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Phylogeographic Insights and Genetic Divergence of *Trogoderma granarium*: A Comprehensive Analysis Using Consensus and Concatenated Gene Trees

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Key words
***Trogoderma granarium*, Stored grain pest, Phylogenetic analysis, Concatenated, DNA, 16SrDNA, Cytochrome oxidase**

ABSTRACT

The presence of the globally devastating agricultural pest *Trogoderma granarium* (Khapra beetle) in Pakistan poses a serious threat to food security and stored grains, especially wheat. Wheat is a crucial economic and nutritional staple for the citizens of Pakistan. Effective management of this beetle relies on its accurate identification, as it closely resembles other Dermestidae species. Therefore, precise identification and understanding of the interspecific and intraspecific relatedness of the pest are essential for its management. This report focuses on the accurate detection and phylogenetic analysis of *T. granarium*. Molecular markers are the best tools for diagnosing and analyzing the phylogeny of the pest in any life phase and condition. For this purpose, partial sequences of two mitochondrial genes, 16S rDNA and cytochrome oxidase I, were used. Samples for this study were collected from various areas of Punjab, Pakistan. Phylogenetic trees, constructed from individual and concatenated gene sequences using neighbor-joining and maximum likelihood algorithms, indicate that this pest has a monophyletic origin.

INTRODUCTION

Addressing global food security in the twenty-first century requires multi-pronged approaches. It is crucial to identify and enumerate the major contributors to annual yield losses. Among these factors, post-harvest losses caused by various infestations account for up to one-fifth of the total yield (Pimentel, 2019). The potential for such infestation is manifold when the pest in question is invasive. Invasive pests can survive in new habitats (Mack *et al*., 2000). The Khapra beetle, *Trogoderma granarium* Everts, is one such invasive pest with a significant impact on stored grain losses. Its destructive activity is due to several inherent characteristics.

The Khapra beetle has 142 known hosts (Athanassiou *et al*., 2019), an expansive temperature range (EPPO, 2013), a wide geographic range, the ability to outcome other species (Kavallieratos *et al*., 2017) and the abilities to undergo diapause (Banks, 1977). These abilities make pest management strategies challenging Pakistan is an agrarian economy where wheat is the main cereal staple for domestic use and export. The country, part of the Indian peninsula, is the origin of the Khapra beetle (Stibick, 2007) and has a tropical climate conducive to its proliferation.

An outbreak of this beetle can cause significant losses to food security and exports. Pakistan ranks fourth in Khapra beetle interception frequency during shipment pretreatments by the US (Athanassiou *et al*., 2019), following India, Saudi Arabia, and Sudan, which is alarming. In addition to its destructive capabilities, the Khapra beetle is challenging to identify. Expertise is required to identify individuals at the adult or larval stages to the species level of the genus *Trogoderma*, sometimes necessitating dissection to inspect genitals (Castalanelli *et al*., 2011).

Available identification keys are often limited to specific habitats or developmental stages and do not cover

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all species of the genus *Trogoderma* (EPPO, 2013). The lack of accurate characterization can lead to the failure of pest management strategies (Mack *et al*., 2000). Therefore, developing new characterization strategies is more critical than developing new management strategies. Molecular techniques offer an alternative for identification with options including nuclear DNA or mitochondrial DNA. The *16S rDNA* and cytochrome oxidase subunit I (*CO1*) genes from the mitochondrial genome evolve at a higher rate than other mitochondrial genes (Hwang and Kim, 1999). This study has utilized the two molecular markers for characterization of Khapra beetle populations in Pakistan.

MATERIALS AND METHODS

Sampling and rearing of beetles

The Khapra beetle is prevalent across Punjab, Pakistan. Seventeen population samples were collected from various wheat warehouses in districts including Gujranwala, Okara, Sialkot, Lahore, Dera Ghazi Khan, Kasur, Shakargarh, Lodhran, Shaikhupura, KahnaNau, Sahiwala, Khushab, Gujrat, Multan, Jhang, Faisalabad and Layyah of Punjab province. A cluster sampling technique was used, visiting both government-run and privately owned warehouses. Beetle samples were collected in labeled plastic zipper bags with wheat grains and brought to the *Trogoderma* culture room at the Institute of Zoology, University of the Punjab, Lahore for further culturing.

The beetles were reared in the laboratory according to the pre-established protocol (Riaz *et al*., 2014). Fifth instar larvae of third generation were processed for isolation of DNA and amplification of 16S rRNA and cytochrome oxidase 1 (*CO1*) genes.

DNA extraction

The CTAB method of Doyle and Doyle (1990) was used with minor modifications for isolation of DNA. Larvae were homogenized in mixture of CTAB buffer (pH 7.8) and β-mercaptoethanol (5:1) incubated at 65°C for thirty min followed by cooling at room temperature for ten min. A chloroform: iso-amyl alcohol mixture (24:1) was then added to the tubes, which were centrifuged at 11,000 rpm for 10 min. The supernatant from each tube was transferred to sterile eppendorfs to which chilled absolute ethanol was then added for precipitation of DNA. The tubes were then centrifuged at 14,000 rpm for 10 min. The supernatant was discarded, and 70% ethanol was added to each tube. After another centrifugation at 14,000 rpm for 3 min, the supernatant was discarded. The resulting pellet was dried at 60 °C for 30 min in an incubator. Finally, 10 µl of nuclease-free water was added to each tube, and the DNA pellet was dissolved with gentle tapping.

Amplification of 16S rRNA *and mitochondrial* CO1 *genes*

Once extracted, the DNA was amplified using species-specific mitochondrial 16S rDNA and cytochrome oxidase 1 (*CO1*) gene primers (Olson *et al*., 2014).

16S rDNA

Forward = 5'CTAAAATTGAAAATTTCTATACT3'
Reverse = 5'CTAGCCTGCTCCCTGATTGA3'.

CO1

Forward = 5'CAACATTTATTTTGATTTTTTGG3'
Reverse = 5'TCCAATGCACTAATCTGCCATATTA3'

Each DNA sample was subjected to PCR amplification in a 50 µl reaction mixture. Each PCR tube contained 5.0 µl of dNTPs (2.0 mM), 1.0 µl of 100 nM forward primer, 1.0 µl of 100 nM reverse primer, 4.0 µl of template DNA, 5.0 µl of 10X Taq buffer, 3.0 µl of 25 mM magnesium chloride, and 0.50 µl of Taq polymerase (Thermo Scientific cat# 00855243). The volume was adjusted to 50 µl with 30.50 µl of sterilized distilled water.

The PCR cycles (Bio Rad T100) included an initial denaturation at 95°C for fifteen min, followed by forty cycles of denaturation at 94°C for one minute, annealing at 42°C for one minute, and extension at 72°C for one minute, with a final extension at 72°C for five min. The amplified product was separated on a 2% agarose gel (Weal Tec MD-20) and sequenced by Macrogen, Korea.

The 250bp PCR product amplified from 16S rDNA of Khapra beetle populations collected from Gujranwala (TR1), Okara (TR2), Sialkot (TR3), Lahore (TR4), Dera Ghazi Khan (TR5), and Layyah (TR6) were deposited in GenBank with accession number MN535884, MN537148, MN537149, MW049034, MW049033 and MW049032, respectively. Likewise the 800bp PCR product amplified from mitochondrial *CO1* gene of Khapra beetle populations of Gujranwala (IR-01), Okara (IR-02), Sialkot (IR-03), Lahore (IR-04), Dera Ghazi Khan (IR-05), Mughalpura (IR-06), Kasur (IR-07), Shakargarh (IR-08), Lodhran (IR-09), Khairpur (IR-10), Layyah (IR-11), Shaikhupura (IR-12), Manawa (IR-13), Wassan Pura (IR-14), Kahna Nau (IR-15), Khairpur (IR-16) and Layyah (IR-17) were deposited in GenBank with accession number OP341260, OP345940, OP346112, OP346574, OP349051, OP349099, OP349101, OP361281, OP352898, OP353625, OP354417, OP361318, OP354510, OP354511, OP354512, OP359420 and OP360011, respectively.

DNA sequence analysis

The sequences obtained from Macrogen were edited using DNASTAR Lasergene 7v7.1.0 software and aligned using the built-in version of CLUSTAL W (Thompson *et al*., 1994). Phylogenetic relationships were analyzed by

constructing a phylogenetic tree using the neighbor-joining method (Tamura *et al*., 2013) on phylogeny.fr, funded by Reseau National des Genopoles (RNO). The percentage identity and divergence between the study group sequences and published sequences were calculated using genetic distance values from MagAlign, a DNASTAR software project (version 7v7.1.0).

RESULTS

Figures 1 and 2 show the percentage identity of the consensus tree of *16S rDNA* and *MCOI* genes, respectively. Highlighted sequences in the gene tree represent the individuals of interest. The phylogenetic analysis of

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		100.0	100.0	100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	1	KJ930487_T.granarium_USA
2	0.0		100.0	100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	2	KJ930432_T.granarium_USA
3	0.0	0.0		100.0	100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	3	ON725096_T.granarium_Turkey
4	0.0	0.0	0.0		100.0	99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	4	OM388538_T.granarium_Iraq
5	0.0	0.0	0.0	0.0		99.6	99.6	99.6	100.0	100.0	100.0	100.0	95.8	96.2	74.2	5	MZ571636_T.granarium_Australia
6	0.4	0.4	0.4	0.4	0.4		99.2	99.2	99.6	99.6	99.6	99.6	95.8	96.2	74.2	6	KJ930452_T.granarium_USA
7	0.4	0.4	0.4	0.4	0.4	0.8		99.2	99.6	99.6	99.6	99.6	95.4	95.7	74.2	7	KJ930433_T.granarium_USA
8	0.4	0.4	0.4	0.4	0.4	0.8	0.8		99.6	99.6	99.6	99.6	95.4	95.7	74.2	8	KJ930431_T.granarium_USA
9	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4		100.0	100.0	100.0	95.8	96.2	74.2	9	MN535884_T.granarium_PK
10	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0		100.0	100.0	95.8	96.2	74.2	10	MN537148_T.granarium_PK
11	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	0.0		100.0	95.8	96.2	74.2	11	MN537149_T.granarium_PK
12	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.4	0.0	0.0	0.0		95.8	96.2	74.2	12	MW049032_T.granarium_PK
13	3.8	3.8	3.8	3.8	3.8	3.8	4.3	4.3	3.8	3.8	3.8	3.8		98.6	71.3	13	MW049033_T.granarium_PK
14	2.9	2.9	2.9	2.9	2.9	2.9	3.4	3.4	2.9	2.9	2.9	2.9	0.0		71.1	14	MW049034_T.granarium_PK
15	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	31.7	35.7	35.4		15	AJ438144_T.castaneum
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Fig. 1. Table showing the divergence and percent identity of *16S rDNA* genes.

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
1		99.9	99.9	99.1	99.6	99.5	99.6	99.6	99.6	99.6	100.0	99.6	99.6	99.9	99.9	99.9	99.6	99.7	99.7	99.2	99.9	99.7	99.5	99.9	99.6	98.6	99.6	99.8	99.8	99.8	48.2	1
2	0.1		100.0	99.0	99.5	99.4	99.5	99.5	99.5	99.5	99.9	99.5	99.5	100.0	99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	2
3	0.1	0.0		99.0	99.5	99.4	99.5	99.5	99.5	99.5	99.9	99.5	99.5	100.0	99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	3
4	0.4	0.5	0.5		99.0	98.9	99.0	99.0	99.0	99.0	99.4	99.0	99.0	99.2	99.2	99.2	99.0	99.1	99.1	98.6	99.2	99.1	98.8	99.1	98.9	97.9	98.8	99.2	99.2	99.0	48.1	4
5	0.4	0.5	0.5	0.5		99.9	100.0	100.0	100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	5
6	0.5	0.6	0.6	0.6	0.1		99.9	99.9	99.9	99.9	99.5	99.9	99.9	99.4	99.6	99.4	99.9	99.5	99.5	99.0	99.6	99.5	99.2	99.6	99.3	98.3	99.3	99.5	99.5	99.5	48.1	6
7	0.4	0.5	0.5	0.5	0.0	0.1		100.0	100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	7
8	0.4	0.5	0.5	0.5	0.0	0.1	0.0		100.0	100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	8
9	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0		100.0	99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	9
10	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0		99.6	100.0	100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	10
11	0.0	0.1	0.1	0.1	0.4	0.5	0.4	0.4	0.4	0.4		99.6	99.6	99.9	99.9	99.9	99.6	99.7	99.7	99.2	99.9	99.7	99.5	99.9	99.6	98.6	99.6	99.8	99.8	99.8	48.2	11
12	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4		100.0	99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	12
13	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4	0.0		99.5	99.7	99.5	100.0	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	13
14	0.1	0.0	0.0	0.3	0.5	0.6	0.5	0.5	0.5	0.5	0.1	0.5	0.5		99.7	100.0	99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	14
15	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3		99.7	99.7	99.9	99.9	99.4	100.0	99.9	99.6	100.0	99.7	98.7	99.7	99.8	99.8	99.8	48.4	15
16	0.1	0.0	0.0	0.3	0.5	0.6	0.5	0.5	0.5	0.5	0.1	0.5	0.5	0.0	0.3		99.5	99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	16
17	0.4	0.5	0.5	0.5	0.0	0.1	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.5	0.3	0.5		99.6	99.6	99.1	99.7	99.6	99.3	99.7	99.4	98.5	99.4	99.7	99.7	99.7	48.2	17
18	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4		100.0	99.5	99.9	100.0	99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.4	18
19	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4	0.0		99.5	99.9	100.0	99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.4	19
20	0.8	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9	0.9	0.8	0.9	0.9	0.9	0.6	0.9	0.9	0.5	0.5		99.5	99.6	99.3	99.4	99.7	98.7	99.7	99.5	99.2	99.5	48.1	20
21	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3	0.0	0.3	0.3	0.1	0.1	0.5		99.9	99.6	100.0	99.7	98.7	99.7	99.8	99.8	99.8	48.7	21
22	0.3	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.1	0.4	0.4	0.0	0.0	0.4	0.1		99.7	99.9	99.9	98.9	99.9	100.0	99.7	100.0	48.7	22
23	0.5	0.7	0.7	0.7	0.7	0.8	0.7	0.7	0.7	0.7	0.5	0.7	0.7	0.7	0.4	0.7	0.7	0.3	0.3	0.7	0.4	0.3		99.6	99.6	98.6	99.6	99.7	99.4	99.7	48.9	23
24	0.1	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.1	0.3	0.3	0.3	0.0	0.3	0.3	0.1	0.1	0.6	0.0	0.1	0.4		99.7	99.0	99.7	99.8	99.8	99.8	48.1	24
25	0.4	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.4	0.6	0.6	0.6	0.3	0.6	0.6	0.1	0.1	0.3	0.3	0.1	0.4	0.3		99.3	100.0	99.8	99.5	99.8	47.9	25
26	0.7	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9	0.9	0.7	0.9	0.9	0.9	0.6	0.9	0.9	0.4	0.4	0.6	0.6	0.4	0.7	0.3	0.0		99.3	99.0	98.6	99.0	47.8	26
27	0.4	0.6	0.6	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.4	0.6	0.6	0.6	0.3	0.6	0.6	0.1	0.1	0.3	0.3	0.1	0.4	0.3	0.0	0.0		99.8	99.5	99.8	48.0	27
28	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.0	0.0	0.5	0.2	0.0	0.3	0.2	0.2	0.2	0.2		99.7	100.0	48.7	28
29	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.8	0.2	0.3	0.7	0.2	0.5	0.5	0.5	0.3		99.7	48.9	29
30	0.2	0.3	0.3	0.3	0.3	0.5	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.0	0.0	0.5	0.2	0.0	0.3	0.2	0.2	0.2	0.2	0.0	0.3		48.5	30
31	89.4	89.4	89.4	89.0	89.4	89.9	89.4	89.4	89.4	89.4	89.4	89.4	89.4	89.4	88.9	89.4	89.4	88.9	88.9	90.0	87.7	87.7	87.1	89.6	90.6	89.8	90.2	88.1	87.2	88.4		31
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	

1	IR01_T.granarium_PK
2	IR02_T.granarium_pk
3	IR03_T.granarium_PK
4	IR04_T.granarium_PK
5	IR05_T.granarium_PK
6	IR06_T.granarium_PK
7	IR07_T.granarium_PK
8	IR08_T.granarium_PK
9	IR09_T.granarium_PK
10	IR10_T.granarium_PK
11	IR11_T.granarium_PK
12	IR12_T.granarium_PK
13	IR13_T.granarium_PK
14	IR14_T.granarium_PK
15	IR15_T.granarium_PK
16	IR16_T.granarium_PK
17	IR17_T.granarium_PK
18	KP331457_T.granarium
19	KP331450_T.granarium
20	FJ589736_T.granarium
21	HQ419097_T.granariur
22	HQ419089_T.granariur
23	HQ419090_T.granariur
24	JN872145_T.granarium
25	JN872147_T.granarium
26	JN872148_T.granarium
27	JN872146_T.granarium
28	HM243454_T.granariur
29	HQ419105_T.granariur
30	KM578752_T.granarium
31	OP604562.1_T.castane

Fig. 2. Table showing the divergence and percent identity of *MCOI* genes.

consensus and concatenated trees indicates that *T. granarium* individuals are monophyletic in origin (Figs. 3, 4). However, the consensus trees (Figs. 3, 4) for *16S rDNA* sequences showed some differences. The NJ consensus tree revealed a separate clade for MW049033 and MW049034, while the ML consensus tree did not show this clade. Similarly, a separate clade for 16S sequences was observed in the NJ concatenated tree. The percent identity table (Fig. 2) showed no significant divergence among the reported and GenBank sequences for the *MCOI* gene. However, the percent identity table (Fig. 1) showed no prominent divergence among MN535884, MN537148, MN537149, and MW049032. In contrast, MW049033 and MW049034 showed divergences of 3.8% to 4.3% and 2.9% to 3.4%, respectively, for the *16S rDNA* gene. Therefore, the individuals in the current study are closely related to each other and to individuals reported worldwide, except for the 16S rDNA sequences of MW049033 and MW049034.

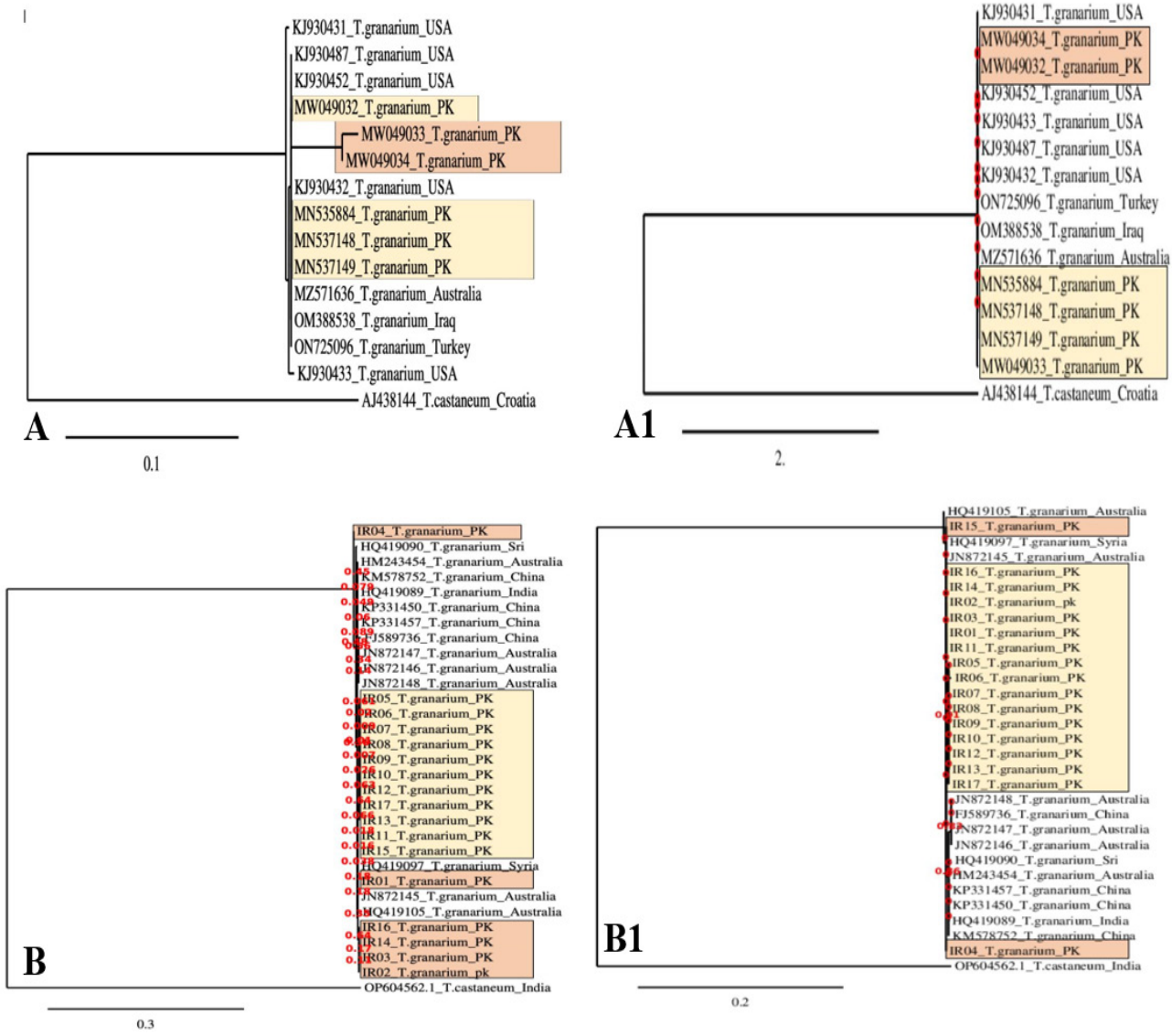

Fig. 3. The evolutionary history of *T. granarium* traced using neighbour joining and maximum likelihood methods based on 16S and *MCOI* sequences. Highlighted boxes represent the sequences reported from current study. Phylogenetic tree constructed through neighbour joining method (A) and maximum likelihood methods (A1), respectively 250 bp *16S rDNA* gene indicates the clustering of species at one node showing minimum divergence among the organisms (B, B1). Phylogenetic tree constructed through neighbour joining method (B) and maximum likelihood method (B1) of 800 bp MCOI indicates the clustering of species at one node showing minimum divergence among the individuals. OP604562.1 *T. castaneum* was used an outgroup.

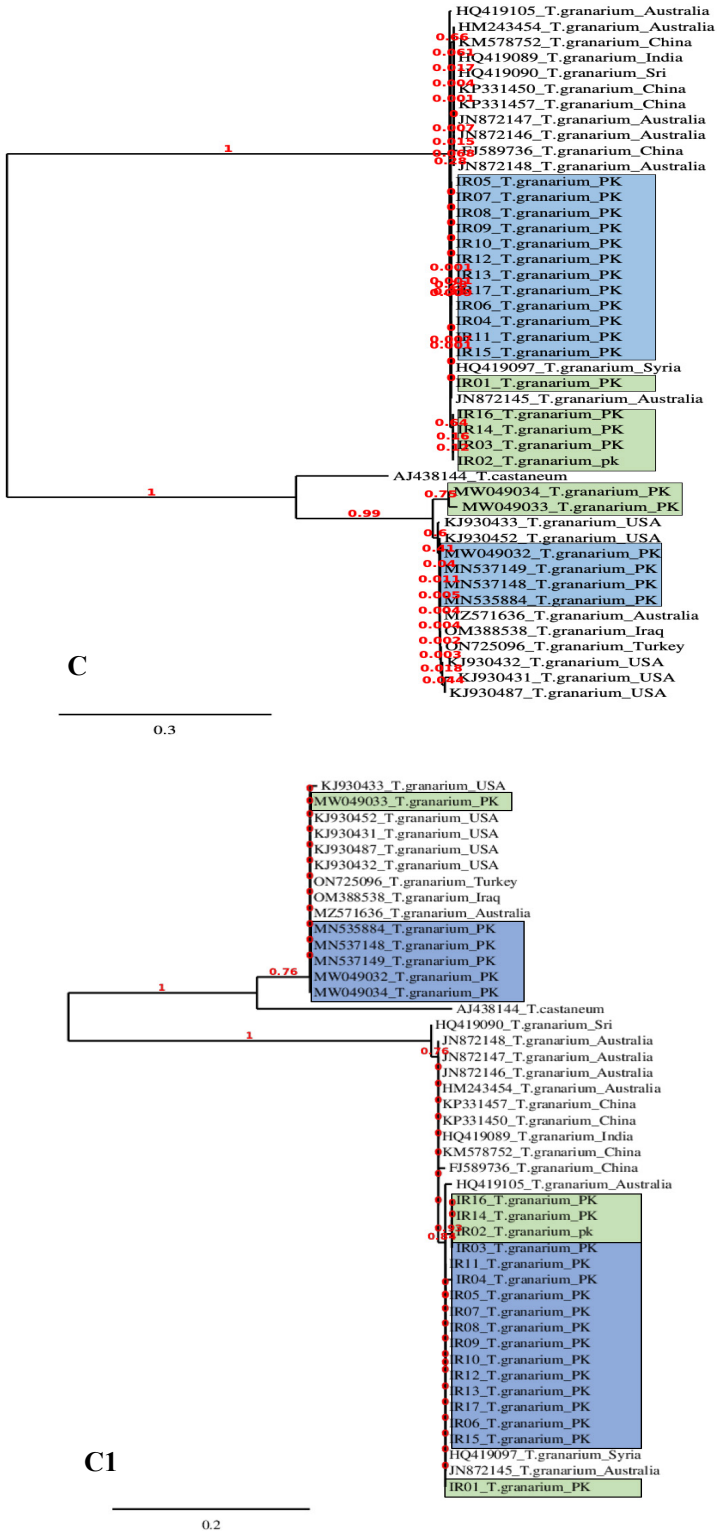

Fig. 4. Phylogenetic tree based on concatenation of *16S rDNA, MCOI* sequences of 23 isolates with 21 reference strains. The sequences were aligned using Neighbour Joining methods (C) and maximum (C1) likelihood methods. AJ438144 *T. castaneum* was used as an outgroup.

DISCUSSION

The utility of mitochondrial markers (16S and MCOI) for khapra beetle identification

Morphological keys are geographically restricted, of limited use without a verified voucher list, and challenging to identify without deep expertise because of the nuances in morphological landmarks, further complicating identification (Beal, 2003). However, studies on other invasive insect species has shown that molecular markers can provide useful information about population structure, gene flow, and dispersal pathways (Mikac and Clarke, 2006). This, in turn, helps establish the existence of cryptic species and provides insight into species dispersal (Loxdale and Lushai, 1998; Mikac and FitzSimmons, 2010; Mikac and Clarke, 2006). However, it is not possible to identify all animal species with universal molecular assays (Adam and Palmer, 2003; Damgraad, 2008; Dentinger *et al*., 2011). Olson *et al*. (2014) described that *CO1* and *16S rDNA* gene sequences from mitochondria can be diagnostic for *T. granarium*. *T. granarium* species associated with grain stores can be confirmed as Khapra Beetle by sequence data alone (Olson *et al*., 2014). Therefore, for this study, specific primers of *16S rDNA* and *MCO1* genes were used to analyze the collected species of the pest.

Phylogenetic diagnosis

Phylogenetic analysis of *16S rDNA* and *MCO1* genes provides a definitive means for diagnosing *T. granarium,* especially in cases of PCR products suspected of primer mis-annealing or contamination. Current study analysis shows that *T. granarium* collected from different locations in Punjab are monophyletic. In Pakistan, no work on the phylogenetic relationships of *T. granarium* has been reported yet. Thus, we take the opportunity to comment on the phylogeny of the insect pest and found that the individuals from Punjab are closely related and likely come from the same population.

CONCLUSION

This study provides a basis for both accurate molecular identification and divergence analysis of *T. granarium* based on partial DNA sequences of mitochondrial *16S rDNA* and *CO1* genes. Molecular data from other Khapra populations across this part of the globe may help integrate a bigger picture.

DECLARATION

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Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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