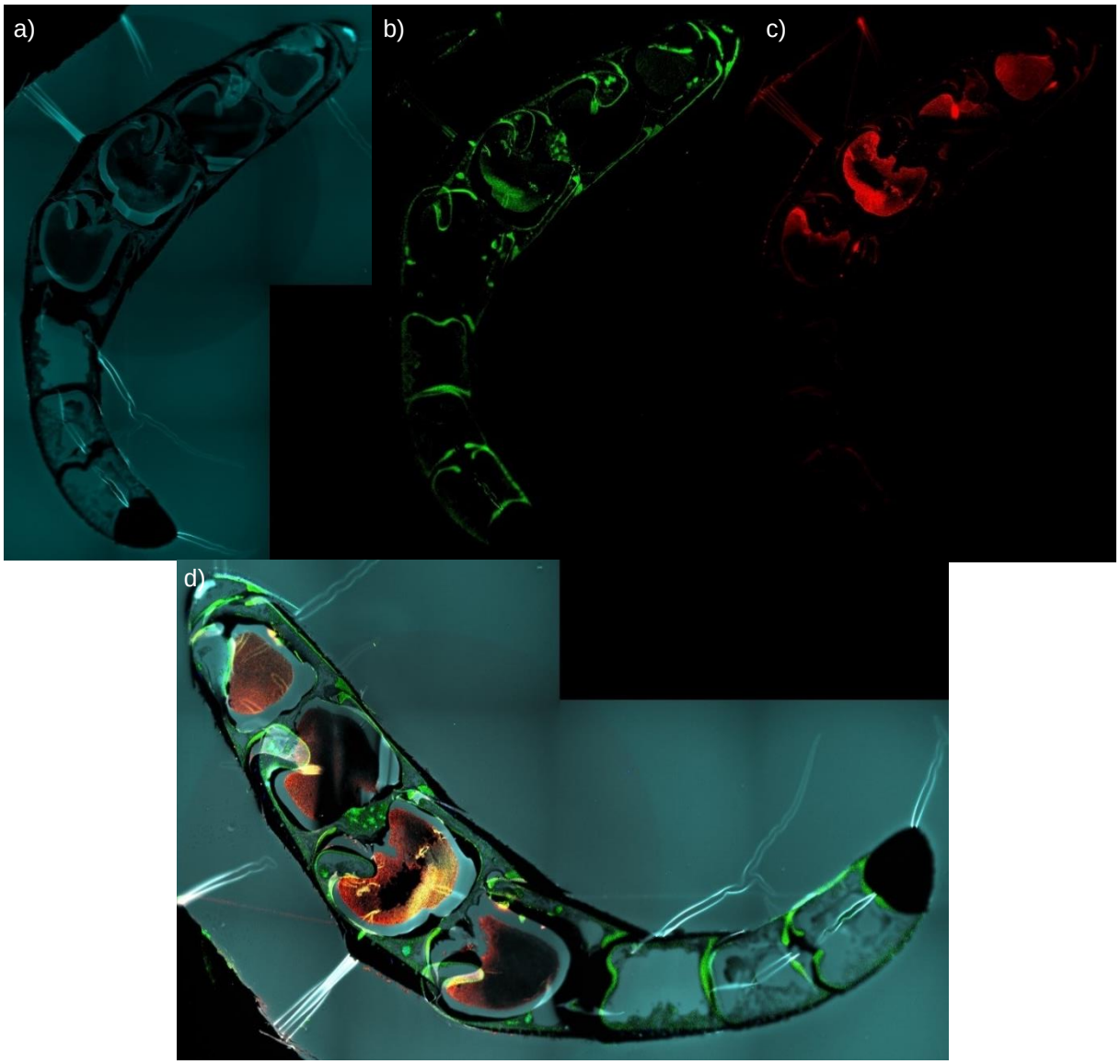

Figure 16. Whole structure of female beewolf antenna of H4, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Merged image of antenna targeted by all probes including DAPI

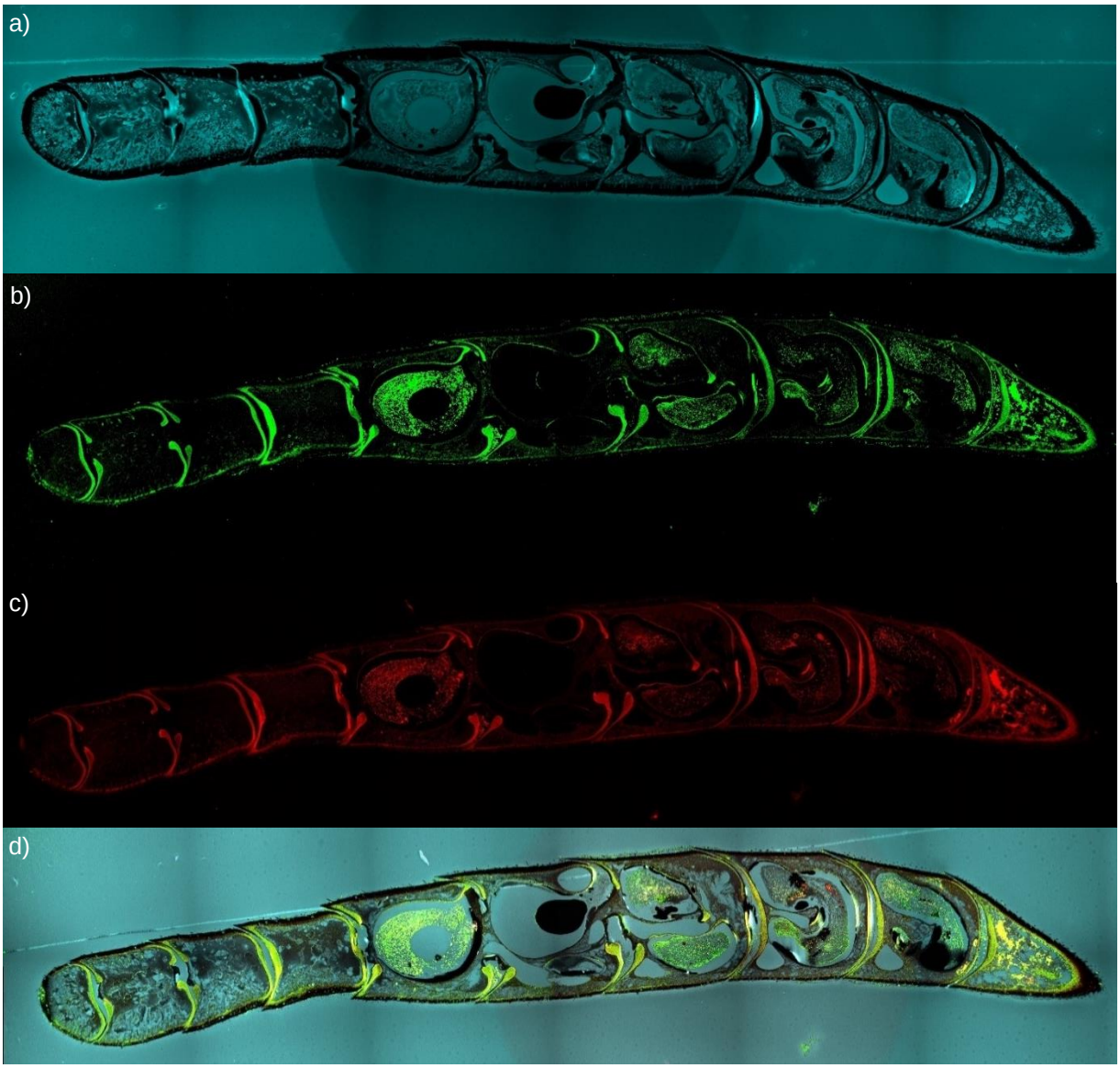

Figure 17. Whole structure of female beewolf antenna of BR2-25, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3-SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Merged image of antenna targeted by all probes including DAPI

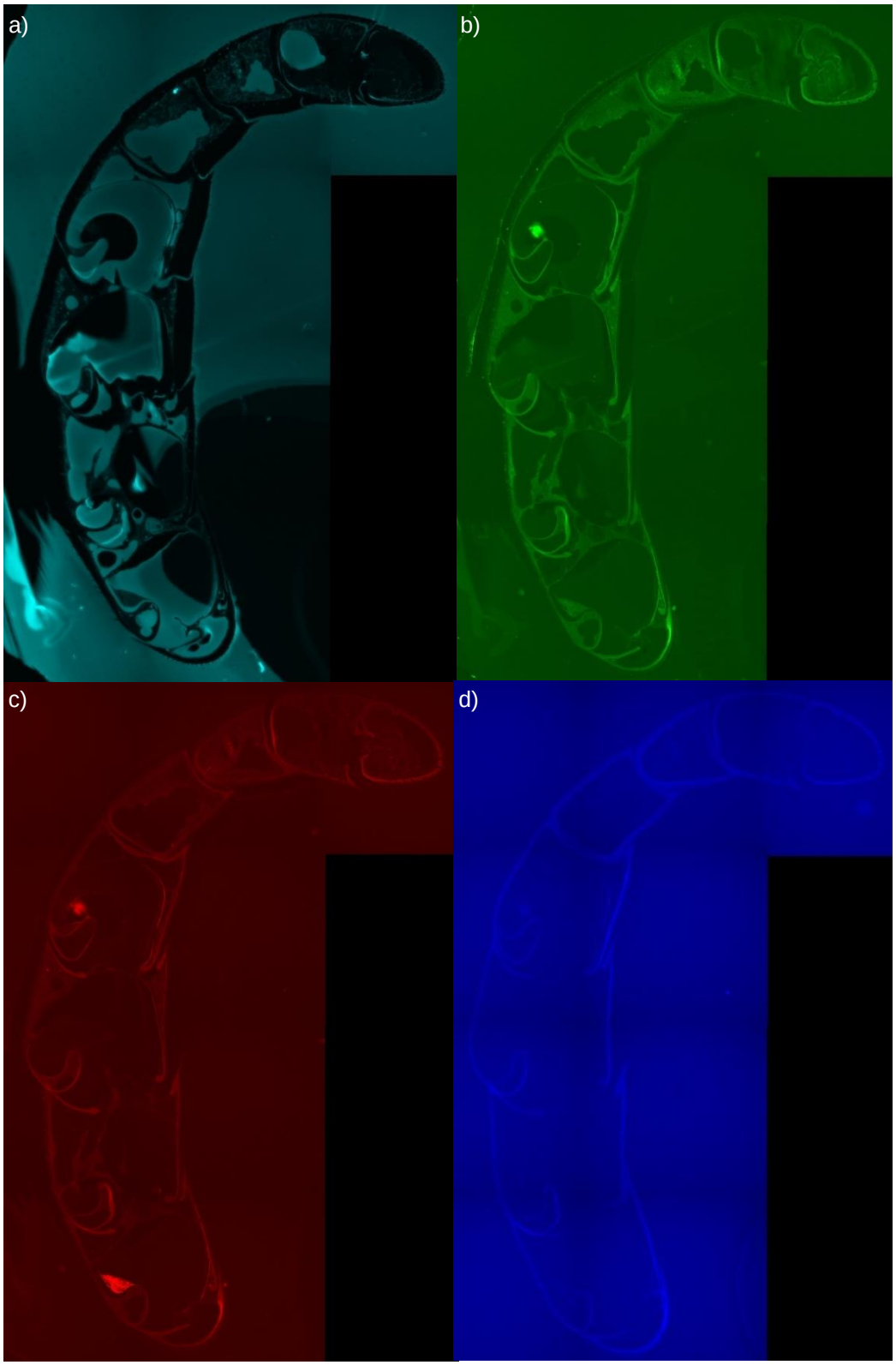

Figure 18. Whole structure of female beewolf antenna of BR-05, showing the empty gland reservoirs could not be labeled by FISH probes within the gland reservoirs of antennomeres (Green: Cy3-SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI)

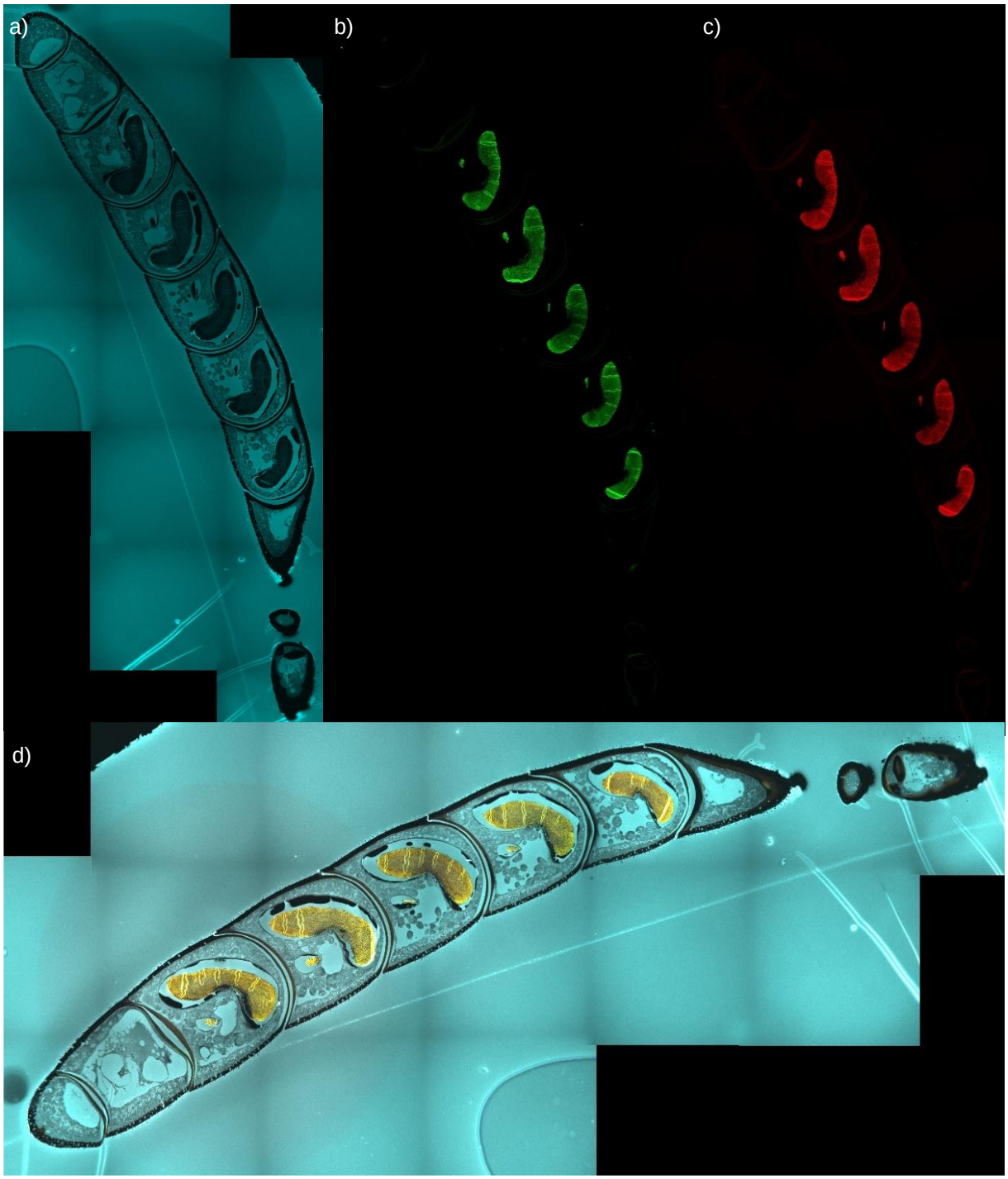

Figure 19. Whole structure of female beewolf antenna of BR2-14, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Merged image of antenna targeted by all probes including DAPI

FISH applied antennae of female *Philanthus ventilabris*

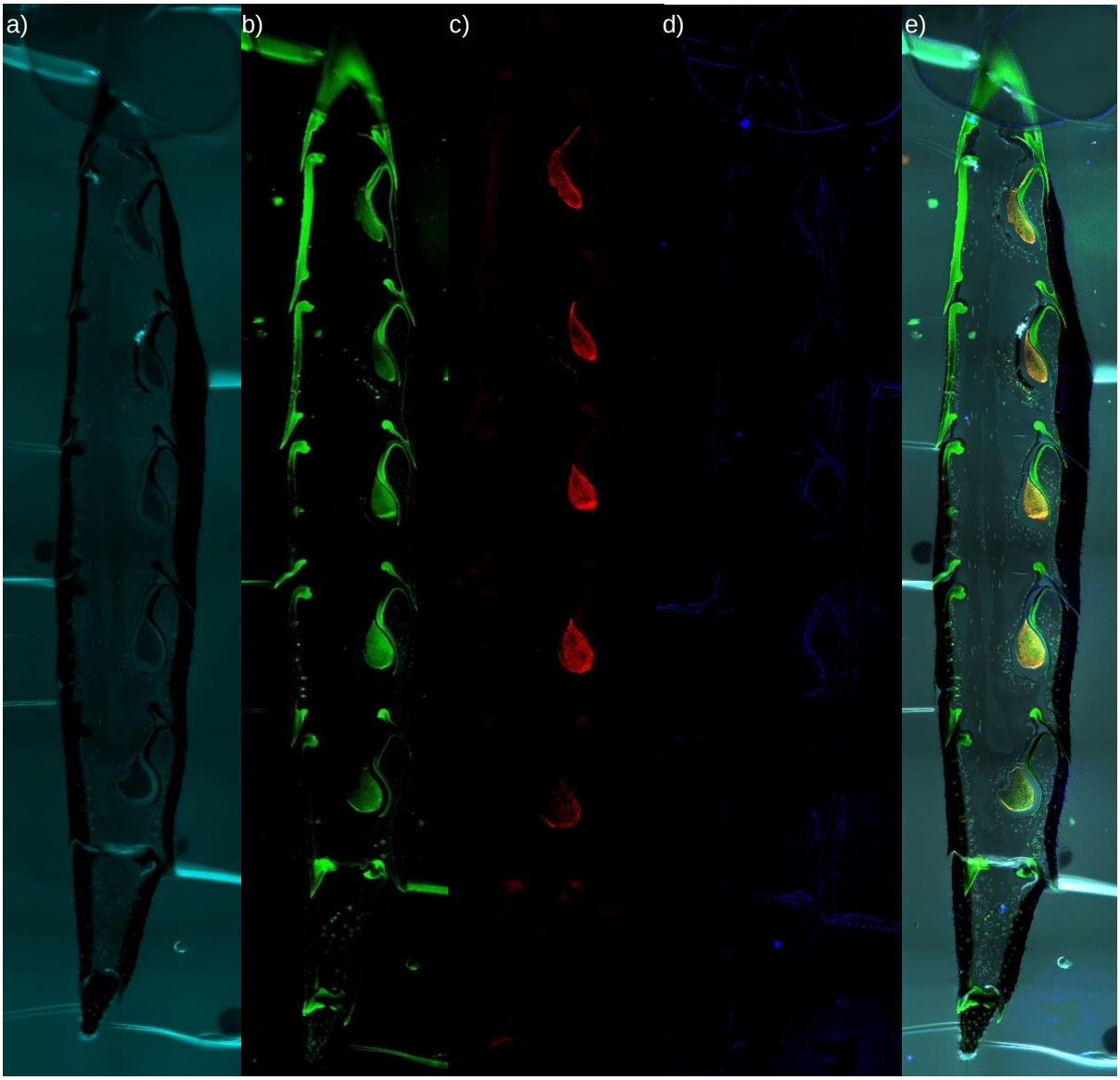

Figure 20. Whole structure of female beewolf antenna of UT-E54, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Cy5-Act-A19 d) Bacteria in gland reservoirs labeled by Eub338-Cy7 e) Merged image of antenna targeted by all probes including DAPI

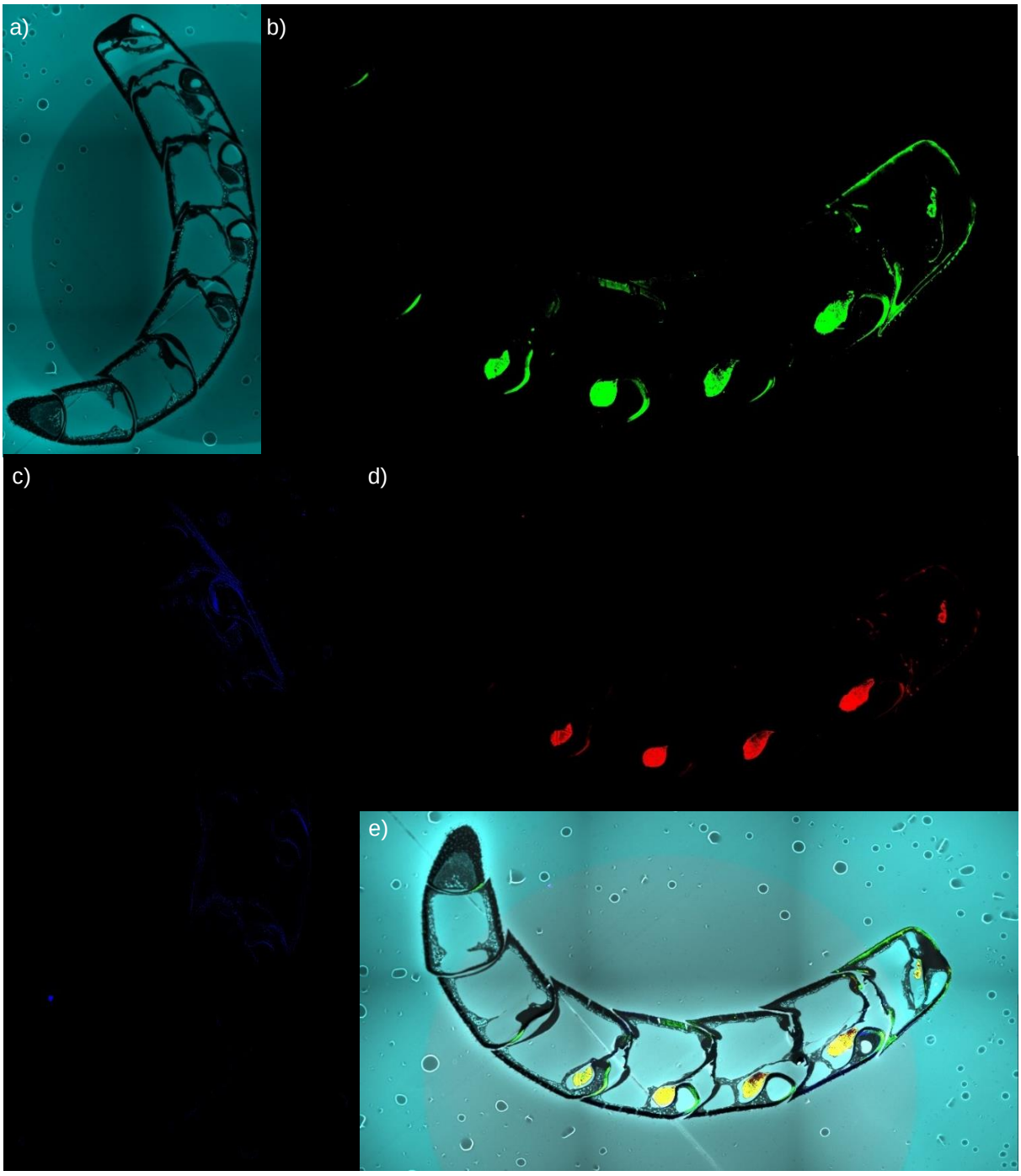

Figure 21. Whole structure of female beewolf antenna of UT-E61, showing the bacteria labeled by FISH probes within the gland reservoirs of antennomeres. (Green: Cy3- SPT177 Red: Cy5-Act-A19 Dark Blue: Eub338-Cy7 Blue: DAPI) a) Antennal structures labeled by DAPI b) Bacteria in gland reservoirs labeled by Cy3- SPT177 c) Bacteria in gland reservoirs labeled by Eub338-Cy7 d) Bacteria in gland reservoirs labeled by Cy5-Act-A19 e) Merged image of antenna targeted by all probes including DAPI

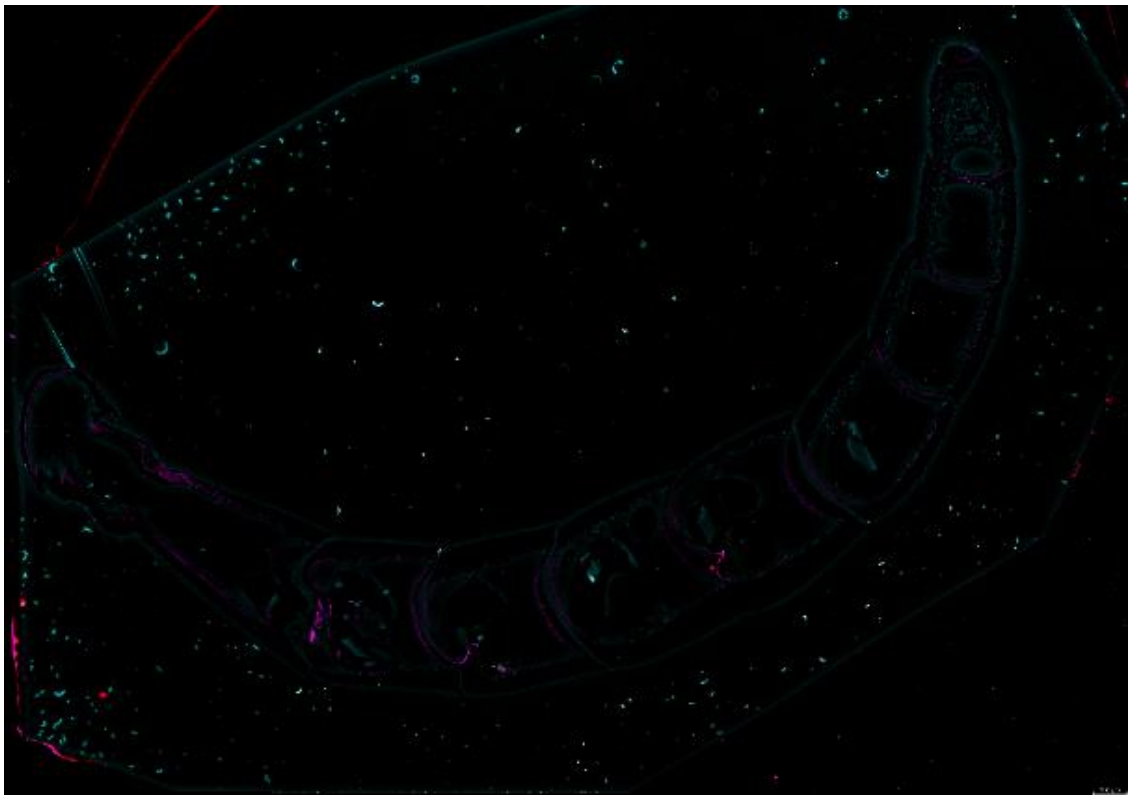

Figure 22. Whole structure of female beewolf antenna of BR2-04, showing the merged image of empty gland reservoirs could not be labeled by FISH probes within antennomeres.

4. DISCUSSION

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) possesses a unique endosymbiotic association with its natural symbiont, *Streptomyces philanthi* that is cultivated within gland reservoirs of the antenna. The female beewolf possesses a white secretion primarily composed of symbiont cells embedded in a matrix of hydrocarbons (Ingham *et al*. 2023). This secretion provides chemical protection for its offspring through the presence of several antibiotics.

There is a powerful vertical transmission mechanism between the symbionts and beewolves, involving the brood cell and cocoon surface (Kaltenpoth *et al*. 2014). This mechanism was acquired from soil ancestors of the bacteria at least 68 million years ago, resulting in a strong evolutionary endosymbiotic association. (Kaltenpoth *et al*. 2014). However, although the antennal gland reservoirs are mostly occupied by its natural symbiont, *Streptomyces philanthi*, the female beewolf is always in contact with abundant numbers of microorganisms in the soil. Because of the high abundance of soil bacteria, female beewolves may encounter bacteria, particularly from the *Streptomyces* genus in the natural habitat of these wasps. The infection of the antennal gland reservoirs by another bacteria was tested and only proven with FISH in vitro (Kaltenpoth *et al*. 2014).

To evaluate the frequency of colonization by free-living bacteria, separate from the symbionts, within the antennal gland reservoirs, we employed a combination approach. This approach involved characterizing bacteria colonized in the gland reservoirs through culture-dependent methods, along with FISH with the use of specific and general probes targeting the 16S rRNA of Eubacteria, Actinobacteria, and symbionts.

4.1 Isolation of Bacteria from gland reservoirs

We have tested the prevalence of symbiont or other Actinobacteria colonization in the antennal gland reservoirs using two methods for each antenna of the same sample. Our experimental design aimed to separate antennae from the same female. One antenna was designated for characterizing bacteria colonizing the gland reservoir using a culture-dependent approach, while the other was allocated for the specific diagnosis of bacteria colonizing the gland reservoir using FISH. However, it was not easy to make a comparative analysis of each antenna from the same female beewolf sample due to the difficulties in axenic cultivation of the bacteria from the segments of the antennae (Nechitaylo *et al*. 2014).

One of the major challenges in axenic cultivation was separating the segments of the antennae to obtain the bacterial cultures. We disinfected the surface antenna samples with 70% ethanol, but this method may not provide complete sterility of the antennae surface while eliminating all contaminants, excluding spores from fungi or sporulated bacteria. It is important to note that the antennae samples from female beewolves used in our experiments were likely in contact with sporulated bacteria such as *Streptomyces* in the soil (Seipke *et al*. 2012). Consequently, the disinfection of the antennae did not effectively eliminate the potential for spore formation, which could impact both cultivation and sequencing results. Furthermore, another limitation in cultivation based analysis from symbiotic bacteria-host interaction is the difference in condition for *in vitro* cultivation compared to *in vivo*, which may lead to findings that are incompatible with the natural dynamics of this interaction (Nechitaylo *et al*. 2014).

In previous studies, the antennal glands of the beewolf were infected with an Actinobacteria, *Amycolatopsis* sp., indicating positive results for the colonization within the antennal glands as a free-living bacteria (Kaltenpoth *et al*. 2014). However, in the antennal samples obtained from the field, which were already infected with *Streptomyces*, and in experimentally infected antennae with *Amycolatopsis*, the bacteria did not exhibit the secretion of the protective white substance, as symbionts typically do (Kaltenpoth *et al*. 2014). This evidence suggests that the colonization of bacteria in the gland reservoirs is controlled by unknown mechanisms, likely involving interactions and evolutionary adaptations between the host and symbiont. Our isolation findings indicate that the antennal gland reservoirs may be colonized by other free-living bacteria, primarily *Streptomyces* spp. However, these isolates obtained from vegetative bacterial cells or spores present on the surface of the antennae, which were not eliminated by exposure to 70% ethanol. Therefore, the origin of these isolates potentially might be contaminants.

Analysis of 16S rRNA sequences from bacterial isolates with different morphotypes obtained from gland reservoirs revealed that 19 isolates are closely related to *Streptomyces spp*. (*Streptomyces venezuela*, *Streptomyces ardesiacus*, *Streptomyces tanashiensis*, *Streptomyces coelicolor*, *Streptomyces tendae*, and *Streptomyces rubrogriseus*). Furthermore, we identified the *Saccharothrix hoggarensis* strain in one antennomere, along with sporulated *Mesobacillus* spp. from two different antennomeres. This finding suggests the possibility of contamination during the isolation process from antennal segments due to sporulation of *Mesobacillus* spp. and *Streptomyces* spp.

The host-symbiont interaction is strictly maintained through a robust vertical transmission mechanism, passing from mother to offspring, which serves as protection against other free-living bacteria that may colonize the gland reservoirs (Engel *et al*. 2013; Grunseich *et al*. 2019). In addition to the complicated nature of partner fidelity between the host and symbionts, the secreted white substance, including symbionts, is also exposed to toxic nitric oxide released by the beewolf egg to sanitize the brood cell (Ingham *et al*. 2023). This exposure results in the elimination of potential antagonistic microorganisms, while the symbionts are protected from the diffusion of toxic concentrations of nitric oxide (Ingham *et al*. 2023). The complex structures of these protective strategies seem to be highly efficient in preventing the uptake of non-symbiotic Actinobacteria from the soil. Additionally, the low amount of symbionts is taken by the adult beewolf from the cocoon during or shortly before the emergence, resulting in the uptake of the symbionts into the gland reservoirs within the antenna (Kaltenpoth *et al*. 2014). However, in this scenario, there is a risk of contamination by pathogenic bacteria or the introduction of another free-living bacteria into the gland reservoirs due to the low number of symbionts being taken up (Nechitaylo *et al*. 2014). Intriguingly, some Actinobacteria were naturally found in the antennal gland reservoirs of some of the female beewolves (Kaltenpoth *et al*. 2014). However, a strong barrier is generally formed by the symbiotic association between *Philanthus triangulum* and *Streptomyces philanthi* to block the invasion of opportunistic (likely *Streptomyces* from our results) bacteria. The female beewolf firstly provides a selective environment to eliminate the colonization of opportunistic bacteria in gland reservoirs (Scheuring and Yu 2012). Moreover, if the gland reservoirs are occupied by free-living bacteria, generally Actinobacteria, the secretion of bacteria from gland reservoirs of the beewolf is strictly inhibited, leading to the blocking of vertical transmission of bacteria other than the symbionts (Kaltenpoth *et al*. 2014).

When considering the partner fidelity mechanisms and protective strategies employed by female beewolves to prevent the potential colonization of their antennal gland reservoirs by free-living opportunistic bacteria, the results of isolates obtained from the analysis of 16S rRNA sequences strongly indicate the possibility of contamination, which may have occurred through sporulation even after surface sterilization of the antennae.

4.2 Fluorescence in situ hybridization (FISH) of beewolf antennae

The endosymbionts in the gland reservoirs can be analyzed with FISH by using specific oligonucleotide probes. The DNA probes can be fluorescently labeled and detected with signals under the fluorescence microscope (Kliot *et al*. 2014). The localization of bacteria in the antennal glands depends on the proper application of the fixation method for probe penetration and improved visualization of the samples (Kliot *et al*. 2014). In addition to fixation, the specificity of the probe design and washing steps in the protocol would influence the signal detection of the bacterial targets for the insect samples (Kliot *et al*. 2014).

Among 16 antenna samples from different female beewolves, only 2 antenna samples did not show any signal detection from the 3 probes used after FISH application. This might be caused by the absence of bacteria inside gland reservoirs or FISH probes could not hybridize to the corresponding 16S rRNA regions, despite the presence of bacteria colonizing the antennal gland reservoirs. Furthermore, although the bacteria in the gland reservoirs of some antennae samples are targeted by Cy3-SPT177 and Cy5-Act-A19 to detect the symbiont *Streptomyces philanthi* and Actinobacteria, respectively, the Eub338-Cy7 probe designed for Eubacteria did not display any hybridization signal. The probe might be damaged due to several reasons such as repeating the freezing and thawing, excessive light exposure, systematical error for probe recognition by software, or depletion of the fluorescence feature of the probe. In addition to the loss of signal of the Eub338-Cy7 probe, some of the gland reservoirs displayed no or slight signal detection by Cy3-SPT177 and Cy5-Act-A19 probes, which might lead to a low amount of bacterial accumulation in the gland reservoirs naturally or probe signaling loss that may have occurred due to several reasons during the application of FISH protocol, as mentioned above.

The majority of the FISH results of the antennae samples showed that the bacteria within the gland reservoirs are targeted by Cy3-SPT177, Cy5-Act-A19, and Eub338-Cy7, indicating the bacteria localize in the gland reservoirs of the FISH applied antennae refer to symbiont *Streptomyces philanthi*. When the symbiont transmission mechanism of the beewolf is taken into account, which needs to be selected against the uptaking of other opportunistic or pathogenic bacteria, the sequencing results of the experimentally isolated bacteria from the gland reservoirs do not match with the result of the antennae analyzed with FISH. Nevertheless, the antennae of a beewolf may be different from each other in terms of the bacterial composition within the antennal gland reservoirs. Thereby, the potential presence of the bacterial content might show differences for each of the antennae.

In consequence, we have tried to address our hypothesis to investigate the bacterial composition of antennae of female *Philanthus triangulum* individuals by providing genetic analysis of isolates and application of fluorescence in situ hybridization (FISH) to the antennae. Although our findings from bacterial isolation from antennal gland reservoirs did not match with FISH results of antennae, the isolation of free-living *Streptomyces* spp. from the gland reservoirs of different antennae may potentially stem from contamination originating from the surface of antennae.

In addition to our work in this project, more detailed research studies might be focused on the colonization of free-living bacteria (mostly *Streptomyces*) and their frequency within the antennal glands compared to natural symbiont, *Streptomyces philanthi* by collecting more female beewolves from the field. This should be followed with the processing of antennae to enlighten the potential gaps of interactions between free-living bacteria and *Philanthus triangulum*. Moreover, contamination risks during the genetic analysis of bacterial isolates and experimental steps in FISH protocols, including the efficacy of probes should be extremely reconsidered.

5. Summary

Symbiosis is a powerful evolutionary interaction commonly found in nature among organisms that enhance fitness, resulting in increased survival and reproductive success. The European beewolf (*Philanthus triangulum*) uses that strategy by collaborating with its natural symbiont, *Streptomyces philanthi*, which is located within the gland reservoirs of the female's antenna. Female beewolf transmits the symbionts to its offspring vertically, resulting in the uptaking of symbionts by larvae during the cocoon spinning. The larvae incorporate the symbiont on the cocoon surface, which leads to the protection of the cocoon through the antibiotics produced by the symbiont during hibernation periods. This significantly improves the wasp's chances of survival. Due to the environmental conditions of the habitats of female beewolves, they are continuously in contact with other bacteria, especially *Streptomyces* in the soil. It has also been shown that the gland reservoirs can be infected by filamentous Actinobacteria other than the beewolf's natural symbiont. Is this common in nature, or do beewolves and their symbionts have such an intimate association that somehow the infection of another bacteria, such as filamentous *Streptomyces*, is highly prohibited by that symbiotic association? To address this question, we have investigated the bacterial contents in the specialized gland reservoirs by culture-dependent techniques. Moreover, bacterial prevalence within the gland reservoirs was also examined by fluorescence in situ hybridization (FISH) using 16 different antennae of female beewolves. 33 bacterial isolates were cultivated and identified by 16S rRNA sequencing. The closest relatives of these isolates were *Streptomyces* sp. such as *Streptomyces venezuela*, *Streptomyces ardesiacus*, *Streptomyces tanashiensis*, *Streptomyces coelicolor*, *Streptomyces tendae*, and *Streptomyces rubrogriseus*. FISH applied to antennae from 16 female beewolves, 14 from Europe and 2 from the US, has indicated that the bacteria within the antennal gland reservoirs belong to *Streptomyces philanthi*. To gain a deeper understanding of the interaction between free-living bacteria and *Philanthus triangulum*, additional studies can focus on the colonization frequency of other Actinobacteria, specifically *Streptomyces*, aside from *Streptomyces philanthi*, within the specialized antennal gland reservoirs of female beewolves. Furthermore, improving the conditions for axenic cultivation of free-living bacteria from the antennal segments of antennae may potentially impact the reliability of future research studies.

6. Zusammenfassung

Symbiose ist eine starke evolutionäre Interaktion, die in der Natur häufig zwischen Organismen vorkommt und die Fitness verbessert, was zu einem erhöhten Überlebens- und Fortpflanzungserfolg führt. Der Europäische Bienenwolf (*Philanthus triangulum*) nutzt diese Strategie, indem er mit seinem natürlichen Symbionten, *Streptomyces philanthi,* zusammenarbeitet, der sich in den Drüsenreservoiren der Antenne des Weibchens befindet. Das Weibchen überträgt die Symbionten vertikal auf seine Nachkommen, was dazu führt, dass die Larven die Symbionten während des Spinnens des Kokons aufnehmen. Die Larven nehmen den Symbionten auf der Kokonoberfläche auf, was zum Schutz des Kokons durch die vom Symbionten produzierten Antibiotika während der Überwinterungszeit führt. Dies verbessert die Überlebenschancen der Wespe erheblich. Aufgrund der Umweltbedingungen in den Lebensräumen der weiblichen Bienenwölfe stehen diese ständig in Kontakt mit anderen Bakterien, insbesondere mit Streptomyces im Boden. Es hat sich auch gezeigt, dass die Drüsenreservoirs von anderen fadenförmigen Actinobakterien als dem natürlichen Symbionten des Bienenwolfs infiziert werden können. Ist dies in der Natur üblich, oder haben Bienenwölfe und ihre Symbionten eine so enge Beziehung, dass die Infektion mit einem anderen Bakterium, wie z. B. fadenförmigen Streptomyces, durch diese symbiotische Beziehung stark behindert wird? Um dieser Frage nachzugehen, haben wir den Bakteriengehalt in den spezialisierten Drüsenreservoiren mit kulturabhängigen Techniken untersucht. Darüber hinaus wurde das Bakterienvorkommen in den Drüsenreservoiren auch durch Fluoreszenz-in-situ-Hybridisierung (FISH) an 16 verschiedenen Fühlern von weiblichen Bienenwölfen untersucht. 33 bakterielle Isolate wurden kultiviert und durch 16S rRNA-Sequenzierung identifiziert. Die engsten Verwandten dieser Isolate waren *Streptomyces* sp. wie *Streptomyces venezuela, Streptomyces ardesiacus, Streptomyces tanashiensis, Streptomyces coelicolor*, *Streptomyces tendae* und *Streptomyces rubrogriseus*. Die FISH-Untersuchung der Antennen von 16 weiblichen Wölfen, 14 aus Europa und 2 aus den USA, hat ergeben, dass die Bakterien in den Antennendrüsenreservoiren zu Streptomyces philanthi gehören. Um ein tieferes Verständnis der Interaktion zwischen freilebenden Bakterien und Philanthus triangulum zu erlangen, könnten sich weitere Studien auf die Besiedlungshäufigkeit anderer Actinobakterien, insbesondere Streptomyces, neben Streptomyces philanthi, in den spezialisierten Antennendrüsenreservoiren von weiblichen Bienenwölfen konzentrieren. Darüber hinaus könnte die Verbesserung der Bedingungen für

die axenische Kultivierung von freilebenden Bakterien aus den Antennensegmenten der Antennen die Zuverlässigkeit künftiger Forschungsstudien beeinflussen.

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Declaration of Self-Dependence

Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

Jena, Germany, 12.10.2023