The University of Sheffield

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STUDIES ON MICROBES INCLUDING POTENTIAL HUMAN PATHOGENS FROM INSECTS AND OTHER INVERTEBRATES

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Dedication

For my father God forgive him, my mother, may God prolong her age, my wonderful wife, my sons Abdulrahman and Abdulaziz, my brothers, my relatives, friends and colleagues.

ACKNOWLEDGMENTS

I would like to express my gratitude to Almighty Allah

I extend my thanks and appreciation to my supervisor Professor Milton Wainwright for his support, helpful advice guidance, encouragement given to me during this study and completion of my thesis.

I would also like to special thanks to Dr Jim Gilmour for his advice, throughout my project, I am also grateful to Dr Lynda Partridge for her advice.

Finally, I would like to thank The Kingdom of Saudi Arabia Government, King Fahd Security College for financial scholarship and supports.

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Summary

A wide range of bacteria were obtained from the exterior, and from the body fluids, of insects collected locally, and Lepidoptera species obtained from an Entomological supplier. The insects were found to contain a wide range of bacteria, both internally and externally including *Bacillus thuringiensis*, a bacterium used in the biocontrol of larval pests. Although not major pathogens, many of the bacterial isolates can cause infection in immune-compromised patients, a possibility which is discussed.

Larvae of the Peacock butterfly (*Vanessa io*) were fed nettle leaves which were deliberately covered with a range of bacteria. Feeding with *B. thuringiensis* not surprisingly, lead to the death of all of the larvae after 4 hours. The results show that feeding with *B. subtilis* and *E. coli* can lead to larval death, while MRSA was shown to be less toxic. Feeding the larvae with the other bacteria killed some larvae, with the death rate after feeding *B. subtilis* and *E. coli* being identical. *Bacillus cereus* was isolated from the larvae fed *B. thuringiensis* and *B.subtilis*.

Bacteria were isolated from the Dermestidae (beetle larvae) obtained from human cadavers. The dominant species of bacteria was *Enterococcus faecalis* which was isolated from inside the larvae extracted from a human corpse. Two species of *Clostridium* were also isolated; *Clostridium cochlearium* was isolated from the Dermestid larva, the other, *Clostridium paraputrificum* was isolated from inside the larva. *Brevibacterium ravenspurgense, Staphylococcus hominis, Lishizhenia tianjinensis* and *Bacillus safensis*, were also isolated from inside larvae, extracted from human body.

The biocontrol agents *Bacillus thuringiensis* and *Paenibacillus popiliae* were shown to be capable of mediating *in vitro*, transformations which are important in the major environmental mineral cycles These bacteria are likely to reach the agriculture soils following treatment and, on germination can presumably participate in mineral cycling Both bacteria

were shown to be capable *in vitro* hydrolysis of urea, and were shown to oxidize ammonium and elemental sulphur and also to solubilize a source of insoluble phosphate. It is not clear however, to what extent the ability of these bacteria to participate in these reactions *in vitro* correlates with the same activity in soils and other environments.

Insects were sampled at a height of 120 meters using a drone–towed fabric sleeve and their microbial content studied. The major point of interest behind this work is the use of a drone-towed sleeve to sample the insects. As far as can be determined, this is the first reported use of this approach to sample high flying insects in relation to a study of their microbiology. The use of a drone was shown to be ideal for the high altitude sampling of insects since it proved to be both powerful and highly manoeuvrable and there is no doubt that the drone used could have been used to sample at greater heights than the 120 m used here. The results relating to the microbiology of the insects sampled using the drone are not surprisingly similar to those obtained using other sampling methods, since the drone, of course, does not necessarily sample insects which differ from those obtained using more traditional approaches.

An octanol-based midge sampler (Predator) was used to obtain large numbers of midges from the air, in relations to studying their microbiology this approach appears to be novel. The midge-biomass collected was found to contain microbes and was shown to break down in an agricultural soil to release ammonium and nitrate. The potential use of this material as an agricultural or home fertilizer is discussed. Finally, larger moths were trapped using a Robinson UV light trap. The moths were found to carry filamentous fungi on their bodies, some of which are plant pathogens, notably of trees.

CHAPTER ONE: ISOLATION OF BACTERIA FROM INVERTEBRATE SURFACES

1: Introduction

Countless numbers of insect species occur around the world. With the exception of some pest species, surprisingly little is known however, about the relationship between insects and bacteria (Broderick *et al*. 2004, Robinson *et al*, 2010). Insect comprise some 53.1 percent (751,000 species) of all the known species (1.4 million) living species found on this planet. Furthermore, beetles constitute 20.5 percent of all living species (290,000) and Lepidoptera like those shown in Fig1.1 make up approximately 9.9 percent (140,000) of insects (Shalaway, 2004).

Fig.1:1. An Elephant Hawk Moth (*Deilephila elpenor*) and a Silk Moth (*Hyalophora cecropia*) (obtained from an entomological supplier).

Insects possess very efficient immune system allowing them to deal with pathogenic infections and consists of a wide range of defence mechanisms which can act individually or in combination in order to stop foreign organisms entering or to suppress pathogens after they have gained access to their tissues. The epithelium is the first line of defence, which acts as a barrier and produces local antimicrobial peptides (AMP) following infection or wounding (Davis and Engström, 2012). The innate immune system provides a second line of defence which involves a) the systemic production of AMP largely from the fat body (the insect equivalent to the mammalian liver) (Ganesan *et al*., 2011); b) cellular responses by insect haemocytes (equivalent to mammalian white blood cells) that are involved in immune surveillance, c) phagocytosis, and the encapsulation of foreign intruders (Marmaras and Lampropoulou, 2009); d) melanization and clotting or coagulation of the haemolymph (equivalent to vertebrate blood), which needs active phenoloxidase and the involvement of both humoral and cellular factors that bring about the rapid production and deposition of melanin around wounds (Eleftherianos and Revenis, 2011); e) the generation of large amounts of reactive oxygen species (ROS) and AMP in epithelial cells and the production of nitric oxide (NO) which is involved in the regulation of innate immune responses both to bacteria and parasites (Ryu *et al*.,2010) and which is stimulated by the gut microbiota; and finally f) RNA interference (RNAi) and inducible innate immune responses against invading viruses (Kemp and Imler, 2009).

In addition to their native microflora, insects carry symbiotic bacteria which can occupy specialized cells and tissues within the host. These symbiotic microbes live under an active immune system and therefore must devise approaches allowing them to avoid the negative effects of the host's immune defence systems (*Gross et al.,* 2009). Such symbiotic bacteria present in various insect species are associated with increased host resistance to both pathogens and parasites.

Although we have made advances in the field of insect innate immunity, our recognition of the part played by endosymbiotic bacteria in the host immune response to pathogenic infections is incomplete. Studies have begun to determine the phenotypic response of a variety of insects carrying endosymbionts to infection by pathogenic bacteria, viruses as well as parasites. Substantially more detailed and comprehensive knowledge is needed to show exactly how endosymbiotic bacteria regulate insect immune defence mechanisms against pathogens and parasites. A further challenge is to characterize the interplay between different endosymbionts, including *Wolbachia*, which co-exist in an insect host, and effectiveness of the immune function. It is also important to determine the precise mechanisms used by endosymbiotic bacteria to modulate insect immune signalling. From a more applied viewpoint, the discovery that the presence of *Wolbachia* endosymbionts in mosquitoes has a direct effect on insect sensitivity to pathogens has suggested the possibility that they might be used in medicine (Hancock *et al.,* 2011), including potential implementation in the field of practices that are effective disruption of dengue transmission by mosquitoes.

1:1 A**im of the work described in Chapter 1**

The aim of the research discussed in this Chapter is to study the relationship between insects and bacteria, by isolating bacteria from insects and identifying the isolates using 16S rRNA gene sequencing via PCR amplification for the identification and characterization of the isolates. The study initially focused on moths and butterflies which are likely to be associated with *Bacillus thuringiensis* (du Rand, 2009, Roh, *et al*, 2007), and other bacteria and determine if these occur throughout the developmental life cycle of Lepidoptera, i.e. ova, larva, pre-pupa, pupa and adult (imago).

1:2 PCR techniques

DNA is required for all cellular life cell and contains four nitrogen bases (pyrimidine) cytosine (C), thymine (T) and (purine) guanine (G), adenine (A). DNA bases pair up with

each other, A with T and C with G, to form units called base pairs. Each base is attached to a sugar molecule and also a phosphate molecule. A base, sugar, and phosphate molecule are together called a nucleotide. Nucleotides are organized in two long strands forming a spiral. i.e. a double helix. The structure of the double strand helix resembles a ladder, with the base pairs forming the rungs of the ladder and the sugar and phosphate molecules forming the vertical components of the ladder (Sinden, 1994, Baker *et al*., 2006).

The PCR (polymerase chain reaction) is a tool now routinely used in most diagnostic and medical and biological research laboratory. This technique has been used in forensic investigation, sequencing mutations and pathogens, eukaryotic classification, and the sequencing of the human genome (Prada-Arismendy, 2011, Hadidi and Candresse, 2003, Erlich, 1988). It is an excellent method for use in the detection of nucleic acids, eukaryotic species, human identification, disease identification, forensic science and to the identification of pathogens (Chambers, *et al*. 2014, Randall *et al*., 1985). The identification of organisms to the species level has usually been allocated to specialist field of taxonomists, providing a classification as key prerequisite and mainstay for numerous biological studies. In 1990s there emerged the idea of a consolidated molecular identification system with the development of PCR-based approaches for species identification, particularly. The great benefit appeared in molecular identification Studies and surveys on microbial biodiversity is done by the use of bacteria in these applications (e.g. Kyrpides, 1996 and Zhou *et al.*, 1997) and the routine identification of pathogens (e.g. Maiden *et al*., 1996, Sugita *et al*., 1998 and Wirth *et al*., 2006), all of which are based on the need for systems based on culture-independent identifications. Methods which are based on the use of PCR have also been regularly applied to taxonomy, also food such is Food and Drug agencies around the world and forensic molecular identification (Teletchea *et al*., 2008) and for eukaryotic pathogens and vector identification.

1:3 Materials and Methods

The entomological samples were obtained from the local environment. Moths were collected using light traps, while others were sampled from vegetation and flowers using nets (Fig.1:2).

Fig.1:2. Example of moth traps and an entomological net used in these studies

After the insects were collected they were photographed to aid in future identification.

1:4 Bacterial isolations

A wet cotton swab was used to isolates bacteria from the surface of insects, arthropods, a shield bug and slugs. The swab was then spread onto the surface of the isolation medium, in addition by using a fine hypodermic needle to isolate bacteria from haemolymph.

1:5 Nutrient agar media

Nutrient agar medium was prepared by dissolving 23 g of Nutrient Agar (Oxoid) in 1000 ml of distilled water in a flask and autoclaving at 121°C for 30 min.; the medium was then poured in Petri dishes and allowed to cool.

Identification of isolates

1:6 Gram stain

Bacteria are divided into two types depending on the interaction with the Gram Stain. Bacteria that retain the primary stain crystal violet (purple) are "Gram-positive," while those that de-stain and are coloured red with safranin or carbol fuchsin are "Gram-negative". This response is based on the staining chemical composition and structural walls of the cells from both species of bacteria. Gram-positives have, relatively impermeable thick wall resists the removal of colour and consists of peptidoglycan polymers and mucopeptide. Gram-negatives have a thin layer of peptidoglycan in addition to the bi-layer overlying fat, protein, known as the outer membrane, which can be disrupted to allow by the removal of colour.

1:7 LB media

LB is one of the most commons used bacterial culture medium used today. It was developed by Guiseppi Bertani while attempting to optimize plaque formation on a *Shigella* indicator strain. LB broth was prepared here from 10 g peptone, 5 g yeast extract and 5 g sodium chloride in 1000 ml distilled water, with autoclaving at 121°C for 30 min.

1:8 DNA quality and quantity

Agarose gel electrophoresis

Agarose (1%) is use to separate DNA fragments. The gels were prepared as follows: 0.5g of molecular biology grade agarose was dissolved in 50 ml of 1x TAE buffer by heating in a microwave on a medium high power for approximately 2 minutes and then 2.5 µl ethidium bromide (after the temperature decline to 60°C to avoid ethidium bromide steam) to visualize the DNA before setting the solution in a gel tray and then the gel was poured in the gel rack. The comb was inserted at one side of the gel and left at room temperature. The gel then was immersed in TAE buffer 1x and the DNA samples (10 µl) were added, mixed with 2 µl loading dye, to wells. To determine the size of fragments, 6 µl of Hyper Ladder was used.

The samples were then electrophoresed for 40 minutes at 80V. The DNA was visualized on the gel and all PCR products were analysed on agarose gels to check for the successful amplification of the 16S rRNA gene in the samples band and a digital image was taken using UVitec ‘‘Uvidoc”, attached to a digital camera (Fig.1:3; 4).

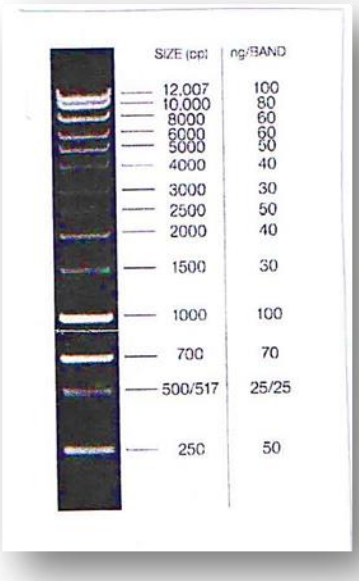

Fig.1:3. A standard hyper ladder I with 14 lanes indicating higher intensity bands, 1000 and 10,000 and each lane (5µl) provides 720ng of DNA (BIOLINE supplier)

Fig.1:4. Shows a successful 16-DNA amplification genomic DNA from bacteria extraction from ten bacteria (taken from haemolymph) which were unknown prior to DNA sequencing *Bacillus licheniformis, Bacillus thuringiensis, Bacillus cereus, Bacillus* sp, *Stenotrophomonas, Microbacterium* sp, *Bacillus weihenstephanensis, Bacillus safensis, Bacillus pumilus* and *Bacillus mycoides.*

Nanodrop

DNA concentration and purity was determined by electrophoresis after DNA extraction for quality and quantity of the genomic DNA was evaluated spectrophotometrically by using a Nanodrop 1000 spectrophotometer. A 1 ul sample was used to measure the quantity of DNA (NanoDrop Technologies, Wilmington, DE, USA); DNA absorbance was measured at 260 nm.

1:9 PCR Techniques

PCR is a molecular procedure for amplifying the target gene and permit accessing to the genomic information from eukaryotic and prokaryotic cells. Three main stages are performed in PCR, which are repeated for a number of cycles to significantly increase the number of copies of a specific goal area (Henson and French, 1993).

1: Initial denaturation (melting of DNA), involves the denaturation of the double stranded DNA into two single strands of template DNA by heating the DNA to 94 °C during 3 min.

2: annealing of primers (annealing of two oligonucleotide primers to the denatured DNA strands), encompass lowered the temperature around 60°C during 1 min to allow the primers to match DNA fragments.

3: involves the extension by a polymerase (primer extension by a

thermo-stable DNA polymerase) involves the incorporation of (dNTPs; A, C, G, T), thereby extending the DNA sequence in the 5 ` to 3 ` directions by raised the temperature (72-75°C) .5 min time which depends on the period DNA polymerase used**.**

1:10 Methods for DNA extraction used QIA prep Spin miniprep kit from Qiagen.

The following is provided in a "manual style":

The DNA extraction procedure was as follows: Add lyse Blue reagent to Buffer P1 at ratio of 1 to 1000.Add RNase A solution (200µl) to Buffer P1at ratio 1-100 and store at 2-8 °C. Add ethanol (96-100%) to buffer PE. After being incubated, in LB overnight take 1-5 ml from LB culture then centrifuge at 8,000 rpm for 3 min at room temperature. Decant the supernatant and keep residual (suspended).Add 250 µl Buffer P1 (Resuspension buffer) mixed the solution gently by inverting 4-6 time. Add 250 µl buffer P2(Lysis buffer it contains salts used to break down the cell and nuclear membranes allowing the DNA to be released); mix the solution gently by inverting 4-6 time the solution became blue colour (protein denaturation)

Add 350 µl Buffer N3 (After the addition of acetate-containing neutralization buffer the large and less supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNA plasmids stay in solution) and mix immediately by inverting 4-6 time the solution became colourless. (Homogenization)Centrifuge for 10 min at 13,000 rpm.

Apply the supernatant from previous step to QIA prep spin column (filter) by decanting. Centrifuge for 30-60 s and discard the flow through. (Loading to column)Wash the filter by adding 750 µl Buffer PE (washing buffer) Centrifuge for 30-60 s and discard the flow through. Centrifuge for 1 min to remove residual wash Buffer. Transfer the filter to the new 1.5 ml micro centrifuge tube. Add 50 µl buffer EB (Elution buffer) to elute DNA incubate for 1 min then centrifuge for 1 min. Through the filter and the DNA extract was stored at -4 °C (DNA elution).

Glass bead

Glass beads were used for the extraction DNA:

add a small amount of cultured of bacteria by loop to Eppendorf tube containing 100 µl of molecular water and mixed by Pipette up and down the suspension then added glass beads diameter of 0.1 mm (ten balls) then rubbing gently by pipetting up-and-down repeatedly. Vortex for ten second subsequently centrifuges at 13 rpm for 5 minutes then carefully discharges the supernatant to new Eppendorf tube

1:11 PCR Amplification

The 16S rRNA gene was amplified with the universal bacterial forward primer (5` CCG AAT TCG TGG ACA ACA GAG GAT CCT GGC TCA G 3`) (34) and universal reverse primer (5` CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T 3`) Table 1:1) (Weisburg *et al*., 1991)

A typical PCR mixture (20 µl in volume) contained the following components: 6 µl sterile molecular water, 10 µl Ampli Taq Gold or Master Mix, 1 µl forward Primer, 1 µl Reverse primer (before using the primer need to diluted to 90% with molecular water by add 90 µl molecular water to 10 µl from primer stock) 2 µl dNTPs. The following standard conditions were used for bacterial 16S rRNA gene amplification: initial denaturation at 94ºC for 3 min; 35 cycles of denaturation for 1 min at 94 ºC, annealing for 1 min at 60ºC, elongation for 1 min at 72ºC; and a final extension at 72ºC for 5 min. various process are available now to isolate DNA from many different samples. Such as, tissues or cells are broken then the cells are lysed by using enzymes or detergents then centrifuged to separate the DNA from other components followed by cleaning from other molecules (Amann *et al.*, 1995).

1:12 Quantities

Add 47 µl molecular water for volume 100pmol/µl to 16s reverse primer Add 69 µl molecular water for volume 100pmol/µl to 16s forward primer Storage in the freezer at -4°C (Stock)

Steps	**Temperature**	**Time**	**Cycle No**
Initial denature	94°C	3 min to separate the double strand of DNA to single strand	1
RNA.denature	94°C	1 min. Annealing	
Annealing	60°C	1 min to allow primers to match DNA fragments	**35**
Elongation	72°C	1 min	
Final elongation	72°C	5 min by using Tag polymerase	1
Hold	4° C	Until continue procedure	

Table 1:1. The PCR process

1:13 16SrRNA sequencing

DNA was extracted from bacteria then amplification and copy to bacteria by using a PCR protocol with the suitable primers to produce large quantities of the 16SrRNA gene then by genotyping machine and protocols we can identify the genus or species from specific loci in DNA strand. Genomic DNA was isolated by using (Qiagen- Bacterial DNA Extraction) the following procedures. DNA was extracted from the bacterial cells using a commercial kit (Qiagen)

1:14 Identification of unknown bacteria:

This stage was achieved by sending samples to the University of Sheffield Medical School Genetics Unit for more sequencing. Finch TV software was used to check the nitrogen base sequencing result after that used BLAST software to identify gene sequences of bacteria determination from 16SrRNA (NCBI) (http://www.ncbi.nlm.nih.gov). The bacteria, isolated from the relevant species of Lepidoptera, and their potential pathogenicity are shown in Table 1:2.

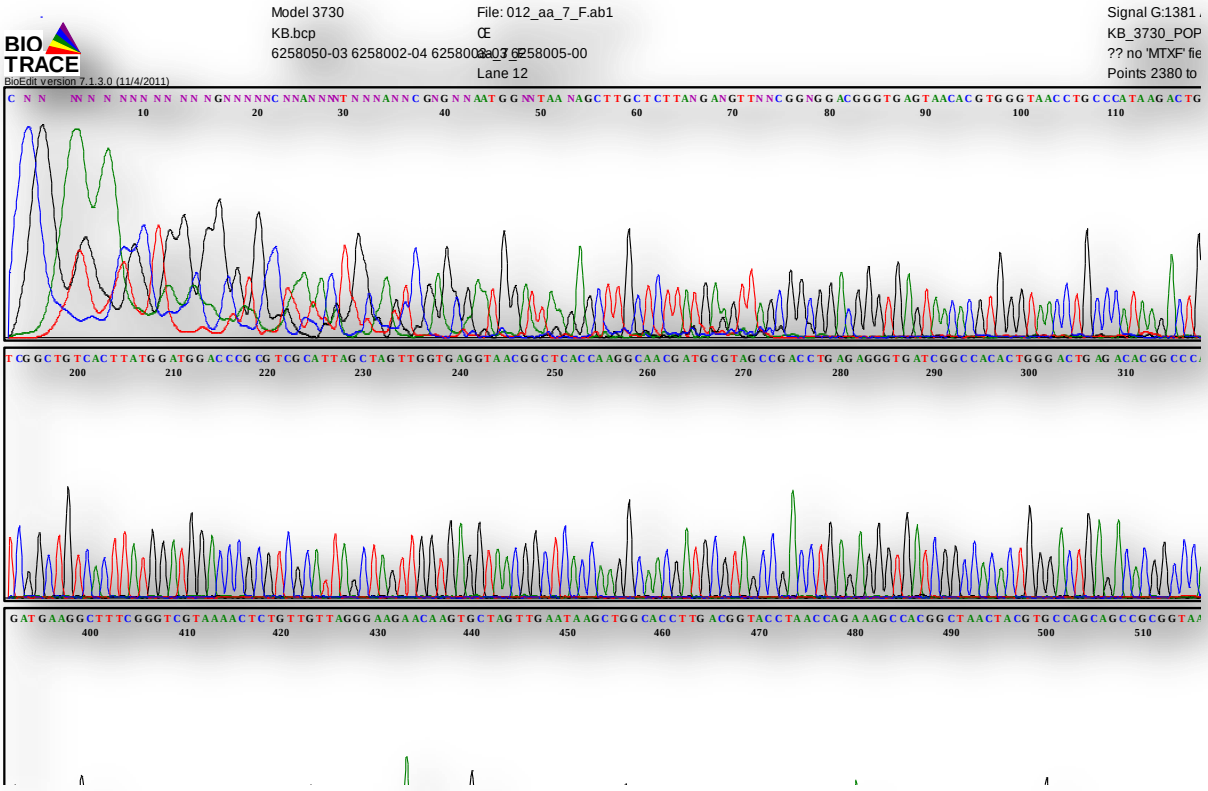

Fig.1:5. Shows Finch TV software as ladder for nitrogen base pair

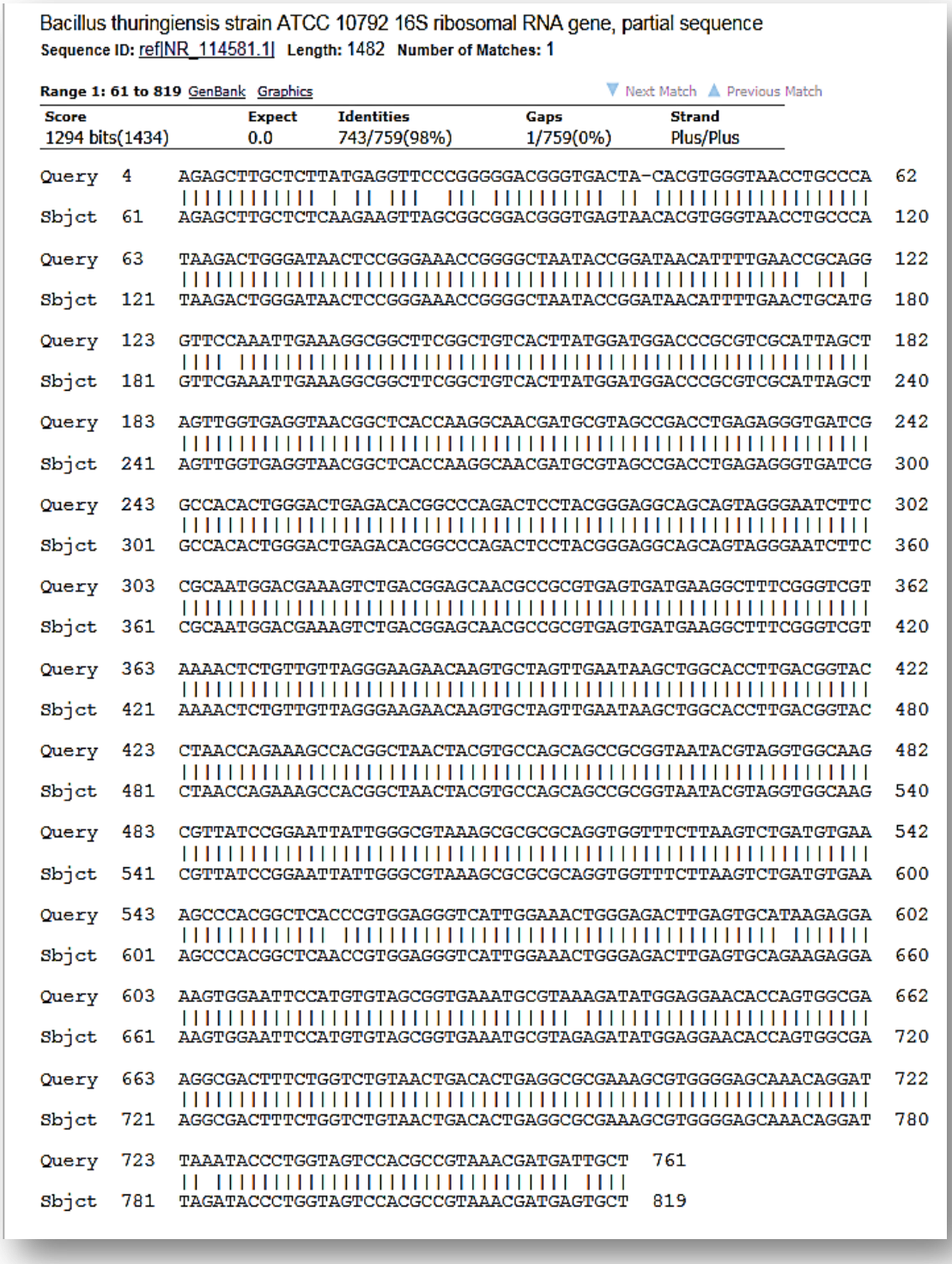

Fig.1:6. Shows example of *Bacillus thuringiensis* gene sequences

1:15 RESULTS AND DISCUSISON

No	Bacteria	Insects and arthropods	Repetition
1	*Bacillus thuringiensis*	Butterfly pupa and butterfly (*Aglais io*) larva, Garden Snail(*Cornus asperum*) and slug (*Lehmannia valentiana*)- external wet swab, Shield Bug (*Paromena prasina*)	**7**
2	*Bacillus cereus*	Snail, Housefly, Earthworm (*Lumbricus terrestre*)	**7**
3	*Stenotrophomonas maltophilia*	External butterfly larva swab (*Aglais io*), Shield bug(*Paromena prasina*)	**2**
4	*Microbacterium* sp	Moth (*Abraxas grossulariata*)	**2**
5	*Bacillus weihenstephanensis*	Moth (*Abraxas grossulariata*	1
6	*Bacillus* sp.	Snail (*Cornus asperum*)	1
7	*Enterococcus* sp.	Slug (*Lehmannia valentiana*)-	1
8	*Bacillus licheniformis*	Moth (*Abraxas grossulariata*	1
9	*Bacillus safensis*	Ladybird (*Adalia bipuncta*)	1
10	*Bacillus pumilus*	Ladybird (*Adalia bipuncta*)	1
11	*Exiguobacterium sibiricum*	Butterfly larva(*Aglais io*)	1
12	*Staphylococcus succinus*	Moth (*Abraxas grossulariata*	1
13	*Vagococcus* sp.	External swab butterfly pupav(*Aglais io*)	1
14	*Bacillus mycoides*	Moth (*Abraxas grossulariata*	1
15	*Clostridium litorale*	External swab butterfly pupa (*Aglais io*)	1
16	*Enterococcus mundtii*	Slug, external dry swab (*Lehmannia valentiana*)	1

Table 1:2. Bacteria isolated from external surface of insects and arthropods.

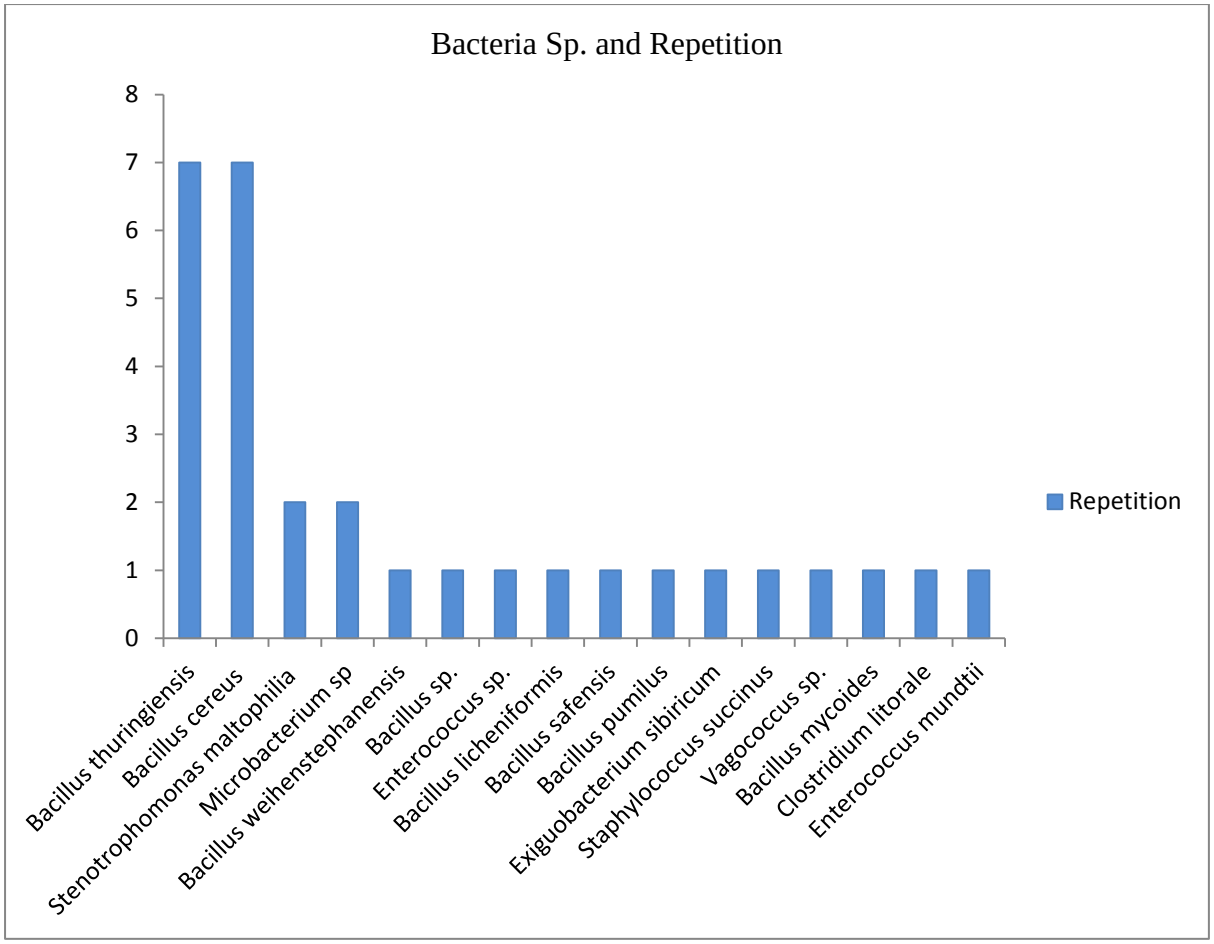

Fig.1:7. Bacteria (number of isolates) obtained from the surface and from haemolymph of a range of insects and arthropods species.

As the results given in Table 1:2 and Fig.1.5 show, *Bacillus thuringiensis* was the most commonly isolated bacterial species from the insects and the other invertebrates studied (being isolated from all samples). The next most frequently isolated species of bacterium was *B. cereus* (which is closely related to *B. thuringiensis*). Bacilli in general were widely represented amongst the isolates, with *Bacillus weihenstephanensis, Bacillus licheniformis* and *Bacillus mycoides* being isolated from moths and *Bacillus safensis* and *Bacillus pumilus* from a Ladybird and *Bacillus* sp from the snail sample. Other bacterial species isolated include *Stenotrophomonas maltophilia, Microbacterium* sp, *Enterococcus* sp, *Exiguobacterium sibiricum, Staphylococcus succinus, Vagococcus sp* and *Clostridium*

litorale all these bacteria were found outside the bodies of butterflies and moths and other arthropods. Most of the bacteria shown in Table 1:2 are capable of causing opportunistic bloodstream infections, and problems with the respiratory tract, urinary tract and surgical-sites (Cunha, 2011). The association of Enterococci with a variety of insects points to the potential of these organisms as transmitters of gastroenteritis, i.e. food poisoning. *Enterococcus* sp bacteria also cause important clinical urinary tract infections, bacterial endocarditis, bacteraemia, meningitis, and diverticulitis. The Gram positive bacterium, *Staphylococcus succinus* is not generally regarded as a pathogen, but could doubtless cause problems in immunocompromised patients. The Gram positives, *Exiguobacterium sibiricum* and *Vagococcus* sp are similarly not reported to be human pathogens, the same applying to *Bacillus safensis* and *Bacillus pumilus.* In contrast, *Bacillus thuringiensis and Bacillus cereus* can cause non-serious infections of the digestive tract; again, any problem they may cause in immunocompromised patients is not immediately evident.

The work described relates to the isolation of bacteria from a wide range of entomological specimens obtained from the local environment. This initial screening programme allowed the author to become acquainted with the handling of insects and arthropods and with their anatomy. The results show that bacteria are present in all of the entomological specimens sampled. Some of these bacteria are potential pathogens of humans and therefore could present a risk in hospital settings, especially in relation to immunocompromised patients. The most frequently isolated bacteria were two bacilli, *B.thuringiensis* and *B. cereus.* This is perhaps not surprising, since Bacillus species are spore formers and are known to survive in a wide variety of environments. The finding that *B. thuringiensis* is widely distributed amongst insects and arthropods is of particular importance because they produce an anti-larval protein which may impact on the survival of these entomological specimens (du Rand, 2009, Roh, *et*

al.2007). The finding of bacteria (*Bacillus thuringiensis*, *Enterococcus mundtii* and *Clostridium litorale*) in the intestines of the slug confirms early work by Walker *et al*. (1999).

It is surprising that relatively little attention has been given to the microbiology of insects, especially, since these organisms are frequently found in medical settings, both as living and dead specimens. Of course it is unlikely that large insects like the ones studied here would provide a major focus, and route, for infection when compared with, for example, blowflies or biting insects. A perhaps surprising means of transfer of potentially pathogenic bacteria from insects to man relates to the entomophagy, i.e. the consumption of living or dead insects (Ramous Elorduy 2009). The human consumption of insects provides a major source of nutrition for people in some 130 countries. In Mexico, for example, some 100 varieties of edible insects have been consumed over a period of some 500 years, from the Spanish conquest until modern times. The consumption of insects is becoming increasingly fashionable in rich Western societies and has even been considered as a source of nutrition during space flight, and colonization planets like mars (Katagama *et al*., 2005).

CHAPTER TWO-ISOLATION OF BACTERIA FROM THE HAEMOLYMPH OF LEPIDOPTERA

The Lepidopteran life cycle

There is generally no problem in distinguishing between butterflies and moths, because:

1. Most butterflies are active during the day, while most moths are active at dusk or at night (there are however day flying moths)

2-Most butterflies have clubbed antenna, while in moths they tend to be feather-like.

3-Most butterflies have slender bodies covered with hair, while most moths have fuller bodies, and have a fur-like covering.

4. Most butterflies rest with upright wings, while the majority of moths rest with their wings flat.

Butterflies and moths have a worldwide occurrence, with the majority being found in tropical rainforests. These insects however, range from fields and forests and some live on cold mountain peaks or even hot deserts. Many butterflies (and fewer moths) migrate to spend the winter in warmer areas, the classic example being the annual migration of the Milkweed or Monarch butterfly from the USA and Canada to central Mexico. The life cycle of butterflies and moth (Fig.2:1) begins with the egg which is produced after the female is fertilized by sperm which is stored in her body after mating; eggs vary in size from one to two millimetres and are usually deposited, from an ovipositor, onto the underside of a food plant leaf or stem. Within the egg the embryo grows quickly and hatches, usually within a week, although some species overwinter in the egg stage. The eggs eventually hatch and produce tiny larvae (i.e. caterpillars) which then feed voraciously and grow rapidly.

Fig. 2:1.The Lepidopteran life cycle (showing a Monarch Butterfly, *Danaus plexippus*) (F.W. Frohawk, 1914, out of Copyright).

Larvae have soft bodies and covered by hair or spines; on the head they also have small eyes, tiny antennae and relatively massive jaws consisting of keratin which allows for chewing of even tough plants. The larval stage is essentially an "eating machine". The first meal is the shell of the egg, followed by the food plant on which it was deposited. Larvae often eat their equivalent weight several times per day. Metamorphosis, the transformation from the egg via the larva and pupa, to the adult then occurs; the caterpillar often shedding its skin three to five times. Eventually hormonal changes take place which stop the larva from eating and allow its digestive system to empty before it finds a suitable pupation site. Moth pupae are often covered in a silk cocoon while butterfly pupae (i.e. chrysalides) are usually naked and attached to the substratum by a single silk thread. The pupal stage may overwinter, or occupy only a few days. The pupal-case then splits and the adult butterfly or moth emerges. The

wings unfurl and harden in several hours to allow the imago to begin its primary mission to life, i.e. reproduction. Most butterfly and moths have a short life span depending to the species, with some living only days to a few weeks. Some species also exhibit more than one brood over a single year and may overwinter as eggs, pupae or adults (Shalaway, 2004). The male Emperor moth (*Saturnia pavonia*) can detect a female-released pheromone over a distance of some 1.5 km.

Relatively little is known is about the microbiology of insects, notably species of Lepidoptera (Goff, 1987, Schoenly, Reid, 1987, Ashworth and Wall, 1994).

What is known relates largely to species which are pests or which transmit disease in humans. For example, it is unclear if bacteria and other microbes persist for long periods within the growth stages of Lepidoptera (ova, larva or pupa and imago and whether or not bacteria, for example bacteria can be transmitted throughout the Lepidopteran life cycle.

2:1The aim of the work described here

The aim of this research was a) to determine the bacteria present in Lepidoptera-imago-haemolymph, b) to study the bacteria in the haemolymph of larvae and pupae and c) the passage of bacteria supplied on the food plant to the larvae.

2:2 Study focus

The study initially focused on moths and butterfly species (i.e. Lepidoptera) in order to determine if bacteria can be isolated throughout the developmental life cycle, i.e. ova, larva, pre-pupa, pupa and adult (imago). Various species and life-stages of Lepidoptera were obtained locally or from entomological suppliers. Where killing was required, ethyl acetate was used in a standard glass killing bottle. Samples from the inside of the organisms were obtained using a fine, sterile, hypodermic syringe (the surface was first sterilized with a bleach (10%v/v) swab. Bacteria were then isolated from the extracted contents and subsequently identified.

After obtaining the haemolymph, the imagoes were immediately set for future identification and use. If the imagoes could not be directly set they were stored and then relaxed, using relaxing fluid (Worldwide Butterflies), in a relaxing box. The gut contents were then transferred to Nutrient Agar plates which were incubated at 37°C overnight.

2:3 PCR Techniques

PCR (polymerase chain reaction) is a tool used routinely in every diagnostic and medical and biological research laboratory. This technique has been used in the detection of forensic investigations, sequencing mutations and pathogens, eukaryotic classification, human genome (Prada-Arismendy,2011) to exponentially DNA sequencing profiling involved and producing millions copies from specific area on DNA profiling or specific gene to be able for us to study the genetic information (Hadidi and Candresse,2003, Erlich,1988) it is an excellent method for the detection of nucleic acids, eukaryotic species, human identification, disease identification, forensic science and to the discovery of pathogens their use in determining multiple paternity, relies on Alec Jeffreys' pioneering work (Chambers, *et al*. 2014, Randall *et al*., 1985). In 1990s the idea emerged of using a consolidated molecular identification system with the development of PCR-based approaches for species identification. The great benefit appeared in Molecular identification applied to bacterial studies, microbial biodiversity surveys (e.g. Kyrpides, 1996 and Zhou *et al*., 1997) and routine pathogenic strains diagnoses (e.g. Maiden et al., 1996, Sugita *et al*., 1998 and Wirth *et al*., 2006) due to a need for culture-independent identification systems. PCR-based methods have also been frequently applied to areas of study related to taxonomy, food and forensic identification (Teletchea *et al*., 2008) and for the identification of eukaryote which are pathogens and vectors.

2:4 Materials and Methods

Fig.2:2. Author collecting samples using a sweep net (The Ponderosa Park Sheffield).

The samples where obtained locally in the Sheffield region from vegetation (Fig.2.2) and where required killed using ethyl acetate in a killing bottle (Fig.2.3); other species obtained from Entomological supply houses.

Fig.2:3. A killing bottle used with ethyl acetate to kill imagoes (Showing European Swallowtail butterfly).

Chrysalids, pupae and larvae were kept in perspex boxes contains a wetted wad of cotton wool to maintain humidity (Fig.2:4); examples of typical moth pupae and a butterfly chrysalid are shown in Fig 2:5.

Fig.2:4. A perspex container showing a variety of chrysalids.

2:5. Setting of imagoes for future use

The imagoes were set using standard entomological setting techniques (Fig.2:4, 2:5, 2:6). A steel entomological pin was inserted into the thorax at a slight forward angle and the imago was then pinned into the grove of an adjustable pinning board (Worldwide Butterflies). The wings were then spread and tracing paper strips and pins were used to keep them in place. After an appropriate period of drying the images were transferred to a mite-tight collection box for storage.

Fig.2:5. A European Swallowtail butterfly being prepared for setting, showing setting board groove to left.

A

B

Fig.2:6. A, a moth undergoing setting and B, the resultant on-going collection of insects, mainly Lepidoptera.

2:6 Isolation of bacteria from larvae and chrysalids of the Peacock Butterfly (*Aglais io,* formally *Vanessa io*)

The larvae (first instar) were collected from local nettle patches and transferred to Perspex boxes in which were placed fresh nettle leaves and beaker containing water (added to maintain the desired humidity). When the larvae reached the second instar their stomach contents were removed by using a fine hypodermic needle (Fig.2:7) and the contents were spread on Nutrient Agar (as above) and any bacteria which grew were isolated. The same procedure was used for chrysalids obtained from pupae obtained by allowing members of the same batches of larvae to pupate (Fig: 2.8).

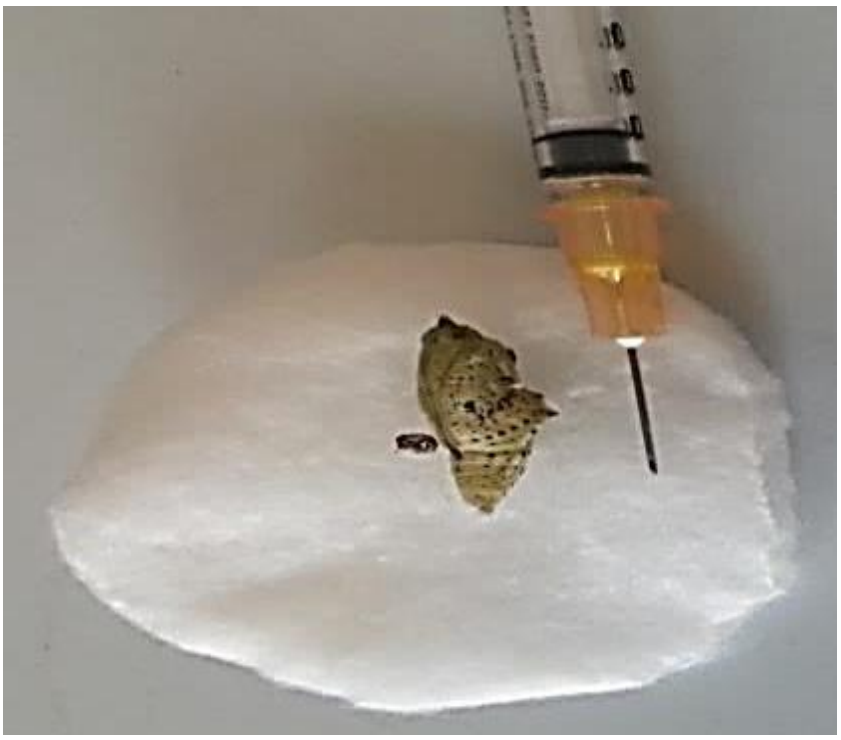

Fig.2:7. The isolation of bacteria from chrysalis body fluid.

Fig.2:8. Moth pupae (first three) and a butterfly chrysalis (extreme right).

2:7 Feeding of Peacock larvae with bacteria and subsequent isolation of the same bacteria from the larval gut contents

Samples of fresh nettle leaves from local nettle patches were soaked in Nutrient Broth cultures of various bacteria (MRSA *Staphylococcus aureus, Bacillus thuringiensis*, *Bacillus subtilis,* and *Escherichia coli)* and allowed to dry at room temperature. The bacteria-treated leaves were then transferred to separate plastic food containers (which had fine holes made in the lid to allow for gas exchange). Ten, third instar larvae were then added to each container. Control leaves, which were not treated with bacteria, were also included in a separate container. After 48 hours, fresh nettles which had not been treated with bacteria were added. After a further 24 hours, the bacterial contents of the larvae were then determined as described above.

2:8 Nutrient agar media

Nutrient agar media was prepared by weighing 23 g nutrient agar then dissolved in 1000 ml Distilled water in a flask with magnetic spin-bar until the solution dissolved than transfer class flask with covered or lid into autoclave at 120°C for 30 min for sterilization. Then the

solution was poured in Petri dishes carefully when solution temperature decline to 60-55 C. Preliminary identification is based on the colour the isolated bacterial colony developed when samples are grown on nutrient agar medium.

2:9 LB medium

LB is a most common used bacterial culture medium today. LB broth media was prepared by dissolving 10 g Peptone 140, 5 g yeast extract and 5 g of sodium chloride in 1000 ml distilled water in flask, followed by autoclaving at 121`°C for 30 min. When cooled to room temperature, it was the inoculated with a loopful of culture and incubated at 37^0C (Sambrook and Russell, 2001 Gerhardt, *et al*. 1994).

2:10 Nutrient Broth medium

Nutrient Broth medium (25g) was dissolved in a litter of sterile water, mixed thoroughly and then autoclaved at 121 °C for 30 minutes. Then it will be ready to grown the bacteria which is transformed from nutrient agar media cultured, by using loop attach with bacteria culture and dipping in nutrient broth after that incubate at 37 °C for 18 hours**.**

2:11 Agarose gel electrophoresis

Agarose gel electrophoresis was conducted as described above.

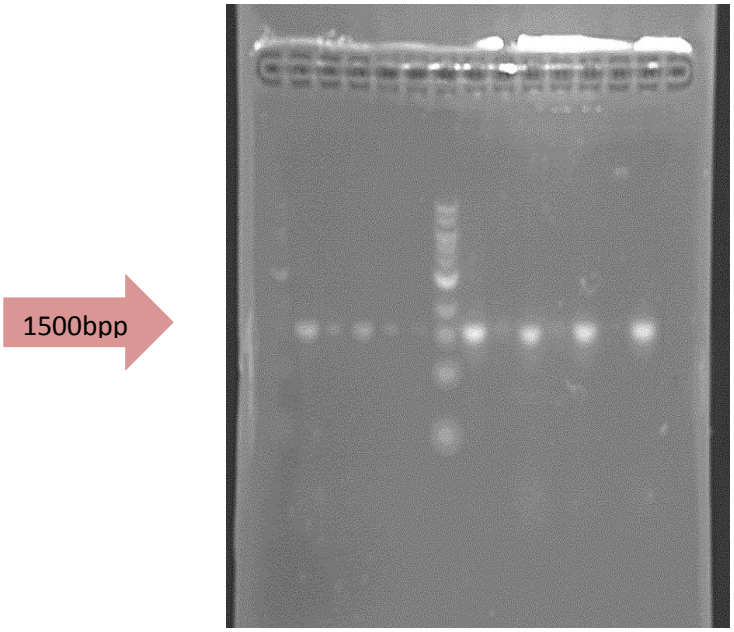

Fig.2:.9. An example of electrophoresis band in expected region 1500 bp.

2:12. Results and Discussion

The following Tables show that bacteria were isolated from the pupae and imago-body fluids of both butterflies and moths. Table 2:1(see also Appendix), shows that *Bacillus subtilis* was isolated from the chrysalid of the European Swallowtail. Species of the broad family of Streptococci, *Staphylococcus* (including MRSA) and *Stenotrophomonas* were also isolated from the body fluid of the imago of this butterfly has the highs repetition. Species of these Genera also predominate in the LGS of moths as shown in Table 2.1 and Fig.2.9.

No	Bacteria sp.	Repetition	Isolate
1	*Bacillus subtilis* strain 168	3	Pupa(chrysalis) European Swallowtail Butterfly (*Papilio machaon*)
2	*Granulicatella elegans* strain B1333	1	Body fluid, European Swallowtail Butterfly (*Papilio machaon*)
3	*Enterococcus mundtii* QU 25	1	Body fluid, European Swallowtail Butterfly (*Papilio machaon*)
4	*Staphylococcus saprophyticus* strain ATCC 15305	5	Body fluid, European Swallowtail Butterfly (*Papilio machaon*)
5	*Staphylococcus saprophyticus* subsp	5	Body fluid, European Swallowtail Butterfly
6	*Staphylococcus capitis* strain ATCC 27840	2	Body fluid long tail Zebra Swallowtail Butterfly.
7	*Staphylococcus capitis* strain JCM 2420	2	Body fluid European Swallowtail butterfly
8	*Staphylococcus aureus* subsp. aureus N315 strain	3	Body fluid European Swallowtail butterfly yellow and black
9	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Body fluid European Swallowtail butterfly yellow and black
10	*Stenotrophomonas maltophilia* R551-3 strain	8	Body fluid European Swallowtail butterfly, yellow and bright green

	R551-3		
11	*Brevibacterium frigoritolerans* strain DSM 8801	1	Body fluid from Eyed Hawk-Moth (*Smerinthus ocellatus*).
12	*Staphylococcus aureus* subsp. aureus N315 strain N315	3	Pupa chrysalis Eyed Hawk Moth *Smerinthus ocellatus*
13	*Bacillus subtilis strain 168*	3	Pupa chrysalis Eyed Hawk Moth (*Smerinthus ocellatus*).
14	*Staphylococcus sciuri strain* DSM 20345	3	Body fluid Elephant Hawk Moth (*Deilephila elpenor*).
15	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Body fluid, Silk Moth *Bombyx mori*
16	*Solibacillus silvestris* strain HR3-23	1	Body fluid from Atlas Moth (*Attacus atlas*)
17	*Staphylococcus saprophyticus* strain ATCC 15305	5	Body fluid, Elephant Hawk Moth (*Deilephila elpeno*r).
18	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Body fluid Elephant Hawk Moth (*Deilephila elpeno*r).
19	*Stenotrophomonas pavanii* strain LMG 25348	1	Body fluid, Elephant Hawk Moth (*Deilephila elpenor*).
20	*Staphylococcus succinus*	1	Body fluid, Elephant Hawk Moth (*Deilephila*

	strain AMG-D1		*elpenor*).
21	Staphylococcus sciuri subsp. carnaticus strain GTC 1227	3	Body fluid, Elephant Hawk Moth (*Deilephila elpenor*).
22	Staphylococcus saprophyticus strain ATCC 15305	5	Body fluid Eyed Silk moth
23	*Staphylococcus saprophyticus* strain ATCC 15305	5	Body fluid Small White
24	*Pantoea agglomerans* strain ATCC 27155	1	Body fluid, Atlas Moth (*Attacus atlas*).
25	*Bacillus subtilis* strain 168	3	Body fluid Silk moth
26	*Micrococcus yunnanensis* strain YIM 65004	1	Body fluid Silk moth
27	*Bacillus licheniformis* strain DSM 13	1	Body fluid, Comma Butterfly (*Polygonia c-album*)
28	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Wet swab from butterfly larvae(outside)
29	*Staphylococcus sciuri* subsp. *carnaticus* strain GTC 1227	3	Wet swab from butterfly larvae(outside)
30	*Lysinibacillus fusiformis* strain NBRC15717	1	Isolated from inside butterfly larvae body

31	*Stenotrophomonas rhizophila* strain e-p10	1	Isolated from inside butterfly larvae body
32	*Bacillus cereus* ATCC 14579	3	Isolated from inside butterfly larvae body
33	*Lysinibacillus macroides* strain LMG 18474	1	Isolated from inside butterfly larvae body
34	*Bacillus cereus* ATCC 14579	3	Isolated from inside die butterfly larvae body treated by *B. thuringiensis*
35	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Isolated from inside die butterfly larvae body treated by *E.coli*
36	*Bacillus cereus* ATCC 14579	3	Isolated from inside butterfly larvae body treated by *B. subtilis*
37	*Staphylococcus aureus* subsp. aureus N315 strain N315	3	Isolated from inside die butterfly larvae body treated by MRSA bacteria
38	*Stenotrophomonas maltophilia* strain IAM 12423	8	Isolated from inside butterfly larvae body(Control)
39	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Isolated from inside butterfly adult body(Control)

Table 2:1. Bacteria isolated from the Lepidoptera (butterfly) haemolymph.

Unusual species of bacteria such as *Granulicatella elegans, Solibacillus silvestris, Pantoea agglomerans* and *Lysinibacillus fusiformis* were also isolated.

Figure 2:10 provides a summary of the bacterial species isolated from butterfly haemolymph, which emphasises the predominance of species of *Staphylococcus. Stenotrophomonas maltophiliait* is a Gram negative species which is an important cause of nosocomial infection in the respiratory tract and urinary catheters. *Staphylococcus saprophyticus* despite its name can cause urinary tract infections and *Staphylococcus sciuri* bacteria species is an important human pathogen responsible for endocarditis, peritonitis, septic shock, urinary tract infection. (Chen, *et al*., 2007). *Granulicatella elegans* is a part of the normal microflora of the oral cavity, the genitourinary and intestinal tracts (Luca, 2013), and *Enterococcus mundtii*, is a gram positive non-pathogen (Esteban, 2012). *Brevibacterium frigoritolerans*, is a gram positive species which is of interest because it is present on the human skin, where it causes foot odour. *Micrococcus yunnanensis*, is a gram positive bacteria which is also found in human skin, animal and dairy products. *Lysinibacillus fusiformis* is gram positive and causes infections in humans relating to tropical ulcer formations and dermal and respiratory infections (Calandrini, *et al*., 2014). *Pantoea agglomerans* is gram negative and causes wound, blood, and urinary-tract infections. It is frequently isolated from the surface of a variety of plants and is linked with bacteraemia associated with the use of catheters. (Cruz, *et al*.,2007). Finally, *Lysinibacillus macrolides* is associated with infections such as periodontitis and has previously been isolated from butterfly larvae (Coorevits, *et al*.2012).

Nearly all of these bacteria are capable of causing pathogenicity in humans, especially amongst immunocompromised patients. Lepidopteran species do not, of course, generally interact closely with humans, so it is unlikely that these insects will form reservoirs of pathogenic bacteria which could infect patients having reduced immunity, who reside in

hospitals, so the pathogenicity of these isolates is likely to be of passing interest only, except perhaps where dead Lepidopteran imagoes are not removed from the medical setting

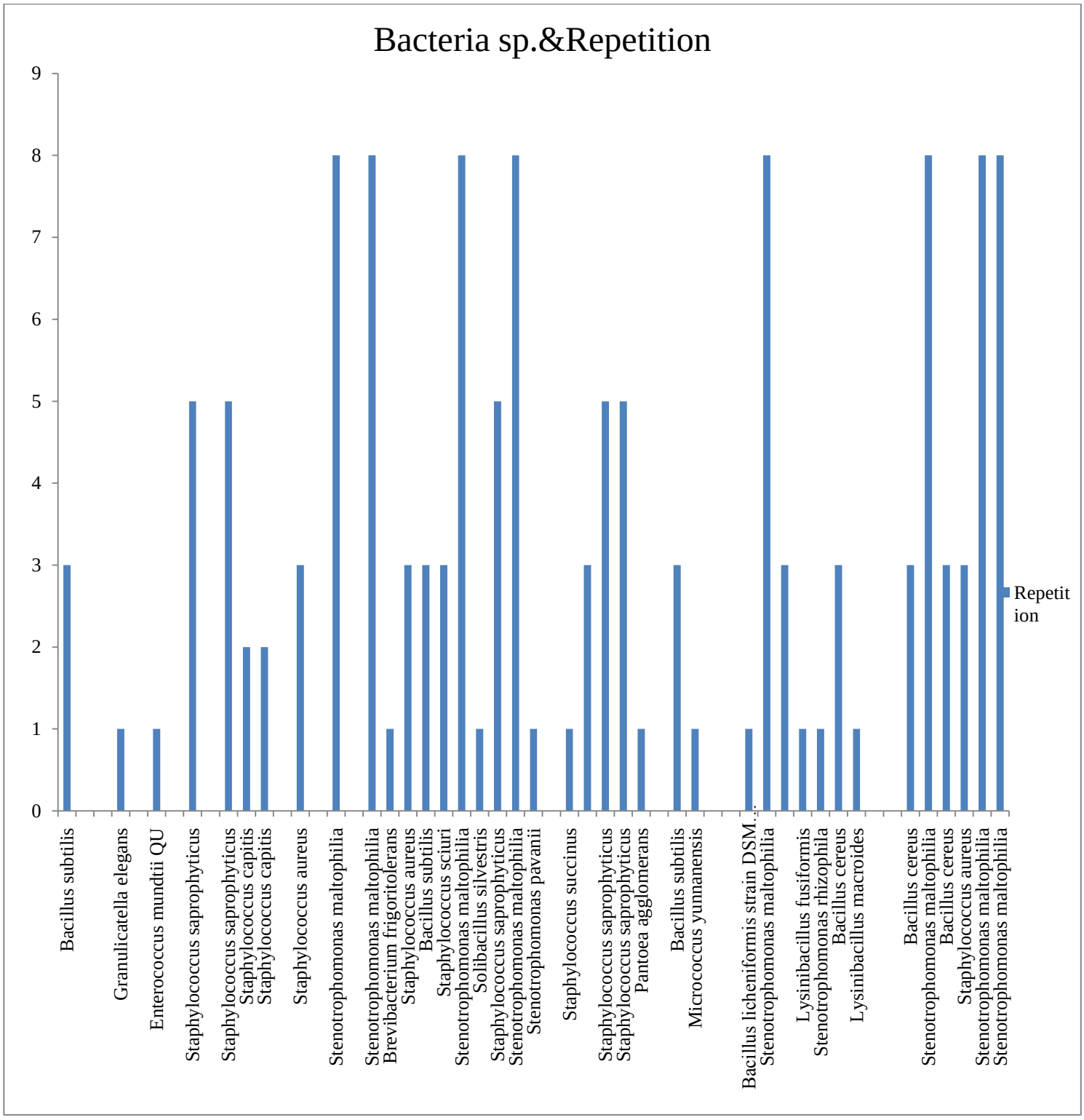

Fig.2.10. Summary of bacteria isolated from various butterflies and moth species (for more detail see appendix table 2).

Feeding larvae from different bacteria species

Perhaps of more scientific interest is the question of how these bacteria survive inside the body fluids of chrysalids and imagoes. This is especially true of the pupal stage, where complex body fluid transformations which involve a complex mixture of enzymes are taking place. As a result of these changes, one might assume that the LGS is sterile, which is clearly not the case. Again, it might be incorrectly assumed that the only bacteria which can survive inside Lepidopteran growth stages would be species of Bacilli, which produce resistant endospores. Again, the prevalence of non-spore forming species of *Staphylococcus* runs contrary to this view (Fig.2:10).

2:13 Results of feeding larvae from different bacteria species:

Bacteria Fed To Larvae	**Larvae Alive after 24hours**	**Larvae Alive after 48hours**	**Larvae Alive after72hours**	***Result of isolation (from 2 dead larvae)***
MRSA	10	10	5	*Staphylococcus aureus*
Bacillus thuringiensis	1	0	0	*Bacillus cereus.*
Bacillus subtilis	8	6	3	*Bacillus cereus*
E. coli	8	6	3	*Stenotrophomonas maltophilia*
Control	10	10	10	*Stenotrophomonas maltophilia*

Table: 2.2. Results obtained following the feeding of larvae of the Peacock butterfly with leaves covered in various bacteria.

Larvae of the Peacock butterfly were fed nettle leaves covered with a range of bacteria (Table 2:2). The number of larvae reaming alive after 24, 48 and 72 hours varied with the type of bacteria used. None of the control larvae died when fed non-contaminated nettles over the length of the experiment. Feeding of the larvae with MRSA covered leaves lead to a fifty per cent death rate after 72 hours, although no larvae were killed after 24 and 48 hours; *S. aureus* was isolated. Not surprisingly, since it is toxic to many insect larvae, feeding with *B. thuringiensis* lead to the death of all of the larvae after 4hours. The results how that feeding with *B. subtilis* and *E. coli* can lead to larval death, while MRSA was shown to be less toxic. Feeding the larvae with the other bacteria killed some larvae, with the death rate after feeding *B. subtilis* and *E. coli* being identical. *Bacillus cereus* was isolated from the larvae fed *B. thuringiensis* and *B. subtilis.*

CHAPTER THREE-ISOLATION OF BACTERIA FROM A DERMESTIDAE LARVA OBTAINED FROM HUMAN CADAVERS DURING FORENSIC ANALYSIS IN SAUDI ARABIA

3:1 Introduction

Forensic entomology is the study of the application of insects and other arthropods to criminal investigations (Catts and Goff, 1992). Insects or arthropods are located on or within decomposing vertebrate corpse or carrion (LeBlanc, 2010) These insect colonizers can be used to make a crude determination of a) the time of death i.e., the time interval between the corpse and its discovery which is generally referred to as the post-mortem index (PMI), b) whether or not the corpse has been moved at the scene of death. As soon as death occurs, cells start dying and enzymes begin to digest the body cells from the inside via the process of autolysis, i.e. the body starts decomposing. Bacteria which are present in the gastrointestinal tract begin destroying the soft tissue producing liquids and gases such as hydrogen sulphide, carbon dioxide, methane, ammonia, sulphur dioxide and hydrogen. Volatile molecules referred to as apeneumones escape from the decomposing body and attract insects. Researchers have been able to isolate these volatile chemicals which are liberated at different stages of cadaver- decomposition. Volatiles, released during each stage, can also modify insect behaviour and putative sulphur compounds are responsible for initiating the process which attracts flies to the decomposing carcass. Subsequent egg laying by flies is induced by ammonium-rich compounds present on the carrion (Ashworth *et al.*1994).

Four categories of insects can be found on decomposing bodies: a) necrophagous species which feeding on the carrion; b) predators and parasites feeding on the necrophagous species; c) omnivorous species feeding on the carrion and other arthropods such as ants, wasps and some beetles; d) species such as springtails and spiders which use the corpse as an extension of their environment. The first two groups are generally most important in relation to forensic

entomology, i.e. mainly species of the order Diptera (flies) and Coleoptera (beetles) (Fig.3:1) and the waves of succession whereby arthropods colonize the carrion relates to the decomposition state of the carrion (LeBlanc, 2010). The insects which are mainly involved in forensic investigations are true flies or Diptera, with main species in this order being Calliphoridae (blow flies), Sacrophagidae (flesh flies) and Muscidae (house flies). Calliphoridae (blow flies), Sacrophagidae (flesh flies) often arrive within minutes after death. Muscidae (house flies) do not however, begin colonizing until the body reaches the bloat stage of decomposition. The fresh stage (Days 1-2) begins at the point of death and terminates with the observation of carcass-bloating. Autolysis occurs at this stage, but this process is not associated with gross morphological changes at this juncture. The estimation of the time of death using entomological data (after a period of 24 hours) is notably more accurate than is soft tissue examination by a medical examiner. Insects arrive at the cadaver within ten minutes of death. Insect larvae which then develop on the dead body can be used to determine the post-mortem interval (PMI) for up to a period of one month (LeBlanc, 2010). The initial step of the forensic process is the correct identification of insect species, since different species vary in their growth rates and maturation, so that it is necessary to determine the correct age of the cadaver-invading larvae. The age of the larvae can be estimated by measuring the length or dry weight of the most mature larvae and comparing it with the reference data; larval development rates are recognized as being dependent on the surrounding ambient temperatures

3:2 Using insect data for determining the site of crime

There occur differences in the range of insects involved with decomposing corpses located in a variety of habitats and environments and such differences can often be used to determine the geographical location of a corpse at time of death if it has been transported long distances and into different ecological zones following death.

3:3 Entomotoxicology

Those fly larvae which feed on carrion can accumulate drugs ingested by the dead person. However, it is almost impossible to determine the presence of toxicological substance such as drugs when the cadaver is in an advanced state of decomposition, or is skeletonized. Toxins can have a major impact on the stages of development of larvae; cocaine and heroin for example are both known to accelerate the development of larvae while poisons, including Malathion often reduce the rate of insect colonization of carrion and cadavers (Tullis, Goff, 1987, Schoenly, Reid, 1987, Ashworth and Wall, 1994).

Fig.3:1. Dermestid larvae and Blow fly obtained from human cadaver.

3:4 Aims

The aim of the work reported in this section of the Report was to determine the surface and gut-related bacteria of larvae associated with a human cadaver (sampled in Saudi Arabia). This work is currently preliminary in nature and relates to the possibility of using insect-associated bacteria in Forensic Entomology.

3:5. Materials and Methods

Dermestid fly larvae (*Dermestidae*) and adult Blow fly (*calliphoridae*) were obtained from a decomposed human body by a forensic entomologist working in Riyadh, Saudi Arabia. They were then separately placed in a sterilised plastic tube and stored at – 20°C. Bacteria were isolated from the surface of the imagoes and larvae using a cotton wool swab, moistened with sterile distilled water. Some of the samples were then surface-sterilized by rapid transfer to sterile ethanol (70% v/v).In addition by using a fine hypodermic needle to isolate bacteria from haemolymph the contents were removed and their bacterial content determined as described above.

3:6 Results and Discussion

Table 3:1Results of bacteria species isolate from larvae of *Dermestidae* and Blow fly obtained from a human cadaver.

No	Bacteria sp.	Repetition	Isolate
1	*Brevibacterium ravenspurgense* strain 20	1	Isolated from inside larvae of *Dermestidae* extracted from human dead body.
2	*Staphylococcus hominis* subsp. *novobiosepticus* strain GTC 1228	1	Isolated from inside larvae of *Dermestidae* extracted from human dead body.
3	*Enterococcus faecalis* V583 strain V583	1	Isolated from inside adult blow fly *(Calliphora)* extracted from human dead body.
4	*Lishizhenia tianjinensis* strain H6	1	Isolated from inside larvae of *Dermestidae* extracted from human dead body
5	Clostridium cochlearium strain JCM 1396	1	Isolate from inside larvae of *Dermestidae* extracted from human dead body.
6	*Bacillus safensis* strain NBRC 100820	1	Isolated from inside larvae of *Dermestidae* extracted from human dead body
7	*Enterococcus faecalis* strain NBRC 100480	1	Isolated from inside larvae of *Dermestidae* extracted from human dead body.
8	*Clostridium paraputrificum* strain JCM 1293	1	Isolated from inside blow fly (*Calliphora*)

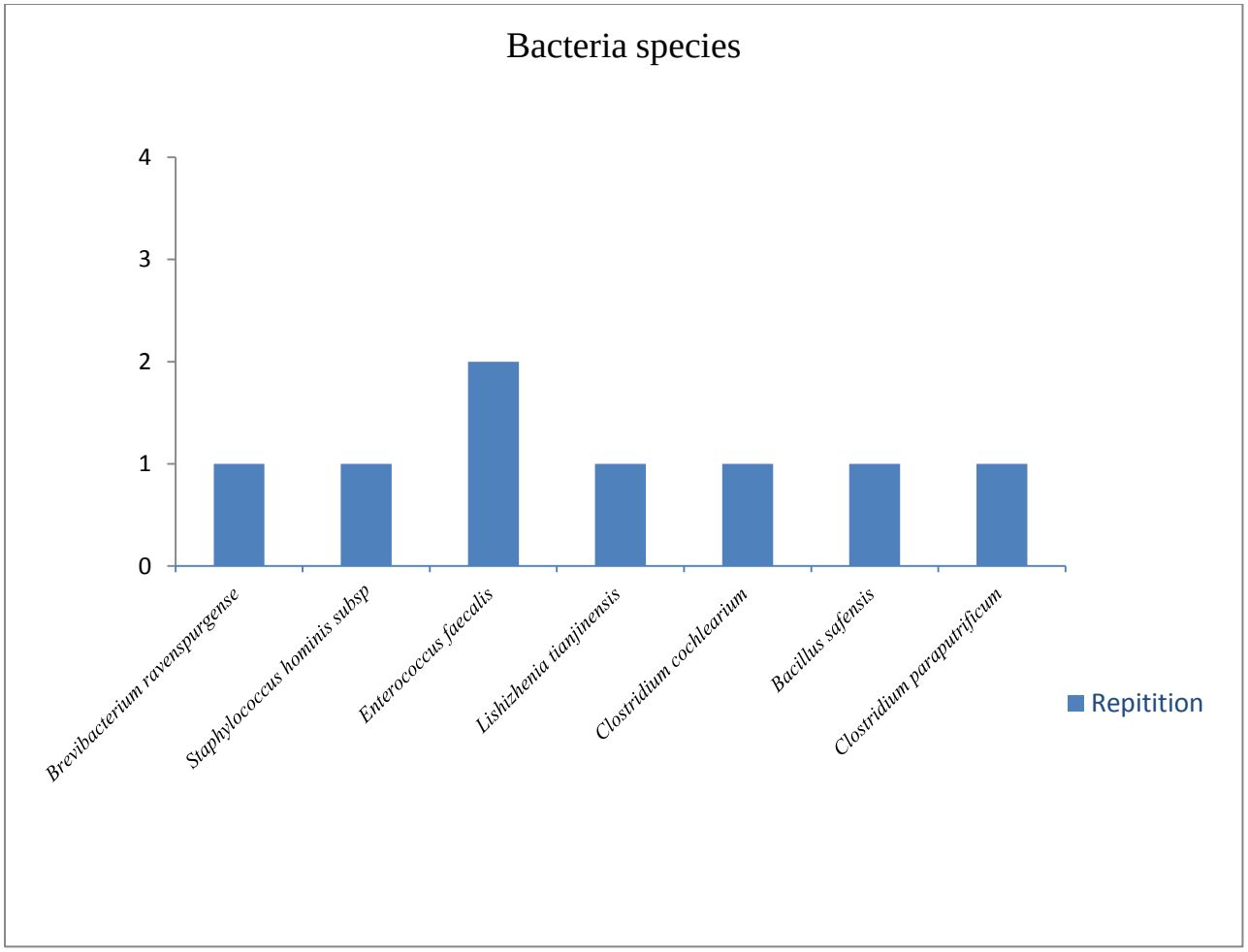

Fig.3:2. Bacteria extracted from the Dermestid larva and Blow fly isolated from a human cadaver.

Insects have considerable potential position in this science for use in apprehending criminals, especially those involving toxins and drug intake by the victim; more commonly, they can be used to determine time and broad location of death. Dermestidae like the ones studied here are a family of beetles (Coleoptera) commonly known as skin, larder, leather or hide beetles, of which there are 500 to 700 species worldwide. They can range in size from 1–12 mm. Most Dermestids scavenge on dry animal or plant material, including skin, pollen, animal hair, feathers, dead insects and natural fibers and are found in animal carcasses, and a variety

of nests. These beetles are significant in forensic entomology since some are associated with decaying carcasses, which aids criminal investigations. *Dermestes maculatus*, hide beetles, can offer investigators an estimation of the time since death in homicide or questionable cases. This use is based on the fact that the arrival to carrion and cadavers of *D. maculatus* generally takes place in a regular succession; for example, adult *D. maculatus* beetles usually arrive some five to ten days after death. The appearance of Dermestids is temperature dependent, being optimal at 30˚C. Of particular interest to forensic scientists is the fact that the feaces and shed larval skins of this beetle can be analyzed for toxins, including drugs.

Figure 3:2 and Table 3:1 show eight bacteria species were extracted from the Dermestid larvae obtained from a human decomposed body. The dominant species of bacteria was *Enterococcus faecalis* which was isolated from inside the adult blow fly and from inside larvae extracted from the human corpse. Two species of *Clostridium* were also isolated, *Clostridium cochlearium* was isolated from outside larvae blow fly (*Calliphora*) removed from the human cadaver; the other, *Clostridium paraputrificum* was isolated from inside the blow fly (*Calliphora*) larvae obtained from the cadaver. *Brevibacterium ravenspurgense, Staphylococcus hominis, Lishizhenia tianjinensis* and *Bacillus safensis*, were also isolated from outside and inside larvae, extracted from the human body.

Microbes play major and sometimes essential roles in the growth and development of insects. Insects which harbour endosymbionts depend on them for reproduction, digestion and for the supply of essential nutrients and also in the production of pheromones (Gil and Moya, 2004, Wernegreen, 2002). Bacteria present on the gut of some specialized niche feeders like termites and aphids, have been widely studied because of interest in the diverse microbial enzymes involved (Brauman *et al*.1992). In comparison, relatively little is known about the microbiology of foliage–feeding insects which involve no strict symbiotic

interactions. Since most lepidopteran larvae are herbivores their gut content (food bolus) is far from sterile (Dillon and Dillon, 2004). The gut flora of lepidopteran and other insects plays a role in detoxifying harmful secondary metabolites (Morrison *et al.,* 2009) and also protects the host against the pathogen colonization. The gut flora is also involved in a) the aggregation pheromones of locusts (Dillon *et al*.2000) b) maintenance of the host fitness (Freitek *et al*.2007) and c) homeostasis of plant defence elicitors in certain lepidopteran larvae (Ping *et al*.2007).

Whether or not autochthonous bacterial strains exist in these insect guts is largely unknown (Dillon and Dillon,2004).The problems of isolating the total microbiota of insect guts is illustrated as follows: Less than half of the bacterial phylotypes identified with terminal-restriction fragment-length polymorphism of 16S rRNA genes from gypsy moth (*Lymantria dispar*) have been found to grow in the laboratory (Broderick *et al*.,2004),while none of the bacteria isolated from the laboratory-bred tobacco hornworm (*Manduca sexta*) (van der Hoeven, 2008) belong to the abundant phylotypes revealed by PCR-single-strand conformation polymorphism of the 16S rRNA genes (Brinkman,2008). Denaturing gradient gel electrophoresis coupled with 16S rRNA gene sequencing has shown that 72% of the mid gut bacteria of the “old world” cotton bollworm (*Helicoverpa armigera*) share less than 98% sequence identities to known species (Xiang *et al*. 2006).

CHAPTER FOUR-POTENTIAL ROLE OF *B. THURINGIENSIS* AND *PAENIBACILLUS POPILLIAE* IN ENVIRONMENTAL MINERAL CYCLING

4:1 INTRODUCTION

As has already been mentioned, *Bacillus thuringiensis* is widely used as an insecticide, notably in the USA. As a result, it is inevitable that this bacterium will reach soils, particularly those of the rhizosphere, and here can grow and be able to participate in mineral cycling. Martin and Travers (1999) found that *Bacillus* thuringiensis. *Bacillus thuringiensis* occurs naturally, and has a world-wide distribution and was found in some three quarters of all soils tested; over 60% being toxic to the larvae of Lepidoptera and Diptera (Martina and Travers, 1999) Saleh et al (1970) isolated the bacterium from muck soil which had been treated with Thuricde (Fig.4:1) to control insect pests on lettuce and cabbage; spores of the bacterium added to soil, remained viable for at least a month. The fact that *Bacillus thuringiensis* remains viable in treated soils means that it can germinate, grow and contribute to the major biochemical cycles involving the most important plant nutrients, i.e. nitrogen phosphorus and sulphur.

Fig.4:1. Containers showing commecial Thuricide and Milky Spore powders.

4:2 *Bacillus thuringiensis*

Bacillus thuringiensis (or Bt) is a gram positive, soil-living bacterium which is frequently used as a biological pesticide (du Rand, 2009, Roh, *et al*.2007) During sporulation many Bt strains produce insecticidal crystal protein inclusions called δ-endotoxins which can be used to control caterpillars on crops, especially when genetically modified (Yamamoto and Dean, 2000, da Silva and Valicente, 2013). *Bacillus thuringiensis* and several strains of *B. cereus* cause gastrointestinal diseases in insect larvae that are attributed to enterotoxins (Hansen and Hendriksen, 2001). This bacterium is common throughout the world (Vilas-Boas *et al*. 2002). *Bacillus thuringiensis* is a gram positive, bacterium which is readily isolated from soil and in the gut of a variety of caterpillars of Lepidoptera species; it can also occur on leaf surfaces, in aquatic environments, animal faeces, insect-inhabited environments, and in grain-storage facilities and on associated dead insects. *B. thuringiensis* was first isolated from a silkworm larva in Japan, 1901 by Ishiwata Shigetane; some ten years later it was rediscovered in Germany, by Ernst Berliner who isolated it from the Mediterranean flour moth in the town of Thuringia. *Bacillus thuringiensis* species have been isolated and

classified into subspecies primarily based on the flagella antigenicity. *B. thuringiensis* is closely related to *B. cereus,* a soil bacterium, and *B. anthracis,* the cause of anthrax in Man and animals, the three organisms differing mainly in their plasmid content; all three grow aerobically and reproduce by the production of endospores. On sporulation, *B. thuringiensis* forms crystals of the protein insecticide δ-endotoxins (also referred to as crystal proteins or cry proteins), which are encoded by cry genes. In most strains of *B. thuringiensis,* the cry genes are located on a plasmid and the gene is generally not chromosomal. Cry toxins specifically attack insect species of the orders Lepidoptera, Diptera, Coleoptera, Hyymenoptera, and nematodes. This bacterium provides an important reservoir of Cry toxins, which can be utilized to produce biological insecticides and genetically modified crops in which insect-resistant is induced. Insects consume the toxin crystals insoluble crystals are denatured in their digestive tract making them soluble and able to be cut by proteases present in the insect gut, thereby liberating the toxin from the crystal. The cry toxin then enters the insect gut cell membrane and causes marked paralysis of the digestive tract and produces a pore which prevents the insect eating, leading to death by starvation. An active mid-gut bacterial population of susceptible larvae needs to be present in order to induce *B. thuringiensis* insecticidal activity. Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used in insect to control since the 1920s and are today, are often applied in the form of liquid sprays under trade names such as the formulations, DiPel and Thuricide. Because of their specificity, these bio-pesticides are considered to be environmentally friendly, and appear not to affect humans, wildlife and, insect pollinators, and other useful insects and so can be utilized in organic farming. *Bacillus thuringiensis* serovar israeliensis is now extensively employed as a larvicide for the control of mosquito larvae and is considered to provide an environmentally friendly form of mosquito control (Hellmich, *et al*. 2001)

4:3 Uses of Bt in agriculture

In 1995, potato plants producing CRY 3A *Bacillus thuringiensis* toxin were approved safe for use by the US Environmental Protection Agency, thereby making it the first human-modified pesticide-producing plant to be successfully approved in the USA. A number of naturally growing plants are also able to produce the pesticide, including tobacco, coffee and cocoa. In 1996, a corn plant was genetically modified to produce Cry protein which killed the European corn borer and related species; subsequent *Bacillus thuringiensis* genes were introduced which endowed it with the ability to kill corn rootworm larvae. The *Bacillus thuringiensis* genes which have been engineered into crops and approved for release include, singly and stacked: Cry1A.105, CryIAb, CryIF, Cry2Ab, Cry3Bb1, Cry34Ab1, Cry35Ab1, mCry3A, and VIP, while the engineered crops include, corn and cotton; corn, genetically modified to produce VIP was first approved in the USA in 2010. Monsanto then produced a soybean which expresses Cry1Ac and the glyphospate -resistance gene which was accepted for use in Brazil. (Roh, *et al*. 2007) (Ars et al, 2014)

4:4 Development of insect resistance

Monsanto-based scientists found that, in India, the pink bollworm has developed resistance to the first-generation *Bacillus thuringiensis* cotton- which expresses one Bt gene, Cry1Ac. This turned out to be the first case of *Bacillus thuringiensis* resistance anywhere in the world. The company immediately introduced second-generation cotton with a number of *Bacillus thuringiensis* proteins, which was rapidly adopted. Bollworm resistance to first-generation *Bacillus thuringiensis* cotton has also been reported in Australia, China, Spain, and the USA (Cheng and Thomas, 1984)

4:5 Potential Lepidopteran toxicity

The most publicised problem which is claimed to be associated with the use of Bt crops is that pollen derived from Bt maize can kill the Monarch Butterfly. However, by 2001 the

USDA had shown that the most common types of Bt maize pollen are not toxic to Monarch larvae in concentrations the insects would encounter in the field environment.

4:6 Milky Spore (*Paenibacillus popilliae*)

Milky Spore contains *Paenibacillus popilliae* (formerly known as *Bacillus popilliae*) is a gram positive, rod shaped soil bacterium (Glare and Callaghan, 2003; Fig.4:1). It is responsible for milky spore disease of the white grubs of the Japanese beetle. The adult beetles feed on flowers and leaves of shrubs and garden plants, where they mate, with the female laying eggs under the soil in late July – early August. The eggs hatch soon afterwards and in the larval (grub) stage, feeds on the roots of grasses and other plants. At the approach of winter, the grubs move deeper in the soil and stops feeding in order to over-winter. It is during August when the grubs are close to the surface and actively feeding that they succumb to infestation by Milky Spore. The Milky Spore biological control agent is applied during this period. The spores contained in the product are swallowed by grubs as they feed on roots. The spore then germinates and reproduces rapidly to produce internal bacteria which eventually kill the grub. Within a period of 1-3 weeks, the grub dies and as it decomposes vast numbers of new spores are liberated into the soil. The presence of Milky Spore in the soil does not however, harm beneficial insects, birds, bees, pets or man. Milky Spore-derived bacteria are capable of surviving in drought conditions but suffer when exposed to low temperatures.

The aim of the work described in this Chapter was therefore to determine if *Bacillus thuringiensis* (as Thuricide) and *Paenibacillus popilliae* (as Milky Spore) can participate in major nutrient transformations *in vitro*, namely urea hydrolysis, the oxidation of ammonium to nitrate (nitrification) and the solubilisation of a source of insoluble phosphate

4:7 Nitrogen cycles

Nitrogen is the predominant component of amino acids which are themselves the building blocks of peptides and proteins, and since this element is essential for growth and reproduction in both plants and animals the N-cycle is regarded being the most important biogeochemical cycle (Pidwirny, 2004). Nitrogen is part of the genetic make-up of cells, the nucleic acids and as a result, makes up about 80% of the Earth's atmosphere and some 12% of cell dry weight (Maier *et al*, 2009).

Five main processes operate in the N-cycle (Harrison, 2003):

1) Nitrogen fixation: the process by which atmospheric nitrogen (N_2) is converted to ammonia (NH_3).
2) Nitrogen uptake (organismal growth or assimilation): where microorganisms make use of ammonium to build organic nitrogen compounds
3) Nitrogen mineralisation (decay): which organic nitrogen is converted to inorganic nitrogen (ammonium NH_4^+).
4) Nitrification: the ammonium (NH_4^+) is oxidized to nitrate (NO_3^-).
5) Denitrification: the reduction of nitrate (NO_3^-) and nitrite (NO_2^-), to nitrous oxide (N_2O), then to a nitrogen gas into the atmosphere (Figure 2.1).

4:8 Ammonification

In the decomposition processes, the nitrogen component of proteins is transformed to ammonia (NH_3) or ammonium (NH_4) by a wide range of microorganisms (bacteria and fungi). This brings about the release of N from the organic matter which makes up decomposing plants and dead animals or dung (Hart *et al*., 1994).

4:9 Nitrification

Nitrification is of major importance for the N-cycle in both aquatic and terrestrial environments; it involves the oxidation of ammonia (NH_4^+) to nitrite. (NO_2^-) then nitrite to

nitrate (NO_3^-) by chemoautotrophic bacteria and by some heterotrophic organisms including, fungi and bacteria, which can also bring about these oxidations (Maier et al., 2009). Two types of nitrification exist (Killham, 1994): The first relies upon the activity of chemoautotrophic nitrifying bacteria (*Nitrosomonas)* by which ammonia (NH_3) or ammonium (NH_4^+) ions are oxidised to nitrite (NO_2^-).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H+ + H_2O \qquad \Delta G = -267.5 \text{ kJ.mol}^{-1}$$

The second, involves the activities of chemoautotrophic Gram-negative bacteria which oxidize nitrite (NO_2^-) is oxidized to nitrate (NO_3^-) *Nitrobacter*.

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \qquad \Delta G = -86.96 \text{ kJ.mol}^{-1}$$

4:10 Urea hydrolysis

Urea has a highly water-soluble fertilizer whose nitrogen content is higher than that of ammonium, nitrate and ammonium sulphate (Ferguson *et al*., 1984). Ureases are enzymes which are released by microorganisms into soil, plants and animals. Urea is converted to carbon dioxide and ammonia by soil ureases by a diverse range of microorganisms which are able to hydrolyse urea, including bacteria, predominantly *Pseudomonas, Achromobacter, Bacillus, Micrococcus* and some fungi, including *Penicillium* species as well as most other members of the Deuteromycetes (Maier *et al*., 2009).

4:11 Sulphur cycle

Sulphur, the tenth most abundant element in the earth's crust is an essential element for the growth of all organisms being essential for the synthesis of the amino acids, cysteine and methionine, and vitamins such as vitamin B1 (thiamine), hormones, including biotin, coenzymes and lipoid acid (*Maier et al*, 2009). The S-cycle can be summarized as a) the mineralization of organic sulphur to inorganic sulphate, b) the oxidation of reduced, inorganic forms to sulphate, c) the anaerobic reduction of sulphate to sulphides, and finally d) the immobilisation of sulphate as organic sulphur.

Filamentous fungi also play a major role in the S- cycle; the soil fungus *Fusarium solani* for example, oxidizes elemental sulphur to polythionates, thiosulphate and sulphate (Wainwright, 1984; Wainwright and Killham, 1980). Fungi are also able of oxidizing sulphur to sulphate with the production of tetrathionate and thiosulphate. It has been suggested that these oxyanions protect fungi from the toxic effects of heavy metals (Wainwright *et al*., 1997).

Several factors affect sulphur oxidation in environment, including:

1. pH: sulphur oxidation can take place between pH 2 and 9 and sulphur oxidation increases with increasing pH (Vitolins and Swaby, 1969).
2. Temperature: the optimum temperature for S-oxidation range is between 25°C to 40°C, while some thermophilic bacteria and fungi can also grow at 55°C (Wainwright, 1984).
3. Microbial composition: S-oxidation is influenced by the size and composition of the soil microbial community (Soomro, 2000).
4. Moisture and aeration: the moisture content for most rapid sulphur oxidation processes is near field capacity (Mahfouz, 2005).

4:12 Phosphorus cycle

Phosphorus is an element which is essential to the growth of all organisms and as a result it is essential that we gain a thorough understanding of how it is cycled in the environment (Goldstein, 1994). This is vital because phosphorus plays a central role in many important biomolecules, especially adenosine triphosphate (ATP), DNA (deoxyribonucleic acid) and in phospholipids (Hyland et al., 2005). Bacteria, actinomycetes and fungi, can all solubilise insoluble phosphates (Hattori, 1973; Paul and Clark, 1996). Such microbes release phosphorus when growing in culture amended with calcium phosphate, apatite or other insoluble source of phosphate; phosphate solubilizing fungi include species of *Aspergillus*,

Fusarium, and *Penicillium* (Al-Turk, 1990). Microbial processes which play a role in the transformation of phosphorus into an available nutrient source include:

1) Altering the solubility of inorganic P compounds.

2) The mineralization of organic compounds to form inorganic phosphorus.

3) The immobilisation of inorganic phosphorus into cell components.

The aim of the work presented below was to determine if *Bacillus thuringiensis* (as Thuricicde) and *Paenibacillus* popiliae (as Milkyspore formulation) can participate in some of the important nutrient cycling transformations when grown *in vitro* in soil.

4:13 Materials and Methods

The two bacteria were separately inoculated into 100 of autoclaved medium (Nutrient broth) in 250 ml sterile Erlenmeyer flasks. The medium was then amended with 0.25 gm of the individual substrate, i.e. urea, ammonium sulphate, elemental sulphur and calcium phosphate. The flasks were set up in triplicate and un-amended controls were also included (No element added to inoculate). Triplicates were used throughout and the flasks were incubated at 25˚C. At seven day intervals the presence of the various ions in the medium was checked using the relevant Quantofix dipstick.

. A B

Fig. 4:2.(A). Dipsticks container containing dipsticks used for ion determination showing concentration chart, 22.(B), Dipsticks used for ion determination showing concentrtation chart set used for determination of phosphate.

4:14 Results and Discussion

A note on the use of Dipsticks for ion analysis. Over the last forty years, the standard approach in this laboratory to the determination of ions (such as nitrate and sulphate) which are important in environmental geochemistry has been to use colorimetric methods of analysis. These approaches have served us well, but of late we have gone over to the use of Dipsticks (Fig.4.2). These cheaper, less dangerous (i.e., when replacing the use of chromotropic acid) and can be used to quickly test a large number of samples. They also tend to be less influenced by interference. For example, the use of chromotrophic acid to measure nitrate is often hindered by the presence of carbohydrates in the soil or medium sample; this is not the case with Dipsticks.

It could be argued that the use of this approach sacrifices accuracy, but in most case, as when determining if a bacterium or fungus participates in a particular biogeochemical cycle, the result needed is generally a plus or minus, and it is not relevant to measure the exact

concentration to one or two decimal places. Tests showed that results from Dipsticks were between 5 percent, plus or minus, of those obtained by the colorimetric methods previously employed. Of course it assumed here that the colorimetric methods provide the gold standard, and this may not be the case; in any event, the closeness of the results shows that, for the purpose of the experiments described here; dipstick analysis is both simple and appropriate.

Table 4:1 provides data showing that *Bacillus thuringiensis* (as Thuricicde): a) hydrolyze urea to ammonium, b) oxidize ammonium to nitrate, c) oxidize elemental sulphur to sulphate and d) solubilize insoluble phosphate. In all cases, the amount of ion released increased over the incubation period and these increase were significantly different from the control ($p=0.05$), where small amounts of the products were produced as the result of non-microbial processes. The data given in Table 4:2 shows that *Paenibacillus popiliae*, growing from the Milky spore formulation, was also able to a) hydrolyze urea to ammonium, b) oxidize ammonium to nitrate, c) oxidize elemental sulphur to sulphate and d) solubilize insoluble phosphate. In all cases, the amount of ion released increased over the incubation period and these increase were significantly different from the control ($p=0.05$), where small amounts of the products were produced as the result of non-microbial processes.

Table 4:1. Effect of *Bacillus thuringiensis* (Thuricide) on *in vitro* transformations relevant to the major nutrient cycles (all changes after day 0 are significantly different from control, p=0.05) µg ml^{-1}

Days	control	Hydrolysis of urea to ammonium	control	Oxidation of Ammonium to Nitrate (Nitrate N)	control	Oxidation of Elemental Sulphur to Sulphate	control	Solubilisation of Insoluble Phosphate to Soluble Phosphate
0	1	1	5	5	2	5	2	15
7	2	15	5	12	5	15	4	15
14	5	25	5	35	5	30	5	35
21	5	35	5	50	5	40	2	60
28	10	40	10	55	15	65	10	80

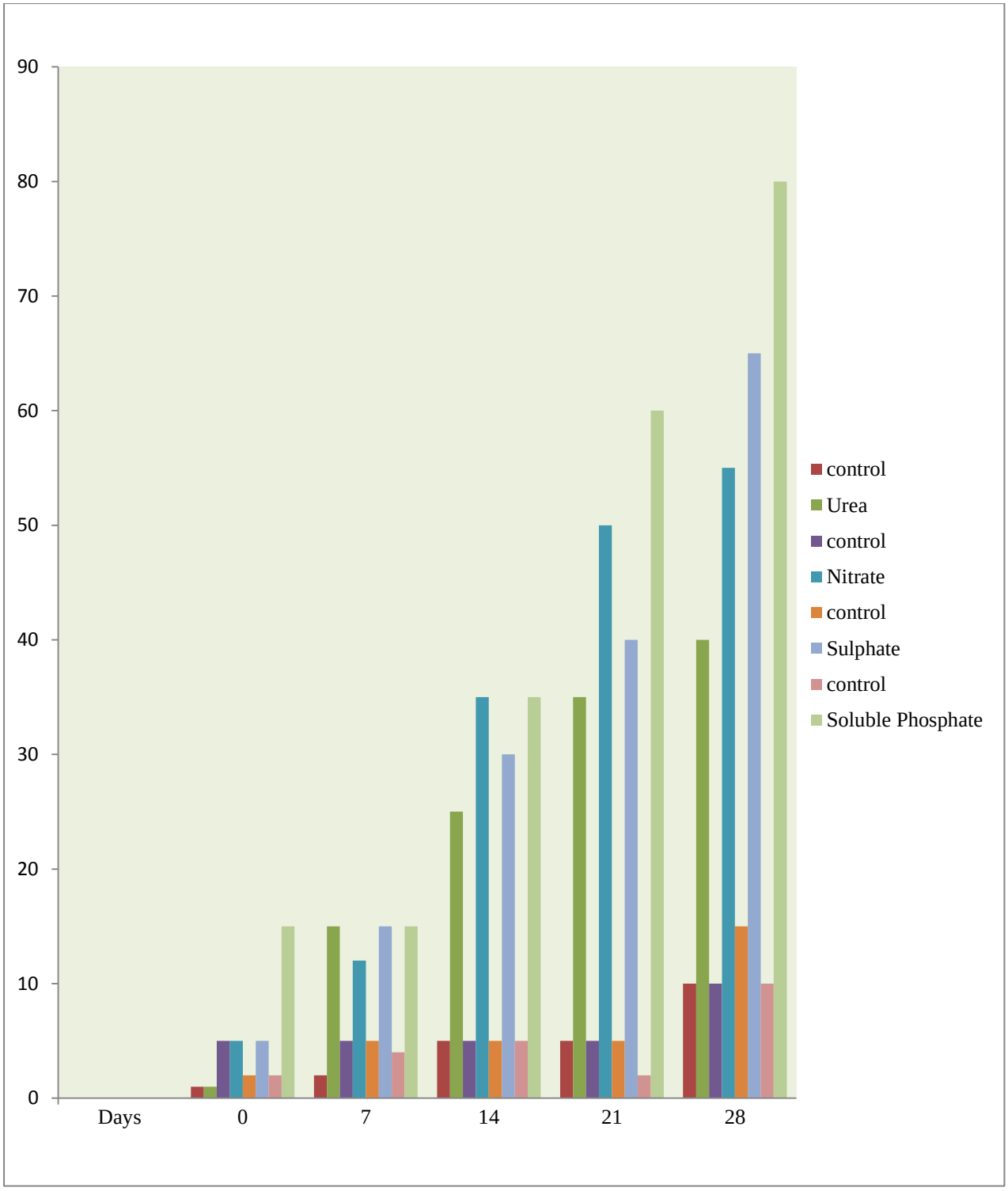

Fig. 4:3The Effect of *Bacillus thuringiensis* (Thuricide) on *in vitro* transformations relevant to the major nutrient cycles.

Table 4:2. Effect of *Paenibacillus popiliae* (Milky Spore) on *in vitro* transformations relevant to the major nutrient cycles (all changes after day 0 are significantly different from control, p=0.05). µg ml^{-1}

Days	control	Hydrolysis of urea to ammonium	control	Oxidation of Ammonium to Nitrate (Nitrate N)	control	Oxidation of Elemental Sulphur to Sulphate	control	Solubilisation of Insoluble Phosphate to Soluble Phosphate
0	5	1	5	15	2	5	2	5
7	5	30	2	20	2	35	2	35
14	5	35	5	35	5	60	5	65
21	8	40	10	70	5	80	2	80
28	10	60	10	85	10	120	5	110

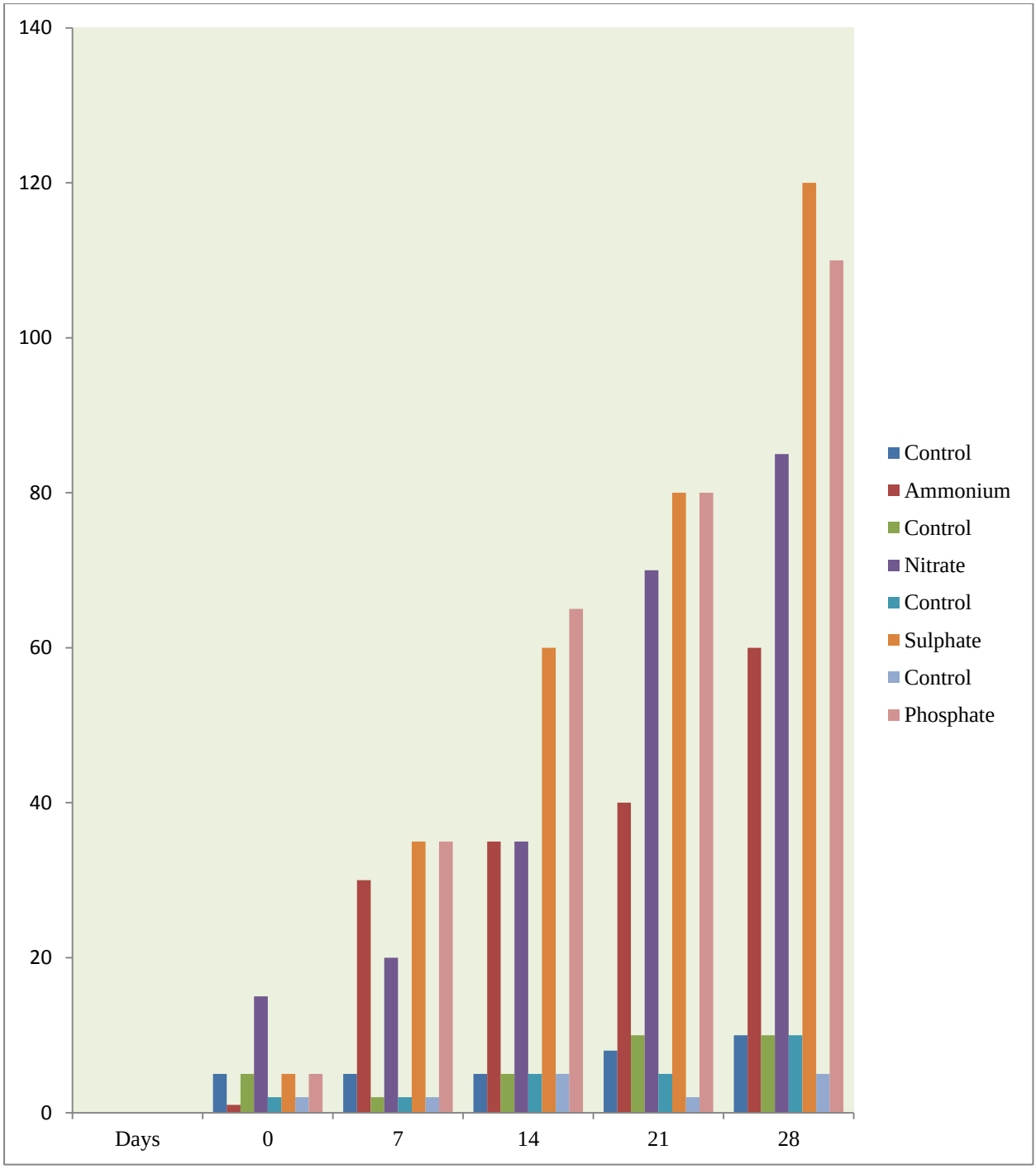

Fig.4:4 The Effect of *Paenibacillus popiliae* (Milky Spore) on *in vitro* transformations relevant to the major nutrient cycles.

The use of analytical dipsticks (Quantifix) as opposed to the more complicated colorimetric analysis proved highly successful in providing data on the ability of these two bacteria to participate (in vitro) in some important process involved in the major biogeochemical transformations which occur in the environment. While not as accurate as the colorimetric analysis techniques used previously in this laboratory, this approach, as was mentioned above, is less time consuming and does not involve the use of dangerous reagents (such as chromotropic acid), nor are the results readily subject to interference from media constituents. The dipsticks used have been checked for accuracy by previous workers in this laboratory and have been shown to give results which are broadly comparable to colorimetric analysis. As a result, for merely demonstrating the ability of microorganisms to participate in these important environmental reactions in vitro, this approach proved ideal.

The rationale behind these experiments is that, following spraying onto crops and other plants, some spores of *Bacillus thuringiensis* and *Paenibacillus popiliae* will reach soils (and other environments, including fresh waters) either directly in the sprays, by rain wash off, or in when the plant degrades. This means that potentially significant numbers of spores of these bacteria will reach the soil and be able to germinate, from where cells can potentially participate in various component parts of the major biogeochemical cycles, including carbon, nitrogen, sulphur and phosphorus.

While showing the potential to participate in reactions in the soil, in vitro studies do not provide confirmatory evidence that a bacterium, or other microorganism which can, for example oxidise ammonium in culture medium will do so in soil. A variety of factors will of course influence the ability of any microbe introduced into the soil, or environment in general (by accident or by purposeful inoculation) to grow and participate in biogeochemical transformations. Factors such a competition from indigenous organisms, environmental parameters such as a suitable ambient temperature and water regime, will markedly influence

microbial growth. However, the fact that the in vitro studies discussed above show that the bacteria under investigation can perform essential environmental reactions in vitro show that they have the potential to do so in the environment. Clearly if these organisms were shown to be incapable of mediating these transformations when growing in culture it would be highly unlikely that they would be able to do so in the environment where conditions are likely to be far more challenging. Two important features of in vitro work which are likely to be far more variable in most environments is the presence of large amounts of carbon and a constant temperature. Most natural environments are considered to contain only small amounts of available nutrients for which both indigenous and introduced bacteria will have to compete. In contrast, large, often "pathological" amounts of carbon substrates are generally provided in nutrient media and bacteria growing in the presence of such large amounts of carbon are unlikely to show the same physiological responses likely to be seen in the highly rigorous, low nutrient, conditions present in most environments.

Table 4:3. Effect of *Bacillus thuringiensis Paenibacillus popiliae* (Thuricide and Milky Spore respectively) on transformations relevant to the major nutrient cycles when added to an agricultural soil and incubated for 28 days at 25°C (all changes are significantly different from control, p=0.05) µg ml^{-1}

Process	**Hydrolysis of urea to ammonium**	**Oxidation of Ammonium to Nitrate (Nitrate N)**	**Oxidation of Elemental Sulphur to Sulphate**	**Solubilisation of Insoluble Phosphate to Soluble Phosphate**
Control	22	15	15	30
Bacillus thuringiensis	50	25	40	50
Paenibacillus popiliae	120	40	65	90

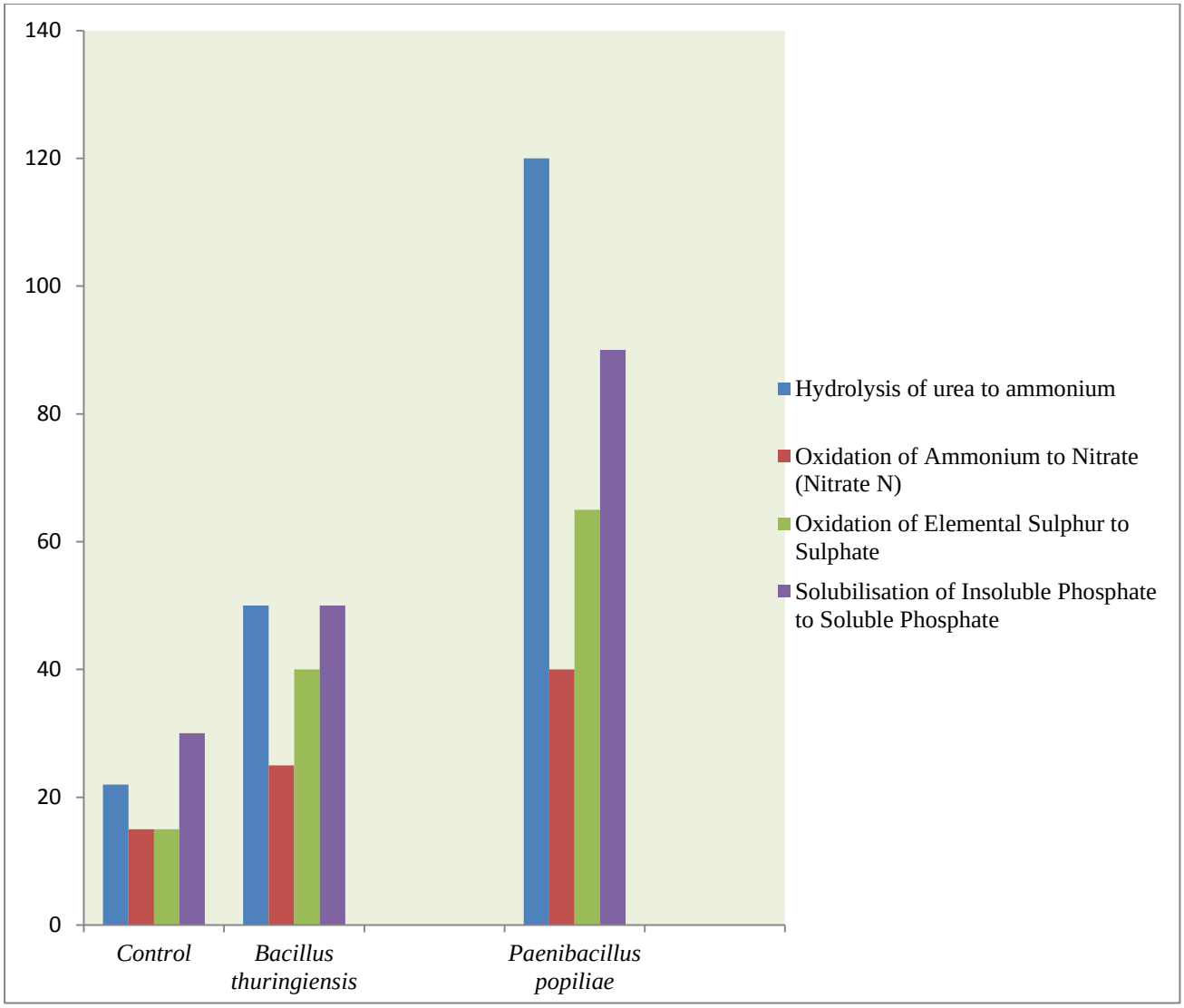

Fig.4:5 The Effect of *Bacillus thuringiensis Paenibacillus popiliae* (Thuricide and Milky Spore respectively) on Transformations Relevant to the major nutrient cycles when added to an agricultural soil and incubated for 28 days at 25°C

Table 4:3 shows that, when added to soil, the two bacteria used here were able to participate in the nutrient transformations. This suggest that when these bacterial formulations are applied to vegetation an additional benefit is potentially provided (when the spores reach the soil) in that the bacteria can contribute to mineral cycling and improve soil fertility following the spraying of these biological insecticides.

CHAPTER FIVE- ISOLATION OF BACTERIA AND FILAMENTOUS FUNGI FROM INSECTS SAMPLED DURING FLIGHT

5:1 Introduction

Up to this point, this Thesis has described studies of Lepidopteran larvae, sampled in the field, and imagoes sampled in the same way or else obtained from dealers. The following discussion is devoted to an evaluation of two novel techniques used for the isolation of insects in flight and a subsequent study of their bacterial and yeast outer body flora. The main rationale behind this work was to determine if these techniques can be usefully employed in studies such as these. The following techniques were used for in-flight sampling:

1) A drone elevated sampler.
2) An octanol-based midge/mosquito sampler.

These are novel approaches to sampling airborne insects. The rationale behind the studies is based on the fact that 1) insects are known to be transported over long distances in the upper atmosphere and can thereby transmit a wide variety of human, animal and plant pathogens over long distance, including across continents and (2) that vast amounts of midges and mosquitoes are hatched each year (in the UK, being mainly found in Western Wales and the Scottish Highlands) and act as carriers of microbes.

5:2 Aims- The aim of the work discussed here is to evaluate the use of the two sampling approaches mentioned above to isolate bacteria, filamentous fungi and yeasts from the surface of airborne insects. Using the drone sampler, insects were isolated at a height of 120 meters (the drone had remote-activated solenoid opener/closure devise), while the octanol-based sampler isolates a large number of female midges/mosquitoes from the air when placed at ground level.

Glick (1939) collected insects from high altitudes by means of special traps fitted to various types of airplanes over Southern USA during 1926 to 1931, Some 30,000 specimens of insects were sampled from altitudes ranging from 20 to 4500 metres. Eighteen orders of insects and the orders of spiders and mites were collected and represented 216 families, 824 genera, 4 new genera, 700 species, and 24 new species. The order Diptera was the most abundant order in the air, nearly three times as many specimens being taken than any other order. Coleoptera followed next after Diptera, Homoptera and Hymenoptera were sampled at 4,270 meters, the highest altitude at which insects were found, while the highest altitude at which any specimen was taken was 4,570 meters, at which a spider was caught. Not surprisingly, insect numbers decreased with sampling height. The size, weight, and buoyancy of an insect were shown to contribute directly to the height to which it is carried by air currents. Many species of the other orders represented at high altitudes were also small insects. Evidence showed that insects taken in the upper air were alive at the time of sampling. The relative distribution and abundance of insects in the upper altitudes depended on weather conditions with temperature being undoubtedly the most important The intensity of air currents is a great factor in the distribution and dispersal of insects. Most insects were taken at the lower altitudes when the surface wind velocity was from 5 to 6 miles per hour, and fewest when it was calm. The direction of the wind has influenced to a great extent the migrations of insects. In the airplane flights at Tallulah it was found that the greatest numbers of insects were taken when the surface wind direction was from the north-northeast, southeast, or southwest. Some insects were apparently moving with the wind during the spring and summer when the surface prevailing winds were from a southerly direction, and again with the wind from a northerly direction in the fall. Convection and turbulence was shown to play an important role in determining the insect population in the upper air.

As has already been noted, millions of metric tons of insects exist in the Earth's atmosphere at any given moment, most of which comprises insects involved in high-altitude, wind-borne migration, often at heights several hundred meters above ground level, where they take advantage of the strong winds found in this region to cover considerable distances, frequently tens or even hundreds of kilometres (Drake and Farrow, 1988, Holyhoak, 1997). This vast aerial "bioflow" has major implications for ecological, physiological, and genetic studies of insects, and added applications relevant to pest management, conservation, and environmental change programs (Drake and Gatehouse, 1995). In the past, the study of insect migration has relied primarily on data from long-distance flights, catches in light traps and other ground-based observations. Maintaining sampling platforms in the air) is however, expensive and impracticable over long periods. The insect fauna flying at high altitude can now be monitored continuously and for long time periods, using autonomous vertical looking radar systems (VLR systems) (Smith *et al*. 2000). Combined with aerial sampling technology and sources of bio-meteorological information, these systems have considerable potential for area-wide monitoring of economically important pests and could clearly be used for pest management and forecasting systems (Smith *et al*.2000). VLR is clearly a powerful new tool that will revolutionize the study of insect migration and provide us with significant new information on both pure and applied entomology.

5:3 Methods used to detect airborne microbes

Microbiological air quality is critical in the medical and pharmaceutical sectors, where maintaining sterility is the aim. Both passive and active methods exist for monitoring the microbial population of the air (extensive details are given in Gregory 1973 and Schulster and Chinn, (2003). Active sampling methods have become an essential environmental monitoring

tool in the pharmaceutical and medical device sectors, but much of the food industry still relies on passive monitoring.

Passive monitoring

Passive monitoring typically employs ‘settle plates’ – petri dishes containing culture media, which are opened and exposed for a given time and then incubated. This approach is obviously only capable of monitoring biological particles that sediment out of the air and settle over the exposure time period and, as a result, they do not detect smaller particles or droplets remaining suspended in the air. They are also unable to sample specific volumes of air, so the results, at best, can only be considered semi-quantitative. Settle plates are also vulnerable to interference and contamination and may become easily overgrown in heavily contaminated conditions. Settle plates are however, inexpensive and easy to use and require no specialised equipment. By employing a range of culture media, they can also estimate the numbers of specific groups of micro-organisms in the air. They are generally useful for the qualitative analysis of airborne microbes and they produce data which indicates underlying trends in airborne contamination and they can provide an early warning of problems.

5:4 Active monitoring

An Octanol-based midge sampler was used for the following experiments. However, as the following discussion shows, other airborne-insect sampling devises is available.

Active monitoring requires the use of a microbiological air sampler to physically draw a pre-determined volume of air over, or through, a particle collection device. Two main types are in general use:

Impingers

Impingers use a liquid medium for particle collection, sampled air being drawn by a suction pump through a narrow inlet tube into a small flask containing the collection medium; speeds

up the air towards the surface of the collection medium, the flow rate being determined by the diameter of the inlet tube. When the air hits the surface of the liquid, it changes direction abruptly and any suspended particles impinge into the collection liquid. Once the sampling is deemed finished, the collection liquid can be cultured to determine the number of viable micro-organisms in the sample. Since the sample volume can be calculated using the flow rate and sampling time, the result is quantitative.

Impactors

Impactor samplers use a solid or adhesive medium, such as agar gel, rather than a liquid for particle collection. Typically, air is drawn into a sampling head by a pump or fan and accelerated, usually through a perforated plate (sieve samplers), or through a narrow slit (slit samplers). This produces a laminar air flow onto the collection surface, generally a normal agar plate filled with a suitable medium. Air velocity is determined by the diameter of the holes in sieve samplers and the width of the slit in slit samplers. When the air hits the collection surface it changes its direction and any suspended particles are thrown out by inertia, impacting onto the collection surface. When the correct volume of air has been passed through the sampling head, the agar plate is removed and incubated directly without further treatment. Following incubation, the number of visible colonies gives a direct quantitative estimate of the number of colony forming units in the volume of air sampled. Impaction samplers are convenient and can handle the higher flow rates and large sample volumes necessary to monitor air quality in controlled environments where microbial numbers are likely to be low; microbial cells may however, be damaged by stress induced by the sampling process and become less viable.

One of the best known impact samplers is the Andersen sampler, a multi-stage ‘cascade’ sieve sampler that uses perforated plates with progressively smaller holes at each stage, allowing particles to be separated according to size. Another is the Casella slit sampler, in

which the slit is positioned above a turntable on which is placed an agar plate. Air is drawn through the slit and an e agar plate rotates, so that particles are deposited evenly over its surface. Automated air sampling

Semi-automated systems usually based on sieve type impaction samplers, for monitoring clean rooms and controlled production areas. Such devices typically use a number of sampler heads linked to a central control unit, which can be programmed to follow a pre-set sampling programme.

5:5 MATERIALS AND METHODS

Fungi media

Czapek Dox Agar media was prepared by suspend 50 g Czapek Dox agar then dissolved in 1000 ml Distilled water in a flask with magnetic spin-bar until the solution dissolved than transfer class flask with covered or lid into autoclave at 120°C for 30 min for sterilization. Then the solution was poured in Petri dishes carefully when solution temperature decline to 60-55 °C. Preliminary identification is based on the colour the isolated Fungi colony developed when samples are grown on Czapek Dox agar medium.

5:6 Drone samplers

The drone sampler consisted of a piece of muslin sleeve (length) (used for a pipe cover) sealed at one end and held open by a circular piece of wire (diam.). The sampling sleeve was attached to the drone which possessed a thin plastic circular cover which, on command from the ground, could open and close the circular end (i.e. aperture) of the sampling sleeve; small flying insects were caught inside the sleeve and sampled on its return to the surface. The drone was fitted with a camera, an altimeter and GPS (Fig.5:1). The done was launched as

single event in open field near Bakewell**,** Derbyshire to a high 120 meters and horizontal distance of 500 meters (to comply with current regulations) above the town for 3 minutes.

A  **B** 

Fig.5.1. (A).Drone used to capture airborne insects and (B). muslin sleeve (length).

5:7 Octanol-based midge samplers

A commercial midge sampler (Predator Dynamic) (Fig.5:2) was used to catch large quantities of airborne midges and mosquitoes. It contains a strong vacuum fan which sucks the insects into the trap and dehydrates them.

The Predator Dynamic midge collector mimics a perspiring person by producing the following 6 cues to attract the female:

Breath-carbon dioxide, moisture and heat

Heat-body skin temperature

Sweat –Rapid Action Attractant.

Movement-Blinking LED light

Moisture–wet tray in collector

Light UV-LED Lights

Fig.5:2. The Predator midge collector.

The manufacturers claim that it can collect vast numbers of females (i.e. the ones that bite) from a large area of up to 5000 square meters. It uses octanol, which is produced by heat evaporation, as a lure and to mimic human breath and sweat. The machine was located at Tan y Bedw, (OS 254, 437508), Caernarfon, Gwynedd, North

Wales and left running for 7 days, following which time the contents were transferred to polythene bags; 20 g. of biomass were isolated.

5:8 Bacterial isolation

The total bacterial load of the midges and high flying insects was obtained by macerating the whole body in a small amount of sterile ¼ strength Ringers' solution, plating onto Nutrient Agar and then incubating overnight at 25^0C. Identification was achieved by the use of 16SrRNA and classical methods following Bergey's Manual (aided by Professor Wainwright).

5:9 Fungi isolation

Using a cotton wool swab, moistened with sterile distilled water to isolates fungi from the surface of midges. The swab was then spread onto the surface of the isolation Czapek Dox Agar media.

5:10 Results and Discussion

Three types of insects were obtained at a height of 120 meters using the drone–towed sampler, namely a Hoverfly, a Vinegar Fly and an Aphid (Table 5:1). It is likely that these insects were carried to this height by a combination of flying and uplift on wind currents. Table 5:1 also shows the bacteria obtained from these sampled insects. The bacteria are commonly isolated environmental organisms, showing a preponderance of spore forming Bacilli, an expected finding considering the high level of resistance to adverse conditions shown by Bacilli-endospores.

Table 5:1. Insects and associated bacteria isolated using a drone from a height of 500 meters. Bacteria (identified using classical methods).

Insects	***Hoverfly (Eristalis intracarius)***	***Vinegar fly (Drosophila funebris)***	***Aphid (Pemphigus burarius)***
Bacteria	*Acetobacter aurantius*	*Azotobacter vinelandii*	*Bacillus licheniformis*
	Actinomyces israelii	*Bacillus licheniformis*	*Bacillus megaterium*
	Bacillus brevis	*Bacillus megaterium*	*Enterococcus faecium*
	Bacillus licheniformis	*Bacillus mycoides*	*Psedomona aeruginosa*
	Bacillus megateroium	*Enterococccus durans*	*Rothia dentocariosa*
	Bacillus mycoides		*Streptococcus sanguis*
	Wolbachia		*S. sobrinus*

In contrast, no Bacilli were isolated from the midge samples sampled at ground level (Table 5:2), the reasons for which are not immediately apparent.

Table 5:2. Bacteria isolated from midges-identified to species level using classical methods.

Insects	Midges
Bacteria	*Pseudomonas aeruginosa*
	Wolbachia sp.
	Moraxella catarrhalis
	Staphylococcus pasteuri
	Acinetobacter baumannii
	Stenotrophomonas maltophillia
	Comommonas terrigena
	Flavobacterium columnare
	Chryseobacterium indologenese
	Citrobacter brakkii
	Ehrlichia ewinii

Of interest is the isolation of *Stenotrophomonas maltophillia*, a bacterium which is increasingly being recognized as an important pathogen of immunocompromised patients. *Stenotrophomonas maltophillia. Stenotrophomonas* infections have been associated with high morbidity and mortality in severely immunocompromised and debilitated individuals (Denton and Kerr, 1998). Risk factors associated with this pathogen include: HIV infection, malignancy, cystic fibrosis, the use of catheters, recent surgery and trauma. It is also enhanced by the use of broad spectrum antibiotics

Table: 5:3. Fungi isolated from midges-identified using classical methods.

Insects	**Midges**
Fungi	*Aspergillus niger*
	Penicillium brevicopmatcum
	Penicllium citrinum
	Penicllium chrysogenum
	Aternaria tenuis
	Fusarium oxysporum
	Fusarium solanum

Fungi were also isolated from the midges. Table 5:3 shows that the isolates are common spore-forming Deuteromycetes, all of which are commonly found on most environmental samples.

5:11 Fertilizer Potential of collected midge biomass

The vast quantity of midges which can be collected by octanol-based midge collectors, like the one used here, opens up the potential of using midge biomass as a fertilizer. The aim of the following experiment was to test this possibility.

5:12Materials and Methods

A sieve less than (5 mm) was sued to screen an agricultural loam soil (previous crop potatoes, pH 6.4) which used in these studies. It was amended with fresh midge biomass (5g per 100 g soil) and moistened (circa 10% water content) and incubated at 25°C in polythene bags with a small hole to allow for gas exchange. Controls without biomass were included and all treatments were set up in triplicate. At weekly intervals, 5g of soil was transferred to 1M KCl (100ml) to extract N-ions; the container was then vigorously shaken by hand. On settling, the concentration of ammonium and nitrate in the extract was determined using dipsticks as described above.

5:13 Results and Discussion

Octanol- based midge collectors are used in areas of the world which have very large midge and mosquito populations, i.e. wet, relatively warm mountainous areas, such as N. Western Scotland, North Wales and British Columbia and Nova Scotia, Canada. Most of the large amounts of biomass caught by individual traps are likely to be casually dumped, sent to landfill or incinerated.

Days	Amended soil Ammonium	Amended soil Nitrate	Control (un-amended soil)Ammonium	Control (un-amended soil)Nitrate
0	25	10	20	10
7	60	25	25	15
14	105	30	30	10
21	110	45	25	12
28	125	60	30	20

Table 5:4. Concentration of ammonium and nitrate (µg ml^{-1} dry weight) extracted from soil amended with midge-biomass (Means of triplicates, all treatment values significantly different from control, p=0.05).

The results of this short study suggest however, that such biomass could have fertilizer potential. Table 5:4 shows that the addition of fresh midge biomass to an agricultural loam

led to a substantial increase in the concentration of the two main fertilizer sources of nitrogen, i.e. ammonium and nitrate, with ammonium predominating. It is likely that dried biomass would provide even larger amounts of fertiliser-nitrogen. This practice would have the advantage of reducing transport costs, but of course the amount of such dried material would be limited by the high costs of heat-drying. For this reason, only wet midge biomass was evaluated here for its fertilizer potential. Such biomass could be applied to soils directly or after a period of composting and could be used alone or together with waste plant materials. One could envisage large amounts of such biomass being produced by individuals or perhaps council-run midge collectors (and co-operatives) and, as a result, relatively large amounts of material could be made locally available to farmers and the public. Transport costs might however, limit the wide-spread collection and use of midge biomass on an industrial scale. Certainly however, an individual octanol-based collector, when located in a high midge area, could supply useable nitrogen fertiliser to homes, allotments, and even small to medium sized fruit and commercial fruit and vegetable growers. The production costs of midge biomass could be offset by local authorities, hotels or other tourist locations, where the waste is produced when attempts are being made to reduce the tourist-nuisance potential of vast numbers of midges or mosquitoes. The fact that this study shows that the midge of biomass collected here does not contains major pathogenic bacteria means that its use need not be limited by safety reasons and, as a result, there is no obvious need for it to undergo expensive sterilization; heat-based sterilization could however, be advantageous in producing a concentrated product, capable of being economically transported, which could be bagged and sold by garden–supply shops.

5:14 DISCUSSION

The UK supports some 40 species of biting midge, but only five are thought to regularly attack people, with the Highland midge, *Culicoides impunctatus,* being the most troublesome, and only the bloodthirsty female causing problems (Hendry, 2011). This midge is particularly common around dawn and dusk and in the Highlands and north-west Wales, where damp conditions provide it with perfect breeding grounds. Individual midges are almost invisible to the human eye, at about a millimetre long. The male feeds on plants and nectar, while his mate requires blood in order to form her eggs. Midges become aware of humans when they detect carbon dioxide on the breath and a swarm can inflict about 3000 bites each hour using a distinctive feeding technique. While mosquitos pierce the skin and suck up blood through a syringe-like mouthpiece, midges cut the skin, and then lick up the resultant pool of blood. A midge's saliva stops the blood in the wound from clotting allowing it keep on drinking indefinitely. It is the saliva which irritates the human body and leads to skin reactions and swelling at the site of a bite. Some people appear immune to midge bites; women tend to react more badly than men to the bites and the tendency to be targeted is hereditary. Midges also attack cattle, deer, sheep, cats, dogs, rabbits and mice, and spread bluetongue, a debilitating disease affecting sheep and cattle caused by a virus belonging to the family Reoviridae. Midges prefer damp, sheltered conditions, woodland and forest areas, avoid breeze, and unlike most other insects, prefer dark-coloured clothing to light (Hendry, 2011).

Chemical solutions are available to deter midges, including insect repellents containing DEET, or the natural alternative, citronella, a product of lemongrass extract which can be bought as a spray or infused into candles. These work by blocking the insect’s odour receptors on the antennae and mouthparts, thereby confusing the midge so that it avoids the person. Homemade repellents include bog myrtle which grows wild in the Highlands, and thyme. There are also several traps on the market, including the Predator (i.e. the trap

employed here), which it is claimed, simulates a large smelly cow and attracts midges by replicating breath, heat, body odour and movement - then catching the creatures on sticky paper. In trials during 2010, a single Predator trap collected 800,000 midges over a five-day period. Midges cost Scotland's tourist industry an estimated £286m per year and midges are threatening the economy of the Lake District and North Wales and as far south as Cornwall. However, midges play a crucial role in the Scottish ecosystem, providing food for bats, birds and even carnivorous plants like sundews and butterworts, and they may have been partially responsible for restricting the development of the Highlands, and thereby maintaining this area as a remarkable wilderness (Hendry, 2011).

Of particular interest was the isolation of *Wolbachia* from the trapped midges. *Wolbachia* is a bacterial genus which infects arthropods, including insects and nematodes and is therefore one of the most common parasites in the biosphere (Werren *et al*., 2008). It sets up a mutualist, rather than parasitic relationship with its host, some of which cannot survive and reproduce in its absence. It is estimated that some 25 to 70 percent of all insects are potential hosts. The genus was first identified in 1924 by Hertig and Wolbach in the common house mosquito and is now of considerable interest, not least as a potential biocontrol agent. *Wolbachia*. Bacteria can infect many different organs, but most notably the testes and ovaries. They are ubiquitous in mature eggs, but not mature sperm and as a result, only infected females pass the infection on to their offspring (Werren *et al*., 2008).

Wolbachia has been linked to viral resistance in *Drosophila* and mosquito species, flies infected with the bacteria being more resistant to RNA viruses, including the West Nile virus and can also confer insecticide resistance (Li et al., 2014). In species of *Phyllonorycter blancardella* (leaf miners), *Wolbachia* bacteria help produce green islands on yellowing tree leaves, allowing the hosts to continue feeding while developing into their adult forms and larvae treated with an antibiotic which kills *Wolbachia*, lose this ability and as a result only

13% emerge as adult moths. In parasitic filarial nematodes which cause elephantitis, *Wolbachia* has become an obligate endosymbiont and supplies the host with the chemicals required for its reproduction and survival; elimination of the *Wolbachia* symbionts by antibiotics therefore prevents nematode reproduction, and eventually results in death. Some *Wolbachia* that are infect arthropods and mediate iron metabolism under nutritional stress, and can also help the host to synthesize vitamin B.

Wolbachia species infect a variety of isopods, including spiders, mites and filarial nematodes including those causing River Blindness and elephantitis in humans and heat worms in dogs. The elimination of *Wolbachia* from filarial nematodes generally results in either death or sterility of the nematode; as a result, these diseases can be controlled using the antibiotic doxycycline to kill the bacterium (Li *et al*., 2014). *Wolbachia* can also be used to control dengue and malaria and a recent study has shown that *Wolbachia* can prevent the spread of Zika virus in mosquitos in Brazil.

CHAPTER SIX-FUNGI ASSOCIATED WITH THE SURFACE OF LARGER MOTHS CAUGHT USING A MERCURY VAPOUR LAMP TRAP

6:1 Introduction

The aim of the work was to isolate fungi from the body surfaces of some larger moths isolated using a Robinson mercury vapour light trap. This type of moth trap is very successful at trapping some of our most attractive, large moths. It relies on the use of a mercury vapour lamp. The lamp is housed in a large, clear plastic funnel which is itself contained in a plastic bowl, inside which is placed foam pieces or broken cardboard egg boxes which provide cover for the trapped moths (Fig.6:1). The lamp is covered by a circular piece of perspex which protects it from rain.

6:2 Materials and Methods

The moth trap was set up during fine weather during June, 2016 at Tann y Bedw, Caernarfon in North Wales and left running overnight. The trapped moths were removed using sterile forceps and their bodies were gently rubbed onto the surface of either Potato Dextrose or Czapek Dox agar in petri dishes, which were incubated at 25^0C for 7 days. Any isolated fungi were then identified using traditional morphological characteristics (Aided by Professor Wainwright).

Fig.6:1 Mercury Vapour (Robinson) Trap used to catch large night flying moths.

Fig. 6:2. Lesser Yellow Underwing Moth (*Noctua comes*).

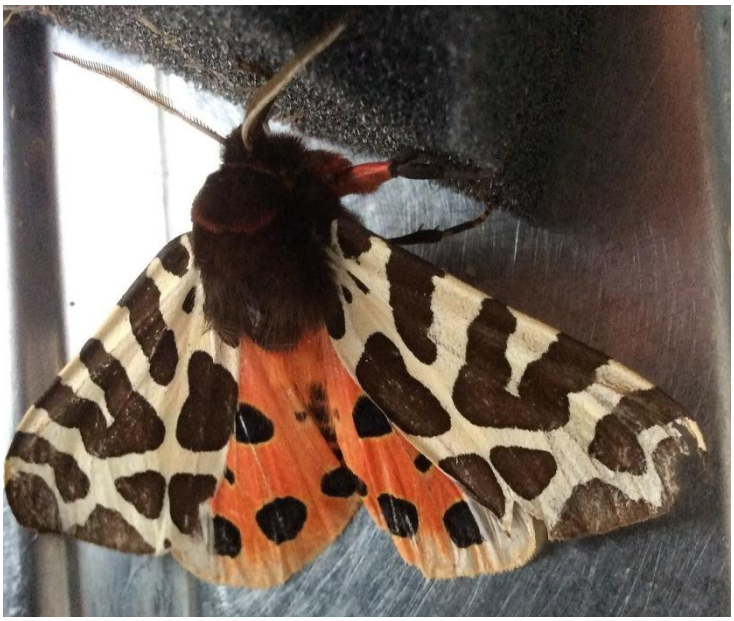

Fig. 6.3. Garden Tiger Moth (*Artia caja*).

Fig.6.4. Small Elephant Hawk Moth (*Deilephilia porcellus*).

RESULTS AND DISCUSSION

Fungal species isolated from the body surfaces of three larger moths, caught during June 21016 (Figs.6.2-4), are shown in Table 6:1. Fungi were found to be associated (presumably mainly as spores) on all of the moths examined. Of particular interest are the human pathogen *Aspergillus flavus* (found on the Lesser Underwing and the Garden Tiger) and the plant pathogens *Botrytis cinerea* (Garden Tiger), *Erysiphe alphitoides, Marssonina betulae* and *Ceratocystis fimbriata,* The spores of *Aspergillus flavus* cause farmer's lung, but only when inhaled in large quantities over extended periods, so its presence on individual moths is unlikely to be of any pathogenic significance to humans (Reboux *et al*.,2001) The plant pathogen *Botrytis cinerea* is of economic importance as a nectrotroph on soft fruits such as strawberries, as well as tomatoes; it could therefore be spread by the moths examined. Interestingly, *Botrytis cinerea* on grapes can also cause "winegrower's lung", a rare form of allergic reaction in predisposed individuals (Williamson *et al*. 2007). The moth-associated fungi which are likely to be of most importance are the three tree pathogens, *Erysiphe alphitoides*, *Marssonina betulae* and *Ceratocystis fimbriata*:

Table 6:1. Filamentous fungi isolated from the body surface of larger moths.

Insects	***Lesser Yellow Underwing Moth (Noctua comes)***	***Garden Tiger Moth (Artia caja)***	***Small Elephant Hawk Moth (Deilephilia porcellus)***
Fungi isolated	*Aspergillus niger*	*Botrytis cinerea*	*Aspergillus flavus*
	Aspergillus flavus	*Erysiphe alphitoides*	*Erysiphe alphitoides*
	Ceratocystis fimbriata	*Verticillium albo-atrum*	*Marssonina betulae*
	Erysiphe alphitoides		*Penicillium brevicompactum*
	Fusarium oxysporum		*Penicillium italicum*
	Penicillium citrinum		

Erysiphe alphitoides

This is a species of fungus which causes powdery mildew on oak trees. Oak powdery mildew is one of the most common fungal diseases in the forests of Europe (Mougou *et al*., 2008). Only young developing leaves are susceptible to colonization by *E. alphitoides* and it only causes necrosis when infection occurs during the very earliest periods of leaf development. It also tends to be more common on the second and third flushes of leaves which appear in July and August, a feature which reduces the severity of the disease on mature trees. The disease can be very severe on *Quercus robur* and *Quercus petraea*, notably on young trees, while in

mature trees the disease is usually less severe. A study of the effects of *E. alphitoides* on *Quercus robur* found that it led to impairment of stomatal conductance by some 15–30%, decreased leaf N-content and stimulated dark respiration. Carbon fixation was also impaired by about 40–50% in fully infected leaves and these tended to be shed earlier than uninfected ones. Generally speaking, the disease has only moderate consequences on tree health despite the general appearance of heavy infections (Mougou *et al*., 2008)

Marssonina betulae

Birch Leaf Spot is a leaf disease affecting Birch, Aspen and Cottonwood trees and other members of the *Betulaceae* family. Dissemination is usually considered by wind-borne spores which overwinter in dormant buds and twigs, to become active during the following spring. Early symptoms include the presence of small black spots that grow bigger and join to form a mass of necrotic tissue which can lead to complete defoliation during summer in affected trees. Untreated trees tend to weaken over consecutive years of defoliation allowing them to become open to secondary insect pests and diseases.

Ceratocystis fimbriata

Ceratocystis fimbriata causes oak wilt a major disease of forest trees.

The fungus achieves entry into the xylem vessels of trees through newly formed wounds to which it is transferred by air or insects as well as via natural root grafts. Tree parts beyond then point of infection begin to wilt, become brown, wilt and die, while dark streaks appear in newly infected wood. The fungus then spreads to uninfected trees by nitidulid beetles such as *Carpophilus lugubris*, *Colopterus niger* and *Cryptarcha ample*, and several species of *Glischrochilus.*

The fact that moths carry these tree pathogens on their bodies obviously means that they could act as vectors of these diseases. This is of particular interest in the case of oak wilt,

since this fungus causes obvious signs of this disease on some small oak trees at Tan y Bedw. Although insects (notably wood boring beetles) can transfer pathogenic fungi to trees, there appears to have been no published interest in the potential role of moths in transmitting such infections.

CHAPTER SEVEN-GENERAL DISCUSSION

While the microbiology of insects, such as mosquitoes and house flies, which carry important human pathogens, has been widely studied, the distribution of saprophytic microbes on non-disease carrying insects has been largely ignored. This is of course not surprising, since the study of the epidemiology of major disease is a fundamental subject for study and non-pathogenic microbes, unless they are of biotechnological importance are of lesser interest. The insects studied in the first Chapter of this thesis are not generally regarded as disease carriers and no evidence was found here to show that that the species of Lepidoptera studies, for example, carry major disease-causing bacteria on their surface or within their body fluids. They do however carry organisms which could cause problems in immune-compromised patients. The question then becomes; since Lepidoptera do not regularly interact with humans in the same way as mosquitoes, midges or house flies is the fact that thy carry bacteria of any significance to human health. House flies in contrast are commonly found in the home and also, if not controlled, in hospital settings, where they can contaminate surfaces and food with bacteria which cause food poisoning. Butterflies and moths, while entering these indoor environments do not usually settle on foodstuffs and are therefore not important vectors of intestinal disease. In the same way, Lepidoptera species are not biting insects and therefore do not directly transmit disease. So while the results of this study show that insects carry bacteria they are not transmitters of major human disease and their ability to carry organism causing disease in immune-compromised patients is likely to be of limited importance. Nevertheless, it remains of academic interest to study the relationship between insects and microorganisms, not least because of the possibility of finding new bacteria which have the potential for controlling insect pests. This study confirmed the fact that *Bacillus thuringiensis* is a commonly carried by insects and the fact they are not killed or impaired by such

contamination presumably suggest that they are either immune to such pathogens, or that the bacteria are not present in sufficiently high numbers to cause pathogenicity.

Larvae of the Peacock butterfly were fed nettle leaves which were deliberately covered with a range of bacteria. Not surprisingly, since it is toxic to many insect larvae, feeding with *B. thuringiensis* lead to the death of all of the larvae after 4 hours. The results how that feeding with *B.subtilis* and *E.coli* can lead to larval death, while MRSA was shown to be less toxic. Feeding the larvae with the other bacteria killed some larvae, with the death rate after feeding *B.subtilis* and *E.coli* being identical. *Bacillus cereus* was isolated from the larvae fed *B.thuringiensis* and *B.subtilis*.

As had already been discussed, insects have considerable potential position in forensic science for use in apprehending criminals. The dominant species of bacteria was *Enterococcus faecalis* which was isolated from inside the adult blow fly and from inside larvae extracted from the human corpse. Two species of *Clostridium* were also isolated, *Clostridium cochlearium* was isolated from outside larvae blow fly (*Calliphora*) removed from the human cadaver; the other, *Clostridium paraputrificum* was isolated from inside the blow fly (Calliphora) larvae obtained from the cadaver. *Brevibacterium ravenspurgense, Staphylococcus hominis, Lishizhenia tianjinensis* and *Bacillus safensis,* were also isolated from outside and inside larvae, extracted from human body.

The biocontrol agents *Bacillus thuringiensis* and were shown to be able to mediate, in vitro, transformations which are important in the major environmental mineral cycles The background to these experiments is that, following spraying onto crops and other plants, spores of *Bacillus thuringiensis* and *Paenibacillus popiliae* will reach soils (and other environments, including fresh waters) either directly in the sprays, by rain wash off, or in when the plant degrades. As a result, potentially significant numbers of spores of these bacteria will reach the soil and be able to germinate, from where bacterial cells can

potentially participate in the various reactions which make up the major biogeochemical cycles, including carbon, nitrogen, sulphur and phosphorus. As was discussed above while the potential to participate in reactions in the soil was demonstrated, in vitro studies do not provide direct confirmatory evidence that a bacterium, or other microorganism which can, for example oxidise ammonium in culture medium, will do so in soil. Numerous factors will of course influence the ability of any microbe introduced into the soil, or environment in general (by accident or by inoculation) to grow and participate in biogeochemical transformations. These factors include competition from indigenous organisms; environmental parameters such as a suitable ambient temperature and water regime will markedly influence microbial growth. However, the fact that the in vitro studies discussed above show that the bacteria a under investigation can perform essential environmental reactions in vitro show that they have the potential to do so in the environment. Clearly if these organisms were shown to be incapable of mediating these transformations when growing in culture it would be impossible for them to do so in the environment where conditions are likely to be far more challenging. Two important features of in vitro work which are likely to be far more variable in most environments is the presence of large amounts of carbon and a constant temperature. Natural environments are generally considered to contain only small amounts of available nutrients for which both indigenous and introduced bacteria will have to compete. In contrast, large, often "pathological" amounts of carbon substrates are generally provided in nutrient media and bacteria growing in the presence of such large amounts of carbon are unlikely to show the same physiological responses likely to be seen in the highly rigorous, low nutrient, conditions present in most environments.

The latter part of this Thesis was devoted to a study of microorganisms associated with insects sampled from a height of 120 meters. The main point of interest behind this work is the use of a drone-towed sleeve to sample the insects. As far as can be determined, this is the

first reported use of this approach to sample high flying insects in relation to a study of their microbiology. A discussion is given above of the standard approaches to sampling flying insects and while these approaches have been widely and successfully used over a long period there is always utility in the use of different sampling approaches. The use of a drone was shown to be ideal for the high altitude sampling of insects since it proved to be both powerful and highly manoeuvrable. There is no doubt that the drone used could have been used to sample at greater heights than the 120 meters used here. The results relating to the microbiology of the insects sampled using the drone are not surprisingly similar to those obtained using other sampling methods, since the drone, of course, does not necessarily sample insects which differ from those obtained using more traditional approaches. The use of drones for high-altitude sampling of microorganisms, insects and other organisms (such as pollen) is of considerable future potential. The use of an octanol-based midge sampler (Predator) to obtain large numbers of midges from the air, in relation to studying their microbiology, also appears to be novel. This approach worked extremely well and although the biomass of midge's samples did not approach that reported by the makers of the Predator machine it was sufficient to study the microbiology of these insects and also to suggest a possible agriculture use for such waste biomass. As was mentioned in the Introduction of Chapter five, the presence of vast numbers of biting midges in the Highlands of Scotland and other damp mountainous regions of the world which support an active tourist industry acts as an important economic loss that runs into millions. While Predator-like machines are used locally by homes and hotels to successfully reduce midge populations locally, it would appear uneconomic to use them on an industrial scale in order to completely remove the midge population of a large area, mainly because of the cost of octanol and the butane (propane) fuel which is required. One could imagine however that large midge collector could be powered by their own wind turbine or river-based electric generators. Midge biomass could

also be fermented to produce methane which could then be burned directly or used to generate electricity to power the midge collectors, so that large–scale collection systems could be self-reliant in energy. While the reduction, or better still, elimination of midges from tourist regions would be of enormous benefit to the economy it might have a negative environmental impact because of the use of midges as a food source for wildlife. There might therefore be considerable opposition to the use of large-scale midge collectors from environmentalists or planners. As was pointed out above, biomass collected could be put to good use as a fertiliser either directly or following composting. While this approach could be used on an agricultural scale it is more likely to be used locally on a small scale for gardens and allotments. The fact that midge biomass can be used as a fertilizer and or soil conditioner in this way has an obvious positive environmental impact in that it reduces the amount sent to landfill.

Finally, larger moths were trapped using a Robinson UV light trap. The moths were found to carry filamentous fungi on their bodies, some of which are plant pathogens, notably of trees.

In conclusion, this Thesis contains results relating to the isolation of bacteria and fungi from insects. The work described has implications for the transfer of potential human pathogenic bacteria, notably to immunocompromised patients and also plant pathogenic fungi, in this case notably of trees. The use of, what appear to be, novel insect collectors, i.e. a drone-carried sampler and the Predator midge collector has been described; further studies will determine if these methods can be added to, and improved upon, the large number of insect collectors already in use.

Suggestion for Future Work

As was mentioned in the Introduction to this Thesis, relatively little is known about the interplay between bacteria and non-disease carrying insects (e.g. mosquitos); As a result this Thesis has taken a broad brush approach where various aspects of this topic have been studied. As a result, nearly every Chapter could be revisited and the work detailed within could be studied in greater detail and could even form the basis of an individual Thesis. Of particular potential interest are:

1) The possibility that bacteria, and other microbes, could be used in forensic studies to determine the time of death of a cadaver. Because of the ubiquitous distribution of bacteria, it seems unlikely however, that microbes could be used to determine the previous location of a body.
2) It would be of particular interest to study the ways in which moths and larvae can transmit tree diseases. While then major insect-related diseases are, in the main, caused by tree boring beetles and their larvae it is likely, as has been shown here, that moths transmit plant diseases on their outer surfaces.
3) It would also be interesting to study the transmission of bacteria through the life cycle using mutants or bacterial cell which have in some way been labelled, so as to properly determine if they can be carried from the egg through the larval and pupal stages into the imago. Similarly, it would be interesting to determine how bacteria evade any immune protection afforded at each stage of the life cycle.

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APPENDIX

Table 1. Bacteria species brief demonstrate in relation to pathogenicity isolate from Invertebrate surfaces and internal body fluids

No	Bacteria sp.	Repetition	Activity	Note
1	*Bacillus thuringiensis*	7	Gram positive	The active ingredient in some insecticides.
2	*Bacillus cereus*	7	Gram positive	Diarrheal and emetic
3	*Stenotrophomona*s	2	Gram negative	Present in the hospital environment and may cause infections including those that affect the bloodstream, respiratory tract, urinary tract and surgical-sites(Cunha.2011)
4	*Microbacterium* sp	2	Gram positive	TB TB bacteria can attack any part of the body such as the kidney, spine, and brain. If not treated properly, TB disease can be fatal.
5	*Bacillus weihenstephanensis*	1	Gram positive	Diarrhea(Lechner.1998)
6	*Bacillus* sp	1	Gram positive	Some species are pathogens
7	*Enterococcus* sp	1	Gram positive	Important clinical infections caused by Enterococcus include urinary tract infections, bacteremia, bacterial endocarditis, diverticulitis, and meningitis.
8	*Bacillus licheniformis*	1	Gram positive	*B. licheniformis* is not a human pathogen nor is it toxigenic cultured in order to obtain protease for use in biological laundry detergent. well adapted to grow in alkaline conditions

9	*Bacillus safensis*	1	Gram positive	Highly resistant to salt.not a pathogen in humans
10	*Bacillus pumilus*	1	Gram positive	Salt tolerance and inhibits the growth of marine pathogens.Not pathogenic to human
11	*Exiguobacterium sibiricum*	1	Gram positive	Not a recored pathogen
12	*Staphylococcus succinus*	1	Gram negative	Not a pathogen
13	*Vagococcus* sp	1	Gram positive	Not a pathogen
14	*Bacillus mycoides*	1	Gram positive	Found in common pesticides
15	*Clostridium litorale*	1	Gram positive	Generates ethanol. It is able to utilize amino acids such as glycine, sarcosine, proline, and betaine as sole carbon and energy sources via Stickland reactions (Poehlein A,2014)
16	*Enterococcus mundtii*	1	Gram positive	Endophthalmitis caused by Enterococcus mundtii (Tomomi,2005) and non-pathogen (Esteban,2012)

No	Bacteria sp.	Repetition	Activity	Note	Isolate
1	*Bacillus subtilis* strain 168	3	Gram positive	Endospore-forming, not a recorded pathogen	Pupa(chrysalis) European Swallowtail Butterfly (*Papilio machaon*)
2	*Granulicatella elegans* strain B1333	1	Gram positive	This bacterium was first described as a member of a family of nutritionally variant streptococci. Part of the normal flora of the oral cavity, the genitourinary tract, and the intestinal tract (Luca, 2013).	Body fluid, European Swallowtail Butterfly (*Papilio machaon*)
3	*Enterococcus mundtii* QU 25	1	Gram positive	Endophthalmitis caused by *Enterococcus mundtii* (Tomomi,2005) and non-pathogen (Esteban,2012)	Body fluid, European Swallowtail Butterfly (*Papilio machaon*)
4	*Staphylococcus saprophyticus* strain ATCC 15305	5	Gram positive	urinary tract infections	Body fluid, European Swallowtail Butterfly

					(*Papilio machaon*)
5	*Staphylococcus saprophyticus* subsp	5	Gram positive	urinary tract infections	Body fluid, European Swallowtail Butterfly, yellow spot
6	*Staphylococcus capitis* strain ATCC 27840	2	Gram positive	Coagulase negative staphylococci are the principal cause of prosthetic valve endocarditis but are a rare cause of native valve infections	Body fluid long tail Zebra Swallowtail Butterfly .
7	*Staphylococcus capitis* strain JCM 2420	2	Gram positive	Coagulase negative staphylococci are the principal cause of prosthetic valve endocarditis but are a rare cause of native valve infections	Body fluid European swallowtail butterfly
8	*Staphylococcus aureus* subsp. aureus N315 strain N315	3	Gram positive	Methicillin-resistant *Staphylococcus aureus* (MRSA) responsible for many infections such as skin and heart valve	Body fluid European swallowtail butterfly yellow and black
9	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial	Body fluid European swallowtail butterfly

				infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al*. (2009).	yellow and black
10	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	Present in the hospital environment and may cause infections including those that affect the bloodstream, respiratory tract, urinary tract and surgical-sites(Cunha.2011)	Body fluid European swallowtail butterfly, yellow and bright green
11	*Brevibacterium frigoritolerans* strain DSM 8801	1	Gram positive	Catalase positive .non spore and non-motile and aerobic .Present on the human skin which is causes foot odour also, report infections in immunocompromised patients.(Bal,et	Body fluid from Eyed Hawk-Moth (*Smerinthus ocellatus*).

				al,2015)	
12	*Staphylococcus aureus* subsp. aureus N315 strain N315	3	Gram positive	Methicillin-resistant Staphylococcus aureus (MRSA) responsible for many infections such as skin and heart valve.	Pupa chrysalis Eyed Hawk Moth *Smerinthus ocellatus*
13	*Bacillus subtilis strain 168*	3	Gram positive	endospore-forming, , Not a recorded pathogen	Pupa chrysalis Eyed Hawk Moth (*Smerinthus ocellatus*).
14	*Staphylococcus sciuri strain* DSM 20345	3	Gram positive	Important human pathogens responsible for endocarditis, peritonitis, septic shock, urinary tract infection.(Chen,et,al. 2007)	Body fluid Elephant Hawk Moth (*Deilephila elpenor*).
15	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, et al. (2009).	Body fluid, Silk Moth *Bombyx mori*

16	*Solibacillus silvestris* strain HR3-23	1	Gram positive	Not pathogen undetermined, rod-shaped, yellow, non-motile, non-spore-forming (Shivaji, et al. 2014)	Body fluid from Atlas Moth (*Attacus atlas*)
17	*Staphylococcus saprophyticus* strain ATCC 15305	5	Gram positive	Urinary tract infections	Body fluid, Elephant Hawk Moth (*Deilephila elpeno*r).
18	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al.* *(*2009).	Body fluid Elephant Hawk Moth (*Deilephila elpeno*r).
19	*Stenotrophomonas pavanii* strain LMG 25348	1	Gram negative	Non-motile and do not form spores. Catalase-positive and oxidase-negative. Growth is observed at 20–37 °C (Ramos, *et al.*, 2011).	Body fluid, Elephant Hawk Moth (*Deilephila elpenor*).

20	*Staphylococcus succinus* strain AMG-D1	1	Gram positive	Not a pathogen	Body fluid, Elephant Hawk Moth (*Deilephila elpenor*).
21	*Staphylococcus sciuri* subsp. carnaticus strain GTC 1227	3	Gram positive	Important human pathogens responsible for endocarditis, peritonitis, septic shock, urinary tract infection. (Chen,*et,al*.2007)	Body fluid, Elephant Hawk Moth (*Deilephila elpenor*).
22	*Staphylococcus saprophyticus* strain ATCC 15305	5	Gram positive	Urinary tract infections	Body fluid Eyed Silk moth
23	*Staphylococcus saprophyticus* strain ATCC 15305	5	Gram positive	Urinary tract infections	Body fluid Small White moth
24	*Pantoea agglomerans* strain ATCC 27155	1	Gram negative	Causing wound, blood, and urinary-tract infections. It is commonly isolated from plant surfaces Associated with penetrating trauma by vegetative material and catheter-related bacteraemia. (Cruz,*et al.*,2007)	Body fluid, Atlas Moth (*Attacus atlas*).

25	*Bacillus subtilis* strain 168	3	Gram positive	endospore-forming, , Not a recorded pathogen	Body fluid Silk moth
26	*Micrococcus yunnanensis* strain YIM 65004	1	Gram positive	Found in human skin, animal and dairy products catalase positive. non-spore-forming spheres (Bergan, and Kocur, (1982) Cause infection and hosts with compromised immune systems Public Health Agency of Canada www.publichealth.gc.ca.	Body fluid Silk moth
27	*Bacillus licheniformis* strain DSM 13	1	Gram positive	*B. licheniformis* is not a human pathogen nor is it toxigenic cultured in order to obtain protease for use in biological laundry detergent. well adapted to grow in	Body fluid, Comma Butterfly (*Polygonia c-album*)

				alkaline conditions	
28	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al.* (2009).	Wet swab from butterfly larvae(outside)
29	*Staphylococcus sciuri* subsp. *carnaticus* strain GTC 1227	3	Gram positive	Important human pathogens responsible for endocarditis, peritonitis, septic shock, urinary tract infection.(Chen,*et,al.* 2007)	Wet swab from butterfly larvae(outside)
30	*Lysinibacillus fusiformis* strain NBRC15717	1	Gram positive	causes infection in humans relating to tropical ulcer formations and dermal and respiratory infections(Calandrini ,*e,al,*2014)	Isolate from inside butterfly larvae body
31	*Stenotrophomonas rhizophila*	1	Gram	Attack Plant roots	Isolate from inside

	strain e-p10		negative	rhizospheres Multiple resistances against antibiotics are not only found with clinical strains but also with strains isolated from the rhizosphere (Alavi,*et al.*2013)	butterfly larvae body
32	*Bacillus cereus* ATCC 14579	3	Gram positive	Diarrheal and emetic	Isolate from inside butterfly larvae body
33	*Lysinibacillus macroides* strain LMG 18474	1	Gram positive and Gram negative	Strictly aerobic, Gram-positive and Gram-negative motile rods. (Coorevits, *et al.*2012). The association of infections such as periodontitis with atherosclerotic diseases is well documented. In spite of the high diversity of the human oral microbiota, and its close contact with	Isolate from inside butterfly larvae body

				the circulatory system	
34	*Bacillus cereus* ATCC 14579	3	Gram positive	Diarrhea and emetic	Isolate from inside die butterfly larvae body treated by *B. thuringiensis*
35	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al.* *(*2009).	Isolate from inside die butterfly larvae body treated by E.coli
36	*Bacillus cereus* ATCC 14579	3	Gram positive	Diarrhea and emetic	Isolate from inside butterfly larvae body treated by *B. subtilis*
37	*Staphylococcus aureus* subsp. aureus N315 strain N315	3	Gram positive	Methicillin-resistant Staphylococcus aureus (MRSA) responsible for many infections such as skin and heart valve. (Deurenberg, *et al.,(*2007).	Isolate from inside die butterfly larvae body treated by MRSA bacteria
38	*Stenotrophomonas maltophilia* strain IAM 12423	8	Gram negative	An important cause of nosocomial	Isolate from inside butterfly larvae

				infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al.* (2009).	body(Control)
39	*Stenotrophomonas maltophilia* R551-3 strain R551-3	8	Gram negative	An important cause of nosocomial infection. The respiratory tract and indwelling urinary catheters. Denton and Kerr (1998) Looney, *et al.* (2009).	Isolate from inside butterfly adult body(Control)

Table 2. Bacteria isolated from Lepidoptera and information regarding pathogenesis.

Table 3. Results of bacteria species isolate from larvae of *Dermestidae* from a human cadaver.

No	Bacteria sp.	Repetition	Activity	Note	Isolate
1	*Brevibacterium ravenspurgense* strain 20	1	Gram_positive	They are catalase-positive, non-spore-forming, non-motile, aerobic Present on the human skin, where it causes foot odor. Reports of infections in immunocompromised patients had been published.(Bal, *et al.*2015)	Isolate from inside larvae of *Dermestidae* extracted from human dead body.
2	*Staphylococcus hominis* subsp. *novobiosepticus* strain GTC 1228	1	Gram_positive	Found on human skin and is usually harmless, but can sometimes cause infections in people with abnormally weak immune systems.(Pfaller, et al.1999)	Isolate from inside larvae of *Dermestidae* extracted from human dead body.
3	*Enterococcus faecalis* V583 strain V583	1	Gram positive	Commensal bacteria inhabiting the gastrointestinal tracts of	Isolate from inside adult blow *Dermestidae*

				humans and other mammals (Ryan KJ, Ray CG 2004).	extracted from human dead body.
4	*Lishizhenia tianjinensis* strain H6	1	Gram negative	isolated from coastal seawater of Tianjin City, China(Chen,*et al.*,2009)	Isolate from inside larvae of *Dermestidae* extracted from human dead body
5	*Clostridium cochlearium* strain JCM 1396	1	Gram positive	Generates ethanol. It is able to utilize amino acids such as glycine, sarcosine, proline, and betaine as sole carbon and energy sources via Stickland reactions (Poehlein,2014)	Isolate from inside larvae of *Dermestidae* extracted from human dead body.
6	*Bacillus safensis* strain NBRC 100820	1	Gram positive	Highly resistant to salt. Not a pathogen in humans	Isolate from inside larvae of *Dermestidae* extracted from human dead body
7	*Enterococcus faecalis* strain NBRC 100480	1	Gram positive	Important clinical infections caused by Enterococcus include urinary tract infections, bacteraemia, bacterial endocarditis,	Isolate from inside larvae of *Dermestidae* extracted from human dead body.

				diverticulitis, and meningitis.	
8	*Clostridium paraputrificum* strain JCM 1293	1	Gram positive	Generates ethanol. It is able to utilize amino acids such as glycine, sarcosine, proline, and betaine as sole carbon and energy sources via Stickland reactions Poehlein,2014)	Isolate from inside blow fly (*Calliphora*) larvae extracted from human dead body.

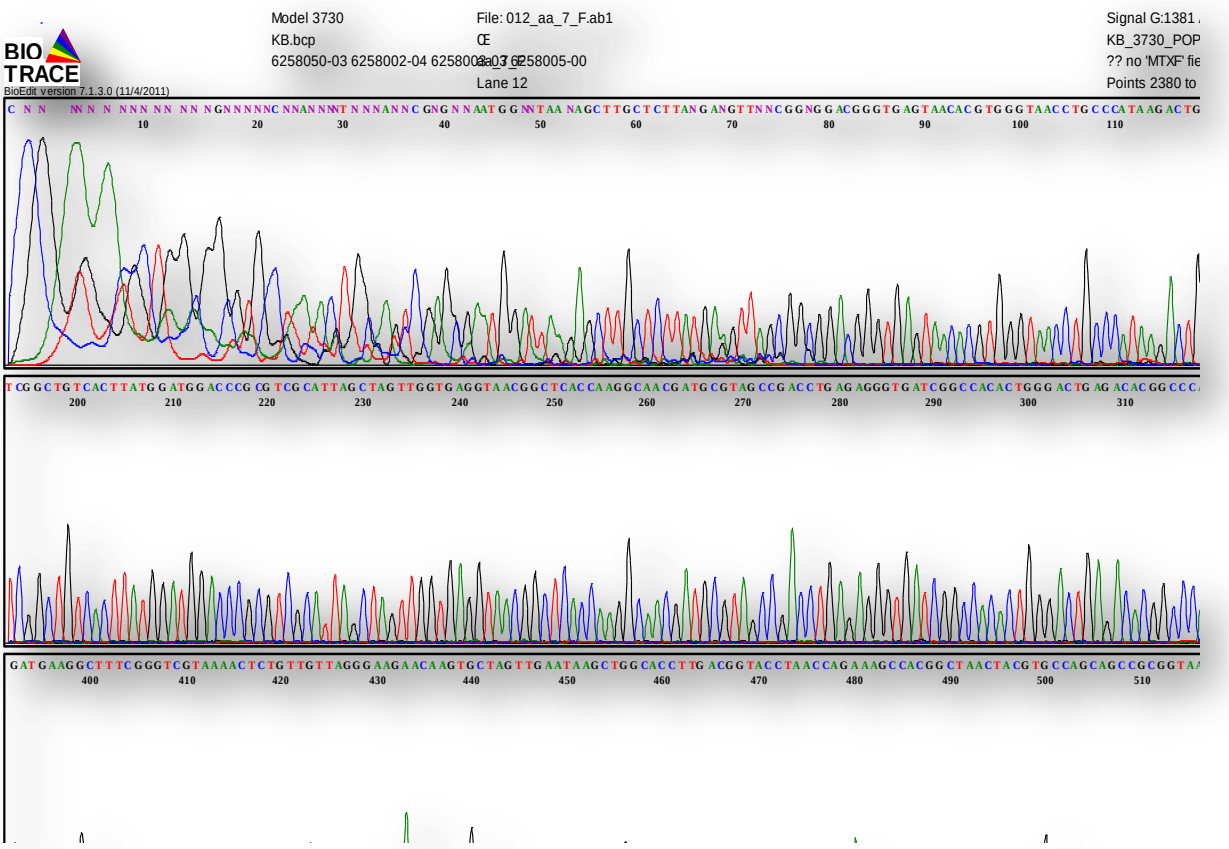

Fig.1 Shows Finch TV software as ladder for nitrogen base pair

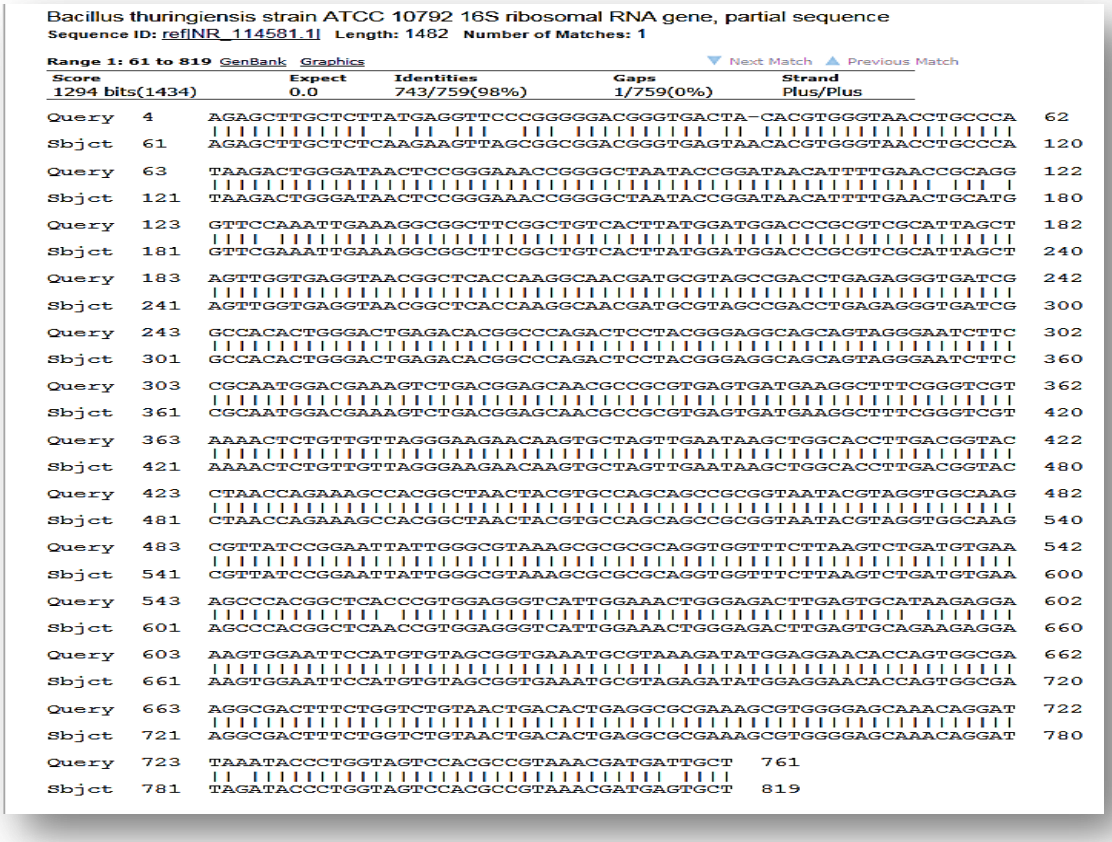

Fig. 2 Shows *Bacillus thuringiensis* gene sequences

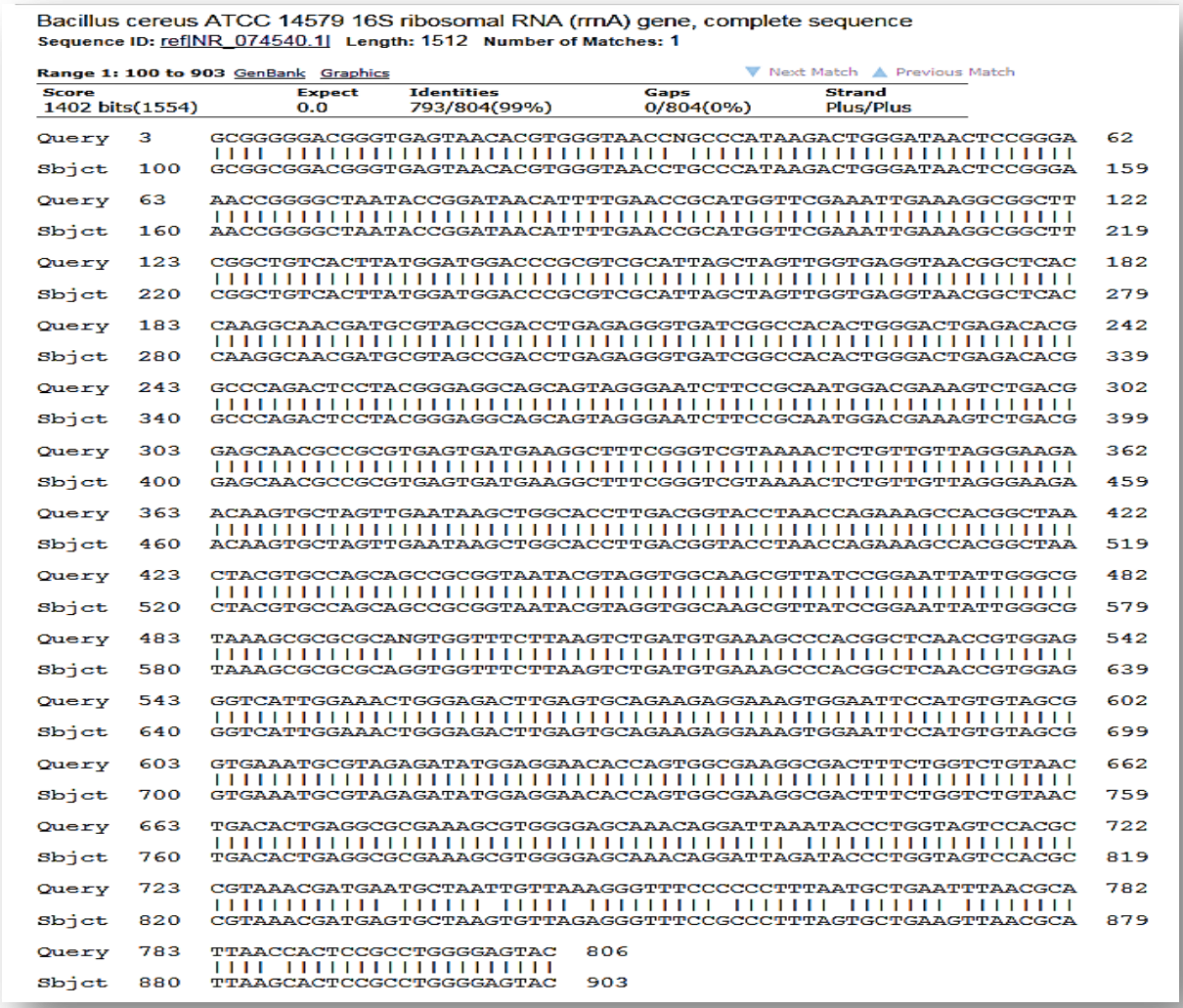

Fig. 3 Shows *Bacillus cereus* gene sequences

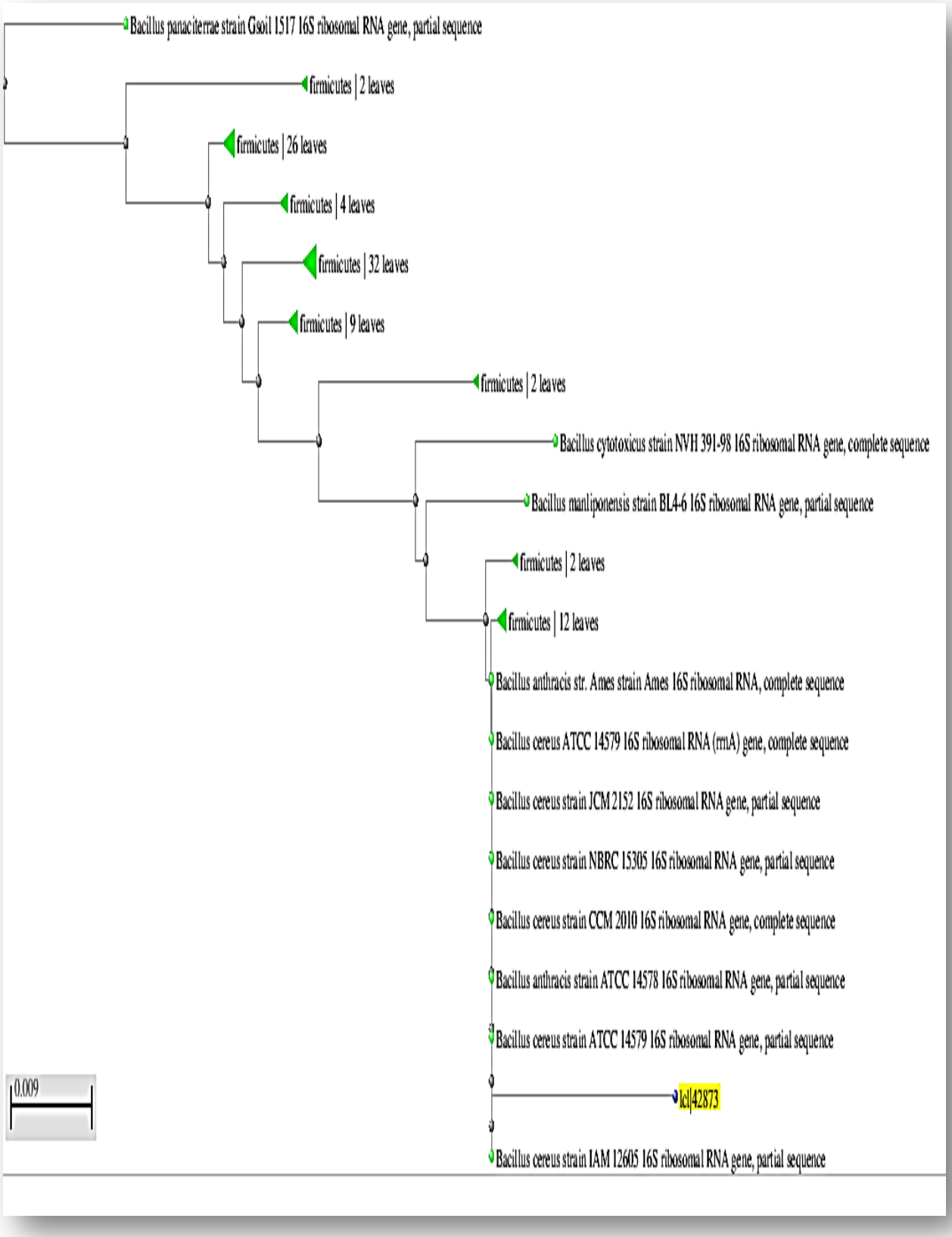

Fig.4 Shows *Bacillus cereus* tree

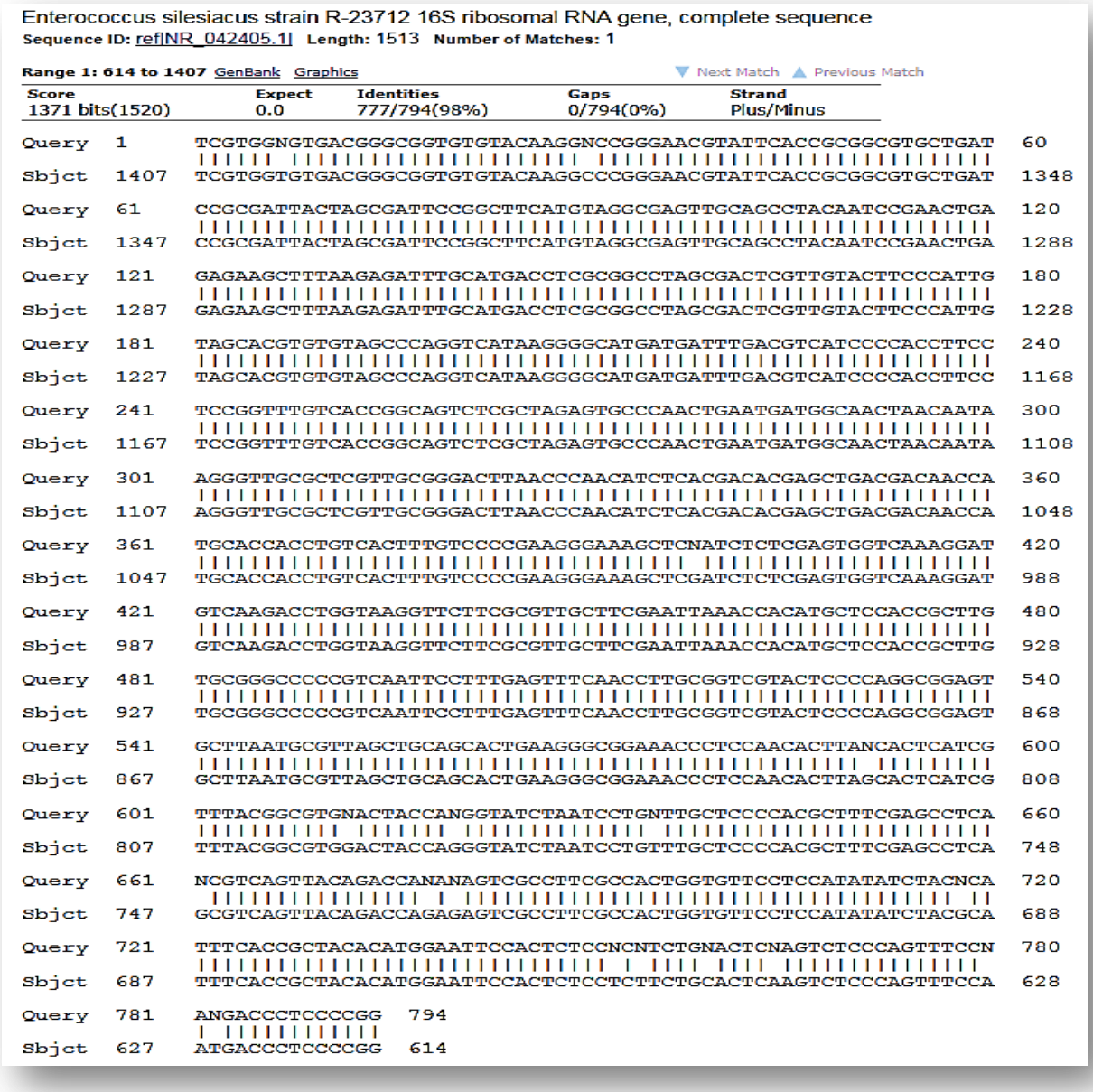

Fig. 5 Shows *Enterococcus silesiacus* gene sequences

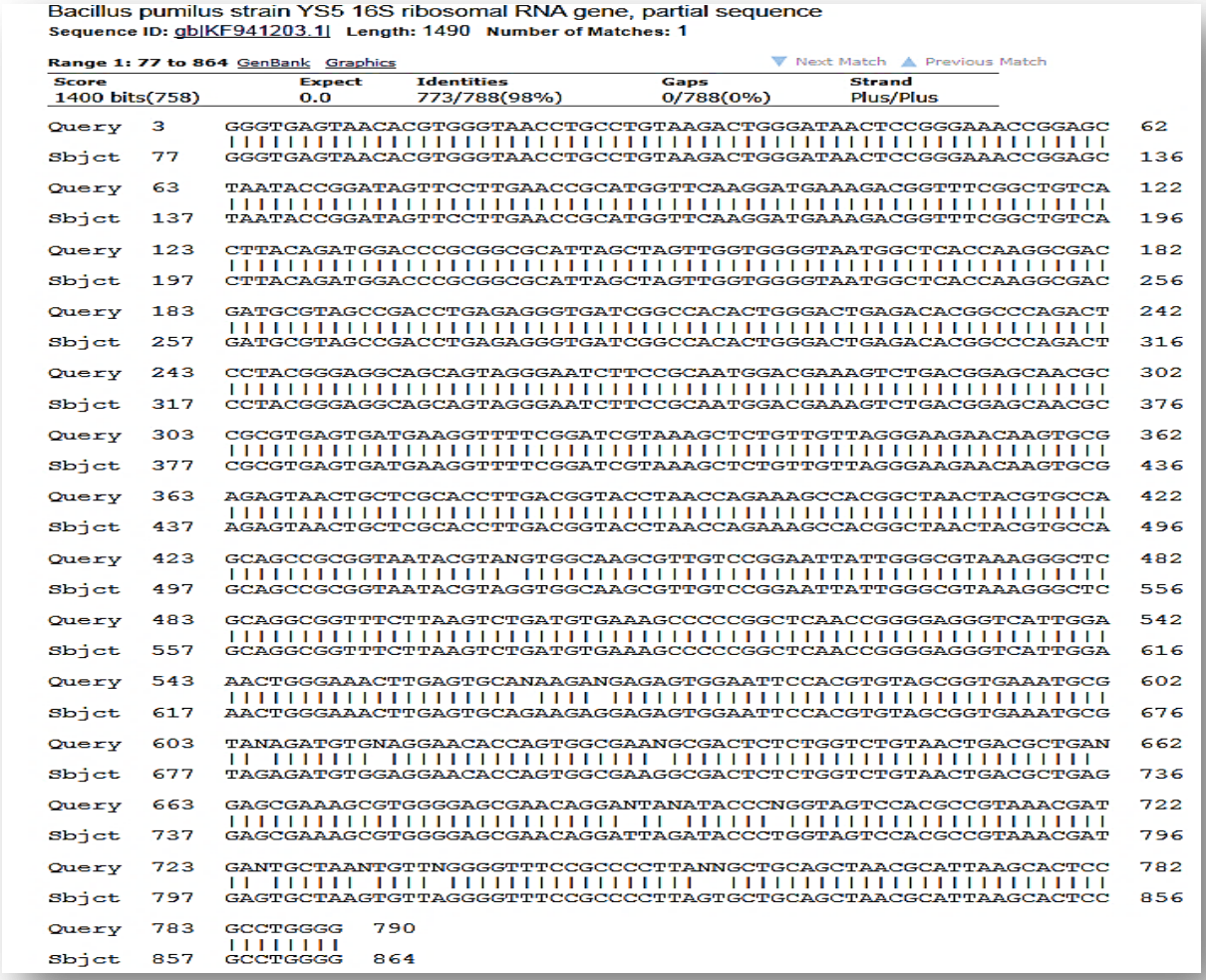

Fig. 6 Shows *Bacillus pumilus* gene sequences

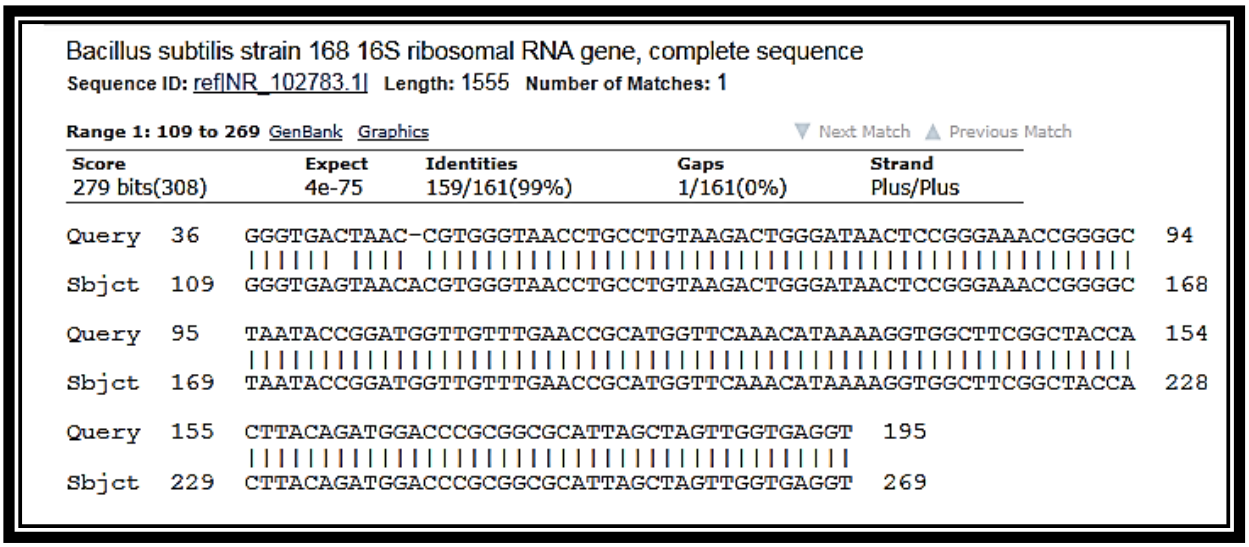

Fig. 7 Shows *Bacillus subtilis* gene sequences

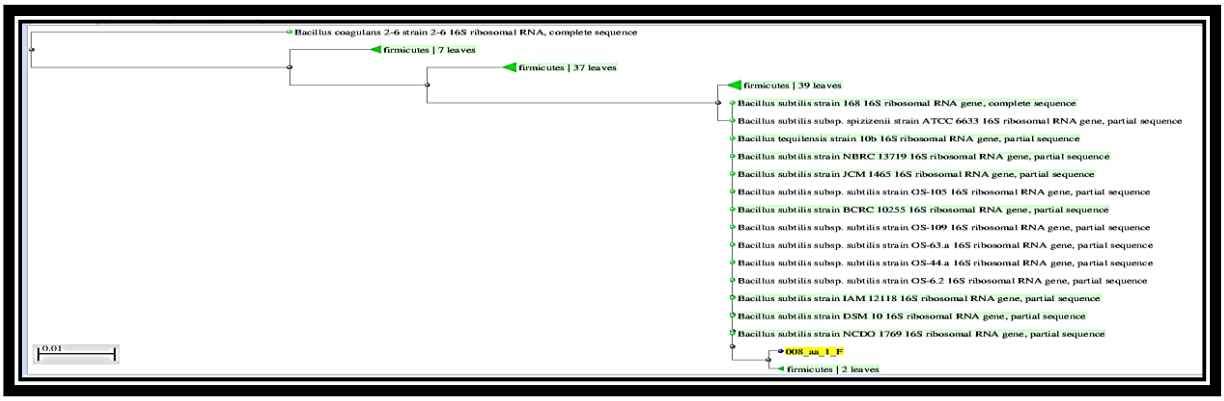

Fig. 8 Shows *Bacillus subtilis* tree

Fig. 9 Shows Butterfly larvae which used to isolate the bacteria(author's image picture)

Bacillus subtilis strain 168 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_102783.1| **Length:** 1555 **Number of Matches:** 1

Range 1: 109 to 269 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  109  GGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGC  168

Query  95   TAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCA  154
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Sbjct  169  TAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCA  228

Query  155  CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT  195
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Sbjct  229  CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGT  269
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Fig. 10 Shows *Bacillus subtilis* gene sequences

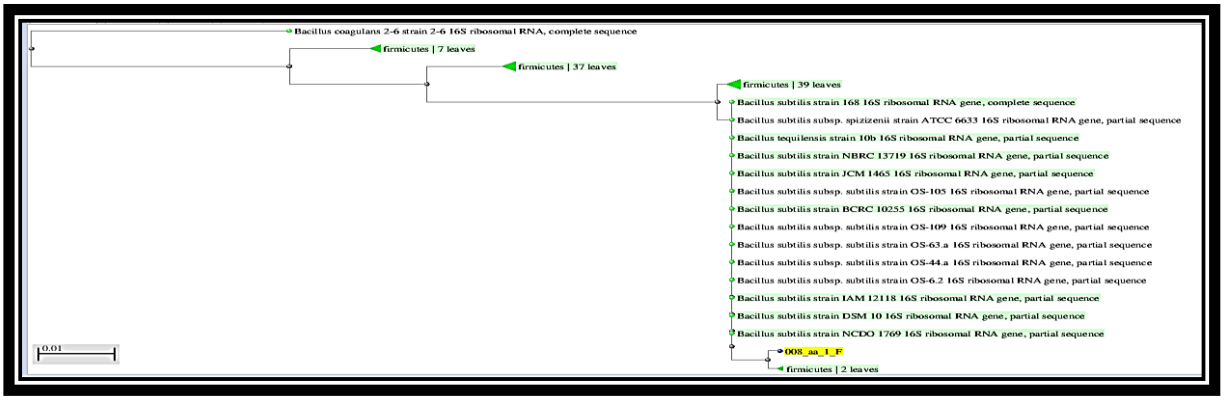

Fig. 11 Shows *Bacillus subtilis* tree

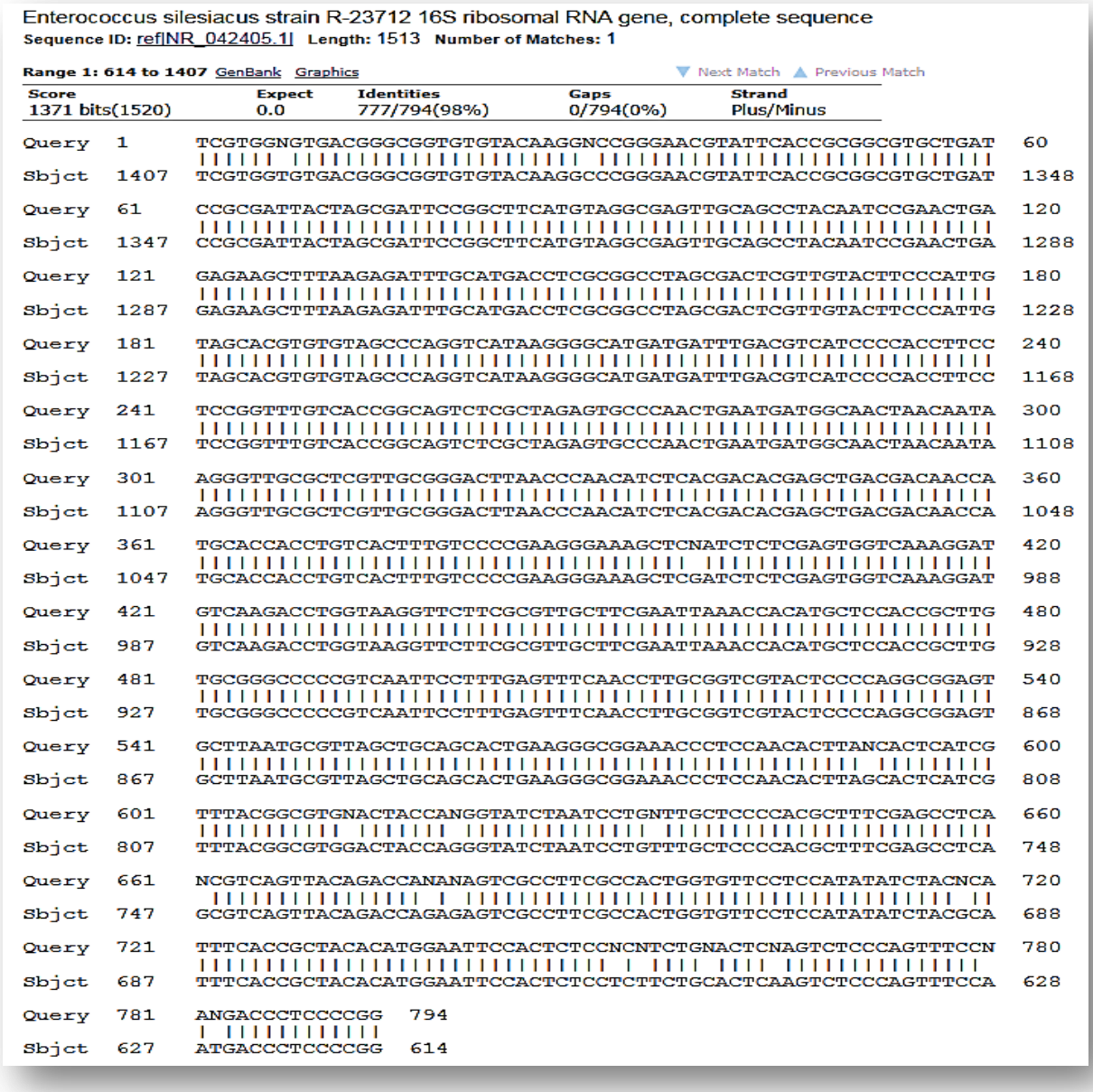

Fig. 12 Shows *Enterococcus silesiacu*s gene sequences

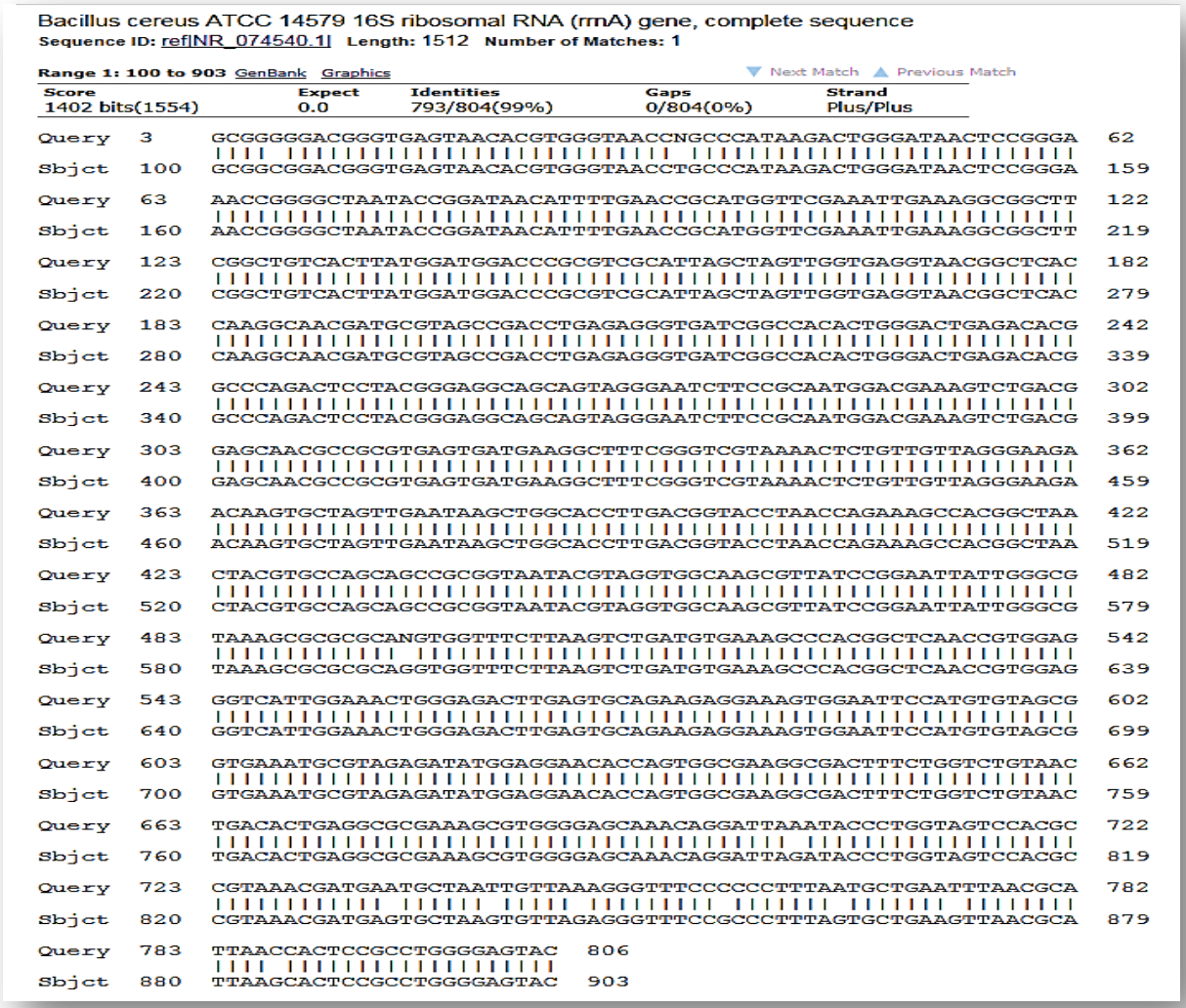

Fig.13 Shows *Bacillus cereus* gene sequences

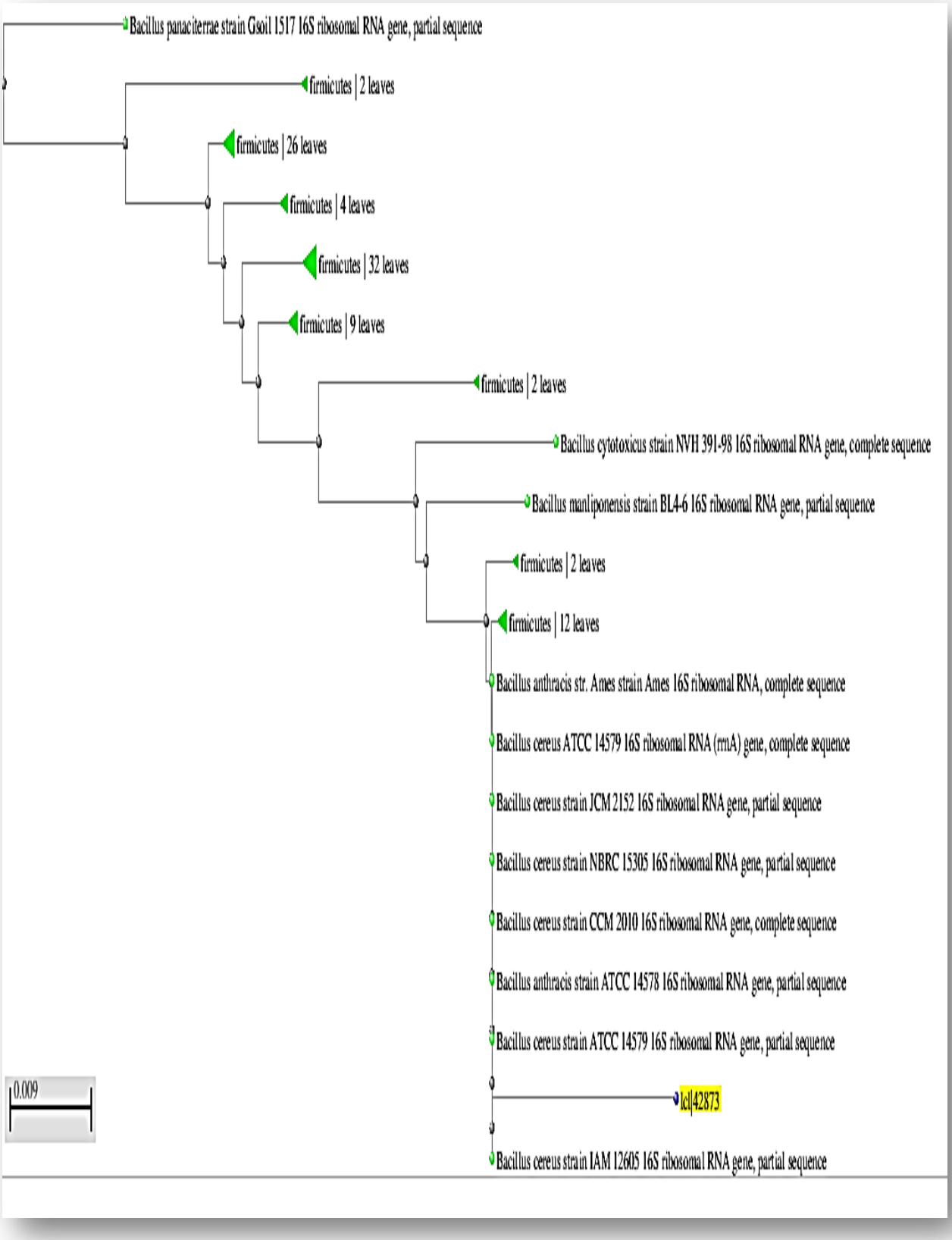

Fig .14 Shows *Bacillus cereus* tree

Fig.15 Shows European Swallowtail butterfly used to isolate the bacteria(authouir's image)

Granulicatella elegans strain B1333 16S ribosomal RNA gene, complete sequence

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Range 1: 863 to 1382 GenBank Graphics Next Match Previous Match

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Query  142   AGTTGCAGCCTACAATCCGAACTGAGACTGGCTTTCAGAGATTCGCTTGCCCTCGCGAGT  201
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Sbjct  1322  AGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTCGCTTACCCTCGCGAGT  1263

Query  202   TTGCTGCTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGA  261
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Sbjct  1262  TCGCTGCTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAAGTCATAAGGGGCATGA  1203

Query  262   TGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCC  321
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Sbjct  1202  TGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCC  1143

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Sbjct  1142  AACTCAATGCTGGCAACTAGTAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATC  1083

Query  382   TCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTTTGGCCCCGAAGGGAATTC  441
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Sbjct  1082  TCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCTTTGTCCCCGAAGGGAATGC  1023

Query  442   TCTATCTCTAAAGTGGTCTCGGGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGCTTCGA  501
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Sbjct  1022  TCTATCTCTAGAGTGGTCAAAGGATGTCAAGACTTGGTAAGGTTCTTCGCGTTGGTTCGA  963

Query  502   ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCT  561
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Sbjct  962   ATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCT  903

Query  562   TGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAAC  601
             |||||||||||||||||||||||||||||||||||||||
Sbjct  902   TGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAAC  863
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Fig. 16 Shows *Granulicatella elegans* gene sequences

Fig. 17 Shows *Granulicatella elegans* tree

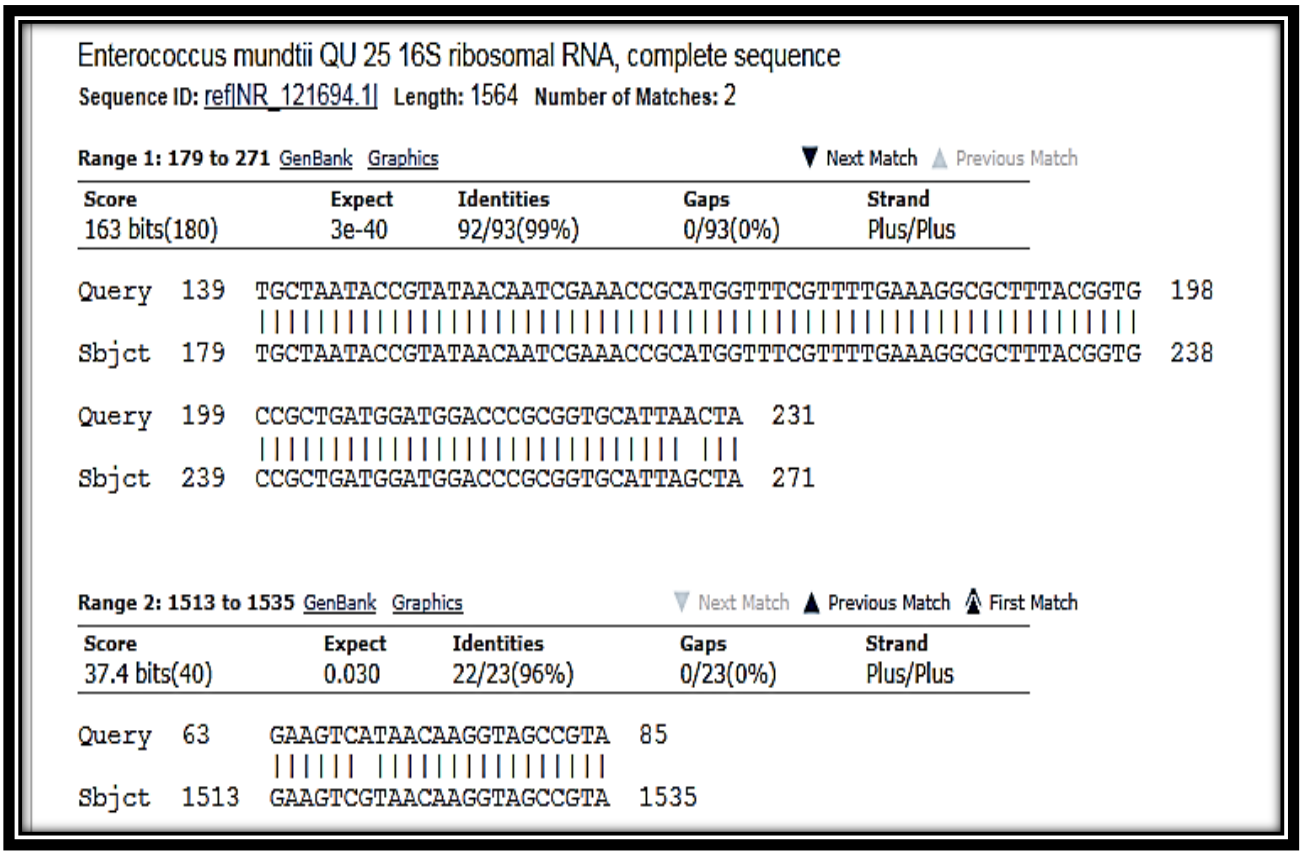


Fig.18 Shows *Enterococcus mundtii* gene sequences

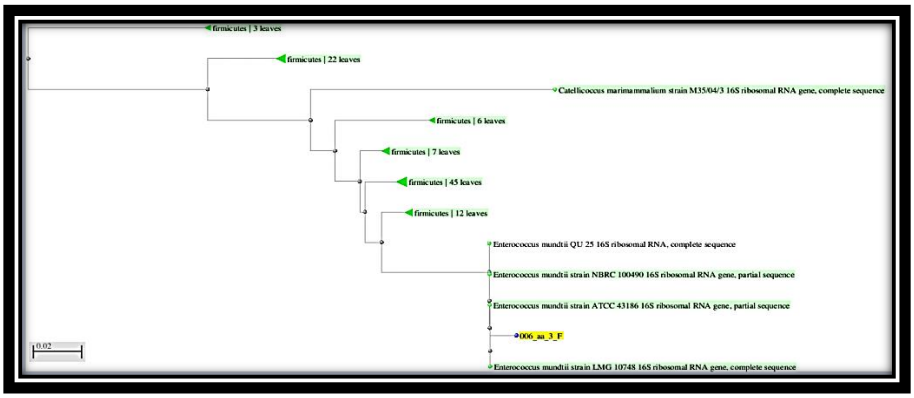


Fig.19 Shows *Enterococcus mundtii* tree

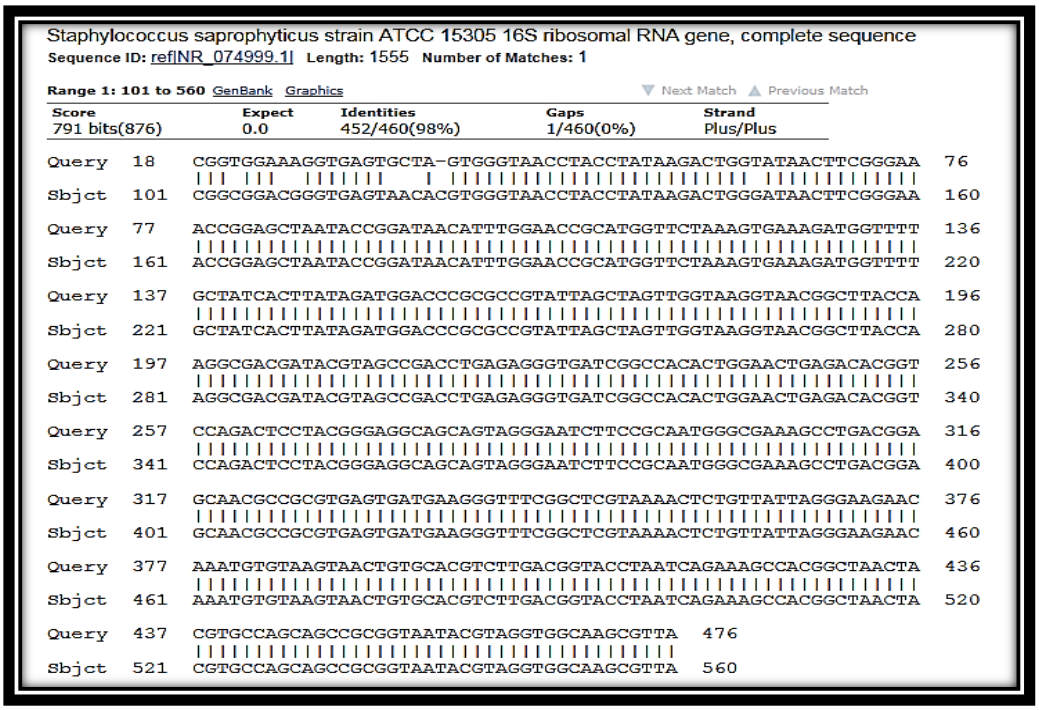


Fig.20 Shows *Staphylococcus saprophyticus* gene sequences

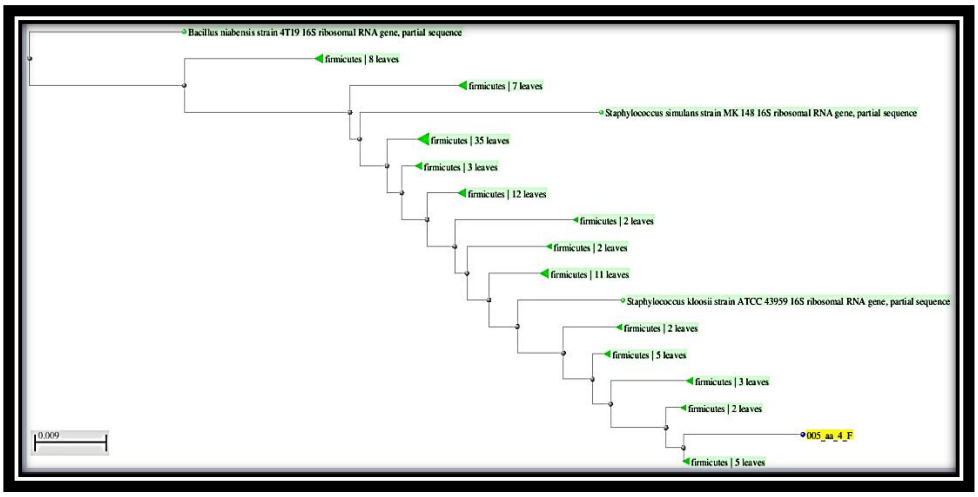


Fig.21 Shows *Staphylococcus saprophyticus* tree

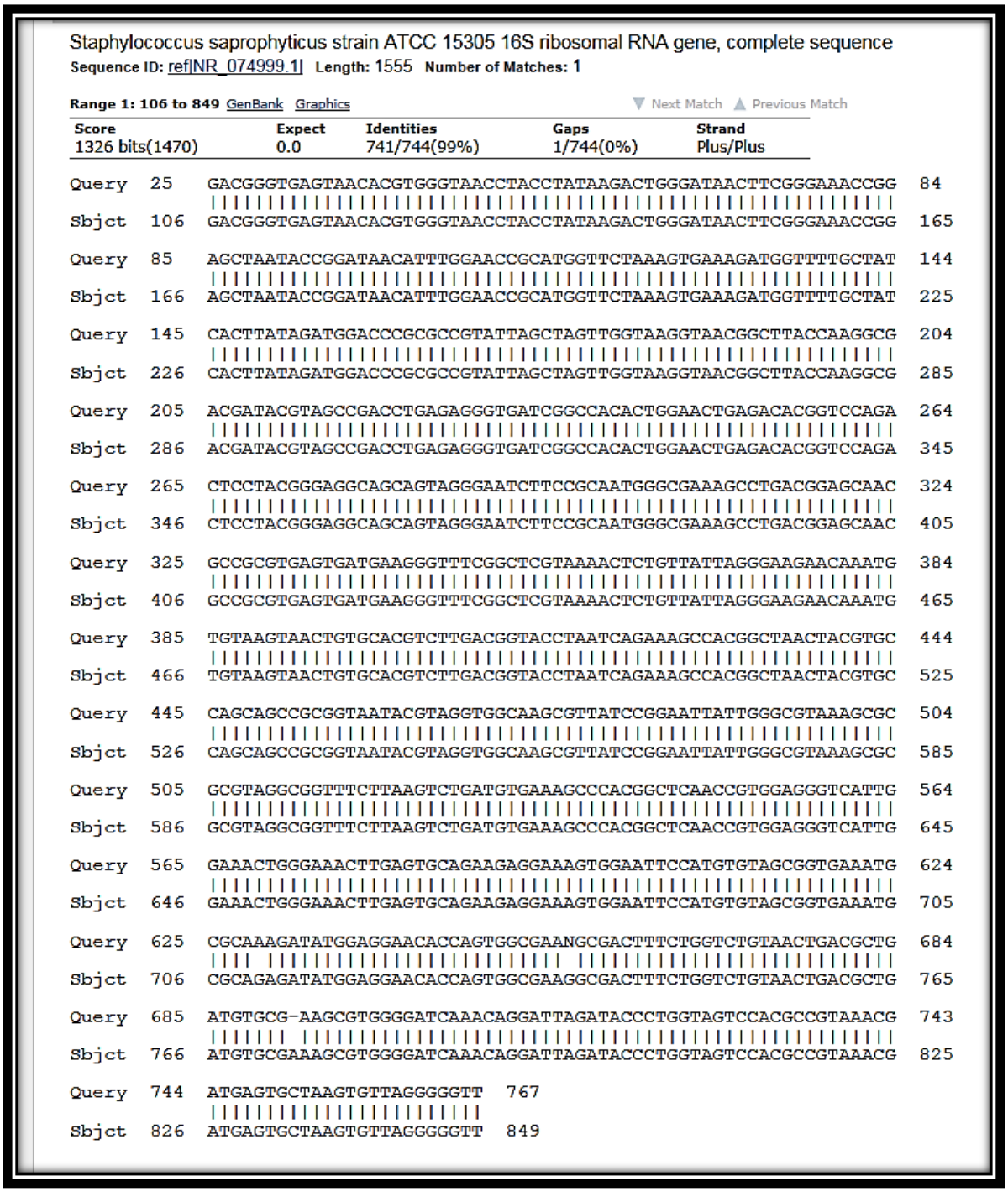
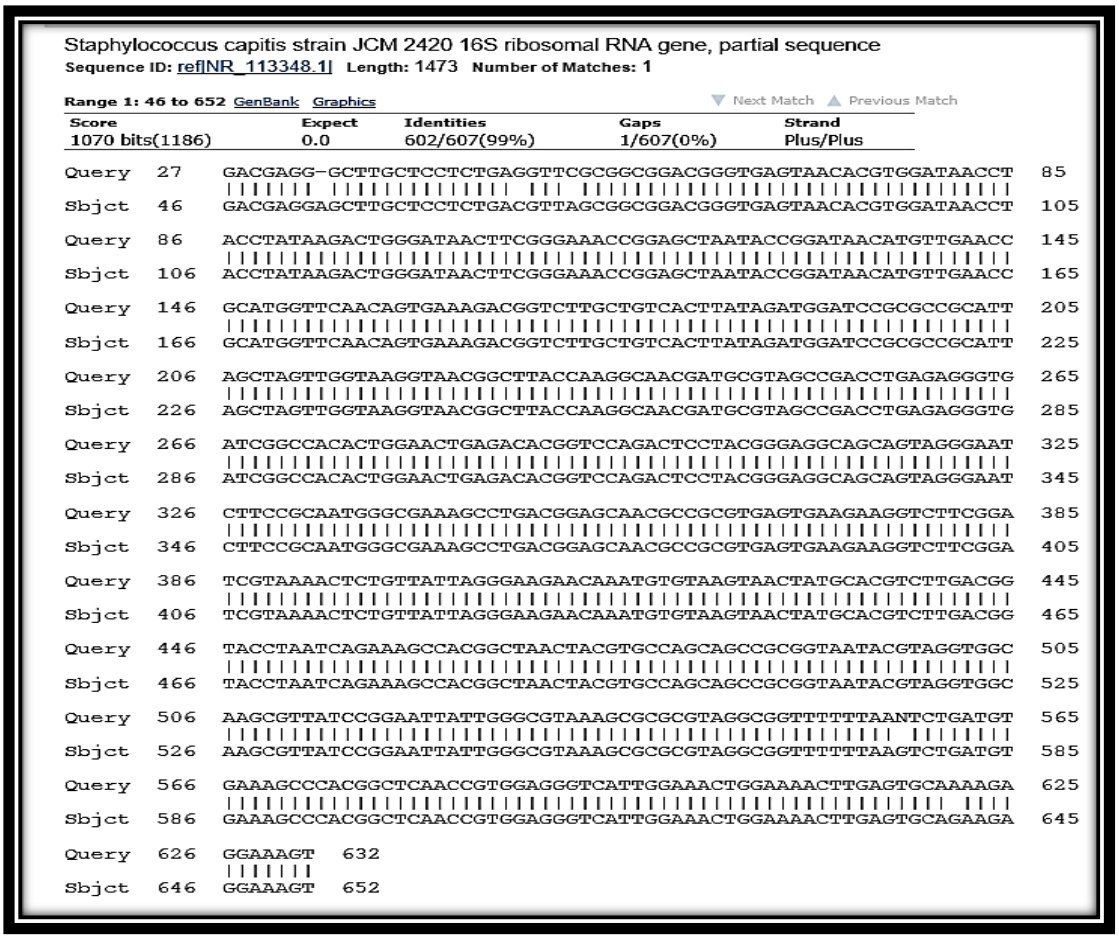


Fig.22 Shows *Staphylococcus saprophyticus* gene sequences



Staphylococcus capitis strain JCM 2420 16S ribosomal RNA gene, partial sequence

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Sbjct  166  GCATGGTTCAACAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCCGCATT  225

Query  206  AGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTG  265
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Sbjct  286  ATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAAT  345

Query  326  CTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGA  385
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Fig.23 Shows *Staphylococcus capitis* gene sequences

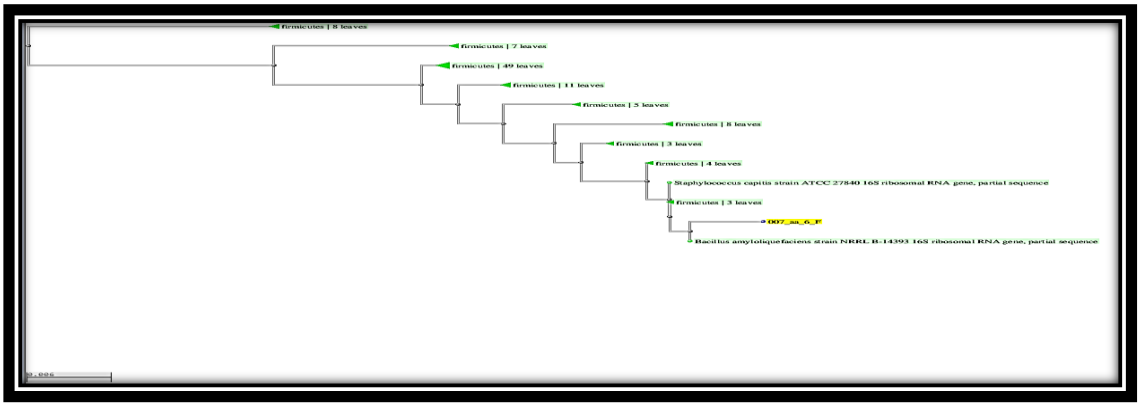
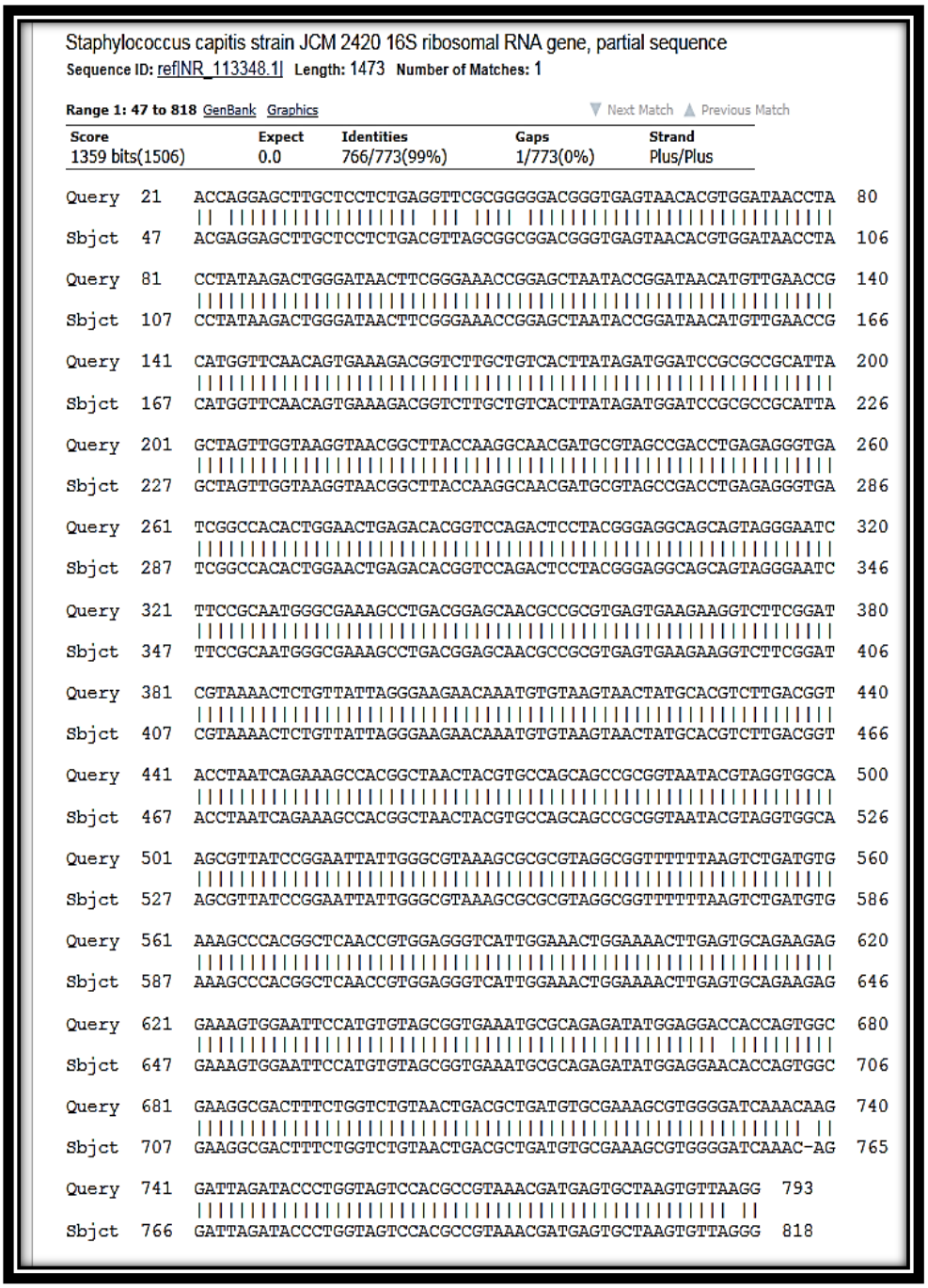


Fig.24 Shows *Staphylococcus capitis* tree



Staphylococcus capitis strain JCM 2420 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_113348.1| Length: 1473 Number of Matches: 1

Range 1: 47 to 818 GenBank Graphics ▼ Next Match ▲ Previous Match

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Sbjct  47   ACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTA  106

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Sbjct  107  CCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAACATGTTGAACCG  166

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Query  201  GCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA  260
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Sbjct  227  GCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGA  286

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Sbjct  287  TCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC  346

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Sbjct  347  TTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTCTTCGGAT  406

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  467  ACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA  526

Query  501  AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTG  560
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  527  AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTG  586

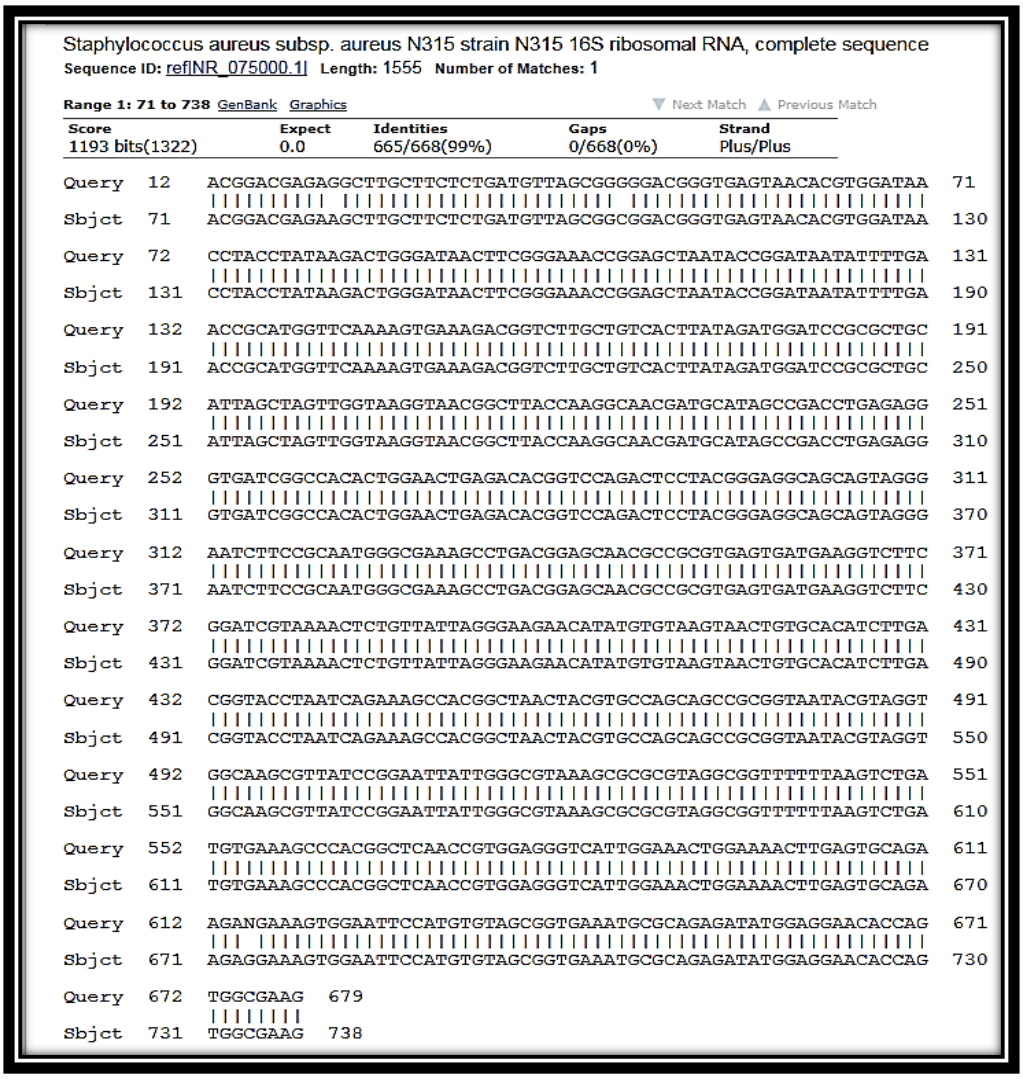
Query  561  AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAG  620
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  587  AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAG  646

Query  621  GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGACCACCAGTGGC  680
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  647  GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGC  706

Query  681  GAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAAG  740
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  707  GAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAAC-AG  765

Query  741  GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAGG  793
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  766  GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGG  818
```

Fig.25 Shows *Staphylococcus capitis* gene sequences



Staphylococcus aureus subsp. aureus N315 strain N315 16S ribosomal RNA, complete sequence

Sequence ID: refINR_075000.1| Length: 1555 Number of Matches: 1

Range 1: 71 to 738 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1193 bits(1322)	0.0	665/668(99%)	0/668(0%)	Plus/Plus

```
Query  12   ACGGACGAGAGGCTTGCTTCTCTGATGTTAGCGGGGGACGGGTGAGTAACACGTGGATAA  71
Sbjct  71   ACGGACGAGAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAA  130

Query  72   CCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGA  131
Sbjct  131  CCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGA  190

Query  132  ACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGC  191
Sbjct  191  ACCGCATGGTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGC  250

Query  192  ATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGG  251
Sbjct  251  ATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGG  310

Query  252  GTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG  311
Sbjct  311  GTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGG  370

Query  312  AATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTC  371
Sbjct  371  AATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTC  430

Query  372  GGATCGTAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGA  431
Sbjct  431  GGATCGTAAAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGA  490

Query  432  CGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT  491
Sbjct  491  CGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT  550

Query  492  GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGA  551
Sbjct  551  GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGA  610

Query  552  TGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGA  611
Sbjct  611  TGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGA  670

Query  612  AGANGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAG  671
Sbjct  671  AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAG  730

Query  672  TGGCGAAG  679
Sbjct  731  TGGCGAAG  738
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Fig.26 Shows *Staphylococcus aureus* gene sequences

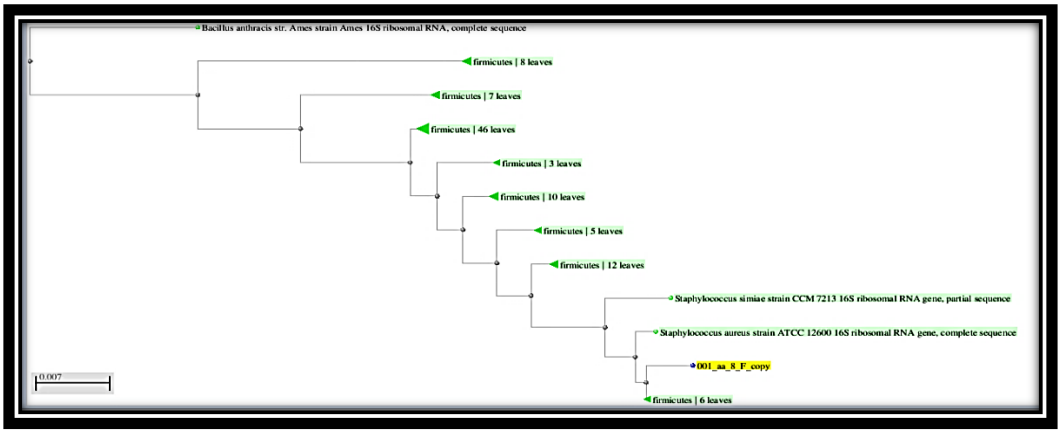


Fig.27 Shows *Staphylococcus aureus* tree

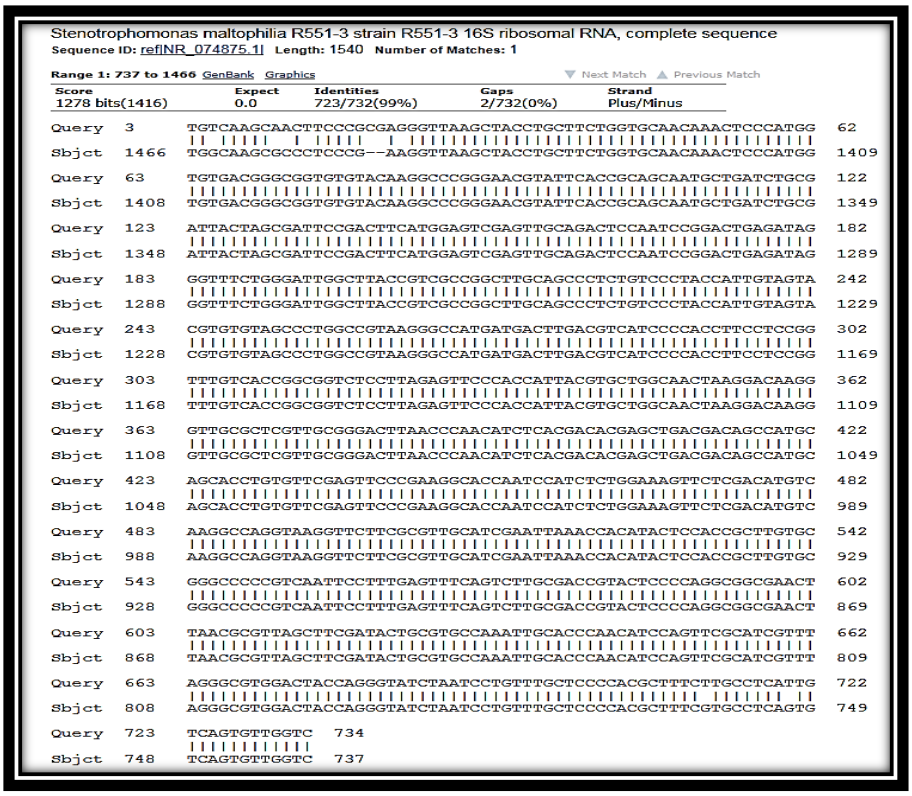


Fig.28 Shows *Stenotrophomonas maltophilia* gene sequences

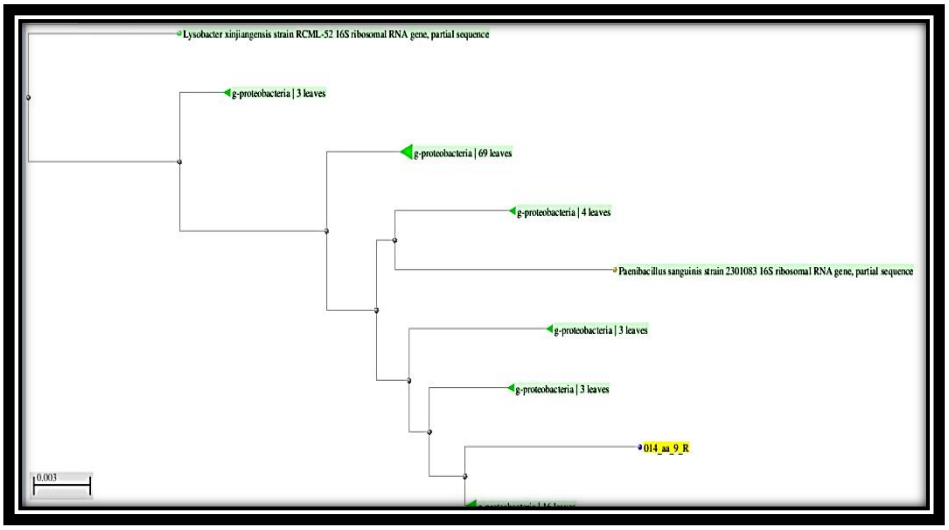


Fig.29 Shows *Stenotrophomonas maltophilia* tree

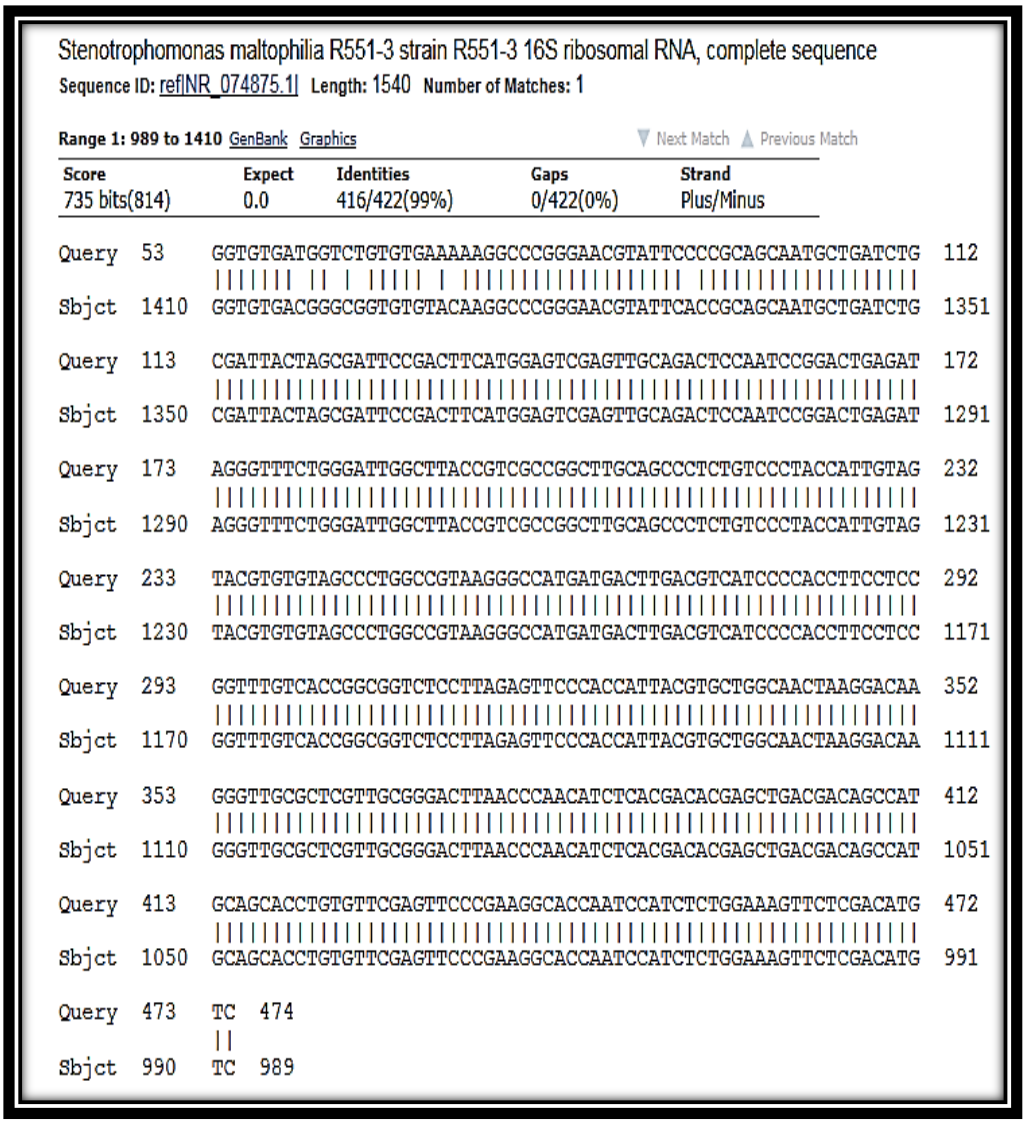
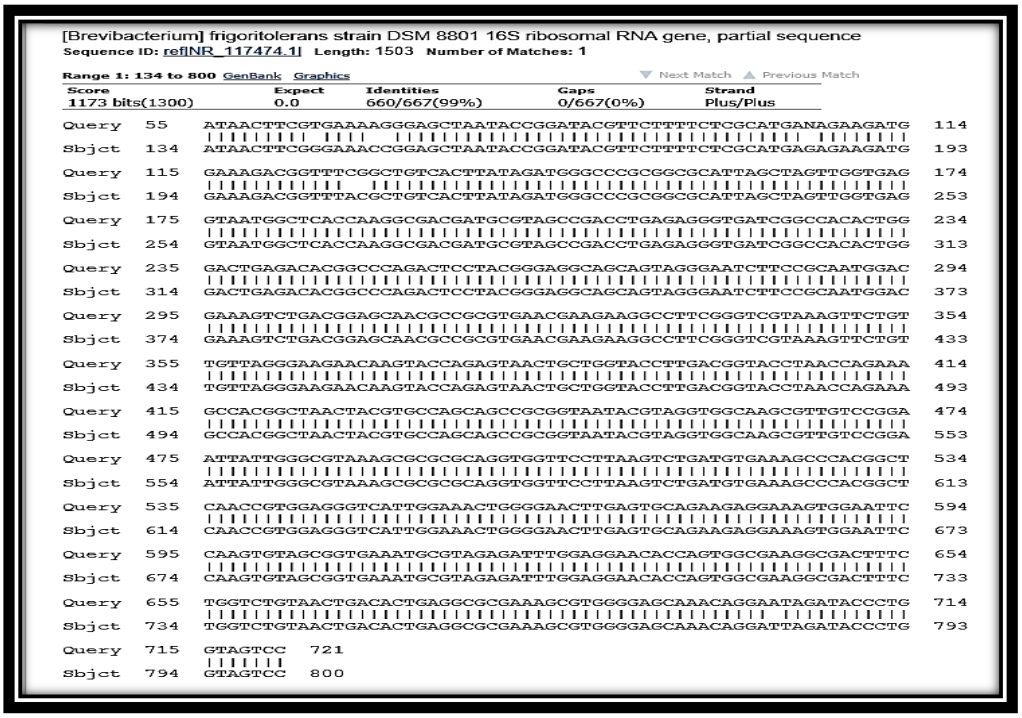


Fig.30 Shows *Stenotrophomonas maltophilia* gene sequences

Fig.31 Shows eyed hawk-moth used to isolate the bacteria (authour's image)



[Brevibacterium] frigoritolerans strain DSM 8801 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_117474.1| Length: 1503 Number of Matches: 1

Range 1: 134 to 800 GenBank Graphics

Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1173 bits(1300)	0.0	660/667(99%)	0/667(0%)	Plus/Plus

```
Query  55   ATAACTTCGTGAAAAGGGAGCTAATACCGGATACGTTCTTTTCTCGCATGANAGAAGATG  114
            ||||||||| ||||   ||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  134  ATAACTTCGGGAAACCGGAGCTAATACCGGATACGTTCTTTTCTCGCATGAGAGAAGATG  193

Query  115  GAAAGACGGTTTCGGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAG  174
            ||||||||||||   ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  GAAAGACGGTTTACGCTGTCACTTATAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAG  253

Query  175  GTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG  234
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  GTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG  313

Query  235  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC  294
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314  GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC  373

Query  295  GAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCGGGTCGTAAAGTTCTGT  354
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374  GAAAGTCTGACGGAGCAACGCCGCGTGAACGAAGAAGGCCTTCGGGTCGTAAAGTTCTGT  433

Query  355  TGTTAGGGAAGAACAAGTACCAGAGTAACTGCTGGTACCTTGACGGTACCTAACCAGAAA  414
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434  TGTTAGGGAAGAACAAGTACCAGAGTAACTGCTGGTACCTTGACGGTACCTAACCAGAAA  493

Query  415  GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA  474
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494  GCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGA  553

Query  475  ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCT  534
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  554  ATTATTGGGCGTAAAGCGCGCGCAGGTGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCT  613

Query  535  CAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTC  594
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  614  CAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTC  673

Query  595  CAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTC  654
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674  CAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTC  733

Query  655  TGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAATAGATACCCTG  714
            ||||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||
Sbjct  734  TGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG  793

Query  715  GTAGTCC  721
            |||||||
Sbjct  794  GTAGTCC  800
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Fig.32 Shows *Brevibacterium frigoritolerans* gene sequences

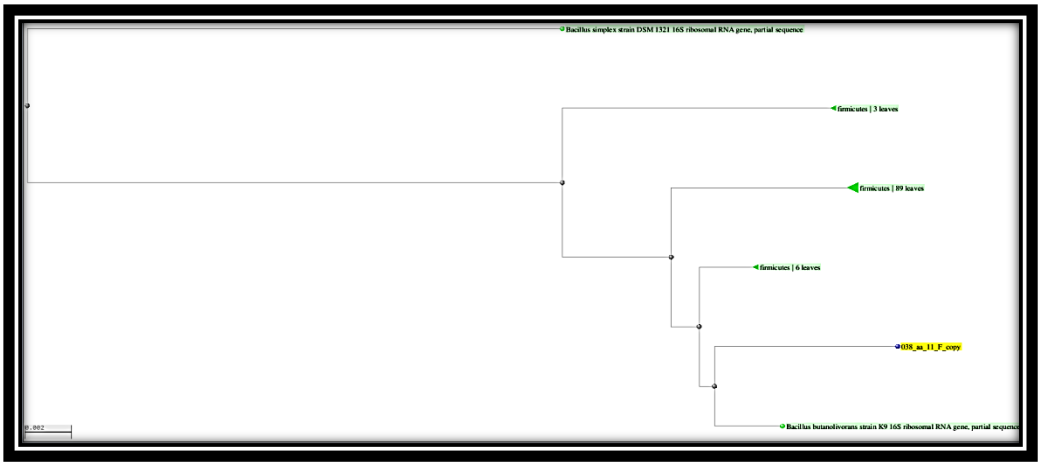
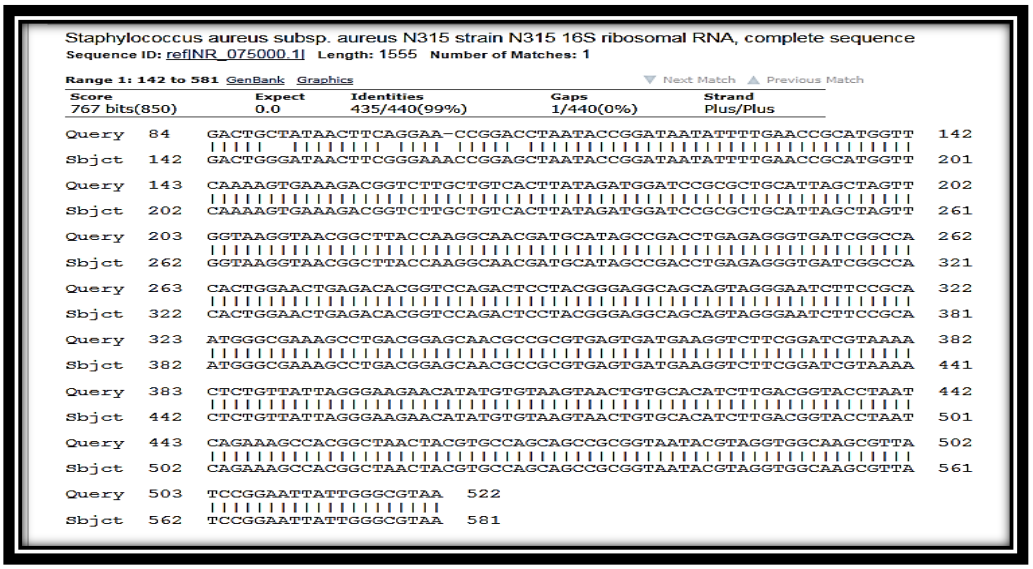


Fig.33 Shows *Brevibacterium frigoritolerans* tree

Fig.34 Shows Pupa of Eyed Hawk-moth used to isolate the bacteria(authour's image)



Staphylococcus aureus subsp. aureus N315 strain N315 16S ribosomal RNA, complete sequence

Sequence ID: refINR_075000.1| Length: 1555 Number of Matches: 1

Range 1: 142 to 581 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
767 bits(850)	0.0	435/440(99%)	1/440(0%)	Plus/Plus

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Query  84    GACTGCTATAACTTCAGGAA-CCGGACCTAATACCGGATAATATTTTGAACCGCATGGTT  142
             |||||  ||||||||  ||||  |||||  ||||||||||||||||||||||||||||
Sbjct  142   GACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATGGTT  201

Query  143   CAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTT  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  202   CAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTAGTT  261

Query  203   GGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCA  262
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262   GGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCA  321

Query  263   CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  322
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322   CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  381

Query  323   ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAA  382
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382   ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTAAAA  441

Query  383   CTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAAT  442
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442   CTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCTAAT  501

Query  443   CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA  502
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502   CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA  561

Query  503   TCCGGAATTATTGGGCGTAA  522
             ||||||||||||||||||||
Sbjct  562   TCCGGAATTATTGGGCGTAA  581
```

Fig.35 Shows *Staphylococcus aureus* gene sequences

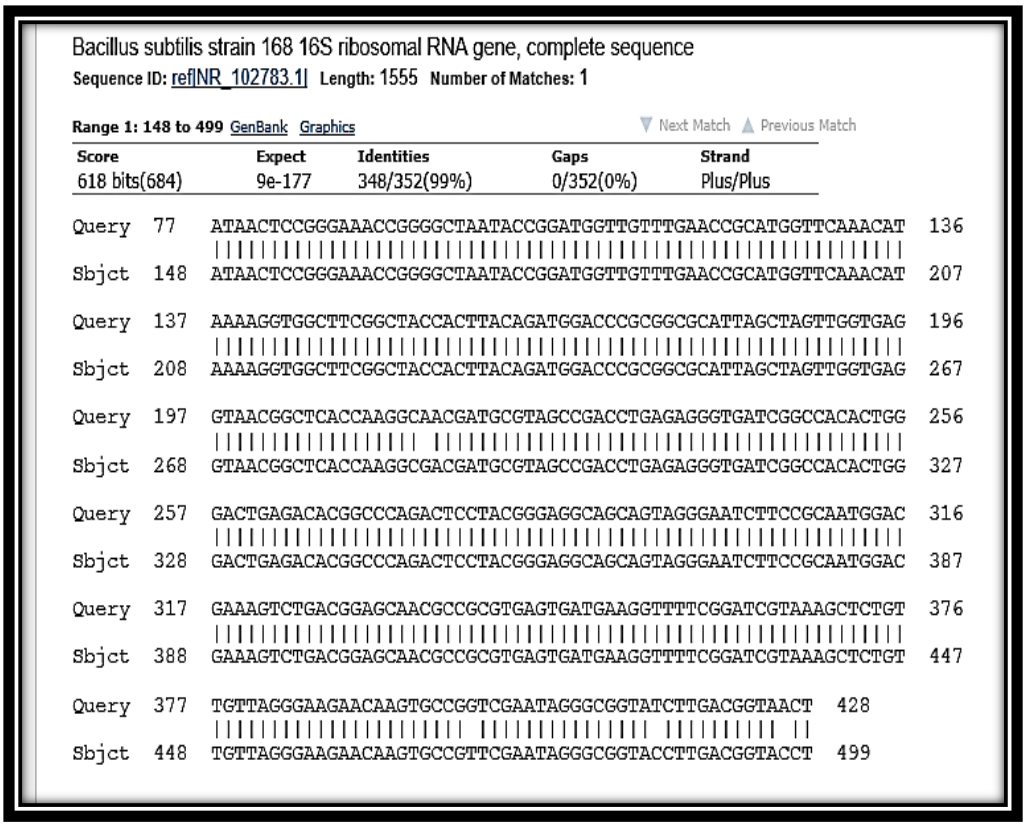


Fig.36 Shows *Bacillus subtilis* gene sequences

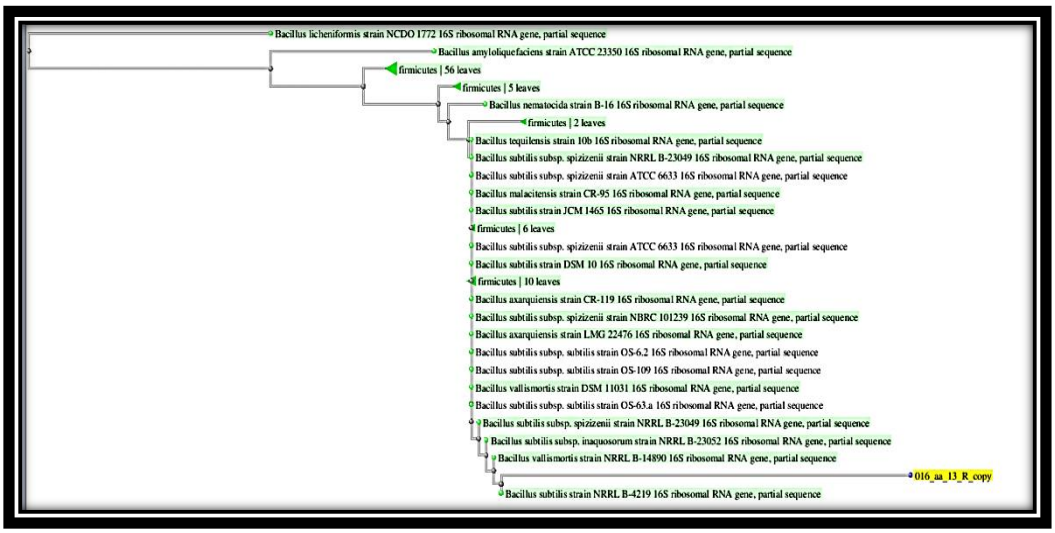
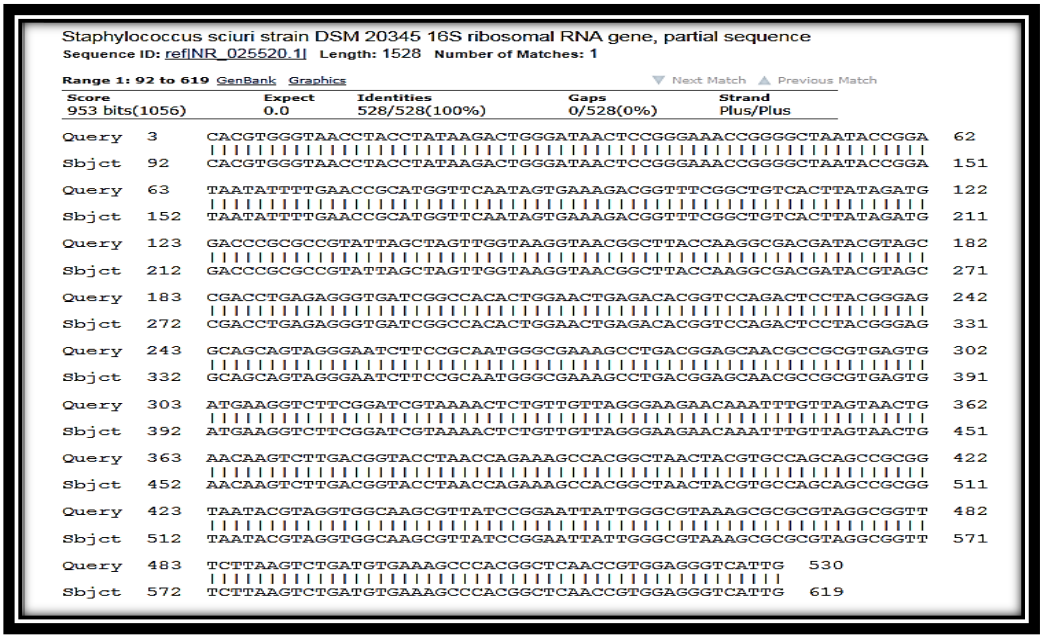


Fig.37 Shows *Bacillus subtilis* tree

Fig.38 Shows Elephant Hawk- moth used to isolate the bacteria (authour's image)



Staphylococcus sciuri strain DSM 20345 16S ribosomal RNA gene, partial sequence
Sequence ID: refNR_025520.1| Length: 1528 Number of Matches: 1

Range 1: 92 to 619 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
953 bits(1056)	0.0	528/528(100%)	0/528(0%)	Plus/Plus

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Query  3    CACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGA  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92   CACGTGGGTAACCTACCTATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGA  151

Query  63   TAATATTTTGAACCGCATGGTTCAATAGTGAAAGACGGTTTCGGCTGTCACTTATAGATG  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  TAATATTTTGAACCGCATGGTTCAATAGTGAAAGACGGTTTCGGCTGTCACTTATAGATG  211

Query  123  GACCCGCGGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGC  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  GACCCGCGGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACGTAGC  271

Query  183  CGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  CGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG  331

Query  243  GCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTG  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  GCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTG  391

Query  303  ATGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTG  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  392  ATGAAGGTCTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAATTTGTTAGTAACTG  451

Query  363  AACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  452  AACAAGTCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG  511

Query  423  TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTT  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  512  TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTT  571

Query  483  TCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTG  530
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  572  TCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTG  619
```

Fig.39 Shows *Staphylococcus sciuri* gene sequences

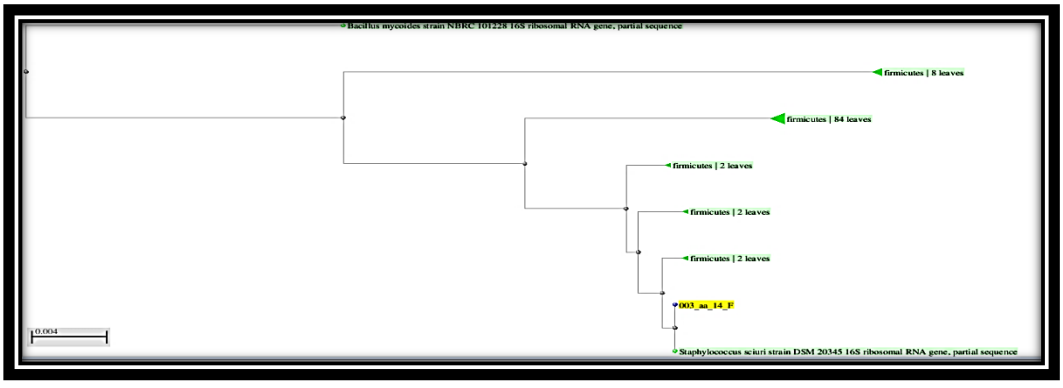
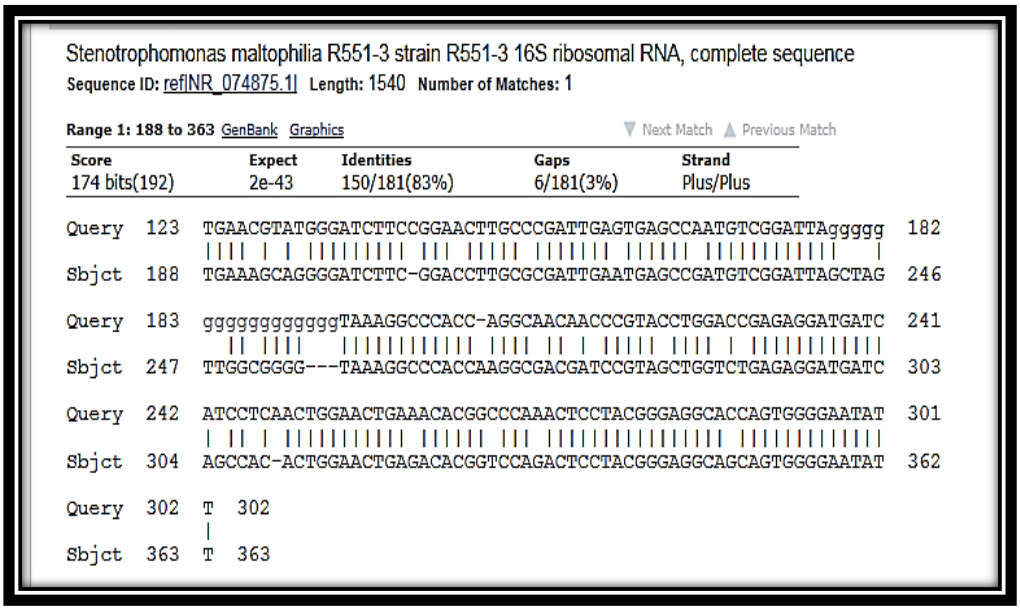


Fig.40 Shows *Staphylococcus sciuri* tree

Fig.41 Shows Silk moth used to isolate the bacteria(authour's image)



Stenotrophomonas maltophilia R551-3 strain R551-3 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074875.1| **Length:** 1540 **Number of Matches:** 1

Range 1: 188 to 363 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
174 bits(192)	2e-43	150/181(83%)	6/181(3%)	Plus/Plus

```
Query  123  TGAACGTATGGGATCTTCCGGAACTTGCCCGATTGAGTGAGCCAATGTCGGATTAggggg  182
            ||||  |  | |||||||||| |||  |||||  |||||||  ||||||  ||||||||||||   |
Sbjct  188  TGAAAGCAGGGGATCTTC-GGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAG  246

Query  183  gggggggggggTAAAGGCCCACC-AGGCAACAACCCGTACCTGGACCGAGAGGATGATC  241
              ||  ||||    ||||||||||||  ||||  ||  |  |||||  ||||  |  ||||||||||||
Sbjct  247  TTGGCGGGG---TAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATC  303

Query  242  ATCCTCAACTGGAACTGAAACACGGCCCAAACTCCTACGGGAGGCACCAGTGGGGAATAT  301
            |  ||  |  ||||||||||| ||||||  |||  ||||||||||||||||  ||||||||||||
Sbjct  304  AGCCAC-ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT  362

Query  302  T  302
            |
Sbjct  363  T  363
```

Fig.42 Shows *Stenotrophomonas maltophilia* gene sequences

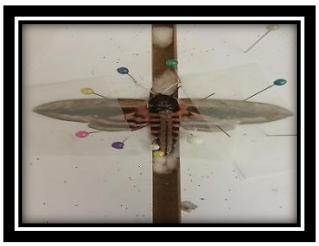


Fig.43 Shows Atlas moth used to isolate the bacteria(authour's image)

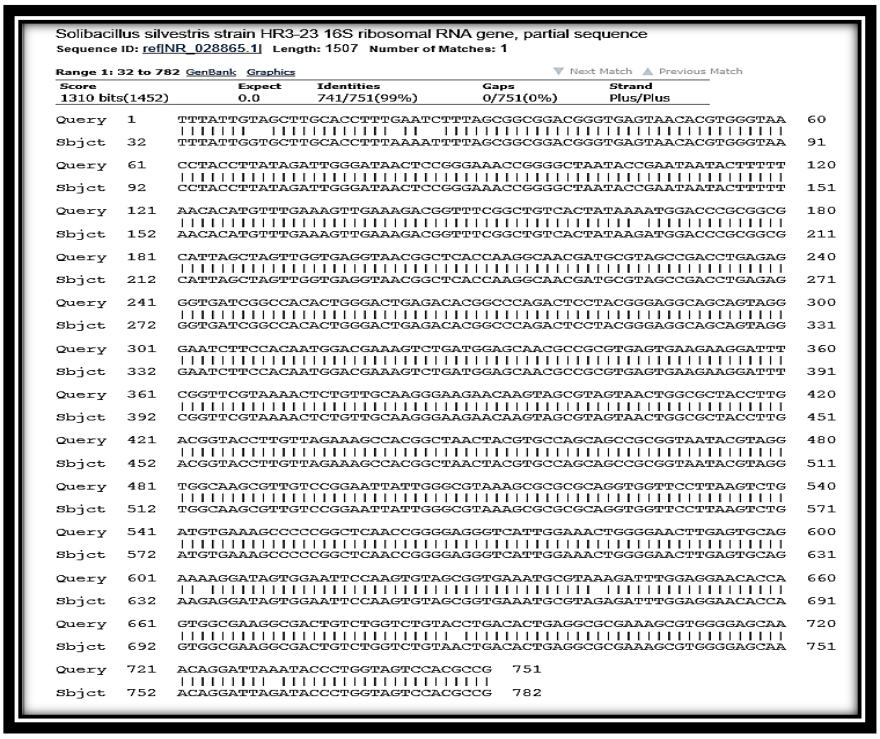


Fig.44 Shows *Solibacillus silvestris* gene sequences

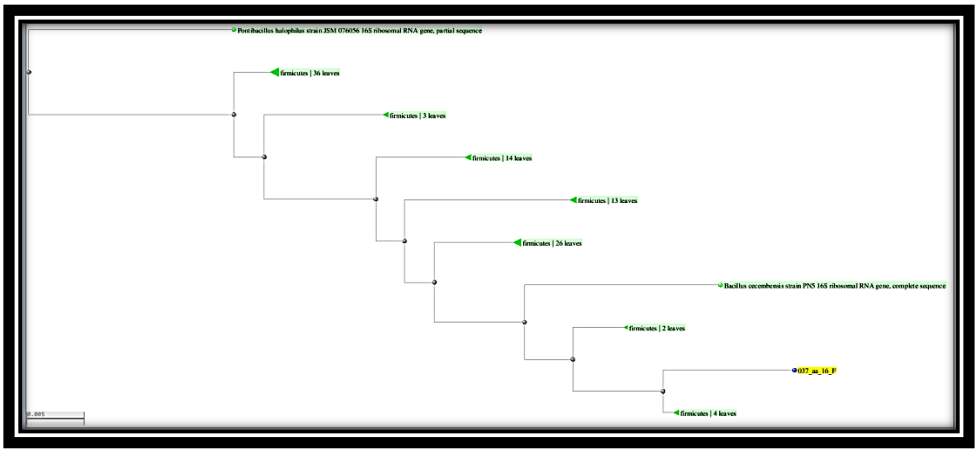


Fig.45 Shows *Solibacillus silvestris* tree

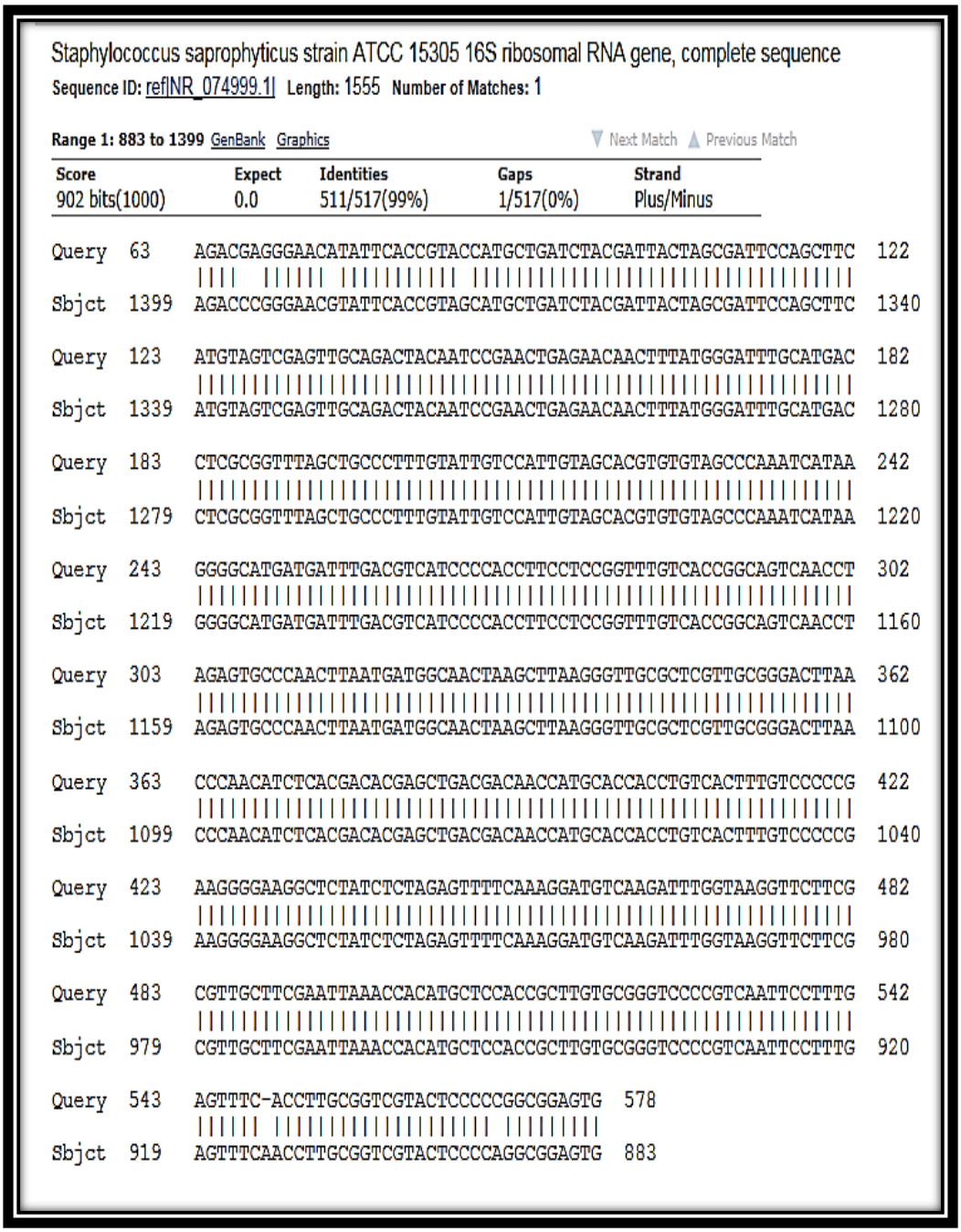


Fig.46 Shows *Staphylococcus saprophyticus* gene sequences

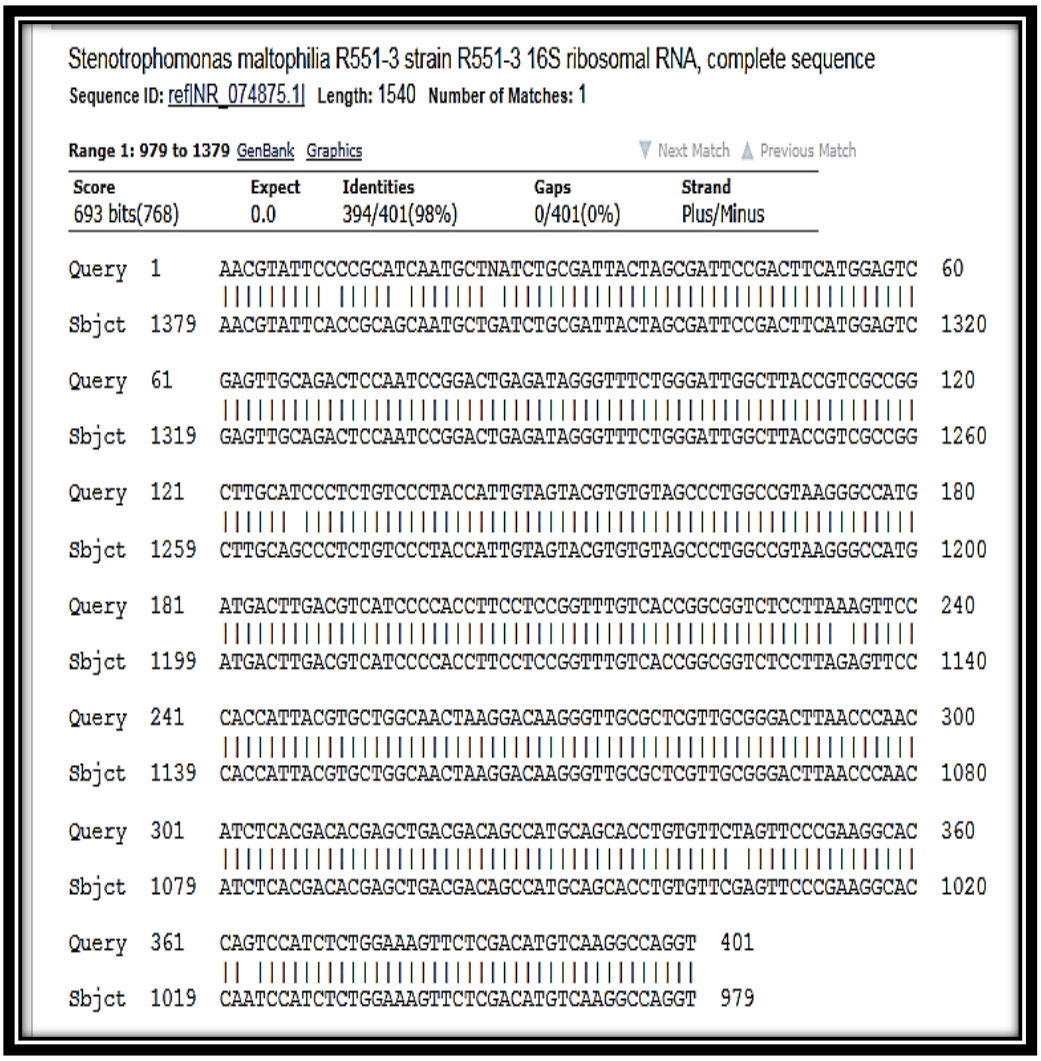
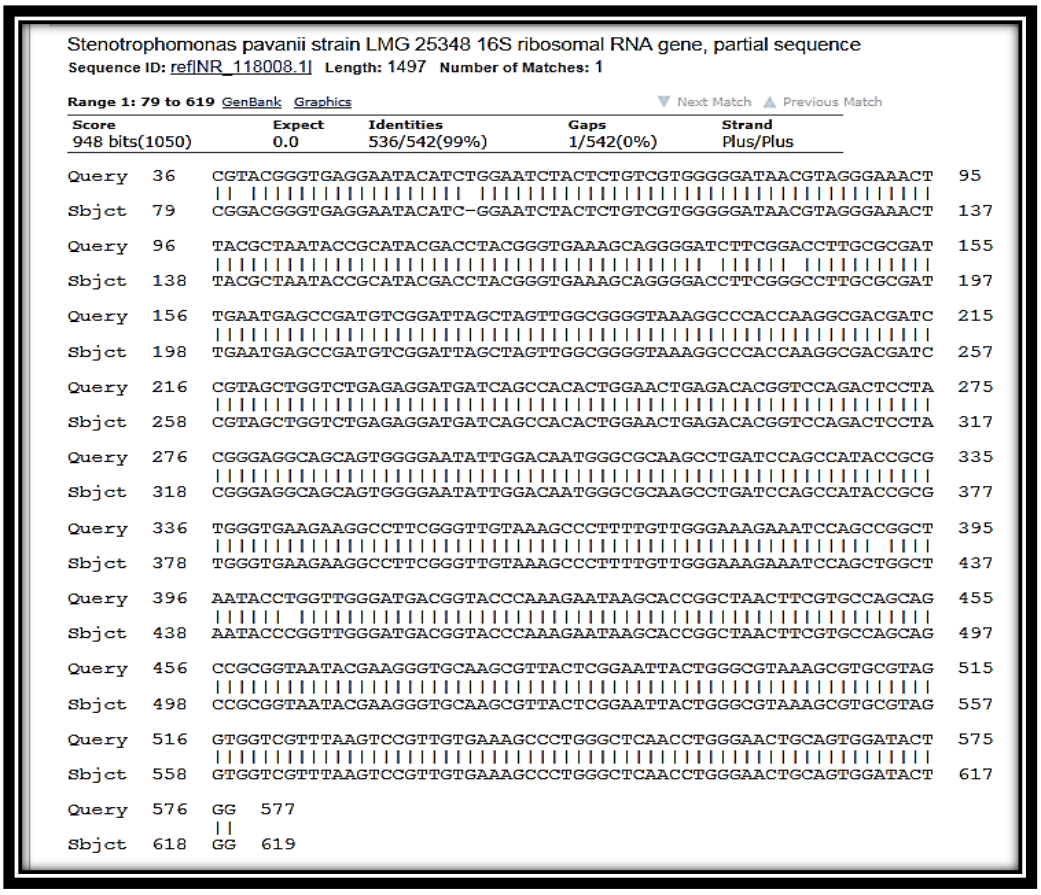


Fig.47 Shows *Stenotrophomonas maltophilia* gene sequences



Stenotrophomonas pavanii strain LMG 25348 16S ribosomal RNA gene, partial sequence

Sequence ID: refNR_118008.1| Length: 1497 Number of Matches: 1

Range 1: 79 to 619 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
948 bits(1050)	0.0	536/542(99%)	1/542(0%)	Plus/Plus

```
Query  36   CGTACGGGTGAGGAATACATCTGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACT  95
            ||  ||||||||||||||||||  ||||||||||||||||||||||||||||||||||||
Sbjct  79   CGGACGGGTGAGGAATACATC-GGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACT  137

Query  96   TACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGAT  155
            ||||||||||||||||||||||||||||||||||||||||||||  ||||||  ||||||||||
Sbjct  138  TACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGAT  197

Query  156  TGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATC  215
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  198  TGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATC  257

Query  216  CGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA  275
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  258  CGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTA  317

Query  276  CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCG  335
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  318  CGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCG  377

Query  336  TGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCT  395
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||||
Sbjct  378  TGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCT  437

Query  396  AATACCTGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAG  455
            ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  438  AATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAG  497

Query  456  CCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAG  515
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  498  CCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAG  557

Query  516  GTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT  575
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  558  GTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACT  617

Query  576  GG  577
            ||
Sbjct  618  GG  619
```

Fig.48 Shows *Stenotrophomonas pavanii* gene sequences

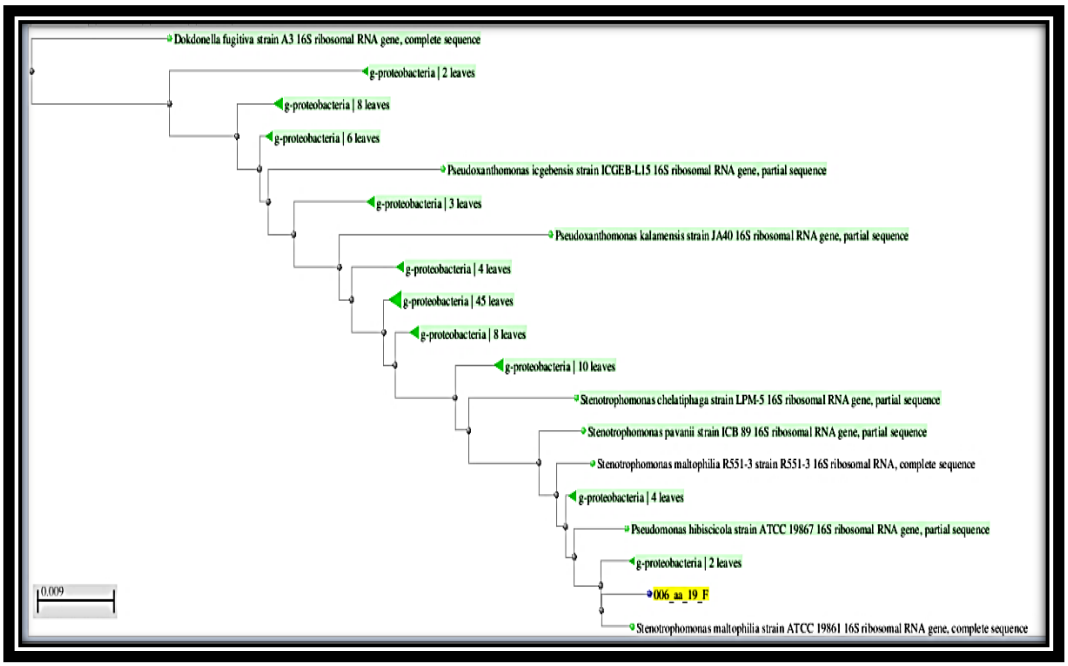


Fig.49Shows *Stenotrophomonas pavanii* tree

Fig.50 Shows Elephant Hawk-moth Deilephila elpenor used to isolate the bacteria(authour's own image)

Staphylococcus succinus strain AMG-D1 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_028667.1| **Length:** 1548 **Number of Matches:** 1

Range 1: 137 to 773 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1108 bits(1228)	0.0	628/637(99%)	0/637(0%)	Plus/Plus

```
Query  68   GACAGCACTAACTTCTGCCAACCTGATCTAATGCCGGATAACATATAGAACCGCATGGTT  127
            ||| | | |||||||| |   |||| || ||||||||||||||||||||||||||||||
Sbjct  137  GACTGGAATAACTTCGGGAAACCGGAGCTAATGCCGGATAACATATAGAACCGCATGGTT  196

Query  128  CTATAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTT  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  CTATAGTGAAAGATGGTTTTGCTATCACTTATAGATGGACCCGCGCCGTATTAGCTAGTT  256

Query  188  GGTAAGGTAAAGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCA  247
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  GGTAAGGTAATGGCTTACCAAGGCGACGATACGTAGCCGACCTGAGAGGGTGATCGGCCA  316

Query  248  CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  CACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  376

Query  308  ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAA  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAA  436

Query  368  CTCTGTTATTAGGGAAGAACAAATGCGTAAGTAACTGTGCGCATCTTGACGGTACCTAAT  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  CTCTGTTATTAGGGAAGAACAAATGCGTAAGTAACTGTGCGCATCTTGACGGTACCTAAT  496

Query  428  CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTA  556

Query  488  TCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC  547
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  557  TCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCC  616

Query  548  ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTG  607
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  617  ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTG  676

Query  608  GAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCG  667
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  677  GAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCG  736

Query  668  ACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGC  704
            |||||||||||||||||||||||||||||||||||||
Sbjct  737  ACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGC  773
```

Fig.51 Shows *Staphylococcus succinus* gene sequences

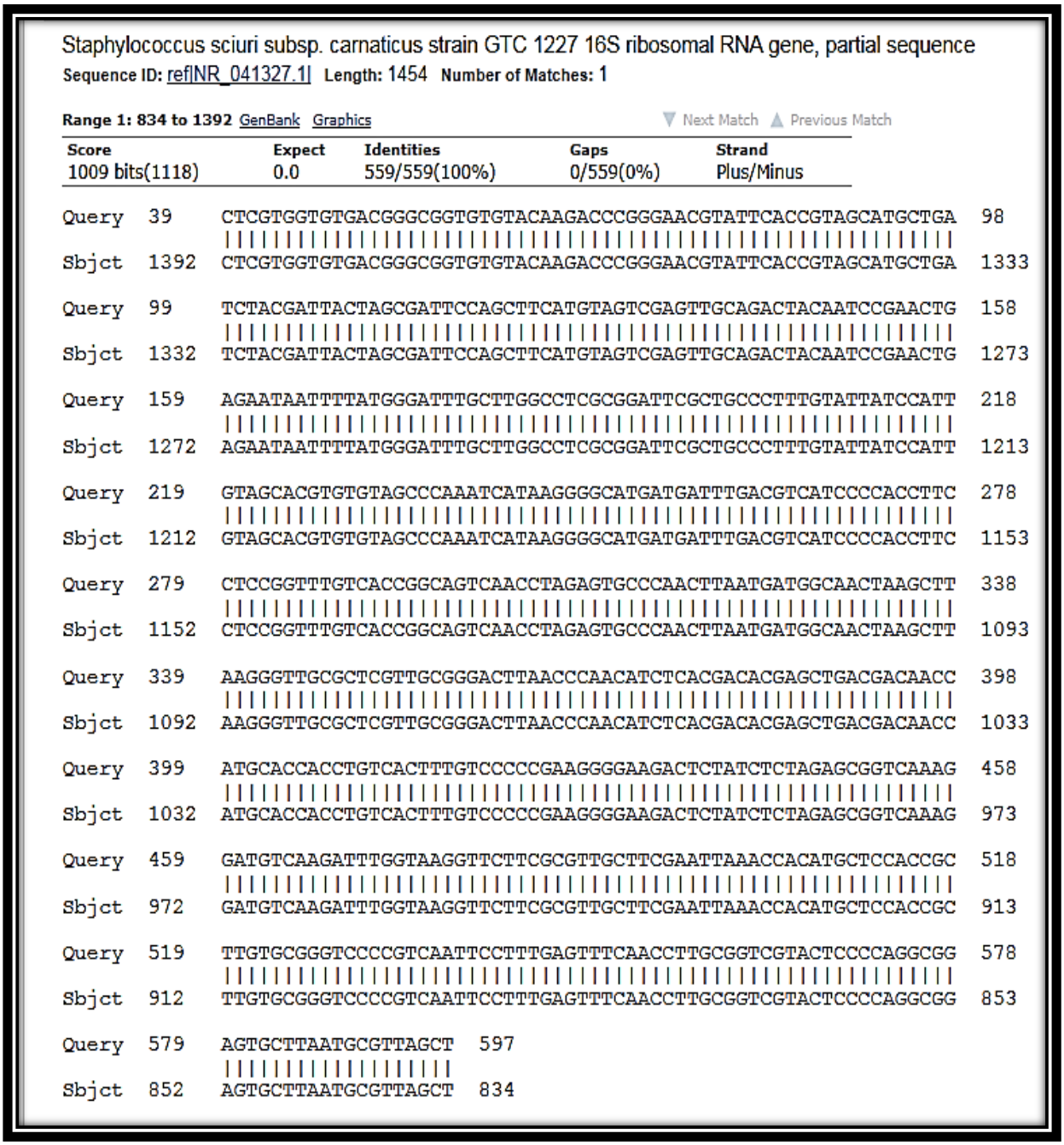


Fig.52 Shows *Staphylococcus sciuri* gene sequences

Fig.53 Shows Rosy Maple Moth (Dryocampa rubicunda) moth used to isolate the bacteria(authour's own image)

Staphylococcus saprophyticus strain ATCC 15305 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_074999.1| **Length:** 1555 **Number of Matches:** 1

Range 1: 114 to 839 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1297 bits(1438)	0.0	723/726(99%)	0/726(0%)	Plus/Plus

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Query  1    AGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  AGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATA  173

Query  61   CCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  CCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATA  233

Query  121  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACG  180
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  234  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACG  293

Query  181  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  353

Query  241  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  413

Query  301  AGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  AGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTA  473

Query  361  ACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  420
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  ACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  533

Query  421  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  593

Query  481  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG  653

Query  541  GAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  GAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA  713

Query  601  TATGGAGGAACACCAGTGGCGAANGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGA  660
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  714  TATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGA  773

Query  661  AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC  833

Query  721  TAAGTG  726
            ||||||
Sbjct  834  TAAGTG  839
```

Fig.54 Shows *Staphylococcus saprophyticus* gene sequences

Fig.55 Shows Small white moth used to isolate the bacteria(authour's own image)

Staphylococcus saprophyticus strain ATCC 15305 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_074999.1| **Length:** 1555 **Number of Matches:** 1

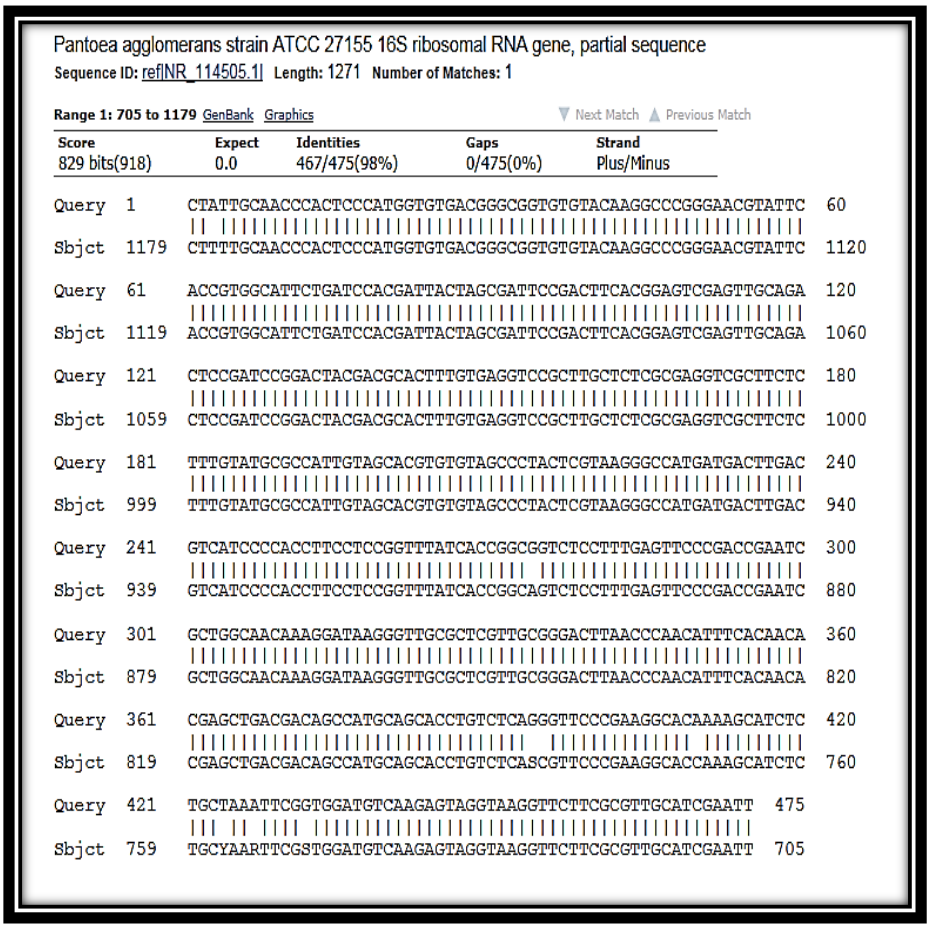
Range 1: 114 to 839 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1297 bits(1438)	0.0	723/726(99%)	0/726(0%)	Plus/Plus

```
Query  1    AGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  AGTAACACGTGGGTAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATA  173

Query  61   CCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  CCGGATAACATTTGGAACCGCATGGTTCTAAAGTGAAAGATGGTTTTGCTATCACTTATA  233

Query  121  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATACG  180
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  234  GATGGACCCGCGCCGTATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCGACGATACG  293

Query  181  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  TAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCAGACTCCTACG  353

Query  241  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  GGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTG  413

Query  301  AGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  AGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTA  473

Query  361  ACTGTGCACATCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  420
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  ACTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCC  533

Query  421  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534  GCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGC  593

Query  481  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  594  GGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGG  653

Query  541  GAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  654  GAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGA  713

Query  601  TATGGAGGAACACCAGTGGCGAANGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGA  660
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  714  TATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGA  773

Query  661  AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  AAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC  833

Query  721  TAAGTG  726
            ||||||
Sbjct  834  TAAGTG  839
```

Fig.56 Shows *Staphylococcus saprophyticus* gene sequences

Fig.57 Shows Atlas moth (*Attacus atlas*) moth used to isolate the bacteria(authour's own image)



Pantoea agglomerans strain ATCC 27155 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_114505.1| Length: 1271 Number of Matches: 1

Range 1: 705 to 1179 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
829 bits(918)	0.0	467/475(98%)	0/475(0%)	Plus/Minus

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Query  1     CTATTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC  60
             ||                                                          
Sbjct  1179  CTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC  1120

Query  61    ACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCACGGAGTCGAGTTGCAGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1119  ACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCACGGAGTCGAGTTGCAGA  1060

Query  121   CTCCGATCCGGACTACGACGCACTTTGTGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1059  CTCCGATCCGGACTACGACGCACTTTGTGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTC  1000

Query  181   TTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGAC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  999   TTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGAC  940

Query  241   GTCATCCCCACCTTCCTCCGGTTTATCACCGGCGGTCTCCTTTGAGTTCCCGACCGAATC  300
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  939   GTCATCCCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCTTTGAGTTCCCGACCGAATC  880

Query  301   GCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  879   GCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACA  820

Query  361   CGAGCTGACGACAGCCATGCAGCACCTGTCTCAGGGTTCCCGAAGGCACAAAAGCATCTC  420
             |||||||||||||||||||||||||||||||  ||||||||||||||| ||||||||||
Sbjct  819   CGAGCTGACGACAGCCATGCAGCACCTGTCTCASCGTTCCCGAAGGCACCAAAGCATCTC  760

Query  421   TGCTAAATTCGGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATT  475
             ||| || |||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  759   TGCYAARTTCGSTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATT  705
```

Fig.58 Shows *Pantoea agglomerans* gene sequences

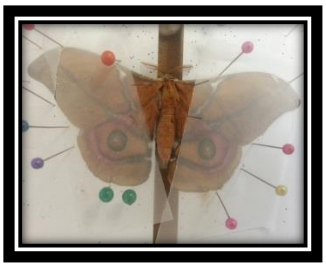
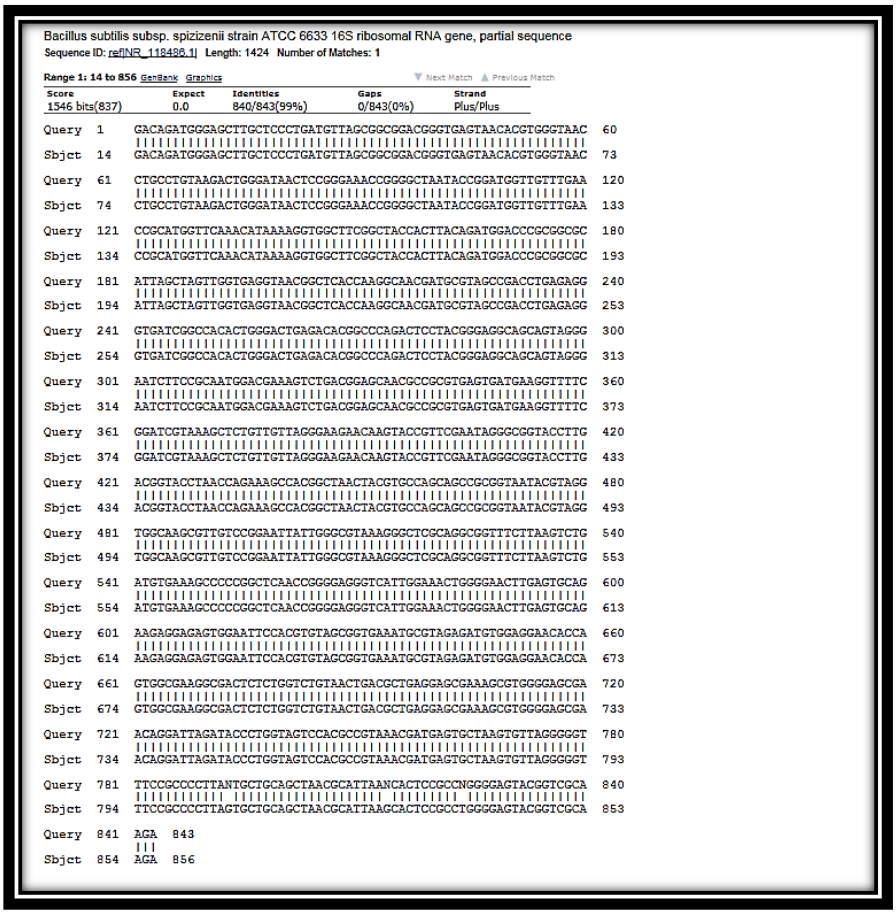


Fig.59 Shows Slik moth used to isolate the bacteria(authour's own image)



Bacillus subtilis subsp. spizizenii strain ATCC 6633 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_118486.1| Length: 1424 Number of Matches: 1

Range 1: 14 to 856 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1546 bits(837)	0.0	840/843(99%)	0/843(0%)	Plus/Plus

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Query  1    GACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14   GACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC  73

Query  61   CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  74   CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAA  133

Query  121  CCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  CCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGC  193

Query  181  ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGG  253

Query  241  GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG  313

Query  301  AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314  AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC  373

Query  361  GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374  GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG  433

Query  421  ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434  ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  493

Query  481  TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTG  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  494  TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTG  553

Query  541  ATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  554  ATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG  613

Query  601  AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  614  AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA  673

Query  661  GTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  674  GTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA  733

Query  721  ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT  780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  734  ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT  793

Query  781  TTCGCGCCCCTTANTGCTGCAGCTAACGCATTAANCACTCCGCCNGGGGAGTACGGTCGCA  840
            ||||||||||| |||||||||||||||||||| |||||||| ||||||||||||||||||
Sbjct  794  TTCGCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCA  853

Query  841  AGA  843
            |||
Sbjct  854  AGA  856
```
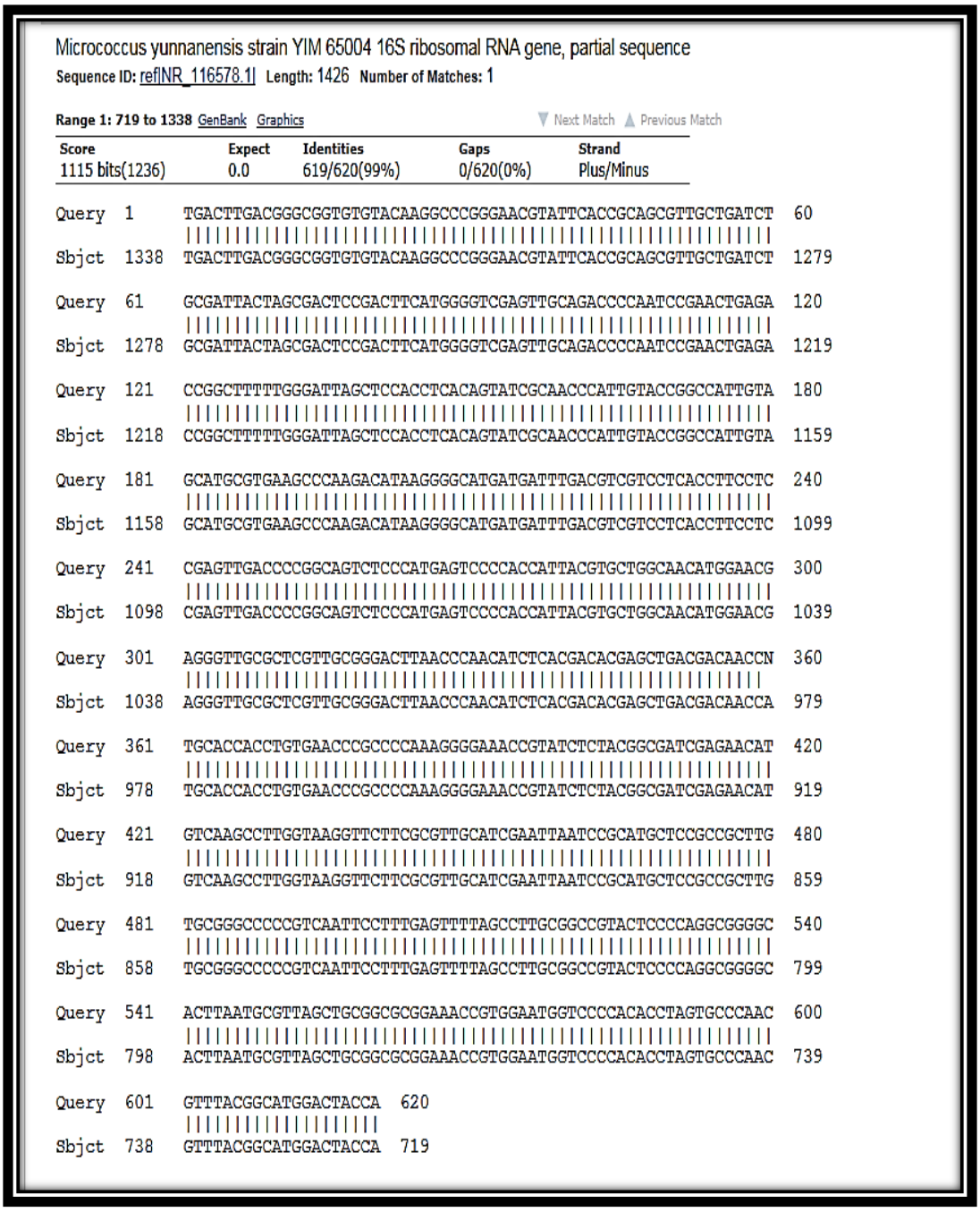

Fig.60 Shows *Bacillus subtilis* gene sequences



Micrococcus yunnanensis strain YIM 65004 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_116578.1| Length: 1426 Number of Matches: 1

Range 1: 719 to 1338 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1115 bits(1236)	0.0	619/620(99%)	0/620(0%)	Plus/Minus

```
Query  1     TGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1338  TGACTTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCT  1279

Query  61    GCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278  GCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCCAATCCGAACTGAGA  1219

Query  121   CCGGCTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCATTGTACCGGCCATTGTA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1218  CCGGCTTTTTGGGATTAGCTCCACCTCACAGTATCGCAACCCATTGTACCGGCCATTGTA  1159

Query  181   GCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCGTCCTCACCTTCCTC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1158  GCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCGTCCTCACCTTCCTC  1099

Query  241   CGAGTTGACCCCGGCAGTCTCCCATGAGTCCCCACCATTACGTGCTGGCAACATGGAACG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1098  CGAGTTGACCCCGGCAGTCTCCCATGAGTCCCCACCATTACGTGCTGGCAACATGGAACG  1039

Query  301   AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCN  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1038  AGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCA  979

Query  361   TGCACCACCTGTGAACCCGCCCCAAAGGGGAAACCGTATCTCTACGGCGATCGAGAACAT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  978   TGCACCACCTGTGAACCCGCCCCAAAGGGGAAACCGTATCTCTACGGCGATCGAGAACAT  919

Query  421   GTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTG  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  918   GTCAAGCCTTGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTG  859

Query  481   TGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  858   TGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGC  799

Query  541   ACTTAATGCGTTAGCTGCGGCGCGGAAACCGTGGAATGGTCCCCACACCTAGTGCCCAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  798   ACTTAATGCGTTAGCTGCGGCGCGGAAACCGTGGAATGGTCCCCACACCTAGTGCCCAAC  739

Query  601   GTTTACGGCATGGACTACCA  620
             ||||||||||||||||||||
Sbjct  738   GTTTACGGCATGGACTACCA  719
```

Fig.61 Shows *Micrococcus yunnanensis* gene sequences

Fig.62 Shows Eastern Comma(Polygonia comma) moth used to isolate the bacteria(authour's own image)

Bacillus licheniformis strain DSM 13 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_118996.1| **Length:** 1545 **Number of Matches:** 1

Range 1: 62 to 854 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1459 bits(790)	0.0	792/793(99%)	0/793(0%)	Plus/Plus

```
Query  1    GACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   GACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAAC  121

Query  61   CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAA  181

Query  121  CCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  CCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCG  241

Query  181  CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  CATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG  301

Query  241  GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGG  361

Query  301  GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  GAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT  421

Query  361  CGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  CGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTT  481

Query  421  GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG  541

Query  481  GTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCT  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  GTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCT  601

Query  541  GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA  661

Query  601  GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC  721

Query  661  AGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCG  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722  AGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCG  781

Query  721  AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAAAGGG  780
            |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  782  AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGG  841

Query  781  TTTCCGCCCTTTA  793
            |||||||||||||
Sbjct  842  TTTCCGCCCTTTA  854
```

Fig.63 Shows *Bacillus licheniformis* gene sequences

Fig.64 Shows Eastern Comma(Polygonia comma) which used to isolate the bacteria (authour's own image)

Stenotrophomonas maltophilia R551-3 strain R551-3 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074875.1| **Length:** 1540 **Number of Matches:** 1

Range 1: 759 to 1386 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1128 bits(1250)	0.0	627/628(99%)	0/628(0%)	Plus/Minus

```
Query  1     GCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1386  GCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCA  1327

Query  61    TGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1326  TGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTACCG  1267

Query  121   TCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1266  TCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAG  1207

Query  181   GGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1206  GGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTA  1147

Query  241   GAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1146  GAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTA  1087

Query  301   ACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1086  ACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCG  1027

Query  361   AAGGCACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1026  AAGGCACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGC  967

Query  421   GTTGCATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  966   GTTGCATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGA  907

Query  481   GTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGATACTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  906   GTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGATACTG  847

Query  541   CGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGGACTACCAAGGTAT  600
             |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  846   CGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTTAGGGCGTGGACTACCAGGGTAT  787

Query  601   CTAATCCTGTTTGCTCCCCACGCTTTCG  628
             ||||||||||||||||||||||||||||
Sbjct  786   CTAATCCTGTTTGCTCCCCACGCTTTCG  759
```

Fig.65 Shows *Stenotrophomonas maltophilia* gene sequences

Fig.66 Shows Butterfly larvea which used to isolate the bacteria(authour's own image)

Staphylococcus sciuri subsp. carnaticus strain GTC 1227 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_041327.1| Length: 1454 Number of Matches: 1

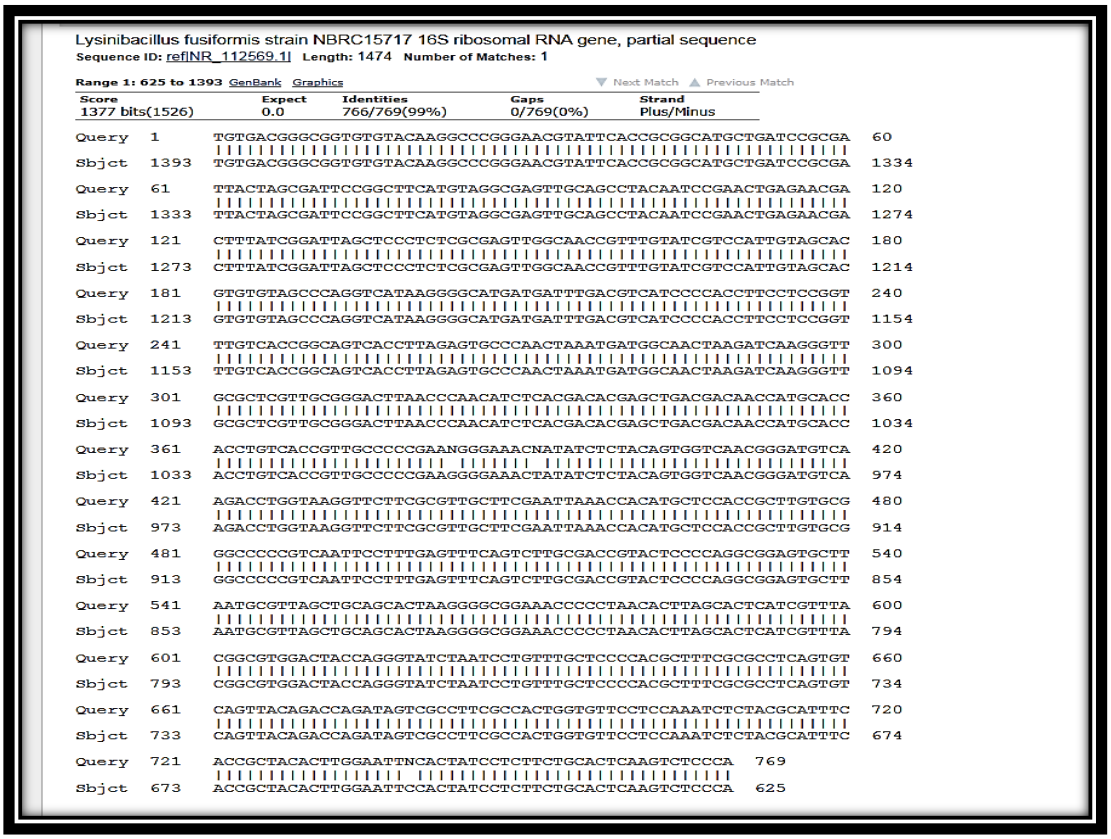
Range 1: 855 to 1398 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
895 bits(992)	0.0	525/544(97%)	0/544(0%)	Plus/Minus

```
Query  3     CAAGCGCTCGTGGGGTGACGGGCGGTGTGTACAAGACCCGGAAACGTATTCACCGTACCA  62
             ||| | |||||||| ||||||||||||||||||||||||||| ||||||||||||||| ||
Sbjct  1398  CAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGTAGCA  1339

Query  63    TGCTGATCTACGATTACTAGCGATTCCAACTTCATGTAGTCGAGTTGCAGACTACAATCC  122
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  1338  TGCTGATCTACGATTACTAGCGATTCCAGCTTCATGTAGTCGAGTTGCAGACTACAATCC  1279

Query  123   GAACTGAGAATAATTTTATGGGATTTGCTTGGCCTCGCGGATTCGCTGCCCTTTGTATTA  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1278  GAACTGAGAATAATTTTATGGGATTTGCTTGGCCTCGCGGATTCGCTGCCCTTTGTATTA  1219

Query  183   TCCATTGTAGCACGTGTGTAGCCCAGATCATAAGGGGCATGATGATTTGACGTCATCCCC  242
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  1218  TCCATTGTAGCACGTGTGTAGCCCAAATCATAAGGGGCATGATGATTTGACGTCATCCCC  1159

Query  243   ACCTTCCTCCGGTTTGTCACCGGCAGTCTACCTAGAGTGCCCAACTTAATGAGGGGAACT  302
             |||||||||||||||||||||||||| |||||||||||||||||||| || ||||
Sbjct  1158  ACCTTCCTCCGGTTTGTCACCGGCAGTCAACCTAGAGTGCCCAACTTAATGATGGCAACT  1099

Query  303   AAACTTAAGGGTTGCGCTCGTTGCGGGACTTATCCCAACATCTCACGACACGAGCTGACA  362
             || |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  1098  AAGCTTAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACG  1039

Query  363   ACAACCATGCGCCACCTGTGAGTTTGACCCCCGAAAGGGAACACTCTATCTCTAGAGCGG  422
             |||||||||| |||||||| | |||| |||||||| ||||| ||||||||||||||||||
Sbjct  1038  ACAACCATGCACCACCTGTCACTTTGTCCCCCGAAGGGGAAGACTCTATCTCTAGAGCGG  979

Query  423   TCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  978   TCAAAGGATGTCAAGATTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC  919

Query  483   CACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCC  542
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  918   CACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCC  859

Query  543   AGGC  546
             ||||
Sbjct  858   AGGC  855
```

Fig.67 Shows *Staphylococcus sciuri* gene sequences

Fig.68 Shows Butterfly larvea which used to isolate the bacteria(authour's own image)



Lysinibacillus fusiformis strain NBRC15717 16S ribosomal RNA gene, partial sequence

Sequence ID: refINR_112569.1| Length: 1474 Number of Matches: 1

Range 1: 625 to 1393 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1377 bits(1526)	0.0	766/769(99%)	0/769(0%)	Plus/Minus

```
Query  1     TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA  60
Sbjct  1393  TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGA  1334

Query  61    TTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGA  120
Sbjct  1333  TTACTAGCGATTCCGGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGA  1274

Query  121   CTTTATCGGATTAGCTCCCTCTCGCGAGTTGGCAACCGTTTGTATCGTCCATTGTAGCAC  180
Sbjct  1273  CTTTATCGGATTAGCTCCCTCTCGCGAGTTGGCAACCGTTTGTATCGTCCATTGTAGCAC  1214

Query  181   GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGT  240
Sbjct  1213  GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGT  1154

Query  241   TTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGATGGCAACTAAGATCAAGGGTT  300
Sbjct  1153  TTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAATGATGGCAACTAAGATCAAGGGTT  1094

Query  301   GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC  360
Sbjct  1093  GCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACC  1034

Query  361   ACCTGTCACCGTTGCCCCCGAANGGGAAACNATATCTCTACAGTGGTCAACGGGATGTCA  420
Sbjct  1033  ACCTGTCACCGTTGCCCCCGAAGGGGAAACTATATCTCTACAGTGGTCAACGGGATGTCA  974

Query  421   AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG  480
Sbjct  973   AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCG  914

Query  481   GGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTT  540
Sbjct  913   GGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTT  854

Query  541   AATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTA  600
Sbjct  853   AATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTA  794

Query  601   CGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGT  660
Sbjct  793   CGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGT  734

Query  661   CAGTTACAGACCAGATAGTCGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTC  720
Sbjct  733   CAGTTACAGACCAGATAGTCGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTC  674

Query  721   ACCGCTACACTTGGAATTNCACTATCCTCTTCTGCACTCAAGTCTCCCA  769
Sbjct  673   ACCGCTACACTTGGAATTCCACTATCCTCTTCTGCACTCAAGTCTCCCA  625
```

Fig.69 Shows *Lysinibacillus fusiformis* gene sequences

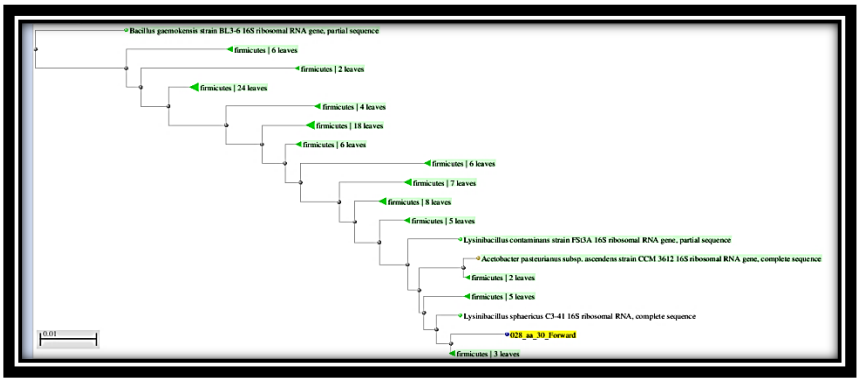


Fig.70 Shows *Lysinibacillus fusiformis* bacteria Tree

Fig.71 Shows Butterfly larvea which used to isolate the bacteria(authour's own image)

Stenotrophomonas rhizophila strain e-p10 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_121739.1| **Length:** 1546 **Number of Matches:** 1

Range 1: 888 to 1389 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
906 bits(1004)	0.0	502/502(100%)	0/502(0%)	Plus/Minus

```
Query  70    CGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGG  129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1389  CGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGATTCCGACTTCATGG  1330

Query  130   AGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTGCCCTCG  189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1329  AGTCGAGTTGCAGACTCCAATCCGGACTGAGATAGGGTTTCTGGGATTGGCTTGCCCTCG  1270

Query  190   CGGGTTTGCAGCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGTCGTAAGGGC  249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269  CGGGTTTGCAGCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGTCGTAAGGGC  1210

Query  250   CATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAG  309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1209  CATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAG  1150

Query  310   TTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACC  369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1149  TTCCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACC  1090

Query  370   CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAG  429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1089  CAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAG  1030

Query  430   GCACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGACCAGGTAAGGTTCTTCGCGTT  489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1029  GCACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGACCAGGTAAGGTTCTTCGCGTT  970

Query  490   GCATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTT  549
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  969   GCATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTT  910

Query  550   TCAGTCTTGCGACCGTACTCCC  571
             |||||||||||||||||||||
Sbjct  909   TCAGTCTTGCGACCGTACTCCC  888
```

Fig.72 Shows *Stenotrophomonas rhizophila* gene sequences

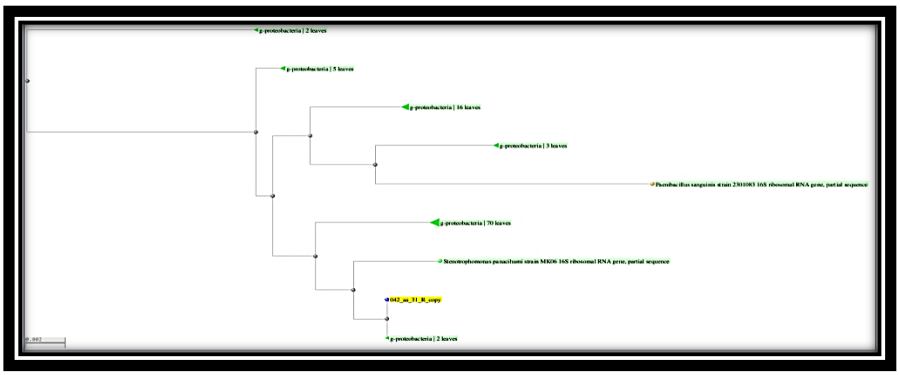


Fig.73 Shows *Stenotrophomonas rhizophila* tree

Fig.74 Shows Butterfly larvea which used to isolate the bacteria(authour's own image)

Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence

Sequence ID: ref|NR_074540.1| **Length:** 1512 **Number of Matches:** 1

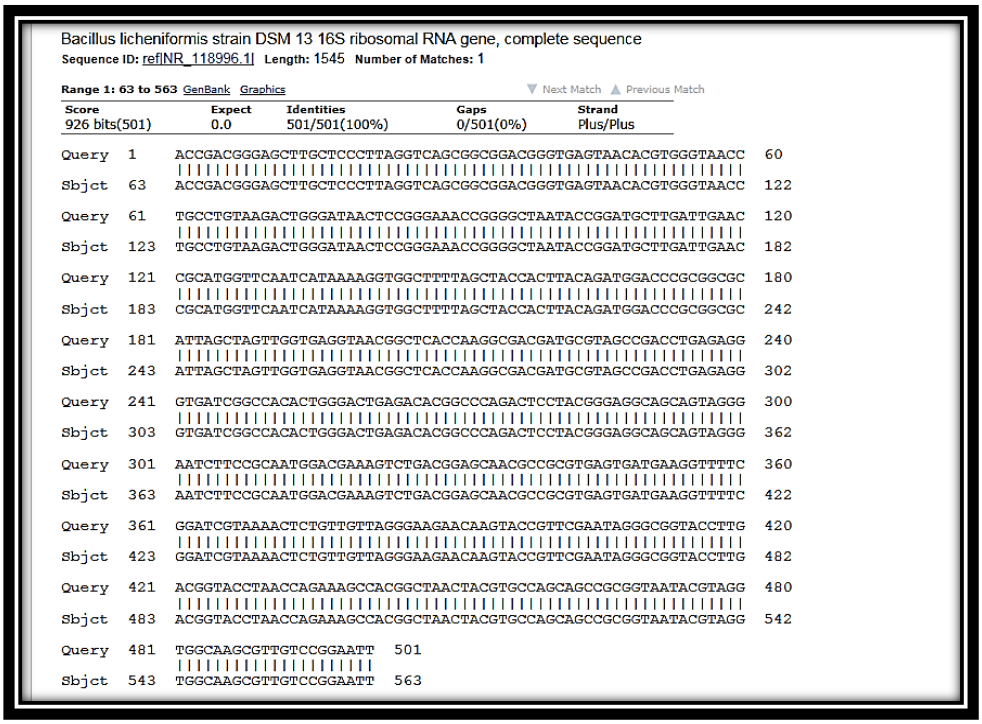
Range 1: 120 to 637 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
852 bits(944)	0.0	502/518(97%)	3/518(0%)	Plus/Plus

```
Query  63   ACCTGGGTATC-TGCCCGGA-GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT  120
            ||  |||||||  |  |||||   |  ||||||||||||||||||||||||||||||||||
Sbjct  120  ACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGAT  179

Query  121  AACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  AACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGG  239

Query  181  ACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  ACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCC  299

Query  241  GACCTGACAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  300
            |||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG  359

Query  301  CAGC-GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCATCGCCGCGTGAGTGA  359
            ||||  ||||||||||||||||||||||||||||||||||||||  ||||||||||||||
Sbjct  360  CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA  419

Query  360  TGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  TGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG  479

Query  420  CTGGCACCTTGACGGTACCTAACCACAGAGCCACGGCTAACTACGTGCCAACATCCGCGG  479
            ||||||||||||||||||||||||  |  ||||||||||||||||||||||  ||  |||||||
Sbjct  480  CTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG  539

Query  480  TAATACTTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTT  539
            ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  540  TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTT  599

Query  540  TCTTAAATCTGATGTGAAATCCCACGGCTCAACCGTGG  577
            ||||||  ||||||||||||  ||||||||||||||||
Sbjct  600  TCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG  637
```

Fig.75 Shows *Bacillus cereus* gene sequences

Fig.76 Shows Butterfly larvea which used to isolate the bacteria(authour's own image)



Bacillus licheniformis strain DSM 13 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_118996.1| Length: 1545 Number of Matches: 1

Range 1: 63 to 563 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
926 bits(501)	0.0	501/501(100%)	0/501(0%)	Plus/Plus

```
Query  1    ACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   ACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACC  122

Query  61   TGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  TGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAAC  182

Query  121  CGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  CGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGCGC  242

Query  181  ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGG  302

Query  241  GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG  362

Query  301  AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC  422

Query  361  GGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  GGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTG  482

Query  421  ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG  542

Query  481  TGGCAAGCGTTGTCCGGAATT  501
            |||||||||||||||||||
Sbjct  543  TGGCAAGCGTTGTCCGGAATT  563
```

Fig.77 Shows *Lysinibacillus macroides* gene sequences

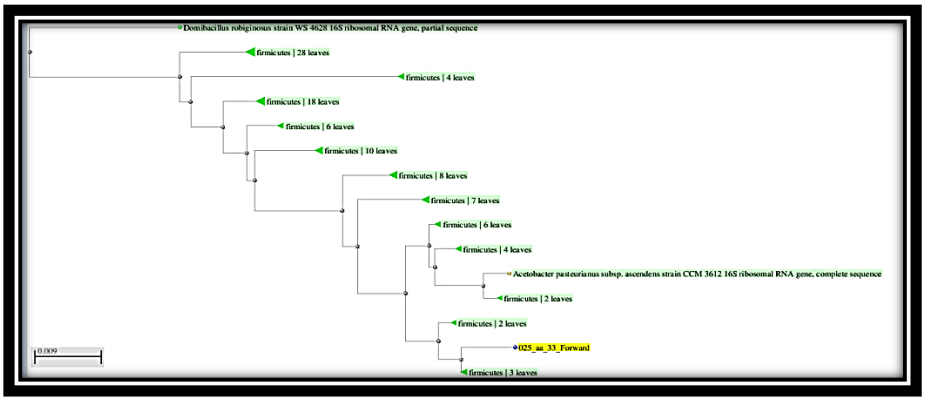


Fig.78 Shows *Lysinibacillus macroides* tree

Fig.79 Shows Nettle leaf which treated by *Bacillus thuringiensis* used to feed butterfly larveas(authour's own image)

Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence

Sequence ID: ref|NR_074540.1| **Length:** 1512 **Number of Matches:** 1

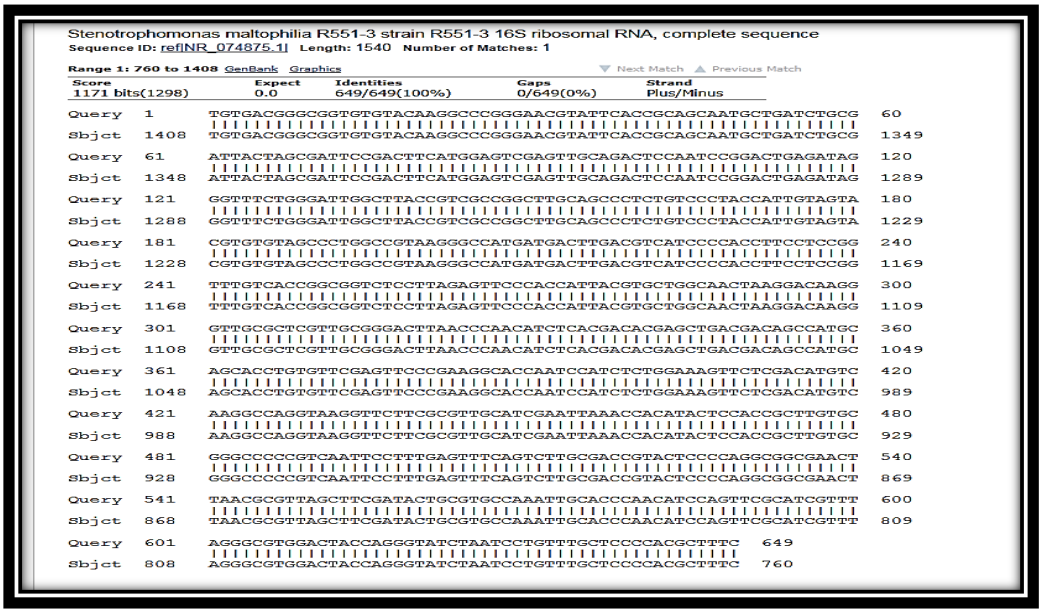
Range 1: 82 to 794 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1261 bits(1398)	0.0	707/713(99%)	0/713(0%)	Plus/Plus

```
Query  1    CTTGCTCTTATGAGGTTCGCGGGGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAG  60
            ||||||||||||| ||| |||| ||||||||||||||||||||||||||||||||||||
Sbjct  82   CTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAG  141

Query  61   ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  142  ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC  201

Query  121  GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  202  GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTT  261

Query  181  GGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  262  GGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA  321

Query  241  CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322  CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA  381

Query  301  ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382  ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAA  441

Query  361  CTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442  CTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA  501

Query  421  CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502  CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT  561

Query  481  ATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  562  ATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCC  621

Query  541  CACGGCTCACCCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGT  600
            ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  622  CACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGT  681

Query  601  GGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGN  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  682  GGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC  741

Query  661  GANTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAG  713
            || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  742  GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAG  794
```

Fig.80 Shows *Bacillus cereus* gene sequences

Fig.81 Shows Nettles leave treated by *E.coli* and feed butterfly larvae then Isolate from inside larvea died body (authour's own image)



Stenotrophomonas maltophilia R551-3 strain R551-3 16S ribosomal RNA, complete sequence

Sequence ID: refINR_074875.1| Length: 1540 Number of Matches: 1

Range 1: 760 to 1408 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1171 bits(1298)	0.0	649/649(100%)	0/649(0%)	Plus/Minus

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Query  1     TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCG  60
Sbjct  1408  TGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCG  1349
Query  61    ATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAG  120
Sbjct  1348  ATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTGAGATAG  1289
Query  121   GGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTA  180
Sbjct  1288  GGTTTCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCTCTGTCCCTACCATTGTAGTA  1229
Query  181   CGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGG  240
Sbjct  1228  CGTGTGTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGG  1169
Query  241   TTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGG  300
Sbjct  1168  TTTGTCACCGGCGGTCTCCTTAGAGTTCCCACCATTACGTGCTGGCAACTAAGGACAAGG  1109
Query  301   GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGC  360
Sbjct  1108  GTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGC  1049
Query  361   AGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGACATGTC  420
Sbjct  1048  AGCACCTGTGTTCGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCGACATGTC  989
Query  421   AAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC  480
Sbjct  988   AAGGCCAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATACTCCACCGCTTGTGC  929
Query  481   GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACT  540
Sbjct  928   GGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGCGAACT  869
Query  541   TAACGCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTT  600
Sbjct  868   TAACGCGTTAGCTTCGATACTGCGTGCCAAATTGCACCCAACATCCAGTTCGCATCGTTT  809
Query  601   AGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTC  649
Sbjct  808   AGGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTC  760
```

Fig.82 Shows *Stenotrophomonas maltophilia* gene sequences

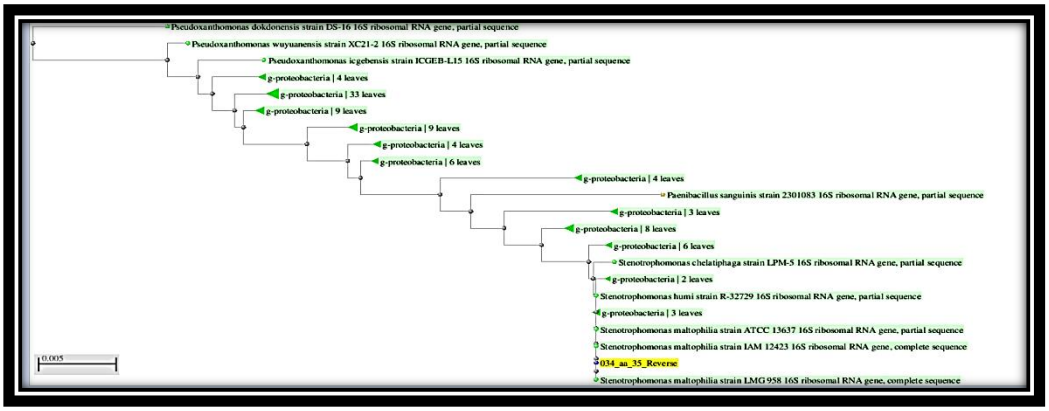


Fig.83 Shows *Staphylococcus saprophyticus* tree

Fig.84 Shows Nettles leave treated by by *B. subtilis* and feed butterfly larvae then Isolate from inside larvea died body (authour's own image)

Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence

Sequence ID: ref|NR_074540.1| **Length:** 1512 **Number of Matches:** 1

Range 1: 81 to 830 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1339 bits(1484)	0.0	747/750(99%)	0/750(0%)	Plus/Plus

```
Query  1    GCTTGCTCTTTATGAGGTTCGCGGGGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAA  60
           ||||||||||||||| ||| |||| ||||||||||||||||||||||||||||||||||||
Sbjct  81   GCTTGCTCTTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAA  140

Query  61   GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTT  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTT  200

Query  121  CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGT  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGT  260

Query  181  TGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  TGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC  320

Query  241  ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC  380

Query  301  AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAA  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAA  440

Query  361  ACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTA  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  ACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTA  500

Query  421  ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT  560

Query  481  TATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561  TATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC  620

Query  541  CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAG  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621  CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAG  680

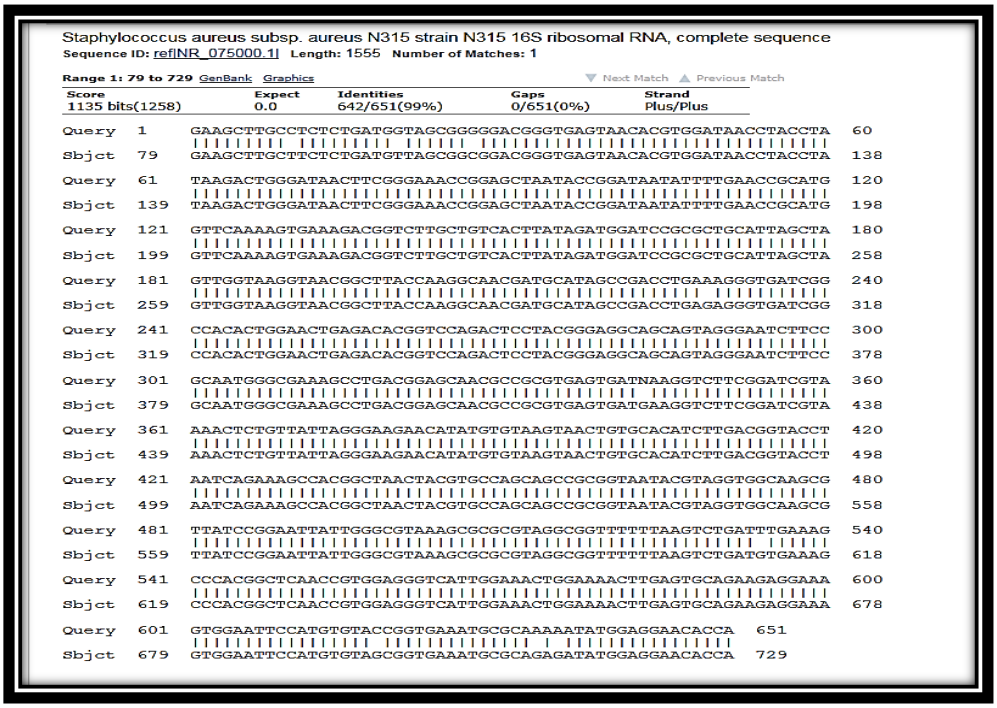
Query  601  TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG  660
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681  TGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGG  740

Query  661  CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG  720
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741  CGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAG  800

Query  721  ATACCCTGGTAGTCCACGCCGTAAACGATG  750
           ||||||||||||||||||||||||||||||
Sbjct  801  ATACCCTGGTAGTCCACGCCGTAAACGATG  830
```

Fig.85 Shows *Bacillus cereus* gene sequences

Fig.86 Shows Nettles leave treated by MRSA bacteria and feed butterfly larvae then Isolate from inside larvae died body (author's own image)



Staphylococcus aureus subsp. aureus N315 strain N315 16S ribosomal RNA, complete sequence

Sequence ID: refINR_075000.1| Length: 1555 Number of Matches: 1

Range 1: 79 to 729 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1135 bits(1258)	0.0	642/651(99%)	0/651(0%)	Plus/Plus

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Query  1    GAAGCTTGCCTCTCTGATGGTAGCGGGGGACGGGTGAGTAACACGTGGATAACCTACCTA  60
            ||||||||| |||||||||  |||||||  ||||||||||||||||||||||||||||||
Sbjct  79   GAAGCTTGCTTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGATAACCTACCTA  138

Query  61   TAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  TAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATTTTGAACCGCATG  198

Query  121  GTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  GTTCAAAAGTGAAAGACGGTCTTGCTGTCACTTATAGATGGATCCGCGCTGCATTAGCTA  258

Query  181  GTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAAAGGGTGATCGG  240
            |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  259  GTTGGTAAGGTAACGGCTTACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGG  318

Query  241  CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  CCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC  378

Query  301  GCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATNAAGGTCTTCGGATCGTA  360
            |||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  379  GCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTCTTCGGATCGTA  438

Query  361  AAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  AAACTCTGTTATTAGGGAAGAACATATGTGTAAGTAACTGTGCACATCTTGACGGTACCT  498

Query  421  AATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499  AATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG  558

Query  481  TTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATTTGAAAG  540
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  559  TTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAG  618

Query  541  CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAA  600
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  619  CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAA  678

Query  601  GTGGAATTCCATGTGTACCGGTGAAATGCGCAAAAATATGGAGGAACACCA  651
            |||||||||||||||| ||||||||||||| | ||||||||||||||||
Sbjct  679  GTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCA  729
```

Fig.87 Shows *Staphylococcus aureus* gene sequences

Fig.88 Shows Nettles leave (Control)feed butterfly larvae then Isolate from inside larvea died body (author's own image)

Stenotrophomonas maltophilia strain IAM 12423 16S ribosomal RNA gene, complete sequence

Sequence ID: ref|NR_041577.1| Length: 1538 Number of Matches: 1

Range 1: 183 to 722 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
933 bits(1034)	0.0	531/540(98%)	0/540(0%)	Plus/Plus

```
Query  6    GGGTGAAAGCATGTGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCT  65
           ||||||||||| | ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  GGGTGAAAGCAGGGGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCT  242

Query  66   AGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCA  125
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  AGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCA  302

Query  126  GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  185
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG  362

Query  186  GACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGT  245
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  GACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGT  422

Query  246  AAAGCCCTTTTGTTGGGAAAGAAATCCAGCCGGCTAATACCGGGTTGGGATGACGGTACC  305
           ||||||||||||||||||||||||||||||| || ||||||| ||||||||||||||||||
Sbjct  423  AAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGTTAATACCCGGTTGGGATGACGGTACC  482

Query  306  CAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC  365
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  CAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGC  542

Query  366  GTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAA  425
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  543  GTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAA  602

Query  426  GGCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGT  485
           | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603  GCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGT  662

Query  486  AGCGAAATTCCTGGTGTAGCAGTGAAATGCGTAAAGATCAAGAGGAACATCCATGGCGAA  545
           |||| |||||||||||||||||||||||||| |||||| ||||||||||||||||||||||
Sbjct  663  AGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAA  722
```

Fig.89 Shows *Stenotrophomonas maltophilia* gene sequences

Fig.90 Shows Nettles leave (Control)feed butterfly larvae then Isolate from inside larvea died body (author's own image)

Stenotrophomonas maltophilia R551-3 strain R551-3 16S ribosomal RNA, complete sequence

Sequence ID: ref|NR_074875.1| **Length:** 1540 **Number of Matches:** 1

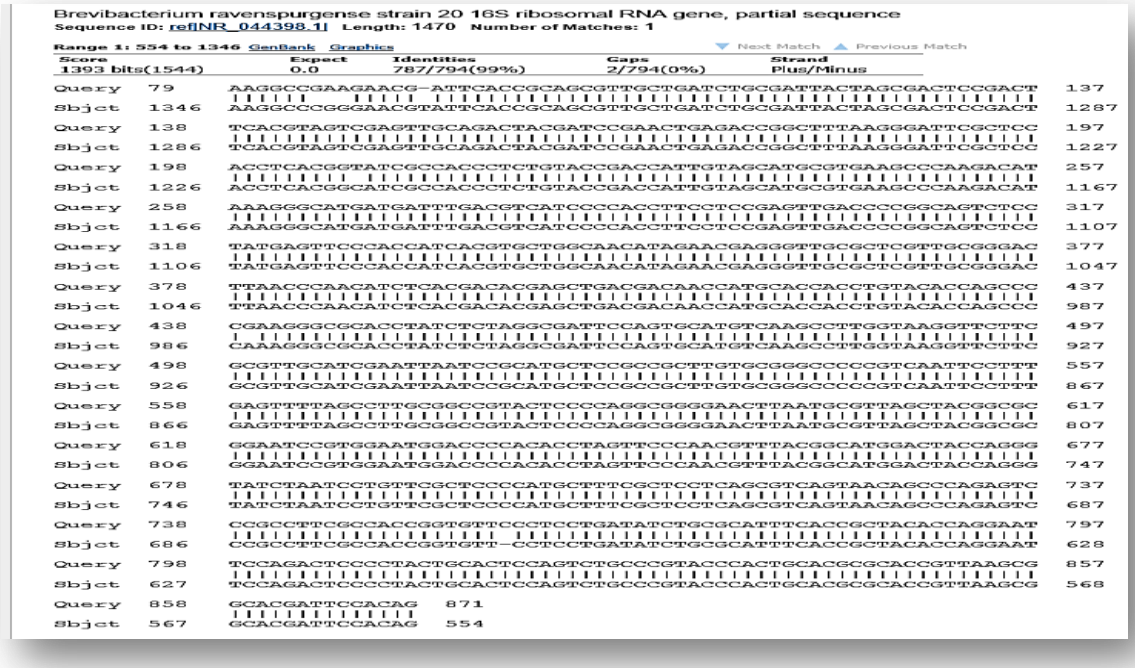
Range 1: 828 to 1261 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
551 bits(298)	2e-156	389/434(90%)	1/434(0%)	Plus/Minus

```
Query  185   GGCGTGCAACCCTTTTTCTTTACCACGGGAATACTTGTGCAGCCCTGGCCGTATCGGCCA  244
             ||| |||| |||| | ||  |||||  | | ||| |||| ||||||||||||||  |||||
Sbjct  1261  GGCTTGCAGCCCTCTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGCCGTAAGGGCCA  1202

Query  245   CGAGGAAATGACATCATCCCCACCTTCCTCCGGTTTGTCACGGGTAGTCTCCTTAGAGTT  304
              || ||  |||| ||||||||||||||||||||||||||||| ||  ||||||||||||||
Sbjct  1201  TGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTT  1142

Query  305   CCCACCATTATGTGCTGGGAACTAACGACAAAGGTTGCGCTCGTTGCGGGACTTAAACCA  364
             |||||||||| ||||||| ||||||  |||||  ||||||||||||||||||||||||| |||
Sbjct  1141  CCCACCATTACGTGCTGGCAACTAAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCA  1082

Query  365   ACATCTCACGACACGAGCTGACGACCGCCATGCACCACCTGTGTTCGAGTTCCCAAAGGC  424
             |||||||||||||||||||||||||| |||||||| |||||||||||||||||||| |||||
Sbjct  1081  ACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTTCGAGTTCCCGAAGGC  1022

Query  425   ACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGGCAAGGTAACGCTCTTCACGATGC  484
             |||||||||||||||||||||||||||||||||||||||| |||||| | ||||| || |||
Sbjct  1021  ACCAATCCATCTCTGGAAAGTTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGC  962

Query  485   GTCAAATTTAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTC  544
              || |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   ATCGAATTAAACCACATACTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTC  902

Query  545   TTTCTTGCGACCGTAC-CCCCAGGCGGTGAACTTAACGCGTTAGCTTCGATACTGCCTGC  603
              |||||||||||||| |||||||||| |||||||||||||||||||||||||||| |||
Sbjct  901   AGTCTTGCGACCGTACTCCCCAGGCGGCGAACTTAACGCGTTAGCTTCGATACTGCGTGC  842

Query  604   AAAATTGAACCCAA  617
              |||||| ||||||
Sbjct  841   CAAATTGCACCCAA  828
```

Fig.91 Shows *Stenotrophomonas maltophilia* gene sequences

Fig.92 Shows larvae blow fly (Calliphora) appeared from human body(author's own image)



Brevibacterium ravenspurgense strain 20 16S ribosomal RNA gene, partial sequence

Sequence ID: refNR_044398.1| Length: 1470 Number of Matches: 1

Range 1: 554 to 1346 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1393 bits(1544)	0.0	787/794(99%)	2/794(0%)	Plus/Minus

```
Query   79     AAGGCCCGAAGAACG-ATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACT  137
               ||||||  |||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1346   AAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACT  1287

Query   138    TCACGTAGTCGAGTTGCAGACTACGATCCGAACTGAGACCGGCTTTAAGGGATTCGCTCC  197
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1286   TCACGTAGTCGAGTTGCAGACTACGATCCGAACTGAGACCGGCTTTAAGGGATTCGCTCC  1227

Query   198    ACCTCACGGTATCGCCACCCTCTGTACCGACCATTGTAGCATGCGTGAAGCCCAAGACAT  257
               ||||||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1226   ACCTCACGGCATCGCCACCCTCTGTACCGACCATTGTAGCATGCGTGAAGCCCAAGACAT  1167

Query   258    AAAGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCC  317
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1166   AAAGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCC  1107

Query   318    TATGAGTTCCCCACCATCACGTGCTGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGGAC  377
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1106   TATGAGTTCCCCACCATCACGTGCTGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGGAC  1047

Query   378    TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTACACCAGCCC  437
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1046   TTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTACACCAGCCC  987

Query   438    CGAAGGGCGCACCTATCTCTAGGCGATTCCAGTGCATGTCAAGCCTTGGTAAGGTTCTTC  497
               |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   986    CAAAGGGCGCACCTATCTCTAGGCGATTCCAGTGCATGTCAAGCCTTGGTAAGGTTCTTC  927

Query   498    GCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGGCCCCCGTCAATTCCTTT  557
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   926    GCGTTGCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGGCCCCCGTCAATTCCTTT  867

Query   558    GAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGAACTTAATGCGTTAGCTACGGCGC  617
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   866    GAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGAACTTAATGCGTTAGCTACGGCGC  807

Query   618    GGAATCCGTGGAATGGACCCCACACCTAGTTCCCAACGTTTACGGCATGGACTACCAGGG  677
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   806    GGAATCCGTGGAATGGACCCCACACCTAGTTCCCAACGTTTACGGCATGGACTACCAGGG  747

Query   678    TATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGTAACAGCCCAGAGTC  737
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   746    TATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGTAACAGCCCAGAGTC  687

Query   738    CCGCCTTCGCCACCGGTGTTCCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAAT  797
               ||||||||||||||||||  |||||||||||||||||||||||||||||||||||||||||
Sbjct   686    CCGCCTTCGCCACCGGTGTT-CCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAAT  628

Query   798    TCCAGACTCCCCTACTGCACTTCCAGTCTGCCCGTACCCACTGCACGGCGCACCGTTAAGCG  857
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   627    TCCAGACTCCCCTACTGCACTCCAGTCTGCCCGTACCCACTGCACGCGCACCGTTAAGCG  568

Query   858    GCACGATTCCACAG  871
               ||||||||||||||
Sbjct   567    GCACGATTCCACAG  554
```

Fig.93 Shows *Brevibacterium ravenspurgense* gene sequences

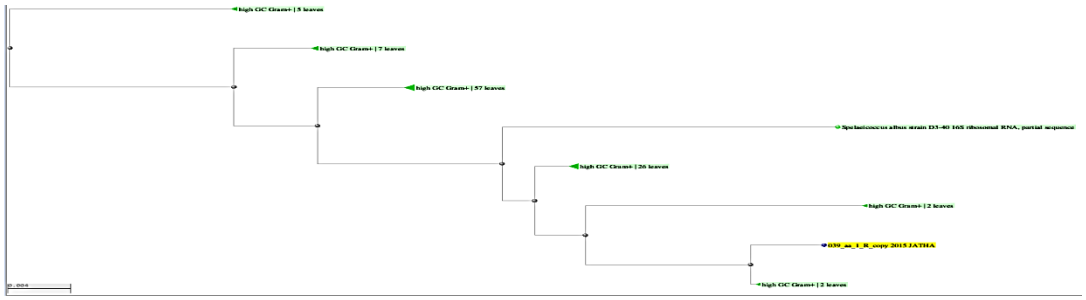


Fig.94 Shows *Brevibacterium ravenspurgense* tree

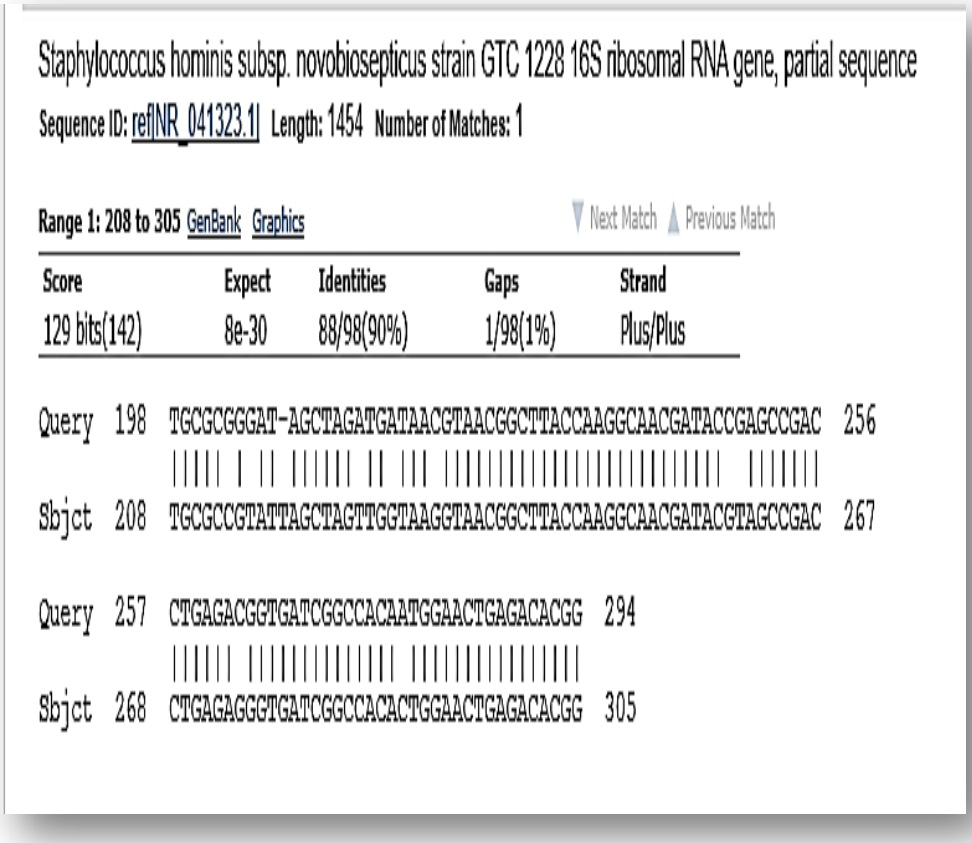


Fig.95 Shows *Staphylococcus hominis* gene sequences

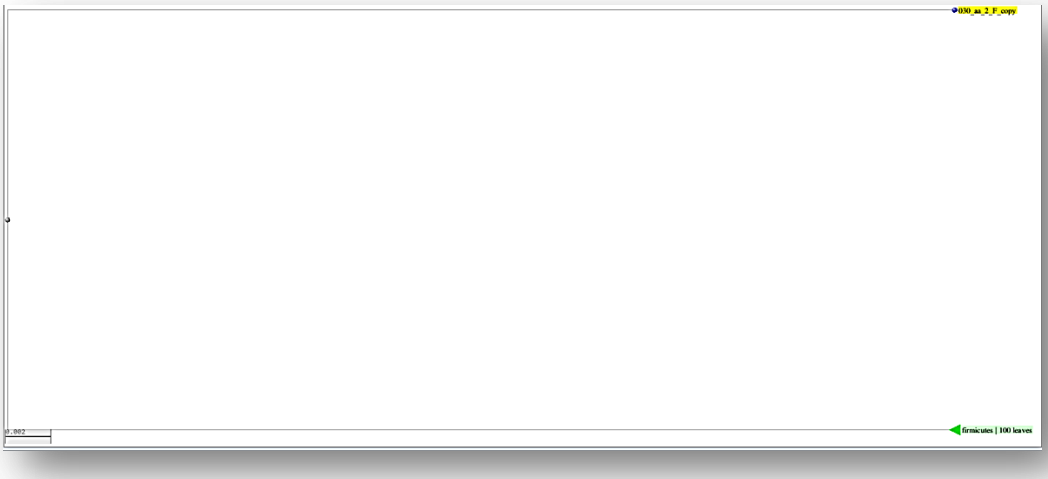


Fig.96 Shows *Staphylococcus hominis* tree

Fig.97 Shows adult blue fly (Calliphora) appeared from human body(author's own image

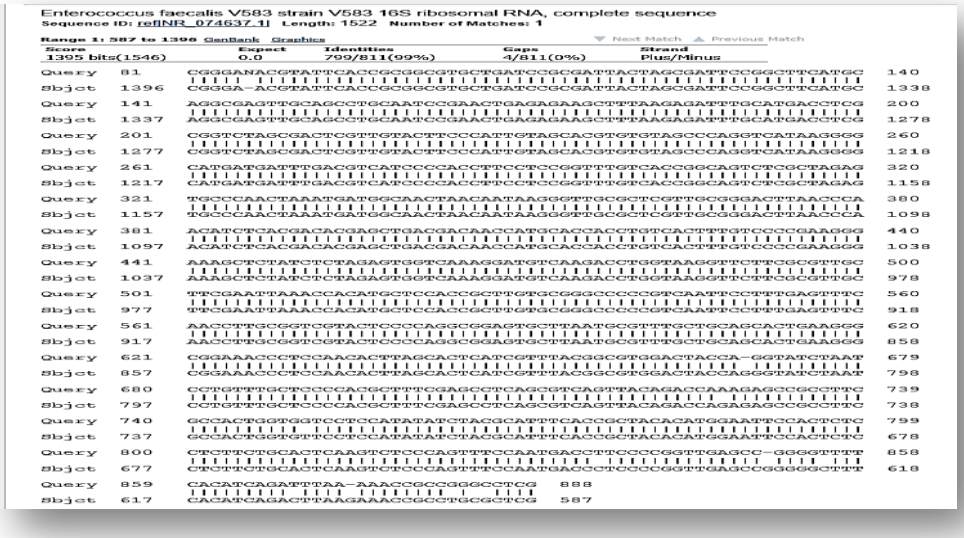


Fig.98 Shows *Enterococcus faecalis* gene sequences

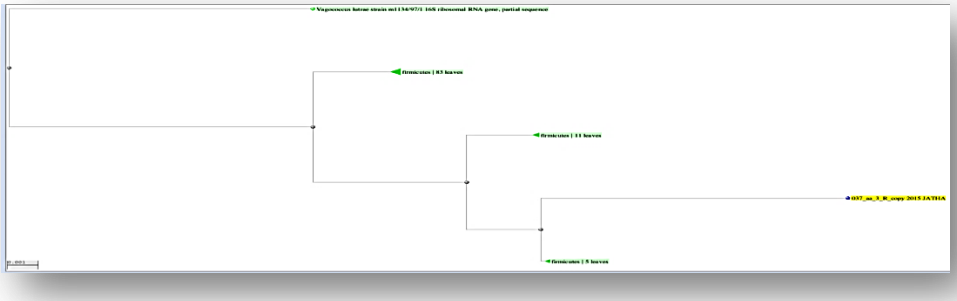


Fig.99 Shows *Enterococcus faecalis* tree

Fig.100 Shows Larvae blow fly (*Calliphora*) appeared from human body(author's own image

Lishizhenia tianjinensis strain H6 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_116229.1| **Length:** 1423 **Number of Matches:** 1

Range 1: 211 to 245 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
37.4 bits(40)	0.033	29/35(83%)	0/35(0%)	Plus/Plus

```
Query  215  ATTAACTAATTAGTGAGGTAACTGTTCAGCTAGGC  249
            ||||  |||  ||  ||||||||||||  |||  |  ||||
Sbjct  211  ATTAGCTAGTTGGTGAGGTAACTGCTCACCAAGGC  245
```

Fig.101 Shows *Lishizhenia tianjinensis* gene sequences

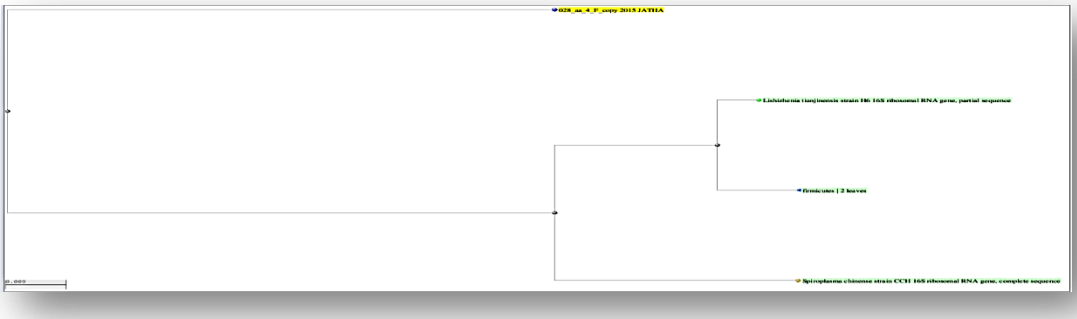


Fig.102 Shows *Lishizhenia tianjinensis* tree

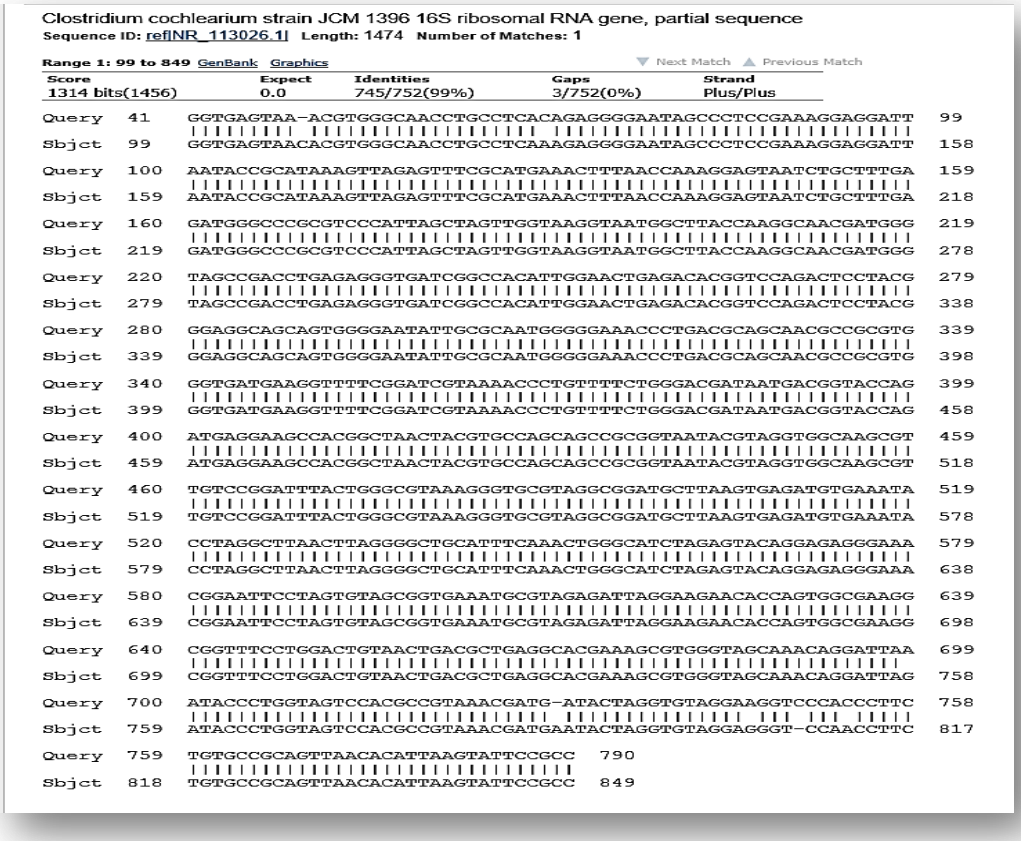


Fig.103 Shows *Clostridium cochlearium* gene sequences

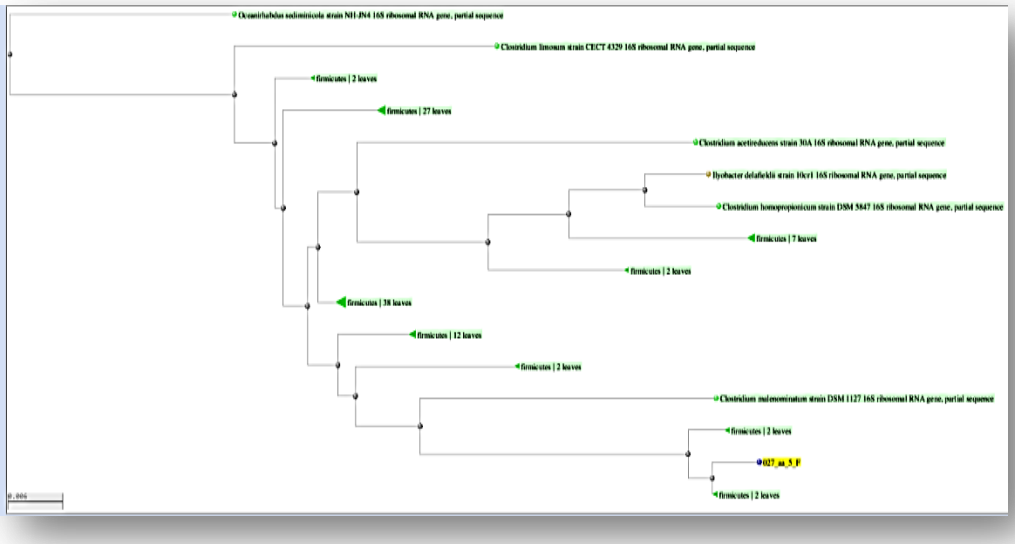
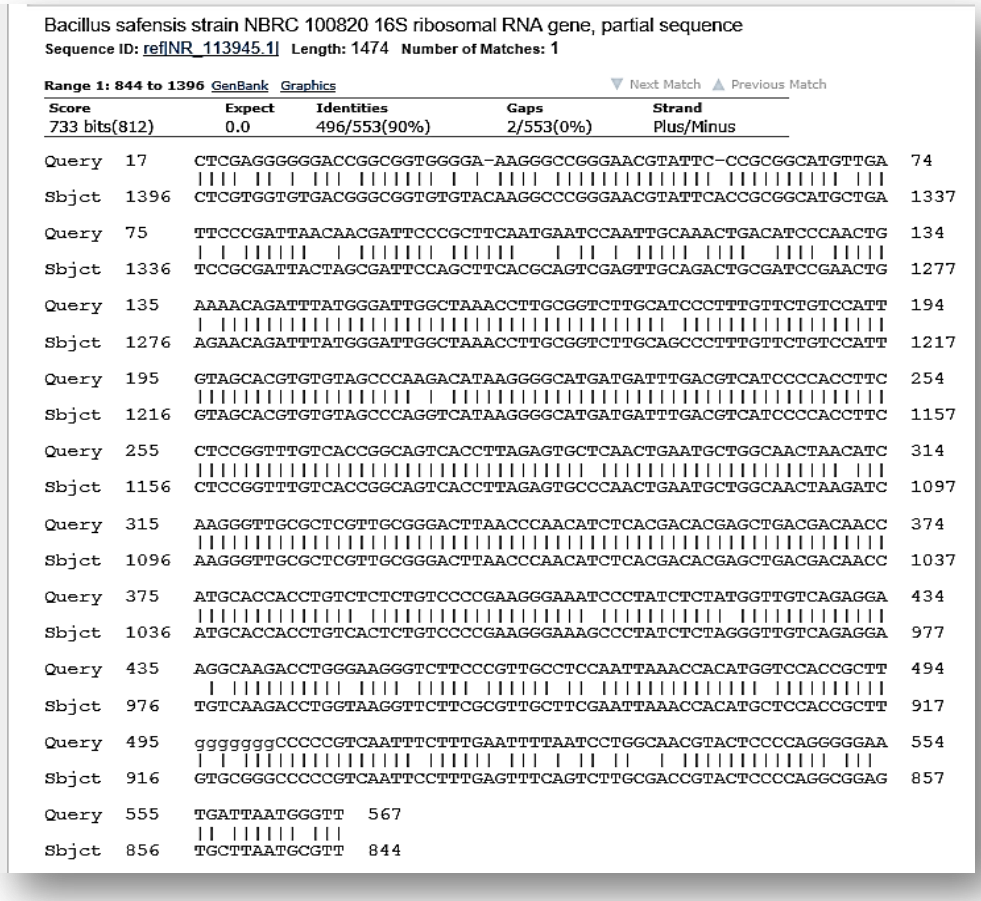


Fig.104 Shows *Clostridium cochlearium* tree



Bacillus safensis strain NBRC 100820 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_113945.1| **Length:** 1474 **Number of Matches:** 1

Range 1: 844 to 1396 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
733 bits(812)	0.0	496/553(90%)	2/553(0%)	Plus/Minus

```
Query  17    CTCGAGGGGGGACCGGCGGTGGGGA-AAGGGCCGGGAACGTATTC-CCGCGGCATGTTGA  74
             |||| || | ||| ||||||| | | |||| ||||||||||||||| |||||||||| |||
Sbjct  1396  CTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGA  1337

Query  75    TTCCCGATTAACAACGATTCCCGCTTCAATGAATCCAATTGCAAACTGACATCCCAACTG  134
             | | ||||||  | ||||||| ||||||   | || | ||||| |||| |||| |||||
Sbjct  1336  TCCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTG  1277

Query  135   AAAACAGATTTATGGGATTGGCTAAACCTTGCGGTCTTGCATCCCTTTGTTCTGTCCATT  194
             | ||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  1276  AGAACAGATTTATGGGATTGGCTAAACCTTGCGGTCTTGCAGCCCTTTGTTCTGTCCATT  1217

Query  195   GTAGCACGTGTGTAGCCCAAGACATAAGGGGCATGATGATTTGACGTCATCCCCACCTTC  254
             |||||||||||||||||| | ||||||||||||||||||||||||||||||||||||||
Sbjct  1216  GTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTC  1157

Query  255   CTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCTCAACTGAATGCTGGCAACTAACATC  314
             |||||||||||||||||||||||||||||||||| |||||||||||||||||||| |||
Sbjct  1156  CTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTGGCAACTAAGATC  1097

Query  315   AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1096  AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACC  1037

Query  375   ATGCACCACCTGTCTCTCTGTCCCCGAAGGGAAATCCCTATCTCTATGGTTGTCAGAGGA  434
             |||||||||||||| |||||||||||||||||| |||||||||| ||||||||||||
Sbjct  1036  ATGCACCACCTGTCACTCTGTCCCCGAAGGGAAAGCCCTATCTCTAGGGTTGTCAGAGGA  977

Query  435   AGGCAAGACCTGGGAAGGGTCTTCCCGTTGCCTCCAATTAAACCACATGGTCCACCGCTT  494
               | |||||||||| |||| ||||| |||||| || |||||||||||||| ||||||||||
Sbjct  976   TGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT  917

Query  495   gggggggCCCCCGTCAATTTCTTTGAATTTTAATCCTGGCAACGTACTCCCCAGGGGGAA  554
             | | ||||||||||||||| |||||| ||| | || ||  | ||||||||||||| |||
Sbjct  916   GTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAG  857

Query  555   TGATTAATGGGTT  567
             || |||||| |||
Sbjct  856   TGCTTAATGCGTT  844
```

Fig.105 Shows *Bacillus safensis* gene sequences

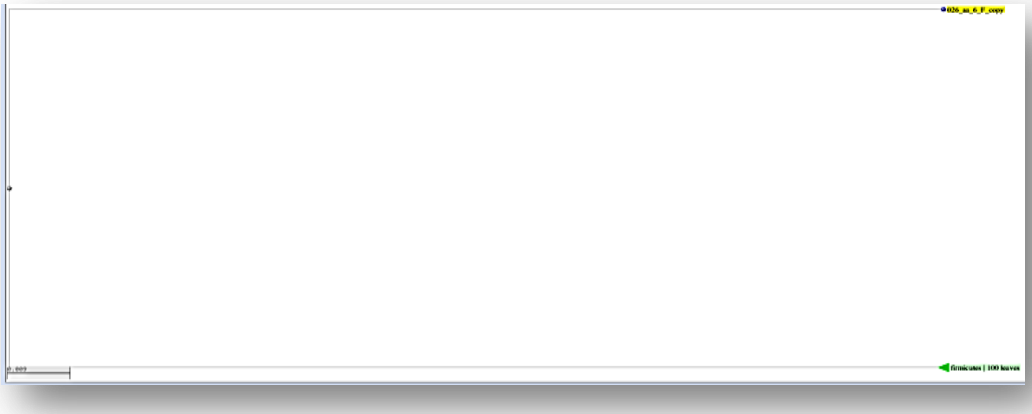


Fig.106 Shows *Bacillus safensis* tree

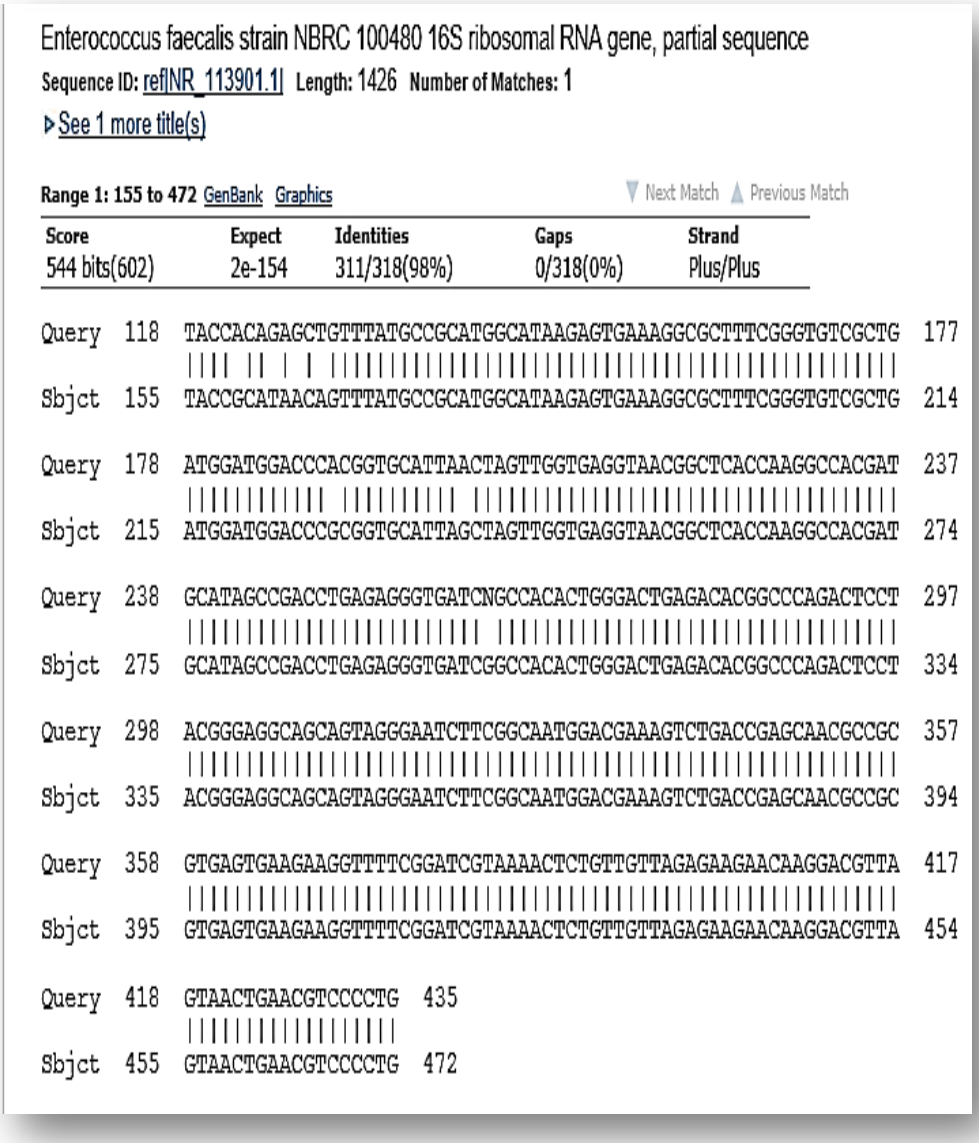
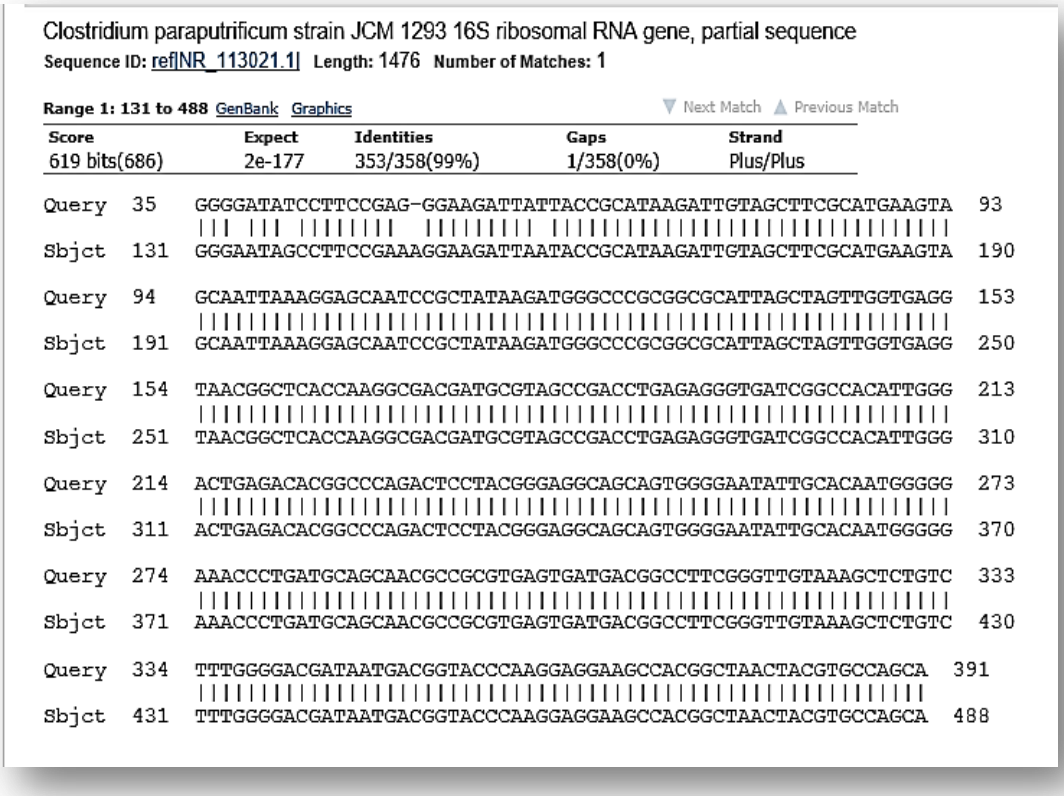


Fig.107 Shows *Enterococcus faecalis* gene sequences



Clostridium paraputrificum strain JCM 1293 16S ribosomal RNA gene, partial sequence

Sequence ID: ref|NR_113021.1| Length: 1476 Number of Matches: 1

Range 1: 131 to 488 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
619 bits(686)	2e-177	353/358(99%)	1/358(0%)	Plus/Plus

```
Query  35   GGGGATATCCTTCCGAG-GGAAGATTATTACCGCATAAGATTGTAGCTTCGCATGAAGTA  93
           ||| ||| ||||||||  ||||||||| ||||||||||||||||||||||||||||||||
Sbjct  131  GGGAATAGCCTTCCGAAAGGAAGATTAATACCGCATAAGATTGTAGCTTCGCATGAAGTA  190

Query  94   GCAATTAAAGGAGCAATCCGCTATAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGG  153
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  191  GCAATTAAAGGAGCAATCCGCTATAAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGG  250

Query  154  TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGG  213
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGG  310

Query  214  ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGG  273
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGG  370

Query  274  AAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTAAAGCTCTGTC  333
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  AAACCCTGATGCAGCAACGCCGCGTGAGTGATGACGGCCTTCGGGTTGTAAAGCTCTGTC  430

Query  334  TTTGGGGACGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACGTGCCAGCA  391
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  TTTGGGGACGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACGTGCCAGCA  488
```

Fig.108 Shows *Clostridium paraputrificum* gene sequences

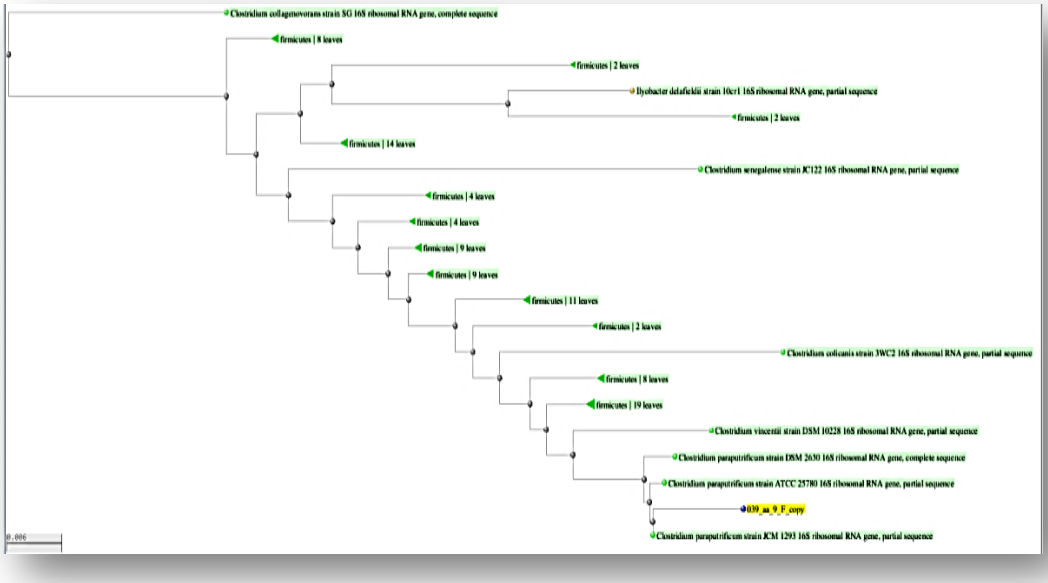


Fig.109 Shows *Clostridium paraputrificum* tree