

# **Diversity of Actinobacteria associated with *Nasutitermes costalis* termite and their ability to inhibit the growth of entomopathogenic fungi**

by

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## Abstract

The Actinobacteria are Gram-positive bacteria with high GC content, spore producers, and have the ability to produce antimicrobial agents. They are capable of degrading structural polysaccharides such as cellulose, lignocellulose and chitin. Previous studies have found this group of bacteria associated with different groups of insects including ants, bees, wasps, beetles, and termites. Actinobacteria have two primary roles in these associations: (1) defend the host from pathogenic organisms and (2) decompose organic matter in the gut. However, it is known that the genus *Streptomyces* (Actinobacteria) is naturally selected to perform these symbiotic relationships. In Attine ants and Beewolf wasps, Actinobacteria protect them against pathogenic microbes. At present it is unknown which Actinobacteria could be associated with termites and if they possess the ability of inhibiting the micelial growth of entomopathogenic fungus *Metarhizium anisopliae* and *Beauveria bassiana*, which also affect termites. On the other hand, the termite gut has been extensively explored. In termites gut have been identified Actinobacteria of the genus *Streptomyces* are believed to help degrade organic matter. In this study we characterized the diversity of Actinobacteria associated with *Nasutitermes costalis*, a common arboreal termite in Puerto Rico, and evaluated their antifungal capacity against *Metarhizium anisopliae* and *Beauveria bassiana*. Two castes of termites (workers and soldiers) and nest material were collected in Miradero forest in Mayagüez, Puerto Rico. We used culture-dependent methods (chitin agar, YMEA) for isolation of Actinobacteria from the nest material, the gut, and the termites exoskeleton. Morphological and molecular techniques (sequencing of 16S rDNA gene) were later used for identification. We isolated 1342 Actinobacteria represented by the genera *Streptomyces*, *Amycolatopsis*, *Lentzea*, *Saccharothrix*, *Pseudonocardia*, *Microbacterium*, and *Gordonia*. Sequence analysis revealed that the most common genus found in this association is *Streptomyces* from 73% to 97% of frequency among samples and presents great morphological variation. Phylogenetic analysis shows 15 monophyletic clades that separate the *Streptomyces* sequences isolated from *N. costalis* with no match to GenBank database. The most frequent and constant strains in the nest material and exoskeleton of termites were closely related to *Streptomyces cavourensis* (GenBank, HQ610450.1). Strains closely related to *Streptomyces cavourensis* (HQ610450.1) were not found in nests abandoned by termites. All selected strains were able to inhibit the growth of *M. anisopliae* and *B. bassiana*, but 73% of them were effective in inhibiting micelial growth of *B. bassiana* from 90% to 100%. The strain W1OE185[6] closely related to *Streptomyces cavourensis* (HQ610450.1) and the second most abundant in worker exoskeletons was the highest ability to inhibit both fungi. The results indicate that the 15 clades of *Streptomyces* can be unique in termites, but another gene should be used to confirm this hypothesis. Our results support the existence of *Streptomyces* symbionts in the system of the termite *N. costalis* and that one of its roles is the production of antifungal agents.

## Resumen

Las Actinobacterias son bacterias Gram-positivas con alto contenido de GC, productores de esporas, con la capacidad de producir agentes antimicrobianos. Estas son capaces de degradar polisacáridos estructurales como la celulosa, la lignocelulosa y la quitina. En estudios previos han encontrado a éste grupo de bacterias en asociación con diferentes grupos de insectos como las hormigas, abejas, avispas, escarabajos y termitas. Las Actinobacterias tienen dos funciones principales en estas asociaciones: (1) defender al huésped de organismos patógenos y/o (2) descomponer materia orgánica en el intestino. Además se conoce que el género *Streptomyces* (Actinobacterias) es seleccionado naturalmente para llevar a cabo esta relación simbiótica. En las hormigas *Attine* y en las avispas *Beewolf*, las Actinobacterias juega un rol importante contra microorganismos patógenos. En la actualidad se desconoce la comunidad de Actinobacterias asociadas con las termitas, y si éstas poseen la capacidad de inhibir hongos entomopatógenos como *Metarhizium anisopliae* y *Beauveria bassiana*, que afectan también a las termitas. Por otro lado, se ha estudiado ampliamente el intestino de las termitas. Se conoce que las Actinobacterias del género *Streptomyces* ayudan a degradar la materia orgánica en el intestino de las termitas. En éste estudio el objetivo es conocer la diversidad de Actinobacterias asociadas a *Nasutitermes costalis*, el cual es una termita arbórea común en Puerto Rico. Además se evaluó a través de bioensayos la capacidad antimicótica contra *Metarhizium anisopliae* y *Beauveria bassiana*. Para esto se tomaron termitas obreras, soldados y material de nido en una región boscosa en Miradero, Mayagüez, Puerto Rico. Se utilizó métodos dependientes de cultivos (medio de quitina, YMEA) para aislar las Actinobacterias asociadas al material del nido, el intestino y el exoesqueleto de las termitas. Luego se utilizó caracterización morfológica y métodos moleculares (secuenciación de gen del 16S rDNA) para la identificación de los aislados. Durante el proceso se aislaron 1342 Actinobacterias representados por los géneros *Streptomyces*, *Amycolatopsis*, *Lentzea*, *Saccharothrix*, *Pseudonocardia*, *Microbacterium*, y *Gordonia*. Al analizar las secuencias revelamos que el género más común es *Streptomyces* que fluctúa entre 73% a 97% de frecuencia entre las muestras y presenta una gran variación morfológica. El análisis filogenético revela 15 clados monofiléticos que separan nuestras secuencias con las parecidas a las de GenBank. Las cepas más frecuentes y constantes en el material del nido y exoesqueleto de las termitas fueron las cercanamente relacionadas a la cepa de GenBank identificada como *Streptomyces cavourensis* (HQ610450.1). Además, las cepas cercanamente relacionadas a *Streptomyces cavourensis* (HQ610450.1) no fueron encontradas en nidos abandonados por las termitas. También, todas las cepas seleccionadas fueron capaces de inhibir el crecimiento de *M. anisopliae* y *B. bassiana*, pero el 73% de éstas fueron eficaces en la inhibición del crecimiento micelial de *B. bassiana* de 90% a 100%. Pero la cepa W10E185[6] cercanamente relacionada a *Streptomyces cavourensis* (HQ610450.1) y la segunda más abundante en exoesqueletos de obreras fue la de mayor

habilidad en inhibir ambos hongos. Estos resultados nos indican que los 15 clados de *Streptomyces* pueden ser únicos en las termitas, pero es necesario utilizar otro gen para confirmarlo. Nuestros resultados apoyan que existen posibles *Streptomyces* simbioses en el sistema de termitas de *N. costalis* y que una de las funciones que pueden estar realizando sería la producción de agentes antimicrobianos.



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## **Dedication**

I dedicate this work to my family...

...To my nephew Waldyn Ramírez-Méndez.

...To my brothers Noé Alberto, Alberto Francisco and my sister Wanda Angélica.

...To my parents Alberto Méndez Avilés and Wanda Pilar Morales La Santa for unconditional support, understanding and love.

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# Table of contents

	Page
Abstract .....	ii
Resumen .....	iii
Dedication .....	vi
Acknowledgments .....	vii
Table of contents .....	viii
List of tables .....	x
List of figures .....	xi
List of appendices .....	xiv
1. Introduction .....	1
1.1 Literature review .....	2
1.1.1 Phylogeny and classification of termites .....	2
1.1.2 Lifestyle of termites .....	4
1.1.3 Bacteria associated with termites .....	5
1.1.4 Role of the Actinobacteria associated with insects .....	8
1.1.5 <i>Nasutitermes costalis</i> (Isoptera: Termitidae: Termitinae: Nasutitermitinae) .....	11
1.2 Questions and objectives .....	11
1.2.1 Questions .....	11
1.2.2 Objectives .....	12
2. Diversity of Actinobacteria associated with <i>Nasutitermes costalis</i> termite (Isoptera: Termitidae) .....	13
2.1 Introduction .....	13
2.2 Materials and methods .....	14
2.2.1 Sampling area .....	14
2.2.2 Sample collection .....	14
2.2.3 Sample processing .....	15
2.2.4 Isolation of Actinobacteria .....	16
2.2.5 Macroscopic characterization of colonies .....	17
2.2.6 Selection of strains for molecular identification .....	17
2.2.7 Genomic DNA extraction .....	17
2.2.8 Polymerase chain reaction (PCR) .....	17
2.2.9 DNA sequencing .....	18
2.2.10 Data analysis .....	18
2.3 Results .....	19
2.3.1 Colonies isolated from <i>N. costalis</i> .....	19
2.3.2 Genera of Actinobacteria associated with <i>N. costalis</i> .....	21
2.3.3 Actinobacteria associated with <i>N. costalis</i> workers in wet season .....	22
2.3.4 Actinobacteria associated with <i>N. costalis</i> soldiers in wet season .....	24
2.3.5 Actinobacteria associated with <i>N. costalis</i> workers in dry season .....	25
2.3.6 Actinobacteria associated with <i>N. costalis</i> soldiers in dry season .....	31

2.3.7 Actinobacteria associated with <i>N. costalis</i> nest material in wet and dry seasons .....	31
2.3.8 Actinobacteria diversity associated with <i>N. costalis</i> .....	32
2.3.9 Phylogenetic analysis of <i>Streptomyces</i> spp. associated with <i>N. costalis</i> .....	35
2.4 Discussion .....	38
3 Ability to inhibit the growth of <i>Metarhizium anisopliae</i> and <i>Beauveria bassiana</i> by <i>Streptomyces</i> strains isolated from <i>Nasutitermes costalis</i> .....	43
3.1 Introduction .....	43
3.2 Materials and methods .....	44
3.2.1 Selection of <i>Streptomyces</i> strains associated with <i>N. costalis</i> .....	44
3.2.2 Bioassay: confrontation Actinobacteria-fungus .....	45
3.2.3 Data Analysis .....	46
3.3 Results .....	47
3.3.1 Growth controls of fungi and <i>Streptomyces</i> strains .....	47
3.3.2 Growth of fungi in confrontation bioassays .....	47
3.3.3 Growth of <i>Streptomyces</i> strains in presence of fungi .....	48
3.3.4 Inhibition of fungal growth .....	57
3.4 Discussion .....	61
4 Conclusions .....	63
References .....	64
Appendices .....	71

## List of tables

	Page
Table 1.1- Classification of the order Isoptera. ....	4
Table 1.2- Genera and species of bacteria identified in the gut of different species of termites. ....	9
Table 2.1- Index of abundance, richness, and diversity of Actinobacteria obtained for each sample in the wet season. ....	34
Table 2.2- Index of abundance, richness, and diversity of Actinobacteria obtained for each sample in the dry season. ....	35
Table 2.3- Identification of <i>Streptomyces</i> strains and their phylogenetic relationships (Figure 2.11). ....	42
Table 3.1- Information of <i>Streptomyces</i> strains used to bioassay confrontation with fungi. ....	45
Table 3.2- ANOVA of <i>Metarhizium anisopliae</i> micelial growth in presence of <i>Streptomyces</i> strains isolates from <i>N. costalis</i> . ....	60
Table 3.3- ANOVA of <i>Beauveria bassiana</i> micelial growth in presence of <i>Streptomyces</i> strains isolates from <i>N. costalis</i> . ....	60

## List of figures

	Page
Figure 2.1- <i>Nasutitermes costalis</i> : worker termite (A), soldier termite (B), and nest (C).	15
Figure 2.2- Isolated colonies percentage per season (A) and the relative frequency of the isolated colonies per sample during the season (B). .....	19
Figure 2.3- Relative frequency of morphotypes isolated in different samples in wet and dry seasons. ....	20
Figure 2.4- Relative frequency of Actinobacteria genera isolated in wet and dry seasons. ....	21
Figure 2.5- Relative frequency of Actinobacteria isolated from the different anatomical regions of the workers, the soldiers and the nest material of <i>N. costalis</i> in wet (A) and dry (B) seasons. ....	23
Figure 2.6- Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the worker termites <i>N. costalis</i> for the wet season. Identification of Actinobacteria was performed using BLAST® in GenBank. ....	26
Figure 2.7- Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the soldiers termites <i>N. costalis</i> for the wet season. Identification of Actinobacteria was performed using BLAST® in GenBank. ....	27
Figure 2.8- Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the workers termites <i>N. costalis</i> for the dry season. Identification of Actinobacteria was performed using BLAST® in GenBank. ....	28
Figure 2.9- Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the soldiers termites <i>N. costalis</i> for the dry season. Identification of Actinobacteria was performed using BLAST® in GenBank. ....	29
Figure 2.10- Frequency of similarity of the Actinobacteria isolated from the nest material of <i>N. costalis</i> for wet (A) and dry (B) season. Identification of Actinobacteria was performed using BLAST® in GenBank. ....	30
Figure 2.11- Rarefaction curve of total samples of Actinobacteria in <i>N. costalis</i> . The $S_{obs}$ (Mao Tau) statistic was used to calculate the expected number of strains in the samples. The dashed lines represent the upper and lower limits of the confidence intervals at 95 % of $S_{obs}$ (Colwell <i>et al.</i> 2004). ....	34

Figure 2.12- Neighbor-joining phylogenetic tree of 16S rDNA for *Streptomyces* isolated from worker exoskeleton (■), worker gut (□), soldier exoskeleton (■), soldier gut (■) and, nest material (■) of *N. costalis*. Monophyletic clades A, B and C are the principal groups of sequences that are not represented in GenBank. Monophyletic clades 1 to 12 are unresolved small groups in this analysis. Bootstrap support values after 5000 pseudoreplicates are shown. For more details see Appendices L, M and N. ... 37

Figure 3.1- Technique used to confrontations bioassays. Inoculated at the center *Streptomyces* strain (●). Fungi (■) were inoculated to 21 mm away from inoculation site of *Streptomyces* strain. .... 46

Figure 3.2- Control growth of fungi: *M. anisopliae* (■) and *B. bassiana* (■) (n = 4). .... 49

Figure 3.3- Control growth of *Streptomyces* strains W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■) during the incubation period. .... 50

Figure 3.4- Growth of *M. anisopliae* (A) and *B. bassiana* (B) for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■). .... 51

Figure 3.5- Growth of *Metarhizium anisopliae* for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (A), W2SE43[7] (B), W1SI94[3] (C), W1SI96[3] (D), W1SI107[4] (E), W2SE112[4] (F), W1OE136[3] (G), W1SE179[1] (H), W1SE180[1] (I), W1OE185[6] (J), W1OE186[1] (K), W1OE187[2] (L), W1OE190[3] (M), W1OE191[1] (N) and D1OE213[8] (O). .... 52

Figure 3.6- Growth of *Beauveria bassiana* for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (A), W2SE43[7] (B), W1SI94[3] (C), W1SI96[3] (D), W1SI107[4] (E), W2SE112[4] (F), W1OE136[3] (G), W1SE179[1] (H), W1SE180[1] (I), W1OE185[6] (J), W1OE186[1] (K), W1OE187[2] (L), W1OE190[3] (M), W1OE191[1] (N) and D1OE213[8] (O). .... 54

Figure 3.7- Growth of *Streptomyces* strains W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■) during the incubation period with *M. anisopliae* (A) and *B. bassiana* (B). .... 56

Figure 3.8- Zone of inhibition (ZOI) (mm) of *Metarhizium anisopliae* (A) and *Beauveria bassiana* (B) in presence of *Streptomyces* strains isolated from *N. costalis* (n = 4). .... 58



Figure 3.9- Percentage of inhibition of *Metarhizium anisopliae* (■) and *Beauveria bassiana* (■) in presence of *Streptomyces* strains isolated from *N. costalis* (n = 4). ..... 59

## List of appendices

	Page
Appendix A- General information and colony characteristics of isolates .....	71
Appendix B- Morphotypes isolated from worker gut in wet season .....	79
Appendix C- Morphotypes isolated from worker exoskeleton in wet season .....	80
Appendix D- Morphotypes isolated from soldier gut in wet season .....	81
Appendix E- Morphotypes isolated from soldier exoskeleton in wet season .....	82
Appendix F- Morphotypes isolated from nest material in wet season .....	83
Appendix G- Morphotypes isolated from worker gut in dry season .....	84
Appendix H- Morphotypes isolated from worker exoskeleton in dry season .....	85
Appendix I- Morphotypes isolated from soldier gut in dry season .....	85
Appendix J- Morphotypes isolated from soldier exoskeleton in dry season .....	86
Appendix K- Morphotypes isolated from nest material in dry season .....	86
Appendix L- General information of strains and molecular identification .....	87
Appendix M- Phylogenetic trees of <i>Streptomyces</i> strains isolated from termite workers (A), soldiers (B) and nests material (C) .....	95
Appendix N- Phylogenetic tree of <i>Streptomyces</i> strains isolated in this research .....	98

# 1 Introduction

Numerous groups of bacteria are known to naturally occur in association with other organisms such as insects (Kaltenpoth 2009). There have been several studies (Kaltenpoth 2009) on bacterial communities associated with insects and their role in these hosts. Some of these roles/functions include: (1) host protection from pathogenic organisms (Kaltenpoth 2009, Haeder *et al.* 2009), (2) degradation of organic matter when the host does not have the metabolic capacity to do it (Pasti *et al.* 1990, Visôto *et al.* 2009), and (3) nitrogen fixation (French *et al.* 1976, Pinto-Tomás *et al.* 2009). Thus, over thousands of years of interaction, bacteria have established mutualistic associations with various insects (Margulis and Fester 1991, Maynard-Smith 1989), such as bacteria living in the gut of higher termites, which are essential for degradation of cellulose (Husseneder *et al.* 2010, Schmitt-Wagner *et al.* 2003, Watanabe *et al.* 2003).

Termites are divided into several families in the order Isoptera (Table 1.1), which are grouped into lower and higher termites. The latter are all in the family Termitidae, which is the only group that does not have protists that help termites break down their food, mostly cellulose. Instead, degradation is performed by bacterial communities in the gut (Pasti *et al.* 1990). Diverse bacterial communities have been described in the gut of both, higher and lower termites, whose role is to degrade organic matter into nutrients that can be utilized by the host (Husseneder *et al.* 2010, Schmitt-Wagner *et al.* 2003, Watanabe *et al.* 2003). Among these bacteria, Actinobacteria, mostly in the genus *Streptomyces*, are believed to be responsible for degradation of cellulose and lignocellulose (Watanabe *et al.* 2003).

In Puerto Rico, two species of the genus *Nasutitermes* (Family Termitidae, subfamily Nasutitermitinae), *N. acajutlae* and *N. costalis*, have been reported, with the latter being the most

common (Scheffrahn *et al.* 2003). Based on the above considerations, we anticipate that Actinobacteria are part of the gut microflora of the *Nasutitermes* termites. To date no studies have been performed on the presence of Actinobacteria associated to termite exoskeleton. These Actinobacteria communities that are associated with the termite gut and exoskeleton may have different functions other than cellulose degradation. At present, the only other known association of Actinobacteria with the exoskeleton of other social insects, especially with *Streptomyces*, occurs in ants (Haeder *et al.* 2009), wasps (Kaltenpoth and Göttler 2005) and beetles (Scott *et al.* 2008). The function of these Actinobacteria is to protect the insect against pathogenic microorganisms, producing secondary metabolites such as antibiotics (Currie *et al.* 2003a, Currie *et al.* 1999b, Cafaro and Currie 2005, Kaltenpoth and Göttler 2005, Scott *et al.* 2008, Patil *et al.* 2010).

The present research was aimed to describe the association of Actinobacteria communities with the termite species *N. costalis*. We compared Actinobacteria associated with different termite castes and with different anatomical parts (exoskeleton and gut). In particular, we determined if this community was dominated by the genus *Streptomyces* as with other social insects. In addition, we evaluated the role of Actinobacteria as potential termite protection against fungal pathogens and their possible symbiotic relationship.

## **1.1 Literature review**

### **1.1.1 Phylogeny and classification of termites**

Termites are generally classified in the order Isoptera, although there is a debate about where they are positioned within the Hexapoda. Inward *et al.* (2007a) propose, based on

morphological and genetic evidence, that termites belong to the order Blattodea (cockroaches) forming a clade with the family Cryptocercidae. Regardless, Isoptera is divided into four clades: Euisoptera, Neoisoptera and Cratomastotermitidae (fossil) and Mastotermitidae families (Table 1.1). Families belonging to clade Euisoptera are: Termopsidae (fossil), Hodotermitidae, Archotermopsidae, Stolotermitidae (Stolotermitinae and Porotermitinae subfamilies) and Kalotermitidae. While families belonging to clade Neoisoptera are: Archeorhinotermitidae (fossil), Stylotermitidae, Rhinotermitidae (Coptotermitinae, Heterotermitinae, Prorhinotermitinae, Psammotermitinae, Termitogetoninae and Rhinotermitinae subfamilies), Serritermitidae and Termitidae (Sphaerotermitinae, Macrotermitinae, Foraminitermitinae, Sytermitinae, Nasutitermitinae, Apicotermitinae, Cubitermitinae and Termitinae subfamilies) (Engel *et al.* 2009).

In addition to the morphological and genetic methods, termites are classified by co-cladogenesis, defined as “the parallel process of speciation between host and symbiont, such that phylogenetic trees of each partner are equivalent, or approximately so” (Bignell *et al.* 2011). Co-cladogenesis refers to whether different genera of termites have symbiotic bacteria and protists in the gut, which are transferred vertically through trophallaxis, which is the direct transfer of oral and/or gut fluids of a donor to the mouth of a receiver (Bignell *et al.* 2011). These microorganisms exert a major role in the degradation of the main food component: cellulose and lignocellulose. Symbiotic protists of termites belong to the genera *Trichonympha*, *Euconympha* and *Teranympha* (Eggleson 2001, Kambhampati and Eggleson 2000). According to Pearce and Waite (1994), termites that have protists that break down cellulose are classified as low termites. On the other hand, if cellulose degradation is carried out only by bacteria then the termites are considered higher termites.

**Table 1.1-** Classification of the order Isoptera.

Clade	Family	Subfamily	
	Cratomastotermitidae (fossil)		
	Mastotermitidae		
Euisoptera	Termopsidae (fossil)		
	Hodotermitidae		
	Archotermopsidae		
	Stolotermitidae	Stolotermitinae Porotermitinae	
	Kalotermitidae		
Neoisoptera	Archeorhinotermitidae (fossil)		Lower
	Stylotermitidae		
	Rhinotermitidae	Coptotermitinae Heterotermitinae Prorhinotermitinae Psammotermitinae Termitogetoninae Rhinotermitinae	
	Serritermitidae		
	Termitidae	Sphaerotermitinae Macrotermitinae Foraminitermitinae Sytermitinae Nasutitermitinae Apicotermitinae Cubitermitinae Termitinae	

Modified from Engel *et al.* 2009

### 1.1.2 Lifestyle of termites

Termites are one of the organisms responsible for the decomposition of organic matter being most abundant in the tropics and subtropics (Bandeira and Torres 1985, Bignell *et al.* 2011). Termites are eusocial organisms that are characterized by an organized social structure. This social organization is divided in three castes: workers, soldiers, and breeders. The reproductive caste in turn is subdivided into winged, primary reproductive or royal couple and secondary reproductive (Bandeira and Torres 1985, Bignell *et al.* 2011).

Termite nests have several types of architecture depending on the ecosystem, food and species. There are several different nests: arboreal, subterranean and epigeal (Ohkuma 2003). The nest structure indicates the type of feeding the termites performed. Overall, arboreal termite nests are organisms that feed on wood, leaves and branches of dead trees. Subterranean nest termites feed on soil organic matter and roots of dead trees (Bignell *et al.* 2011). Moreover, epigeal termite nests, which are found only in Africa and Asia, have a different feeding strategy. Epigeal termites collect and pre-digest organic matter and then deposit it in the center of the nest where they grow fungi that serve as their primary food source (Kroodsma and Roosen 2006, Meyer *et al.* 2000).

### **1.1.3 Bacteria associated with termites**

Microorganisms (bacteria, archaea and protists) associated with the termite gut have been extensively studied (Schmitt-Wagner *et al.* 2003, Friedrich *et al.* 2001, Ohkuma 2008). Recent works describe microbial communities in the gut of almost all major groups of termites (Table 1.2). Nonetheless, there has only been one study of bacteria associated to the nest (Fall *et al.* 2007) and no studies of microorganisms associated with the exoskeleton of termites.

In the Mastotermitidae family, the most studied species is *Mastotermes darwiniensis*, which harbors the bacteria *Citrobacter freundii* and *Blattabacterium* sp. (French *et al.* 1976, Sabree *et al.* 2011). Both bacteria have an important role in nitrogen fixation in the gut of *M. darwiniensis*, which is essential for the production of amino acids and vitamins (Sabree *et al.* 2011).

There have also been studies in cellulolytic bacteria in the termite *Zootermopsis agusticollis* (Archotermopsidae). According to Wenzel *et al.* (2002), in *Z. agusticollis* the role of cellulose degradation is performed by Actinobacteria, Bacillales (Firmicutes),  $\alpha$ -Proteobacteria and Flexibacteriaceae (Bacteroidetes) (Table 1.2). Watanabe *et al.* (2003) also recognize the capacity of cellulose degradation of *Streptomyces* sp. in the gut of *Hodotermopsis japonica* (Archotermopsidae) and *Neotermes kashunensis* (Kalotermitidae). While Okuma *et al.* (2007) studied the Termite Group 1 (TG1) bacteria associated with *Hodotermopsis sjoestedti* (Table 1.2). Termite Group 1 is an intracellular symbiont bacteria in the protist (which are in the termite gut) and its role is to produce amino cofactors, H<sub>2</sub> and acetate, which are essential for the termite and the protist (Okuma *et al.* 2007, Hongoh *et al.* 2008).

Furthermore, in the family Rhinotermitidae (higher termites) the bacteria associated with gut of the termites *Coptotermes* (Coptotermitinae) and *Reticulitermes* (Rhinotermitinae) were also studied. Boopathy and Adams (2005) identified the enteric bacteria *Serratia marcescens*, *Enterobacter aerogenes*, *E. cloacae* and *Citrobacter farmer* in the gut of the termite *Coptotermes formosanus* (Table 1.2). Also in *C. formosanus* the bacteria *Streptomyces* sp. (Watanabe *et al.* 2003), *Burkholderia* sp. and *Citrobacter* sp. (Harazono *et al.* 2003) were isolated. According to Bugg *et al.* (2010) these bacteria have the ability to degrade cellulose and lignin, which is consumed by *C. formosanus*. Through culture-independent methods, Shinzato *et al.* (2005) have identified bacterial communities associated with the gut of *C. formosanus* composed as follows: Bacteroidales (72.4%), Clostridiales (9.2%), Spirochaetes (6%), Mycoplasmatales (4.8%), Actinobacteria (2%), Bacillales/Lactobacillales (1.6%), Synergistes (0.8%), Planctomycetes (0.8%) and Verrucomicrobia (0.8%) (Table 1.2). In the termite *Coptotermes lacteus* the bacteria



*C. freundii* was also isolated and it is believed to be essential for nitrogen fixation (French *et al.* 1976).

Using culture-independent methods different groups of bacteria associated with the gut termites of the genus *Reticulitermes* were identified. Hongoh *et al.* (2005) produced a clone library of bacteria associated with the gut of the termites *R. speratus*, *R. amamianus* and *R. okinawanus*. The authors found that composed samples of the three termites were dominated by Firmicutes (44.6%), Proteobacteria (10.6%), Bacteroidetes (12.5%) and Spirochaetes (20.4%). Additionally, Nakajima *et al.* (2005) studied only the bacteria associated with the gut of *R. speratus* finding Bacteroidales (19.4%), *Treponema* (17.4%), Propionibacteriaceae (16.3%), Rhodocyclales (9.2%) and TG1 (8.2%) as the most prevalent groups. Also in *R. speratus* and *R. flavipes* several bacteria were identified in relation to several processes: nitrogen fixation, sulfate reduction, CO<sub>2</sub> to acetate reduction, and cellulose and lignin degradation in the termite gut (Ohkuma and Kudo 1996, Watanabe *et al.* 2003, Hongoh *et al.* 2007, Chung *et al.* 1994, Schultz and Breznak 1978).

In the family Termitidae, the genera *Odontotermes* (Macrotermitinae), *Nasutitermes* (Nasutitermitinae) *Cubitermes* (Cubitermitinae) and *Microcerotermes* (Termitinae) have been studied (Watanabe *et al.* 2003, Tokuda *et al.* 2000, Kato *et al.* 1998, French *et al.* 1976, Paster *et al.* 1995, Grech-Mora *et al.* 1996). Watanabe *et al.* (2003) identified the bacterium *Streptomyces* sp. in the gut of *Odontotermes formosanus* (Table 1.2), which has the ability to degrade cellulose *in vitro*. On the other hand, the bacteria *Clostridium piliforme* (Tokuda *et al.* 2000) and *Burkholderia cepacia* (Kato *et al.* 1998) (Table 1.2) were identified in *Nasutitermes takasagoensis*, and both bacteria are capable of degrading lignin. While in *Nasutitermes lujae* and

*Nasutitermes exitiosus* the bacteria *C. freundii* (French *et al.* 1976), *Termite spirochete* (Paster *et al.* 1995) and *Sporobacter termitidis* (Grech-Mora *et al.* 1996) (Table 1.2) were isolated.

Culture-independent methods have been used to identify groups and genera of bacteria associated with the gut of *Microcerotermes crassus*, *Microcerotermes minutus*, *Cubitermes orthognathus*, *Cubitermes ugandensis* and *Cubitermes niokoloensis*. (Hongoh *et al.* 2005, Schmitt-Wagner *et al.* 2003, Fall *et al.* 2007). In *M. crassus* and *M. minutus*, the predominant bacterial groups are: Firmicutes (20.3 %), Proteobacteria (4.5 %), Bacteroidetes (5.9 %) and Spirochaetes (56.8 %) (Hongoh *et al.* 2005). Furthermore, in *C. orthognathus* and *C. ugandensis*, the bacteria of low content of guanine and cytosine are predominant in the majority of the gut (proctodeal segments 1, 3, 4 and 5), while the group of Cytophaga-Flexibacter-Bacteroides and Actinobacteria are frequent in proctodeal segments 5 and 4, respectively (Schmitt-Wagner *et al.* 2003). Additionally, the bacterial communities associated with *C. niokoloensis* were studied and the predominant groups are: Firmicutes, Clostridiales, Actinobacteria and Proteobacteria. While in the nest material of *C. niokoloensis* the following bacterial groups have been identified: Nocardioideae (Actinobacteria), Chloroflexi, Firmicutes and Proteobacteria (Fall *et al.* 2007).

#### **1.1.4 Role of the Actinobacteria associated with insects**

Actinobacteria are Gram positive bacteria with high content of guanine and cytosine (Ventura *et al.* 2007). Morphologically, this group of bacteria varies between cocci, coccobacilli or filamentous forms. These organisms are recognized for their physiological diversity and metabolic properties. The Actinobacteria are well-known for producing extracellular enzymes and secondary metabolites that are potent antibiotics (Ventura *et al.* 2007).

**Table 1.2- Genera and species of bacteria identified in the gut of different species of termites.**

Family	Species	Bacteria	Phylum	Reference
Mastotermitidae	<i>Mastotermes darwiniensis</i>	<i>Citrobacter freundii</i>	Proteobacteria	French <i>et al.</i> 1976
Archotermopsidae	<i>Zootermopsis angusticollis</i>	<i>Blattabacterium</i> sp.	Bacteroidetes	Sabree <i>et al.</i> 2011
		<i>Cellulomonas</i> sp.	Actinobacteria	Wenzel <i>et al.</i> 2002
		<i>Oerskovia</i> sp.	Actinobacteria	
		<i>Microbaterium</i> sp.	Actinobacteria	
		<i>Kocuria</i> sp.	Actinobacteria	
		<i>Bacillus melitensis</i>	Firmicutes	
		<i>Brevibacillus</i> sp.	Firmicutes	
		<i>Paenibacillus</i> sp.	Firmicutes	
		<i>Afipia</i> sp.	Proteobacteria	
		<i>Agrobacterium</i> sp.	Proteobacteria	
		<i>Rhizobium</i> sp.	Proteobacteria	
		<i>Brucella</i> sp.	Proteobacteria	
		<i>Ochrobactrum</i> sp.	Proteobacteria	
		<i>Pseudomonas</i> sp.	Proteobacteria	
		<i>Sphingomonas</i> sp.	Proteobacteria	
		<i>Zymomonas</i> sp.	Proteobacteria	
		<i>Spirosoma</i> sp.	Bacteroidetes	
		<i>Streptomyces</i> sp.	Actinobacteria	Watanabe <i>et al.</i> 2003
		TG1	TG1	Okuma <i>et al.</i> 2007
		<i>Streptomyces</i> sp.	Actinobacteria	Watanabe <i>et al.</i> 2003
Kalotermitidae	<i>Hodotermopsis japonica</i>			
Rhinotermitidae	<i>Hodotermopsis sjoestedti</i>			
Coptotermitinae	<i>Neotermes kashunensis</i>			
	<i>Coptotermes formosanus</i>	<i>Marinilabilia salmonicolar</i>	Bacteroidetes	Shinzato <i>et al.</i> 2005
		<i>Tannerella forsyhensis</i>	Bacteroidetes	
		<i>Bacteroides</i>	Bacteroidetes	
		<i>Eubacterium sulci</i>	Firmicutes	
		<i>Clostridium leptum</i>	Firmicutes	
		<i>Clostridium propionicum</i>	Firmicutes	
		<i>Mycoplasma</i> sp.	Firmicutes	
		<i>Leuconostoc mesenteroides</i>	Firmicutes	
		<i>Lactococcus</i>	Firmicutes	
		<i>Aminobacterium</i>	Synergistetes	
		<i>Treponema</i>	Spirochaetes	
		<i>Spirochaeta</i>	Spirochaetes	
		<i>Eggerthella lenta</i>	Actinobacteria	
		<i>Acidimicrobium ferrooxidans</i>	Actinobacteria	
		<i>Actinomyces meyeri</i>	Actinobacteria	
		<i>Desulfovibrio termitidis</i>	Proteobacteria	
		<i>Rhodoplanes elegans</i>	Proteobacteria	
		<i>Propionivibrio pelophilus</i>	Proteobacteria	
		<i>Victivallis vadensis</i>	Lentisphaerae	
		<i>Opitutus terrae</i>	Verrucomicrobia	
		<i>Serratia marcescens</i>	Proteobacteria	Adams and Boopathy 2005
		<i>Enterobacter aerogenes</i>	Proteobacteria	
		<i>Enterobacter cloacae</i>	Proteobacteria	
		<i>Citrobacter farmer</i>	Proteobacteria	
		<i>Streptomyces</i> sp.	Actinobacteria	Watanabe <i>et al.</i> 2003
		<i>Burkholderia</i> sp.	Proteobacteria	Harazono <i>et al.</i> 2003
		<i>Citrobacter</i> sp.	Proteobacteria	
		<i>Citrobacter freundii</i>	Proteobacteria	French <i>et al.</i> 1976
		<i>Desulfovibrio</i> sp.	Proteobacteria	Ohkuma and Kudo 1996
		<i>Treponema</i> sp.	Spirochaetes	
		<i>Bacteroides</i> sp.	Bacteroidetes	
		<i>Clostridium</i> sp.	Firmicutes	
		<i>Streptomyces</i> sp.	Actinobacteria	Watanabe <i>et al.</i> 2003
		TG1	TG1	Hongoh <i>et al.</i> 2008
		<i>Rhodococcus erythropolis</i>	Actinobacteria	Chung <i>et al.</i> 1994
		<i>Lactobacillus</i> sp.	Firmicutes	Schultz and Breznak 1978
		<i>Fusobacterium</i> sp.	Fusobacteria	
		<i>Streptococcus lactis</i>	Firmicutes	
		<i>Streptococcus cremoris</i>	Firmicutes	
		<i>Citrobacter</i> sp.	Proteobacteria	
		<i>Enterobacter cloacae</i>	Proteobacteria	
Termitidae				
Nasutitermitinae	<i>Nasutitermes takasagoensis</i>	<i>Burkholderia cepacia</i>	Proteobacteria	Kato <i>et al.</i> 1998
		<i>Clostridium piliforme</i>	Firmicutes	Tokuda <i>et al.</i> 2000
	<i>Nasutitermes exitiosus</i>	<i>Citrobacter freundii</i>	Proteobacteria	French <i>et al.</i> 1976
	<i>Nasutitermes lujae</i>	<i>Termite spirochete</i>	Spirochaetes	Paster <i>et al.</i> 1995
		<i>Sporobacter termitidis</i>	Firmicutes	Grech-Mora <i>et al.</i> 1996
Macrotermitinae	<i>Odontotermes formosanus</i>	<i>Streptomyces</i> sp.	Actinobacteria	Watanabe <i>et al.</i> 2003

Recently, genera of Actinobacteria have been found associated with different insects (Seipke *et al.* 2012). Haeder *et al.* (2009) described the association of *Streptomyces* with *Acromyrmex* spp. (leaf-cutter ants). The Actinobacteria associated with the exoskeleton of the ant (Attine), produce antibiotics that combat a pathogenic fungus (*Escovopsis*) that attacks the culture of the fungus (*Leucoagaricus*) that serves as food for these ants. In other research with fungus-growing ants the same type of association was found, but with other Actinobacteria genus: *Pseudonocardia* (Currie *et al.* 2003a, Currie *et al.* 1999b, Cafaro and Currie 2005). Another example of mutualistic association is evident in the beetle *Dendroctonus frontalis*, which has a similar relationship with *Streptomyces* to protect their food source, the fungus *Entomocorticium* (Scott *et al.* 2008, Seipke *et al.* 2012).

In addition, another protecting association with *Streptomyces* and *Nocardiopsis* was found in the beewolf *Philanthus coronatus* and in the honeybee *Apis mellifera*, respectively (Kaltenpoth and Göttler 2005, Patil *et al.* 2010, Seipke *et al.* 2012). Furthermore, the genera *Streptomyces*, *Cellulomonas*, *Oerskovia*, *Microbaterium*, *Kocuria*, *Eggerthella*, *Acidimicrobium*, *Actinomyces* and *Rhodococcus* were also found associated with the termite gut (Wenzel *et al.* 2002, Watanabe *et al.* 2003, Shinzato *et al.* 2005, Chung *et al.* 1994). The function of these Actinobacteria is to produce extracellular enzymes to degrade cellulose and lignocellulose, which are consumed by the termites (Ohkuma and Kudo 1996, Watanabe *et al.*, 2003, Chung *et al.* 1994). At present, it is unknown if Actinobacteria are associated with the exoskeleton of termites. Hence, it is not known if a protective role such as in ants, beetles, wasps and bees exists in termites. Other studies (Sun *et al.* 2003, Sun *et al.* 2002) indicate that *Metarhizium anisopliae* and *Beauveria bassiana* attack the termite nests as occurred to other insects (Hughes *et al.* 2004, Shah and Pell 2003). *Metarhizium anisopliae* and *B. bassiana* serve as biocontrol entomopathogenic fungi and have

been extensively studied in termites (Delate *et al.* 1995, Neves and Alves 2004, Sun *et al.* 2003, Sun *et al.* 2002), but the interaction with Actinobacteria is unknown.

#### **1.1.5 *Nasutitermes costalis* (Isoptera: Termitidae: Termitinae: Nasutitermitinae)**

*Nasutitermes costalis* is a higher termite that lives in tropical and subtropical zones. Generally, *N. costalis* is xylophagous, feeding on dry wood and establishing nests on trees with tunnels that reach into the ground. These termites are divided into three castes: reproductive, soldier and worker (Bignell *et al.* 2011). Soldiers are characterized by dark head and a structure that projects from the head called nasu (Scheffrahn *et al.* 2003), which is used to disperse the chemicals  $\alpha$ -pinene and limonene, which serve as a defense against pathogens and predators (Rosegaus *et al.* 2000, Fuller 2007). *Nasutitermes costalis* is the most common termite species in Puerto Rico (Scheffrahn *et al.* 2003) and has only been studied for its impact as a common plague (Rosegaus *et al.*, 2000).

### **1.2 Questions and objectives**

#### **1.2.1 Questions**

1. Is the community of Actinobacteria associated with *N. costalis* dominated by *Streptomyces*?
2. Is the variation in *Streptomyces* community associated with seasonal changes, termite caste or anatomy region (gut or exoskeleton)?
3. Do *Streptomyces* isolates have activity against fungal pathogens?

### 1.2.2 Objectives

1. Characterize Actinobacteria community associated with *N. costalis* through culture-dependent method (morphological and molecular characterization).
2. Compare communities of *Streptomyces* associated with *N. costalis* using phylogenetic and statistical analyses.
3. Perform bioassays against entomopathogenic fungi.

## **2 Diversity of Actinobacteria associated with *Nasutitermes costalis* termite (Isoptera: Termitidae)**

### **2.1 Introduction**

Recently, many bacterial communities have been studied for their association with different groups of insects (Kaltenpoth 2009). Bacterial communities use their hosts as their habitat and contribute to the degradation process of organic matter (Pasti *et al.* 1990 Visôto *et al.* 2009). Bacteria contribute to nitrogen fixation (French *et al.* 1976, Pinto-Tomás *et al.* 2009) and protect the host from pathogenic organisms (Kaltenpoth 2009, Haeder *et al.* 2009). Consequently, a diverse number of bacteria belonging to the class Actinobacteria have been found associated with different types of insects. In fungus-growing ants (Attine), a symbiotic association with *Streptomyces* and *Pseudonocardia* (Actinobacteria), which secrete compounds preventing the growth of a pathogenic fungus (*Escovopsis*) that attacks the fungus culture (*Leucoagaricus*) that serves as food for these ants was extensively studied (Currie *et al.* 2003a, Currie *et al.* 1999b, Cafaro and Currie 2005, Haeder *et al.* 2009). Other associations with Actinobacteria were also described for several insects including beetles (*Dendroctonus frontalis*), wasps (*Philanthus coronatus*) and bees (*Apis mellifera*) (Scott *et al.*, 2008, Kaltenpoth and Göttler 2005, Patil *et al.* 2010, Seipke *et al.* 2012).

In the gut of termites, several members of the Actinobacteria have already been identified: *Streptomyces*, *Cellulomonas*, *Oerskovia*, *Microbacterium*, *Kocuria*, *Eggerthella*, *Acidimicrobium*, *Actinomyces* and *Rhodococcus* (Wenzel *et al.* 2002, Watanabe *et al.* 2003 Shinzato *et al.*, 2005, Chung *et al.* 1994). Known functions of these Actinobacteria include production of extracellular enzymes that degrade cellulose and lignocellulose consumed by the termites (Ohkuma and Kudo

1996, Watanabe *et al.*, 2003, Chung *et al.* 1994). At present, no data exist about the community of Actinobacteria that live in association with the exoskeleton of termites. Therefore, the aim of this research was to describe and characterize Actinobacteria communities that are associated with *Nasutitermes costalis* using culture-dependent methods. In addition, we determined similarities in communities of Actinobacteria associated with different termite castes (workers and soldiers) and anatomical regions (exoskeleton and gut).

## **2.2 Materials and methods**

### **2.2.1 Sampling area**

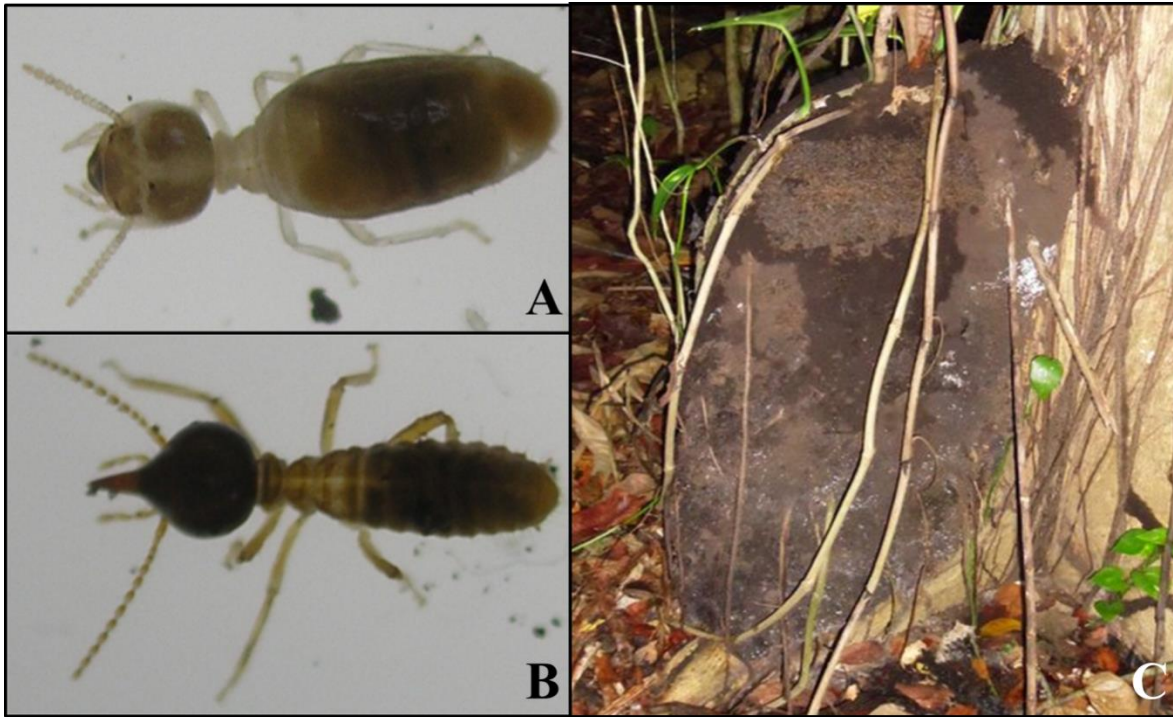
The sampling site selected for this study was an urban forest behind the building of the Biology Department (University of Puerto Rico, Mayagüez Campus) located in Miradero, Mayagüez, Puerto Rico. Sampling was performed during the wet (May to October) and dry (November to April) seasons. Sampling in wet season was on June 28, 2010 with a temperature of  $24.83 \pm 0.21$  °C and rainfall of 0.10 mm and in dry season was on February 18, 2011 with a temperature of  $24.93 \pm 0.12$  °C and rainfall of 0.25 mm (PRISM climate data, NOAA). For every season three different nests of *N. costalis* were selected during collection.

### **2.2.2 Sample collection**

Pieces of each nest were collected using a hacksaw previously sanitized (with 70% ethanol and flamed) and placed in sterile containers. Termites were collected with sterile forceps (10 termites from each caste). Soldier termites are easily identified because they have frontal tube (nasu) that is absent in workers (Figure 2.1). Samples were placed in previously sanitized



containers (70% ethanol) for transportation to the laboratory. During the dry season there was no collection of termite samples in nest 3 as there were no live termites found.



**Figure 2.1-** *Nasutitermes costalis*: worker termite (A), soldier termite (B) and nest (C).

### 2.2.3 Sample processing

Ten termites were collected (per nest) of the worker and soldier caste, respectively. Five of these were separately placed (with sterile forceps) in tubes of 1.5 mL with 500  $\mu$ L of sterile distilled water. Five termites were processed for gut extraction using sterile forceps. We grabbed the termites by the head with sterile forceps to provide support. Then, with another sterile forceps was grabbed the anal area and was pulled to remove the intestine. The guts were macerated (Disposable Pellet Mixers and Cordless Motor, VWR<sup>®</sup>) in 1.5 mL tubes with 500  $\mu$ L of sterile

distilled water. The negative control was a sterile sample tube processed in the same manner for each type of sample (exoskeleton and gut).

All samples were vortexed for 15 seconds twice with a 10 minute sonication (Ultrasonic Cleaner Branson 200, Emerson™) intermediate process at room temperature (25 °C) and 40 Hz, to dislodge bacteria from the substrate (exoskeleton and gut). For nest samples, 3 grams of nest material were taken out using a sterile spatula and placed in sterile centrifuge tubes with 47 mL of sterile distilled water and vortexed for 10 minutes.

#### **2.2.4 Isolation of Actinobacteria**

Isolation was performed by taking out 500 µL of each suspension from gut, exoskeleton and nest material. The spread plate technique was used for isolation by spreading each sample (separately) with a hockey stick loop in a petri dish of 100 mm diameter containing chitin medium as previously described (Cafaro and Currie 2005) The chitin medium was modified by adding the antifungals cyclohexamide (0.05 mg/mL) and nystatin (2 mg/mL). The plates were placed in the incubator at 25 °C and observed weekly for one month. Actinobacteria colonies in the chitin medium are observed as small point ranging in color from gray to white. The striated 4 quadrants technique was used for maintenance of Actinobacteria in yeast malt extract agar (4 g of yeast extract, 10 g of malt extract, 4 g of dextrose, 20 g of agar and 1000 mL of distilled water) supplemented with cyclohexamide (0.05 mg/mL) and nystatin (2 mg/mL).

### **2.2.5 Macroscopic characterization of colonies**

Size, shape, pigmentation, margin and elevation of isolated colonies were recorded for all strains from the gut, exoskeleton, and nest material. Macroscopic observations and Gram staining were performed for all strains.

### **2.2.6 Selection of strains for molecular identification**

Using the previous macroscopic observations and the sample origin, isolates were grouped by morphotype and the frequency for each morphotype was determined. An isolate of each morphotype was selected for molecular identification.

### **2.2.7 Genomic DNA extraction**

For the selected morphotypes total genomic DNA was extracted using the protocol established by Cafaro and Currie (2005) with the following modifications. We used two methods to lyse the cells: physical method with maceration (Disposable Pellet Mixers and Cordless Motor, VWR<sup>®</sup>) and chemical method with lysozyme (0.01g/mL). DNA quality was checked by electrophoresis in 1% agarose gel stained with ethidium bromide.

### **2.2.8 Polymerase chain reaction (PCR)**

Amplification of the 16S rDNA gene was performed using approximately 40 ng of DNA template. We used 25 µL of master mix that contains: 0.8x PCR buffer (5x Colorless GoTaq<sup>®</sup> Flexi Buffer, Promega), 2.5 mM MgCl<sub>2</sub>, 0.3 µM of each primer 0.16 mM dNTPs and 5 units Taq

polymerase (Applied Biological Materials) per reaction. Parameters used in the thermocycler (MyCycler Thermal Cycler PCR, Bio-Rad<sup>®</sup>) were: 95 °C 3', 95 °C 45'', 52 °C 45'', 72 °C 1'3'' and 72 °C 7' for 30 cycles. We use the universal bacterial primers (Lane 1991, Weisburg *et al.* 1991) 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCT TGTTCGACTT-3') for the 16S rRNA gene. A negative control was used included using master mix without DNA template.

### **2.2.9 DNA sequencing**

PCR products were assessed by electrophoresis and sent to be sequenced by the High-Throughput Genomics Unit at the University of Washington, Seattle, Washington. The sequences obtained were analyzed and edited using Sequencher 4.3 (Gene Codes, MI).

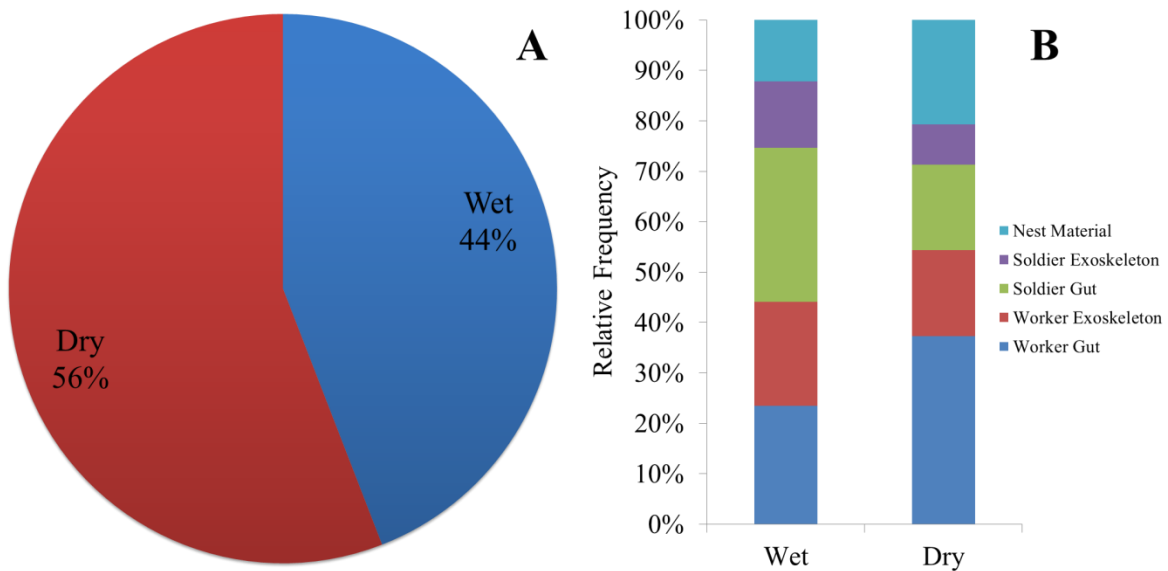
### **2.2.10 Data analysis**

Sequences were analyzed against the database of the National Center for Biotechnology Information (NCBI) using BLAST<sup>®</sup> (Basic Local Alignment Search Tool) in GenBank to determine the percentage of similarity with species and strains already available in the database. The alignment and phylogenetic analysis (Neighbor-Joining) were performed using the program Mega 5 (Tamura *et al.* 2011). In addition, we used the EstimateS 7.5.2 program (Colwell 2005) to determine species richness (Chao1) and diversity (Fisher's alpha, Shannon-Weaver and Simpson) indices of each sample. Rarefaction curves were used to determine the magnitude of sampling ( $S_{obs}$ ) depending on the ability to isolate the rare species (Colwell 2005).

## 2.3 Results

### 2.3.1 Colonies isolated from *N. costalis*

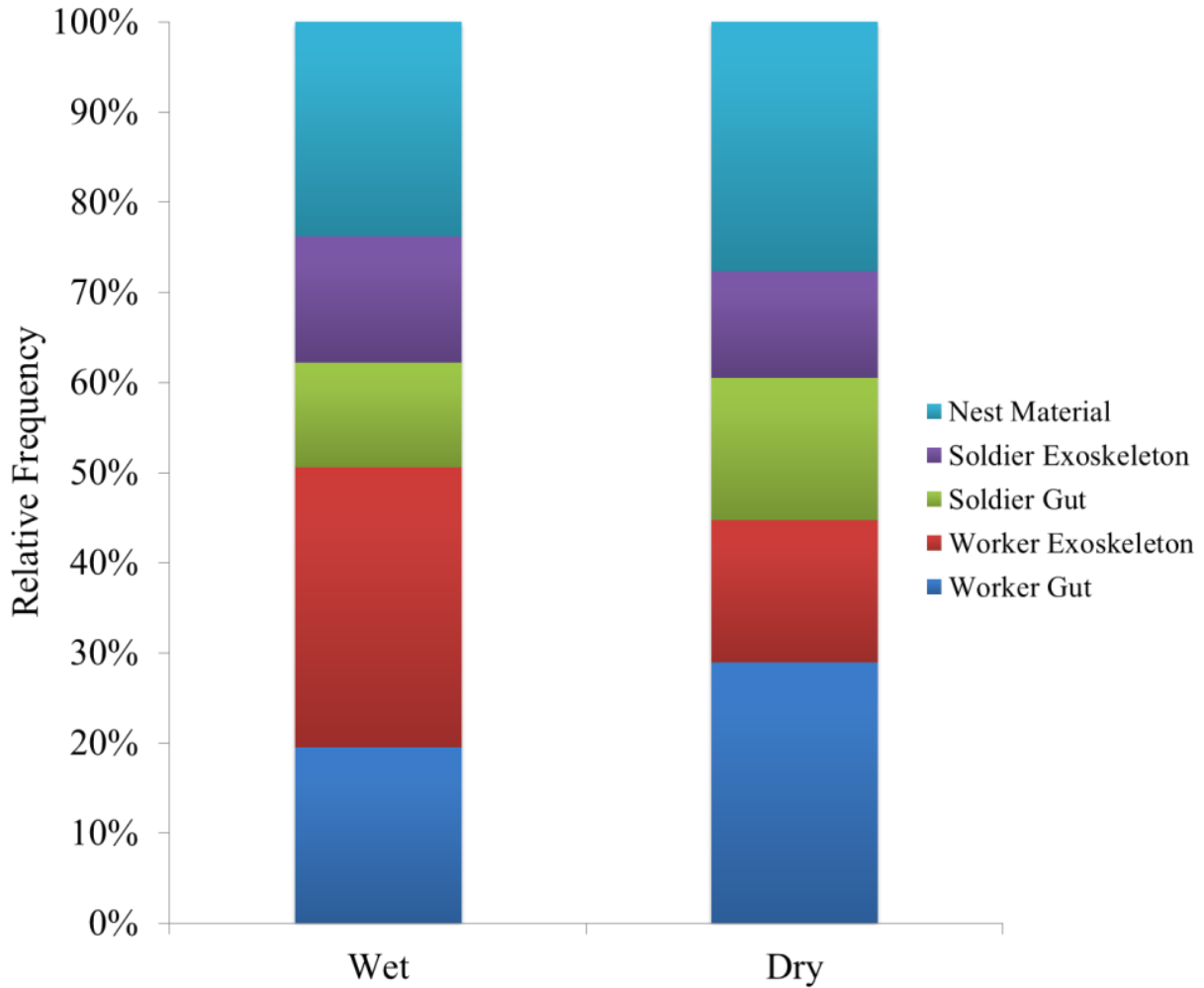
In total we isolated 1342 colonies of bacteria in this study, of which 592 (44%) were isolated in the wet season and 750 (56%) in the dry season (Figure 2.2A). Of the isolates in the wet season, 139 (23%), 122 (21%), 181 (31%), 78 (13%) and 72 (12%) colonies we obtained in the sample of worker gut, worker exoskeleton, soldier gut, soldier exoskeleton and nest material respectively. In the dry season 280 (37%), 128 (17%), 127 (17%), 60 (8%) and 155 (21%) colonies we isolated, respectively (Figure 2.2B).



**Figure 2.2-** Isolated colonies percentage per season (A) and relative frequency of the isolated colonies per sample during the season (B).

After isolation, we proceeded to observe the macroscopic characteristics of colonies in each sample and grouped by morphotypes (Appendix A to K). In the wet season samples were isolated

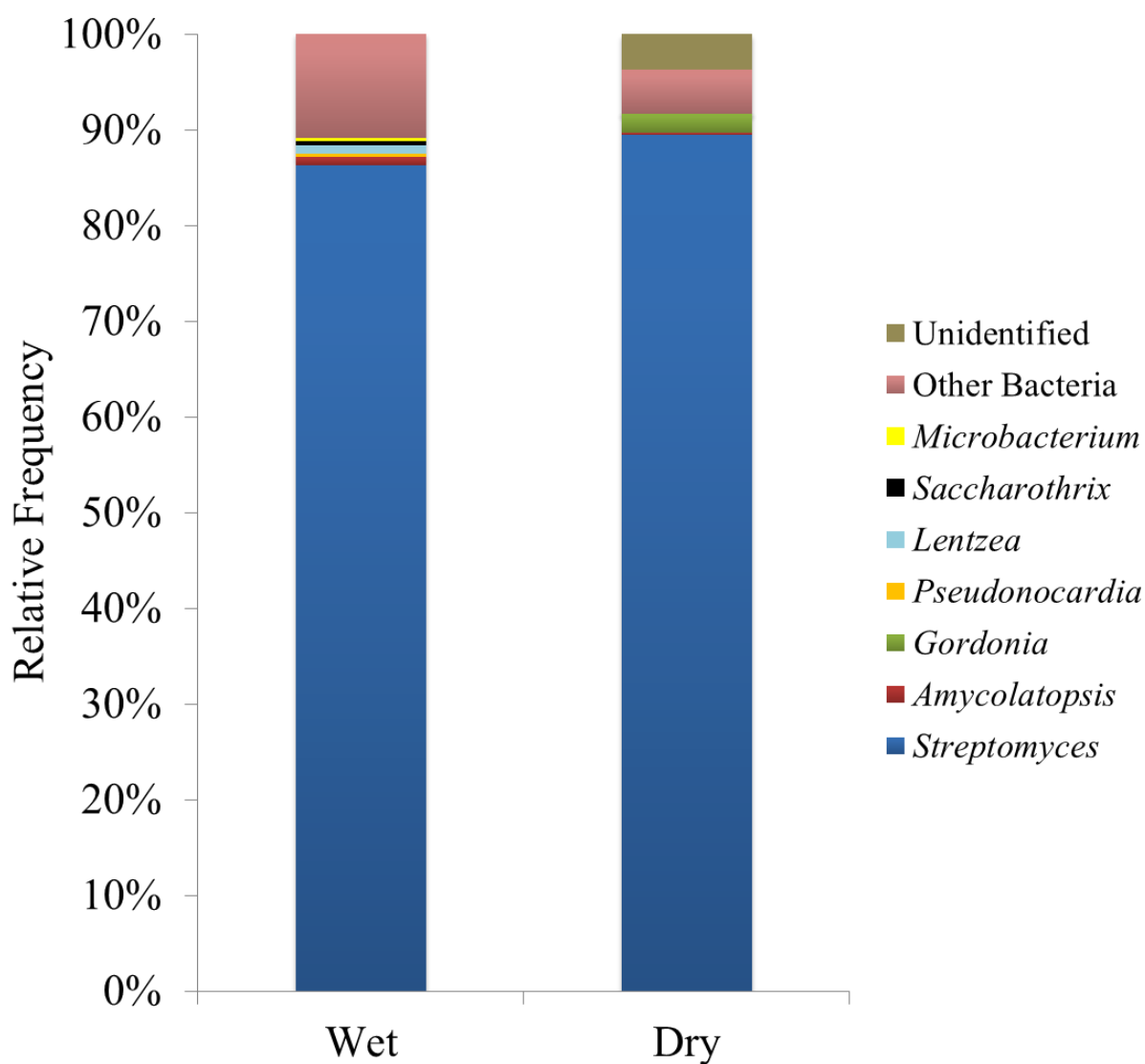
32 (19%), 51 (31%), 19 (12%), 23 (14%) and 39 (24%) morphotypes for worker gut, worker exoskeleton, soldier gut, soldier exoskeleton and nest material respectively. While in the dry season we isolated 22 (29%), 12 (16%), 12 (16%), 9 (12 %) and 21 (27%) morphotypes respectively (Figure 2.2).



**Figure 2.3-** Relative frequency of morphotypes isolated in different samples in wet and dry seasons.

### 2.3.2 Genera of Actinobacteria associated with *N. costalis*

The genera of Actinobacteria that we identified in the wet season are *Streptomyces* (86.3%), *Amycolatopsis* (0.8%), *Pseudonocardia* (0.3%), *Lentzea* (0.8%), *Saccharothrix* (0.5%) and *Microbacterium* (0.3%) (Figure 2.4). In the dry season, only three genera we identified: *Streptomyces* (89.5%), *Amycolatopsis* (0.3%) and *Gordonia* (2.0%) (Figure 2.4).



**Figure 2.4-** Relative frequency of Actinobacteria genera isolated in wet and dry seasons.

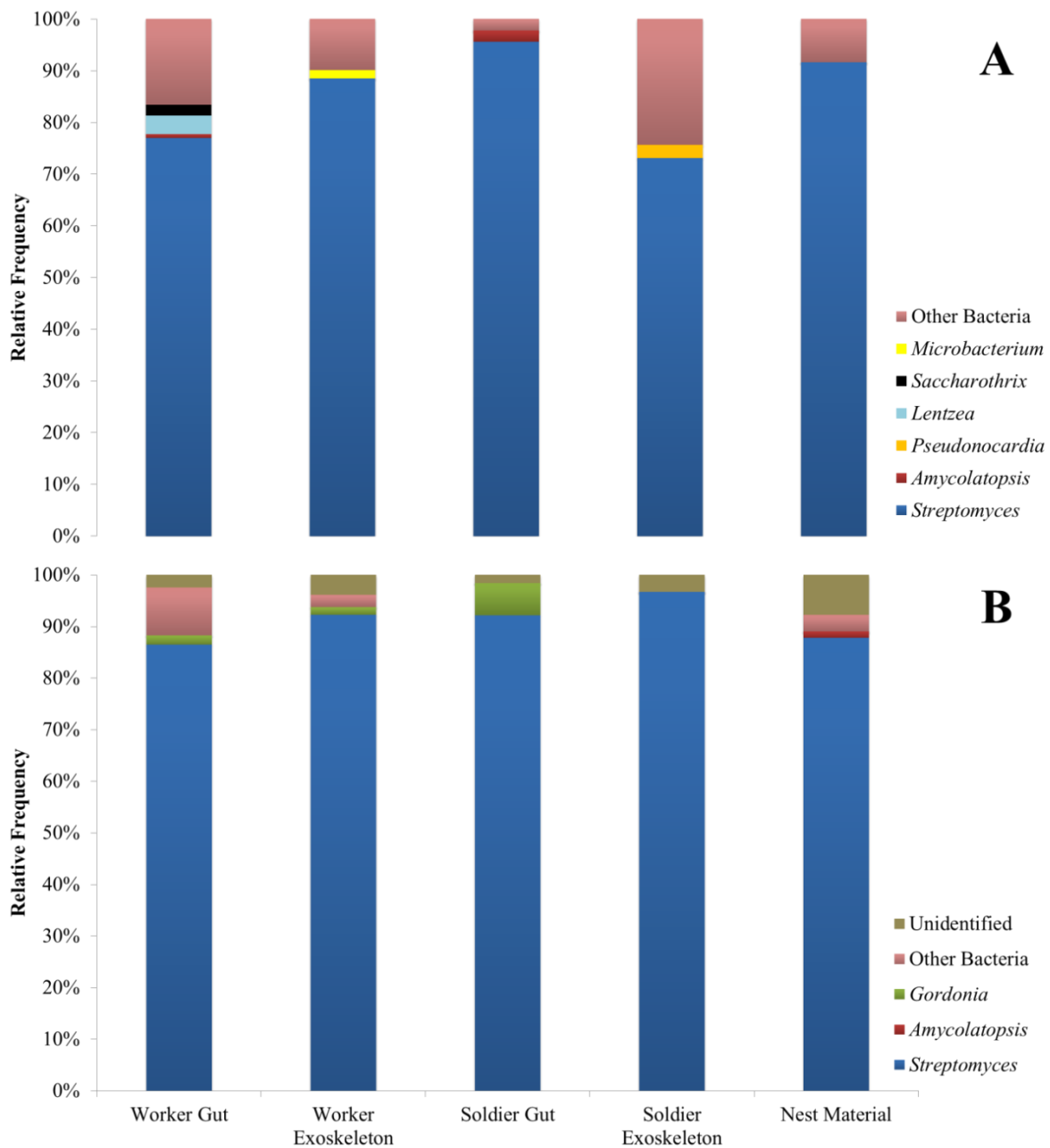
For all communities sampled, the most prevalent genus was *Streptomyces*. In the wet season, *Streptomyces* was isolated in high frequency in all partitions studied of 77%, 89%, 96%, 73% and 92% in worker gut, worker exoskeleton, soldier gut, soldier exoskeleton and nest material respectively (Figure 2.5A). *Amycolatopsis* was found in low frequency only in the gut of workers (1%) and soldiers (2%) (Figure 2.5A). In the gut of termite workers the following genera were also identified in low frequency: *Lentzea* (4%) and *Saccharothrix* (2%) (Figure 2.5A). Furthermore, *Pseudonocardia* and *Microbacterium* were identified in soldier (3%) and worker (2%) exoskeletons, respectively (Figure 2.5A). In the dry season, *Streptomyces* was isolated in high frequency from all samples of 86%, 92%, 92%, 97% and 88% in worker gut, worker exoskeleton, soldier gut, soldier exoskeleton and nest material respectively (Figure 2.5B). *Gordonia* was identified in worker gut (2%), worker exoskeleton (2%) and soldier gut (6%) in relatively low frequency (Figure 2.5B). While *Amycolatopsis* was only isolated in the nest material with 1% (Figure 2.5B).

### **2.3.3 Actinobacteria associated with *N. costalis* workers in wet season**

Sequencing results of the morphotypes W2OE27[1], W3OE48[1], W1OE133[3], W1OE135[4], W1OE139[5], W1OE169[2], W1OE174[3], W1OE187[2], W1OE192[2] and W1OE441[2] isolated from termite worker exoskeleton in the wet season are closely related to *Streptomyces globisporus* (JQ284036.1) (Appendix L) with 23% of frequency (25 strains) (Figure 2.6A). The morphotypes W2OE30[1], W2OE40[1], W1OE136[4], W1OE176[2], W1OE185[6], W1OE186[1], W1OE191[1] and W1OE440[5] are closely related to *Streptomyces cavourensis* (HQ610450.1) (Appendix L) with 19% of frequency (21 strains) (Figure 2.6A). And the morphotypes W2OE32[2], W1OE130[2], W1OE177[3] and W1OE529[5] are similar 99% to



*Streptomyces microflavus* (JF778669.1) (Appendix L) with 11% of frequency (12 strains) (Figure 2.6A). While the remaining 2% (1 morphotype, 2 strains) and 45% (19 morphotypes, 50 strains) of *Microbacterium* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with workers exoskeleton in wet season (Figure 2.6A).



**Figure 2.5-** Relative frequency of Actinobacteria isolated from the different anatomical regions of the workers, the soldiers and the nest material of *N. costalis* in wet (A) and dry (B) season.

Moreover, in the worker gut from wet season, the morphotype with high frequencies is W1OI103[33] with 28% of frequency (33 strains) and similar 99% to *Streptomyces* sp. Av25\_4 (FJ490534.1) (Figure 2.6B, Appendix L). Furthermore the morphotypes W1OI423[14] (similar 99% to *Streptomyces atratus*, JN862838.1) and W3OI56[9] (closely related to *Streptomyces chromofuscus*, FJ486284.1) obtained 12% (14 strains) and 8% of frequency (9 strains) respectively (Figure 2.6B, Appendix L). While the remaining 1% (1 morphotype, 1 strain), 3 % (1 morphotype, 3 strains), 4% (1 morphotype, 5 strains) and 44% (18 morphotypes, 51 strains) of *Amycolatopsis*, *Saccharothrix*, *Lentzea* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with workers gut in wet season (Figure 2.6B).

#### **2.3.4 Actinobacteria associated with *N. costalis* soldiers in wet season**

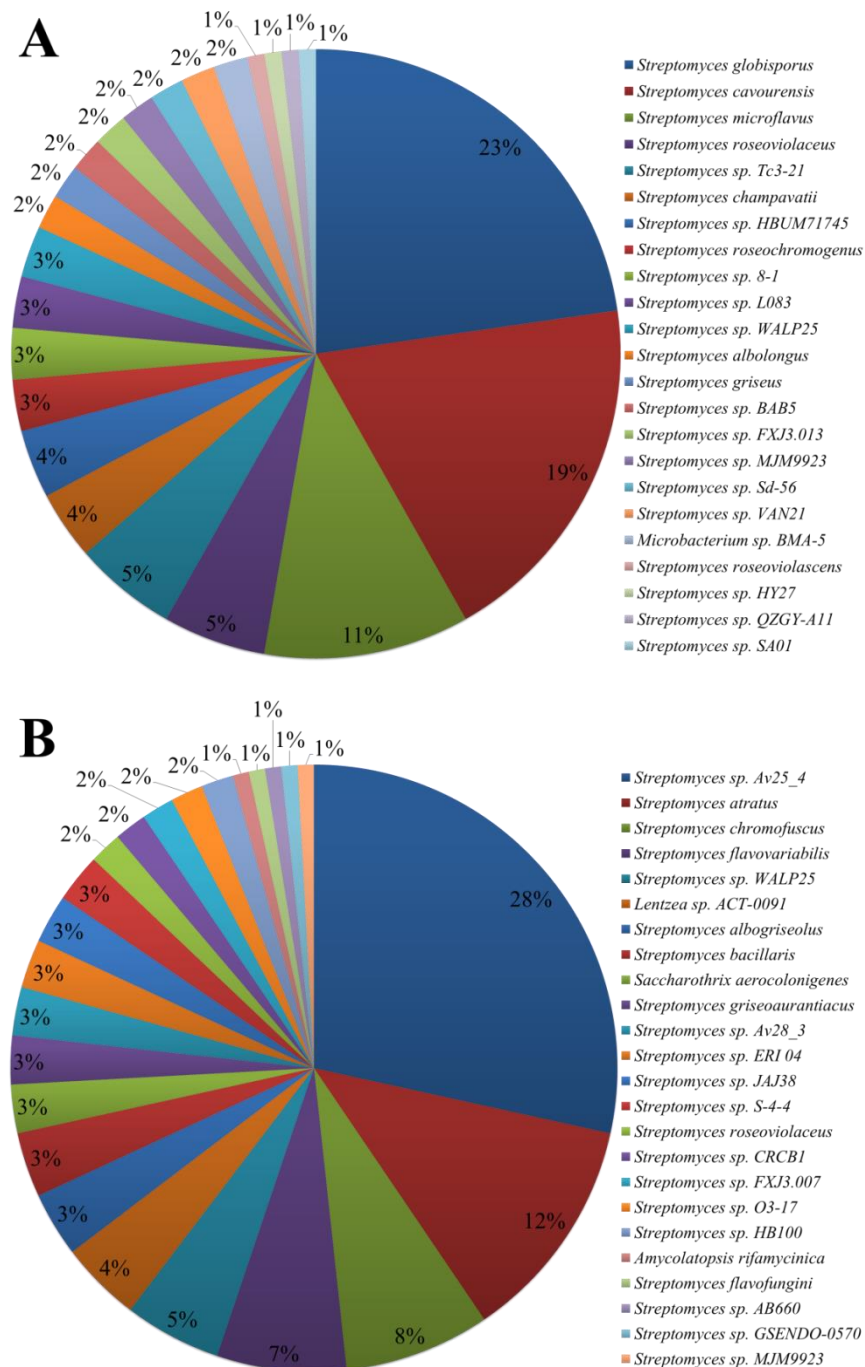
In the soldiers exoskeleton for wet season, the most frequent morphotypes were W2SE111[9] and W2SE43[7] with 15% (9 strains) and 12% of frequency (7 strains) (Figure 2.7A). These two morphotypes are similar 99% to *Streptomyces kanamyceticus* (AB184388.1) and *Streptomyces* sp. CGMCC (JQ824035.1), respectively (Appendix L). Furthermore the morphotypes W1SE147[1], W1SE149[4] (closely related to *Streptomyces* sp. 1x, EU360152.1), W2SE161[5] (similar 99% to *Streptomyces* sp. SA01, GU294692.1) and W1SE182[5] (similar 99% to *Streptomyces* sp. BF-3, JN408756.1) have a total of 8% of frequency individually (Figure 2.7A, Appendix L). While the remaining 3% (1 morphotypes, 2 strains) and 46% (11 morphotypes, 26 strains) of *Pseudonocardia* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with soldier exoskeleton in wet season (Figure 2.7A).

On the other hand, in soldiers gut the most frequent morphotype was W1SI515[85] similar 99% to *Streptomyces* sp. BAB5 (JF799913.1) with 48% (85 strains) of frequency, followed by W2SI113[15] (similar 99% to *Streptomyces mediolani*, FJ792545.1) with 8% (15 strains) of frequency and W1SI93[13] (closely related to *Streptomyces* sp. O3-17, JQ771582.1) with 7% (13 strains) of frequency (Figure 2.7B, Appendix L). While the remaining 2% (1 morphotype, 4 strains) and 35% (12 morphotypes, 60 strains) of *Amycolatopsis* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with soldier gut in wet season (Figure 2.7B).

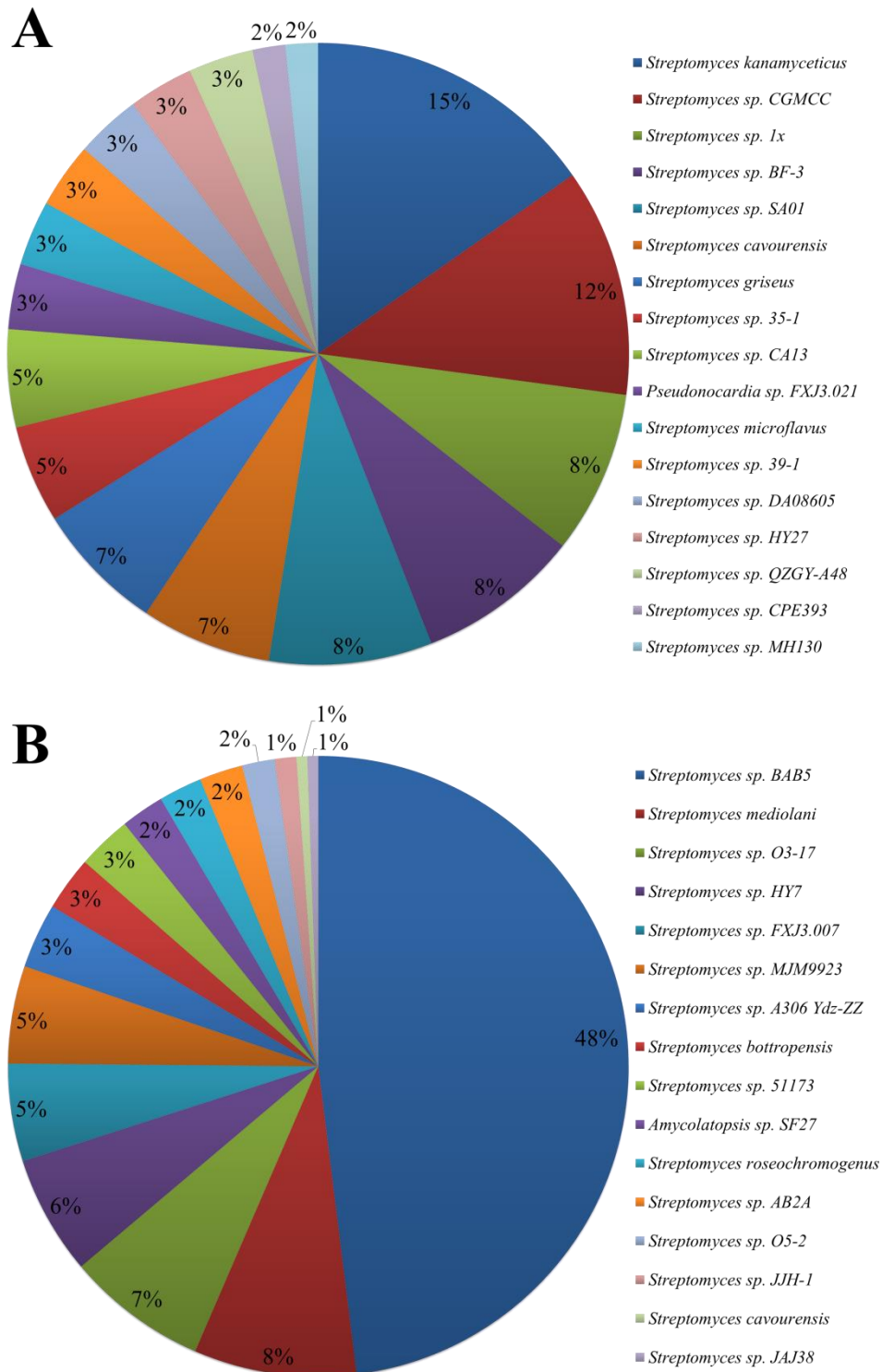
### **2.3.5 Actinobacteria associated with *N. costalis* workers in dry season**

In workers exoskeleton for dry season, the most frequent morphotype (50% of all isolates) was D1OE214[60] closely related to *Streptomyces cavourensis* (HQ610450.1), followed by D1OE213[8] and D1OE447[12] (similar 99% to *Streptomyces microflavus*, JF778669.1) with 17% of frequency (20 strains) and D1OE297[15] (closely related to *Streptomyces* sp. JAJ38, JN859008.1) with 12% of frequency (15 strains) (Figure 2.8A, Appendix L). While the remaining 2% (1 morphotype, 2 strains) and 19% (4 morphotypes, 23 strains) of *Gordonia* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with worker exoskeleton in dry season (Figure 2.8A). But in the workers gut, the most frequent morphotype was D1OI396[160] similar 99% to *Streptomyces* sp. A8Ydz-XM (EU257235.1) with 65% of frequency (160 strains), followed of D1OI321[18] (similar 99% to *Streptomyces atratus*, JN862838.1) with 7% (18 strains) and D2OI337[2], D2OI430[5] and D2OI485[6] (similar 99% to *Streptomyces* sp. 8-1, EU054375.1) with 5% of frequency (13 strains) (Figure 2.8B, Appendix L). While the remaining 2% (1 morphotype, 4 strains) and 21% (10 morphotypes, 52 strains) of

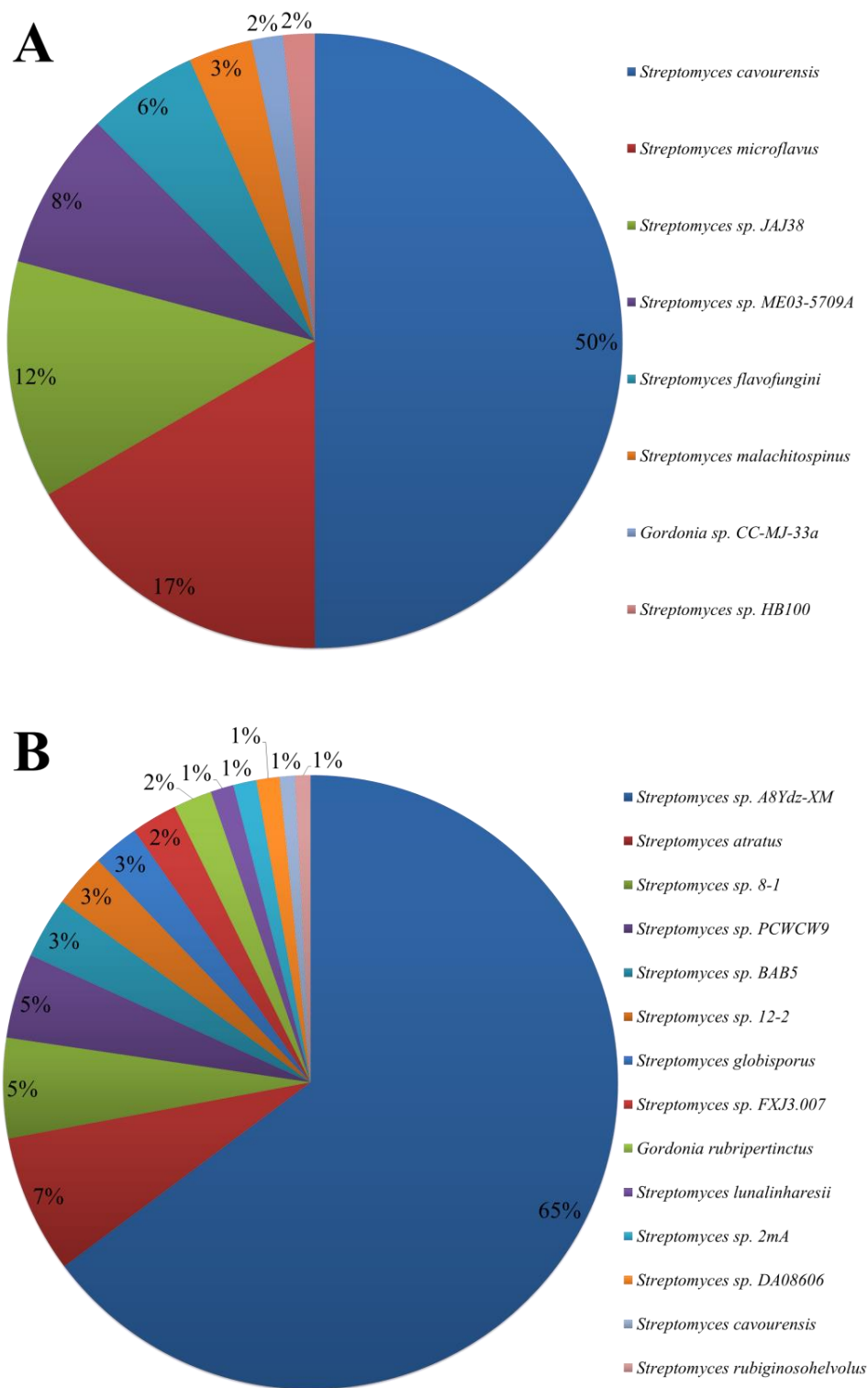
*Gordonia* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with worker gut in dry season (Figure 2.8B).



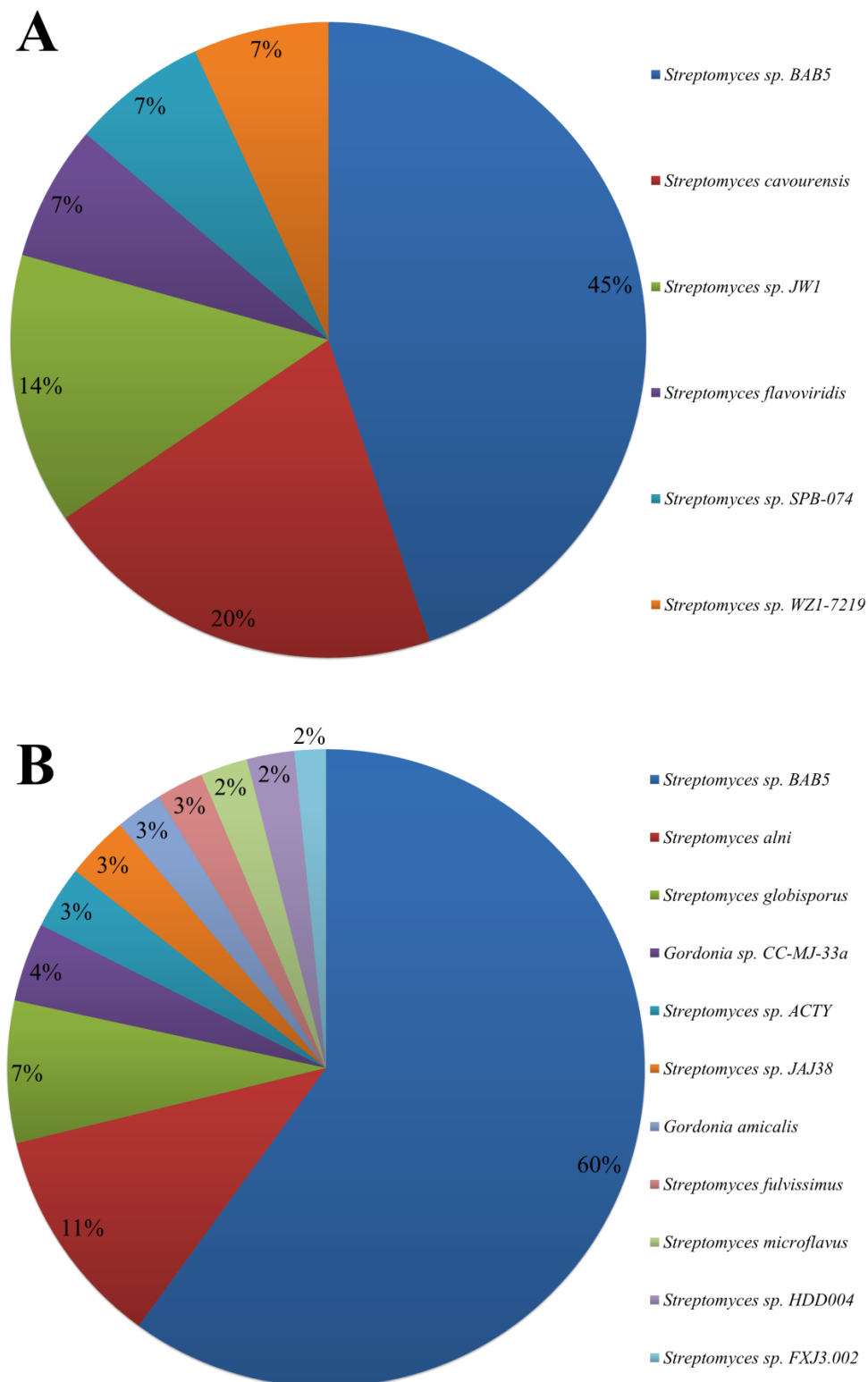
**Figure 2.6-** Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the worker termites *N. costalis* for the wet season. Identification of Actinobacteria was performed using BLAST® in GenBank.



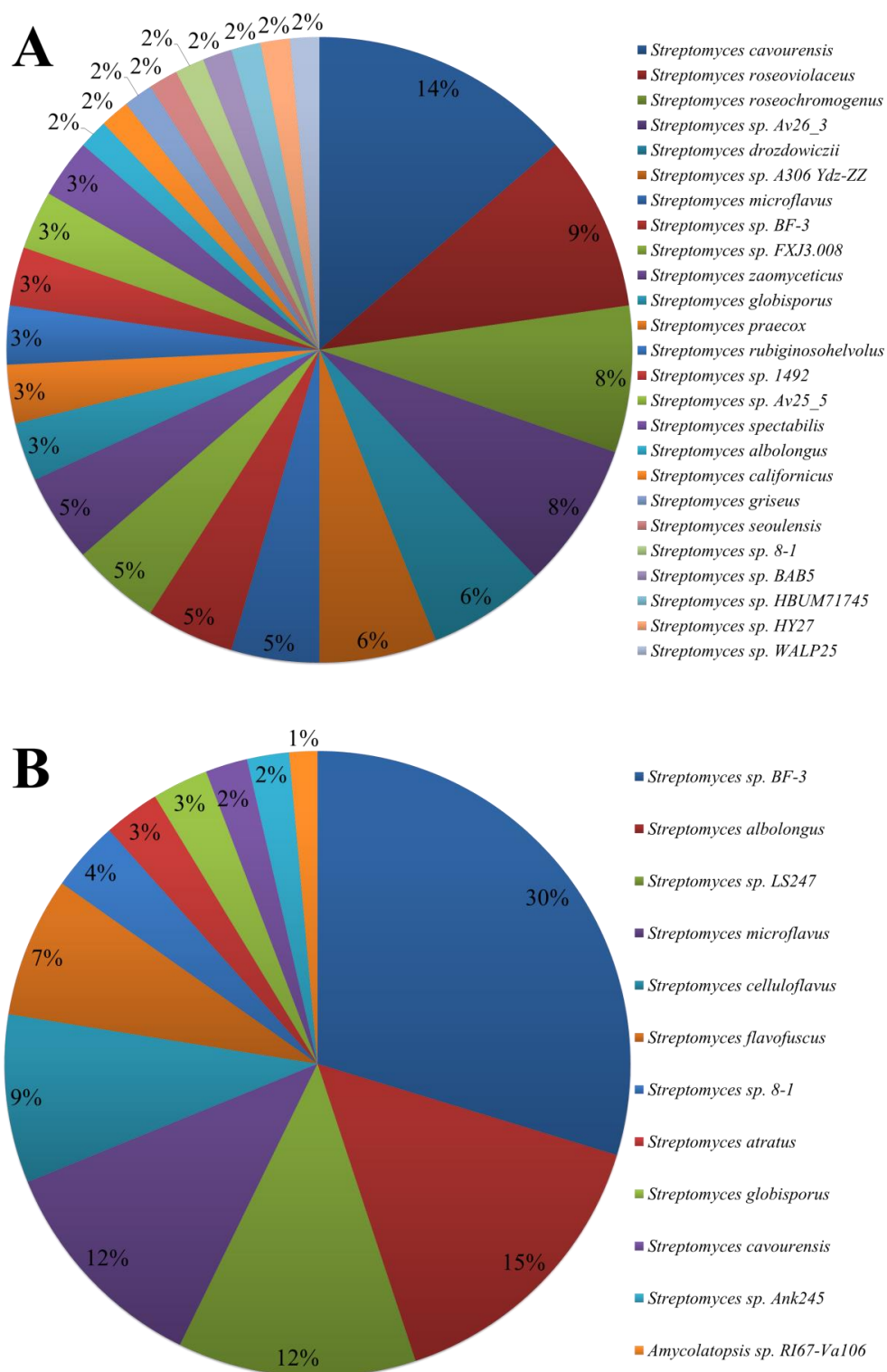
**Figure 2.7-** Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the soldiers termites *N. costalis* for the wet season. Identification of Actinobacteria was performed using the BLAST<sup>®</sup> data base.



**Figure 2.8-** Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the workers termites *N. costalis* for the dry season. Identification of Actinobacteria was performed using BLAST® in GenBank.



**Figure 2.9-** Frequency of similarity of the Actinobacteria isolated from the exoskeleton (A) and gut (B) of the soldiers termites *N. costalis* for the dry season. Identification of Actinobacteria was performed using BLAST® in GenBank.



**Figure 2.10-** Frequency of similarity of the Actinobacteria isolated from the nest material of *N. costalis* for wet (A) and dry (B) season. Identification of Actinobacteria was performed using BLAST® in GenBank.



### 2.3.6 Actinobacteria associated with *N. costalis* soldiers in dry season

In the soldiers exoskeleton for dry season, the most frequent morphotypes were D2SE261[16] and D2SE433[10] these are closely related to *Streptomyces* sp. BAB5 (JF799913.1) with 45% of frequency (26 strains), followed by the morphotypes D1SE216[6] and D2SE257[6] (similar 100% to *Streptomyces cavourensis*, HQ610450.1) with 20% (12 strains) and D2SE262[8] (similar 99% to *Streptomyces* sp. JW1, EU906929.1) with 14% (8 strains) (Figure 2.9A, Appendix L). While the remaining 21% (3 morphotypes, 12 strains) of *Streptomyces* represent the less frequent Actinobacteria associated with soldier exoskeleton in dry season (Figure 2.9A).

With respect to the isolates in the gut of the soldier, the most frequent morphotype was D1SI281[75] similar 99% to *Streptomyces* sp. BAB5 (JF799913.1) with 60% of frequency (75 strains) (Figure 2.9B, Appendix L). Furthermore, the morphotypes D1SI287[14] (similar 100% to *Streptomyces alni*, NR\_043866.1) and D1SI286[9] (similar 99% to *Streptomyces globisporus*, JQ284036.1) was obtained 11% (14 strains) and 7% of frequency (9 strains) respectively. The 7% (2 morphotypes, 8 strains) and 15% (6 morphotypes, 19 strains) of *Gordonia* and *Streptomyces*, respectively, represent the least frequent Actinobacteria isolated in soldiers gut for the dry season (Figure 2.9B).

### 2.3.7 Actinobacteria associated with *N. costalis* nest material in wet and dry seasons

In the nest material for the wet season, the most frequent morphotypes were W2N4[1], W2N6[1], W2N7[1], W2N15[1], W3N72[3], W2N160[1] and W1N196[1] which were closely related to *Streptomyces cavourensis* (HQ610450.1) with a total of 14% of frequency (9 strains)

(Figure 2.10A, Appendix L). These were followed by the morphotypes W3N412[3] and W2N494[3] (similar of 99% to *Streptomyces roseoviolaceus*, JQ682626.1) with 9% of frequency (6 strains) and W2N18[5] (similar 92% to *Streptomyces roseochromogenus*, AB184777.2) with 8% of frequency (5 strains) (Figure 2.10A, Appendix L). The 69% (22 morphotypes, 46 strains) of *Streptomyces* represent the least Actinobacteria isolated in nest material for the dry season (Figure 2.10A).

Moreover, for the dry season, the most frequent morphotypes were D3N424[41] (similar 99% to *Streptomyces* sp. BF-3, JN408756.1), D1N42[21] (similar 99% to *Streptomyces albolongus*, JN609385.1) and D1N421[17] (similar 99% to *Streptomyces* sp. LS247, FJ919601.1) with 30% (41 strains), 15% (21 strains) and 12% (17 strains) of frequency, respectively (Figure 2.10B, Appendix L). While the remaining 1% (1 morphotypes, 2 strains) and 42% (8 morphotypes, 57 strains) of *Amycolatopsis* and *Streptomyces*, respectively, represent the less frequent Actinobacteria associated with nest material in dry season (Figure 2.10B).

### **2.3.8 Actinobacteria diversity associated with *N. costalis***

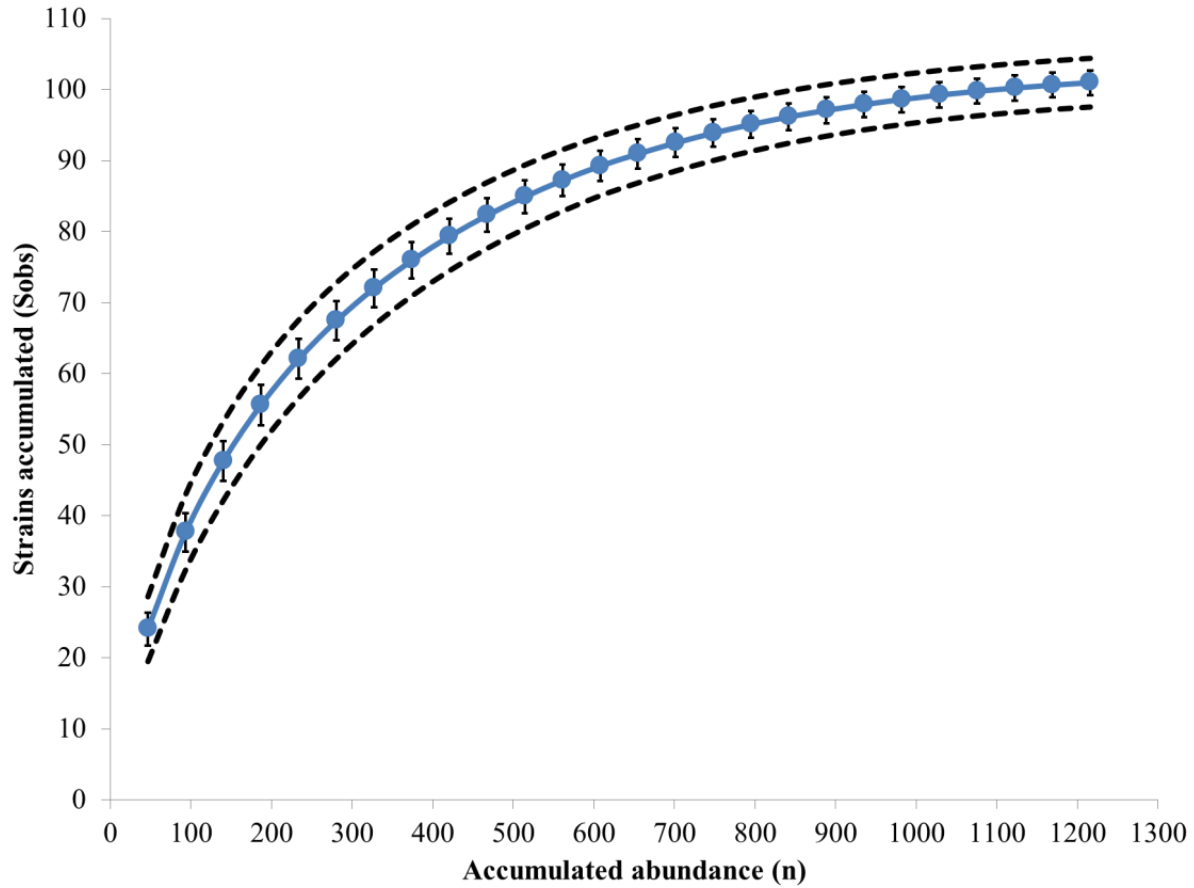
To determine whether the sampling effort was sufficient to establish the diversity of Actinobacteria associated with *N. costalis*, a rarefaction curve was performed. The statistic  $S_{\text{obs}}$  (Mao Tau) was calculated with the relative abundance of isolated strains per sample. The rarefaction curve (Figure 2.11) approaches an asymptote near 100 strains, which means that the sample was satisfactory in this study.

The indices used to compare Actinobacteria communities in *N. costalis* were the number of strains (M), abundance (A), Chao1, Fisher's alpha ( $\alpha$ ), Shannon-Weaver (H) and Simpson (S).

According to the results by season, in the wet season, the indices of species richness and diversity (Table 2.1) are higher than in the dry season (Table 2.2) ( $\text{Chao1}_{\text{wet}} > \text{Chao1}_{\text{dry}}$ ,  $\alpha_{\text{wet}} > \alpha_{\text{dry}}$ ,  $H_{\text{wet}} > H_{\text{dry}}$ ,  $S_{\text{wet}} > S_{\text{dry}}$ ). This is proportional to the amount of strains by seasons ( $M_{\text{wet}} > M_{\text{dry}}$ ), while the number of isolates is lower in the wet season ( $A_{\text{wet}} < A_{\text{dry}}$ ).

Moreover, the indices obtained in each of the samples per season (worker gut, worker exoskeleton, soldier gut, soldier exoskeleton and nest material) have the same pattern as the total indices per season. This indicates that the number of strains in the wet season for each sample (Table 2.1) is higher than the amount of strains in dry season per sample (Table 2.2).

The indices for termite workers in the wet and dry season indicate that species diversity with  $\alpha$  and  $H$  indices are higher in the intestine. But for the  $S$  index, the diversity is higher in the exoskeleton, because the  $S$  index considers the abundance of each species. Therefore the gut community is dominated by one species, which shows a lower  $S$  index (Table 2.1, Table 2.2). While the indices for termite soldiers in the wet season indicate that diversity is higher in the exoskeleton. For the exoskeleton of the soldiers in the wet season, the  $S$  index is higher ( $S = 15.41$ ), this is because the diversity and abundance of species have lower entropy or there is not a highly dominant strain (Table 2.1). In the soldiers for the dry season, the indices  $\alpha$  and  $H$  are higher in the gut, but the  $S$  index is higher in the exoskeleton (Table 2.2).



**Figure 2.11-** Rarefaction curve of total samples of Actinobacteria in *N. costalis*. The  $S_{obs}$  (Mao Tau) statistic was used to calculate the expected number of strains in the samples. The dashed lines represent the upper and lower limits of the confidence intervals at 95 % of  $S_{obs}$  (Colwell *et al.* 2004).

**Table 2.1-** Index of abundance, richness and diversity of Actinobacteria obtained for each sample in the wet season.

Index	Worker		Soldier		Nest Material	Total
	Exoskeleton	Gut	Exoskeleton	Gut		
Strains	23	24	17	16	25	105
Abundance	110	116	59	177	66	528
Chao1	23.67±1.15	25.67±2.21	17.14±0.49	16.50±1.30	30.14±4.65	78.56±2.48
Alpha	8.86±1.36	9.19±1.38	8.00±1.65	4.27±0.6	14.66±2.89	24.35±1.81
Shannon	2.60	2.64	2.66	1.98	2.99	3.68
Simpson	9.40	8.98	15.41	3.96	21.24	21.31

**Table 2.2-** Index of abundance, richness and diversity of Actinobacteria obtained for each sample in the dry season.

Index	Worker		Soldier		Nest Material	Total
	Exoskeleton	Gut	Exoskeleton	Gut		
Strains	8	14	6	11	12	51
Abundance	120	247	58	125	138	688
Chao1	8±0.00	14±0.00	6±0.02	11±0.01	12±0.01	38±0.00
Alpha	1.93±0.36	3.22±0.44	1.68±0.4	2.91±0.49	3.16±0.51	8.66±0.71
Shannon	1.48	1.53	1.50	1.51	2.11	2.8
Simpson	3.34	2.32	3.78	2.64	6.63	9.59

### 2.3.9 Phylogenetic analysis of *Streptomyces* spp. associated with *N. costalis*

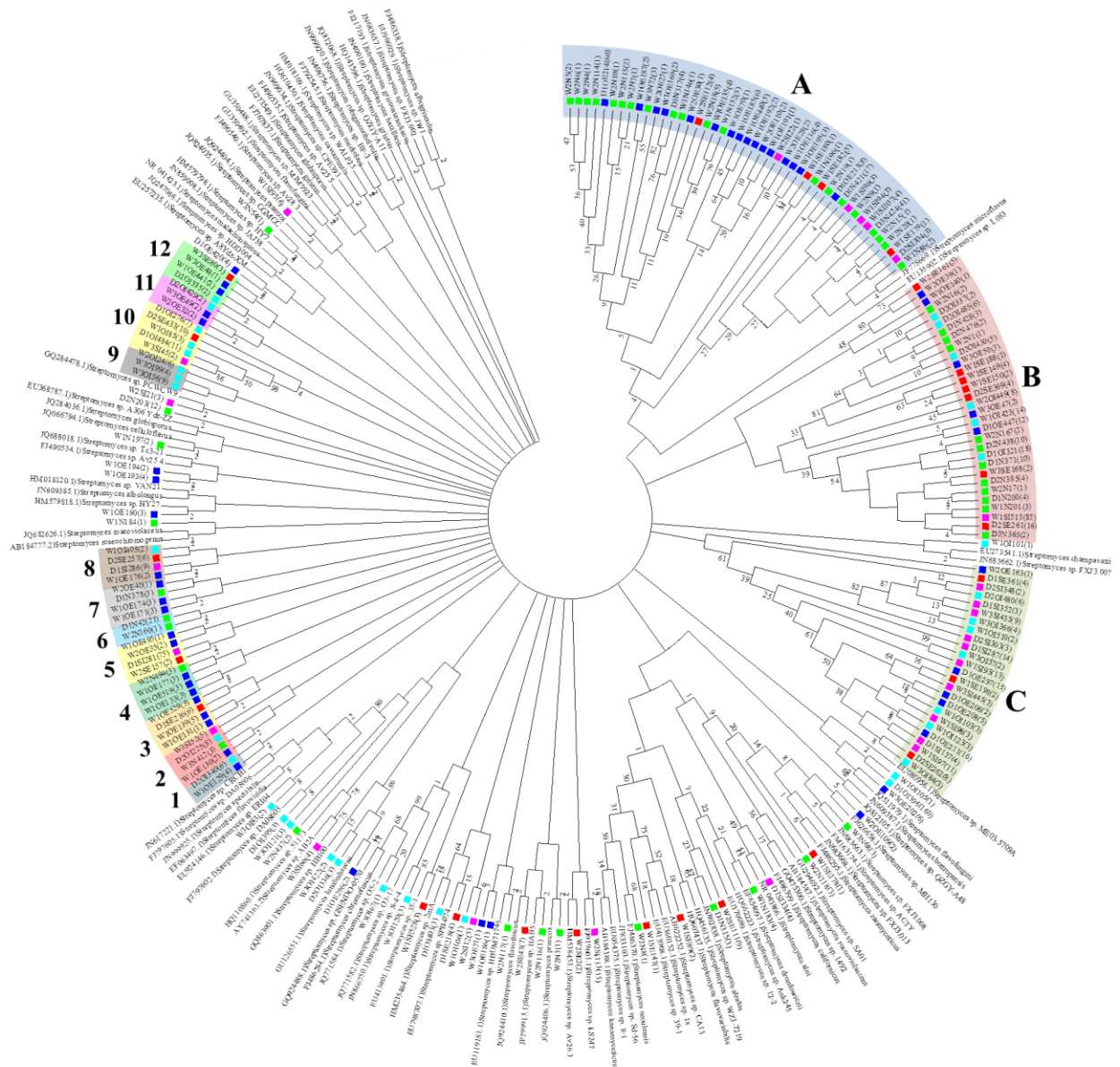
Phylogenetic analysis from different strains of *Streptomyces* show that 15 independent clades of sequences (Figure 2.12) are formed with no known matches to sequences in GenBank indicating that these might represent exclusive associates of termites. Clades A, B and C are monophyletic lineages with different strains, while clades labeled 1 to 12 represent several unresolved monophyletic groups of *Streptomyces* associated with termites. In clade A (Figure 2.12), most of the sequences belong to isolates from nest material (20 morphotypes) and from worker exoskeleton (16 morphotypes). In the worker exoskeleton, morphotype D1OE214[60] (with 60 strains) is the dominant isolate and closely related to *Streptomyces cavourensis* (HQ610450.1) according to the search in GenBank (Appendix L), although in the phylogenetic analysis all morphotypes of clade A are more closely related to *Streptomyces microflavus* (JF778669.1) (Figure 2.12). While within the nest material the dominant strain was D3N424[41] with 41 strains closely related to *Streptomyces* sp. BF-3 (JN408756.1) (Appendix L).

Clade B (Figure 2.12) is composed of sequences from nest material (12 morphotypes) and soldier exoskeleton isolates (7 morphotypes). The morphotype D2N438[10] with 19 isolates from nest material is highly represented and more closely related to *Streptomyces flavofuscus*

(JQ924410.1) according to the search in GenBank (Appendix L). On the other hand, the predominant morphotype in the soldier exoskeleton was D2SE261[16] with 16 isolates and closely related to *Streptomyces cavourensis* (HQ610450.1) in search of GenBank (Appendix L). For soldier gut isolates, with the most common strain W1SI515[85], which had a significant number of isolates (85), were closely related to undescribed *Streptomyces* sp. BAB5 (JF799913.1) (Appendix L).

Monophyletic group C (Figure 2.12) is composed of sequences from strains isolated from soldier (10 morphotypes) and worker guts (7 morphotypes). In soldier guts, the dominant morphotype was D1SI287[14] with 14 strains and closely related to *Streptomyces alni* (NR\_043866.1) in GenBank searches (Appendix L). While in worker guts morphotypes D2OI480[6] (6 strains) and W1OI510[2] (2 strains) were the predominant isolates, which are closely related to *Streptomyces* sp. FXJ3.007 (JN683662.1) (Appendix L).

Moreover, in clades 1 to 8 (Figure 2.12) the predominant morphotypes belonged to the worker exoskeleton (14 morphotypes). But in the clades 5 and 6, the morphotypes with more frequency were D1SI281[75] (closely related to *Streptomyces* sp. BAB5, JF799913.1) with 75 strains and D1N42[21] (closely related to *Streptomyces albolongus*, JN609385.1) with 21 strains, respectively (Figure 2.12, Appendix L). Finally, in clades 9 to 12 (Figure 2.12) the predominant morphotypes belonged to the worker guts (8 morphotypes), among which the dominant one was D1OI484[11] with 11 strains and closely related to *Streptomyces* sp. PCWCW9 (GQ284478.1) according to the search in GenBank (Appendix L).



**Figure 2.12-** Neighbor-joining phylogenetic tree of 16S rDNA for *Streptomyces* isolated from worker exoskeleton (■), worker gut (■), soldier exoskeleton (■), soldier gut (■) and, nest material (■) of *N. costalis*. Monophyletic clades A, B and C are the principal groups of sequences that are not represented in GenBank. Monophyletic clades 1 to 12 are unresolved small groups in this analysis. Bootstrap support values after 5000 pseudoreplicates are shown. For more details see Appendices L, M and N.

## 2.4 Discussion

The phylum Actinobacteria has an important role in aquatic and terrestrial ecosystems. Members of the phylum are recognized as bacteria producing secondary metabolites, which are important in the degradation of biomaterials such as cellulose and chitin, and the production of antimicrobial agents (Ventura *et al.* 2007). Many Actinobacteria are known as symbionts of eukaryotic organisms, which can establish mutualistic or parasitic relationships that are important for development and growth of the host (Margulis and Fester 1991, Seipke *et al.* 2011, Moran 2006). In higher termites, these bacteria play an important role in the degradation of cellulose, which is their food source. Previous studies in higher termite guts have found strains of *Streptomyces* and their ability to degrade cellulose was established (Watanabe *et al.* 2003). Until now, no information was available about the Actinobacteria community associated with the higher termite *N. costalis*; especially, the association of specific strains with the termites exoskeleton and their nest.

In the dry season, we isolated the highest numbers of bacteria colonies (Figure 2.2), but higher diversity in number of morphotypes (164) and in estimated diversity (Chao1 =  $78.56 \pm 2.48$ ,  $\alpha = 24.35 \pm 1.81$ ,  $H = 3.68$ ,  $S = 21.31$ ) was established for the wet season (Figure 2.3, Table 2.1). Actinobacteria are resistant to desiccation and typically their numbers are higher in the dry season (Castro *et al.* 2010), which may explain these results. Our data are consistent, although the particular collecting day of this study for the dry season (February 18, 2011) had higher precipitation (0.25 mm) than the one for the wet season (June 28, 2010) (0.10 mm) (PRISM climate data, NOAA). Although the day of collection of the wet season there was a



lower rainfall, when we look at the average precipitation for the month of June 2010 total rainfall was 203.00 mm, while in February 2011 it was 12.70 mm (PRISM climate data, NOAA).

The dominant genus throughout our study was *Streptomyces* regardless of the source or season sampled. *Streptomyces* isolates composed the majority of isolates from termites (97 %) and from nest material (73 %) (Figures 2.4, 2.5). Therefore, we established that *Streptomyces* dominates the community of Actinobacteria associated with *N. costalis* and constantly interacts with the termite environment. *Streptomyces* has been reported in several symbiotic associations with insects and plants (Seipke *et al.* 2012), which makes it a potential symbiont of the termite system. We speculate that *Streptomyces* has obtained a protected niche with the termites and probably provides the host with different benefits. Current information points to two specific roles played by *Streptomyces* strains in other insects: (1) degrade complex biomaterials that the host cannot digest and (2) the ability to protect them from pathogens by secreting antimicrobial agents (Seipke *et al.* 2012). *Streptomyces* in *O. formosanus* (higher termite) provides the ability to degrade cellulose (Watanabe *et al.* 2003). In *N. costalis* soldier gut, the most common Actinobacteria was closely related to *Streptomyces* sp. BAB5 (JF799913.1), whereas the most predominant among termite worker guts were two different strains: *Streptomyces* sp. Av25\_4, FJ490534.1 (in the wet season) and *Streptomyces* sp. A8Ydz-XM, EU257235.1 (in the dry season) (Figure 2.6, 2.7, 2.8, 2.9) (Appendix L). But in the worker gut, the most frequent strains were those similar 99% to *Streptomyces atratus* (JN862838.1) (Appendix L).

In the exoskeleton of termite workers and soldiers, the four more frequent strains recovered from termites were closely related to *Streptomyces cavourensis* (HQ610450.1) (Figure 2.5, 2.6, 2.7, 2.8) (Appendix L). While in the nest material from the wet season the most common isolated strain was also closely related to *Streptomyces cavourensis* (HQ610450.1), but in the dry season a

close relative of *Streptomyces* sp. BF-3 (JN408756.1) was the dominant strain (Figure 2.10) (Appendix L), although most of the morphotypes that are closely related to both strains belong to the 15 major clades (Table 2.3). *Streptomyces cavourensis* (HQ610450.1) is recognized as a chitinolytic bacteria and *Streptomyces* sp. BF-3 (JN408756.1) as endophytic bacteria. It should be emphasized that the strain with 99% similarity to *Streptomyces* sp. BF-3 (JN408756.1) belongs to the abandoned nest in the dry season and that in the absence of termite activity, the strains closely related to *Streptomyces cavourensis* (HQ610450.1) were not found (Appendix L) at all.

Our phylogenetic analysis determined that many of the isolates from *N. costalis* have no known matches in GenBank (Figure 2.11), but they separated from other sequences forming monophyletic groups. Nonetheless, it is hard to establish if they have other known relatives using solely 16S rDNA gene because the sequences are highly similar (Table 2.3, Appendix L). The majority of strains that are closely related to *Streptomyces* sp. BAB5 (JF799913.1), *Streptomyces atratus* (JN862838.1), *Streptomyces cavourensis* (HQ610450.1) and *Streptomyces* sp. BF-3 (JN408756.1) are within the majority of the fifteen clades identified in this study (Figure 2.11, Table 2.3). Furthermore, these are the most abundant and constant strains among samples. Therefore, these isolated *Streptomyces* strains from *N. costalis* can be unique and specific to these termites, but more data is needed to support this hypothesis.

*Streptomyces* strains isolated in this study are able to use this niche (*N. costalis* and its nest) as habitat. No nest was found sick or affected by pathogens during the study, although nest 3 in the dry season was abandoned. We isolated three morphotypes from nest 3; the most abundant being D3N424[41] 99% similar to *Streptomyces* sp. BF-3 (JN408756.1) with 41 strains isolated from a total of 48 colonies (Appendix L). Strain D3N424[41] is not found or is less common with termite activity, but we were unable to determine its effects in *N. costalis*. In addition, the data

showed that strains closely related to *Streptomyces cavourensis* (HQ610450.1) are in higher abundance when the nest is alive. It seems that these Actinobacteria are related to the presence and activity of the termites, and somehow controlled by them. This idea would explain the high number of isolates that were similar to *Streptomyces cavourensis* (HQ610450.1) that was found in the nest material and the exoskeleton of termites. Our findings support the existence of possible symbionts in this system.

**Table 2.3-** Identification of *Streptomyces* strains and their phylogenetic relationships (Figure 2.11).

Clade	Strain	Sample	Description	Accession Number	Max identity
A	W2N4[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	93%
A	W2N6[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	94%
A	W2N7[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	93%
A	W2N9[1]	NM	<i>Streptomyces</i> sp. BAB5	JF799913.1	99%
A	W2N15[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W2SI22[1]	SG	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W2OE30[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	94%
2	W2OE40[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	100%
3	D1N42[21]	NM	<i>Streptomyces albolongus</i>	JN609385.1	99%
2	W3OE49[2]	WE	<i>Streptomyces albolongus</i>	JN609385.1	99%
----	W3N54[1]	NM	<i>Streptomyces</i> sp. BF-3	JN408756.1	99%
A	W3N72[3]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	96%
1	W3SE80[3]	SE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W1N86[2]	NM	<i>Streptomyces</i> sp. BF-3	JN408756.1	98%
6	W1OE131[1]	WE	<i>Streptomyces</i> sp. BAB5	JF799913.1	99%
A	W1OE136[4]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W1N146[1]	NM	<i>Streptomyces albolongus</i>	JN609385.1	90%
3	W2N160[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
C	W2OE163[1]	WE	<i>Streptomyces</i> sp. BAB5	JF799913.1	87%
1	W1OE176[2]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	100%
A	W1SE180[1]	SE	<i>Streptomyces cavourensis</i>	HQ610450.1	98%
A	W1SE182[5]	SE	<i>Streptomyces</i> sp. BF-3	JN408756.1	99%
A	W1OE185[6]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
---	W1OE186[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W1OE191[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
A	W1N196[1]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	98%
B	D1N200[4]	NM	<i>Streptomyces atratus</i>	JN862838.1	99%
A	D1OE214[60]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	96%
6	D1SE216[6]	SE	<i>Streptomyces cavourensis</i>	HQ610450.1	100%
7	D2OI225[8]	WG	<i>Streptomyces</i> sp. BAB5	JF799913.1	99%
1	D2SE257[6]	SE	<i>Streptomyces cavourensis</i>	HQ610450.1	100%
B	D2SE261[16]	SE	<i>Streptomyces</i> sp. BAB5	JF799913.1	99%
4	D1SI281[75]	SG	<i>Streptomyces</i> sp. BAB5	JF799913.1	98%
---	D1SI287[14]	SG	<i>Streptomyces alni</i>	NR_043866.1	100%
B	D1OI321[18]	WG	<i>Streptomyces atratus</i>	JN862838.1	99%
1	D2OI335[2]	WG	<i>Streptomyces cavourensis</i>	HQ610450.1	100%
---	D1N378[3]	NM	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
B	W1OI423[14]	WG	<i>Streptomyces atratus</i>	JN862838.1	99%
A	D3N424[41]	NM	<i>Streptomyces</i> sp. BF-3	JN408756.1	99%
3	D2SE433[10]	SE	<i>Streptomyces</i> sp. BAB5	JF799913.1	87%
C	W3SI435[9]	SG	<i>Streptomyces</i> sp. FXJ3.007	JN683662.1	100%
B	D2N438[10]	NM	<i>Streptomyces flavofuscus</i>	JQ924410.1	100%
A	W1OE440[5]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
C	D2OI480[6]	WG	<i>Streptomyces</i> sp. FXJ3.007	JN683662.1	99%
3	D1OI484[11]	WG	<i>Streptomyces</i> sp. PCWCW9	GQ284478.1	79%
C	W1OI510[2]	WG	<i>Streptomyces</i> sp. FXJ3.007	JN683662.1	100%
B	W1SI515[85]	SG	<i>Streptomyces</i> sp. BAB5	JF799913.1	99%

NM- Nest Material, SG- Soldier Gut, WE- Worker Exoskeleton, SE- Soldier Exoskeleton, WG- Worker Gut

### **3 Ability to inhibit the growth of *Metarhizium anisopliae* and *Beauveria bassiana* by *Streptomyces* strains isolated from *Nasutitermes costalis***

#### **3.1 Introduction**

Arthropods have developed different physical, chemical, and symbiotic mechanisms to combat pathogens that affect their development as individuals or as a colony. Recent research has shown that several groups of insects have developed mutualistic interactions with a symbiont to obtain the ability to fix nitrogen (French *et al.* 1976, Pinto-Tomás *et al.* 2009), degrade different biomaterials (Pasti *et al.* 1990, Visôto *et al.* 2009) and/or protect them against pathogenic organisms (Kaltenpoth 2009, Haeder *et al.*, 2009, Seipke *et al.* 2012). Among the most studied insects are the Attine ants. In these ants, the pathogenic fungus *Escovopsis* does not directly attack the ants, but rather the symbiotic fungus *Leucoagaricus* they cultivate as food. To combat the pathogen, the Attine ants have coevolved with Actinobacteria that live in the ant exoskeleton and produce antifungal agents against *Escovopsis*. Several studies have shown that this association occurs mainly with the Actinobacteria genus *Pseudonocardia*, although *Streptomyces* has also been reported in this mutualistic relationship (Currie *et al.* 2003a, Currie *et al.* 1999b, Cafaro and Currie 2005, Haeder *et al.* 2009).

In addition, *Streptomyces* has been identified as an organism which is adapted to interact symbiotically with different groups of eukaryotic organisms (Seipke *et al.* 2012). In *Philanthus coronatus* wasps (beewolf) and in *Dendroctonus frontalis* beetles, researchers have observed a mutualistic relationship with *Streptomyces*. In *P. coronatus*, *Streptomyces* has been found colonizing a gland located in the antennae. The wasp transmits *Streptomyces* to the cocoon to

prevent attack by pathogenic fungi during larvae development (Kaltenpoth and Göttler 2005). While in *D. frontalis*, a similar relationship to Attine ants associated with Actinobacteria has been found where *Streptomyces* has presented the ability to inhibit the growth of the pathogenic fungus *Ophiostoma minus* that grows in the beetle galleries (Scott *et al.* 2008).

*Streptomyces* has also been identified in the termite gut. Its primary function in the gut is to degrade cellulose that serves as food for termites (Watanabe *et al.* 2003). Our research has shown that *Streptomyces* is the predominant genus in the Actinobacteria community associated with the termite exoskeleton (Chapter 2), as it has been reported for other insects. Although it is not known whether these bacteria associated with termites have the capacity to protect them against entomopathogenic fungi. Other studies (Sun *et al.* 2003, Sun *et al.* 2002) indicate that the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* attack termite nests as occurred in other insects (Hughes *et al.* 2004, Shah and Pell 2003). In addition, *M. anisopliae* and *B. bassiana* have been used as biocontrol agents of termites (Delate *et al.* 1995, Neves and Alves 2004). Therefore, in this research the objective was to determine whether *Streptomyces* strains associated with *N. costalis* possess the ability to inhibit the growth the most common entomopathogenic fungi *M. anisopliae* and *B. bassiana*.

## **3.2 Materials and Methods**

### **3.2.1 Selection of *Streptomyces* strains associated with *N. costalis***

We randomly selected fifteen *Streptomyces* strains associated with termite workers and soldiers: W2OE28[2], W2SE43[7], W1SI94[3], W1SI96[3], W1SI107[4], W2SE112[4], W1OE136[4], W1SE179[1], W1SE180[1], W1OE185[6], W1OE186[1], W1OE187[2],

W1OE190[3], W1OE191[1] and D1OE213[8] (see Table 3.1). Most of the selected strains belonged to monophyletic clade A of the phylogenetic tree in Figure 2.11 (Chapter 2).

**Table 3.1-** Information from *Streptomyces* strains used for bioassay confrontation with fungi

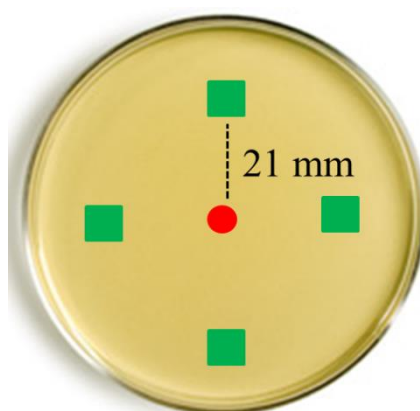
Strain	Sample	Description	Accession Number	Max identity
W2OE28[2]	WE	<i>Streptomyces</i> sp. VAN21	HM018120.1	99%
W2SE43[7]	SE	<i>Streptomyces</i> sp. CGMCC	JQ824035.1	99%
W1SI94[3]	SG	<i>Streptomyces</i> sp. A306 Ydz-ZZ	EU368787.1	99%
W1SI96[3]	SG	<i>Streptomyces</i> sp. A306 Ydz-ZZ	EU368787.1	99%
W1SI107[4]	SG	<i>Streptomyces roseochromogenus</i>	AB184777.2	99%
W2SE112[4]	SE	<i>Streptomyces griseus</i>	FJ767837.1	93%
W1OE136[4]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
W1SE179[1]	SE	<i>Streptomyces</i> sp. CPE393	JN969034.1	99%
W1SE180[1]	SE	<i>Streptomyces cavourensis</i>	HQ610450.1	98%
W1OE185[6]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
W1OE186[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
W1OE187[2]	WE	<i>Streptomyces globisporus</i>	JQ284036.1	97%
W1OE190[3]	WE	<i>Streptomyces roseochromogenus</i>	AB184777.2	99%
W1OE191[1]	WE	<i>Streptomyces cavourensis</i>	HQ610450.1	99%
D1OE213[8]	WE	<i>Streptomyces microflavus</i>	JF778669.1	99%

SG- Soldier Gut, WE- Worker Exoskeleton, SE- Soldier Exoskeleton,

### 3.2.2 Bioassay confrontation Actinobacteria-fungus

This methodology was modified from Cafaro and Currie (2005), Currie *et al.* (2006) and Sen *et al.* (2009). Initially, *Streptomyces* strains were inoculated separately in the center of a Petri dish (100 mm of diameter) with 25 mL of yeast malt extract agar (YMEA) (Chapter 2, pp. 16) and one uninoculated plate as negative control. Plates were incubated at 25 °C for 21 days, because Actinobacteria grow slowly and; also, we allowed radial diffusion of secondary metabolites in the media during that time. The fungi *M. anisopliae* and *B. bassiana* were inoculated into separated Petri dishes (100 mm of diameter) with 25 mL of YMEA and incubated at 25 °C for 7 days.

After 21 days of incubation for the *Streptomyces* strains, we proceeded to cut four pieces of 5 mm<sup>2</sup> from 7-day old cultures of each fungus (*M. anisopliae* and *B. bassiana*) to confront against bacterial strains and the negative control. These four pieces (replicates) were placed at 21 mm of distance from the inoculation site of the *Streptomyces* strain. Bioassay confrontations and controls were incubated at 25 °C for four weeks. Finally, micelial growth of fungus was measured weekly in the presence of each *Streptomyces* strain.



**Figure 3.1-** Technique used to confrontations bioassays. Inoculated at the center *Streptomyces* strain (●). Fungi (■) were inoculated to 21 mm away from inoculation site of *Streptomyces* strain.

### 3.2.3 Data analysis

Data was analyzed through ANOVA and the Tukey multiple comparison method (significance level of 0.01). This analysis was performed with the software InfoStat version 2008 (Di Rienzo *et al.* 2008). The percent of inhibition (I) of fungi was calculated using the formula from Bendahmane *et al.* (2012) and Hmouni *et al.* (1996):

$$I (\%) = \left(1 - \frac{F_A}{F_C}\right) \times 100$$



Where  $F_A$  is the measure of fungal growth in the presence of the Actinobacteria and  $F_C$  is the measure of the fungus growing alone (control). To estimate the zone of inhibition (ZOI), the following formula was used:

$$\text{ZOI} = 21 \text{ mm} - F_G$$

Where  $F_G$  is the radius of the fungus colony and 21 mm is the distance between the site of inoculation of *Streptomyces* strain and the fungus.

### 3.3 Results

#### 3.3.1 Growth controls of fungi and *Streptomyces* strains

The control for *M. anisopliae* and *B. bassiana* grew to an approximately radius of 21 mm in 13 days and 9 days, respectively (Figure 3.2). The controls for *Streptomyces* strains kept growing progressively during 51 days (Figure 3.3). The maximum growth was obtained by *Streptomyces* W1OE186[1] with  $53.5 \pm 0.7$  mm of diameter. In contrast, the minimum growth was obtained by *Streptomyces* W2SE43[7] with  $31.2 \pm 0.3$  mm of diameter (Figure 3.3).

#### 3.3.2 Growth of fungi in confrontation bioassays

*Metarhizium anisopliae* growth varied among confrontation with different strains of *Streptomyces*. The maximum growth varied between a radius of  $11.6 \pm 1.7$  to  $3.3 \pm 0.9$  (Figure 3.4A). *Metarhizium anisopliae* growth was higher in confrontation with *Streptomyces* W1SI96[1], while minimum growth was observed with *Streptomyces* W1OE187[5] (Figure 3.4A, Figure 3.5). In growth curves of *M. anisopliae* confronted with *Streptomyces* W1SI96[1],

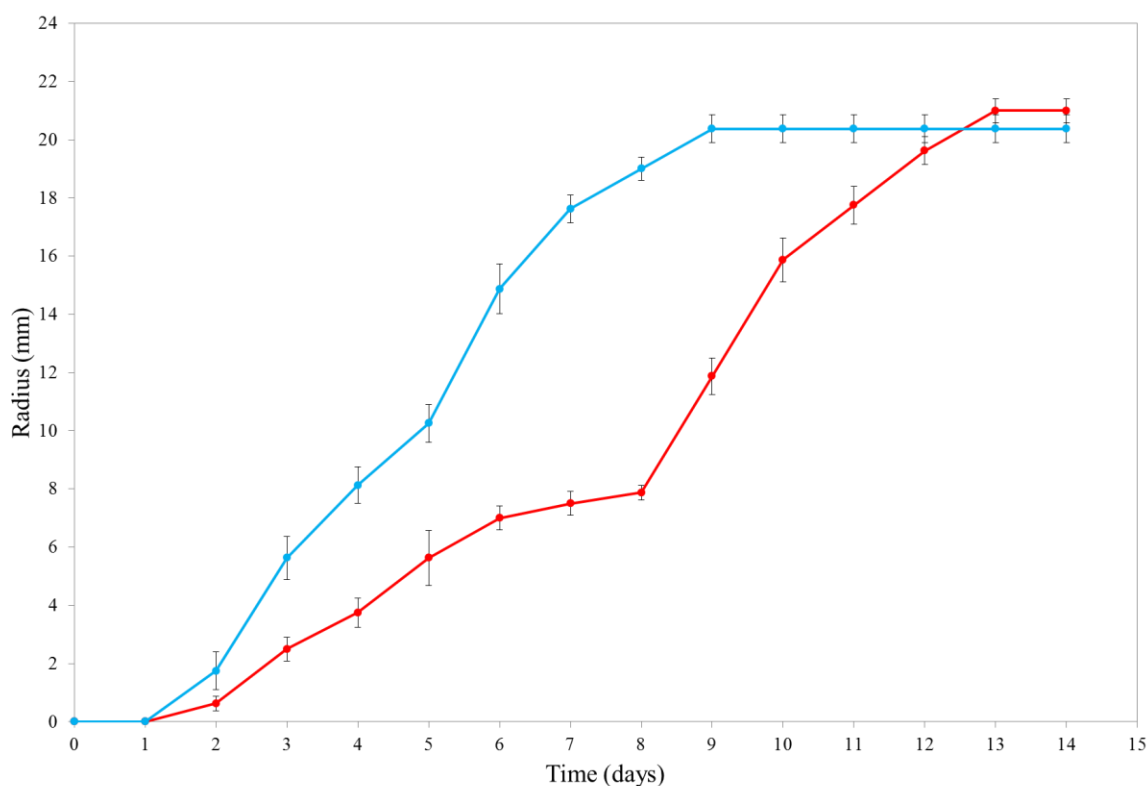
W1OE186[1], W1OE190[3] W2SE112[1], W1SE179[1], W1OE191[1] and W1OE185[3] we observed decreasing points of micelial growth (Figure 3.4A, Figure 3.5). These reductions are due to micelial necrotic regions close to *Streptomyces* strains. *Streptomyces* D1OE213[8] W1SE179[1], W1OE191[1], W1SE180[1], W2OE2 [2], W1SI107[4], W1SI94[3], W2SE43[7], W1OE187[2] and W1OE136[4] stopped the growth of *M. anisopliae* on day 12 of the test (maximum growth) (Figure 3.3A, Figure 3.5).

*Beauveria bassiana* growth was significantly less than *M. anisopliae* when confronted to *Streptomyces* strains. Maximum growth reached a radius of  $6.6 \pm 0.5$  mm, while some confrontations did not grow at all (Figure 3.4B). The maximum and minimum growth were obtained in the presence of *Streptomyces* W2SE43[5] and W1SI96[1], respectively (Figure 3.4B, Figure 3.6). The majority of *Streptomyces* strains inhibited *B. bassiana* growth after 12 days, but confrontations with *Streptomyces* strains W2SE43[5], D1OE213[5], W1OE191[1], W1OE185[3], W2OE28[1] and W1SE180[1] showed an increase in growth on day 24 (Figure 3.4B, Figure 3.6). The majority of the growth curves of *B. bassiana* obtained a reduction in micelial growth on day 18 (Figure 3.4B, Figure 3.6).

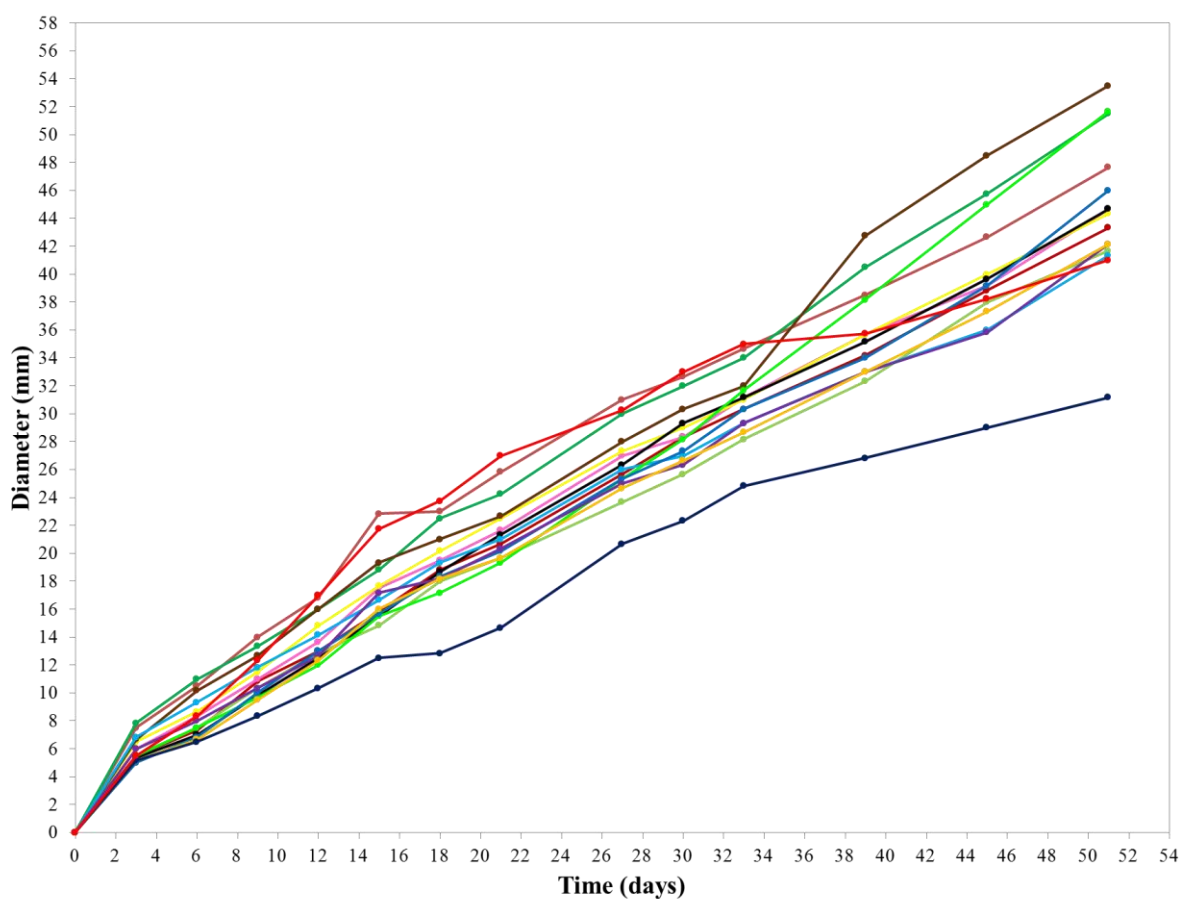
### **3.3.3 Growth of *Streptomyces* strains in presence of fungi**

Growth of *Streptomyces* strains stopped in the presence of *M. anisopliae* (Figure 3.7A). These were observed in day 33 of the incubation period (Figure 3.7A). The *Streptomyces* strain with maximum growth was W1SI96[3] with 30.0 mm of diameter, while the minimum growth was D1OE213[8] with 18.0 mm of diameter (Figure 3.7A).

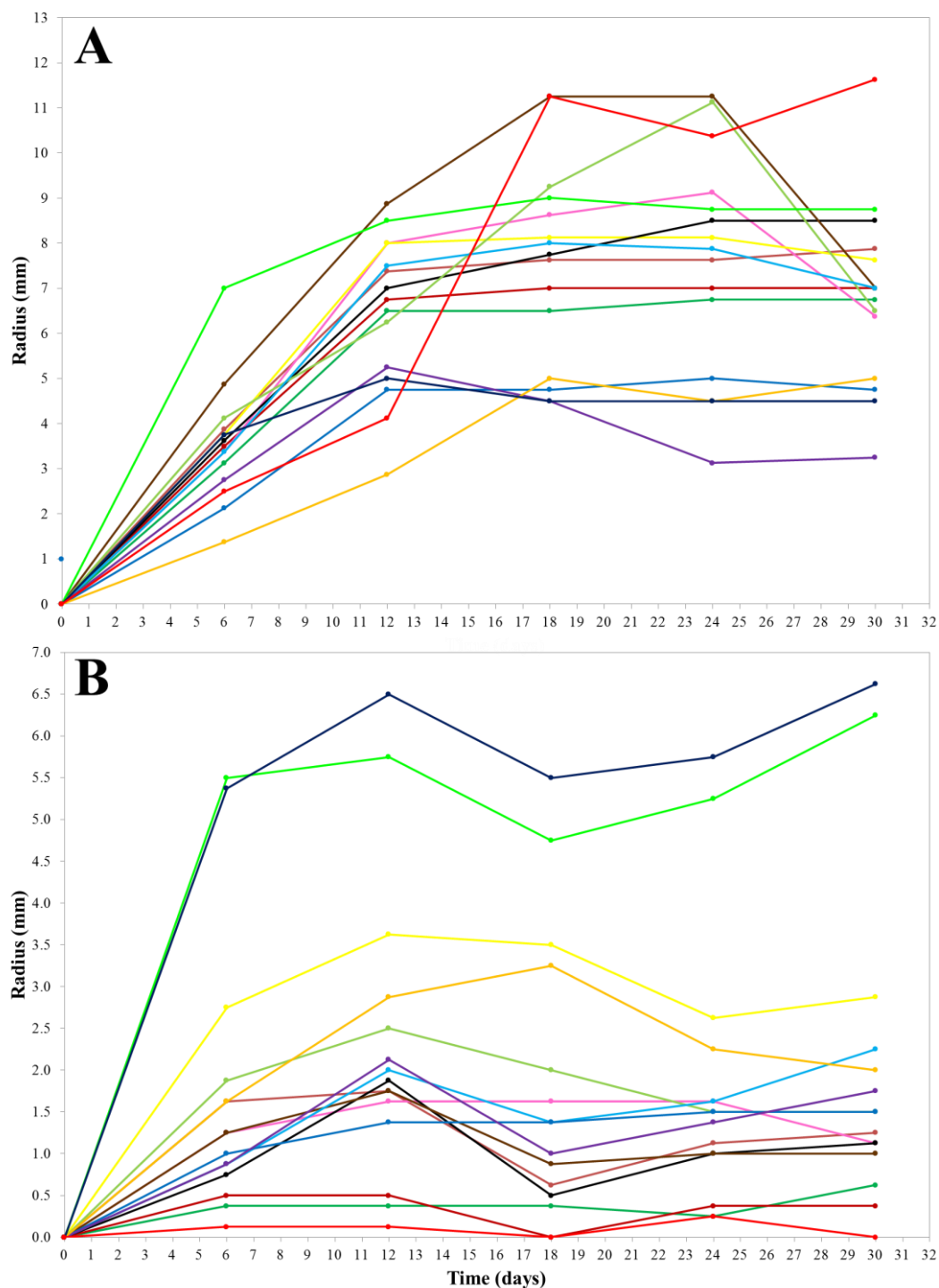
In the presence of *B. bassiana*, the majority of *Streptomyces* strains continued to grow (Figure 3.7B, Figure 3.6). *Streptomyces* strain with maximum (49.0 mm) and minimum (23.5 mm) growth were W2OE28[2] and W2SE43[7], respectively (Figure 3.7B). *Streptomyces* D1OE213[8] stopped growing in the presence of *B. bassiana* on day 33 of incubation (Figure 3.7B).



**Figure 3.2-** Control growth of fungi: *M. anisopliae* (■) and *B. bassiana* (■) (n = 4)



**Figure 3.3-** Control growth of *Streptomyces* strains W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■) during the incubation period.



**Figure 3.4-** Growth of *M. anisopliae* (A) and *B. bassiana* (B) for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■).

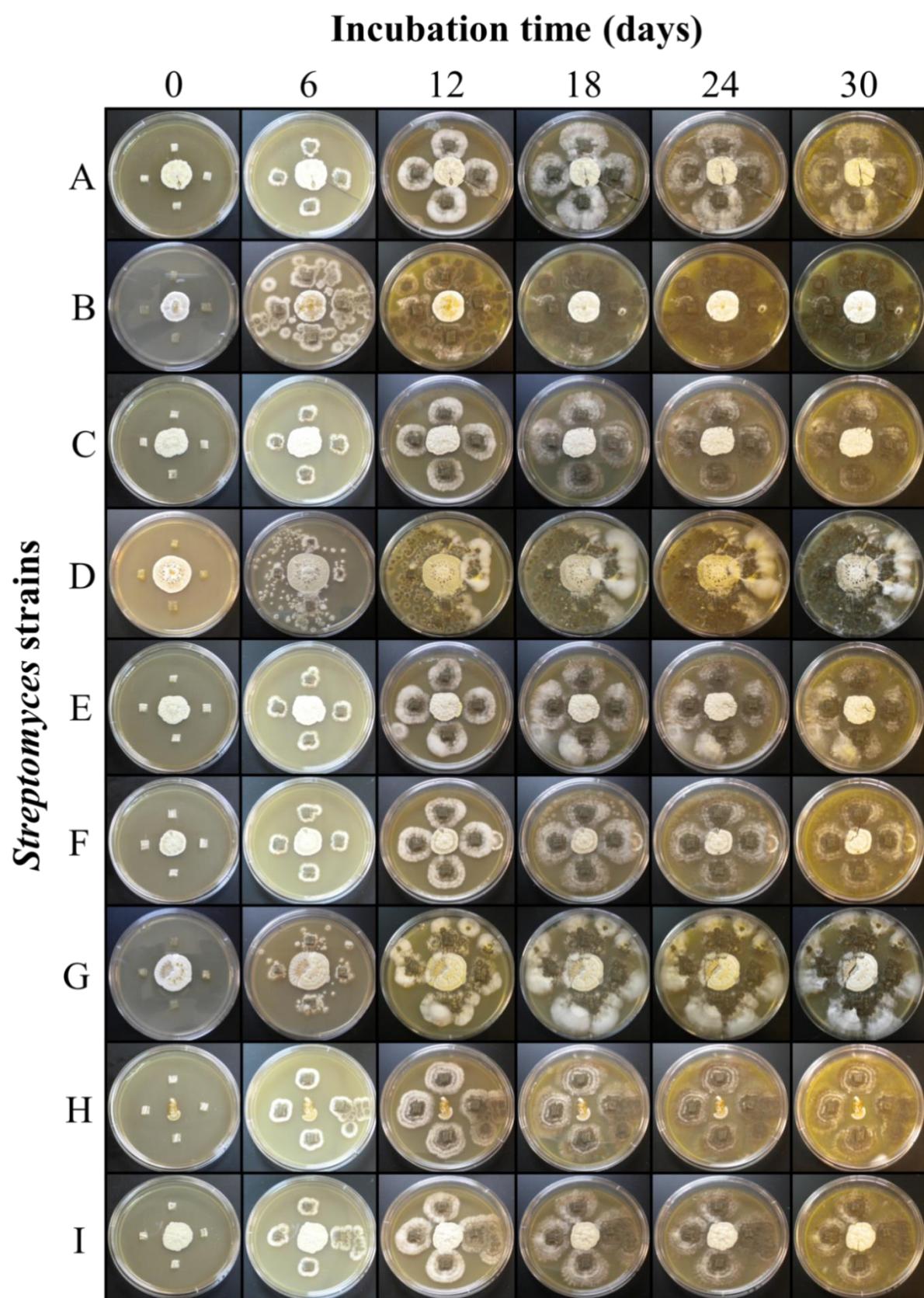
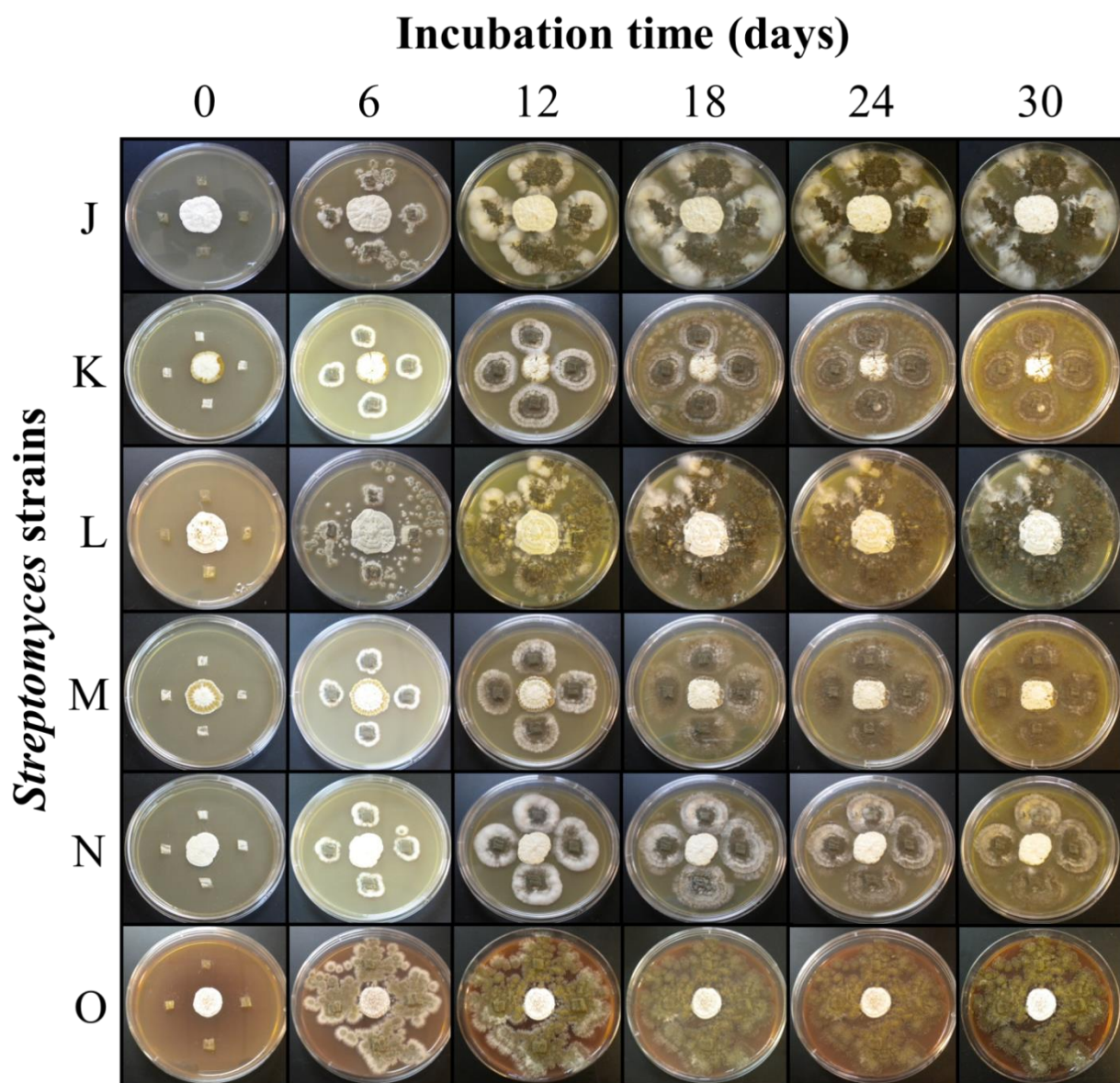
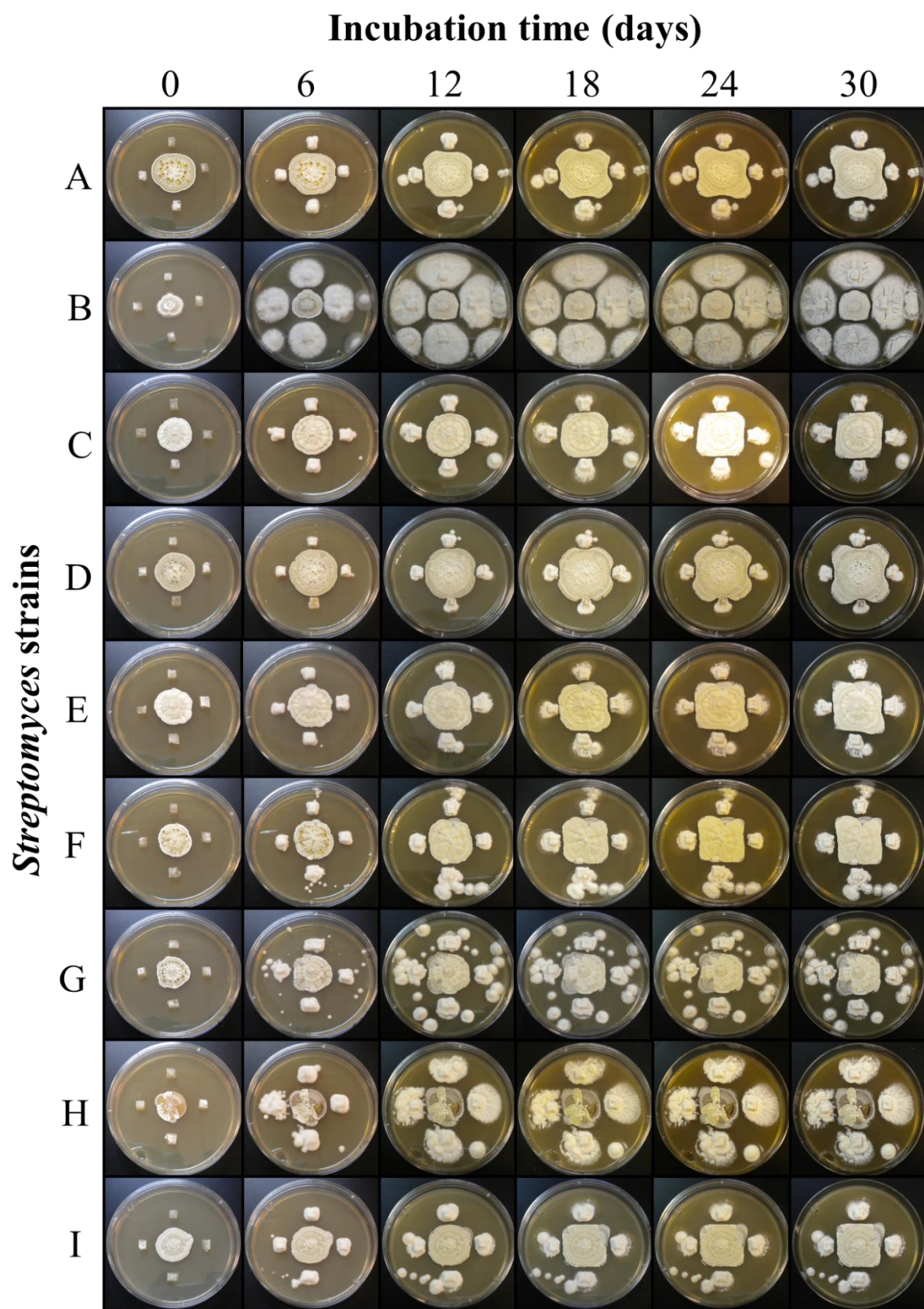


Figure 3.5



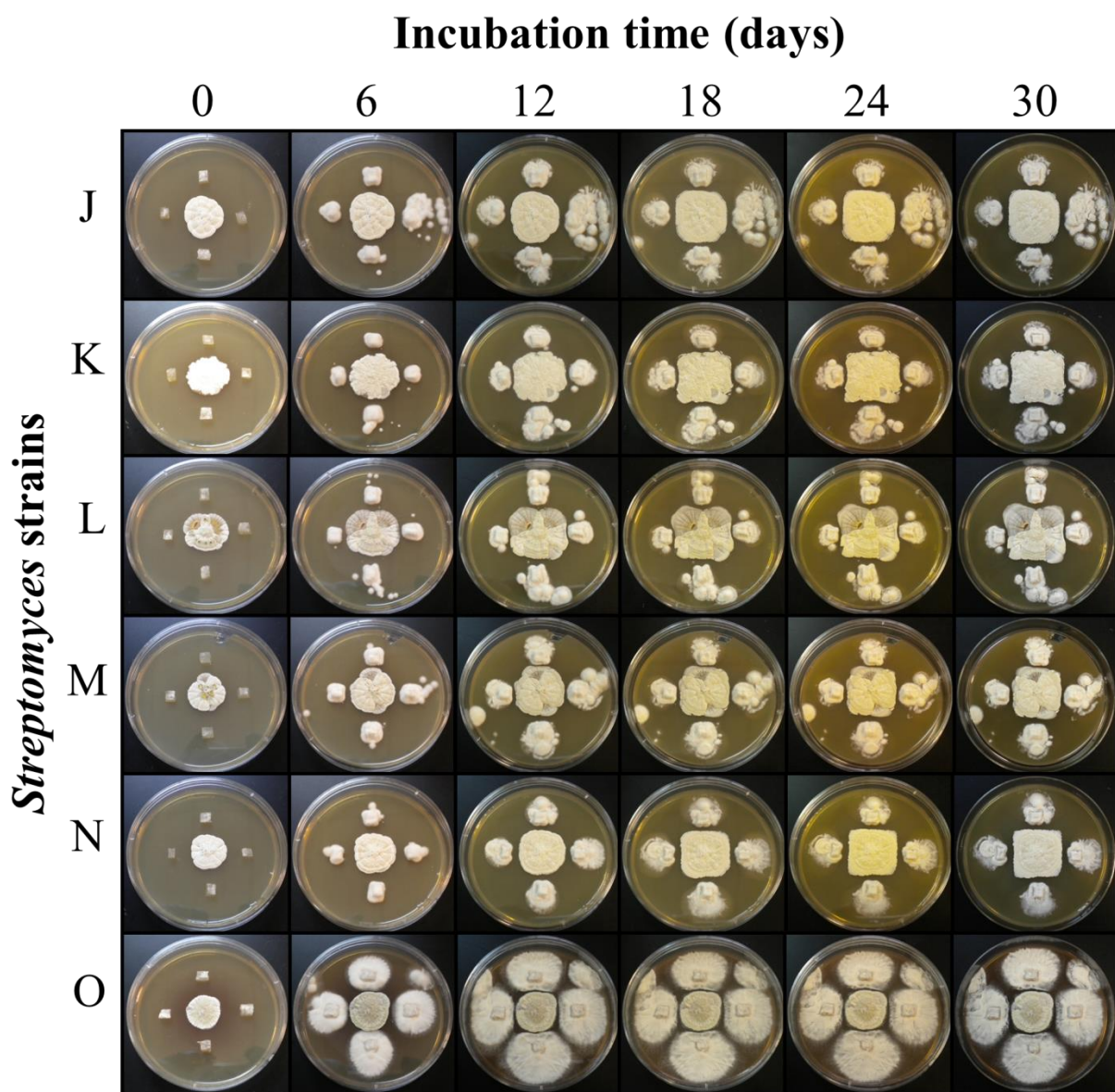


**Figure 3.5-** Growth of *Metarhizium anisopliae* for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (A), W2SE43[7] (B), W1SI94[3] (C), W1SI96[3] (D), W1SI107[4] (E), W2SE112[4] (F), W1OE136[3] (G), W1SE179[1] (H), W1SE180[1] (I), W1OE185[6] (J), W1OE186[1] (K), W1OE187[2] (L), W1OE190[3] (M), W1OE191[1] (N) and D1OE213[8] (O).

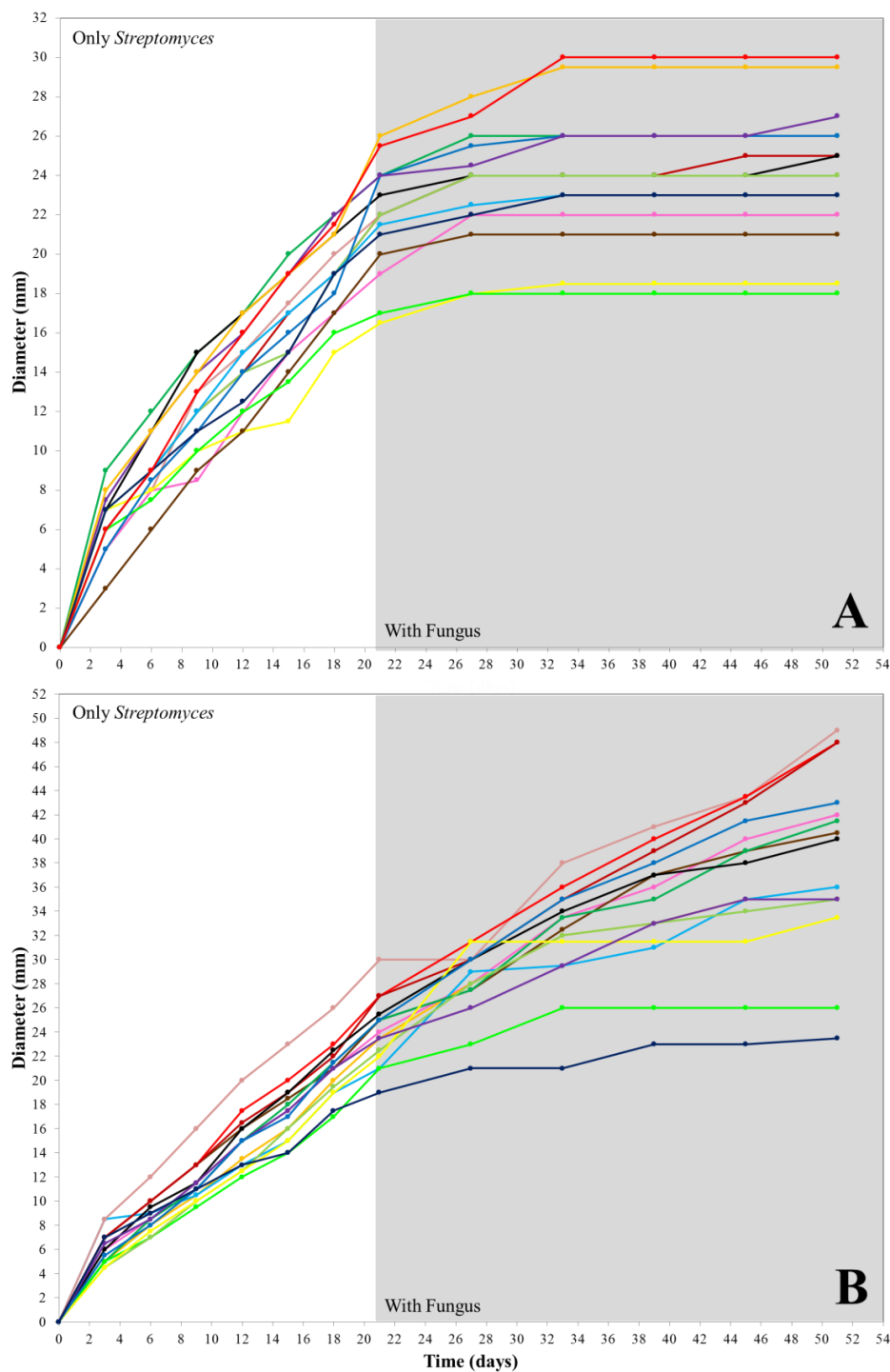


**Figure 3.6**





**Figure 3.6-** Growth of *Beauveria bassiana* for 30 days of incubation (25 ° C) in the presence of *Streptomyces* strains: W2OE28[2] (A), W2SE43[7] (B), W1SI94[3] (C), W1SI96[3] (D), W1SI107[4] (E), W2SE112[4] (F), W1OE136[3] (G), W1SE179[1] (H), W1SE180[1] (I), W1OE185[6] (J), W1OE186[1] (K), W1OE187[2] (L), W1OE190[3] (M), W1OE191[1] (N) and D1OE213[8] (O).



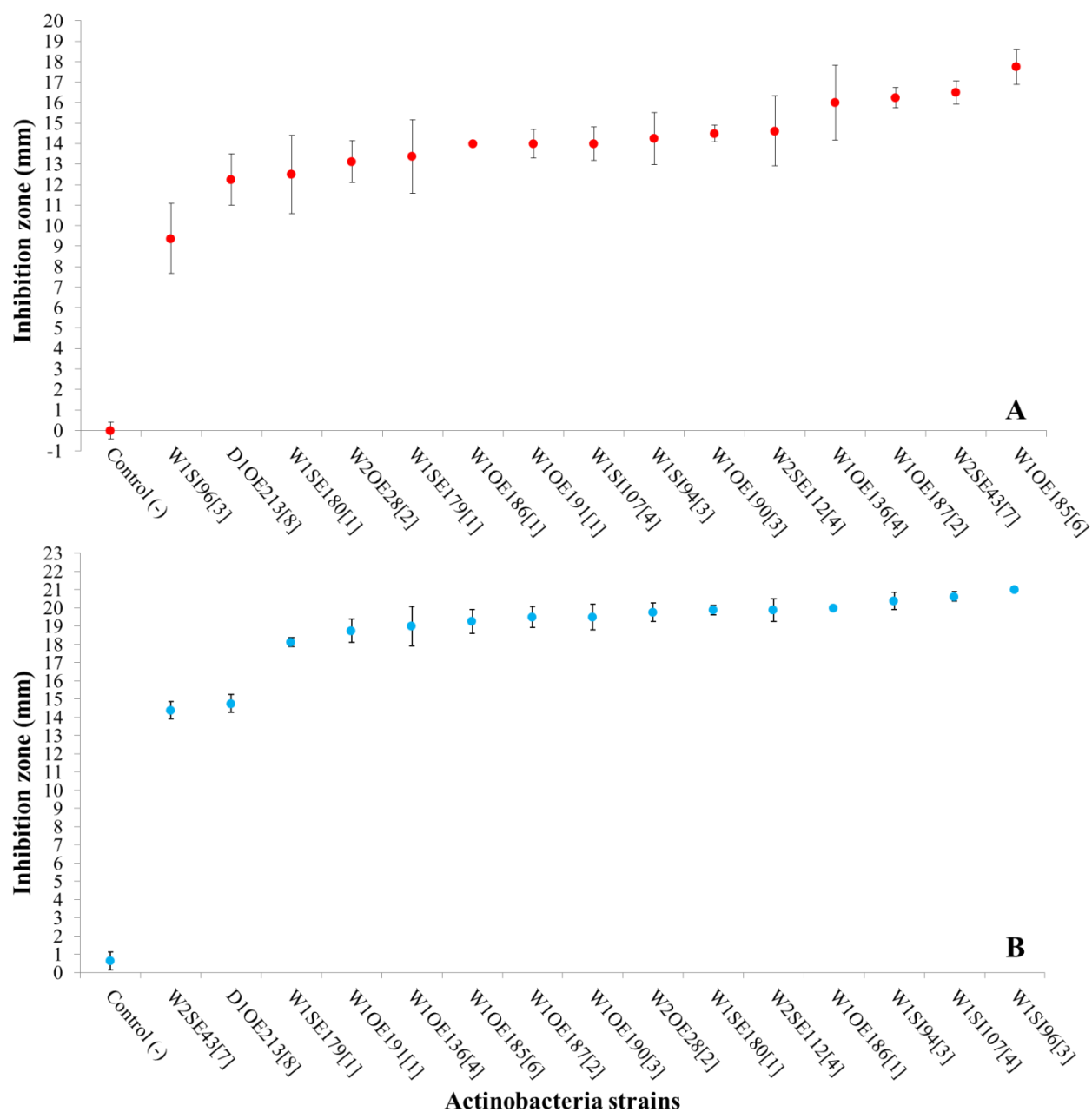
**Figure 3.7-** Growth of *Streptomyces* strains W2OE28[2] (■), W2SE43[7] (■), W1SI94[3] (■), W1SI96[3] (■), W1SI107[4] (■), W2SE112[4] (■), W1OE136[4] (■), W1SE179[1] (■), W1SE180[1] (■), W1OE185[6] (■), W1OE186[1] (■), W1OE187[2] (■), W1OE190[3] (■), W1OE191[1] (■) and D1OE213[8] (■) during the incubation period with *M. anisopliae* (A) and *B. bassiana* (B).

### 3.3.4 Inhibition of fungal growth

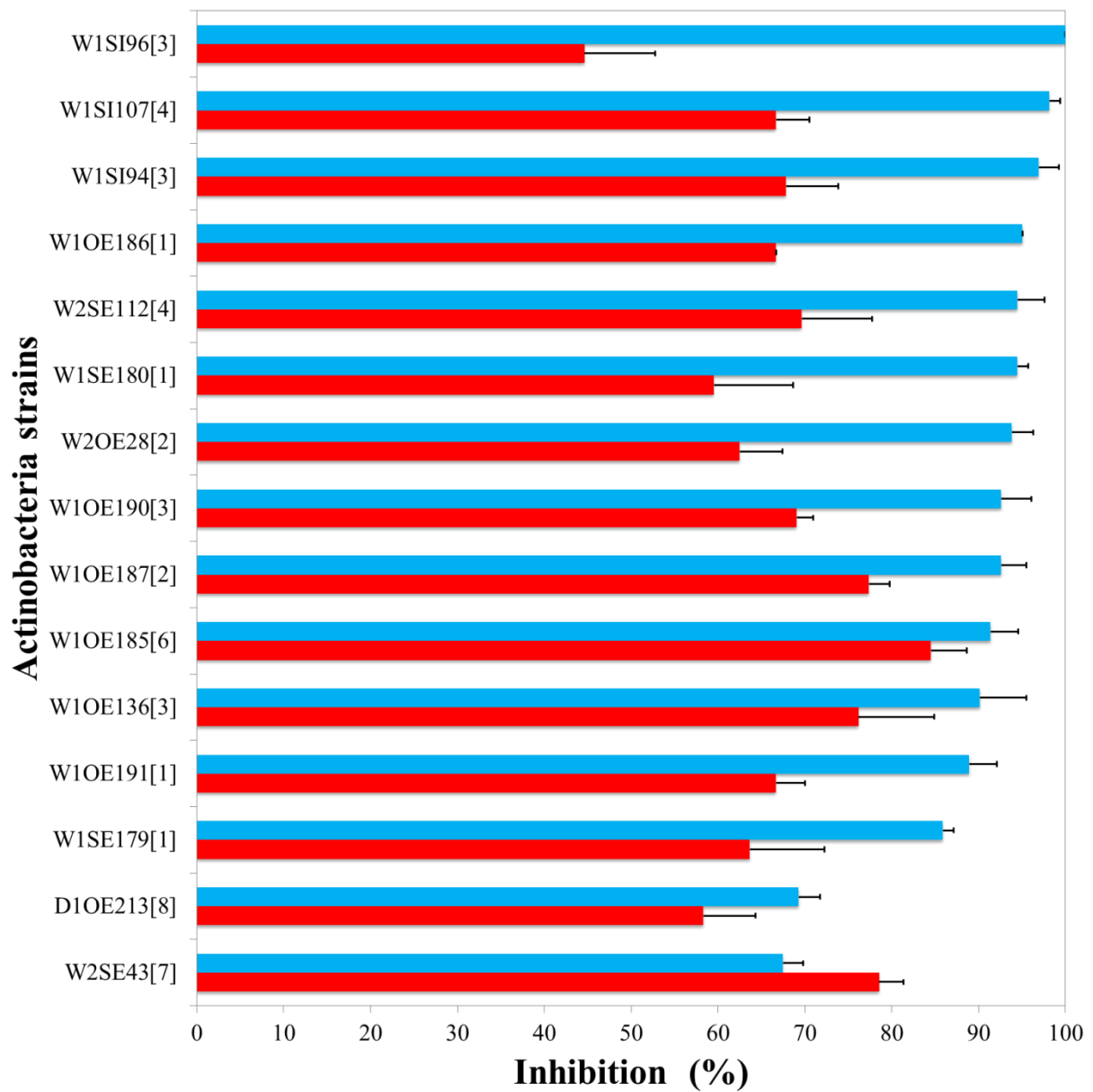
The zone of inhibition (ZOI) of the fungi (*M. anisopliae* and *B. bassiana*) was calculated using the difference of micelial growth and inoculation distance from the *Streptomyces* strains (21 mm). The ZOI of *M. anisopliae* with *Streptomyces* strains ranged from  $9.4 \pm 1.7$  to  $17.8 \pm 0.9$  mm (Figure 3.8A). For *M. anisopliae* we observed maximum ZOI ( $17.8 \pm 0.9$  mm) with *Streptomyces* W1OE185[6], while the minimum ZOI ( $9.38 \pm 1.70$  mm) was observed with *Streptomyces* W1SI96[3] (Figure 3.8A). For *B. bassiana* ZOI varied from  $14.4 \pm 0.5$  mm to  $21.0 \pm 0.0$  mm (Figure 3.8B). *Beauveria bassiana* showed maximum ZOI ( $21.0 \pm 0.0$  mm) with *Streptomyces* W1SI96[3], while the minimum ZOI ( $14.4 \pm 0.5$  mm) was observed with *Streptomyces* W2SE43[7] (Figure 3.8B).

In calculating the percentage of inhibition of *M. anisopliae* and *B. bassiana* in the presence of *Streptomyces* strains, it was observed that the percentage of inhibition of *M. anisopliae* was less than the percentage of inhibition of *B. bassiana* (Figure 3.9). *Beauveria bassiana* growth was inhibited 90% to 100% by 11 of the *Streptomyces* strains tested (73%). While *M. anisopliae* growth was inhibited only 62% to 85% by 12 *Streptomyces* strains (80%) (Figure 3.9).

The variance analysis indicates that there are significant differences between fungal growths in the presence of *Streptomyces* strains (Table 3.2, 3.3). The Tukey test also shows that for *M. anisopliae* (Table 3.2) there are 5 different groups of growth patterns (A, B, C, D and E), varying from 3.25 mm to 11.63 mm. In *B. bassiana* (Table 3.3), there are 6 different groups of growth (A, B, C, D, E and F) varying from 0.00 mm to 6.63 mm.



**Figure 3.8-** Zone of inhibition (ZOI) (mm) of *Metarhizium anisopliae* (A) and *Beauveria bassiana* (B) in presence of *Streptomyces* strains isolated from *N. costalis* (n = 4).



**Figure 3.9-** Percentage of inhibition of *Metarhizium anisopliae* (■) and *Beauveria bassiana* (■) in presence of *Streptomyces* strains isolated from *N. costalis* (n = 4).

**Table 3.2-** ANOVA of *Metarhizium anisopliae* micelial growth in presence of *Streptomyces* strains isolates from *N. costalis*.

<i>Streptomyces</i> strains	Means $\pm$ 0.60 (mm)	
W1OE185[6]	3.25	A
W2SE43[7]	4.50	A B
W1OE187[2]	4.75	A B
W1OE136[4]	5.00	A B C
W2SE112[4]	6.38	A B C D
W1OE190[3]	6.50	A B C D
W1SI94[3]	6.75	A B C D
W1SI107[4]	7.00	B C D
W1OE186[1]	7.00	B C D
W1OE191[1]	7.00	B C D
W1SE179[1]	7.63	B C D
W2OE28[2]	7.88	B C D
W1SE180[1]	8.50	C D E
D1OE213[8]	8.75	D E
W1SI96[3]	11.63	E
Control (-)	21.00	F

n = 4,  $F = 45.41$ ,  $p < 0.0001$

**Table 3.3-** ANOVA of *Beauveria bassiana* micelial growth in presence of *Streptomyces* strains isolates from *N. costalis*.

<i>Streptomyces</i> strains	Means $\pm$ 0.27 (mm)	
W1SI96[3]	0.00	A
W1SI107[4]	0.38	A B
W1SI94[3]	0.63	A B C
W1OE186[1]	1.00	A B C D
W1SE180[1]	1.13	A B C D
W2SE112[4]	1.13	A B C D
W2OE28[2]	1.25	A B C D
W1OE187[2]	1.50	A B C D E
W1OE190[3]	1.50	A B C D E
W1OE185[6]	1.75	B C D E
W1OE136[3]	2.00	C D E
W1OE191[1]	2.25	D E
W1SE179[1]	2.88	E
D1OE213[8]	6.25	F
W2SE43[7]	6.63	F
Control (-)	20.38	G

n = 4,  $F = 341.42$ ,  $p < 0.0001$

### 3.4 Discussion

Some bacteria are recognized by their association with other eukaryotic organisms and their ability to inhibit host pathogens (Kaltenpoth 2009, Seipke *et al.* 2012). Several researchers have studied different bacteria-insect associations such as ants (Sen *et al.*, 2009, Currie *et al.* 1999b, Haeder *et al.*, 2009), wasps (Kaltenpoth and Göttler 2005) and beetles (Scott *et al.* 2008) that have Actinobacteria as symbionts (Seipke *et al.* 2012). Symbiotic interactions have also been observed in plants (Taechowisan *et al.*, 2003, Franco *et al.* 2007), sponges (Khan *et al.* 2011) and marine snails (Peraud *et al.*, 2009, Lin *et al.* 2010). In vitro studies have shown the ability of these Actinobacteria in inhibiting the growth of pathogens affecting different groups of eukaryotes. *Streptomyces* has been shown to be more dominant in these types of symbiotic relationships (Seipke *et al.* 2012). As shown in Chapter 2, *Streptomyces* is found in high frequency associated with *N. costalis*, compared with other genera of Actinobacteria.

The growth of *M. anisopliae* and *B. bassiana* confronted with *Streptomyces* strains is lower than the growth in the controls. Bioassay data shows that most strains of *Streptomyces* inhibit growth of *B. bassiana* from 67.5% to 100.0%, with the majority of inhibition above 90%. While the same strains of *Streptomyces* inhibit the growth of *M. anisopliae* from 44.6% to 84.5%. Therefore *Streptomyces* strains associated with *N. costalis* are highly efficient in inhibiting *B. bassiana*. *Streptomyces* W1SI96[3] isolated from soldier gut was more efficient in inhibiting the growth of *B. bassiana* with 100.0%, while strain W1OE185[2] isolated from worker exoskeleton was the most efficient at inhibiting *M. anisopliae* with 84.5%. *Streptomyces* W1SI96[3], which is the seventh most frequent in soldier gut, may be specific to inhibiting *B. bassiana* (100.0%), because it was less efficient at inhibiting *M. anisopliae* (44.6%). Although *Streptomyces*

W1OE185[2], which is the second most frequent in worker exoskeleton, was more efficient in inhibiting both fungi with 84.5% for *M. anisopliae* and 91.4% for *B. bassiana*. On the other hand, the majority of the *M. anisopliae* confrontations show mutual inhibition between fungus and *Streptomyces* strains. This *in vitro* study shows the high efficiency to produce antifungal agents of *Streptomyces* strains and antibacterial agents by the fungi.

The data obtained in this *in vitro* study suggest that *Streptomyces* W1OE185[2] (closely related to *Streptomyces cavourensis*, HQ610450.1) associated with the exoskeleton of *N. costalis* have a possible defense role since they are effective at inhibiting *M. anisopliae* and *B. bassiana*. Both fungi are common entomopathogenic that attack many insects, including termites (Hughes *et al.* 2004, Shah and Pell 2003). We suggest there may be selection for particular *Streptomyces* strains, which have the ability to inhibit pathogenic microorganisms such as *M. anisopliae* and *B. bassiana* in the association. Although the isolated strains in this study are not highly efficient for *M. anisopliae*, they could represent a trade-off between high pathogen inhibition specificity and breadth of pathogen effects. *Streptomyces* strains could be selected to inhibit, to a lesser extent, a greater variety of pathogens, instead of being specific. We only tested for two common fungal pathogens, but there are bacteria, fungi, protists and other insects that can attack the nest (Connick *et al.* 2001, Sung-Oui *et al.* 1998, Lcal *et al.* 1995, Prestwich 1984). Thus, *N. costalis* can maintain an optimized and protected nest environment for the development and survival of the colony.



## 4 Conclusions

- The Actinobacteria community in *N. costalis* is dominated by *Streptomyces*, regardless of the season, castes or anatomical regions.
- The strains closely related to *Streptomyces cavourensis* (HQ610450.1) are the most frequent strains of the termite exoskeleton, regardless of castes and season.
- *Streptomyces* BAB5 (JF799913.1) is closely related to the strains most abundant in the gut of termite soldiers, while in the gut of termite workers it varies.
- In the nest material the dominant strains of *Streptomyces* varies between seasons, but the strains closely related to *Streptomyces cavourensis* (HQ610450.1) is among the ten most common strains.
- The strains closely related to *Streptomyces cavourensis* (HQ610450.1) in the nest material, were only found in the presence and activity of termites, this indicates that there may be some type of symbiotic relationship in this system.
- All selected strains inhibit the micelial growth of *B. bassiana* and *M. anisopliae*, but 73% of the strains inhibit the growth of *B. bassiana* among 90% to 100%.
- The *Streptomyces* strains isolated from *N. costalis* in particular the strain W1OE185[2] performs the role of pathogenic protection against microorganisms as it occurs in other eukaryotic organism.

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## Appendices

### Appendix A- General information and colony characteristics of isolates.

General information							Colony characteristic						
Code	Short Code	Season	Nest	Caste	Sample	Frequency	Form	Margin	Elevation	Size (mm) Average	Colony top color	Colony bottom color	Change color of the medium
W2N1[1]	1	Wet	2	-----	Nest	1	Irregular	Lobate	Cerebroide	1.8	Yellow	Cream/Yellow	No
W2SE2[2]	2	Wet	2	Soldier	Exoskeleton	2	Irregular	Filamentous	Crateriform	5.7	White/Yellow	White	No
W2N3[1]	3	Wet	2	-----	Nest	1	Circular	Undulated	Cerebroide	4.5	Brown	Brown	No
W2N4[1]	4	Wet	2	-----	Nest	1	Circular	Filamentous	Convex	7.3	White	Yellow	No
W2N5[2]	5	Wet	2	-----	Nest	2	Circular	Filamentous	Convex	2.5	White	White	No
W2N6[1]	6	Wet	2	-----	Nest	1	Circular	Filamentous	Convex	6.7	White	White/Yellow	No
W2N7[1]	7	Wet	2	-----	Nest	1	Circular	Entire	Flat	3.2	Cream	Cream	No
W2N8[1]	8	Wet	2	-----	Nest	1	Circular	Entire	Convex	1.2	Cream/White	Cream/White	No
W2N9[1]	9	Wet	2	-----	Nest	1	Circular	Filamentous	Pulvinate/Crateriform	4.3	White/Cream	Orange	No
W2N10[1]	10	Wet	2	-----	Nest	1	Circular	Filamentous	Convex	8.2	White	Cream	No
W2SI12[5]	12	Wet	2	Soldier	Gut	5	Circular	Undulated	Convex	6.3	Cream/White	Cream	No
W2N15[1]	15	Wet	2	-----	Nest	1	Puntiform	Entire	Flat	4.3	Cream	Cream	No
W2N16[2]	16	Wet	2	-----	Nest	2	Circular	Entire	Convex	1.8	Cream	Cream/White	No
W2N17[1]	17	Wet	2	-----	Nest	1	Circular	Entire	Convex	1.0	Yellow/White	Yellow/White	No
W2N18[5]	18	Wet	2	-----	Nest	5	Circular	Entire	Umbonate	11.7	White	Cream	No
W2N19[1]	19	Wet	2	-----	Nest	1	Circular	Filamentous	Convex	7.8	White	Cream	No
W2N20[1]	20	Wet	2	-----	Nest	1	Circular	Curled	Convex	1.3	White	Cream	No
W2SI21[3]	21	Wet	2	Soldier	Gut	3	Circular	Filamentous	Flat	2.0	Cream	Cream	No
W2SI22[1]	22	Wet	2	Soldier	Gut	1	Spindle	Curled	Convex	13.7	White	Orange	No
W2OI24[6]	24	Wet	2	Worker	Gut	6	Circular	Filamentous	Umbonate	6.0	Green Gray	Cream	No
W2OI25[4]	25	Wet	2	Worker	Gut	4	Circular	Filamentous	Umbonate	1.7	Brown Gray with white spots	Brown	No
W2OE27[1]	27	Wet	2	Worker	Exoskeleton	1	Circular	Filamentous	Convex	4.5	White	Gray	No
W2OE28[2]	28	Wet	2	Worker	Exoskeleton	2	Circular	Undulated	Convex	9.7	Cream/White	Yellow	No
W2OE30[1]	30	Wet	2	Worker	Exoskeleton	1	Circular	Entire	Convex	4.7	White	Cream	No
W2OE31(3)	31	Wet	2	Worker	Exoskeleton	3	Circular	Undulated	Convex	4.2	White	Brown	No

W2OE32[2]	32	Wet	2	Worker	Exoskeleton	2	Circular	Entire	Umbonate	6.7	White	White	No
W2OE33[2]	33	Wet	2	Worker	Exoskeleton	2	Circular	Entire	Convex	1.5	Cream	Cream	No
W2OE34[3]	34	Wet	2	Worker	Exoskeleton	3	Circular	Entire	Convex	3.5	Cream/White	Cream/White	No
W2OE35[2]	35	Wet	2	Worker	Exoskeleton	2	Circular	Filamentous	Pulvinate	3.3	Cream/White	Cream	No
W2OE39[1]	39	Wet	2	Worker	Exoskeleton	1	Circular	Entire	Convex	0.8	Yellow	Yellow	No
W2OE40[1]	40	Wet	2	Worker	Exoskeleton	1	Circular	Entire	Pulvinate	3.8	Yellow White	Yellow	No
W2OE41[1]	41	Wet	2	Worker	Exoskeleton	1	Circular	Undulated	Convex	3.7	Yellow	Yellow	No
D1N42[21]	42	Dry	1	-----	Nest	21	Irregular	Erose	Cerebroide	2.0	Cream	Cream	No
W2SE43[7]	43	Wet	2	Soldier	Exoskeleton	7	Irregular	Lobate	Cerebroide	1.2	Cream	Cream	No
W3SI45[2]	45	Wet	3	Soldier	Gut	2	Circular	Entire	Convex	1.5	White	White	No
W3OE47[2]	47	Wet	3	Worker	Exoskeleton	2	Circular	Undulated	Cerebroide/Pulvinate	6.3	Cream	Cream	No
W3OE48[1]	48	Wet	3	Worker	Exoskeleton	1	Circular	Entire	Convex	1.3	Cream	Cream	No
W3OE49[2]	49	Wet	3	Worker	Exoskeleton	2	Circular	Entire	Convex	1.2	Cream	Cream	No
W3OE50[3]	50	Wet	3	Worker	Exoskeleton	3	Circular	Filamentous	Convex	5.3	White	Cream	No
W3SI52[5]	52	Wet	3	Soldier	Gut	5	Circular	Filamentous	Cerebroide	5.7	Gray	Brown	No
W3AE53[3]	53	Wet	3	Winged	Exoskeleton	3	Circular	Filamentous	Cerebroide	-----	White	Yellow	No
W3N54[1]	54	Wet	3	-----	Nest	1	-----	-----	-----	-----	-----	-----	-----
W3OI56[9]	56	Wet	3	Worker	Gut	9	Circular	Filamentous	Convex	2.0	Gray	Gray	No
W3OI57[2]	57	Wet	3	Worker	Gut	2	Circular	Entire	Convex	1.3	Cream	Cream	No
W3OI63[2]	63	Wet	3	Worker	Gut	2	Circular	Entire	Convex	1.3	Cream	Cream	No
W3OI64[7]	64	Wet	3	Worker	Gut	7	Circular	Undulated	Convex	1.3	Cream	Cream	No
W3SI66[4]	66	Wet	3	Soldier	Gut	4	Circular	Entire	Convex	1.5	White	White	No
W3OI67[1]	67	Wet	3	Worker	Gut	1	Puntiform	Entire	Convex	6.3	White	Cream	No
W3N68[3]	68	Wet	3	-----	Nest	3	-----	-----	-----	-----	-----	-----	-----
W3OE70[1]	70	Wet	3	Worker	Exoskeleton	1	Circular	Entire	Convex	0.7	Yellow	Yellow	No
W3OE71[1]	71	Wet	3	Worker	Exoskeleton	1	Circular	Entire	Umbonate	7.3	White	Cream	No
W3N72[3]	72	Wet	3	-----	Nest	3	Circular	Filamentous	Flat	4.3	White	Cream	No
W3SE79[5]	79	Wet	3	Soldier	Exoskeleton	5	-----	-----	-----	-----	-----	-----	-----
W3SE80[3]	80	Wet	3	Soldier	Exoskeleton	3	Puntiform	Entire	Convex	0.5	Yellow	Yellow	No
W3OI83[2]	83	Wet	3	Worker	Gut	2	Circular	Entire	Umbonate	2.7	Gray with white spots	Cream	No
W3OI84[3]	84	Wet	3	Worker	Gut	3	Circular	Undulated	Flat	3.2	Gray	Brown	No
W1OI85[3]	85	Wet	1	Worker	Gut	3	Circular	Filamentous	Flat	2.0	White	White	No

W1N86[2]	86	Wet	1	-----	Nest	2	Circular	Curled	Crateriform	7.8	White	Orange	No
W1SE89[2]	89	Wet	1	Soldier	Exoskeleton	2	Circular	Undulated	Crateriform	11.0	White	Brown	No
W1SI93[13]	93	Wet	1	Soldier	Gut	13	Circular	Filamentous	Convex	3.2	Cream	Cream	No
W1SI94[3]	94	Wet	1	Soldier	Gut	3	Circular	Entire	Crateriform	1.0	White	Yellow	No
W1SI95[6]	95	Wet	1	Soldier	Gut	6	Circular	Filamentous	Flat/Crateriform	3.3	Cream/White	Cream	No
W1SI96[3]	96	Wet	1	Soldier	Gut	3	Circular	Entire	Convex	2.3	Cream/White	Cream	No
W1SI97[11]	97	Wet	1	Soldier	Gut	11	Circular	Erode	Convex	2.3	White Gray	Brown	Brown
W1SI98[3]	98	Wet	1	Soldier	Gut	1	Puntiform	Entire	Flat	0.8	Cream	Cream	No
W1OI99[4]	99	Wet	1	Worker	Gut	4	Circular	Entire	Pulvinate	3.0	White	Cream	No
W1OI100[3]	100	Wet	1	Worker	Gut	3	Circular	Entire	Convex	1.0	Cream	Cream	No
W1OI101[1]	101	Wet	1	Worker	Gut	1	Circular	Filamentous	Convex	2.2	White/Cream	White	No
W1OI103[33]	103	Wet	1	Worker	Gut	33	Circular	Filamentous	Flat	7.3	Gray/White	Brown/Cream	No
W1OI104[5]	104	Wet	1	Worker	Gut	5	Circular	Erode	Flat	1.2	Gray/White	White	No
W1OI105[1]	105	Wet	1	Worker	Gut	1	Circular	Filamentous	Flat	1.7	Cream	Cream	No
W1OI106[1]	106	Wet	1	Worker	Gut	1	Circular	Undulated	Flat	6.5	White/Green	Green/Cream	Green
W1SI107[4]	107	Wet	1	Soldier	Gut	4	Puntiform	Filamentous	Convex	6.0	White	Cream	No
W2OE109[2]	109	Wet	2	Worker	Exoskeleton	2	Puntiform	Entire	Convex	5.0	White	Cream	No
W2OE110[2]	110	Wet	2	Worker	Exoskeleton	2	Circular	Curled	Flat	10.7	White	Brown	Brown
W2SE111[9]	111	Wet	2	Soldier	Exoskeleton	9	Circular	Filamentous	Crateriform	3.3	White/Cream	Cream	No
W2SE112[4]	112	Wet	2	Soldier	Exoskeleton	4	Circular	Filamentous	Convex	6.3	White	White	No
W2SI113[15]	113	Wet	2	Soldier	Gut	15	Circular	Filamentous	Crateriform	4.0	Gray	Cream	No
W2N114[1]	114	Wet	2	-----	Nest	1	Circular	Entire	Flat	1.0	White	White	No
W2N115[2]	115	Wet	2	-----	Nest	2	Puntiform	Entire	Flat	0.5	Cream	Cream	No
W2N116[1]	116	Wet	2	-----	Nest	1	Circular	Filamentous	Convex	3.7	White	Cream	No
W2N117[1]	117	Wet	2	-----	Nest	1	Circular	Filamentous	Flat	3.0	Cream/White	Cream	No
W2N118[3]	118	Wet	2	-----	Nest	3	Circular	Undulated	Convex	3.7	White/Yellow	Yellow	No
W3OI120[3]	120	Wet	3	Worker	Gut	3	Circular	Entire	Convex	2.0	White	Orange/Brown	Brown
W3OI121[3]	121	Wet	3	Worker	Gut	3	Circular	Entire	Convex	7.0	Gray	Brown	No
W1OI123[3]	123	Wet	1	Worker	Gut	3	Circular	Undulated	Convex	5.8	White	Cream	No
W1OI124[1]	124	Wet	1	Worker	Gut	1	Circular	Curled	Flat	3.8	Gray	Brown	Brown
W1OI125[3]	125	Wet	1	Worker	Gut	3	Circular	Filamentous	Convex	4.5	Yellow	Orange	Orange
W1OI126[1]	126	Wet	1	Worker	Gut	1	Circular	Filamentous	Convex	1.5	Cream White	Cream	No

W1OE129[4]	129	Wet	1	Worker	Exoskeleton	4	Circular	Entire	Pulvinate	4.5	Yellow/White	Cream	No
W1OE130[2]	130	Wet	1	Worker	Exoskeleton	2	Circular	Entire	Convex	4.7	Cream/Gray	Brown	No
W1OE131[1]	131	Wet	1	Worker	Exoskeleton	1	Circular	Undulated	Pulvinate	12.7	White	White	No
W1OE133[3]	133	Wet	2	Worker	Exoskeleton	3	Circular	Filamentous	Pulvinate	2.7	White	White	No
W1OE135[4]	135	Wet	1	Worker	Exoskeleton	4	Circular	Filamentous	Pulvinate	3.3	White	Cream	No
W1OE136[4]	136	Wet	1	Worker	Exoskeleton	4	Circular	Filamentous	Convex	4.3	White	Cream	No
D1SI137[4]	137	Dry	1	Soldier	Gut	4	Irregular	Curled	Flat	7.0	White	White	No
W1SI138[4]	138	Wet	1	Soldier	Gut	4	Circular	Entire	Convex	3.5	White	Orange	No
W1OE139[5]	139	Wet	1	Worker	Exoskeleton	5	Circular	Filamentous	Crateriform/Convex	4.7	White	Cream	No
W1OE140[1]	140	Wet	1	Worker	Exoskeleton	1	Irregular	Undulated	Crateriform	3.7	White/Orange	Dark Cream	No
W1N143[3]	143	Wet	1	-----	Nest	3	Circular	Undulated	Convex	3.0	White/Cream	Cream	No
W1N146[1]	146	Wet	1	-----	Nest	1	Circular	Filamentous	Convex	2.0	White	White	No
W1SE147[1]	147	Wet	1	Soldier	Exoskeleton	1	Irregular	Undulated	Cerebroide	2.3	Cream/White	Cream	Yellow
W1SE149[4]	149	Wet	1	Soldier	Exoskeleton	4	Circular	Filamentous	Convex	6.7	Gray White	Brown	No
W1SE150[2]	150	Wet	1	Soldier	Exoskeleton	2	Circular	Filamentous	Flat	2.8	White	Cream	No
W1N152[5]	152	Wet	1	-----	Nest	5	Circular	Entire	Convex	4.7	Yellow/White	Yellow	No
W2SE157[2]	157	Wet	2	Soldier	Exoskeleton	2	Circular	Entire	Convex	1.0	Cream	Cream	No
W2N160[1]	160	Wet	2	-----	Nest	1	Circular	Undulated	Convex	5.2	White	Brown	No
W2SE161[5]	161	Wet	2	Soldier	Exoskeleton	5	Circular	Filamentous	Convex	1.8	Yellow	Yellow	No
W2OE163[1]	163	Wet	2	Worker	Exoskeleton	1	Circular	Filamentous	Convex	3.5	White/Cream	White	No
W2N167[2]	167	Wet	2	-----	Nest	2	Circular	Undulated	Convex	4.7	White/Cream	Cream	No
W3SE168[2]	168	Wet	3	Soldier	Exoskeleton	2	Circular	Filamentous	Convex	7.8	Cream/White	Cream	No
W1OE169[2]	169	Wet	1	Worker	Exoskeleton	2	Circular	Entire	Convex	4.3	Yellow/White	Yellow	No
W1OE171[3]	171	Wet	1	Worker	Exoskeleton	3	Circular	Entire	Convex	7.0	White	Yellow	No
W1OE173[1]	173	Wet	1	Worker	Exoskeleton	1	Circular	Entire	Convex	5.0	Green/Gray	Brown/Cream	Brown
W1OE174[3]	174	Wet	1	Worker	Exoskeleton	3	Circular	Curled	Convex	5.0	Cream	Cream	No
W1OE176[2]	176	Wet	1	Worker	Exoskeleton	2	Circular	Filamentous	Convex	3.3	White	White	No
W1OE177[3]	177	Wet	1	Worker	Exoskeleton	3	Circular	Filamentous	Convex	3.3	Cream/White	Cream	No
W1SE178[1]	178	Wet	1	Soldier	Exoskeleton	1	Circular	Undulated	Convex	3.5	Cream/White	cream	No
W1SE179[1]	179	Wet	1	Soldier	Exoskeleton	1	Circular	Filamentous	Convex	2.2	Yellow	Cream	No
W1SE180[1]	180	Wet	1	Soldier	Exoskeleton	1	Circular	Undulated	Convex	7.0	Yellow/White	Cream	No
W1SE181[2]	181	Wet	1	Soldier	Exoskeleton	2	Circular	Undulated	Convex	3.7	White	Brown	No

W1SE182[5]	182	Wet	1	Soldier	Exoskeleton	5	Circular	Entire	Convex	2.7	Yellow	Yellow	No
W1N183[4]	183	Wet	1	-----	Nest	4	Circular	Entire	Pulvinate	2.3	Gray	Brown	Brown
W1N184[1]	184	Wet	1	-----	Nest	1	Circular	Filamentous	Convex	9.2	White/Cream/Green	Yellow	No
W1OE185[6]	185	Wet	1	Worker	Exoskeleton	6	Circular	Entire	Convex	2.8	White/Cream	Cream	No
W1OE186[1]	186	Wet	1	Worker	Exoskeleton	1	Circular	Undulated	Cerebroide	7.3	Yellow	Yellow	No
W1OE187[2]	187	Wet	1	Worker	Exoskeleton	2	Circular	Filamentous	Convex	3.7	White	Orange	No
W1SE188[1]	188	Wet	1	Soldier	Exoskeleton	1	-----	-----	-----	-----	-----	-----	-----
W1N189[1]	189	Wet	1	-----	Nest	1	Circular	Undulated	Cerebroide	4.3	Cream/White	Cream	No
W1OE190[3]	190	Wet	1	Worker	Exoskeleton	3	Circular	Filamentous	Pulvinate	7.7	White	Yellow	No
W1OE191[1]	191	Wet	1	Worker	Exoskeleton	1	Circular	Curled	Flat	8.3	White	Brown	Brown
W1OE192[2]	192	Wet	1	Worker	Exoskeleton	2	Circular	Filamentous	Convex	4.3	Cream/White	Yellow	No
W1OE193[4]	193	Wet	1	Worker	Exoskeleton	4	Circular	Undulated	Crateriform	5.7	Cream/White	Cream	No
W1OE194[2]	194	Wet	1	Worker	Exoskeleton	2	Puntiform	Curled	Flat	8.3	Cream	Cream	No
W1OE195[3]	195	Wet	1	Worker	Exoskeleton	3	Circular	Filamentous	Raised	1.7	Yellow White	Yellow	No
W1N196[1]	196	Wet	1	-----	Nest	1	Circular	Undulated	Convex	4.0	White/Yellow	Cream	No
W1N197[2]	197	Wet	1	-----	Nest	2	Filamentous	Filamentous	Convex	3.3	Cream	Cream	No
W1SE198[2]	198	Wet	1	Soldier	Exoskeleton	2	Circular	Entire	Convex	1.3	Yellow Cream	Cream	No
W3OI199[3]	199	Wet	3	Worker	Gut	3	Circular	Filamentous	Convex	4.8	Gray	Green	No
D1N200[4]	200	Dry	1	-----	Nest	4	Circular	Entire	Flat	4.3	Cream/White	Cream	No
W1N201[3]	201	Wet	1	-----	Nest	3	Circular	Filamentous	Convex	5.0	Cream/White	Cream	No
W3OE202[6]	202	Wet	3	Worker	Exoskeleton	6	Circular	Filamentous	Flat	4.8	Gray	Brown	No
D2N203[12]	203	Dry	2	-----	Nest	12	Circular	Undulated	Pulvinate	3.8	White	Brown	Dark Brown
D1OE206[2]	206	Dry	1	Worker	Exoskeleton	2	Circular	Undulated	Crateriform	7.3	Gray	Brown	No
D1OE208[5]	208	Dry	1	Worker	Exoskeleton	5	Circular	Lobate	Crateriform	6.7	Gray	Dark Brown	No
D1OE211[10]	211	Dry	1	Worker	Exoskeleton	10	Irregular	Entire	Crateriform	3.3	Gray	Gray	Brown
D1OE213[8]	213	Dry	1	Worker	Exoskeleton	8	Circular	Filamentous	Flat	10.0	White	Brown	Dark Brown
D1OE214[60]	214	Dry	1	Worker	Exoskeleton	60	Circular	Filamentous	Flat	1.7	White/Gray	Cream	No
D1SE216[6]	216	Dry	1	Soldier	Exoskeleton	6	Circular	Entire	Convex	2.2	Cream	White/Cream	Brown
D1SE218[4]	218	Dry	1	Soldier	Exoskeleton	4	Circular	Filamentous	Convex	1.7	Yellow	Cream	No
D2OI225[8]	225	Dry	2	Worker	Gut	8	Circular	Undulated	Convex	7.0	White	Cream	No
D2OI240[2]	240	Dry	2	Worker	Gut	2	Circular	Filamentous	Cerebroide	6.7	Brown/Yellow	Brown	No
D2N242[1]	242	Dry	2	-----	Nest	1	Circular	Filamentous	Pulvinate	3.0	White	Brown	No

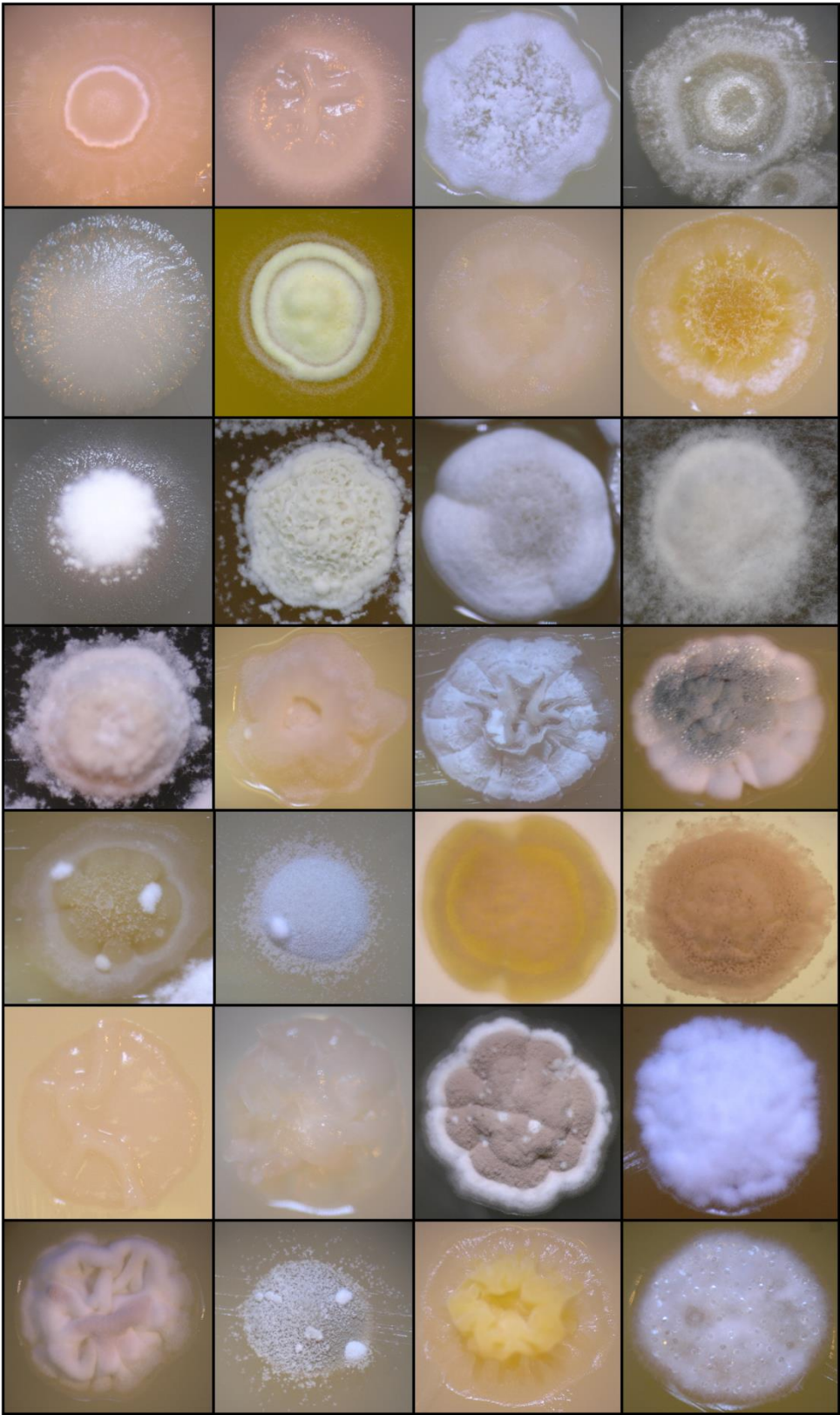
D1OI245[5]	245	Dry	1	Worker	Gut	5	Circular	Filamentous	Convex	4.5	White	Brown	Brown
D2SE257[6]	257	Dry	2	Soldier	Exoskeleton	6	Circular	Entire	Convex	2.0	White/Violet/Yellow	Violet/Cream	Brown
D2SE261[16]	261	Dry	2	Soldier	Exoskeleton	16	Circular	Filamentous	Convex	9.0	White Green	Brown	Brown
D2SE262[8]	262	Dry	2	Soldier	Exoskeleton	8	Circular	Entire	Raised	5.8	White	Cream	Brown
D1N265[2]	265	Dry	1	-----	Nest	2	Circular	Undulated	Pulvinate	2.7	White	Cream/Orange	No
D1OI276[7]	276	Dry	1	Worker	Gut	7	Circular	Filamentous	Convex	3.5	White	White	No
D1SI281[75]	281	Dry	1	Soldier	Gut	75	Circular	Entire	Convex	2.3	Cream Green	Cream	No
D1SI286[9]	286	Dry	1	Soldier	Gut	9	Circular	Filamentous	Flat	7.0	White	Cream	No
D1SI287[14]	287	Dry	1	Soldier	Gut	14	Irregular	Filamentous	Convex	4.0	Gray	Green	No
D1OE296[2]	296	Dry	1	Worker	Exoskeleton	2	Circular	Filamentous	Flat	6.0	Gray	Gray	No
D1OE297[15]	297	Dry	1	Worker	Exoskeleton	15	Circular	Filamentous	Convex	7.8	Dark Gray	Cream	No
D2OE298[2]	298	Dry	2	Worker	Exoskeleton	2	-----	-----	-----	-----	-----	-----	-----
D2SI303[3]	303	Dry	1	Soldier	Gut	3	Circular	Entire	Umbonate	2.7	Yellow	Yellow	No
D2SI304[3]	304	Dry	2	Soldier	Gut	3	Circular	Entire	Flat	1.0	Cream	Cream	No
D2SI305[5]	305	Dry	2	Soldier	Gut	5	Circular	Erose	Convex	1.3	Cream	Cream	No
D1OE310[3]	310	Dry	1	Worker	Exoskeleton	3	Circular	Filamentous	Convex	4.3	White	White	No
D1N312[1]	312	Dry	1	-----	Nest	1	-----	-----	-----	-----	-----	-----	-----
D1N315[3]	315	Dry	1	-----	Nest	3	Circular	Undulated	Crateriform	3.7	Cream	Cream	No
D1N317[4]	317	Dry	1	-----	Nest	4	Circular	Entire	Convex	2.8	Cream	Cream	No
D1OI321[18]	321	Dry	1	Worker	Gut	18	Circular	Filamentous	Convex	4.7	Gray	Dark Gray	Brown
D1OI332[9]	332	Dry	1	Worker	Gut	9	Circular	Undulated	Convex	2.3	White	White	No
D1SI334[4]	334	Dry	1	Soldier	Gut	4	Circular	Erose	Convex	3.0	Brown/White	Brown	Brown
D2OI335[2]	335	Dry	2	Worker	Gut	2	Circular	Undulated	Convex	7.7	White	Cream	No
D2OI336[3]	336	Dry	2	Worker	Gut	3	Circular	Undulated	Convex	3.2	Gray	Gray	No
D2OI337[2]	337	Dry	2	Worker	Gut	2	Irregular	Undulated	Cerebroide	1.7	White/Cream	Cream/Brown	Yellow
D2OI340[6]	340	Dry	2	Worker	Gut	6	Circular	Entire	Convex	3.7	Cream/White	Cream	No
D2OI341[1]	341	Dry	2	Worker	Gut	1	Circular	Curled	Convex	1.7	White Pink	Orange Pink	No
D2SI346[2]	346	Dry	2	Soldier	Gut	2	Circular	Filamentous	Crateriform	9.0	White	Yellow	No
D2SI348[2]	348	Dry	2	Soldier	Gut	2	Circular	Entire	Crateriform	3.5	Gray	White	No
D2SI351[3]	351	Dry	2	Soldier	Gut	3	Circular	Filamentous	Convex	3.0	White	Cream	No
D1SI352[3]	352	Dry	1	Soldier	Gut	3	Circular	Undulated	Cerebroide	5.5	White/Gray	Gray	No
D2SE358[2]	358	Dry	2	Soldier	Exoskeleton	2	Circular	Undulated	Cerebroide	8.2	White/Gray	White	No

D1SE361[4]	361	Dry	1	Soldier	Exoskeleton	4	Circular	Entire	Convex	1.3	Cream	Cream	No
D3N365[2]	365	Dry	1	-----	Nest	2	Circular	Undulated	Umbonate	4.3	White	White	No
W3OI366[4]	366	Wet	3	Worker	Gut	4	Irregular	Undulated	Pulvinate/Crateriform	5.0	Yellow	Yellow	No
D2SE369[4]	369	Dry	2	Soldier	Exoskeleton	4	Circular	Undulated	Convex	5.3	Cream/White	Cream	No
D1N371[10]	371	Dry	1	-----	Nest	10	Irregular	Undulated	Cerebroide	1.0	Yellow/White	Yellow	No
D1N378[3]	378	Dry	1	-----	Nest	3	Irregular	Undulated	Crateriform	6.0	White	Brown	No
D2N385[4]	385	Dry	2	-----	Nest	4	Circular	Filamentous	Umbonate	9.0	White Green	Brown	Brown
W2OI392[2]	392	Wet	2	Worker	Gut	2	Circular	Erose	Cerebroide	4.5	Yellow/White	Cream	No
D1OI395[3]	395	Dry	1	Worker	Gut	3	Irregular	Undulated	Cerebroide	7.0	Yellow	Yellow	No
D1OI396[160]	396	Dry	1	Worker	Gut	160	Circular	Curled	Flat	2.0	White/Cream	Cream	No
W3N397[2]	397	Wet	3	-----	Nest	2	Circular	Entire	Convex	3.8	Cream	Cream	No
W3SI398[4]	398	Wet	3	Soldier	Gut	4	Circular	Undulated	Crateriform	2.8	Yellow	Yellow	No
D1OI403[3]	403	Dry	1	Worker	Gut	3	Circular	Undulated	Umbonate	1.7	White/Pink	Orange	Yellow
W1OI405[2]	405	Wet	1	Worker	Gut	2	Circular	Undulated	Convex	3.3	Yellow	Yellow	No
W3N412[3]	412	Wet	3	-----	Nest	3	Circular	Filamentous	Convex	2.3	White	Yellow	No
W1OI418[1]	418	Wet	1	Worker	Gut	1	Irregular	Undulated	Crateriform	4.7	Cream Gray	Green	No
D1OE420[4]	420	Dry	1	Worker	Exoskeleton	4	Circular	Curled	Crateriform	-----	White/Brown	Dark Brown	Brown
D1N421[17]	421	Dry	1	-----	Nest	17	Irregular	Erose	Flat	4.3	Cream	Cream	No
W3OI422[2]	422	Wet	3	Worker	Gut	2	Circular	Filamentous	Convex	2.0	White/Gray	White	No
W1OI423[14]	423	Wet	1	Worker	Gut	14	Irregular	Filamentous	Cerebroide	4.0	White	Yellow	No
D3N424[41]	424	Dry	3	-----	Nest	41	Circular	Filamentous	Convex	5.0	White Green	Brown	No
D2OI427[4]	427	Dry	2	Worker	Gut	5	Circular	Filamentous	Flat	2.3	White	Orange	No
D1N428[3]	428	Dry	1	-----	Nest	3	Circular	Filamentous	Convex	2.2	Violet/Cream	Violet/Cream	No
D2OI429[2]	429	Dry	2	Worker	Gut	2	Circular	Filamentous	Flat	3.7	White	White	No
D2OI430[5]	430	Dry	2	Worker	Gut	5	Circular	Undulated	Flat	3.0	White	Pink	Pink
D2SE433[10]	433	Dry	2	Soldier	Exoskeleton	10	Circular	Undulated	Crateriform	5.7	Brown/White/Green	Brown	Brown
W3SI435[9]	435	Wet	3	Soldier	Gut	9	Irregular	Filamentous	Convex	7.2	White/Gray	Cream	No
D2OE436[5]	436	Dry	2	Worker	Exoskeleton	5	Circular	Filamentous	Convex	6.2	White	Dark Brown	Dark Brown
W2N437[2]	437	Wet	2	-----	Nest	2	Circular	Entire	Convex	5.7	White	Cream	No
D2N438[10]	438	Dry	2	-----	Nest	10	Circular	Undulated	Convex	1.0	White	Brown	Brown
W1OE440[5]	440	Wet	1	Worker	Exoskeleton	5	Circular	Entire	Umbonate	4.2	Cream/White	Cream	No
W1OE441[2]	441	Wet	1	Worker	Exoskeleton	2	Circular	Undulated	Convex	4.3	Cream/White	Cream	No

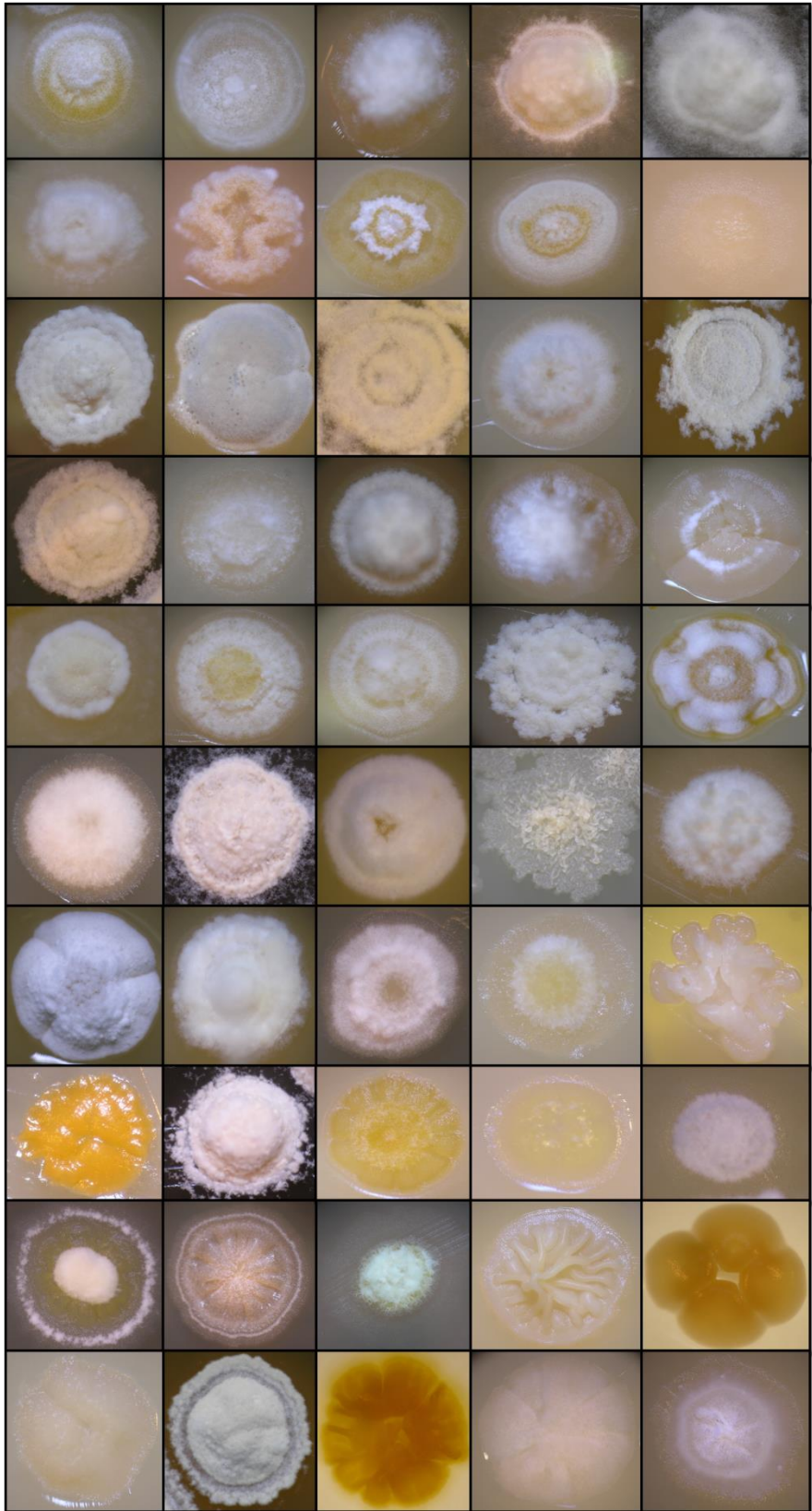
D2N442[4]	442	Dry	2	-----	Nest	4	Circular	Filamentous	Flat	4.0	White	Brown	Brown
W3SI445[3]	445	Wet	3	Soldier	Gut	3	Circular	Undulated	Convex	6.5	Green Gray	Gray	No
D1OE447[12]	447	Dry	1	Worker	Exoskeleton	12	Circular	Undulated	Convex	3.5	Violet	Violet	No
W2OI449[8]	449	Wet	2	Worker	Gut	8	Circular	Entire	Convex	4.3	Green	Brown	No
D3N463[1]	463	Dry	3	-----	Nest	1	-----	-----	-----	-----	-----	-----	-----
D3N469[6]	469	Dry	3	-----	Nest	6	-----	-----	-----	-----	-----	-----	-----
D2N476[2]	476	Dry	2	-----	Nest	2	Irregular	Curled	Crateriform	4.7	White	Dark Orange	No
D2N477[4]	477	Dry	2	-----	Nest	4	Circular	Filamentous	Convex	7.0	White	Brown	Brown
W2OE478[2]	478	Wet	2	Worker	Exoskeleton	2	Circular	Filamentous	Convex	2.0	Yellow White	Yellow	No
D2OI480[6]	480	Dry	2	Worker	Gut	6	Circular	Entire	Convex	1.3	Cream/White	Cream	No
D1OI484[11]	484	Dry	1	Worker	Gut	11	Circular	Filamentous	Umbonate	3.7	Brown with white spots	Cream	No
D2OI485[6]	485	Dry	2	Worker	Gut	6	Circular	Curled	Convex	1.0	White	Dark Orange	No
W1SE491[2]	491	Wet	1	Soldier	Exoskeleton	2	Circular	Erose	Flat	3.7	Cream	Cream	No
W2N494[3]	494	Wet	2	-----	Nest	3	Circular	Filamentous	Convex	13.0	Cream/White	Cream	No
W1OE495[1]	495	Wet	1	Worker	Exoskeleton	1	Circular	Filamentous	Convex	6.7	White	White	No
D1OI496[12]	496	Dry	1	Worker	Gut	12	Circular	Filamentous	Flat	1.7	Cream/White	Cream	No
W3AE498[3]	498	Wet	3	Winged	Exoskeleton	3	Circular	Filamentous	Convex	4.0	White	Yellow	No
W2SE500[12]	500	Wet	2	Soldier	Exoskeleton	12	Circular	Filamentous	Convex	5.8	Yellow/White	Cream	No
W1OI510[2]	510	Wet	1	Worker	Gut	2	Circular	Entire	Pulvinate	1.2	White/Gray	White	No
W1SI515[85]	515	Wet	1	Soldier	Gut	85	Circular	Filamentous	Flat	3.8	White	Cream	No
W1OE519[3]	519	Wet	1	Worker	Exoskeleton	3	Circular	Undulated	Convex	4.3	Yellow/White	Yellow	No
D2OI524[4]	524	Dry	2	Worker	Gut	4	Circular	Filamentous	Convex	5.7	White	Yellow	No
W1SE528[3]	528	Wet	1	Soldier	Exoskeleton	3	Circular	Filamentous	Crateriform	2.3	White	Brown	No
W1OE529[5]	529	Wet	1	Worker	Exoskeleton	5	Circular	Entire	Pulvinate	2.2	White	Cream	No
W1OE530[2]	530	Wet	1	Worker	Exoskeleton	2	Irregular	Erose	Cerebroide	2.8	Cream	Cream	No



**Appendix B-** Morphotypes isolated of worker gut from wet season

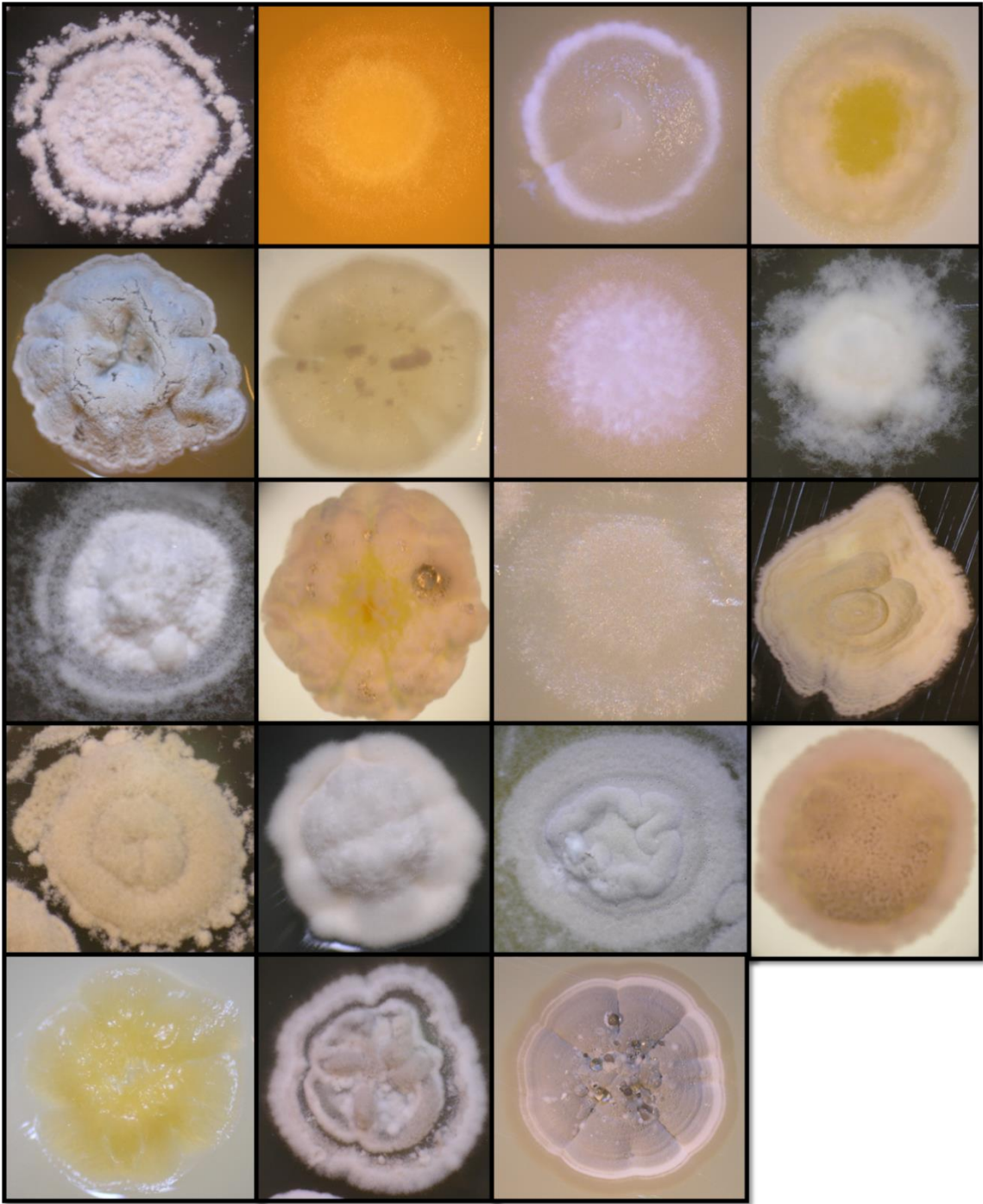


**Appendix C-** Morphotypes isolated of worker exoskeleton from wet season

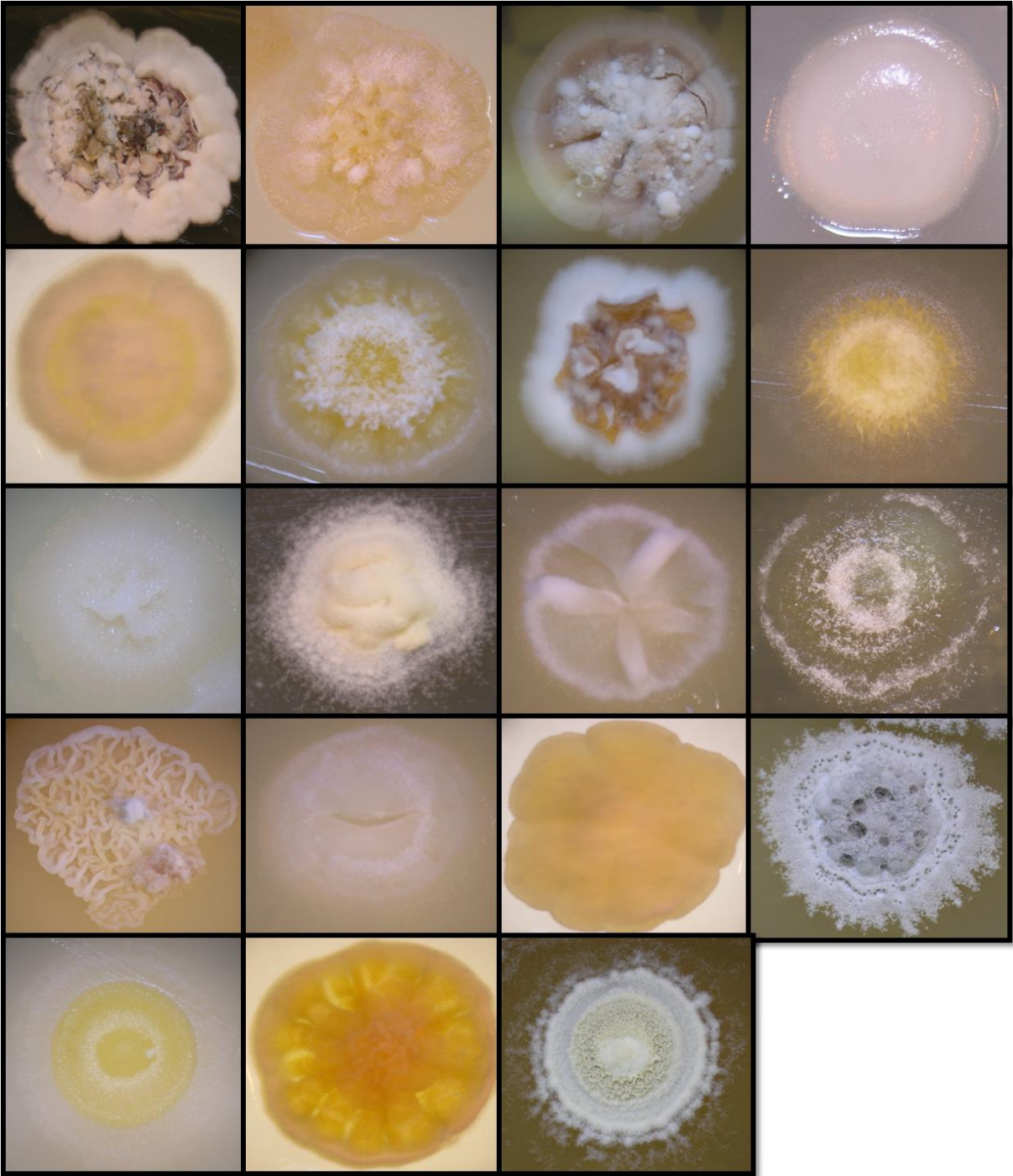




**Appendix D-** Morphotypes isolated of soldier gut from wet season

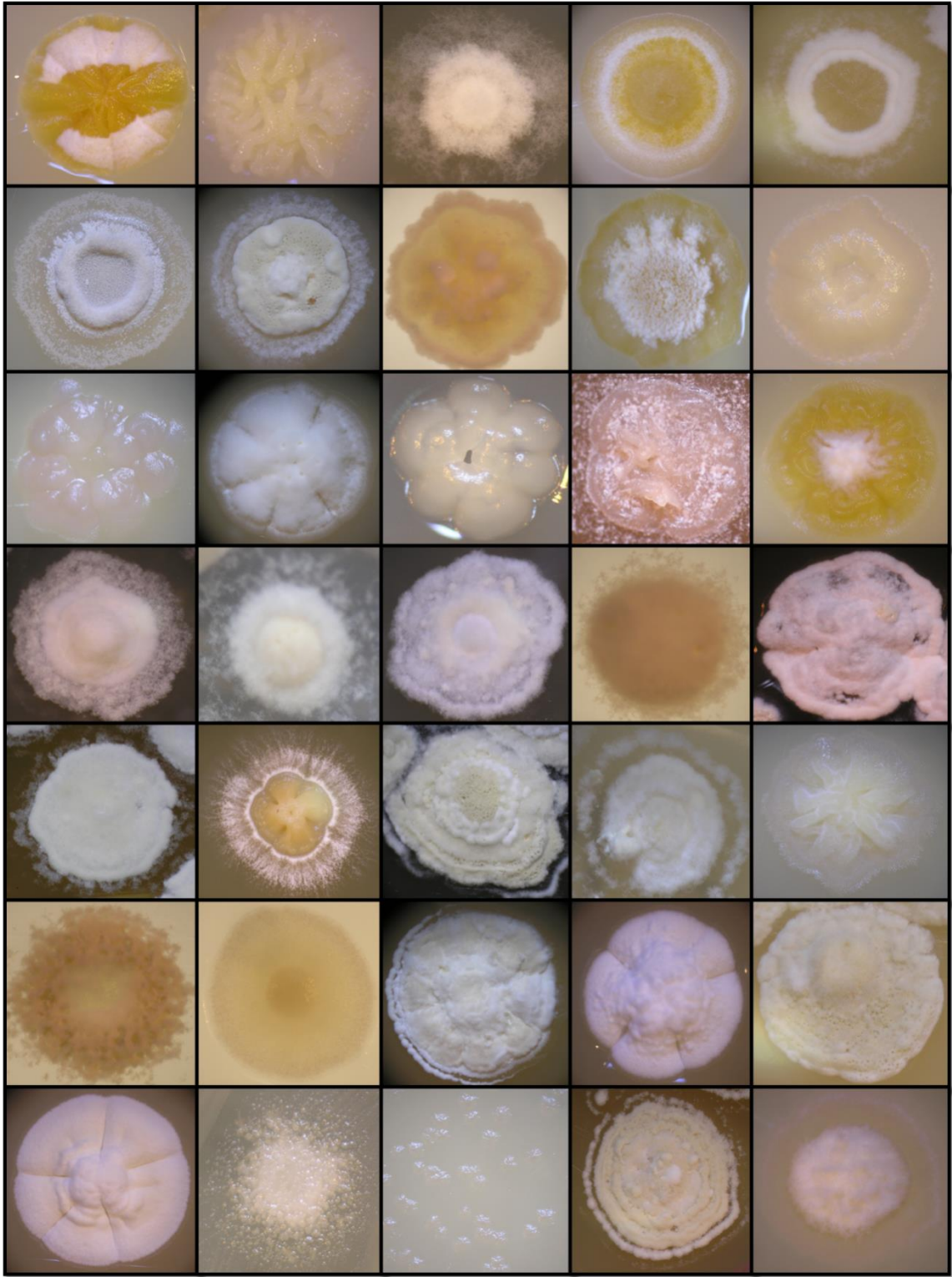


**Appendix E-** Morphotypes isolated of soldier exoskeleton from wet season

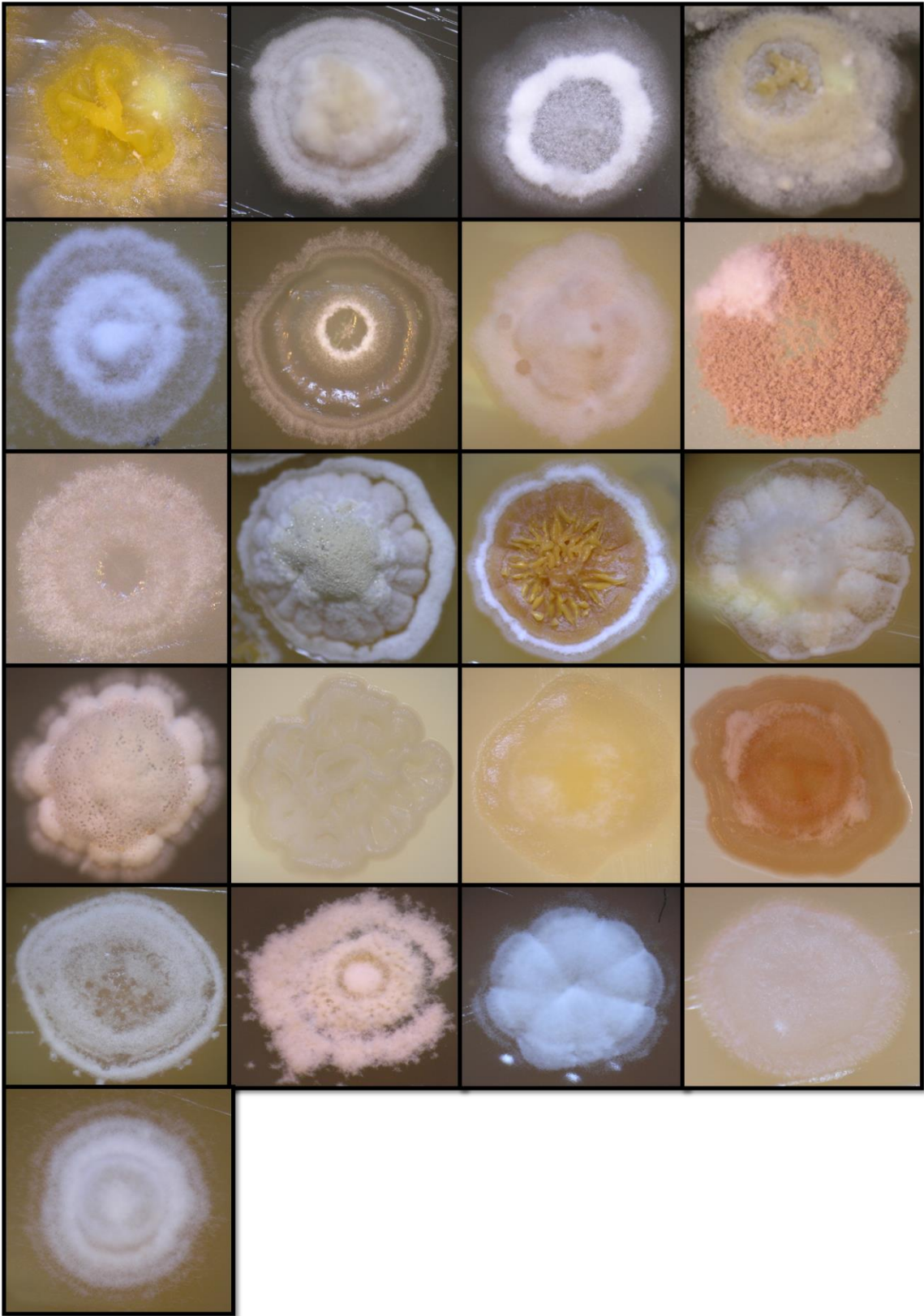




**Appendix F-** Morphotypes isolated of nest material from wet season

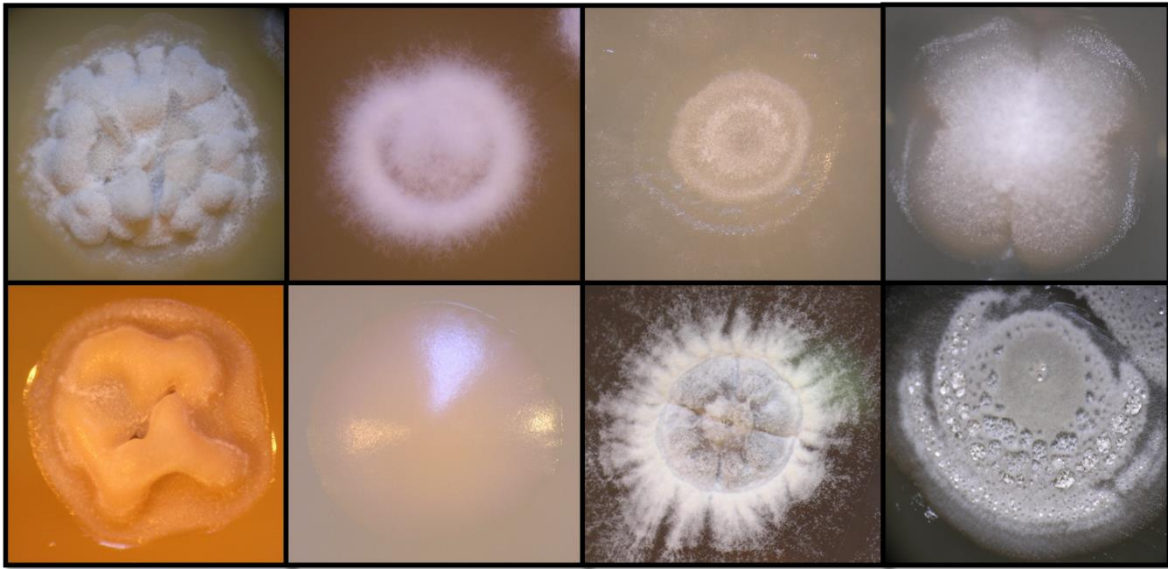


**Appendix G-** Morphotypes isolated of worker gut from dry season

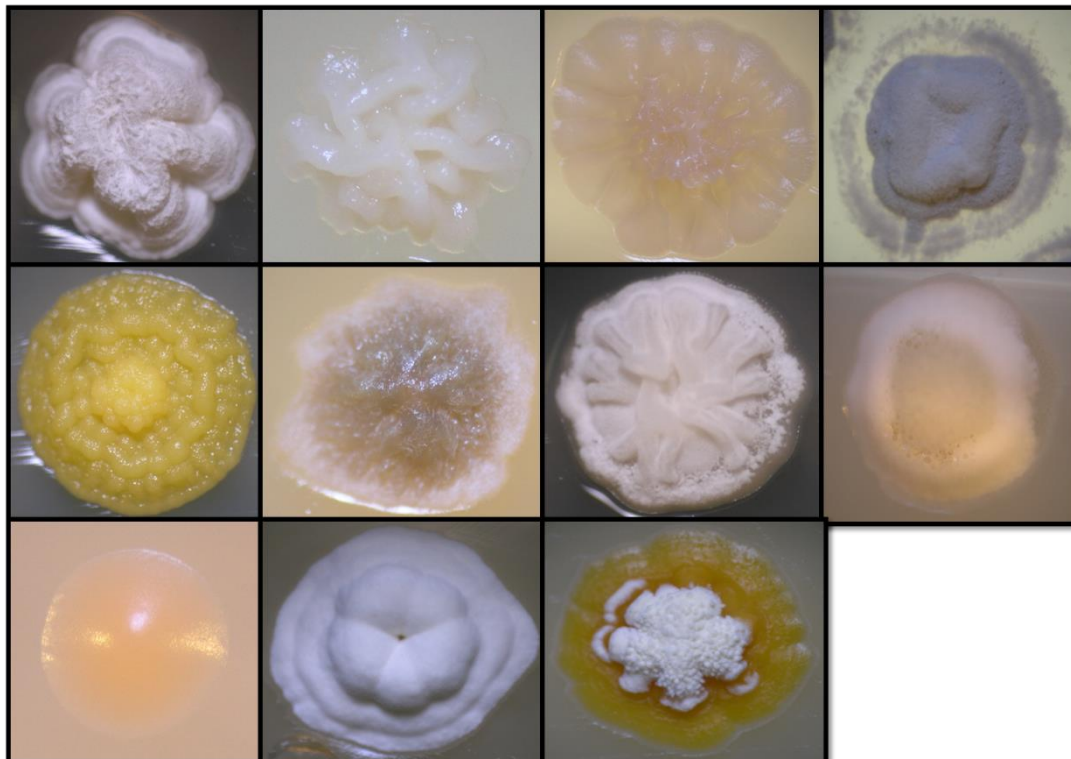




**Appendix H-** Morphotypes isolated of worker exoskeleton from dry season



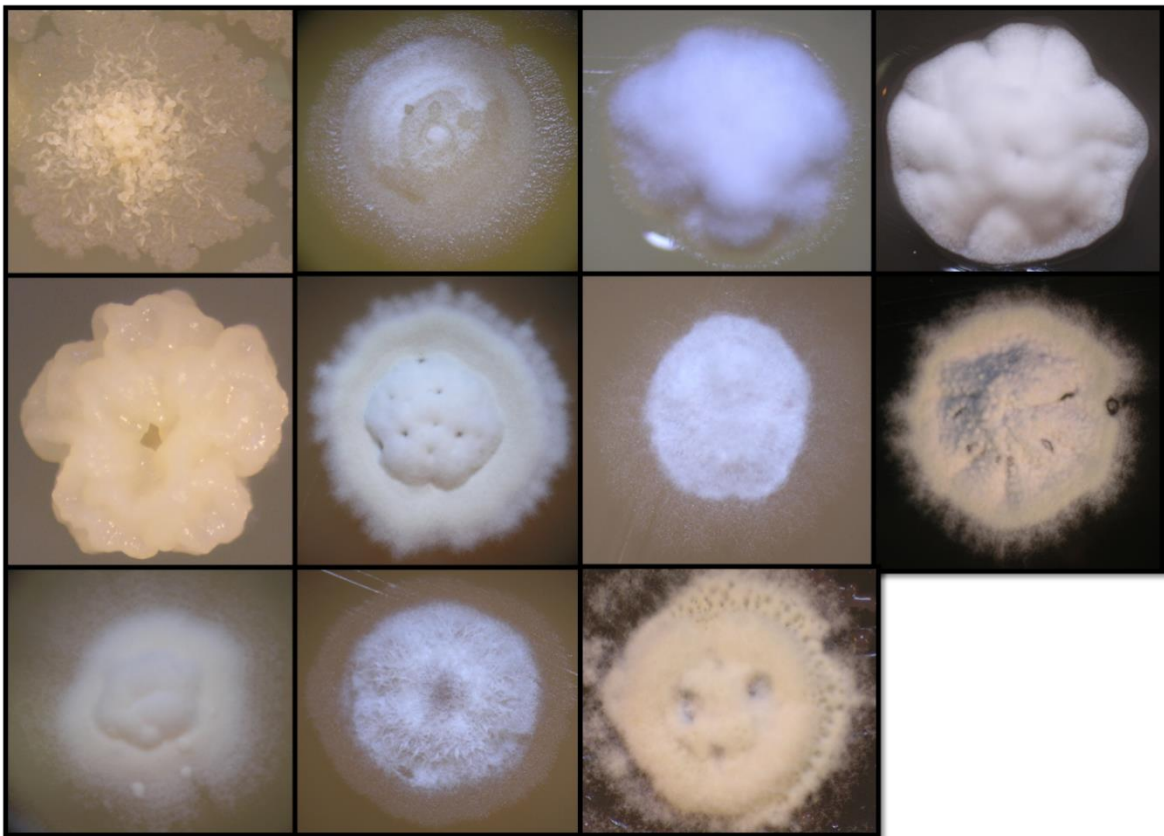
**Appendix I-** Morphotypes isolated of soldier gut from dry season



**Appendix J-** Morphotypes isolated of soldier exoskeleton from dry season



**Appendix K-** Morphotypes isolated of nest material from dry season





## Appendix L- General information of strains and molecular identification

General information							Molecular identification		
Code	Short Code	Season	Nest	Caste	Sample	Frequency	Accession Number	Description	Max identification
W2N1[1]	1	Wet	2	-----	Nest	1	EU054375.1	<i>Streptomyces</i> sp. 8-1	99%
W2SE2[2]	2	Wet	2	Soldier	Exoskeleton	2	HM579818.1	<i>Streptomyces</i> sp. HY27	99%
W2N3[1]	3	Wet	2	-----	Nest	1	JQ924406.1	<i>Streptomyces praecox</i>	99%
W2N4[1]	4	Wet	2	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	93%
W2N5[2]	5	Wet	2	-----	Nest	2	JQ284036.1	<i>Streptomyces globisporus</i>	94%
W2N6[1]	6	Wet	2	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	94%
W2N7[1]	7	Wet	2	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	93%
W2N8[1]	8	Wet	2	-----	Nest	1	FJ486370.1	<i>Streptomyces seoulensis</i>	99%
W2N9[1]	9	Wet	2	-----	Nest	1	JF799913.1	<i>Streptomyces</i> sp. BAB5	99%
W2N10[1]	10	Wet	2	-----	Nest	1	FJ486399.1	<i>Streptomyces californicus</i>	95%
W2SI12[5]	12	Wet	2	Soldier	Gut	5	JN609387.1	<i>Streptomyces bottropensis</i>	95%
W2N15[1]	15	Wet	2	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W2N16[2]	16	Wet	2	-----	Nest	2	GQ475300.1	<i>Streptomyces</i> sp. 1492	96%
W2N17[1]	17	Wet	2	-----	Nest	1	EU119181.1	<i>Streptomyces</i> sp. HBUM71745	99%
W2N18[5]	18	Wet	2	-----	Nest	5	AB184777.2	<i>Streptomyces roseochromogenus</i>	92%
W2N19[1]	19	Wet	2	-----	Nest	1	EU368787.1	<i>Streptomyces</i> sp. A306 Ydz-ZZ	99%
W2N20[1]	20	Wet	2	-----	Nest	1	EU368787.1	<i>Streptomyces</i> sp. A306 Ydz-ZZ	98%
W2SI21[3]	21	Wet	2	Soldier	Gut	3	GU350488.1	<i>Streptomyces</i> sp. MJM9923	99%
W2SI22[1]	22	Wet	2	Soldier	Gut	1	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W2OI24[6]	24	Wet	2	Worker	Gut	6	HM018109.1	<i>Streptomyces</i> sp. WALP25	93%
W2OI25[4]	25	Wet	2	Worker	Gut	4	HM352343.1	<i>Burkholderia</i> sp. CmMC2	99%
W2OE27[1]	27	Wet	2	Worker	Exoskeleton	1	JQ284036.1	<i>Streptomyces globisporus</i>	95%
W2OE28[2]	28	Wet	2	Worker	Exoskeleton	2	HM018120.1	<i>Streptomyces</i> sp. VAN21	99%
W2OE30[1]	30	Wet	2	Worker	Exoskeleton	1	HQ610450.1	<i>Streptomyces cavourensis</i>	94%
W2OE31(3)	31	Wet	2	Worker	Exoskeleton	3	AJ550282.1	<i>Comamonas</i> sp. TK41	90%
W2OE32[2]	32	Wet	2	Worker	Exoskeleton	2	JF778669.1	<i>Streptomyces microflavus</i>	99%
W2OE33[2]	33	Wet	2	Worker	Exoskeleton	2	JN030348.1	<i>Achromobacter</i> sp. RAU-832	89%
W2OE34[3]	34	Wet	2	Worker	Exoskeleton	3	HQ285876.1	<i>Achromobacter xylosoxidans</i>	89%

W2OE35[2]	35	Wet	2	Worker	Exoskeleton	2	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	100%
W2OE39[1]	39	Wet	2	Worker	Exoskeleton	1	GU294692.1	<i>Streptomyces sp. SA01</i>	99%
W2OE40[1]	40	Wet	2	Worker	Exoskeleton	1	HQ610450.1	<i>Streptomyces cavourensis</i>	100%
W2OE41[1]	41	Wet	2	Worker	Exoskeleton	1	DQ868676.1	<i>Halobacillus sp. SMB10</i>	91%
D1N42[21]	42	Dry	1	-----	Nest	21	JN609385.1	<i>Streptomyces albolongus</i>	99%
W2SE43[7]	43	Wet	2	Soldier	Exoskeleton	7	JQ824035.1	<i>Streptomyces sp. CGMCC</i>	99%
W3SI45[2]	45	Wet	3	Soldier	Gut	2	GU132510.1	<i>Streptomyces sp. JJH-1</i>	82%
W3OE47[2]	47	Wet	3	Worker	Exoskeleton	2	JF833103.1	<i>Streptomyces sp. Sd-56</i>	99%
W3OE48[1]	48	Wet	3	Worker	Exoskeleton	1	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W3OE49[2]	49	Wet	3	Worker	Exoskeleton	2	JN609385.1	<i>Streptomyces albolongus</i>	99%
W3OE50[3]	50	Wet	3	Worker	Exoskeleton	3	EU054375.1	<i>Streptomyces sp. 8-1</i>	99%
W3SI52[5]	52	Wet	3	Soldier	Gut	5	HQ110860.1	<i>Streptomyces sp. 51173</i>	99%
W3AE53[3]	53	Wet	3	Winged	Exoskeleton	3	FJ486432.1	<i>Amycolatopsis coloradensis</i>	98%
W3N54[1]	54	Wet	3	-----	Nest	1	JN408756.1	<i>Streptomyces sp. BF-3</i>	99%
W3OI56[9]	56	Wet	3	Worker	Gut	9	FJ486284.1	<i>Streptomyces chromofuscus</i>	90%
W3OI57[2]	57	Wet	3	Worker	Gut	2	JQ771582.1	<i>Streptomyces sp. O3-17</i>	93%
W3OI63[2]	63	Wet	3	Worker	Gut	2	JQ724537.1	<i>Achromobacter xylosoxidans</i>	91%
W3OI64[7]	64	Wet	3	Worker	Gut	7	JN256921.1	<i>Ochrobactrum sp. CRRI 29</i>	88%
W3SI66[4]	66	Wet	3	Soldier	Gut	4	AY741363.2	<i>Streptomyces sp. AB2A</i>	99%
W3OI67[1]	67	Wet	3	Worker	Gut	1	GQ924488.1	<i>Streptomyces sp. GSEUDO-0570</i>	99%
W3N68[3]	68	Wet	3	-----	Nest	3	FJ486295.1	<i>Streptomyces zaomyceticus</i>	99%
W3OE70[1]	70	Wet	3	Worker	Exoskeleton	1	JQ812068.1	<i>Streptomyces sp. QZGY-A11</i>	93%
W3OE71[1]	71	Wet	3	Worker	Exoskeleton	1	HM579818.1	<i>Streptomyces sp. HY27</i>	99%
W3N72[3]	72	Wet	3	-----	Nest	3	HQ610450.1	<i>Streptomyces cavourensis</i>	96%
W3SE79[5]	79	Wet	3	Soldier	Exoskeleton	5	JQ396393.1	<i>Serratia sp. PXG6</i>	94%
W3SE80[3]	80	Wet	3	Soldier	Exoskeleton	3	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W3OI83[2]	83	Wet	3	Worker	Gut	2	JN617221.1	<i>Streptomyces sp. CRCB1</i>	99%
W3OI84[3]	84	Wet	3	Worker	Gut	3	JN859008.1	<i>Streptomyces sp. JAJ38</i>	99%
W1OI85[3]	85	Wet	1	Worker	Gut	3	FJ490540.1	<i>Streptomyces sp. Av28_3</i>	75%
W1N86[2]	86	Wet	1	-----	Nest	2	JN408756.1	<i>Streptomyces sp. BF-3</i>	98%
W1SE89[2]	89	Wet	1	Soldier	Exoskeleton	2	EU413908.1	<i>Streptomyces sp. 39-1</i>	99%
W1SI93[13]	93	Wet	1	Soldier	Gut	13	JQ771582.1	<i>Streptomyces sp. O3-17</i>	94%

W1SI94[3]	94	Wet	1	Soldier	Gut	3	EU368787.1	<i>Streptomyces</i> sp. A306 Ydz-ZZ	99%
W1SI95[6]	95	Wet	1	Soldier	Gut	6	GU350488.1	<i>Streptomyces</i> sp. MJM9923	99%
W1SI96[3]	96	Wet	1	Soldier	Gut	3	EU368787.1	<i>Streptomyces</i> sp. A306 Ydz-ZZ	99%
W1SI97[11]	97	Wet	1	Soldier	Gut	11	HM579798.1	<i>Streptomyces</i> sp. HY7	99%
W1SI98[3]	98	Wet	1	Soldier	Gut	1	JN859008.1	<i>Streptomyces</i> sp. JAJ38	99%
W1OI99[4]	99	Wet	1	Worker	Gut	4	JN400100.1	<i>Streptomyces bacillaris</i>	93%
W1OI100[3]	100	Wet	1	Worker	Gut	3	AB681828.1	<i>Burkholderia fungorum</i>	99%
W1OI101[1]	101	Wet	1	Worker	Gut	1	GU350492.1	<i>Streptomyces flavofungini</i>	99%
W1OI103[33]	103	Wet	1	Worker	Gut	33	FJ490534.1	<i>Streptomyces</i> sp. Av25_4	99%
W1OI104[5]	104	Wet	1	Worker	Gut	5	GQ924531.1	<i>Lentzea</i> sp. ACT-0091	99%
W1OI105[1]	105	Wet	1	Worker	Gut	1	GU350488.1	<i>Streptomyces</i> sp. MJM9923	99%
W1OI106[1]	106	Wet	1	Worker	Gut	1	DQ086242.1	<i>Streptomyces</i> sp. AB660	97%
W1SI107[4]	107	Wet	1	Soldier	Gut	4	AB184777.2	<i>Streptomyces roseochromogenus</i>	99%
W2OE109[2]	109	Wet	2	Worker	Exoskeleton	2	JN683668.1	<i>Streptomyces</i> sp. FXJ3.013	99%
W2OE110[2]	110	Wet	2	Worker	Exoskeleton	2	FJ767837.1	<i>Streptomyces griseus</i>	99%
W2SE111[9]	111	Wet	2	Soldier	Exoskeleton	9	AB184388.1	<i>Streptomyces kanamyceticus</i>	99%
W2SE112[4]	112	Wet	2	Soldier	Exoskeleton	4	FJ767837.1	<i>Streptomyces griseus</i>	93%
W2SI113[15]	113	Wet	2	Soldier	Gut	15	FJ792545.1	<i>Streptomyces mediolani</i>	99%
W2N114[1]	114	Wet	2	-----	Nest	1	HM579818.1	<i>Streptomyces</i> sp. HY27	93%
W2N115[2]	115	Wet	2	-----	Nest	2	FJ490535.1	<i>Streptomyces</i> sp. Av25_5	94%
W2N116[1]	116	Wet	2	-----	Nest	1	JQ924404.1	<i>Streptomyces praecox</i>	99%
W2N117[1]	117	Wet	2	-----	Nest	1	HQ143596.1	<i>Streptomyces griseus</i>	99%
W2N118[3]	118	Wet	2	-----	Nest	3	JN683663.1	<i>Streptomyces</i> sp. FXJ3.008	98%
W3OI120[3]	120	Wet	3	Worker	Gut	3	JN866750.1	<i>Streptomyces</i> sp. S-4-4	98%
W3OI121[3]	121	Wet	3	Worker	Gut	3	FJ217193.1	<i>Streptomyces griseoaurantiacus</i>	99%
W1OI123[3]	123	Wet	1	Worker	Gut	3	EU924146.1	<i>Streptomyces</i> sp. ERI 04	99%
W1OI124[1]	124	Wet	1	Worker	Gut	1	AY134849.1	<i>Burkholderia</i> sp. ICD	99%
W1OI125[3]	125	Wet	1	Worker	Gut	3	AB091181.1	<i>Burkholderia fungorum</i>	99%
W1OI126[1]	126	Wet	1	Worker	Gut	1	AB586071.1	<i>Burkholderia</i> sp. JCM 20553	99%
W1OE129[4]	129	Wet	1	Worker	Exoskeleton	4	EU119181.1	<i>Streptomyces</i> sp. HBUM71745	99%
W1OE130[2]	130	Wet	1	Worker	Exoskeleton	2	JF778669.1	<i>Streptomyces microflavus</i>	99%
W1OE131[1]	131	Wet	1	Worker	Exoskeleton	1	JF799913.1	<i>Streptomyces</i> sp. BAB5	99%

W1OE133[3]	133	Wet	2	Worker	Exoskeleton	3	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W1OE135[4]	135	Wet	1	Worker	Exoskeleton	4	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W1OE136[4]	136	Wet	1	Worker	Exoskeleton	4	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
D1SI137[4]	137	Dry	1	Soldier	Gut	4	JN859008.1	<i>Streptomyces sp. JAJ38</i>	99%
W1SI138[4]	138	Wet	1	Soldier	Gut	4	HQ651730.1	<i>Amycolatopsis sp. SF27</i>	99%
W1OE139[5]	139	Wet	1	Worker	Exoskeleton	5	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W1OE140[1]	140	Wet	1	Worker	Exoskeleton	1	AB184587.1	<i>Streptomyces roseoviolascens</i>	99%
W1N143[3]	143	Wet	1	-----	Nest	3	JN001163.1	<i>Delftia sp. SM-1</i>	99%
W1N146[1]	146	Wet	1	-----	Nest	1	JN609385.1	<i>Streptomyces albolongus</i>	90%
W1SE147[1]	147	Wet	1	Soldier	Exoskeleton	1	EU360152.1	<i>Streptomyces sp. 1x</i>	99%
W1SE149[4]	149	Wet	1	Soldier	Exoskeleton	4	EU360152.1	<i>Streptomyces sp. 1x</i>	100%
W1SE150[2]	150	Wet	1	Soldier	Exoskeleton	2	AB622252.1	<i>Streptomyces sp. CA13</i>	99%
W1N152[5]	152	Wet	1	-----	Nest	5	HM538453.1	<i>Streptomyces sp. Av26_3</i>	90%
W2SE157[2]	157	Wet	2	Soldier	Exoskeleton	2	JF778669.1	<i>Streptomyces microflavus</i>	99%
W2N160[1]	160	Wet	2	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W2SE161[5]	161	Wet	2	Soldier	Exoskeleton	5	GU294692.1	<i>Streptomyces sp. SA01</i>	99%
W2OE163[1]	163	Wet	2	Worker	Exoskeleton	1	JF799913.1	<i>Streptomyces sp. BAB5</i>	87%
W2N167[2]	167	Wet	2	-----	Nest	2	JN999920.1	<i>Streptomyces rubiginosohelvolus</i>	100%
W3SE168[2]	168	Wet	3	Soldier	Exoskeleton	2	JQ812105.1	<i>Streptomyces sp. QZGY-A48</i>	99%
W1OE169[2]	169	Wet	1	Worker	Exoskeleton	2	EU273549.1	<i>Streptomyces globisporus</i>	90%
W1OE171[3]	171	Wet	1	Worker	Exoskeleton	3	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	99%
W1OE173[1]	173	Wet	1	Worker	Exoskeleton	1	AB635400.1	<i>Serratia marcescens</i>	99%
W1OE174[3]	174	Wet	1	Worker	Exoskeleton	3	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W1OE176[2]	176	Wet	1	Worker	Exoskeleton	2	HQ610450.1	<i>Streptomyces cavourensis</i>	100%
W1OE177[3]	177	Wet	1	Worker	Exoskeleton	3	JF778669.1	<i>Streptomyces microflavus</i>	99%
W1SE178[1]	178	Wet	1	Soldier	Exoskeleton	1	FJ626658.1	<i>Streptomyces sp. MH130</i>	99%
W1SE179[1]	179	Wet	1	Soldier	Exoskeleton	1	JN969034.1	<i>Streptomyces sp. CPE393</i>	99%
W1SE180[1]	180	Wet	1	Soldier	Exoskeleton	1	HQ610450.1	<i>Streptomyces cavourensis</i>	98%
W1SE181[2]	181	Wet	1	Soldier	Exoskeleton	2	JN683673.1	<i>Pseudonocardia sp. FXJ3.021</i>	99%
W1SE182[5]	182	Wet	1	Soldier	Exoskeleton	5	JN408756.1	<i>Streptomyces sp. BF-3</i>	99%
W1N183[4]	183	Wet	1	-----	Nest	4	EF654097.1	<i>Streptomyces drozdowiczii</i>	99%
W1N184[1]	184	Wet	1	-----	Nest	1	HM018109.1	<i>Streptomyces sp. WALP25</i>	99%

W1OE185[6]	185	Wet	1	Worker	Exoskeleton	6	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W1OE186[1]	186	Wet	1	Worker	Exoskeleton	1	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W1OE187[2]	187	Wet	1	Worker	Exoskeleton	2	JQ284036.1	<i>Streptomyces globisporus</i>	97%
W1SE188[1]	188	Wet	1	Soldier	Exoskeleton	1	AB622252.1	<i>Streptomyces sp. CA13</i>	99%
W1N189[1]	189	Wet	1	-----	Nest	1	JN001163.1	<i>Delftia sp. SM-1</i>	99%
W1OE190[3]	190	Wet	1	Worker	Exoskeleton	3	AB184777.2	<i>Streptomyces roseochromogenus</i>	99%
W1OE191[1]	191	Wet	1	Worker	Exoskeleton	1	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W1OE192[2]	192	Wet	1	Worker	Exoskeleton	2	JQ284036.1	<i>Streptomyces globisporus</i>	99%
W1OE193[4]	193	Wet	1	Worker	Exoskeleton	4	EU273541.1	<i>Streptomyces champavatii</i>	99%
W1OE194[2]	194	Wet	1	Worker	Exoskeleton	2	GU350488.1	<i>Streptomyces sp. MJM9923</i>	99%
W1OE195[3]	195	Wet	1	Worker	Exoskeleton	3	HM018109.1	<i>Streptomyces sp. WALP25</i>	99%
W1N196[1]	196	Wet	1	-----	Nest	1	HQ610450.1	<i>Streptomyces cavourensis</i>	98%
W1N197[2]	197	Wet	1	-----	Nest	2	EU368787.1	<i>Streptomyces sp. A306 Ydz-ZZ</i>	99%
W1SE198[2]	198	Wet	1	Soldier	Exoskeleton	2	FJ797602.1	<i>Streptomyces sp. DA08605</i>	100%
W3OI199[3]	199	Wet	3	Worker	Gut	3	AB024287.1	<i>Saccharothrix aerocolonigenes</i>	99%
D1N200[4]	200	Dry	1	-----	Nest	4	JN862838.1	<i>Streptomyces atratus</i>	99%
W1N201[3]	201	Wet	1	-----	Nest	3	JF778669.1	<i>Streptomyces microflavus</i>	99%
W3OE202[6]	202	Wet	3	Worker	Exoskeleton	6	JQ688018.1	<i>Streptomyces sp. Tc3-21</i>	99%
D2N203[12]	203	Dry	2	-----	Nest	12	JQ066794.1	<i>Streptomyces celluloflavus</i>	99%
D1OE206[2]	206	Dry	1	Worker	Exoskeleton	2	JQ511979.1	<i>Streptomyces flavofungini</i>	99%
D1OE208[5]	208	Dry	1	Worker	Exoskeleton	5	JQ511979.1	<i>Streptomyces flavofungini</i>	99%
D1OE211[10]	211	Dry	1	Worker	Exoskeleton	10	EU080956.1	<i>Streptomyces sp. ME03-5709A</i>	99%
D1OE213[8]	213	Dry	1	Worker	Exoskeleton	8	JF778669.1	<i>Streptomyces microflavus</i>	99%
D1OE214[60]	214	Dry	1	Worker	Exoskeleton	60	HQ610450.1	<i>Streptomyces cavourensis</i>	96%
D1SE216[6]	216	Dry	1	Soldier	Exoskeleton	6	HQ610450.1	<i>Streptomyces cavourensis</i>	100%
D1SE218[4]	218	Dry	1	Soldier	Exoskeleton	4	EU798707.1	<i>Streptomyces sp. SPB-074</i>	99%
D2OI225[8]	225	Dry	2	Worker	Gut	8	JF799913.1	<i>Streptomyces sp. BAB5</i>	99%
D2OI240[2]	240	Dry	2	Worker	Gut	2	-----	-----	----
D2N242[1]	242	Dry	2	-----	Nest	1	-----	-----	----
D1OI245[5]	245	Dry	1	Worker	Gut	5	JN585718.1	<i>Achromobacter xylosoxidans</i>	97%
D2SE257[6]	257	Dry	2	Soldier	Exoskeleton	6	HQ610450.1	<i>Streptomyces cavourensis</i>	100%
D2SE261[16]	261	Dry	2	Soldier	Exoskeleton	16	JF799913.1	<i>Streptomyces sp. BAB5</i>	99%

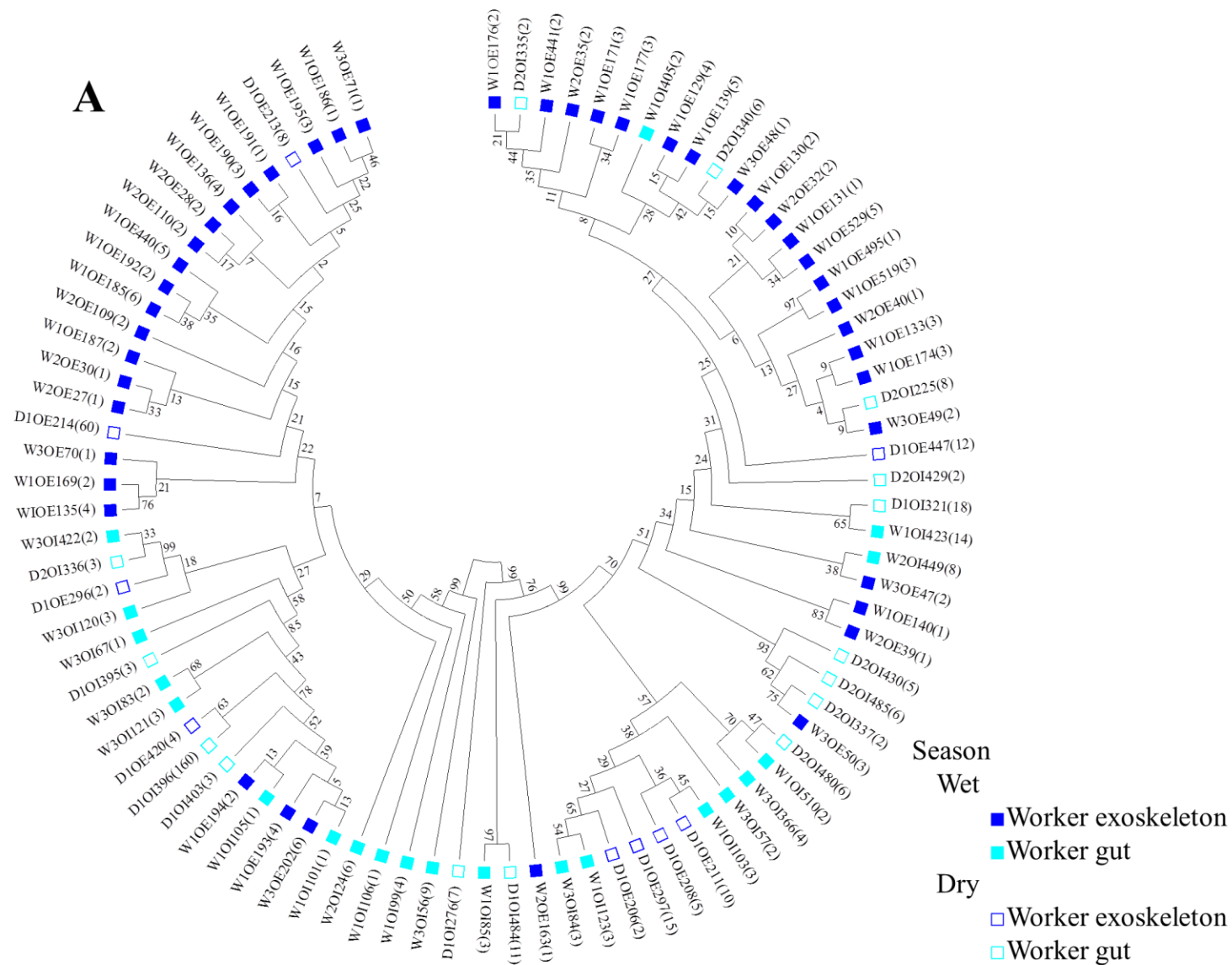
D2SE262[8]	262	Dry	2	Soldier	Exoskeleton	8	EU906929.1	<i>Streptomyces sp. JW1</i>	99%
D1N265[2]	265	Dry	1	-----	Nest	2	AB546274.1	<i>Amycolatopsis sp. RI67-Va106</i>	100%
D1OI276[7]	276	Dry	1	Worker	Gut	7	EU370089.1	<i>Streptomyces sp. 12-2</i>	82%
D1SI281[75]	281	Dry	1	Soldier	Gut	75	JF799913.1	<i>Streptomyces sp. BAB5</i>	98%
D1SI286[9]	286	Dry	1	Soldier	Gut	9	JQ284036.1	<i>Streptomyces globisporus</i>	99%
D1SI287[14]	287	Dry	1	Soldier	Gut	14	NR_043866.1	<i>Streptomyces alni</i>	100%
D1OE296[2]	296	Dry	1	Worker	Exoskeleton	2	GQ863901.1	<i>Streptomyces sp. HB100</i>	99%
D1OE297[15]	297	Dry	1	Worker	Exoskeleton	15	JN859008.1	<i>Streptomyces sp. JAJ38</i>	93%
D2OE298[2]	298	Dry	2	Worker	Exoskeleton	2	EU266487.1	<i>Gordonia sp. CC-MJ-33a</i>	99%
D2SI303[3]	303	Dry	1	Soldier	Gut	3	NR_041210.1	<i>Streptomyces fulvissimus</i>	99%
D2SI304[3]	304	Dry	2	Soldier	Gut	3	JF778669.1	<i>Streptomyces microflavus</i>	99%
D2SI305[5]	305	Dry	2	Soldier	Gut	5	EU266487.1	<i>Gordonia sp. CC-MJ-33a</i>	99%
D1OE310[3]	310	Dry	1	Worker	Exoskeleton	3	AB601654.1	<i>Bradyrhizobium sp. H15-CR</i>	82%
D1N312[1]	312	Dry	1	-----	Nest	1	JQ614022.1	<i>Pseudomonas putida</i>	99%
D1N315[3]	315	Dry	1	-----	Nest	3	HQ662223.1	<i>Streptomyces sp. Ank245</i>	99%
D1N317[4]	317	Dry	1	-----	Nest	4	EU273549.1	<i>Streptomyces globisporus</i>	89%
D1OI321[18]	321	Dry	1	Worker	Gut	18	JN862838.1	<i>Streptomyces atratus</i>	99%
D1OI332[9]	332	Dry	1	Worker	Gut	9	DQ156088.1	<i>Burkholderia sp. mpa1.5</i>	97%
D1SI334[4]	334	Dry	1	Soldier	Gut	4	FM163174.1	<i>Streptomyces sp. ACTY</i>	99%
D2OI335[2]	335	Dry	2	Worker	Gut	2	HQ610450.1	<i>Streptomyces cavourensis</i>	100%
D2OI336[3]	336	Dry	2	Worker	Gut	3	GU126551.1	<i>Streptomyces lunalinharesii</i>	99%
D2OI337[2]	337	Dry	2	Worker	Gut	2	EU054375.1	<i>Streptomyces sp. 8-1</i>	99%
D2OI340[6]	340	Dry	2	Worker	Gut	6	JQ284036.1	<i>Streptomyces globisporus</i>	100%
D2OI341[1]	341	Dry	2	Worker	Gut	1	-----	-----	----
D2SI346[2]	346	Dry	2	Soldier	Gut	2	-----	-----	----
D2SI348[2]	348	Dry	2	Soldier	Gut	2	JN683657.1	<i>Streptomyces sp. FXJ3.002</i>	99%
D2SI351[3]	351	Dry	2	Soldier	Gut	3	GQ848238.1	<i>Gordonia amicalis</i>	99%
D1SI352[3]	352	Dry	1	Soldier	Gut	3	JQ247066.1	<i>Streptomyces sp. HDD004</i>	99%
D2SE358[2]	358	Dry	2	Soldier	Exoskeleton	2	-----	-----	----
D1SE361[4]	361	Dry	1	Soldier	Exoskeleton	4	EF063497.1	<i>Streptomyces flavoviridis</i>	84%
D3N365[2]	365	Dry	1	-----	Nest	2	JF778669.1	<i>Streptomyces microflavus</i>	99%
W3OI366[4]	366	Wet	3	Worker	Gut	4	FJ486338.1	<i>Streptomyces albogriseolus</i>	100%

D2SE369[4]	369	Dry	2	Soldier	Exoskeleton	4	HQ456135.1	<i>Streptomyces</i> sp. WZ1-7219	99%
D1N371[10]	371	Dry	1	-----	Nest	10	JF778669.1	<i>Streptomyces microflavus</i>	99%
D1N378[3]	378	Dry	1	-----	Nest	3	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
D2N385[4]	385	Dry	2	-----	Nest	4	JF778669.1	<i>Streptomyces microflavus</i>	99%
W2OI392[2]	392	Wet	2	Worker	Gut	2	JQ690689.1	<i>Halobacillus</i> sp. Alpha	81%
D1OI395[3]	395	Dry	1	Worker	Gut	3	FJ797605.1	<i>Streptomyces</i> sp. DA08606	99%
D1OI396[160]	396	Dry	1	Worker	Gut	160	EU257235.1	<i>Streptomyces</i> sp. A8Ydz-XM	99%
W3N397[2]	397	Wet	3	-----	Nest	2	AB680343.1	<i>Ochrobactrum</i> sp. NBRC	100%
W3SI398[4]	398	Wet	3	Soldier	Gut	4	GU458286.1	<i>Serratia</i> sp. PT4	99%
D1OI403[3]	403	Dry	1	Worker	Gut	3	HM235464.1	<i>Streptomyces</i> sp. 2mA	99%
W1OI405[2]	405	Wet	1	Worker	Gut	2	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	100%
W3N412[3]	412	Wet	3	-----	Nest	3	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	99%
W1OI418[1]	418	Wet	1	Worker	Gut	1	NR_029049.1	<i>Amycolatopsis rifamycinica</i>	99%
D1OE420[4]	420	Dry	1	Worker	Exoskeleton	4	NR_041423.1	<i>Streptomyces malachitospinus</i>	99%
D1N421[17]	421	Dry	1	-----	Nest	17	FJ919601.1	<i>Streptomyces</i> sp. LS247	99%
W3OI422[2]	422	Wet	3	Worker	Gut	2	GQ863901.1	<i>Streptomyces</i> sp. HB100	99%
W1OI423[14]	423	Wet	1	Worker	Gut	14	JN862838.1	<i>Streptomyces atratus</i>	99%
D3N424[41]	424	Dry	3	-----	Nest	41	JN408756.1	<i>Streptomyces</i> sp. BF-3	99%
D2OI427[4]	427	Dry	2	Worker	Gut	5	X80632.1	<i>Gordonia rubripertinctus</i>	98%
D1N428[3]	428	Dry	1	-----	Nest	3	EU054375.1	<i>Streptomyces</i> sp. 8-1	99%
D2OI429[2]	429	Dry	2	Worker	Gut	2	JN999920.1	<i>Streptomyces rubiginosohelvolus</i>	99%
D2OI430[5]	430	Dry	2	Worker	Gut	5	EU054375.1	<i>Streptomyces</i> sp. 8-1	99%
D2SE433[10]	433	Dry	2	Soldier	Exoskeleton	10	JF799913.1	<i>Streptomyces</i> sp. BAB5	87%
W3SI435[9]	435	Wet	3	Soldier	Gut	9	JN683662.1	<i>Streptomyces</i> sp. FXJ3.007	100%
D2OE436[5]	436	Dry	2	Worker	Exoskeleton	5	-----	-----	----
W2N437[2]	437	Wet	2	-----	Nest	2	JN999925.1	<i>Streptomyces spectabilis</i>	96%
D2N438[10]	438	Dry	2	-----	Nest	10	JQ924410.1	<i>Streptomyces flavofuscus</i>	100%
W1OE440[5]	440	Wet	1	Worker	Exoskeleton	5	HQ610450.1	<i>Streptomyces cavourensis</i>	99%
W1OE441[2]	441	Wet	1	Worker	Exoskeleton	2	JQ284036.1	<i>Streptomyces globisporus</i>	99%
D2N442[4]	442	Dry	2	-----	Nest	4	JQ660216.1	<i>Stenotrophomonas panacihumi</i>	98%
W3SI445[3]	445	Wet	3	Soldier	Gut	3	JQ771584.1	<i>Streptomyces</i> sp. O5-2	99%
D1OE447[12]	447	Dry	1	Worker	Exoskeleton	12	JF778669.1	<i>Streptomyces microflavus</i>	99%

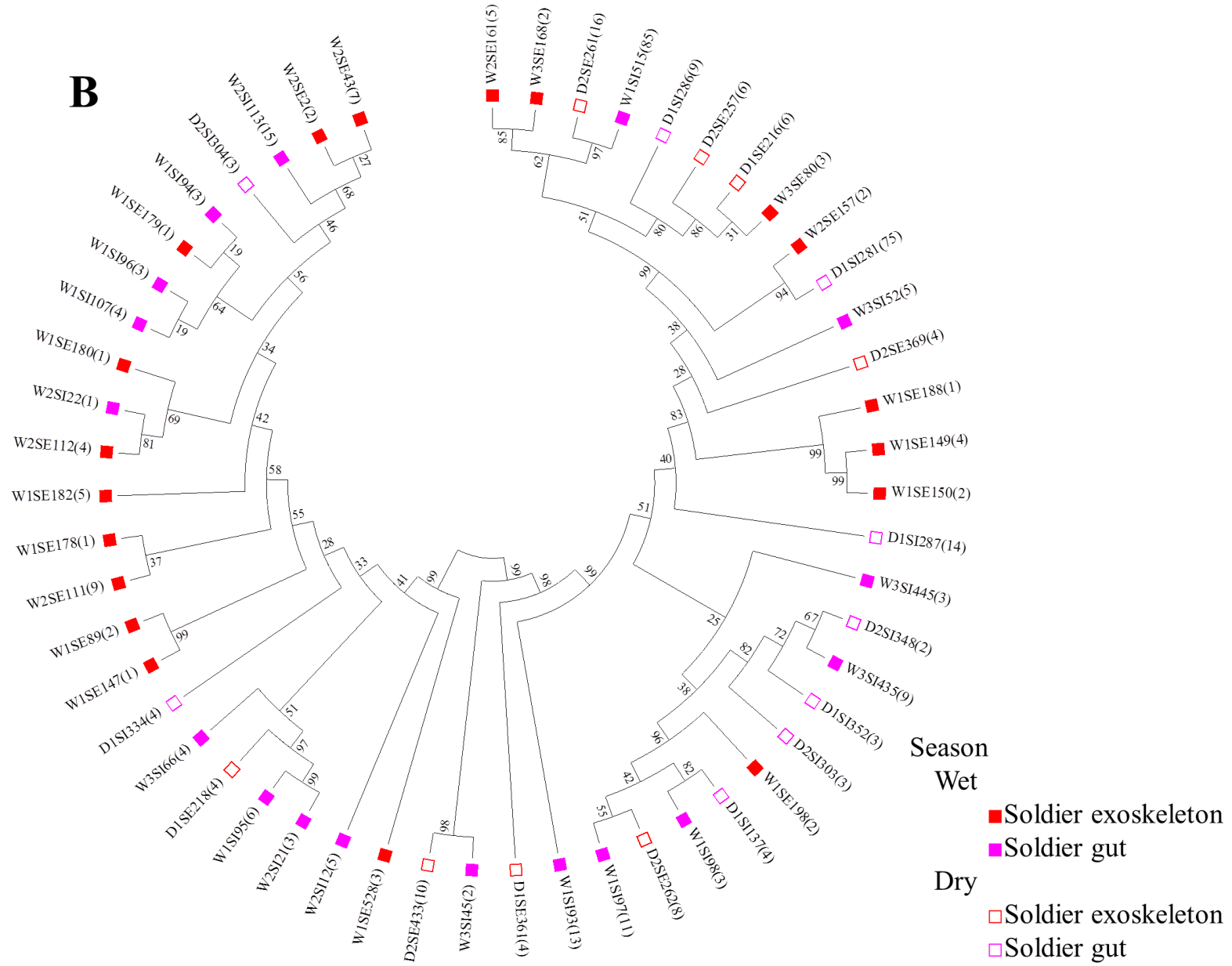
W2OI449[8]	449	Wet	2	Worker	Gut	8	HQ607437.1	<i>Streptomyces flavovariabilis</i>	99%
D3N463[1]	463	Dry	3	-----	Nest	1	-----	-----	----
D3N469[6]	469	Dry	3	-----	Nest	6	-----	-----	----
D2N476[2]	476	Dry	2	-----	Nest	2	EU054375.1	<i>Streptomyces sp. 8-1</i>	99%
D2N477[4]	477	Dry	2	-----	Nest	4	-----	-----	----
W2OE478[2]	478	Wet	2	Worker	Exoskeleton	2	HQ393893.1	<i>Achromobacter xylosoxidans</i>	99%
D2OI480[6]	480	Dry	2	Worker	Gut	6	JN683662.1	<i>Streptomyces sp. FXJ3.007</i>	99%
D1OI484[11]	484	Dry	1	Worker	Gut	11	GQ284478.1	<i>Streptomyces sp. PCWCW9</i>	79%
D2OI485[6]	485	Dry	2	Worker	Gut	6	EU054375.1	<i>Streptomyces sp. 8-1</i>	100%
W1SE491[2]	491	Wet	1	Soldier	Exoskeleton	2	HM352366.1	<i>Pseudomonas sp. HaNA23</i>	96%
W2N494[3]	494	Wet	2	-----	Nest	3	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	100%
W1OE495[1]	495	Wet	1	Worker	Exoskeleton	1	JQ682626.1	<i>Streptomyces roseoviolaceus</i>	99%
D1OI496[12]	496	Dry	1	Worker	Gut	12	FJ386525.1	<i>Halobacillus sp. G-12</i>	86%
W3AE498[3]	498	Wet	3	Winged	Exoskeleton	3	JQ724537.1	<i>Achromobacter xylosoxidans</i>	100%
W2SE500[12]	500	Wet	2	Soldier	Exoskeleton	12	JN585718.1	<i>Achromobacter xylosoxidans</i>	95%
W1OI510[2]	510	Wet	1	Worker	Gut	2	JN683662.1	<i>Streptomyces sp. FXJ3.007</i>	100%
W1SI515[85]	515	Wet	1	Soldier	Gut	85	JF799913.1	<i>Streptomyces sp. BAB5</i>	99%
W1OE519[3]	519	Wet	1	Worker	Exoskeleton	3	EU135902.1	<i>Streptomyces sp. L083</i>	100%
D2OI524[4]	524	Dry	2	Worker	Gut	4	-----	-----	----
W1SE528[3]	528	Wet	1	Soldier	Exoskeleton	3	EU413901.1	<i>Streptomyces sp. 35-1</i>	98%
W1OE529[5]	529	Wet	1	Worker	Exoskeleton	5	JF778669.1	<i>Streptomyces microflavus</i>	99%
W1OE530[2]	530	Wet	1	Worker	Exoskeleton	2	DQ785816.1	<i>Microbacterium sp. BMA-5</i>	98%

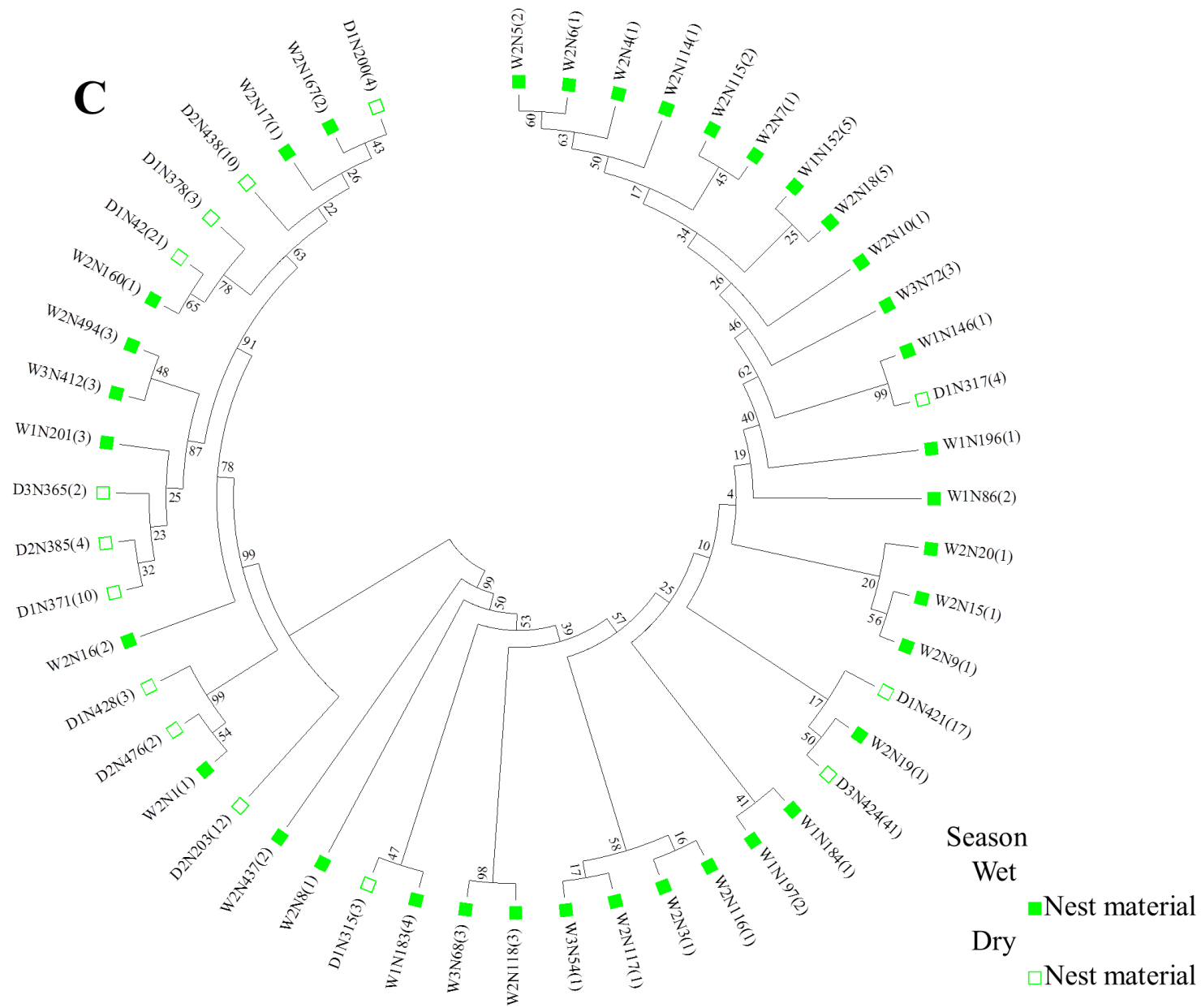


**Appendix M-** Phylogenetic trees of *Streptomyces* strains isolated from termite workers (A), soldiers (B) and nests material (C)



**B**





# **Appendix N-** Phylogenetic tree of *Streptomyces* strains isolated in this research

