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Morphological and molecular identification of rust-red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera, Tenebrionidae) in Basrah Governorate, Iraq

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Abstract

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This study aimed to determine the morphological and molecular characteristics of the red rusty flour beetle, *Tribolium castaneum* (Herbst, 1797). Insects were collected from the stored food items. It included flour, rice, nuts, sugar, wheat grains, sesame, and oats from different areas within the Basra Governorate. It included the Shatt al-Arab, Al-Faw, Abu Al-Khaseeb, Safwan, Al-Madinah, Al-Qurna, and Al-Zubair districts. The collection also included homes in those areas, as well as local markets for the period from November 2023 to October 2024. The results showed differences in morphological characteristics that varied according to the collection area and the type of food material on which the insect was present. Molecular diagnosis was conducted for the insects, and the results showed that all patterns that showed differences in their morphological characteristics belonged to *T. castaneum.* It was registered in the NCBI gene bank for the first time from Basrah Governorate. The first morphism was assigned the genetic sequence number PQ219934.1. The second morphism was assigned the genetic sequence number PQ219935.1.

Keywords: Tribolium castaneum (Herbst, 1797); mtCOX1; molecular identification; morphological identification

Introduction

After harvest, the stored grains are damaged by insects and microorganisms. They may also be damaged by poor storage (Banga et al., 2020). Grains are transported over long distances after harvest and stored for relatively long periods. Grains and their products are important global food supplies (Mason and Strait, 2019). Abbasi et al. (2021) stated that more than 75% of the pests that infect stored grains and foodstuffs belong to the order Coleoptera. *Tribolium castaneum* insects cause severe damage to the stored materials. Infected materials can be contaminated with waste and molted skin. This causes a change in the color of foodstuffs such as flour. It changes its properties, making it lumpy and having an unpleasant odor. Thus, they are unsuitable for human consumption (Afifi, 2020). Therefore, controlling this pest is important and necessary for health and economic reasons. However, it is also an important laboratory model in various disciplines, such as evolutionary ecology (Pointer, 2021). The rusty flour beetle is a heterothermic organism, which has led to its development with different adaptations to resist many pesticides and avoid their toxicity, including phosphine poisoning (Paul et al., 2020). The rusty flour beetle is also used as a model for pest monitoring and management techniques, including the evaluation of insecticide resistance mechanisms (Campbell et al., 2022). This insect is characterized by its ability to move quickly. Thus, they moved from infected to healthy materials. This also leads to infections (Gurdasani, 2018). This insect is characterized by its short life cycle and ease of breeding (Rosner et al., 2020). Insect groups differ in appearance and behavior. This difference

occurs even at the level of the same species, and is also due to differences in complex environmental conditions (Peterson et al., 2012). Polymerase chain reaction (PCR) is used worldwide in insects and other living organisms to provide clear and focused information about genetic diversity. This method is fast and effective in distinguishing between species with very similar characteristics (Abd AL Hseen and Manea, 2020). Molecular genetic research has shown that differences in DNA sequences represent the basis of changes that lead to the evolution of species and the presence of phenotypic differences. Therefore, the polymerase chain reaction (PCR) technique can be used to compare the genetic sequences of closely related species. Therefore, it is an accurate and important taxonomic tool for diagnostic purposes (Ahmed, 2020). This phenotypic and molecular study of the rusty flour beetle was the first in Basra Governorate. However, there are many molecular studies on this insect, such as the study by Kalaf (2021). This study made a molecular diagnosis of the rusty flour beetle, as it is one of the main insects that infects stored grains and causes damage. This affects the economies of several countries. We also studied the genetic variation of two types of storage pests belonging to the order Coleoptera, *Tribolium castaneum* and *Tribolium confusum*. These two species are very similar in appearance, which makes it difficult to distinguish between them. Many studies have been conducted on controlling rusty flour beetles in Basrah Governorate, including the study by (AL-Emara, 2021). The effectiveness of ozone gas against all stages of red rusty flour beetle was tested.

The present study aimed to prove the phenotypic variations and genetic patterns of the red rusty flour beetle *T. castaneum*, so the insect was collected from different areas within Basrah Governorate and from different food materials as well by molecular identification.

Materials and Methods

Sample collection

Samples were collected from the stored food items. These included rice, wheat, flour, nuts, sugar, oats, sesame, and other materials. The samples were from different areas within the Basra Governorate, including the Shatt al-Arab, Al-Faw, Abu Al-Khaseeb, Safwan, Al-Madinah, Al-Qurna, and Al-Zubair districts. The collection included homes and local markets in these areas from November 2023 to Octo-

Fig. 1. Sample collection areas
Source: Authors' own elaboration

ber 2024. The collection was carried out randomly from the areas in the Basra Governorate, (Figure 1). The rusty flour beetle was isolated from the food items using a brush and under a dissecting microscope, and preserved in the laboratory for subsequent steps.

Permanent insect culture in the laboratory

After collecting the insect samples, permanent cultures were obtained. This was done by raising them on culture media consisting of grains free of infection, according to the grains from which they were collected. These grains were previously exposed to 50 °C for 10 min in an oven to ensure that they were free of infection (Ahmady et al., 2016). A total of 250 g of grains free of infection were placed in a 300 ml glass bottle with four replicates for each study area, with a total number of 12. A pair of insects (males and females) was placed inside each bottle. They were then transferred using a small brush, and the mouth of the bottle was covered with a piece of cloth. They were tied with a rubber stopper, and the colonies were renewed after each generation by taking the insects emerging from the eggs to create new colonies for morphological and molecular diagnosis purposes.

Preparation of permanent insect slides

The study samples were placed in a 100 ml glass beaker, half of which was filled with water. They were carried on a piece of cork without touching water. The beaker was then heated to a temperature of (50–60) °C under low heat for 20 min. The mouth of the beaker was covered with a petri dish so that water vapor would not leak out, which would soften the insect parts for easy separation and no breakage. Then, the insects were transferred to another glass beaker, one-third of which contained 15% KOH for 5 min on a hot plate. This facilitated the removal of the muscles attached to some plates. They appeared clear upon examination. The sample was then washed several times with distilled water to remove any remaining KOH. It was then placed in Petri dishes containing ethyl alcohol at different concentrations (30, 50, 70, 90, and 100%) to remove excess water. Then, it was placed in xylene and transferred to a glass slide after adding a small amount of Canada balsam. We surround the sample with a metal or plastic ring in a manner proportional to the height of the sample. The slide cover is placed and then it is placed in the oven at a temperature of 60 °C for 24 hours to get rid of bubbles. It was then examined under a dissecting and compound light microscope.

Molecular study

Molecular diagnosis of adult insects of the rusty red flour beetle was carried out using polymerase chain reaction (PCR). The same samples used in the morphological taxonomy were used in the molecular study. DNA was extracted using an extraction kit (GeneAid). The concentration and purity of the DNA were measured using a NanoDrop device. Polymerase chain reaction (PCR) was performed for the two samples that were previously diagnosed phenotypically. A general primer specific for invertebrates (mtCOX1) was used, a PCR reaction mixture of 25 µL was prepared, and the mixture was placed in a thermocycler device. According to a program with special temperatures for each stage, electrophoresis was carried out on an agarose gel for the COX1 gene according to the method of Sambrook et al. (1989). The samples were then sent to a specialized company for genetic sequence analysis.

Results

The results of the morphological diagnosis study of the rusty flour beetle showed morphological differences between insects from different areas in the Basrah Governorate and from different stored foods. The insects were collected from stored food items, including flour, rice, nuts, sugar, wheat grains, sesame, and oats from different areas within the Basra Governorate. It included the Shatt al-Arab, Al-Faw, Abu Al-Khaseeb, Safwan, Al-Madinah, Al-Qurna, and Al-Zubair districts. Molecular diagnosis proved that all patterns were of one type, *Tribolium castaneum,* despite the morphological differences, and the insect belongs to the following taxonomic position:

Phylum: Animalia
Class: Insecta
Subclass: Pterygota
Division: Endoptergota
Order: Coleoptera
Suborder: polyphage
Super family: Tenebrionoidea
Family: Tenebrionidae
Sub family: Tenebrioninae, Latreille, 1892
Genus: *Tribolium* Macleay, 1825
Triboliumcastaneum (Herbst, 1797)

General description: Its body is elongated, cylindrical, light brown with a reddish tinge, and dark areas at the ends of the wings. It was slightly convex on the dorsal side and flat on the ventral side. Its total length is 4–4.5 mm, and its width is 1–1.5 mm (Table 1) (Fig. 2A and B) and (Fig. 3A and B).

Head

The head is small, smaller than the rest of the body, reddish-brown in color, and has dark areas in front of it. The shape was oval and elongated, and its front end was wider than

Table 1. Morphological Comparison of two *Tribolium castaneum* (Herbst, 1797)

Characteristics	*Tribolium castaneum* first morphism	*Tribolium castaneum* second morphism
Color	Light brown, with some areas appearing darker	Dark brown to reddish brown
General body shape	Length: 4–4.5–4 mm, Width: 1–1.5 mm	Length: 3.5–4 mm, Width: 1.5–1.8 mm
Head	Length: 0.7mm, Width: 0.5 mm	Length: 0.5 mm, Width: 0.6 mm
Antennae	Composed of 11 segments with a gradually increasing size	Composed of 11 segments, the last three segments distinctly enlarged.
Thorax	Shield-like, its anterior end is broad and almost rounded, without sharp margins, and its posterior end is broader and has curved margins.	Broader, with distinct margins and less roundness
Elytra	Light-colored, with pits arranged in longitudinal punctuations	Dark-colored, with deeper pits arranged in an irregular manner
Fore legs	Long and slender, reaching a length of 1.8 mm	Shorter and wider and 1mm long its ends are wider.
Abdomen	Tapered at the end	Its end is wider

Source: Authors' own elaboration

that of the back. The length is 0.7 mm and a width 0.5 mm. Its width was the same as that of the rest of the body. At the dorsal end, where it connects to the chest, there is a flat area with a row of small hairs, as shown in (Fig. 2A and Fig. 3A). On the ventral side, there is a triangular depression in the area where it connects to the chest, as shown in (Fig. 2B and Fig. 3B). The compound eyes were dark black, small, oval, and elongated in color. The distance between them was greater on the dorsal side than on the ventral side. The antennae consisted of 11 segments, and their lengths were greater than the length of the head. These segments were unequal in size. The first five segments were of the same size, whereas the sixth and seventh segments were larger. The last three segments are larger and more distinctive than the rest of the segments. The latter had a distinct spherical to oval shape, whereas the remaining segments were almost square. All segments had soft hair that was densely concentrated in the last three segments.

Thorax

Light reddish-brown with dark edges and pits. They are arranged in rows on each side. It is generally shield-like, with a broad, semi-circular front end and no sharp edges, whereas its rear end is broader.

Wings

Light brown in color, elytra in structure, with dark areas and pits arranged in longitudinal rows at tips and edges. The wings meet in a straight line, above which is a prominent triangular area of the back in the middle of the back. The hind wings cover the front end, which is rectangular, and the back end is pointed.

Legs

The forelegs are 1.8 mm long and have spherical iliums. The thigh is dark at its junction with the ilium. It is elongated and triangular in shape and wide with a pointed anterior end. It is less wide than the middle and hind legs and has soft hair. The leg is longer than the thigh, has prominent protrusions on its sides, and soft hairs. It is thin at its end, connected to the thigh, and widens more at its connection to the tarsus, the last of which has five segments. The first four were small and equal in size, the fifth was clearly elongated, all segments were covered with hair, and the last had a downward-curved claw. The middle legs are similar in shape and length to those of the forelegs. All parts of the legs, except for the anterior end of the thigh, were transverse. The leg had a prominent protrusion at its end connected to the tarsus and consisted

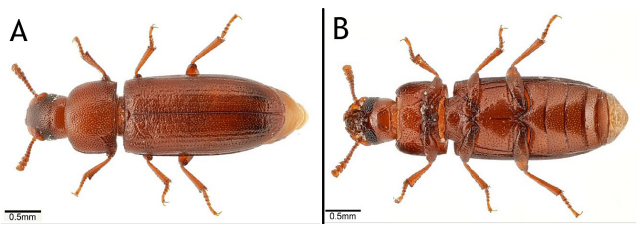

Fig. 2. General appearance of a rusty red flour beetle collected from nuts

A) Dorsal side **B) Ventral side**

Source: Authors' own elaboration

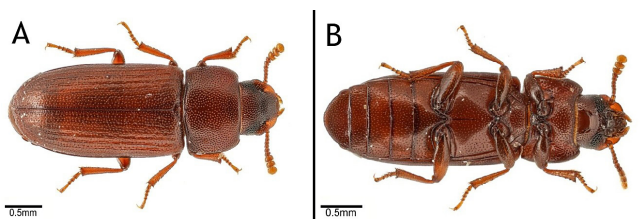

Fig. 3. General appearance of the rusty red flour beetle collected from flour

A) Dorsal side **B) Ventral side**

Source: Authors' own elaboration

of five segments. The thighs of the hind legs were slightly larger than those of the forelegs and middle legs. The thigh and hind legs were similar to the middle leg. The tarsus had four elongated segments that varied in size. The largest segment is the fourth, which is similar to the fifth segment in the claws of the front and middle legs.

Abdomen

It is light reddish-brown and consists of five oblong plates of unequal size. The first four plates have broad anterior and posterior margins. The fifth section had a rounded apex.

Molecular study

1 – DNA extraction and amplification

The concentration and purity of the genetic material were measured using the Nanodrop device.

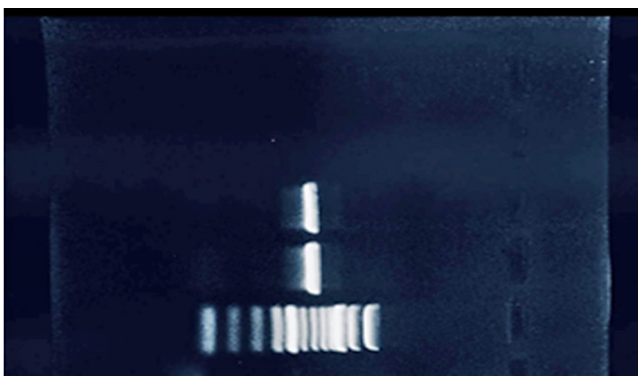

Fig. 4. Amplification product of the mtCOX1 gene primer on agarose gel for the first and second types of *T.castaneum.*

Source: Authors' own elaboration

Table 2. The percentages of matching the genetic sequence of the targeted mtCox1 gene segments in the species with those preserved in the GenBank

Type Name	Target gene	Identical accession numbers in the gene bank	Matching ratio
Type 1	mtCOX1	PQ219934.1	100%
Type 2	mtCOX1	PQ219935.1	100%

Source: Authors' own elaboration

The results obtained using the Nanodrop device showed successful amplification of the cytochrome oxidase gene mtCox1 using specialized primers and DNA bands with molecular weights of approximately 700 base pairs, (Figure 4).

2 – Genetic code analysis

The nucleotide sequence of the mtDNA segments of the mitochondrial gene mtCox1 for the two types of *T.castaneum* was analyzed. The results of sequence analysis after alignment showed a match with the genetic sequence of their counterparts preserved as sources in GenBank at rates ranging from 100% (representing the percentage of identification). All species were subjected to a similarity analysis using the Blast program, showed in (Table 2).

3 – Documentation of genetic sequences

The sequences of the mtCOX1 gene for the studied patterns were documented for the first time in NCBI GenBank as future sources for the collection of stored insects in Basra Governorate and Iraq.

Segments and their lengths were recorded, and an independent accession number was assigned to each segment. In addition, the barcodes of the genetic sequence segments were documented and given a special reference number, showed in (Table 3).

4 – Evolutionary network

Molecular diagnosis is an accurate method for distinguishing closely related insect species using insect primers. The molecular sequence of the gene from T. *T.castaneum* from the Basrah Governorate was documented for the first time in the National Center for Biotechnology Information (NCBI) gene bank database in America. The insect was registered in the GenBank with the molecular sequence number in the GenBank PQ219934.1 for the first type. Second, its genetic number was PQ219935.1. Two samples were collected from the same species. An evolutionary network has been created for *T.castaneum*. The data used in this network were 19 COX1 sequences from different countries that were selected based on 100% coverage, including the sequences of the current study. The results showed that there were 13 T. castaneum genotypes of *T.castaneum* in different coun-

Table 3. Accession numbers in the gene bank for the sequence segments of the mtCOX1 gene and reference numbers for the barcodes

Type Name	Family name	Target gene	Product size after modification	GenBank Accession Number
Type 1	Tenebrionidae	mtCOX1	684	PQ219934.1
Type 2	Tenebrionidae	mtCOX2	684	PQ219935.1

Source: Authors' own elaboration

tries, which were identical to the samples recorded in the USA, China, India, and France. The sequences of the current study sample were collected in genotype H1 with other sequences from India, and the rest of the genotypes were 7 for India (H2, H3, H4, H5, H5, H9, and H12), 3 for China (H7, H8, and H13), 1 for France (H10), and 1 for the USA (H11), showed in (Figure 5).

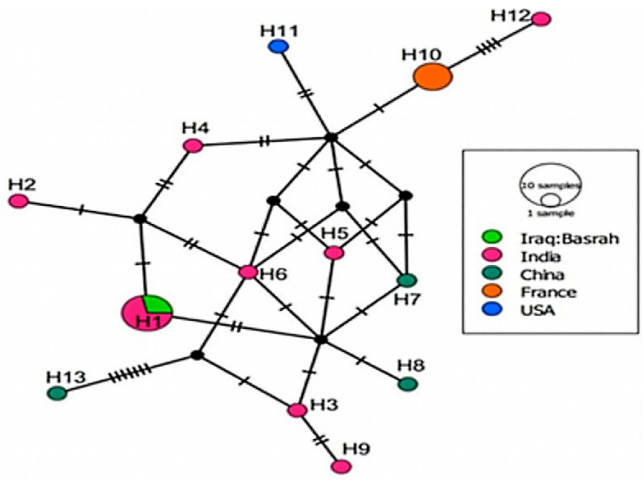

Fig. 5. The evolutionary network of the mtCOX1 gene among the studied strains in Basrah Governorate
Source: Authors' own elaboration

Discussion

This is a morphological study of rusty flour beetles collected from nuts and flour in Basra Governorate. The results of the molecular study showed that the two types belong to *Tribolium castaneum*. However, morphological differences were observed between these two types. The first, collected from nuts and given a genetic sequence number in the gene bank PQ219934.1, is light brown and longer and thinner than the second. Second, it is dark in color. Sequence number PQ219935.1. There was also a difference in the levels of the body parts. The last part of the tentacle was oval and elongated, unlike that in the second sample. It is spherical. The head was elongated, thorax was thinner, and abdomen was oval. The sutures between the abdominal plates were wavy, unlike their counterparts; the abdomen was spherical and the sutures were flat.

Insects have huge genetic diversity that can be determined using molecular techniques that focus on DNA. Insect differences are not limited to phenotypic traits, but also include behavior and size within insect groups and even within individuals of the same species (Peterson et al., 2012). Recently, DNA barcodes have been used to confirm phenotypic diagnoses. This method relies on the diversity of mtCOX1 sequences, known as DNA barcodes (Hebert et al., 2003). Studies using these markers have focused on molecular differences between species (Zufall et al., 2013). Molecular techniques can be used to distinguish between similar species that cannot be distinguished phenotypically, as in a study by Aslam et al. (2019), which distinguished two species that have similar phenotypic traits and are difficult to distinguish between *Tribolium castaneum* and *Tribolium confusum.* Yamauchi et al. (2018) confirmed that *Tribolium castaneum* is characterized by a uniform phenotype, which makes it difficult to visually distinguish between strains that occur in different regions. Polymorphisms in the nucleotide sequences of the mitochondrial genes of this insect were studied, and the samples were collected from Japan, Thailand, and Canada. The study sample *Tribolium castaneum* has been recorded in many countries worldwide. It has been recorded in South and North America, Asia, Africa, Europe, and the Middle East (Rodríguez-Cabo et al., 2021). *Triboliumcastaneum* has also been recorded in several Arab countries such as Egypt, Libya, Lebanon, Sudan, and Palestine (Al-Ali, 1997). The first record of this *castaneum* species was recorded in Iraq in 1918. It has been found in stored wheat grains, rice, and flour (Buxton and Mellan, 1918). Al-Bakr)1962) recorded its appearance on stored dates in the Basrah Governorate.

Conclusion

Molecular diagnosis was conducted for the insects, and the results showed that all patterns that which differences in their morphological characteristics belonged to *T. castaneum.* It was registered in the NCBI gene bank for the first time from Basrah Governorate. The first morphism was assigned the genetic sequence number PQ219934.1. The second morphism was assigned the genetic sequence number PQ219935.1.

Conflict of interest declaration

The authors declare that they have no affiliations with or involvement in any organization, or entity with any financial interest in the subject matter, or materials discussed in this manuscript.

References

Abbasi, I., Halaseh, L., Darwish, H. M. & Matou, I. (2021). Strategy for DNA extraction and detection from insect pests in stored home grain samples. *Al-Quds Journal for Natural Sci., 1*(1), 24 – 32.

Abd AL-Hseen, Z. E. & Manea, A. I. (2020). Effect of biofertilizer and organic extracts in two hybrids of cauliflower (Brassica Oleracea var. Botrytis). *International Journal of Agricultural and Statistical Sci., 16*(Supplement 1), 1651 – 1659.

Afifi, M. (2020). Insects and humams. *Arab Press Agency*, 175 – 178, Egypt.

Ahmady, A., Rahmatzai, N., Hazim, Z. & AA, M. (2016). Effect of temperature on stored product pests Tribolium confusum jaquelin du Val (Coleoptera: tenebrionidae) and Callosobruchus maculatus (F.)(Coleoptera: chrysomelidae: bruchidae). *Journal of Entomology and Zoology Studies, 4*(6), 166 – 172.

Ahmed, H. K. (2020). *A taxonomical study of order odonata and effect of some environmental factors in their occurrence in Basrah province*. A Thesis for the degree of doctor of philosophy in biological science Entomology, University of Basrah, Iraq, 272 – 279.

AL-Ali, A. (1977). Phtophagons and entomophagous insects and mites of Iraq. *Nat. Hist Res. Cent. Pupl*., 142.

Al-Bakr, Ab.-J. (1962). *Iraqi Dates*. Iraqi Government Press, Baghdad, 141.

Al-Emara, M. S, Alyousuf, A. A. & Abass, M. H. (2021). Efficacy of ozone gas against all stages of red flour beetle, Tribolium castaneum (Herbst)(Coleoptera: Tenebrionidae) at different temperatures and exposure periods. *Basrah Journal of Agricultural Sciences, 34*(2), 240 – 252.

Aslam, AF. M., Sultana, S. Das, SR. & Howlader, A. J. (2019). DNA barcoding to resolve the confusion in identifying Triboilum confusum and Tribolium castaneum. *Bangladesh J. Zool., 47*(2), 333 – 342.

Banga, K. S., Kumar, S., Kotwaliwale, N. & Mohapatra, D. (2020). Major insects of stored food grains. *International Journal of Chemical Studies, 8*(1), 2380 – 2384.

Buxton, P. & Mellan, K. (1918). The measurement and control humidity. *Journal Bulletin of scientific Research, 25*(2), 171 – 175.

Campbell, J. F., Athanassiou, C. G., Hagstrum, D. W. & Zhu, K. Y. (2022). Tribolium castaneum: a model insect for fundamental and applied research. *Annual Review of Entomology, 67*(1), 347 – 365.

Gurdasani, K., Rafter, M. A., Daglish, G. J. &Walter, G. H. (2018). Characterising the variables associated with Tribolium castaneum adults that initiate flight in laboratory tests: generating predictions for the field. *Journal Stored Prod. Res., 79*, 123 – 131.

Hebert, P. D. N., Cywinska, A., Ball, S. L. & Dewaard, J. R. (2003). Molecular genetic diagnosis of Tribolium castaneum and Tribolium confusum (C0leoptera: Tenebrionidae) by sequencing of Col gene analysis. *Biochem. Cell. Arch., 21*(2), 3619 – 3624.

Kalaf, J. M. (2021). Genetic diagnosis of the rust red flour beetle Tribolium castaneum (Herbst) (coleoptera) and its mortality, Attraction and replling effect of some vegetabe oil. In: *Agricult. State. Sci., 17*(1), 1867 – 1872.

Mason, L. J. & Strait, C. A. (2019). Stored product integrated pest management with insects and application extreme temperatures. In: *Temperature sensitivity in integrated pest management*, 141 – 177, CRC Press.

Paul, A., Radhakrishnan, M., Anandakumar, S., Shanmugasundaram, S. & Anandharamakrishnan, C. (2020). Disinfestation techniques for major cereals: A status report. *Compr. Rev. Food Sci. Food Saf., 19*(3), 1125 – 1155.

Peterson, B. K., Weber, J. N, Kay, E. H. H., Fisher, H. S. & Hoekstra, H. E. (2012). Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PloS one, 7*(5), e37135.

Pointer, M. D., Gage, M. J. G. & Spurgin, L. G. (2021). Tribolium beetles as a model system in evolution and ecology. *Heredity, 126*, 869 – 883.

Rodríguez-Cabo, T., Moroño, Á., Arévalo, F., Correa, J., Lamas, J. P., Rossignoli, A. E. & Blanco, J. (2021). Paralytic shellfish poisoning (PSP) in mussels from the eastern Cantabrian Sea: toxicity, toxin profile, and co-occurrence with cyclic imines. *Toxins, 13*(11), 761.

Rosner, J., Wellmeyer, B. & Merzendorfer, H. (2020). Tribolium castaneum: a model for investigating the mode of action of insecticides and mechanisms of resistance. *Current pharmaceutical design, 26*(29), 3554 – 3568.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual (No.Ed.2)*. Cold spring harbor laboratory press, 1546.

Yamauchi, H., Harada, M. & Miyanoshita, A. (2018). Polymorphism observed in mitochondrial genes of red flour beetle, Tribolium castaneum (Coleoptera: Tenebrionidae) of different origin in laboratory cultures. *Biosci. Biotechnol. Biochem. Feb., 82*(2), 229 – 231. doi: 10.1080/09168451.2017.1422384. Epub. 2018, Jan 12. PMID: 29327678

Zufall, R. A., Dimod, K. L. & Doeder, F. P. (2013). Restricted distribution and limited gene flow in the model ciliate Tetrahymena thermophila. *Mol. Ecol., 22*(4), 1081 – 1091.

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