The Study of Taiwan Phoridae the addition of Bacillus sk in growth medium elim i nat ed the commonly seen fungal overgrowth, which killed the phoridae generations. Direct observation by scan ning electron microscopy demonstrated that this bacilli were found in the interior of pupa and also in the midgut of larva. Bacillus sk,and other Bacillus spp.DNAs were am pli fied and studied by Random Am pli fi ca tion DNA Polymorphism. Similar but not identical pat terns were found between Bacillus sk and Bacillus subtilis. DNA sequences of 16S rDNA, ITS and 23S rDNA of Ba cil lus sk offered 99% identity to those of Bacillus subtilis. A close evolution relationship with Ba cil lus subtilis was proposed. A protein toxin was secreted by Bacillus sk but not by Bacillus subtilis. Toxin was purified by salt pre cip i ta tion and acetone extraction procedures. This toxin accounts for Bacillus sk's antifungal and worm killing effects. Injections of toxin into larva of ori en tal fruit fly , Bactocera dorsalis, im me di ate ly stop the vessel pulsation and kill the insects. The toxin is nevertheless harmless to Megaselia scalaris larva and fly. Under starvation condition, Megaselia scalaris can even live on washed Bacillus sk bacteria as its only food source. The compensatory symbiosis interactions of Establishment of Artificial Breeding Tech nol o gy The Identification of an Symbiotic Ba cil lus Spe cies 1.Introduction In history,all 54 reported species of Tai wan phoridae had been transferred to Europe, denominated, kept in Hungarian museum , but de stroyed in a big fire in 1965 (1). Accidentally in help ing purification of a bacillus peptide toxin, I ob served a black tiny fly strongly attracted by a protein-rich plate streaked with a toxin-se cret ing Bacillus sk strain. The ex per i ments were repeated several times and 90% of all attracted tiny flies were identified as the long ne glect ed flea fly, Megaselia scalaris. The female Megaselia scalaris flies cop u late with male flies right after meta mor pho sis from pupa and ovulate in 15-45 min utes after reaching the Ba cil lus sk colonies. Further efforts in achieving ar ti fi cial breeding method have dem on strat ed the need of bacillus and high pro tein diet in phoridae growth and maturation.

存活天數	水	牛奶	果蠅培養基	味噌	LB plate	LB plate+ Bacillus SK	水煮鮭肉	乳酪	乳酪+蜂蜜水	蜂蜜水
雄性成蠅	1 天	1-2 天	2 天	2 天	1-2 天	3-5 天	1-2 天	2-3 天	>14 天	>14 天
雌性成蠅	1-2 天	2-3 天	3 天	2 天	2-4 天	3-5 天	2-3 天	3-4 天	>20 天	>20 天

Also during long series of passages, mi cro or gan ism and its insect hosts have been re port ed between flagella protist and termit (2). Re cent ly the identification and analysis of Bacilli DNA from abdominal tissue of some extinct amber-en tombed fossil bees have raised the possibility of bee-Bacillus symbiosis (3). In this study, I presented the evidences from a liv ing example for an acquired symbiotic in ter ac tions be tween Phoridae fly, Megaselia scalaris and a spe cif ic Bacillus strain. Finally for further char ac ter iz ing this very first model of phoridae in Taiwan, its animal behavior, field distribution and rDNA gene struc ture were also issued.2.Objectives Restore the Taiwan phoridae insect modes which had been collected in Hungarian museum, but de stroyed in the 1965 big fire. Identify the characteristic gene structure tion and (c). a dif fer en ti a tion and maturation enhancer. 3.Experiment Procedures Phoridae Artificial Breeding Method: Modified Drosophila's growth gel (a mix.of 900c.c.distilled water, 10 gm agar, 30gm yeast powder,40gm crude sugar,100gm corn starch were heated, honey 2% (v/v) ,0.5c.c alcohol and pro pi on ic acid were added and cooled to gel state. Beef -Bacillus SK broth : a mix. of 100 gram ground lean beef , 100 gram D-glucose and 400 gram distilled water ,121°C autoclaved 30 minutes. The supernatent beef extract mixed with Bacillus SK OD 0.3 was cultured at 32°C 24 hours. For breeding, 0.8 c.c. broth was added into a tube con tain ing 5 c.c. mod i fied gel. DNA

of Fam i ly Phoridae and related gene variation for the dif fer ent genus of this family. Symbiotic toxic Bacillus sk is a close relative of non tox ic Bacillus subtilis. The secreted protein tox in plays an important role for protecting the food from fungal overgrowth, keeping away from nat u ral enemies, saving the larva from animal's intake. Three nontoxic interactions were also iden ti fied in this Phoridae-Bacillus symbiosis interactions: Ba cil lus sk acts as (a). a strong chem i cal attractant, (b). a spare food in stringent sit u a extraction,Random Amplification Poly mor phism DNA and DNA sequencing: These methods were performed as described in all mobiology technology books. DNA data was main ly based of the purification from one larva or adult flea fly instead of many insects. Polymerase chain reaction was perfomed in Thermocycler 480, (Perkin-Elmer, Netherlands). Sequencing the nu cle ar rDNA region containing the internal tran scribed spac er (ITS) regions and 5.8S rRNA gene and nu cle ar small (18S) was performed by primers described in Table 1. and ABI PRISM Model 377 DNA Autosequence.Results were compared in web-site NCBI (www.ncbi.nim.nih.gov/) and run mul

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ti ple alignment with the other species by program Vector NT.4.Results I. Ecology of Megaselia scalarisFigure 1 Three major kinds of phoridae that were attracted by Bacillus sk. 250 Bacillus sk plates were used in 84 differe lo ca tions .Among 2100 attracted phoridae flies, three different phoridae flies were identified and 90% were megaselia scalaris.

Table 1. primers usedd inPCR and RAPD reactions

PCRprimer	Primer sequence(5'→3')	Target site
NS1	GTAGTCATATGCTTGTCTC	18S
NS4	CTTCCGTCAATTCCTTTAAG	18S
NS5	AACTTAAAGGAATTGACGGAAG	18S
NS6	GCATCACAGACCGTGTATTGCCTC	18S
NS7	GAGGCAATAACAGGTCTGTGATGC	18S
ITS1	TCCGTAGGTGAACCTGCGG	ITS
ITS2	GCTGCGTTCTTCATCGATGC	ITS
ITS4	TCCTCCGCTTATTGATATGC	ITS
ITS5	GGAAGTAAAAGTCGTAACAAGG	ITS
FF1	TCGTAACAAGGTTTCCG	ITS
FF2	GTTAGTTTCTTTTCCTC	ITS
RD1	AAGGAGGTGATCCAGCC	16S
FD1	GAGGTTTGATCCTGGCTCAG	16S
16SR	ACTGCTGCCTCCCGTAG	16S
16ScutF	TAATACCGGATAATATTTTCAGC	16S
23SR	CAGCACCGGGCAGGTGTCACCCCC	23S
23ScutF	TAAGTAGGCAAATCCGCTTTACGT	23S
RAPDprimer	Primer sequence(5'→3')	
OPB11	GTAGACCCGT	
OPB12	CCTTGACGCA	
OPB13	TTCCCCCGCT	
OPB16	TTTGCCCGGA	
OPB19	ACCCCCGAAG	

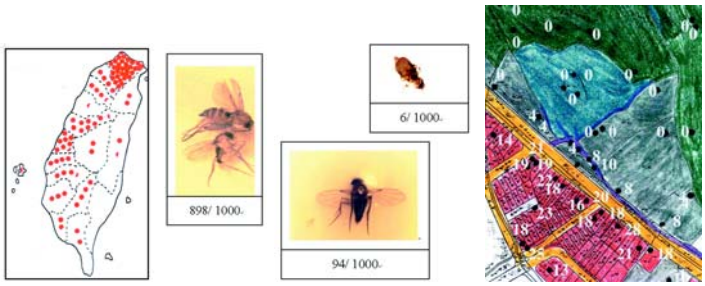
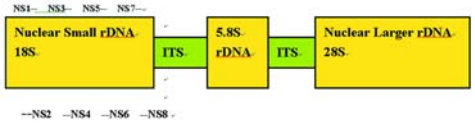


Figure 2 The distribution of Megaselia scalaris in The Taipei Suburban area. 50 traps were evenly sit ed over a mixed area of three or more different regions: red, residential; yellow, public transport paths; purple: a trench; gray: factory; green: farmland; light blue: water pond. The black dots represent the sites of the traps, the white number denotes the number of the Megaselia scalaris flies caught in 6 hours. Although in Taipei city life, healthy-looking and vi va cious Megaselia scalaris are often seen here and there. It is difficult to catch this fly at mountain or farmland. This plus the finding t that Megaselia scalaris often lay eggs at the brim of some opened beef extract cans. Megaselia scalaris may have long adapted to human civilization and become used to live with our artificial waste and junk stuff environment. II.The characteristic phoridae rDNA gene structure In the past 100 years, Taiwan flea flies were col lect ed and transferred to Europe, studied by for eign researchers. Recent study on these reported animal models were extremely difficult due to the huge de struc tion in a big fire. In this paper, the es tab lish ment of artificial breeding method and cross fer til i za tion (at least five generations) provides me large quantities of Megaselia scalaris with iden ti cal mor phol o gy and gene structures. The ap pli ca tion of 18S,28S ribosome DNA probes with PCR method re flects the specific DNA sequence about Megaselia scalaris (Chen,Taipei). To begin with ribosomal DNA primer set FF1: TCG,TAA, CAA,GGT,TTC,CG; FF2:GGT,AGT,TTC,TTT,TTC,TC, I obtained the first DNA segment sequence by an ABI prism Model 377 DNA

autosequencer . Since not any DNA sequence re lat ed to flea fly has been published before, I tried various sets of primers to elucidate the whole se quence of 18S and ITS1 regions(Figure 3). Figure 3 Phoridae Megaselia scalaris 18S rDNA gene structure. Comparison between several different living or gan isms was performed bycomputer search. Care ful study on the gene struc ture of phoridae rDNA has indicated the following events:(1) The closer two organisms appear in evolution, the more their gene structures resemble.(2) There are two kinds of dif fer ent domains in rDNA 18S region,one fair ly con stant (base 300-500) and others very variable among different species. (3) The ITS 1 regions are com posed of many DNA fragments with con ser va tive sequences. Fig 5



>18s rRNA gene of FF

ATTCTGGTTGATCCTCTCCGTCGGGACGGGCCTGTAGNCTATGCTTGTCTGCTTACG
GAATTGACGGAAGATGATCACTATGCTTGTTTCCTTAAGGGAATGGACGGAAGGAGT
ACTGATGTGCTTGTATAGCTAATACATGCATTATAAGCACGGACCTTTTGGAACGTG
TGCTTTTATTAGGCTAAAACCAAGCGATTCTTTCGGGAATCGTTGACTGGTTGAACT
CTAGATAACTTGCAGATCGTATGGTCTTGTACCGACGACAGATCTTTCAAATGTCTG
CCCTATCAACTATTGATGGTAGTATCTAGGACTACCATGGTTGCAACGGGTAACGGG
GAATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAA
GGCAGCAGGCGCGTAAATTACCCACTCCCAGCACGGGGAGGTAGTGACGAAAAATAA
CAATACAGGACTCGTATCCGAGGCCCTGTAATTGGAATGAGTACACTTTAAATCCTT
TAACAAGGACCTATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTT
CAATAGCGTATATTAAAGTTGTTGCGGTTAAAACGTTCGTAGTTGAACTTGTGCTCC
ATACGGGTAGTACACCCATCAATTGGGTTTGTACATTACTCTATGGTATGTGAGCGT
ATTACCGGTGGAGTTCTTATATGTTATGGTACTTGTATTATAGCGTATTCCTCCTAT
TCAAACCTACTTCAGTGCTCTTCATCGAGTGTTGTTGTGGGCCGGTACAATTACTTT
GAACAAATTAGAGTGCTTAAAGCAGGCTTCAAATGCCTGAATATTTGTGCATGGAAT
AATGGAATAAGACCTCTGTTCTACTTTCATTGGTTTATAGATCAAGAGGTAATGATT
AATAGAAGCAGTTTGGGGGCATTAGTATTACGACGCGAGAGGTGAAATTCTTGGACC
GTCGTAAGACTAACTTAAGCGAAAGCATTTGCCAAAGATGTTTTCATTAATCAAGAA
CGAAAGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACCATAAACGAT
GCCAGCTAGCAATTGGGTGTAGCTACTTTTATGGCTCTCTCAGTCGCTTCCGGGAAA
CCAAAGCTTTTGGGCTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAAT
TGACGGAAGGGCACCACCAGGAGTGGAGCCCTGCGGCTTAATTTGACTCAACACGGG
GAAACTCACCAGGTCCAGACTTCTGTAGGATTGACGCATTGGCGATGCTTCATGATT
AGAAGGGTGGTGGTGCATGGCCGTTCTTAGTTCGTGACGTAAGTTGTCTGCTTTATT
GCGATAACGAGCGAGACCTCGGCCGCTAGTTGCCAGAACTCACACTTTGTGAGTCTT
TGGCTCTTAGCGGGACTACCGGAGACAATTCGGGGGAGGTTCGAGGCAATAACAGGT
CTGTGATGCACTTAGATGTCCTGGGCTGCACGCGCGCTACACTGACGCAGCCAGCGA
GTTAATCCTTGTCCGAAAGGAACGGGTAACCTTGTTAAACTGCGTCGTGATGGGGAT
AGAGCTTTGCAATTTTTGCTCTTGAACGAGGAATTCCTAGTAAGCGTAAGTCATCAA
CTTGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGTTACTACCGATTG
AATGGCTTAGTGAGGTCCTAGGAGGATTCTTGCACGAGGGCAACCTCTAGCGAGAAA
CCAAATTCGATCAAACTTGGTCATTTAGAGGAAGTACAAGTCGTAACAAGGTTTCCG
TAGGTGAACCTGCGGAAGGATCATT

ITS1of rRNA gene of FF:

TCTGAAGGCTTTTTGCCAAAACCACTGTGAACAGCTTAGACTTCGGTCTTTGCAATT
GCTTGGGTGTCGAAAGGCGCCCAATCTTTAAAACTTTTATATTTGTTCTGAAACAAT
GAAAATTTAAAACTTTCAACAACGGATCTCTTGGTTCTCGTATCGATGAAGAACGCA
GCAAAGCGCGATAGGTAATGCGAATTGCAGAACGTGAGTCATTGAATTTTTGAACGCA
TATTGCGCTATTAGTTTGTCTAATAGCATGCTTGTTGGAGTGATAATCTTCCTCTCA
ACCATTTTTGGTATGAGGTCTTGCTCCTTTTAGGAGTTAAAATCATGGAAGTGCACA
CGTTAATTAACTCTGTGCAGTTATACACTTTTCATCCTCCAATCAAGCAAGGGTACC
CCCTGAACTTAAGCATATTAATAAGCGGGGAAAAGAAACTAACA*

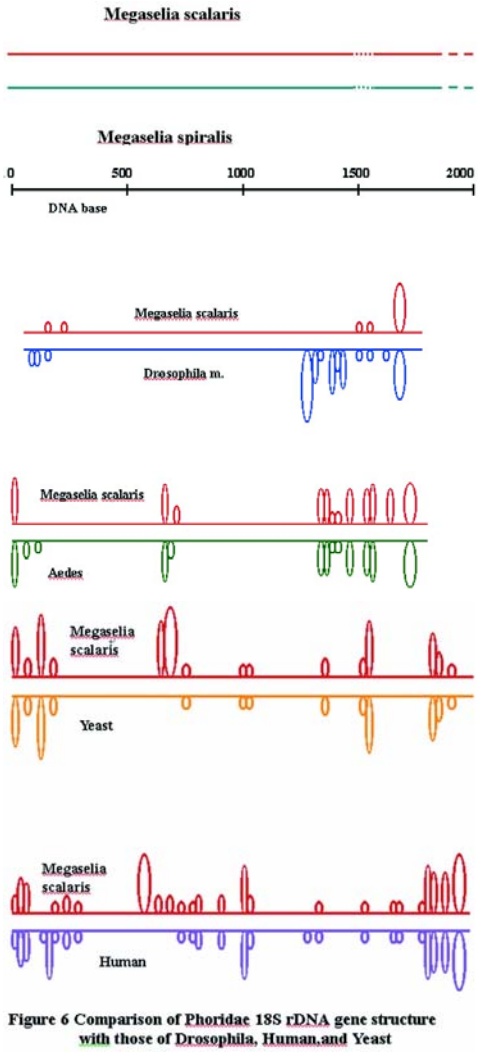


Figure 6 Comparison of Phoridae 18S rDNA gene structure with those of Drosophila, Human,and Yeast

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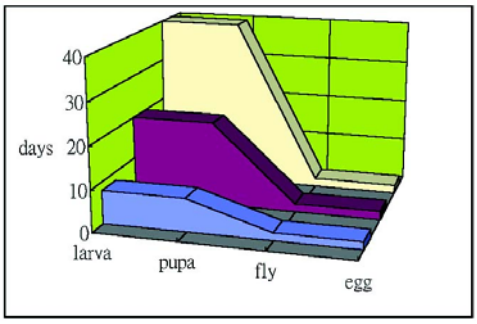
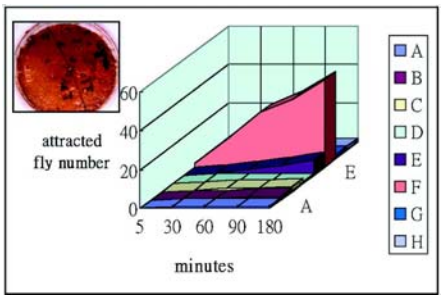
Nontoxic Effects of Bacillus sk Under optimal growth condition, the life cycle of Megasela Scalris can be as short as 18 days. The time intervals in each stages are approximately 2 days for egg to hatch larva, 2 days for new flies to lay eggs, 7 days for pupa development and 7 days for larva growth. The presence of Bacillus sk promotes the rapid maturation and fast growth of Megaselia scalaris at each stages. Beef extract alone or the addition of antibiotic to kill the bacteria significantly slow down the fly growth and maturation. The changes were mainly due to the prolonged pupa and larva stages (7 days to 40 days, 7 days to 35 days). Figure 7 Effect of Bacillus SK on Phoridae Mat u ra tion In each group, 20 fly eggs were incubated with dif fer ent growth gel supplemented with one of the followings: Blue, bacillus sk; Red,beef extract; White: beef extract +ampicillin 0.2mM. Average lengths(Z axis) of each stages (X axis)

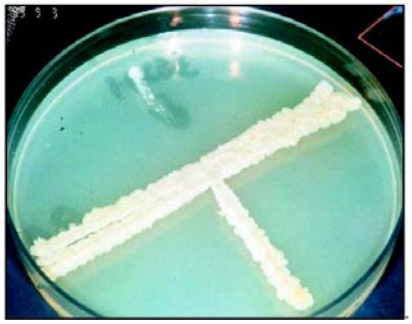


were measured. Bacillus SK has a remakable chemotaxis effect on Megaselia scalaris adult fly. Strepcoccus, Staphylococcus, and different species of Bacillus Escherichia coli,Klebsiella were streaked on each Miller Hinton plate and evaluated their potency on attracting flies. Phenomenal difference was dis cov ered with Bacillus SK ans described in the fol low ing figure. Figure 8 Bacillus sk acts as a strong chemical at trac ta nt toMegaselia scalaris A.Strepcoccus faecalis B.Staphylococcus aureus, C.Bacillus cereus , D.Bacillus thuringiensis, kurstaki, E.Bacillus subtilis F.Bacillus sk G.Es cher i chia coli K12, H.Klebsiella pneumoniae aerogenes were streaked on each Miller Hinton plate and in cu bat ed to 80% saturation. The plate put in a 80 cubic cm plastic box containing 50 male and 50 female Megaselia scalaris flies were examined for their pos i tive chemotaxis effect. The number of trapped flies was measured at different time points. III. Toxic Effects of Bacillus sk In laboratory, it is very common to identify that the contamination of fungi in growth medium will pro hib it the growth and maturation of Drosophila fly larva. Interestingly, during the passages of Megaselia scalaris flies, little if any fungi con tam i na tion has been observed.



In my Electron microscopy study, very short fungi fragments were aa surrounded by a rod bacteria. Further studies reflects an unusally strong an ti fun gus property with Bacillus sk. Tests were per formed to study the antifungal spectrum and all the fol low ings in clud ed Agaricus arvensis CCRC 36371 Aspergillus ochraceus CCRC 300101 Aspergillus fumigatus CCRC 30099 Aspergillus niger ATCC 9642 Aspergillus flavus CCRC 30119 Aspergillus vesicolor CCRC 30225 Aspergillus candidus CCRC31129 Aspergillus terreus CCRC 32664 (terrium production) Aspergillus terreus CCRC32653 Saccharmyces cerevisiae Zygosacchromyces rouxii were prohibited by this specific Bacillus sk. Figure 9 Antifungal effects of Bacillus sk A horizontal streak of Bacillus sk was applied on a Miller Hinton II plate. A second vertical streak of fungus (include Agaricus ,Aspergillus, Saccharmyces and Zygosacchromyces) was ap plied at right angle

across the first Bacillus streak). An ti fun gal Result of Ba cil lus sk was identified as a miss ing upper part of cross + symbol.



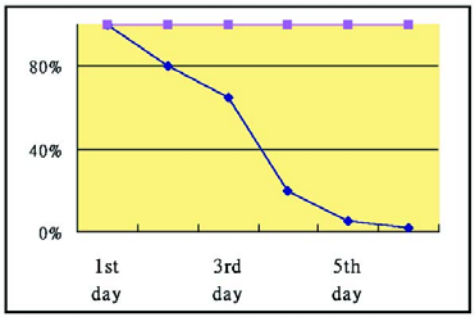
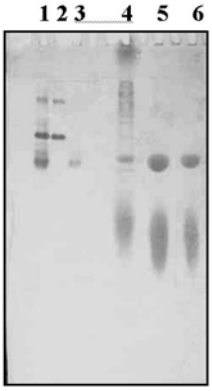


Figure 10 Worm killing effect of Bacillus sk on Bactocera dorsalis larva Bactocera larvae were breed in guajava (Psidium guajava) jelly with (blue dots) or without (pink dots) Bacillus sk bacteria at OD 0.3.The sur viv al per cent age was measured at different time points. (X axis : % of survived Bactocera dorsalis larva , Y axis: days after the addition of Bacillus sk). Figure 11 10% PAGE of Bacillus sk and Bacillus subtilis secreted proteins A toxin with antifungi property was purified by salt precipitation and acetone extraction as described in Materials and Methods in this paper(Chinese Edition). 10% PAGE demonstrates the location of this purified toxin. Well 1:Bacillus sk secreted



proteins, well 2:Bacillus subtilis secreted Proteins, well 3:Acetone washed Bacillus sk protein profile well 4:Salt precipitation of total Bacillus sk se cret ed Proteins well 5:Acetone washed 4. Ppt. 6.As 5. Figure 12 Worm killing effect of purified Bacillus sk toxin on larva of Bactocera dorsalis Larva of Bactocera dorsalis was lightly pressed on a glass plate with a transparent tape and vessel pul sa tion can be clearly observed through a reverse phase contrast microscopy. Purified toxin (100ug/ml) was injected into the tail portion of the worm body. IV. What's Bacillus sk? Bacillus sk is classified as a morphology I, Gram-positive endospore containing rod bacteria. Its col o ny on sheep RBC plate gives a beta-hemolytic picture. Bacillus sk like Bacillus subtilis on many enzymatic activities : glucose fermentation pos i tive and man ni tol fermentation positive. There are many similar PCR bands between Bacillus sk and Ba cil lus subtilis (Fig.); however there are also some distinguished band found in one bacillus but not the other. Study about 16S rDNA of these two re flect two 99% iden ti cal sequences(Fig.).

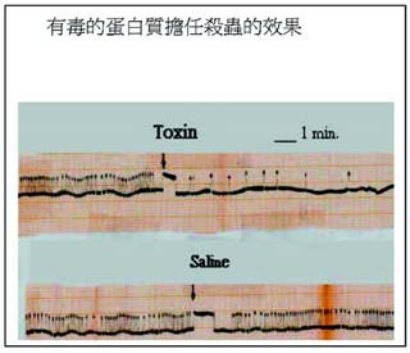
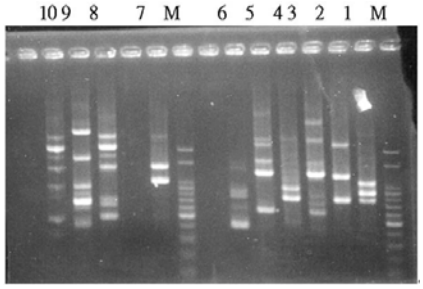


Figure 13 RAPD of Various Bacillus spp. Experiment was performed as described in procedures. M:DNA marker,100bp DNA ladder 1.Bacillus.licheniformis 2.Bacillus.thuringiensis sp.israelensi 3. Bacillus subtilis 4. Bacillus thuringiensis subsp.kurstaki 5 Bacillus SK 6. Bacillus popill 7. Bacillus larvae 8 Bacillus spp. (isolated from Drosophila gut) 9.Bacillus spp. unspecified 10.Bacillus popillae Figure 14,15 RAPD comparison of three Bacillus spp. Differnt PCR probes were used to elucidate the dif fer enc es between Bacillus spp. B.s.: Bacillus subtilis; B.sk: Bacillus sk B.t. :Ba cil lus thuringiensis M: molecular marker



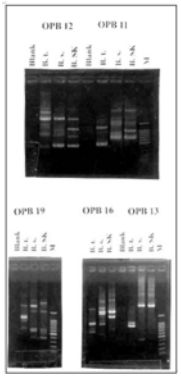
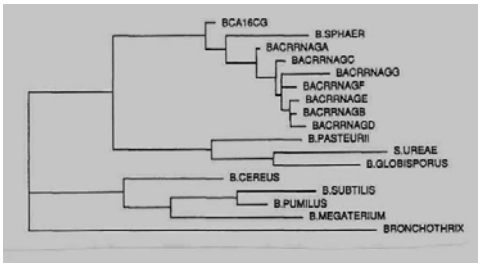


Figure 16, 17 Identical ribosome DNA sequences were found in Bacillus subtilis and Bacillus sk. Bacillus sk:upper; Bacillu subtilis:lower. A The evolution of phoridae makes Megaselia

frag ment of 16S rDNAof Bacillus sk was studiedscalaris take advantage of rotten meat of dead and com put er searched for comparison with thatin sects and animals . In digesting these food of Ba cil lus subtilis. Hypothesis Andstuff, it appears very wise to use bacillus as Discussions Figure 18 Ribosomal DNA seqence ofenzyme source. A sig nif i cant mutation to Bacillus sk ap peared 99% identical to Bacillustolerate hemolysin tox ic i ty not only move subtilis. Bacillus sk is a lately variant located atthis phoridae group ahead of oth er flea flies the twig of the phy lo ge net ic tree. How it(for no longer been killed by toxin),but also becomes a toxin secerting strain? Ob vi ous lyacquire a whole bunch of other advantages not through plasmid transformation. From my(de scribed in conclusions). In nature,a study, toxin by itself is a beta hemolysin which ismutation can sometimes move one spe cies in impotant for bacteria it self to approach its food.one step or few steps to surpass many other close species. How the symbiotic animal and mi cro or gan ism interact each other, is there any gene ex change in between? (Virus was found inside the Megaselia scalaris fat cells and the nature of the vi rus is still unknown.) Interestingly, the energy flow from de com pos er bac te ria and fungi to insects is very significant. Does this shunt porvide a more ef fi cient way than through primary producer ? Many questions left? Can we utilize Bacillus sk as a biology agent for controlling other harmful fly? Is there any potential biohazard to increase the Megaselia scalaris population in our environment? 5.Conclusions 1. Megaselia scalaris appears to be the most abun- dant fle fly species in Taiwan citys. 2. An artificial breeding method is established by adding digested beef extract into starch/yeast



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medium. 3. Besides microscopic difference, the morphology of Taiwan Megaselia scalaris is identical to the de scribed mode. 4. Molecular biology study on the rDNA structure of this bred fly reflects the unique sequence for Megaselia spp. The possible evolution tree is de- scribed at the end of this section. 5. Megaselia scalaris plays an important role in labo- ratory contamination of some bacteria and virus. 6. Bacillus sk acts as a potent chemical attractant for Megaselia scalaris. 7. Bacillus sk enhances the differentiation and matu- ration of Megaselia scalaris. 8. Bacillus sk acts as a spare food in starvation. 9. Bacillus sk inhibits the fungal infection which is detrimental to fly growth. 10. Bacillus sk kills Phoridae's natural enemy :Ants. 11. Bacillus sk toxin kills the larvae of other fly. 12. Bacillus sk promotes the cell lysis and protein digestion in Megaselia scalaris gut. Figure 196.References

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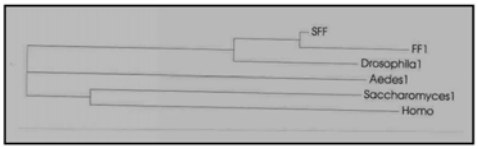

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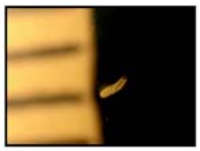
Coin:2.5cm ,House fly: 0.7cm

Size Comparison

Megaselia scalaris: adult fly



egg,larva(just hatched):0.4mm
larva (longest before metamorphosis) 8mm

wing length:1.8-2.0mm

M.scalaris cell size: 8-15 μm