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MOLECULAR CHARACTERIZATION AND IDENTIFICATION OF INSECT POLLINATORS OF *PUNICA GRANATUM* (LINNAEUS) FROM WESTERN HIMALAYA, INDIA

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ABSTRACT

Wild pomegranate *Punica granatum* is one of the most important wild fruit crop, highly pollinated by wild insect pollinators. Present study analyzed the pollinating insect species using molecular method. About 17 pollinator species were characterized and identified using mtCO1. Phylogenetic analysis revealed the close phylogenetic relation between hymenopteran insect species in one clade, whereas another clade showing relationship between lepidoptera, Coleopteran and Diptera.

Key words: *Punica granatum*, pollinators, mtCO1, phylogenetic analysis, genetic diversity, insect species, Hymenoptera, Lepidoptera, Coleoptera, Diptera

Wild pomegranate (*Punica granatum*) is medicinally very important crop and it is distributed in some areas of Jammu and Kashmir, Himachal Pradesh and Uttarakhand in Western Himalaya (Thakur et al., 2011). It has medicinal properties which reduce the risk of heart diseases (Aviram et al., 2004) and cancer (Adams et al., 2006). Phytochemicals have been found from the pomegranate tree as well as from the peel, juice, and seeds of the fruit (Elfalleh et al., 2011). Pollinator diversity is gradually decreasing as a result of human activities. More than 80% of all pollination are carried out by insects. Pollinators are simultaneously vital to supporting both natural ecosystems and human food security has been isolated. Molecular studies on insect pollinators has been isolated. The mtCO1 is used widespreadly as genetic marker (Moritz et al., 1987). Due to high mutation rate of mtDNA makes it most useful for comparisons of individuals within species and for comparisons of species that are closely or moderately-closely related (Jalali et al., 2015). Moreover, phylogenetics obtained with mtCO1 analysis supports conservation biology research by offering details on suitable species comparisons for ecological and genetic factors. This study, therefore focuses in the molecular characterization of pollinators of *P. granatum* through mtCO1 analysis.

MATERIALS AND METHODS

Insect pollinators of *P. granatum* were collected from Western Himalaya from mid April to end of May (2018 to 2021) during the blooming period of the wild pomegranate. Collected samples were preserved at -80^{0}C for molecular characterization. DNA was extracted from the thorax or upper abdominal region of the insect specimen by using DNeasy blood and tissue qiagen kit method by following standardized protocol of the manufacturers. Extracted DNA was preserved in the -20^{0}C for further use. Target DNA from mitochondrial gene, i.e., Cytochrome Oxidase subunit I was amplified using a pair of forward primers LCO1490 5' GGT-CAA CAA ATC ATA AAG ATA TTG G 3' and reverse primer HCO2198 5'- TAA-ACT-TCA-GGG-TGACCA-AAA-AAT-CA-3' (Folmer et al., 1994). PCR reaction was performed in 96-well plates with 20 µℓ reaction volume containing 1µℓ DNA template; 1 µℓ primer forward; 1 µℓ primer reverse; 5 µℓ distilled water; 12 µℓ Emerald PCR master mix in a C10 00™ Thermal Cycler. Thermocycling consisted of an pre-denaturation at temperature of 94^{0}C for 4 min followed by 30 cycles with denaturation reaction conditions at 94^{0}C for 40 sec, annealing at 50^{0}C for 30 sec, and extension at 72^{0}C for 50 sec. Then process of PCR ended with final extension at 72^{0}C for 6 min. The amplified product was analysed on a 1.2% agarose gel electrophoresis and checked under UV light and documented. The amplified DNA fragments were extracted from agarose gels and purified using DNA/RNA purification qiagen kit method standardized by manufacturers. The primers used were the same primers used in PCR amplification and sequencing was done in "big dye terminator version 3.1" cycle sequencing kit with sequencing machine–ABI 3500xL genetic analyser. After completion of sequencing, the results were analyzed by using MEGA X software (Kumar et al., 2018). Analyses were performed on 1000 bootstrapped data sets generated by the program (Felsenstein, 1985). All fasta format sequences obtained

by Sanger sequencing was used for BLAST search to check the sequence homology at NCBI. All the sequences were edited and aligned using bioedit sequence alignment editor software. All the gaps and mismatched were removed and sequences were submitted in the gene bank for accession number. In this study, phylogenetic analysis of 17 sequences was conducted using neighbor-joining method and Kimura-2 parameter in MEGA X. The nucleotide content (A, T, G, C) of all the samples and the total C+G and A+T at first, second and third codon position were calculated using MEGA X software. The AT% at three codon positions was calculated using the same program. Sequences were aligned using the MEGA X software (Kumar et al., 2018).

RESULTS AND DISCUSSION

Previous study also indicates that bee pollination could improve the setting rate and weight of pomegranate fruit significantly compared with self pollination (Derin and Eti, 2001). Wild pomegranate is economically and medicinally very important plant and pollinated by various insect pollinators. In present study, a total of 17 insect pollinator species of *P. granatum* were captured from different areas of Western Himalaya. All pollinator insect species were sequenced by Sanger sequencing using mtDNA markers, the cytochrome oxidase subunit sequence I (COI). MtDNA analysis of COI sequences is one of the useful method to characterized and identify insect pollinator species. MtDNA (COI) has been widely used by investigators all over the world to identify insect species (Jalali et al., 2015). Aslam et al., (2019) also utilized the mitochondrial cytochrome c oxidase subunit I gene sequences to characterized and identified three stored grain pests. Guo et al., (2020) also utilized mitochondrial COI Sequence to analyze the variations within and among geographic samples of the Hemp Pest, *Psylliodes attenuata* from China. The mtCO1 region in almost all the samples was in the range of 550-710bp (Fig. 1, 2). All Sequences were found to be deeply AT-biased due to 3rd codon position, which is expected in insect mtDNA. The high numbers of polymorphic sites were uniformly distributed throughout the third codon position in the COI gene. Genetic distances between the diverse groups showed higher values at the third codon position. Phylogenetic analysis revealed by constructing a phylogeny tree by using the neighbor–joining method (Fig. 3).

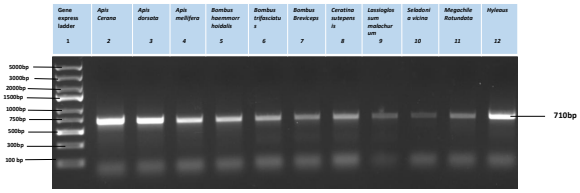

Fig. 1. Analysis of amplified PCR product in 1.2% agarose, Lane 1: Gene ruler express DNA

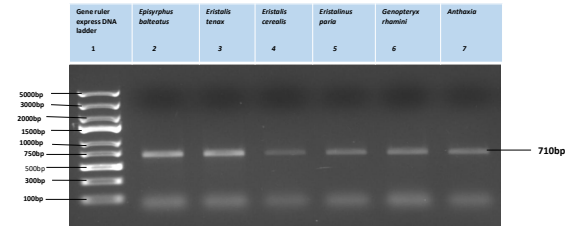

Fig. 2:.Analysis of amplified PCR product in 1.2% agarose, Lane 1: Gene ruler express DNA ladder, Lane 2,3,4,5,6,7: 710 bp size mtCO1 gene.

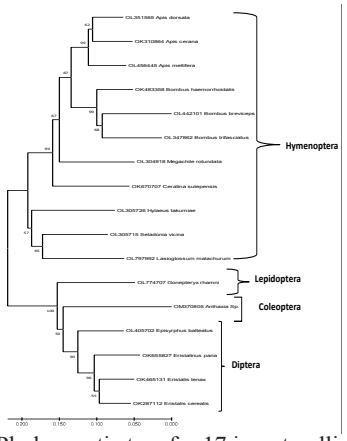

Fig. 3. Phylogenetic tree for 17 insect pollinators of *P. granatum* depicting genetic relationships derived from mtCO1 sequences neighbor-joining method of MEGA X

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AUTHOR CONTRIBUTION STATEMENT

Poonam Kumari contributed to the conception and design of the research work and conducted all the experiments. Mahender Singh Thakur provided guidance and supervision throughout the research process, including experimental design, data interpretation and manuscript revision. All authors contributed to the critical review and editing of the manuscript and approved the final version for publication.

CONFLICT OF INTEREST

No conflict of interest.

Table 1: Sequenced insect pollinator species of *P. granatum* with gene bank accession numbers.

Collecting Area	Altitude (Meter)	Latitude (North)	Longitude (East)	Samples insect species	Genebank Accession Number	Identification with BLASTN results
Alsindi	1132 m	31°-29′33	77°-12′31	*Apis cerana*	OK310864	*Apis cerana* (KU212341.1)
Pottershill	2050 m	31°-12′13	77°-13′41	*Apis dorsata*	OL351565	*Apis mellifera* (MH388489.1)
Naldehra	1887 m	31°-18′39	77°-18′69	*Apis mellifera*	OL456445	*Apis dorsata* (KU212345.1)
Darlaghat	1563 m	31°-13′14	31°-13′14	*Bombus haemorrhoidalis*	OK483358	*Bombus haemorrhoidalis* (MF582594.1)
Ghanahatti	1668 m	31°-08′18	77°-05′04	*Bombus trifaciatus*	OL347862	*Bombus trifasciatus* (KP671646.1)
Naldehra	1887 m	31°-18′39	77°-18′69	*Bombus breviceps*	OL442101	*Bombus breviceps* (MF582618.1)
Dhar	1360 m	31°-62′42	76°-82′85	*Ceratina sutepensis*	OK670707	*Ceratina sutepensis* (MK904769.1)
Naldehra	1887 m	31°-18′39	77°-18′69	*Lasioglossum malachurum*	OL797992	*Lasioglossum* sp. (OK120787.1)
Dhar	1360 m	31°-62′42	76°-82′85	*Seladonia vicina*	OL305715	*Seladonia vicina* (MK904698.1)
Dhar	1360 m	31°-62′42	76°-82′85	*Megachile rotundata*	OL304918	*Megachile rotundata* (MG351161.1)
Dhar	1360 m	31°-62′42	76°-82′85	*Hyleaus tukumiae*	OL305726	*Hylaeus tukumiae* (FJ411762.1)
Ghanahatti	1668 m	31°-08′18	77°-05′04	*Episyrphus balteatus*	OL405702	*Episyrphus balteatus* (KR262632.1)
Darlaghat	1563 m	31°-13′14	31°-13′14	*Eristalis tenax*	OK465131	*Eristalis tenax* (MN868866.1)
Darlaghat	1563 m	31°-13′14	31°-13′14	*Eristalis cerealis*	OK287112	*Eristalis cerealis* (OK465106.1)
Ghanahatti	1668 m	31°-08′18	77°-05′04	*Eristalinus paria*	OK655827	*Eristalinus paria* (OK444104.1)
Darlaghat	1563 m	31°-13′14	31°-13′14	*Gonepteryx rhamni*	OL774707	*Genopteryx rhamini* (KC158375.1)
Dhar	1360 m	31°-62′42	76°-82′85	*Anthaxia* sp.	OM370805	*Buprestinae* sp. (KY835728.1)

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