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**Detection of the presence of insect pests and mite DNA in
samples of home stored grains by molecular techniques**

Lamia Yousef Alhalaseh

M.Sc. Thesis

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**Detection of the presence of insect pests and mite DNA in
samples of home stored grains by molecular techniques**

Prepared by:

Lamia Yousef Alhalaseh

B. Sc. Biology

Al-Quds University- Palestine

Supervisor: Dr. Hisham Darwish

Co-supervisor: Dr. Ibrahim Abbasi

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Al Quds University
Deanship of graduate studies
Biochemistry and Molecular Biology

Thesis Approval

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samples of home stored grains by molecular techniques**

Prepared by: Lamia Yousef Alhalaseh
21012526

Supervisors: Dr. Hisham Darwish
Co-supervisor: Dr. Ibrahim Abbasi

Master thesis submitted and accepted Date: 25/5/2013

The names and signatures of examining committee members:

- | | |
|---|----------------|
| 1. Head of committee: Dr. Hisham Darwish | Signature..... |
| 2. CO- Supervisor : Dr. Ibrahim Abbasi | Signature..... |
| 3. Internal Examiner: Dr. Sameer Barghouthi | Signature..... |
| 4. External Examiner: Dr. Khaldon Nijem | Signature..... |

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Dedication

I dedicate my work to those dearest to me, my family especially my father, mother, sister, brothers and my aunt for their support and advice. To my lovely husband Tamer for his patience and affection for the sake of my success. To the sweetest to my heart my daughter Ruba for taking her allotted time to complete this study.

Thank you all

Declaration

I Certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or Institution.

Signed: _____

Lamia Yousef Alhalaseh

Date: 25 /05/2013

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I would like to express my deep and honest regards to my supervisor; Dr. Ibrahim Abbasi for his support, directing, encouragement and technical training to present this work and also to Dr. Hisham Darwish, both for their patience and kindness; all words can't express my appreciation.

I would like to thank my colleague Ihab Mohamad Nairat for helping me to get ticks samples quickly when I need them.

Abstract

Home stored grains such as wheat, rice, lentils, corn...etc, are usually exposed to contamination with insect pests. Over 60 species of insects infest stored grains where Indian meal moth, flour beetles, saw-toothed grain beetles and granary weevil are the most common. These pests are economically important and are responsible of millions of dollars loss because contamination by these pests reduces grains quality and therefore discarding them. They may also cause several health problems including allergies and gastrointestinal disorders. Insect pests are classified as primary and secondary pests. The primary pests present a bigger problem than secondary pests because they infest grain kernel; feed upon them and reproduce on it leading to major damage of the whole sound grain, while secondary pests feed on grains damaged by primary pests because they are not capable to penetrate grain kernel. The global spreading of these insects occurs as a result of world wide cereal distribution. Infestation might occur during storage, shipping and transportation. Control managements of these insect infestations can be achieved either by chemical (fumigation), physical (thermal control, inert dust, ionizing radiation, light and sound control) or biological treatments (pheromones, growth regulators, microbial control and plant extracts). There are several methods for detection of insect pests in grains. Traditional detection techniques include staining kernels, density separation, uric acid analysis, acoustical sensors, x-ray, near infrared spectroscopy (NIR) and enzyme linked immune-sorbent assay (ELISA). Problems encountered with these methods are that they are laborious, expensive and not sensitive to detect insect contamination at the egg and larvae stages. Therefore, newer methods are needed for rapid and sensitive detection. One obvious approach is to develop a molecular biology technique that utilizing genetic information of the different insects for amplification of specific target gene sequences by polymerase chain reaction [PCR] and real time PCR for that purpose. In this study, used a number of infested grain samples were used to isolate larvae and adult insects from them which serve as positive controls in our work. The isolated insects were subjected to DNA extraction, PCR amplification of defined regions in the cytochrome oxidase gene followed by sequencing to identify each pest species. The sequences were identified according using BLAST generated comparison to the original gene sequence obtained from GeneBank. The sequences of the gene from the different

insects were aligned to design three sets of primers specific for insect mitochondrial cytochrome oxidase subunit I gene. Two primer sets, COI-long1 and COI-long2 for general pest species and COI-mite for the detection of mites. The designed primers were tested for their specificity and sensitivity. A problem was encountered with grain swelling after they were mixed with the aqueous solutions to collect the contaminating insects. This problem was solved by developing and adapting two different methods for grain treatment before DNA extraction using a centrifugation washing method or filtration washing method with the different sample size including either 10 or 50 grams respectively. For PCR optimization, the original DNA sample, 1:10 and 1:100 sample dilution were tested which indicated the best and sample dilution to use was 1:10.. The suitability of PCR primers and DNA extraction methods was evaluated on eleven samples of commercial grains in six separate PCR reactions utilizing each primer set with the two extraction methods. The detection sensitivity varied between the different primers used and extraction method where superiority with COI-long1 primer compared to the COI-long2 primer and the filtration washing method was more efficient over centrifugation washing method giving the best combination is COI-long1 with filtration washing method.

الحبوب المخزنه منزليا مثل القمح، الأرز، العدس، الذره وغيرها. يوجد 60 نوعا من الآفات تصيب الحبوب وأشهرها: العثة الهندية، خنافس الطحين، خنفساء الحبوب المنشارية و سوس الصوامع. هذه الآفات مهمة اقتصاديا وتسبب خسارة آلاف الدولارات سنويا نتيجة لإتلاف كميات كبيرة من الحبوب الملوثة، وتسبب أيضا مشاكل صحية مثل الإضطرابات المعوية وأمراض الحساسية. تصنف هذه الآفات الى آفات أولية وآفات ثانوية والآفات الأولية تشكل خطر أكبر كونها قادرة على مهاجمة نواة الحبوب، التغذية عليها التكاثر داخلها. الآفات الثانوية فتصيب الحبوب التالفة التي سبق وهاجمتها الآفات الأولية لأنها غير قادرة على نواة حبوب سليمة. الانتشار العالمي لهذه الآفات نتج عن تجارة الحبوب في جميع انحاء . يحدث . أماكن التخزين أو أتد .

هذه الآفات إما كيميائي (التبخير بالمبيدات الحشرية)، فيزيائي (المعامله الحرارية الخاملة، الإشعاع المؤين و التحكم بواسطة الضوء و الصوت) أو تحكم حيوي (الفيرمونات، استخدام الميكروبات و استخدام مستخلصات النباتات). هناك طرق عديدة للكشف عن وجود هذه الآفات في الحبوب، الطرق التقليدية تشمل:

الكشف عن وجود حمض اليوريك، المجسات الصوتية، الأشعة السينية، التصوير بالأشعة تحت الحمراء، ومقياسية الممتز المناعي المرتبط بالأنزيم . المشاكل المتعلقة بتلك الطرق هي ان أكثرها دقة شاقة ومكلفة في حين أن الآلية السريعة منها غير قادرة على كشف وجود بيوض ويرقات . يتم حاليا تطوير طرق كشف جديدة سريعة ودقيقة ومنها الكشف بطرق البيولوجيا الجزيئية مثل تقنية تفاعل البلمرة التسلسلي و تفاعل البلمرة التسلسلي الكمي. في هذه الدراسة تم استخدام عينات ملوثة وعزل حشرات منها بغرض استخدامها كمرجع ايجابي.

استخلاص الحمض النووي منها ومضاعفته باستخدام تقنية تحديد قواعد النبتروجينية. عن طريق مقارنتها في بنك الجينات.

يم بادئات متخصصة لأحد جينات الميتوكوندريا "السيتوكروم اوكسيديز" الأحماض النووية. وهي " سيتوكروم اوكسيديز 1 " " سيتوكروم اوكسيديز 2 " " سيتوكروم اوكسيديز - " .

فحص جميع البادئات للتأكد من دقتها و خصوصيتها. تطوير طريقتين جديدتين

حجم الحبوب عند تشربها للسوائل لاجل

النووي وهما طريقة الطرد المركزي وطريقة التصفية ولكل منها ا حجم عينة مختلف يتمثل

10 50 جرام على الترتيب. ولضبط ظروف مثالية لتفاعل البلمرة التسلسلي تم اختيار

العينات بثلاثة تراكيز مختلفة وهي: التركيز الأصلي للحمض النووي بدون تخفيف، حمض نووي

10 100 وكان أفضلها تخفيف 10.

فحص فعالية البادئات وطرق الاستخلاص الجديدة على عينات حبوب تجارية وذلك في ست

تفاعلات منفصلة تمثل كل بادئه مع كلتا الطريقتين.

الحشرات فيما بينها وبين طريقتي الاستخلاص بحيث كانت البادئة " سيتوكروم اوكسيديز 1"

"سيتوكروم اوكسيديز 2" يقة التصفية أفضل من طريقة الطرد المركزي لتكون الأفضلية

لاتحاد البادئه "سيتوكروم اوكسيديز 1 مع طريقة التصفية".

Table of abbreviations

Abbreviation	Full word
Spp.	Species
NIR	Near Infrared Spectriscopy
ELISA	Enzyme Linked ImmunoSorbent Assay
PCR	Ploymerase Chain Reaction
RT-PCR	Real Time- Polymerase Chain Reaction
HPLC	High Performance Liquid Chromatography
CO	Cytochrome Oxidase
COI	Cytochrome Oxidase subunit one
mtDNA	Mitochondrial DNA
rDNA	Ribosomal DNA
Bp	Base pair
Tm	Melting temperature
BLAST	Basic Local Alignment Search Tool
IGRs	Insect Growth Hormones
rpm	Round Per Minute
Ng	Nanogram
Pg	Pictogram
MHz	Mega Hertz
FDA	Food and Drug Administration

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Chapter 1

Introduction

Home storage of food is a traditional skill that has been practiced for thousands of years [1]. People everywhere in the world store food supplies such as: grains, cereals, rice, flour, sorghum and spices either on a small scale in the pantries and kitchens or at large scale for commercial and industrial purposes [2-4]. The importance of grain storage lies in helping people to survive catastrophes, emergencies and during periods of scarcity or famine [5]. Farmers, traders and governments store grains because they are considered as the world's primary staple food and due to their seasonal harvesting [2, 4, 6] since they are not available all the time, they must be stored for different time periods either transit, short-term or long-term storage [7]. Transit storage represents the period during transport from one place to another where grains are stored in bags and arranged in stacks. Short-term-storage is non-airtight bulk storage from harvest to harvest seasons where grains are stored in various structures called: Bukhari, Kothar or mori. Long-term storage is done for large scale trade or by governments to maintain food banks, storing in airtight silos (figure 1.1) or elevators (figure 1.2).



Figure 1.1: Grain elevator in farms



Figure 1.2: Silos of Palestinian Poultry Company

During transit, short and long term storage, grains are exposed to attack by microorganisms including: fungi, mice and insect pests which cause a considerable damage to the stored product [2]. Storage pests constitute a serious problem in all countries; they destroy about 10-20% of agricultural products annually [9, 10]. Grain

infestation might occur in farms, warehouses or during shipping and transportation, thus continues to spread around the world throughout trading [10, 11]. Eventually, they can invade houses through usual methods of entry, reach grains and contaminate them [3].

1. Literature review

1.1 Insects: structure and orders

Insects, belong to phylum Arthropoda, the class insects represents the largest group of organisms on earth [12]. They are commonly grouped into 27 to 32 orders depending upon the classification used (Table 1.1 shows the major orders of insects).

Insects live in every habitat and they have certain features in common, their body is divided into three regions: head, thorax and abdomen (figure 1.3). The **head** contains the brain and bears a pair of sensory antenna, a pair of compound eyes, several simple eyes and the mouthparts which are modified in different groups in relation to their feeding habits [13].

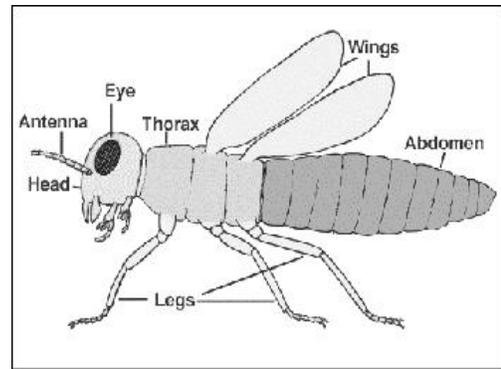


Figure 1.3: insect body plan

The **thorax** consists of three segments that bear three pairs of legs and the wings. The **abdomen** contains most of the internal organs segmented and usually more flexible than the head and thorax [12-14].

Table 1.1 major orders of insects [12]			
Order	Typical example	Figure	Approximate number of named species
Coleoptera	Beetles, weevils		350,000
Diptera	Flies		120,000
Lepidoptera	Butterflies, moths		120,000
Hymenoptera	Bees, wasps, ants		100,000

Hemiptera and Homoptera	True bugs, bedbugs, leafhoppers		60,000
Orthoptera	Grasshoppers, crickets, roaches		20,000
Odonata	Dragonflies		5,000
Isoptera	Termites		2,000
Siphonaptera	Fleas		1,200

Mites belongs to class Arachnida, eight legged arthropods, they have only two body regions, eight legs, do not have wings and they develop by simple metamorphosis and many are crop pests. The class includes spiders' ticks and mites [14].

1.2 Insect's growth and development

The life cycle of insects usually begins when adult female lays eggs -oviposition- which hatches to give an immature stage that develops successively through a process called metamorphosis. There are two types of metamorphosis; simple and complete metamorphosis. Insects are classified

into two main categories according to their developmental stages between eggs and adult: (1) Simple or incomplete metamorphosis: there are only three life forms: egg, nymph and adult (figure 1.4). Nymphs look very similar to the adult except for wings and reproductive organs [14,

15]. (2) Complete metamorphosis: there are four life forms: egg, larva, pupa and adult (figure 1.5). Larvae could be worm-like, grub-like or

Simple metamorphosis

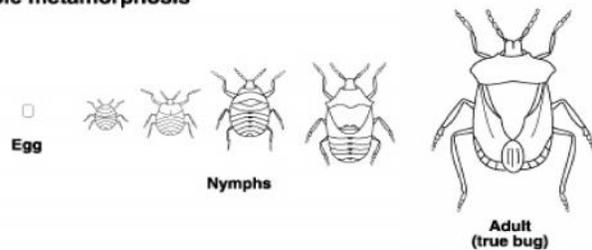


Figure 1.4: A true bug is an example of insects with simple metamorphosis

Complete metamorphosis

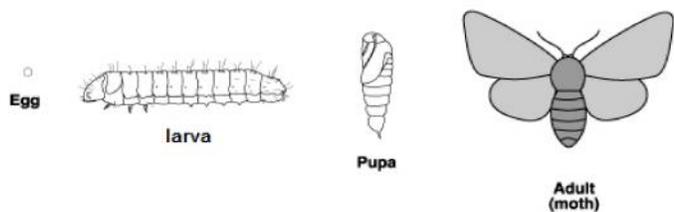


Figure 1.5: codling moth is an example of insects with complete metamorphosis

maggot-like. The length of a generation or the period between stage and another in the life cycle is affected by environmental factors such as temperature, humidity and the availability of food [14]. Temperature is identified as the dominant a biotic factor directly affecting insects [15] because they are cold –blooded and depend on the temperature of their environment for growth and development, most insects prefer warm temperatures (29°-35°) while cooler temperatures result in slow growth [16]. Behavioral activities such as flight, movement, reproduction and feeding are similarly influenced by seasonal temperatures [17]. Relative humidity suitable for insects ranges between 60-80% but most pest are adapted to survive at very low humidity because they obtain water through their food supply [17, 18]. Improper maintenance of storage temperature and humidity results in insect development in grains which lead to biological and chemical damage. Most local grain stores and shops do not take into account temperature and moisture parameters and there are no aeration systems which make storage conditions suitable for insect pest infestations. It is therefore important to examine grains periodically to detect the presence of any insects as early as possible to avoid grain loss.

1.3 Identification of insect pest species

Some insects are considered as pests or vectors because they can feed on or transmit disease to human, animals, plants, food and structures [19, 20]. Over 60 species of insects can infest stored grains [21]. Table (1.2) shows the most common pest species that infest grains. Beetles (order Coleoptera), moths (order Lepidoptera) and mites (Arachnida) are the most common [9, 10, 22, 23]. The principle pests that cause damage are the adult and larval stage of beetles and the larval stage of moths [24]. Each one of these pests prefers a grain type but can live in and contaminate several grains. The grains such as wheat, rice, lentils and chickpeas are major grains in our country and stored in large quantities for long periods, so it is very important to have a good method for detection of insect pests in grains especially if they are processed into other forms where insects are ground with grains and insect fragments are difficult to detect by naked eye or by simple detection techniques.

Table 1.2: The most common pest species [25-29]

	Scientific name	Common name	Host or commodity	Order
1	<i>Tribolium castaneum</i>	Rust-red flour beetle	Whole grain, animal feed	coleoptera
2	<i>Oryzaephilus surinamensis</i>	Sawtoothed grain beetle	Cereals, peanuts	coleoptera
3	<i>Sitophilus oryzae</i>	Rice weevil	Rice, sorghum	coleoptera
4	<i>Sitophilus granaries</i>	Granary weevil	Wheat, barley	coleoptera
5	<i>Trogoderma granarium</i>	Khapra beetle	Cereals, oilseeds, pulses, rice	coleoptera
6	<i>Trogoderma variabile</i>	Warehouse beetle	Cereals, rice, pulses	coleoptera
7	<i>Acanthoscelides obtectus</i>	Bean bruchid	Navy and lima beans	coleoptera
8	<i>Stegobium paniceum</i>	Drugstore beetle	Dried herbs, spices, drugs	coleoptera
9	<i>Rhyzopertha dominica</i>	Lesser grain borer	Wheat, corn	coleoptera
10	<i>Lasioderma serricorne</i>	Cigarette beetle	Cereals, tobacco	coleoptera
11	<i>Plodia interpunctella</i>	Indian meal moth	Cereals, barely, rice, maize	Lepidoptera
12	<i>Sitotroga cerealella</i>	Angoumois grain moth	Maize, wheat, rice	Lepidoptera
13	<i>Corcyra cephalonica</i>	Rice moth	Rice, dried fruits, cereals	Lepidoptera
14	<i>Ephestia kuehniella</i>	Mediterranean flour moth	Cereals, rolled rice, peanuts	Lepidoptera
15	<i>Acarus siro</i>	Flour mite "not insect"	Cereals, flour	Acaridae

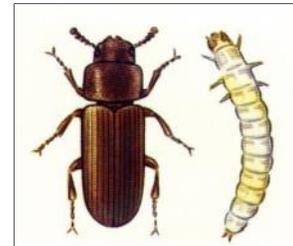
Correct identification of pests is very important because it allows more targeted pest control options. One of the identification methods is based on morphology of the pest as shown below:

(1) ***Tribolium castaneum*** :Rust-red flour beetle

Morphology: adult beetles are 3-4.5 mm long, bright reddish-brown in color when young and darker brown when older, strong fliers.

Distribution: cosmopolitan, active all year.

Survival: adults hide in cracks and crevices in empty storages when food is scarce, reproduction stops below 20C° [30].



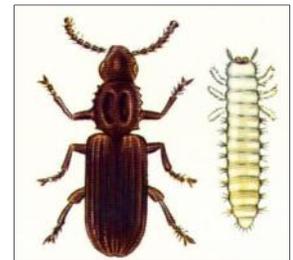
(2) ***Oryzaephilus surinamensis***: Saw-toothed grain beetle

Morphology: adults are 3 mm long, dark brown to black, fast moving.

Life cycle: 21 days from egg to adult at 30-33C° and 60-80% relative humidity.

Distribution: cosmopolitan, active all year [31].

Survival: very cold tolerant but reproduction stops below 17.5C° [32].

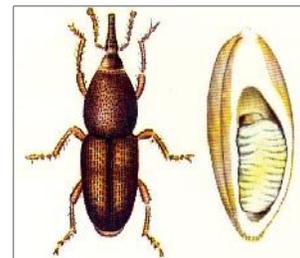


(3) ***Sitophilus oryzae***: Rice weevil

Morphology: adults are 2-4 mm long, dark brownish black with a long weevil snout [33].

Life cycle: 37 days from egg to adult at 26-28C° and 66-72% relative humidity.

Distribution: cosmopolitan, active all year [31].



Survival: fight death by drawing their legs close to the body falling and remaining immobile when disturbed, reproduction stops below 15C° [34].

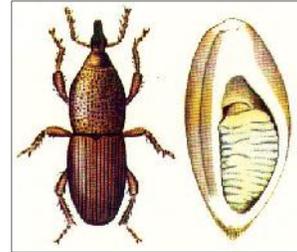
(4) ***Sitophilus granaries***: Granary weevil

Morphology: adults are 3-5 mm long, black-brown, the head ends in a slightly curved proboscis.

Life cycle: 44 days from egg to adult at 26-28C° and 66-72% relative humidity.

Distribution: distributed throughout the temperate regions of the world, uncommon in tropics except in cool upland areas, active all year [27].

Survival: can survive outside grain storage facilities; colonized outdoors and become a new source of infestation [35].



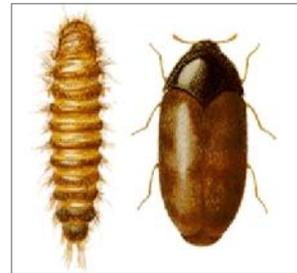
(5) ***Trogoderma granarium***: Khapra beetle

Morphology: adults are 2-3mm long, oval, reddish-brown, hairy.

Life cycle: 30 days from egg to adult at 35C° and 75% relative humidity.

Distribution: originated in India but now introduced to Middle East, Africa and south Asia, active all year.

Survival: larvae can tolerate unfavorable conditions for several months to years [27].



(6) ***Trogoderma variabile***: Warehouse beetle

Morphology: adults are 2-3mm long, oval, brown, hairy.

Life cycle: 27 days from egg to adult at 32C° and 70% relative humidity.

Distribution: originated in central Asia but now established in the northern hemisphere.

Survival: larvae are very cold tolerant and can diapauses up to 2 years but reproduction is inhibited below 20C° [27, 38].



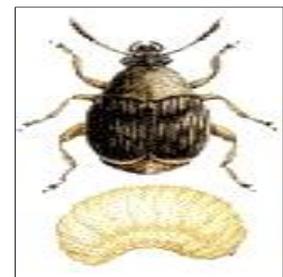
(7) ***Acanthoscelides obtectus***: Bean bruchid

Morphology: adults are 3.5mm long, gray brown, the end of the abdomen is yellow-red.

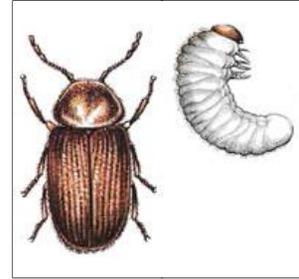
Life cycle: 23 days from egg to adult at 32C° and 70% relative humidity.

Distribution: cosmopolitan, more active during summer.

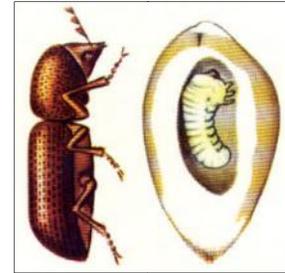
Survival: continue breeding at low temperatures: 18C° [41].



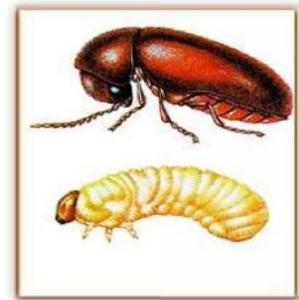
- (8) *Stegobium paniceum*: Drugstore beetle
Morphology: adults are 2-4 mm long, reddish brown, oval body with fine hairs.
Life cycle: 70 days from egg to adult at 28C° and 70% relative humidity.
Distribution: cosmopolitan, active all year.
Survival: young larvae can penetrate even the finest cracks then form a cocoon of nutrient particles [27].



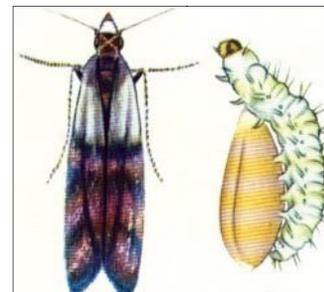
- (9) *Rhyzopertha dominica*: Lesser grain borer
Morphology: adults are 2-3mm long, dark brown, cylindrical body.
Life cycle: 25 days from eggs to adult at 34C° and 70% relative humidity [37].
Distribution: cosmopolitan, active all year [31].
Survival: can survive in grain with moisture content as low as 8-9%, reproduction stops below 15C° [27, 33].



- (10) *Lasioderma serricorne*: Cigarette beetle:
Morphology: adults are 2-4mm long, reddish-brown, covered with fine hairs
Life cycle: 34 days from egg to adult at 30C° and 70% relative humidity.
Distribution: cosmopolitan, but most abundant in the tropics, active during summer months [27].
Survival: can't tolerate food shortage: larvae eat eggs and pupae in the absence of other food sources [36].



- (11) *Plodia interpunctella*: Indian meal moth
Morphology: adults are 5-10mm long, 18-20mm wingspan, dark-reddish-brown on half of the wing and gray at the front [27, 33].
Life cycle: 28 days from egg to adult at 30-35C° and 70-80% relative humidity.
Distribution: cosmopolitan accept Antarctica, active all year [31].
Survival: larvae enter diapauses by spinning a fine pupal cocoon surrounded by dense protective cocoon and remaining inactive for several months; adults are non feeders, short lived [39].



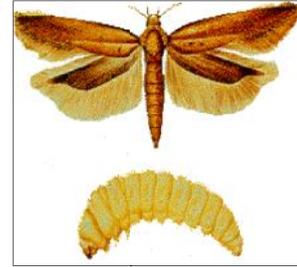
(12) *Sitotroga cerealella*: Angoumois grain moth

Morphology: adults are 5-6mm long, 12mm wingspan, yellowish-brown with small black spots.

Life cycle: 25-30 days from egg to adult at 30C° and 75% relative humidity.

Distribution: cosmopolitan, active during summer months.

Survival: larvae can remain dormant for four to five months under unfavorable conditions; adults only live for 5-10 days [27].



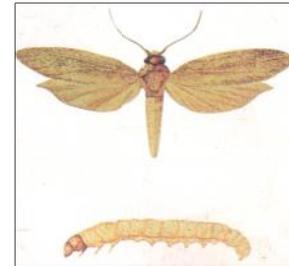
(13) *Corcyra cephalonica*: Rice moth

Morphology: adults are 5-10mm long, 15mm wingspan, light grayish-brown.

Life cycle: 41-59 days from egg to adult at 24-28C° and 70% relative humidity.

Distribution: cosmopolitan, active all year.

Survival: it is the only living species of the genus *Corcyra* [40].



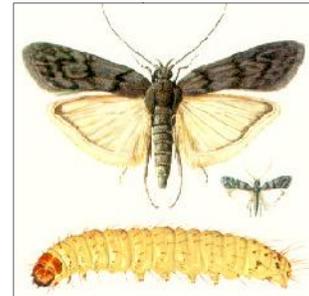
(14) *Ephestia kuehniella*: Mediterranean flour moth

Morphology: adults are 5-7 mm long, 15-16 mm wingspan, brownish-gray crossed with light colored bands.

Life cycle: 6-7 weeks from egg to adult at 25C° and 70% relative humidity [27].

Distribution: cosmopolitan, active during summer months.

Survival: larvae can leave food and crawl about cupboards, walls and ceilings looking for place to pupate [38].



(15) *Acarus siro*: Flour mite

Morphology: adults are 0.5mm long, pearly or grayish white.

Life cycle: 9-12 days from egg to adult at 28-32C° and 80-85% relative humidity.

Distribution: cosmopolitan, active all year.

Survival: under unfavorable conditions mites in the second nymphal stage can enter a highly resistant stage known as the hypopus, become immobile and may remain for months without food, can tolerate very low temperatures: 5C° [42].



1.4 Classification of insect pests

Pests are classified on the bases of feeding capacity into two types: primary and secondary pests. **Primary pests** or internal feeders are economically important because they are capable of breaking down the hard seed coat of sound undamaged grain producing perforated grain and grain dust. These insects can lay their eggs inside or outside the grain. Pests of this type include angoumois grain moth, lesser grain borer, rice weevil and granary weevil [2]. **Secondary pests** or external feeders are less dangerous than the primary insects because they are not able to break through the hard seed coat therefore they cannot attack healthy undamaged grain, but they can cause a considerable damage if the grains are mechanically broken or processed into products like flour or have already been attacked by primary pests [1, 43]. Examples on the secondary pests are saw-toothed grain borer, red flour beetle, Indian meal moth, Mediterranean meal moth, rice moth and mites [2].

1.5 Economic losses and health risks due to insect pest infestations

Storage pests are economically important and are responsible of millions of dollars loss [3] because large amount of food is wasted every year due to insect damage during storage [5]. The presence of insect pests in grains causes a serious reduction in its market value and some developed countries apply zero tolerance policies and will reject grains if a single live insect is founded [1]. The losses are measured in terms of quantitative, qualitative and nutritional value [43]. Quantitative losses are due to direct feeding of insects which reduces grain weight and nutritional value; heavy infestations may convert a solid grain to a mass of powder [24]. Qualitative losses are by contaminating the grains with insect excreta, pupal cocoons, dead bodies and odors. They also produce heat when they reproduce especially weevils which lead to mould development and invasion by other insects [27]. This deterioration of grains makes them unpalatable and unsuitable for processing into food for humans and animals [22, 24].

Pest infestations also reduce germination capacity of the grain by feeding in the endosperm or the kernel of the grain [30]. Moreover, some of these insects like cigarette

beetle feed on and damage furniture, paper, books and clothes [36] and others like warehouse beetle can chew aluminum foil and plastic wrappers [44].

Many health problems are associated with insect pest, the most prominent are allergic reactions caused by direct skin contact with insects especially mites as well as by inhalation of live mites and fragments of dead mites or their excretory pellets. Mites and weevils cause rhinitis, urticaria, pruritus, asthma, dermatitis, intestinal disorders and anaphylactoid reactions [35, 42]. The sharp hairs of the larvae of warehouse beetle break-off the cast skins and can lodge in the throats and intestinal linings of humans and animals and ingestion of the hairs often causes vomiting and diarrhea that lasts for several days [44].

Some of these insects may carry fungi, microorganisms and they act as vectors for the spores of *Aspergillus flavois* and *Aspergillus parasiticus* that produce mycotoxin called aflatoxin which is carcinogenic and fatal to humans and animals [45, 46]. Other insects have been involved in the transmission of pathogenic bacteria such as salmonella as reported by Martin et.al that *Sitophilus oryzae*, *Sitophilus granarius*, *Triboliwn castanewn*, *Oryzaephilus surinamensis*, *Rhyzopertha dominica*, *Tenebroides mauritanicus*, and *Cryptolestes pusillus* transmitted *Salmonella montevideo* from wheat contaminated with 10^6 organisms/g to clean wheat [47]" and *Enterococcus spp.* as reported by Lakshmikantha et.al where 145 enterococcal isolates from 95 stored product insects collected from feed mill, a grain storage silo and a retail store were isolated and identified to the species level using PCR. Enterococcus species are recognized as feared nosocomial pathogens that cause hospital-borne infections worldwide and responsible for the development and transmission of antibiotic resistance traits [48]. Antibiotic resistance in *Enterococcus* species can be transferred by pheromone-mediated conjugative plasmids or transposons. The resistance genes may be passed on not only to antibiotic-susceptible enterococci, but also to other pathogens [49].

Animals may also get hurt if their feed were contaminated with insects and mites. They show reduced feed intake, diarrhea, lesions, digestive ulcers, inflammation of the small intestine and impaired growth [42, 50]. Animal feed is also suspected for *Enterococcus* transmission by insects as reported by Channaiah et.al that adult *Tribolium castanium*

insects were successful in acquiring and transferring *Enterococcus faecalis* from inoculated poultry feed to sterile poultry feed during 7-days test period [51].

1.6 Current methods for detecting the presence of insect pests in grains

There are several methods for the detection of external and internal insect infestations. External infestations could be detected by visual inspection, sampling and sieving methods while internal infestations need more accurate detection methods.

1. **Visual inspection:** many observations can indicate the presence of insects infestations like: the presence of holes in grains, the accumulation of moulted cuticles, webbing or strands of silk and the presence of small fecal pellets. This could be done by naked eye or by microscopy techniques [52, 53].

2. **Sampling and sieving:** it is the oldest, popular commercially and widely practiced method till now due to its rapidity and minimum requirements of equipments. It involves drawing grain sample of 0.5-1 kg using a probe into a standard test sieves, the separation is based on difference in particle size of product and contaminant. The accuracy of this method depends on the quantity of each sample and insect population density in the grain [52, 53].

3. **X-ray micro-tomography:** 3-D imaging technique that operates the same basic principle of medical computed tomography but with higher resolution. Scans are done by rotating the sample perpendicular around x-ray beam while collecting radiographs of the sample [52]. This technique was first used by Michael et.al in 2006 where x-ray computed tomography was used to image hard red winter wheat *Triticum aestivum* samples infested with pupa of *Sitophilus oryzae*. Samples were imaged in a 7.6cm plastic tube containing 0, 50 or 100 infested kernels per kg wheat [54]. This method can even detect eggs inside the kernels, and can determine stage and possibly species of both live and dead insects but it is time consuming to take, process, and examine film X-rays; requires skilled personnel and is expensive.

4. **Acoustical sensors technique:** using acoustical sensors to hear insects feeding inside kernels, this method was used by Mankin et.al in 2010 to detect the crawling and

scraping activities of three stored product pests: *Sitophilus oryzae*, *Tribolium castaneum* and *Stegobium paniceum* were monitored individually by infrared sensors, microphones and a piezoelectric sensor in a small arena. This technique evaluates effect of insect locomotory behavior and size on the ability of an inexpensively constructed instrument to detect insects and distinguish among different species [55]. Accuracy of acoustical methods is correlated with temperature and activity of insect moving and feeding; the number of sounds increased as the temperature increase.

5. Near- Infrared spectroscopy: NIR can measure the chemical composition of biological materials by using the transmittance of the sample at several wave lengths; it can measure the concentration of components having different molecular structures [56]. The detection of insect pests by NIR was reported by Mendoza et.al in 2002 [57], Chen et.al in 2009 were used Near-Infrared image processing in the agricultural engineering [58]. This method is relatively fast and accurate but can't detect insect fragment below FDA level.

7. Enzyme-Linked Immunosorbent Assay: ELISA is a quantitative method based on antigen-antibody reaction. For insect detection by ELISA, an insect specific antigen is required, muscle protein myosin is usually used; it is present in all life stages, except eggs of insects [52]. Detection of infestations by ELISA was first reported by Kitto et.al in 1994 and the method is still used till now. ELISA test detects all major grain insect pests and gives a positive and linear response to larval and pupal life stages and adult insects [59]. This method is accurate for fresh samples because myosin degrades over time and, thus, underestimation of dead insects may occur. Insect eggs can't be detected by this method because they lack myosin [52].

8. Uric acid analysis: uric acid is the principle end product of nitrogen metabolism of almost all terrestrial insects and the major component of their excreta [56]. Uric acid is quantified in infested samples using reversed-phase High Performance Liquid Chromatography HPLC. Detection using uric acid was reported by Ghaedian and Wehling in 1996 where HPLC was used to quantify uric acid content in samples of wheat infested with granary weevil larvae, the samples had about 25µg of uric acid per gram wheat [60]. This method detects uric acid excreted by any insect species in grain

commodity over the entire storage period and may under estimate number of insects that eject their excreta outside grain kernel [61].

Several other methods have been developed to detect hidden infestations like staining of kernels to detect eggs and density separation based on infested grains are lighter than non-infested grains and float in the liquid [61]. Physical traps are also used to detect infestations based on the locomotory activity of insects, these are diverse: sticky traps, pheromone traps, multiple funnel traps and attractant traps [53].

The accuracy of the above-mentioned methods depends on insect species, developmental stage of insects, grain type and can prove less sensitivity to low population densities. The development of better methods of insect detection in grains and processed food is very important. **Molecular biology techniques** are widely used in microbiology for detection of viruses, bacteria and fungi and are being used in entomology, ecological research as well as studies of stored-product insect pests' detection and proved their rapidity, reliability and allow for large scale analysis of multiple samples [62].

1.7 Detection of insect infestations by molecular methods

The significance of molecular methods is the ability to detect primary pests that develop inside grain kernels, causing hidden infestation, which is very difficult to detect, particularly just after oviposition and during the early larval stages and the remnants of insects bodies in processed food. Molecular techniques are based on DNA barcoding which is identified as an identification approach that uses short DNA sequences from a standardized region of the genome as a reference sequence for species identification [63, 64]. DNA barcoding has recently emerged as a rapid method for species identification and discovery without the need for detailed taxonomic knowledge. Identifications are usually made by comparing unknown sequences against known species DNA barcodes via distance-based tree construction or alignment searching (e.g., BLAST) [64, 65]. The standard sequence used is a mitochondrial gene usually cytochrome c oxidase subunits COI and COII, cytochrome b or ribosomal DNA [65]. Mitochondrial DNA is used because of its simple maternally inheritance, average nucleotide composition, patterns of strand asymmetry and the high frequency of codons that encode hydrophobic amino

acids. The sensitivity of molecular detection is very high by the use of mitochondrial genes because they occur in the cell in many copies and approximately all mitochondrial genes sequences are known [67, 68]. These sequences based primers have been used in Polymerase Chain Reaction PCR or real time PCR for the detection and identification of insect pests as reported by Ahrens et.al in 2007 were two mitochondrial genes: cytochrome oxidase subunit 1 (cox1) and 16S ribosomal RNA (rrnL) were amplified using PCR and sequenced for the analysis of different life stages of coleopteran species [69]. Obrepalska et.al in 2008 used standard and real-time PCR in the detection of granary weevil in wheat flour using COI and COII subunits "[70], Wei et.al in 2012 have identify two Psoids species by the analysis of mitochondrial sequences"[67], Virgilio et.al in 2012 have identify insects with incomplete DNA barcode libraries"[71] and Nowaczyk et.al in 2009 used standard PCR with rDNA and COI primers for the detection of *Tribolium confusum* and differentiation between *Tribolium confusum* and *Tribolium castaneum*. They also used real-time PCR for quantitative detection of *Tribolium confusum* in contaminated Oat flakes samples. The authors' brought *Triplium* spp. from laboratory colonies and used DNeasy Blood and Tissue Kit for DNA extraction, plant DNA as negative control and prepared a series of dilutions of oat flakes DNA corresponding to infestation levels of 100 insects per kg, 10 insects per kg, one insect per kg, and one insect per 10 kg. The sensitivity was very high and detection level was up to one insect per 10 kg (figure 1.6) [62].

1.8 Controlling insect infestations in stored grains

• 1.8.1 Preventing insect infestations

There are several precautions and preparation that could be done before grain storage to decrease the chance of having insect infestations, these include:

1. Sanitation: it is a hygienic step before adding grains to the storage facilities which includes cleaning up old grains, trash and insects, walls, ceilings, sills, floors, ledges, the ventilation system and also the outside area [21].

2. Using insecticide sprays: they create a barrier against insect migration, applied after cleaning the storage facility when it is still empty; at least two weeks before adding the grains. Sprays are allowed to dry for 24-hours [8, 21].
3. Using chemical grain protectants: application of insecticides directly to the grains to prevent infestations (not to eliminate existing infestations), may be added during grain filling or to the upper surface of the grain [8, 72].
4. Controlling grain Moisture, Temperature and Aeration: grain temperatures durum storage should be maintained below 20C° to avoid mould and insect reproduction. Controlling temperature itself is difficult because many factors influencing storage temperatures like the sun, the cooling effect of radiation from the store, outside air temperature, heat generated by the respiration of both the food in store and any insect pest present, so aeration is necessary to move air through the grain mass and keep it cool. Aeration is usually achieved by air ducts or fan operation. Grain moisture content is related to the temperature and relative humidity of the air that surrounds it. Under proper conditions of temperature and humidity, grain will neither lose nor gain moisture. Grain moisture should be maintained below 12% at which insects and fungi can't develop [72, 73].

1.8.2 Controlling established infestations

1. **Chemical control: fumigation:** it is one of the most effective control methods in which insect pests are exposed to a poisonous gaseous environment. Fumigants enter the insect body through spiracles and spread to bind the hemolymph components. The most used fumigants are phosphine fumigants and methyl bromide fumigants both are used to protect legumes and cereals [74] they are safe and have no effect on germination [10, 72].
2. **Physical control:** by manipulating the physical environment or applying physical treatments to the infested grains, including:
 - a) **Thermal control:** most insects of stored grains can't tolerate extreme temperatures. Superheating of grains up to 55-65C° for 10-12 h can effectively kill all life stages of insect pests. Heating is applied by hot-air

convection, infrared or microwave radiation. The disadvantage of such heating that it can damage the baking quality of wheat, the malting quality of barely and the germination of most seeds. Low temperatures $< 12^{\circ}\text{C}$ reduce insect development and maintains seed viability [1, 10].

- b) **Inert dust:** un-reactive chemically and kill insects by physical contact. Their killing effect is through desiccation and dehydration of insects by causing moisture to go out of insect body by scratching through or absorbing the waxy coating that normally prevent excessive moisture loss. Types of inert dusts are: sands and soils, Diatomaceous earth, Silica aerogel, non silica dusts and particle films [1].
- c) **Ionizing radiation:** an environmental friendly control strategy. There are two types of ionizing radiation: Gamma radiation produced by radioactive isotope such as Cobalt-60 or Beta radiation which is a beam of electrons that can be generated electrically and it is safer and easier to deal with. Insect damage or death is caused by production of highly reactive free radicals or ions. The radiation kills all life stages of insects and do not affect the nutritional value of the grain [1, 10].
- d) **Light and sound:** light is used to attract and trap flying insects by mass killing and 1MHz sound exposure for 5 minutes can kill some insect species such as *Sitophilus granaries* [10].

3. **Behavioral control using insect pheromones:** males and females pheromones were used to uphold communication disruption or to attract insects for mass trapping. Disruption of mating by pheromones also provides a wide suppression of insect population [10].

4. **Control by using insect growth regulators:** insect hormones or their analogues IGRs are found to be successful against storage insects. IGRs disrupt oviposition behavior and cause impairment of reproduction. Two widely used IGRs are methoprene and hudpene [10].

5. **Microbial control:** alternative to synthetic pesticides in which microbial insecticides in the forms of spores and toxins are used, they show reduction in oviposition and

massive mortality in insect population. The most effective is Beta toxins produced by *Bacillus thuringensis* [10].

6. **Biological control:** using living organisms as parasitoids, predators, pathogens to reductions pest population. Hemimpteran bug *Xylocoris flavipes* is frequently used to control pests of coleoptera and lepidoptera [1, 10].

7. **Control by natural plant products:** botanicals or plant essential oils and their chemical constituents are reported for their developmental inhibitory activities against insect pests, inhibition of oviposition, repellent and insecticidal activity. These molecules could be of a proteic nature, including thionins, defensins, lipid transfer proteins, snakins or protease inhibitors. They may also be produced by the secondary metabolism of plants, and many defense molecules are of an alkaloid, saponin or flavonoid type [75]. Many studies proved the effectiveness of plant products against insect pests Pedro et.al reported about the toxicity and specificity of saponin 3-GlcA-28 AraRhaxyl-medicagenate extracted from *Medicago truncatula* at concentrations down to 100 µg per g of food towards adult of rice weevil *Sitophilus oryzae* [75]. Another study for (Frank et.al) has proven that catmint oil and hydrogenated catmint oil were evaluated as repellents for adult *Tribolium castaneum* and *Tribolium confusum* [76].

Objective

The main focus in this study is to develop a reliable and specific molecular test for the detection of the presence of insect pests in home stored grains. The specific objectives are:

- 1- Standardization of a suitable treatment for grains before DNA extraction.
- 2- Standardization and optimization of a convenient and efficient DNA extraction method suitable for insects in plant seeds.
- 3- Designing and utilization of primers based on selected common genes.

Identification of these pests enables us to decide about implementations of control measures needed for the elimination of identified pests and thereby reducing the loss of grains which will have a major impact on population health.

Chapter 2

Materials and methods

2.1 Samples Used in This Study

A total of 11 grain samples were collected from Bethany region-Jerusalem from local wholesale grocery stores, samples were chosen randomly from sacks which comprised 500 gram of large grains (>0.5cm) and 250 gram of fine grains (<0.5cm). Samples included: corn, wheat, rice, groat, lentils, chickpeas, sesame, cumin, corn flakes, barely and animal feed.

2.2 DNA Extraction

2.2.1 DNA extraction of insects isolated from infested samples

Three infested samples were obtained from home to be used as positive controls: rice, flour and barely samples were heavily contaminated with fully developed larvae and adult insect pests. With the aid of dissecting microscope, larvae from infested rice and adult insects from infested flour and barely samples were directly isolated for DNA extraction. Commercial DNA free distilled water was used as negative control in all experiment.

2.2.2 DNA extraction from the commercially bought samples

The eleven samples showd no signs of infestation neither by naked eye nor under microscope. Grain samples were processed by two different procedures for DNA extraction: **procedure 1: centrifugation washing method** and **procedure 2: filtration washing method**. In the first procedure, 10 grams of grains were put in 50ml sterile plastic tubes containing 20ml distilled water and mixed for 2 minutes. Next 10ml of the turbid water were withdrawn using sterile plastic pipet and transferred into another 50ml plastic tube and centrifuged for 10 min at 4000 rpm. The supernatant was discarded and 400 µl lysis buffer (50 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl pH 7.4, 1% triton X-100, and 200 µg/ml of proteinase K) was added to the pellet for resuspension and transferred to 1.5 eppendorf tube. In the second procedure, 50 gs of grains were put in sterile 100-200 ml glass beakers (according to grain size) containing 70 ml sterile

distilled water and mixed for 2 min. Then 40 ml of the turbid water were drawn into another glass beaker to be filtrated through 47 mm diameter 8 µm pore size nitrocellulose membrane filters (Whatman Inc, Piscatway, NJ) using vacuum filtration system. The membrane filters were left at room temperature to dry and then punched, 4 small disks (0.5 diameter) were taken to 1.5 eppendrofs tube, 400 µl lysis buffer were added.

2.2.3 Phenol based DNA extraction method: eppendorf tubes that contained the samples in 400µl lysis buffer were incubated at 60°C for 2 hours. Equal volumes of TE-saturated phenol (pH 8) were added to the aqueous solution, the mixture was vortexed for few seconds and then centrifuged for 2 minutes at 14,000 rpm. The upper aqueous layer was transferred to new eppendorf tubes and the DNA was precipitated by 0.2M NaCl (addition of 16µl of 5M NaCl to 400 µl aqueous solution) and 2.5 volumes of 100% cold ethanol. The mixture was incubated overnight at -20°C and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet was left to drain by inverting the tubes on tissue paper then dried at 60C° oven for 20 min. Finally, 100 µl sterile double distilled water was added to each tube to dissolve the extracted DNA.

2.3 Bioinformatics and Primers Design

Three previously designed primers for non-specific cytochrome oxidase I DNA (COI) DNA amplification of many species of order Diptera (Table 2.1) were used to amplify DNA extracted from larvae and adult insects isolated from rice, flour and barely infested samples using PCR amplification. PCR amplification products were subjected to sequencing to identify each pest. The obtained 4 sequences were identified using BLAST generated comparison. The original sequences of COI gene of the identified pests were downloaded from Gene Bank and then aligned using ClustalW2 alignment software. The primers are constructed from regions of the highly conserved sequence of COI and should be suitable for amplifying the desired gene in many pest species. The designed primers are listed in (Table 2.2).

Table 2.1: The previously designed primers					
	F/R	Primer sequence 5'-3'	Primer size (bp)	Amplicon size (bp)	Tm (c°)
Comp. CO	F	TCATAAAGATATTGGAAC TTTATAC	25	750	53.1
	R	GATGTCCAAAAAATCAAATAAAT	24		50.7
CO1	F	GGAAC TGGGTGAACAG TTTATCCCC	26	350	66.4
	R	ATGTTGATAAAGAATAGGATCTCCTCC	27		60.4
CO2	F	AATAATATAAGATTTTGACTTCTTCC	25	350	52.8
	R	TATAGTAATAGCTCCAGCTAAA ACTGG	26		52.8

Table 2.2: The newly designed primers					
	F/R	Primer sequence 5'-3'	Primer size	Amplicon size (bp)	Tm (c°)
COI-long1	F	ATTGGAGGATTCGGAAATTGA	21	456	52.0
	R	CCTCCTGCTGGATCAAAAAA	20		55.5
COI-long2	F	TAATCCGATCCGAATTAGGAA	21	370	50.7
	R	ATTGATGAAATTCCTGCTAAATG	23		49.7
COI-mite	F	ATTTATGCTATAACTGCAATTGG	23	308	49.5
	R	GTGCAACTACATAGTAAGTGTC	22		50.9

2.4 Polymerase Chain Reaction (PCR)

PCR amplification reaction was carried out in a volume of 25µl using Ready Mix PCR tubes (Syntezza, Jerusalem, Israel). In each tube 20 µl of sterile water containing 15 pmoles of each primer (reverse and direct) were added followed by the addition of 2 µl from the original DNA extract or 5µl from each DNA diluted sample. The amplification protocol was run as follows: 5 min at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 50°C, 1 min at 72°C, and a final elongation step at 72°C for 10 min.

2.5 Agarose Gel Electrophoresis

The PCR amplified DNA fragments were resolved on 1.5% agarose gels prepared by dissolving 1.5g agarose in 100ml 1x TAE buffer (Tris base, acetic acid and EDTA). The mixture was then heated in a microwave oven until the agarose was completely dissolved. An E-C Apparatus (EC Corporation system) was used to run the gel after the addition of Ethidium Bromide solution. In each lane, 10 μ l of PCR products were loaded in the gel and pUC 8 Mix Marker (Fermentase, Lithuania) was used for sizing PCR amplification products. The gel was run at approximately 80-110 Volts for half an hour. The results were recorder by gel documentation system (DNr Bio-Imaging System).

2.6 DNA Purification

PCR products were purified using an ethanol precipitation procedure where 15 μ l of the remaining PCR product was added to 35 μ l double distilled water in an eppendrofs tube and 2 μ l of 5M NaCl and 150 μ l of 100% cold ethanol were added and the mixture was incubated at -20C° overnight, followed by centrifugation for 10 min at 14,000 rpm. The supernatant was discarded and the DNA pellet was left to dry after inverting the tube on tissue paper and then at 60C° oven for 20 min. Finally 100 μ l double distilled water was adder to each tube to resuspend the purified DNA pellet.

2.7 Sequencing

Purified PCR amplified DNA fragments were sequenced according to the dye terminator method, using Automated DNA Sequencer machine (AB477). Forward PCR amplification primer was used for one direction sequencing.

2.8 DNA Quantitation

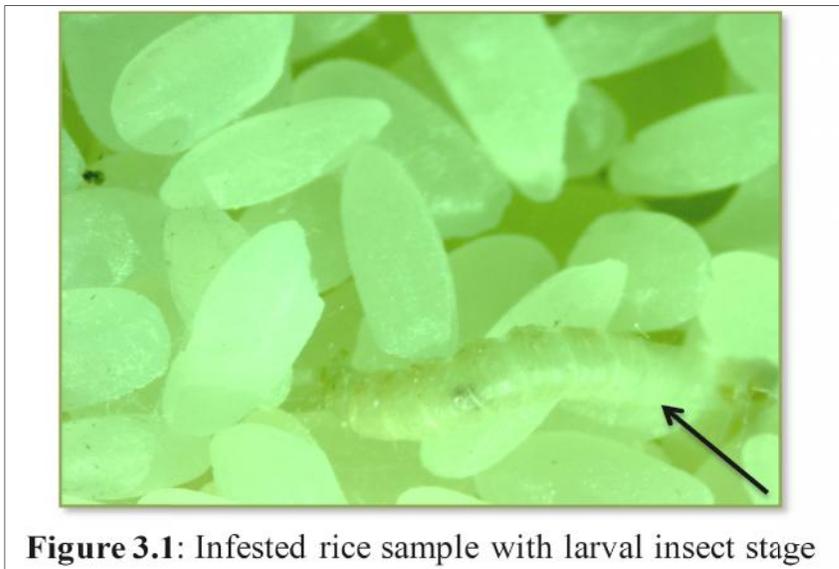
Extracted DNA positive controls were quantified using a NanoDrop instrument (Thermo Fisher scientific Inc, Waltham, Massachusetts, USA). Most of the positive control DNA samples were in the range between 50- 300 ng while in grain samples used in this study DNA concentration ranged between 400 ng to 2 microgram.

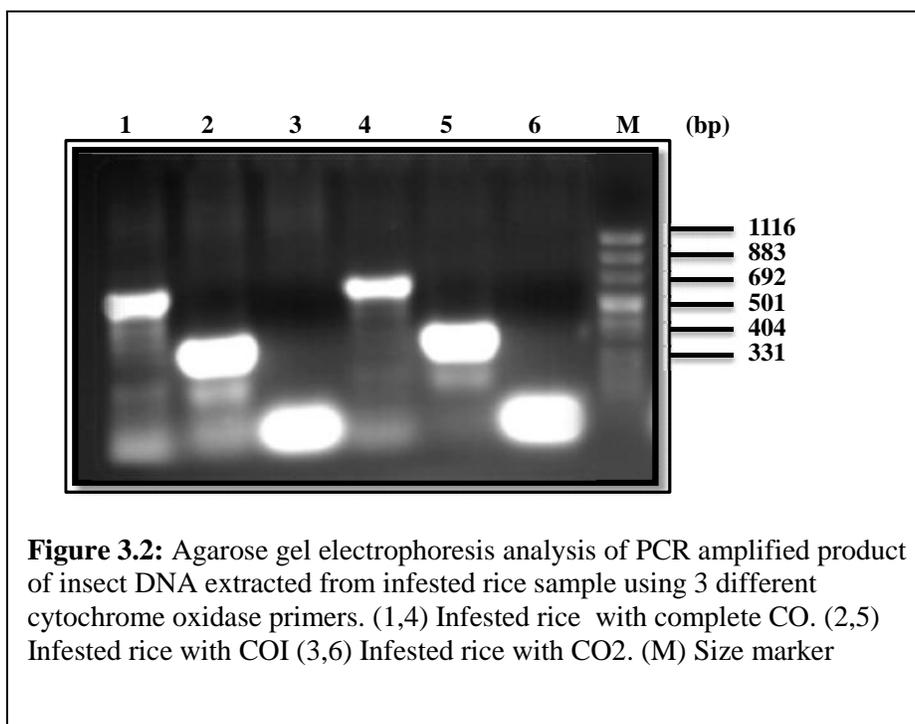
Chapter 3

Results

3.1 Preliminary screening of insects grain pests.

Previously designed primers that amplify insects' cytochrome oxidase I (COI) were used to amplify insect DNA extracted from infested rice sample. For this purpose insects' larvae were directly removed from infested rice by the aid of a dissecting microscope (figure 3.1), then larvae were subjected to DNA extraction followed by PCR assay. Three primer pairs were used; as indicated in table 2.1; these primers were designed with a potential for COI DNA amplification of many species in order Diptera. The results shown in figure 3.2 show that two out of the three primer pairs were able to amplify COI from the two isolated larvae. Specifically the used primers were: complete CO: which were targeting about 700 bp of the COI gene, CO1 and CO2, are two other primers sets that target DNA segment of about 350 bp in the COI gene. Using the three primers sets, COI DNA from insects was amplified only by the aid of the complete and CO1 primers sets.





On the contrary, the COI complete primers were not able to amplify COI DNA from insects isolated from barely and flour infested samples (figure 3.3 and 3.4). While it was possible to amplify COI DNA using the CO1 primers which amplify a shorter DNA segment (about 350 bp); (figure 3.5).

The above primers were used in this study for two main purposes: 1- Direct purpose: to test if any of these primers are able to amplify insect DNA found in grains. 2- Indirect: to identify the COI amplified DNA fragments through DNA sequencing to confirm pest types and to shed more light on the different insects may contaminate rice in our markets. The obtained sequence information will help to design specific primers suitable for rice and grain pests.

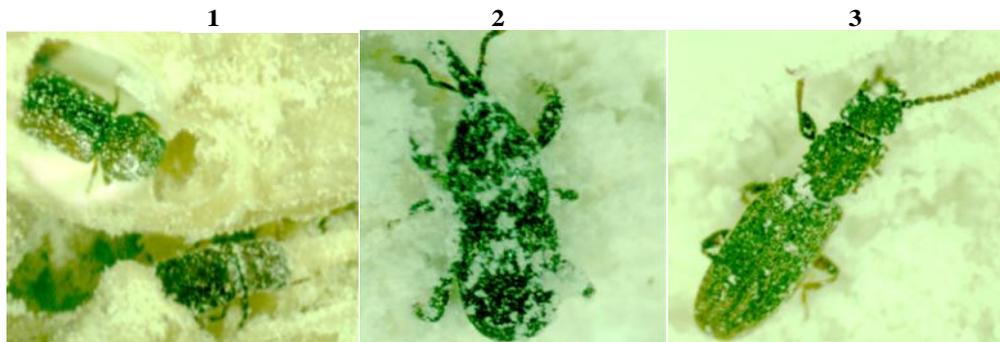


Figure 3.3: Insect pest isolated from barely and flour infested samples which were identified according to their COI DNA sequence: (1) Barely: lesser grain borer (2) flour: granary weevil (3) flour saw-toothed grain beetle

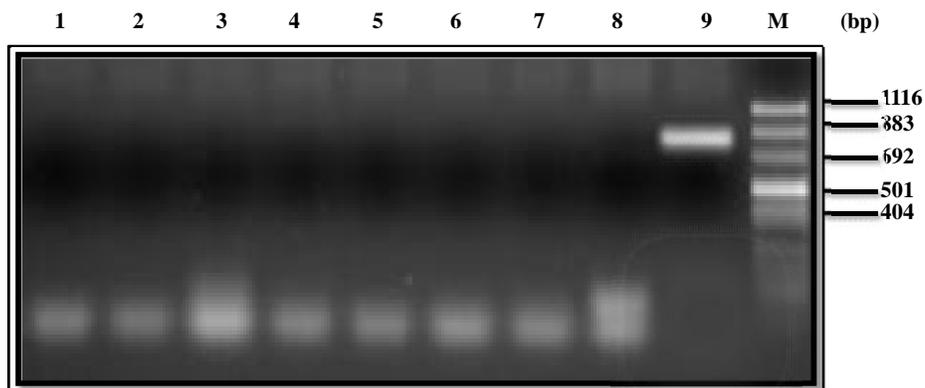
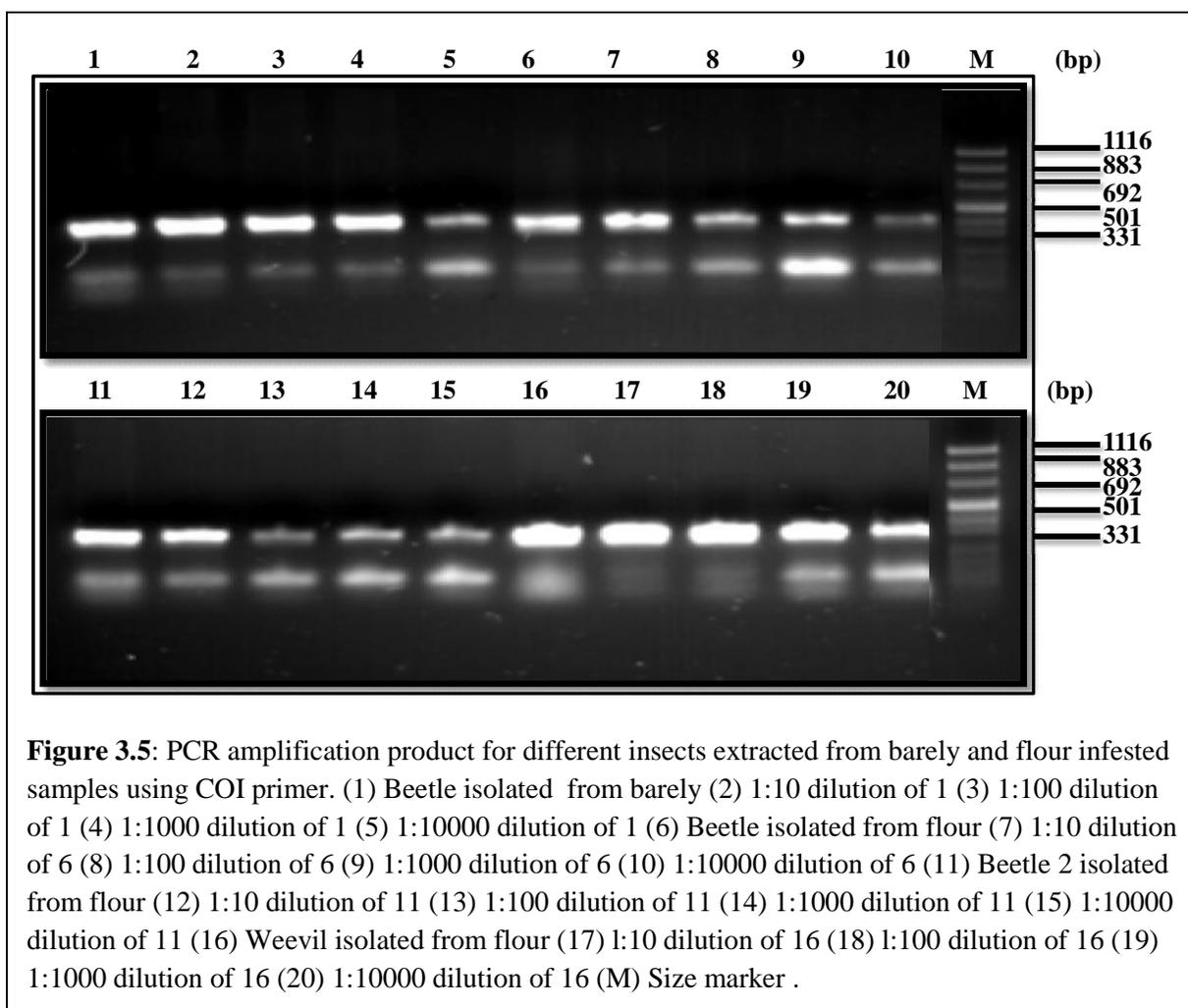
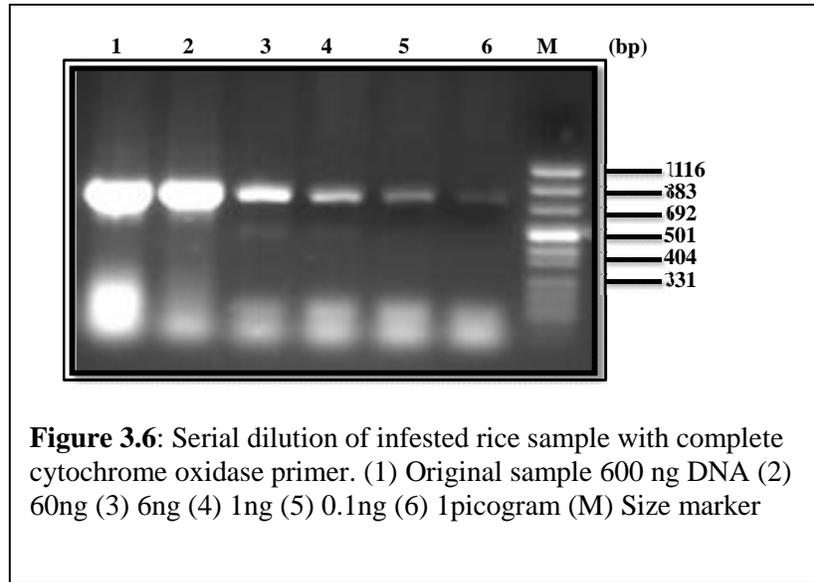


Figure 3.4: Agarose gel electrophoresis analysis of PCR amplification product for different insects extracted from barely and flour infested samples using COI complete primer. (1) Beetle isolated from barely (2) 1:10 dilution of 1 (3) Beetle extracted from flour (4) 1:10 dilution of 3 (5) Beetle2 isolated from flour (6) 1:10 dilution of 5 (7) weevil extracted from flour (8) 1:10 dilution of 7 (9) positive control (M) Size marker



Although the first set of primers that amplify a longer fragment of COI were not used further in this study, still they were analyzed for their sensitivity of DNA amplification targeting different quantities of insects' larval DNA that was isolated from infested rice. As seen in (figure 3.6) the indicated primers were able to amplify down to 1 picogram of the tested insects DNA.



3.2 Strategy for designing new specific primers:

Although in the setting experiments general primers were used with good detection signal however a search for new specific primers was of great importance for this study, a set of primers that can specifically amplify insect pests found in grain food with relatively high sensitivity. For this purpose, all PCR amplified COI segments by the aid of the complete COI primers or the CO1 primers; from rice, barely and flour (figures 3.2, 3.5 respectively) sequenced to identify the insect type (appendix A). Comparison of the obtained DNA sequences with known sequences in the GenBank using BLAST tool comparison method; revealed that the indicated insects are as listed in table (Table 3.1).

Table 3.1: insect pest identified according to BLAST generated comparison				
	Pest scientific name	Best common name	Accession number	Matching %
1	<i>Plodia interpunctella</i>	Indian meal moth	GU096541.1	99%
2	<i>Oryzaephilus spp.</i>	Grain beetle	KC407725.1	85%
3	<i>Sitophilus spp.</i>	Weevil	AY131101.1	76%
4	<i>Rhyzopertha spp.</i>	Grain beetle	KC407718.1	79%

3.2.1 Sequences alignment and primers design

Based on the obtained DNA sequences of the COI segment, new primer sets were designed. Specifically, the original COI DNA sequences of the identified pests were obtained from GenBank and were aligned using ClustalW2 software (Figure 3.7). The aligned DNA sequence areas from both 5' and 3' ends were selected as potential sequences for new primer design. Two primer sets were designed to be used in this study. The first set of primers should amplify a 456 bp segment and the second should amplify a 370 bp segment (Table 2.2).

Due to the importance of mite infestations, and although they were not included in the previous alignments based on the obtained sequences for designing new common primers, a third set of primers that were specific for mite detection were designed. For this purpose, three major mite species were chosen depending on previously published articles [22, 23] and availability of the different mites COI gene sequences in the GenBank data (Table 3.2). The sequences were aligned with two other insect species that have ~ 77% COI DNA sequence similarity according to BLAST generated comparison and the identified sequences of the five organisms were aligned using ClustalW2 software, and another regions of DNA alignment from both 5 ' and 3' ends were selected as potential sites for mites specific primers (figure 3.8). Only one set of primers was selected to be used in this study. All the newly designed primers are listed in (Table 2.2).

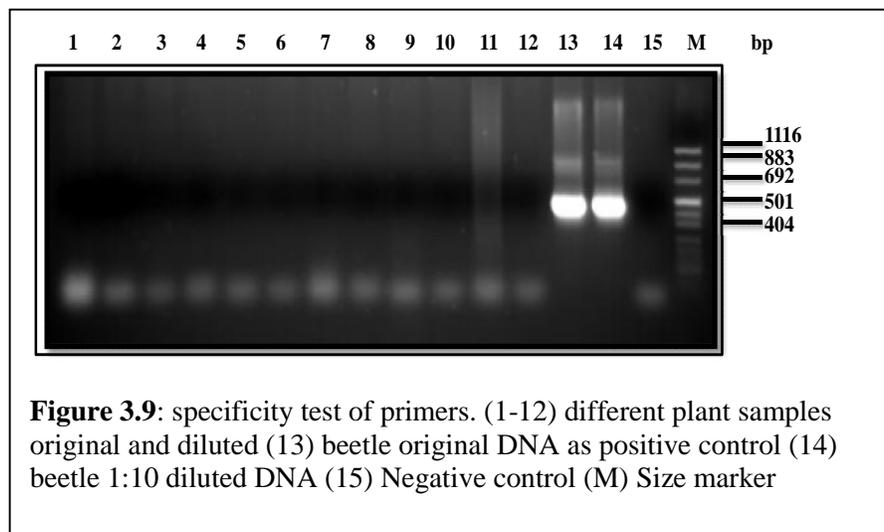
Table 3.2: three mite species and two insect species chosen for primers design

	Scientific name	Common name	Order	Accession number
1	<i>Lepidoglyphus destructor</i>	Storage mite	Acari (non insect order)	EU078972.1
2	<i>Tyrophagus putrescentiae</i>	Mould mite	Acari (non insect order)	EF527826.1
3	<i>Acarus siro</i>	Flour mite	Acari (non insect order)	AY525560.1
4	<i>Tegeticula yuccasella</i>	Yucca moth	Lepidoptera	AF187124.1
5	<i>Orthotomicus caelatus</i>	Bark beetle	Coleoptera	U49028.1

3.3 Experimental testing of the newly designed primers:

The new primers were tested for their specificity and sensitivity of insect detection.

3.3.1 Specificity analysis: the three sets of primers were tested for their specificity against different plant DNA extracted from insect free grains and other plant leaves. Since it was proposed that primers should detect insects contaminating different plant species, it was important to show that these primers will not amplify plant DNA. All the three sets of primers succeeded to amplify insects DNA and none of these primers showed any cross amplification of plant DNA. This analysis utilized six samples of plant DNA in two different dilutions (original extracted DNA, and DNA diluted 1:10) to avoid PCR inhibitors at high DNA concentrations were used. Figure 3.9 shows only the results of one primer (COI long 1), but all the other sets of primers produced similar results.



3.3.2 Sensitivity analysis: Serial dilutions of beetle and ticks DNA samples were used to test the sensitivity and the lower detection limit of the primers. COI long1 and long2 were very sensitive and detected as little as 1 pg DNA (figure 3.10) while Mite cytochrome oxidase detected as low as to 0.005 ng DNA (figure 3.11).

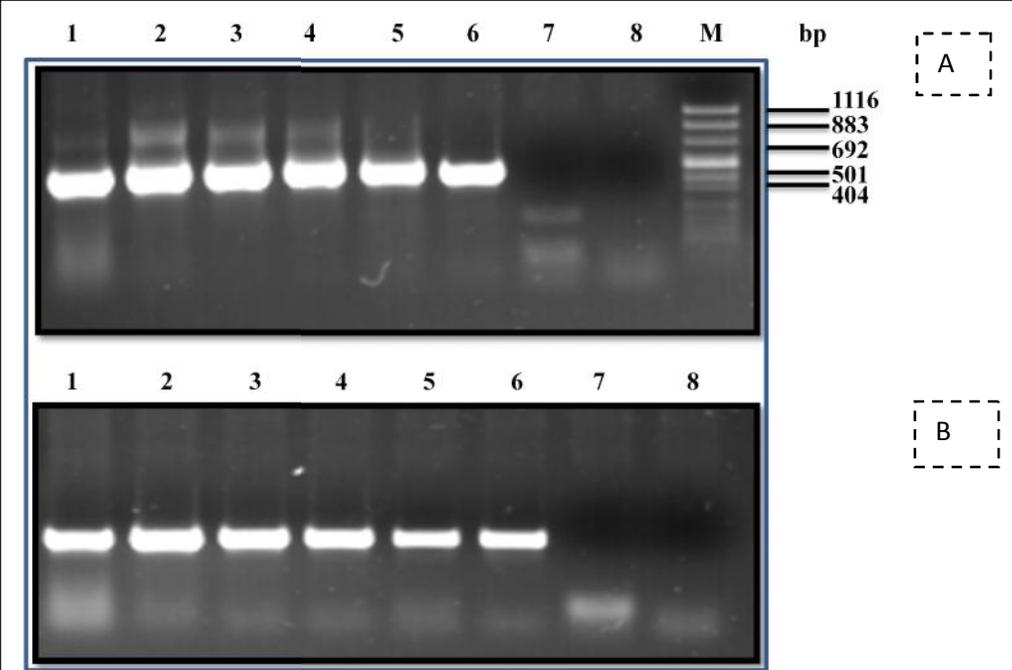


Figure 3.10: sensitivity test of COI- long1 primer (A) and COI-long2 primer (B) using beetle DNA isolated from barely. (1) original 380 ng (2) 40 ng (3) 4 ng (4)1 ng (5) 0.1 ng (6)1 pg (7)negative control (8) negative control (M) size marker

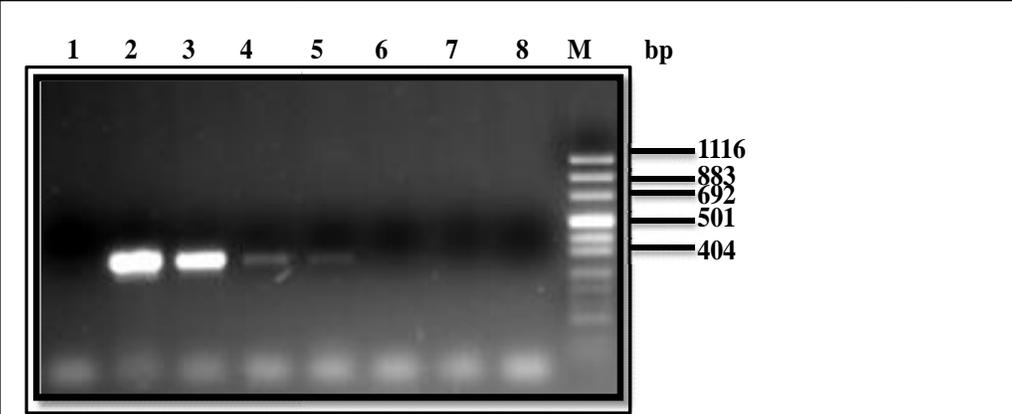


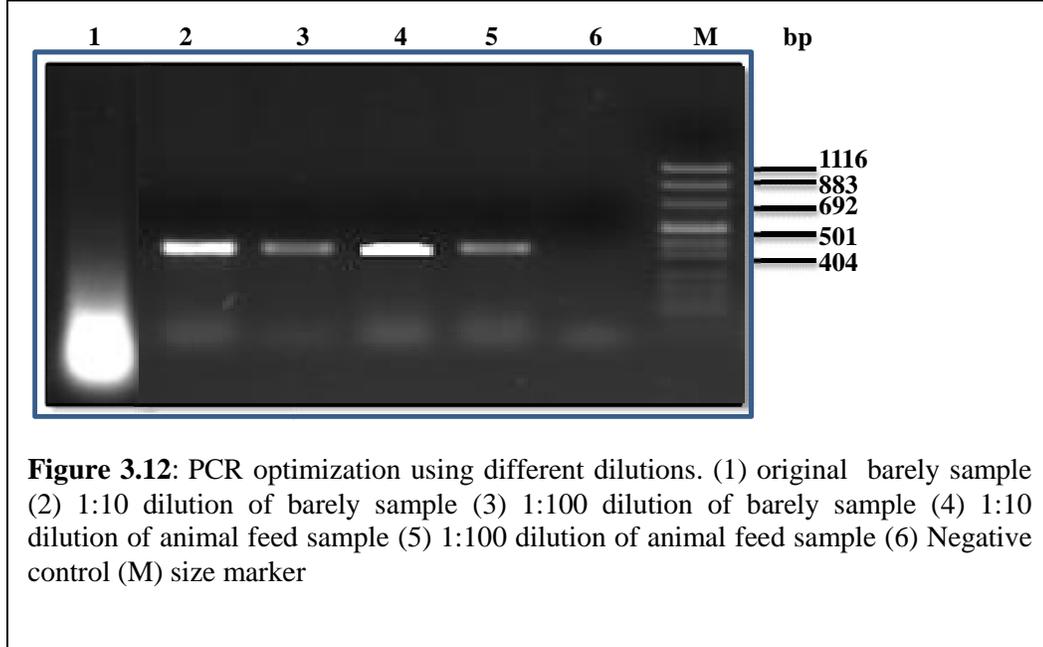
Figure 3.11: sensitivity test of mite cytochrome oxidase primer using ticks DNA. (1) original 50 ng (2) 5 ng (3) 0.5 ng (4) 0.05 ng (5) 0.005 ng (6) 0.5 pg(7) negative control (8) negative control (M) size marker

3.4 Development and adaptation of new extraction methods:

Direct DNA extraction of insects' larvae and eggs found in grains was not successful due to absorption of lyses buffer by grains therefore the importance of developing and adapting new methods has emerged, methods that do not depend on the presence of grains for the final DNA extraction. Two main methods were tested for their usefulness for this purpose: (1) Centrifugation washing method and (2) Filtration washing method, as described in materials and methods.

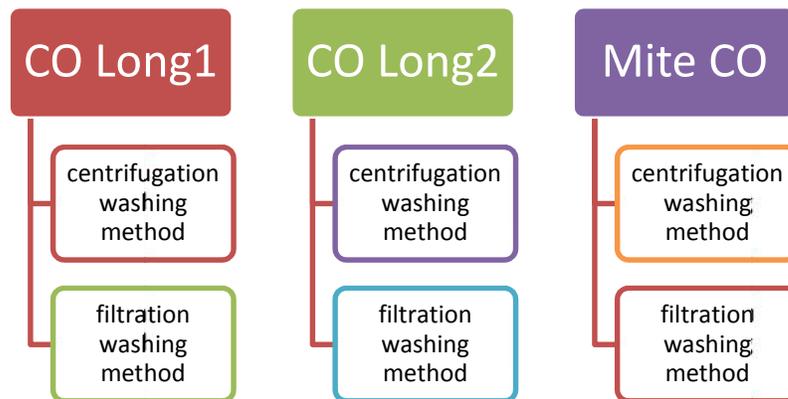
3.5 PCR optimization and its suitability for DNA extraction methods:

It is very difficult to anticipate the exact quantities of the extracted DNA to be used in PCR amplification for insect DNA detection. The obtained DNA is a mixture of insects' DNA (if present) and plant DNA, by experimental experience the use of the original extracted DNA may not be suitable for PCR amplification in some preparations. This situation of (false negative) could be due to presence of unknown PCR inhibitors that normally come out during the extraction procedure. A false negative PCR results are normally resolved after diluting the original extracted DNA samples; and hence diluting the inhibitor effect. Therefore PCR was optimized in terms of the best dilution to be used that definitely will give positive results if insects are found in the extracted samples. For this purpose, several trials were done to detect the suitable DNA dilutions to be used after extraction by any of the two developed methods. Most of the examined samples gave the best PCR amplification results (strong PCR amplicon) at dilution of 1:10 of the original extracted DNA, and faint bands were obtained at 1:100 dilutions. Many of the tested samples gave false negative results upon the use of the originally extracted DNA material (figure 3.12).



3.6 Suitability of PCR primers and DNA extraction methods:

The three newly designed primers pairs: COI Long1, COI Long2 and COI-mite; were used in six separate PCR reactions targeting DNA samples extracted by filtration and centrifugation methods as shown in the following scheme:



Each PCR amplification design with the three indicated primers was used to amplify the target DNA fragment from the extracted DNA from eleven different samples mentioned in materials and methods, one time as original DNA sample without dilution and a second time diluted 1:10 together with a negative control in each amplification set

The results obtained using the three primer sets 1- COI long1, 2- COI long 2, 3-COI-mite combined with DNA extracted by centrifugation washing or filtration washing methods are summarized in table 3.3. The three PCR amplification systems were tested for their effectiveness for insects' COI DNA amplification targeting extracted DNA from the indicated plants prepared by the centrifugation or filtration method: 1- corn, 2- groats, 3- lentils, 4- rice, 5- wheat, 6- commercial serials, 7- chickpeas, 8- cumin, 9- sesame, 10- barely, 11- animal feed.

COI-Long1:

The used primers amplified COI DNA segment of 456 bp, similar amplification results were obtained whether using filtration or centrifugation DNA extraction method. The COI long1 system was able to amplify DNA from all 11 samples after their dilution 1:10 and DNA extraction using filtration washing method (Table 3.3, Figure 3.13A and 3.13B), this compared to successful amplification of only 6 samples out of 11 following centrifugation method and after diluting DNA.

COI-Long2:

The size of the amplicon was 370 bp. Using this PCR system many samples that were detected as positive samples (contaminated samples) were missed; whether using centrifugation method followed by DNA dilution (7/11) or filtration method followed by DNA dilution (5/11) (Table 3.3, Figures 3.14A and 3.14B).

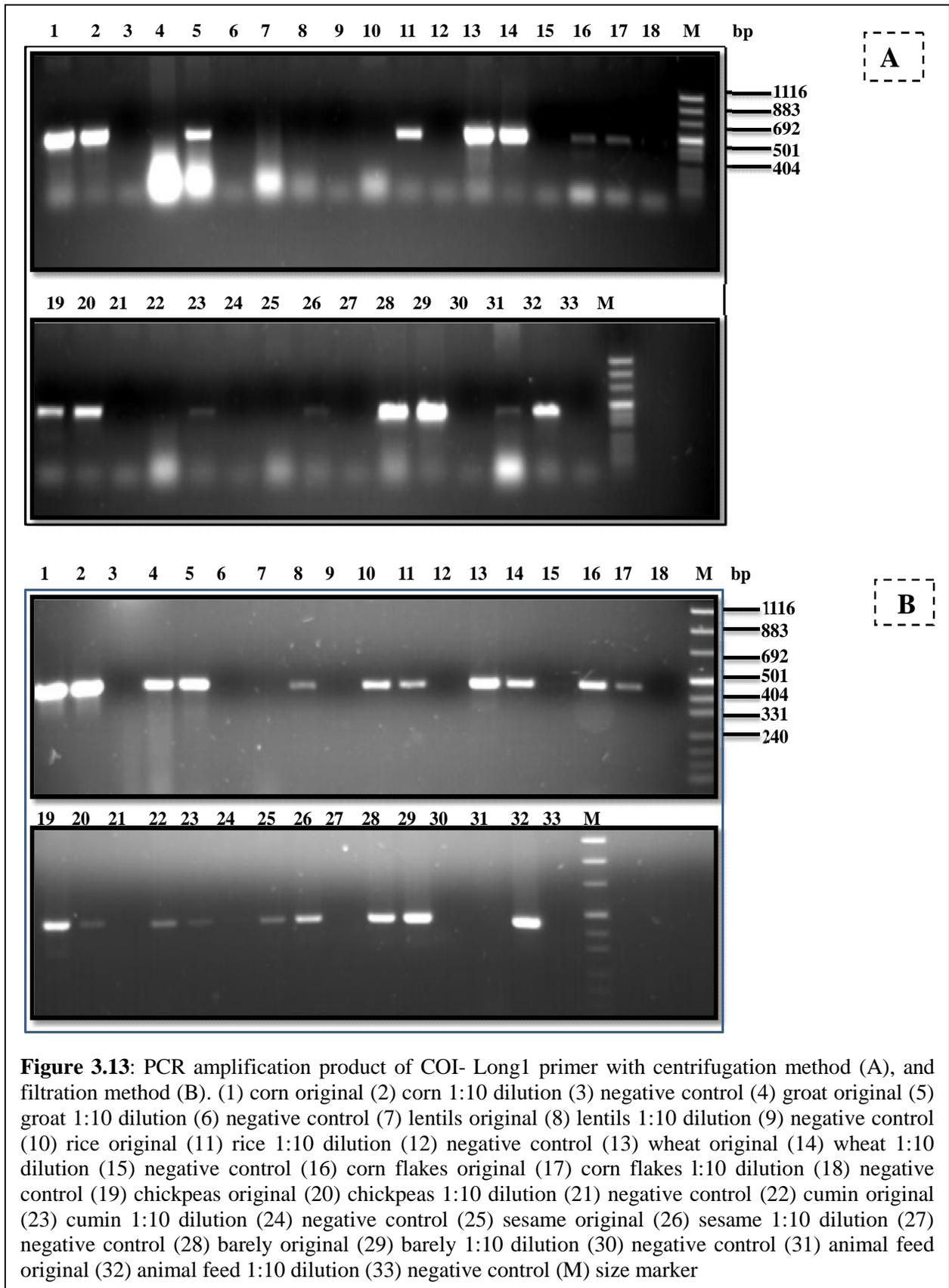
COI-mite:

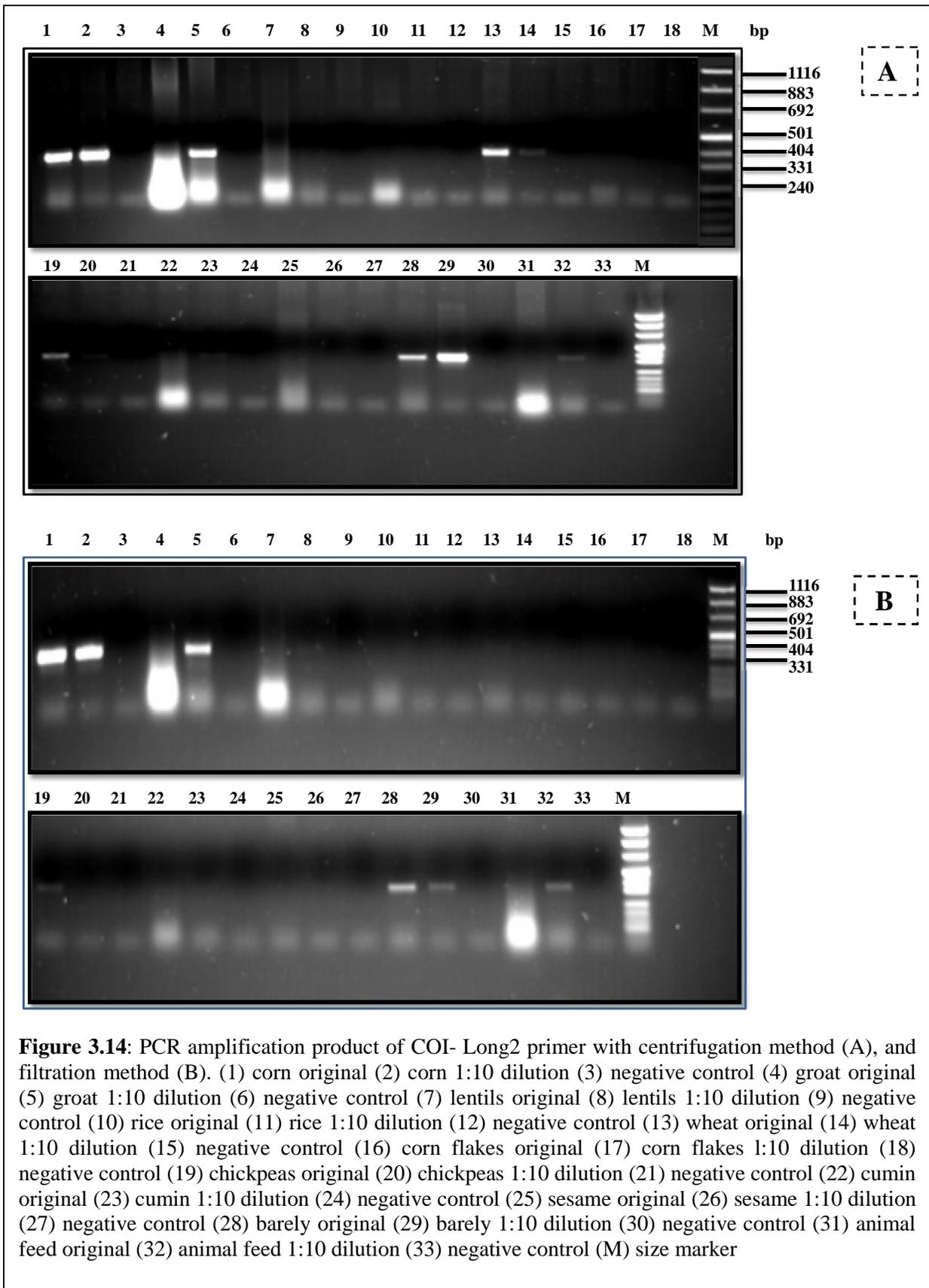
The obtained data were not enough to judge the efficiency of COI-mite PCR system and this may be due to no mite contamination in most tested samples, So few faint bands were seen after COI amplification using this set of primers in barely and animal feed samples which are the most expected to harbor this type of pest. (Table 3.3, Figures 3.15A and 3.15B).

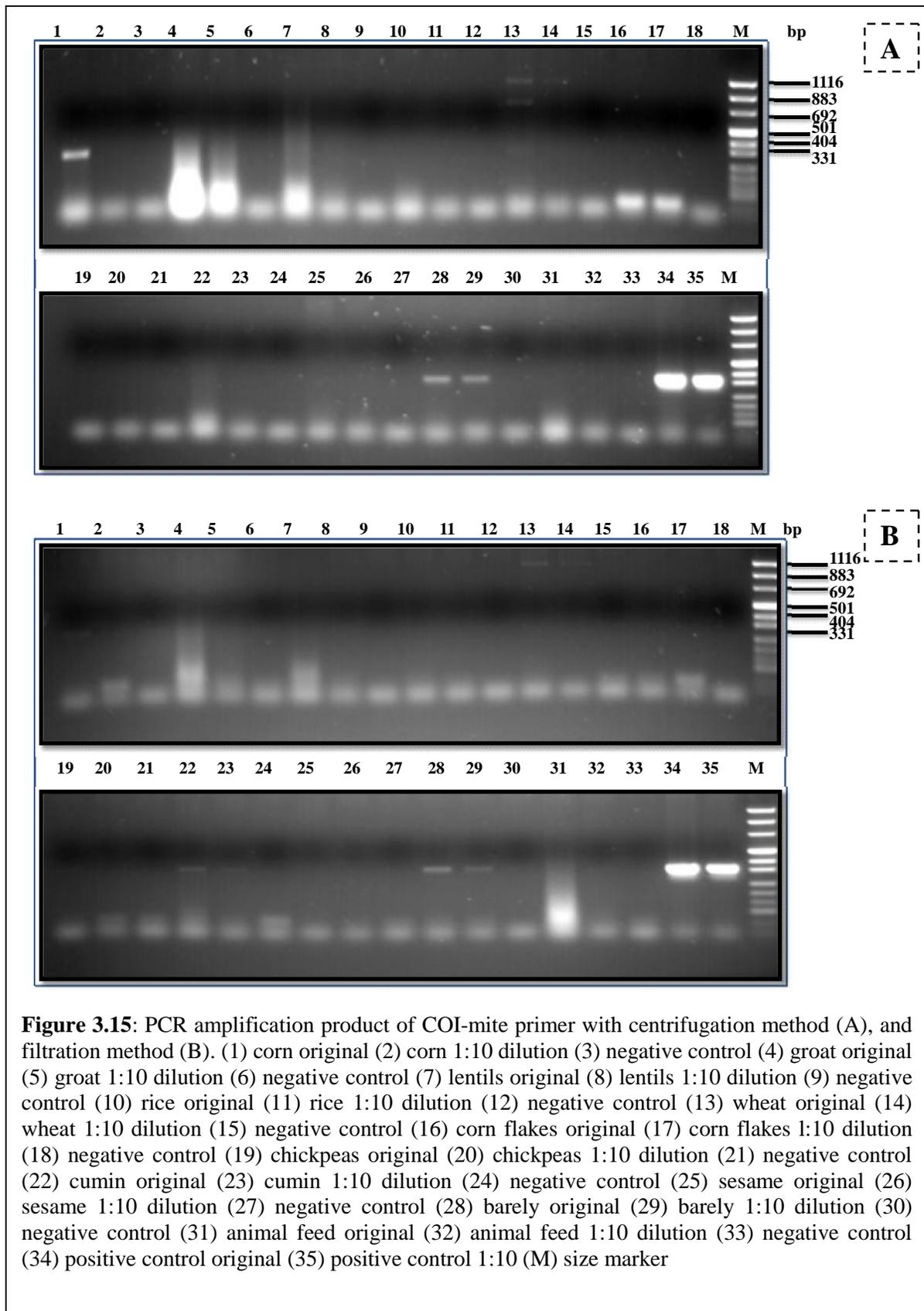
The PCR amplified COI segments by the aid of COI-long1 primer from five samples (cornflakes, chickpeas, animal feed, rice and wheat) extracted by centrifugation method were sequenced for species identification and the resulted sequences were identified according to BLAST generated comparison as shown in table 3.3. (Appendix B).

Table 3.3: insects identified according to BLAST generated comparison (amplified using COI-long1)				
	Pest scientific name	Source (sample)	Accession number	Matching %
1	<i>Rhyzopertha spp.</i>	cornflakes	KC407718.1	89%
2	<i>Rhyzopertha spp.</i>	Chickpeas	KC407718.1	74%
3	Samea spp. (lepidoptera spp.)	Animal feed	HM905018.1	84%
4	<i>Rhyzopertha dominica</i>	Rice	KC407718.1	99%
5	<i>Rhyzopertha dominica</i>	Wheat	KC407718.1	99%

Table 3.4: The results obtained for the three PCR systems: 1- COI long1, 2- COI long 2, 3-COI-mite combined with centrifugation washing or filtration washing methods				
Extraction method	PCR system	Total positives original sample/total	Total positives diluted (1:10)/total	Figure number
Centrifugation	COI-long1	6/11	10/11	3.13A
	COI-long2	4/11	7/11	3.14A
	COI-mite	2/11	1/11	3.15A
Filtration	COI-long1	9/11	11/11	3.13B
	COI-long2	4/11	5/11	3.14B
	COI-mite	2/11	2/11	3.15B







Chapter 4

Discussion and conclusions

Grains are considered as the world's primary staple food and they are stored in stores and warehouses from harvest to harvest season or for longer periods. During storage periods or during shipping and transportation grains are subjected to be attacked by insect pests and mite infestations which cause economical losses by reducing grains quantity and quality. Hence, it is very important to adapt a sensitive method which could detect the presence of these insects at early developmental stage; egg stage and thus decide about implementations of control measures needed for the elimination of these insects and thereby reducing the loss of grains.

There is an increasing demand for insect inspection in food to avoid any hazardous contamination with insects that cause health problems among humans. Insect detection and identification can be considered a simple task that mainly depends on observation of insect developmental stages in stored food items; however, this is not the case. Insects can be found in many food components; that may be stored at cool conditions and hence slow further developmental stages that are easily seen by the naked eye. Also, many edible products may be included in processed food as grind and milled materials together with many types of insects that are hard to identify based on macro- or microscopic examinations. Therefore, it is very important to have a reliable diagnostic test that overpasses most classical method of insect detection that will not be affected by all food processing methods. For that, depending on the detection of insects' genetic material in processed food may be the most ideal approach that could be followed for developing a sensitive and specific examination for food and grain pests.

Grain storage involves more than just placing grain in a suitable container until needed. Grains must be protected from insect pest infestations which cause losses by direct feeding damage, deterioration and contamination of grain. Detecting insect infestations as early as possible enables applying appropriate treatment, and reducing grain losses. Traditional insect detection methods are useful for detection of their

contamination in grains but not for detection of insect fragments in processed food [61]. Molecular biology techniques are characterized for their rapidity and sensitivity. It are used by many researchers in the field of stored- product insect detection.

The main objective of this study was to evaluate the usefulness of molecular biology technique (standard PCR) for the detection of insect pests in stored grains. All previous studies deal with one or two insect pests and there is no established specific primers used for the detection of several species of insects from different orders. For this reason our investigation focused on PCR amplification of defined regions in the insects cytochrome oxidase I genes by the aid of general COI primers that were more specific for dipteran insects. Using these primers, it was possible to detect and eventually identify different insect species from random samples of stored grains. The DNA sequence of the amplified DNA fragments information obtained by these general primers facilitated the design of more specific and sensitive primers for specific identification of the insects detected in the various grain samples.

Direct DNA extraction by adding lyses buffer directly to the grain samples with was long incubation time at room temperature was not successful since the grains absorbed the aqueous lyses buffer and became soaked. Several attempts were tried to overcome this problem, including using of small sample size followed by incubation for short periods at high temperature for a quick extraction step. However, adapting this method for DNA extraction from plant or food materials for the purpose of insect examination was terminated since the small sample size can be misleading and many insect positive samples can be missed. These trials lead to the adaptation of two extraction procedures termed centrifugation washing and filtration washing methods. Both protocols did not involve the addition of lyses buffer for long time in the presence of grain. This was achieved using a washing step with distilled water; and then collecting the wash water which contains particles; dust, eggs and fragments which were re-suspend in the lyses buffer. The filtration method was based on that insect particles will stuck on membrane filters that have pore size smaller than the smallest known pest egg. Insect pest egg size ranges from 0.24-0.72 mm and the membrane filter pore size is 8 μ m [30-42]. Another important complication was resolved after adopting these methods which is the

dramatic reduction of plant DNA and proteins that may interfere in insects' COI DNA amplification.

All positive controls of insects DNA used in this study were extracted from whole insects or their larvae that were directly removed from infested samples. Although it was possible to use previously identified COI DNA sequences for designing specific and sensitive primers, it was preferred to have direct COI DNA sequences from some of our local samples that will identify the specific types of insects isolated from local home or stores of grain samples. Mites primers were designed based on BLAST generated comparison of DNA sequence of the most famous mite species *Acarus siro* aligned with two related mite species together with other far phylogenic sequences to enable finding a primer set that can amplify the maximum number of species that are not completely phylogenetically related.

After establishing the optimal conditions of PCR amplification of the extracted samples, the specificity and sensitivity of the primers were tested. All primers proved to be insect specific and did not amplify different species of plant DNA as original DNA extract or after dilution. The primers proved to be very sensitive to amplify very small amounts of DNA template, CO Long1 and CO Long2 were very sensitive and could detect insect DNA after its dilution down to 1pg, which amplifying a strong DNA band indicating the sensitivity limits was not reached. Mite CO PCR system was also sensitive and it could amplify DNA fragment using up to 0.005 ng of mites positive control DNA. Investigating undiluted sample did not result in any amplification is most probably due to the high concentration of DNA template used which once known for PCR inhibition effect. It is important to indicate here that the new developed PCR systems using COI-long1 and Co-mite are sensitive enough to detect the content of insect DNA in even one egg mixed 10 grams of grain. This spiking experiment was not done, but the total content of DNA in one average sized insect egg is estimated to be about 1 ng, even if the efficiency of DNA extraction by either centrifugation washing or filtration washing methods was only 1% of the total DNA content of the sample, still these PCR systems are able to detect contamination at this limit.

By adapting classical PCR systems, it is not possible to have quantitative results based on one single PCR amplification reaction. A quantitative procedure is needed to determine insect threshold level in grains according to the Federal Grain Inspection Service (FGIS) in the United States which determines the number of insects allowed to be presenting in grains as shown in table 4.1 [77]. The current combination of CO-Long1 PCR system and filtration system based on our data was sufficiently sensitive to detect even lower quantities than the allowed threshold level. However, adapting these primers to a real-time quantitative PCR protocol would be more sensitive for insect level detection limits.

Table 4.1: FGIS infested designation standards.	
Crop	Insects per Kg of grain to receive FGIS "infested" designation
Wheat, rye, triticale	More than 1 weevil, or 1 live weevil plus any other live stored grain insect or no live weevils but 2 or more other live stored grain Insects.
Corn, barley, oats, sorghum and soybeans	More than 1 weevil or 1 live weevil plus any 5 or more other live stored grain Insects, or no live weevils, but 10 or more other live stored grain Insects.

The ability of the developed molecular approach to detect insect pests in samples of stored grains were tested on eleven samples randomly collected from local stores from grain openly sold for the public. Samples were not purposely contaminated by insect pest for research purposes like other studies [62, 69] rather these collected samples were treated by our developed and standardized DNA extraction techniques developed for DNA extraction and amplification in this study; centrifugation method and filtration method. Each sample was extracted by the two procedures for quantitative and qualitative comparison purposes. Each of the newly developed PCR systems were examined against each DNA extraction. The results clearly showed that; the combination of CO-Long1 PCR system with the centrifugation method gave good and reliable results; since ten of eleven collected grain samples were found to be positive including corn, wheat, chickpeas and barely which gave very strong bands using the original and diluted samples, goat, rice and animal feed also gave strong bands for diluted sample formula,

corn flakes samples gave faint bands and very faint bands of cumin and sesame. Lentils was the only negative sample. The second combination of CO-Long1 PCR system with filtration method was shown to be the most superior; since all samples were found to have insect contamination that revealed by strong COI gene amplification. Specifically; corn, groat, rice, wheat, corn flakes, chickpeas, cumin, sesame and barely gave strong bands for both original and diluted samples while lentils and animal feed gave bands for mild dilution only. The data indicated that using the filtration method for DNA extraction was more efficient and gave better results with the CO Long1 PCR system than the centrifugation method. Lentils sample with centrifugation method didn't show positive result with the same PCR system, cumin and sesame samples with filtration method gave better bands than that with centrifugation method. The explanation for these results may be due to sample size taken for filtration method.

The use of the CO-Long2 PCR system in combination with either the centrifugation or filtration methods did not show an effective amplification using CO-Long1 PCR system. Only 5-6 samples out of the 11 tested samples were found to be contaminated, and mainly these samples that gave a strong amplification bands using CO-Long1 PCR system. Considering mite-CO PCR system combined with both centrifugation and filtration methods could amplify mites COI from only few samples such as animal feed, these samples are the most expected to harbor this type of insect.

In conclusion, the data shown in this study represents a major step for the establishment of a rapid, sensitive and reliable molecular method for insect detection in grains based on specific genetic marker information. Further work is needed to optimize the method for the specific identification of the various insects and develop a quantitative assay for the assessment of the degree of contamination at the early stages for the proper handling of contaminated grains. Furthermore, efforts will be directed to develop a multiplex test based on the present results for the detection of the vast majority of insects species known to infest grains. Eventually, his technique will hopefully pave the way for the adoption of a national program to screen all imported and locally store grains for periodic inspection to ensure the safety of grains for human consumption and prevents losses that can have a significant impact on the socioeconomic status of society. Definitely, detection of insects contamination in grains at early stages will allow early

interference to ensure the eradication of all contaminants utilizing effective and reliable methods that are used around the world for this purpose.

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Appendix A

Sequences of COI gene amplified using COI complete and COI primers

Sequence (1): *Plodia interpunctella* insect isolated from rice and identified according to BLAST

TTATTTTTGG ATTTGAGCCG GATAATTGGA ACATCTTTAA GATTATTAAT TCGAGCAGAA
TTAGGAACTC CTGGATCTTT AATTGGAGAT GATCAAATTT ATAATACTAT TGTTACTAGT
CATGCTTTTA TTATAATTTT TTTTATAGTT ATACCAATTA TAATTGGAGG GTTTGGAAAT
TGATTAGTTC CTTTAATATT AGGAGCCCCT GATATAGCTT TCCCCGATT AAATAATATA
AGATTTTGAC TTTTACCCCC CTCTTTAACT CTTTTAATTT CCAGAAGTAT TGTAGAAAAT
GGGGCAGGAA CTGGGTGAAC AGTTTATCCC CCCCTTTCAT CTAATATTGC CCATGGTGGA
AGTTCTGTTG ATTTAGCCAT TTTTCCCTT CATTTAGCGG GTATTTCCCTC CATTCTGGT
GCCATTAATT TTATTACTAC AATTATTAAT ATAAAATTAA ATGGAATAAT ATTTGATCAA
ATACCTTTAT TTGTTTGAGC TGTTGGTATT ACTGCTTTAC TCTTACTTTT ATCACTTCCT
GTTTTAGCAG GTGCTATTAC TATACTTTTA ACAGATCGAA ATCTTAATAC CTCTTTCTTT
GATCCTGCTG GTGGTGGAGA TCCTATTTTA TATCAACATT TATTTTGATT TTTTGGACAT
CA

Sequence (2): *Rhyzopertha spp.* insect isolated from barely and identified according to BLAST

AAGANNCCN TATCGCCNNG GGGGGCNTCC TGTTGCTTTA GCACNTATTA GNTTACATTT
NGCAGGAATT TCATCAATTC TTGGAGCAGN AAACNTTNTN ACTACATATC AGTTAATANA
CGACCGGGAG GAACNACNCN GGGNCGAACC CCCCNTNNG TNTGAACAGA AGGAANCACA
GCTTTNCANT GNTTATTANC TCGTCGGGTT CNNGNNGGAG CGATCACTAT ATTATGNACA
GACCGAACT TCTCANNCTT GGTCTGTGAT ACCNACAGGG GGGGGNNATC CTAGTCNTTN
NCANCNNANN NNNNNNNNNN NN

Sequence (3): *Oryzaephilus spp.* insect isolated from flour and identified according to BLAST

CATNCNGANN NCTNTNTTGG CTCNGGGGGG ACNTCCTGTT GCNTTAGNAC ANATTNGCAT
TACANTTAGC GGGAAATTTN CTCCATNTTA GGAGCAATTA ACNTTATTTT TACAGNTTGC
AATATACGAC CCGGGGGTTN NATCTAGATT AAATNCCTTG GNTCTGTTGN NGNAGNNTGA
TCANNGCCAT TCNNCTGCNN TCANACCGGC NNNNGCTTNC GGCGNGCCGT CNCCNAANGA
NGGCTNAGGC CGNAGAANGN NCCGCTGNNG GNTTNNNCGC TGGNGNGGGG GGGATACTACT
GTTTTCCCAN NCCAA

Sequence (4): *Sitophilus spp.* insect isolated from flour and identified according to BLAST

AGCACACNCC TTTGCCCTGT AGGGGCTTCT GTAGATGCTA GCAATCTTTA GATTACACAT
AGGCAGGAAA TTNNATCTAT CCTTGAGCA ATTAATTTTA GTTACTACNG CATACTANTA
ATACGACCCN CAGGTCTATT ATGCATGATC GANNAAGCAN GGATCTTATT TGAGCTGCTG
TAATTACAGC TATNCTAGNT CNACNGAGAC NTNNNGTCNN AGCTAGGGNA ANTNCNATGC
TTGNTACNGN TCNAAANNCC CCTNCTTGGN NTNTNGANCC TGNGGGAGGG GGAGANNCTG
TTCNCNNTCA NCNNANN

Appendix B

Sequences of COI gene amplified using COI-long1

Sequence 1: *Rhyzopertha spp.* identified according to BLAST generated comparison

```
GNNGCATTCC CTCGAATAAA CANCAGTAGA TTTTGGCTTC TTCCCNCNTC NTTAACCCTT
TTACTAACAA GAAGANTTGT TGAGACAGGN GCAGGAACAG GATGAACAGT TTATCCACCT
CTATCTAACT AATACAAGCC CATAGCAGGA GCTTCTGTTG ATNTAGCAAT TTTTAGATTA
CATTTAGCAG GAATTTTCATC AATTCTNGGN GCAGTAAACT TTATTACTAC ATATCANTTA
ATATACGACG CANNAGGAAT AACNCCANAA CGAATCCCCC TATTTGTNTG AACAGTNNGA
ATCACAGGTT TGCTTTTANT ATNANCNCTT CCGGNTNTAN CNGGNGCTAT CACTATATTA
TTAACCGACC GNAACTTANA TACNTCATT TTTGNACCNG CNGGNGGGTT TGTTNCTTNT
ATNNCTTCNT GGNNTGTCNA GTGCCATNCT TTTACTGCNT NTCNGNTNGG ANCTTTTNTN
```

Sequence 2: *Rhyzopertha spp.* identified according to BLAST generated comparison

```
NCGTCTNNTN ATNGGTGCCC GNNATAGCAT TCCCTCGAAT AAACAACGTA AGCNTTTTGA
CTTCTTCCAC CCTCCTTAAC CCTTNTANTA TACNAGGAAG CAATTGTTGA GTACATGGAG
CAGGGAACAG GGATGAACNT GGTTTATCCC ACCCTCTNTC AAAGTAACTA CAGNCCANAG
NAGGGAGCCA NCTGNTGAAT TAAACAATTT TTANNTTACN TTTAGNAGGG AGTTCANCNN
TTNTTGGGGC NGTANACTTT ANTACCACCA NCANTAATAN ANNANCNAAA GGNANNNNNC
CTTGACNAAT NCCCCTTTTT GGNTGANCAG TAGGGANNAN NGCGCTTGCN TNCATTANNA
TNNCTTNCGG GTCTAGNNGG GGNCATCNCA NATGNGTATN TNACACGACA CNNGAANTTN
TAATANANTC TATATTTTTT TGAGACTCAC ACNGGAGGG
```

Sequence 3: (lepidoptera spp.) identified according to BLAST generated comparison

TCCNTAATTG GCGGCTCGTG TAATAGCCTT TCCCTCCGNA TAAATAATAT AAGCATTTTG
ACTTTTACCC CCCTCTTTAA TTTTATTAAT TTCAAGAAGA ATTGTAGAAA CATGGAGCAG
GAACTGGATG AACTGTTTTAT CCGCCTTTAT CGTCTAATAT TGCACACGGN GGANGATCGG
TNGATTTANC AATTTTTNCT TTNCATTNAG CGGGTATTNC ATCAATTCTN GGANCANTCA
ATTTTATTAC AACAATTATC AATATACGAA TTAATGGACT ANCATTTGAC CAAATACCCT
TATTNGTTGG AGCTGTTGGA ANCACTGCTT NATTATNATN ATTATCTCTA CCNGTATNAN
CNGGNGCTAT TACTATATTA CTAACAGANC GAAATTTAAA TCCATCATTT TTGGANCCAG
CAGGAGGANN

Sequence 4: *Rhizopertha spp.* identified according to BLAST generated comparison

GTTTNNATAT TGGTGGCCCA GATATAGCAT TCCCTCGAAT AAACAACATA AGATTTTGGC
TTCTTCCACC CTCCTTAACC CTTTTACTAA CAAGAAGAAT TGTAGAGACA GGAGCAGGAA
CAGGATGAAC AGTTTATCCA CCTCTATCTA ATAATACAGC CCATAGAGGA GCTTCTGTTG
ATTTAGCAAT TTTTAGATTA CATTTAGCAG GAATTTTCATC AATTCTTGGA GCAGTAAACT
TTATTACTAC AATCATTAAT ATACGACCAA AAGGAATAAC ACCAGAACGA ATCCCCCTAT
TTGTATGATC AGTAGGAATC ACAGCTTTGC TTTTATTATT ATCTCTTCCG GTTCTAGCTG
GAGCTATCAN TATATTATTA ACAGACCGAA ACTTAAATAC TTCATTTTTT GATCCAGCAG
GAGGA

Sequence 5: *Rhizopertha spp.* identified according to BLAST generated comparison

GTTNNATATT GGTGGCCCAG ATATAGCATT CCCTCGAATA AACAACATAA GATTTTGGCT
TCTTCCACCC TCCTTAACCC TTTTACTAAC AAGAAGAATT GTAGAGACAG GAGCAGGAAC
AGGATGAACA GTTTATCCAC CTCTATCTAA TAATACAGCC CATAGAGGAG CTTCTGTTGA
TTTAGCAATT TTTAGATTAC ATTTAGCAGG AATTTTCATCA ATTCTTGAG CAGTAAACTT
TATTACTACA ATCATTAATA TACGACCAA AGGAATAACA CCAGAACGAA TCCCCCTATT
TGTATGATCA GTAGGAATCA CAGCTTTGCT TTTATTATTA TCTCTTCCGG TTCTAGCTGG
AGCTATCACT ATATTATTAA CAGACCGAAA CTTAAATACT TCATTTTTTG ATCCAGCAGG
AGGNNNNN
