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Research Article

Evaluating Genetic Diversity of mtCOI *Bombus haemorrhoidalis* from Different Regions of Western Himalaya

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Abstract

Bumblebees are a diverse group of crucial pollinators for agricultural food production and natural ecosystems worldwide. They are interesting insect pollinators to explore social evolution, behavior and ecology because they have both eusocial and solitary life-cycle stages as well as some social parasite species. Numerous reports of species declines cite the interrelated causes of pathogen spread, habitat loss, pesticide use and global temperature change. Our reliance on a small number of thoroughly researched species for agricultural pollination is particularly hazardous due to these threats to bumblebee diversity. Sanger sequencing was used to analyze the genetic diversity of mtCOI *Bombus haemorrhoidalis*, which was collected on *Punica granatum* (wild pomegranate) from seven different Western Himalayan regions. Omega revealed a total of 6 variable sites in the alignment between all the mtCO1 sequences of *Bombus haemorrhoidalis.* The estimated transition/transversion (Ts/Tv) bias of COI (R) is 1.67. The variation in nucleotide content almost completely exist in the third codon position due to AT rich region, as compared to the first and second position of codons. The transition/transversion (Ts/Tv) ratio is significant in deducing the magnitude and direction of natural selection. Our study reveals clearly signifies the vast difference among the sampled species proves that there is genetic diversity between the samples collected from different areas of Western Himalaya.

Keywords: Genetic diversity of mtCOI *Bombus haemorrhoidalis*; Western Himalaya

Introduction

Wild pomegranate (*Punica granatum* L.) is one of the most important medicinal wild fruit crop and it is highly pollinated by wild insect pollinators viz., bees, bumblebees, beetles, flies etc. Bumblebees (Hymenoptera: Apidae) are pollinators of many wild as well as agricultural plants. They have high thermoregulatory behavior. They are the most reliable and effective pollinators due to their quick pollination, ability to burst the pollen sac with wing vibrations and capacity to forage in low ambient temperatures and light. Therefore, they act as important pollinators, primarily in alpine environments. Different species bumblebees like *Bombus terrestris*, *B. impatiens*, *B. occidentalis* and a number of other are used for commercial pollination of various crops within the world. These species are expensive to import and they compete for nesting locations, food and other resources with native pollinator species [1,2].

They are exceptionally fascinating social insects [3] but some species ranges and abundances have decreased as a result of local and global

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environmental changes, whereas those of other species have remained stable or even increased. Concerns have been raised for bumblebees, the plant species they pollinate, food security and ecosystem stability as a result of the decline in bumblebee abundance and distribution brought on by habitat loss, pathogen transmission, climate change and agrochemical exposure.

There are 250 species of bumblebees that are still alive today, which are divided into 15 subgenera [4-6]. In terms of shape, color patterning, food preference, disease incidence and life histories and ecologies, bumblebees exhibit significant interspecific variation [7-11].

There is still a lot to learn about bumblebees. For instance, little is known about the underlying genetic and chromosomal variety that results in these many phenotypes, particularly how differently they react to shifting circumstances. China is a hotspot of bumblebee species richness, home to around half of the 250 extant species, representing 14 of the 15 *Bombus* subgenera [4,6,12].

The amount of genetic diversity present within a species' populations is a critical component for species survival, as low levels of intraspecific genetic diversity will limit a species' capacity to adapt to present and future environmental changes [13-16].

However, the loss of allelic richness owing to drift can be made up for by high levels of gene flow and better dispersion abilities. Gene flow may be more restricted in smaller, less-connected populations, which reduces its buffering effect and raises the likelihood of brother-sister mating, which increases the likelihood of inbreeding and inbreeding depression [13-15]. The latter dynamics have the potential to further reduce genetic within small populations, potentially resulting in the extinction vortex, a vicious cycle that could eventually result in extinction [13-15].

Material and Methods

Genetic diversity of *Bombus haemorrhoidalis*, an insect pollinator on *Punica granatum* (wild pomegranate), based on mitochondrial COI gene were studied by collecting specimens from seven different areas of Western Himalaya and sequenced by Sanger sequencing. Gaps and mismatch were eliminated from the sequences and submit to NCBI genbank for accession numbers. All the seven sequences of *Bombus haemorrhoidalis* were accessed with accession numbers (Table 1, Figure 1). Alignment of DNA sequences were done by using multiple sequence alignment program CLUSTAL O. MEGA X was used for estimating evolutionary distances and phylogenetic analysis was constructed using Neighbor-Joining method [17]. Analyses were performed on 1000 bootstrapped data sets generated by the program [18]. This analysis involved 7 nucleotide sequences. All the data were calculated by MEGA X software. The nucleotide content of all the samples and the total C+G and A+T were calculated using MEGA software X. DNADIST with the Kimura two parameter distance option was used to estimate divergence between sequences with a transition/transversion ratio.

Results

Current study investigated the genetic diversity of *Bombus haemorrhoidalis* from seven localities of Western Himalaya by using NJ clustering based on COI gene. All the insect pollinators sampled specimens were collected on *Punica granatum* (wild pomegranate) and results are summarized as follow:

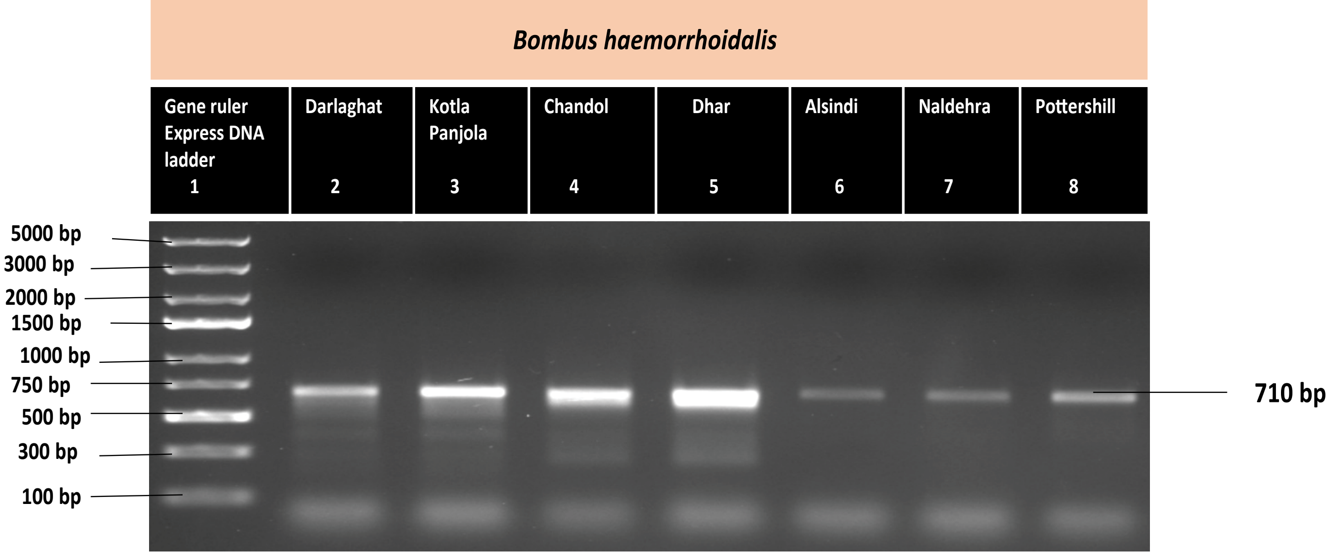

Figure 1: Analysis of amplified PCR product in 1.2% agarose: Lane 1: Gene ruler express DNA ladder, Lane 2, 3, 4, 5, 6, 7, 8: 710 bp size mtCOI gene.

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Multiple sequence alignment by CLUSTAL Omega

Multiple sequence alignment of seven COI sequences of *Bombus haemorrhoidalis* performed by CLUSTAL Omega revealed a total of 6 variable sites in the alignment between all the mtCO1 sequences of *Bombus haemorrhoidalis* (Figure 2).

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OL658828    --GCAATATGATCAGGAATAATTGGATCATCAATAAGGATTTTTAATTCGTATAGAATTA    58
OM432006    ----------------------TGGATCATCAATA-AGATTTTTAATTCGTATAGAATTA    37
OK483358    ------------CAGGAATAATTGGATCATCAATA-AGATTTTTAATTCGTATAGAATTA    47
OL658831    -------------AGGAATAATTGGATCATCAATA-AGATTTTTAATTCGTATAGAATTA    46
OL347869    ---------GATCAGGAATAATTGGATCATCAATA-AGATTTTTAATTCGTATAGAATTA    50
ON303732    ---------------------------------TA-AGATTTTTAATTCGTATAGAATTA    26
OL304916    TTGCAATATGATCAGGAATAATTGGATCATCAATA-AGATTTTTAATTCGTATAGAATTA    59
                                             **  ***********************

OL658828    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    118
OM432006    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    97
OK483358    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    107
OL658831    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTAACTAGACAT    106
OL347869    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    110
ON303732    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    86
OL304916    AGACATCCAGGAATATGAATTAATAATGATCAAATTTATAATTCTTTAGTTACTAGACAT    119
            ************************************************** *********

OL658828    GCATTTATAATAATTTTTTTTATAGTGATACCATTTATAATTGGAGGATTTGGCAATTAT    178
OM432006    GCATTTATAATAATTTTTTTTATAGTAATACCATTTATAATTGGAGGATTTGGCAATTAT    157
OK483358    GCATTTATAATAATTTTTTTTATAGTAATACCATTTATAATTGGAGGATTTGGCAATTAT    167
OL658831    GCATTTATAATAATTTTTTTTATAGTGATACCATTTATAATTGGAGGATTTGGCAATTAT    166
OL347869    GCATTTATAATAATTTTTTTTATAGTGATACCATTTATAATTGGAGGATTTGGCAATTAT    170
ON303732    GCATTTATAATAATTTTTTTTATAGTGATACCATTTATAATTGGAGGATTTGGCAATTAT    146
OL304916    GCATTTATAATAATTTTTTTTATAGTGATACCATTTATAATTGGAGGATTTGGCAATTAT    179
            ************************** *********************************

OL658828    CTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    238
OM432006    TTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    217
OK483358    TTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    227
OL658831    TTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    226
OL347869    CTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    230
ON303732    CTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    206
OL304916    CTAATTCCTTTAATATTAGGATCTCCAGATATAGCATTTCCACGAATAAATAATATTAGA    239
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OL658828    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAACAAATATATTTACTCCAAAT    298
OM432006    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAAGAAATATATTTACTCCAAAT    277
OK483358    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAAGAAATATATTTACTCCAAAT    287
OL658831    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAACAAATATATTTACTCCAAAT    286
OL347869    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAACAAATATATTTACTCCAAAT    290
ON303732    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAACAAATATATTTACTCCAAAT    266
OL304916    TTTTGATTACTTCCTCCTTCATTAATTATATTATTATTAACAAATATATTTACTCCAAAT    299
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OL658828      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      358
OM432006      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCACTCATCACCT      337
OK483358      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      347
OL658831      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      346
OL347869      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      350
ON303732      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      326
OL304916      GTTGGAACAGGATGAACTGTATATCCTCCATTATCTTCATATTTATATCATTCATCACCT      359
              ************************************************** *********

OL658828      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      418
OM432006      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      397
OK483358      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      407
OL658831      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      406
OL347869      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      410
ON303732      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      386
OL304916      TCAGTTGATATTGCAATTTTTTCTCTTCATATAGCTGGAATTTCATCTATTATTGGATCT      419
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OL658828      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      478
OM432006      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      457
OK483358      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      467
OL658831      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      466
OL347869      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      470
ON303732      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      446
OL304916      TTAAATTTCATTGTTACAATTATAATAATAAAAAATAATTCACTAAATTACGATCAAATT      479
              ************************************************************

OL658828      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      538
OM432006      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      517
OK483358      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      527
OL658831      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      526
OL347869      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      530
ON303732      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      506
OL304916      AATTTATTTTCATGATCAGTATCAATTACAGTAATATTATTAATTTTTTCATTACCTGTA      539
              ************************************************************

OL658828      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      598
OM432006      TTAGCTGGGGCTATCACTATATTAATCTTTGATCGAAATTTTAATACATCATTTTTTGAT      577
OK483358      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      587
OL658831      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      586
OL347869      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      590
ON303732      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      566
OL304916      TTAGCTGGAGCTATCACTATATTAATTTTTGATCGAAATTTTAATACATCATTTTTTGAT      599
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OL658828      CCAATAGGAGGAGGAGATCCTATT--------------------------                622
OM432006      CCAATAGGAGGAGGAGATCCTATTCTTTATCAACATTTA-----------                616
OK483358      CCAATAGGAGGAGGAGATCCTATTCTTTATCAACATTTATTTTG------                631
OL658831      CCAATAGGAGGAGGAGATCCTATTCTTTATCAACATTTATTTTT------                630
OL347869      CCAATAGGAGGAGGAGATCCTATTCTTTATCAACATTTATTT--------                632
ON303732      CCAATAGGAGGAG-------------------------------------                579
OL304916      CCAATAGGAGGAGGAGATCCTATTCTTTATCAACATTTATTTTGATTTTT                649
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Figure 2: CLUSTAL O (1.2.4) multiple sequence alignment of COI of *Bombus haemorrhoidalis* from different areas of Himachal Pradesh.

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Table 1: Places of sample collection of *Bombus haemorrhoidalis* with geographical location and the Genbank accession numbers of COI gene.

S. No.	Species name	Sample Location	Geographical Location			Genbank Accession No.
		Locality	Longitude	Latitude	Altitude	COI
1	*Bombus haemorrhoidalis*	Darlaghat	76°-56´50	31°-13´14	1563 m	OK483358
2	*Bombus haemorrhoidalis*	Kotla Panjola	77°-08´51	30°-51´09	1190 m	OL347869
3	*Bombus haemorrhoidalis*	Chandol	77°-34´37	30°-93´60	1418 m	OL658828
4	*Bombus haemorrhoidalis*	Alsindi	77°-12´31	31°-29´33	1132 m	ON303732
5	*Bombus haemorrhoidalis*	Dhar	76°-82´85	31°-62´42	1360 m	OL304916
6	*Bombus haemorrhoidalis*	Pottershill	77°-13´41	31°-12´13	2050 m	OM432006
7	*Bombus haemorrhoidalis*	Naldehra	77°-18´69	31°-18´39	1887 m	OL658831

Table 2: Frequency percentage (%) of transitions and transversions and transition/transversion ratio (Ts/Tv) of mtCOI gene of *Bombus haemorrhoidalis*.

	Transitions (%)				Transversions (%)								Ts/Tv ratio
COI Gene	G/A	C/T	T/C	A/G	A/T	A/C	T/A	T/G	C/A	C/G	G/T	G/C	1.67
	26.38	31.26	9.86	7.92	5.14	1.62	4.25	1.28	4.25	1.28	5.14	1.62	

Table 3: Mean frequencies (%) for base compositions at different codon positions for COI region of *Bombus haemorrhoidalis*.

Samples	First codon				Second codon				Third codon				Total	
Bombus haemorrhoidalis COI	A	C	G	T	A	C	G	T	A	C	G	T	C+G	A+T
OM432006, Pottershill	33.2	15.1	18.5	33.2	21	22.4	11.7	44.9	49	2.9	1	47.1	23.86	76.13
OL304916, Dhar	31.3	15.7	18.4	34.6	22.7	21.8	11.1	44.4	48.6	1.9	1.9	47.7	23.6	76.43
OL658831, Naldehra	32.4	14.8	18.6	34.3	21.9	22.4	11	44.8	49.5	1.9	1.4	47.1	23.36	76.66
ON303732, Alsindi	34.5	13.4	18.6	33.5	18.8	24	11.5	45.8	50.3	2.1	0.5	47.2	23.36	76.7
OL347869, Kotla Panjola	32.2	15.6	19	33.2	22.4	22.4	11	44.3	48.8	1.9	1.4	47.9	23.76	76.26
OL658828, Chandol	32.9	15	19.3	32.9	22.2	22.7	11.6	43.5	49	1.9	1.9	47.1	24.13	75.86
OK483358, Darlaghat	32.2	15.2	18.5	34.1	21.9	21.9	11.9	44.3	49.5	1.9	1	47.6	23.46	76.53
Average	32.6	15	18.7	33.7	21.6	22.5	11.4	44.6	49.2	2.1	1.3	47.4	23.66	76.34

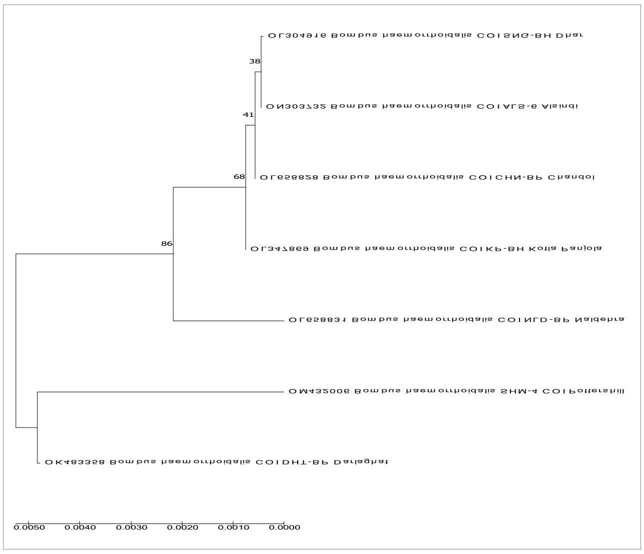

Figure 3: Phylogenetic tree of Bombus haemorrhoidalis showing genetic relationships derived from CO1 sequences by using Neighbor-Joining (NJ) method of MEGA X Software.

Nucleotide content analysis of COI gene: According to the neutral theory, nucleotide polymorphism levels correlate to evolutionary rate, and the transition and transversion ratio within populations should be related to long-term evolutionary rate.

Comparative significance of Transitions and Transversions: The frequencies of transitions and transversions are mentioned in Table 2. The estimated transition/transversion (Ts/Tv) bias of COI (R) is 1.67. The percentage of sites showing transitions (75.42%) is higher than the number of sites showing transversions (24.58%). The nucleotide frequencies are 34.58% (A), 41.84% (T/U), 13.19% (C) and 10.39% (G).

Base composition at each Codon positions

In the present study, the nucleotide content (A,T,G,C) and the total C+G and A+T at first, second and third codon position of all the samples revealed the high numbers of polymorphic sites in the COI gene, evenly distributed among the 3 codon positions. Average A+T percentage (76.34%) were higher than C+T (23.66%) (Table 3). The variation in nucleotide content almost completely exist in the third codon position due to AT rich region, as compared to the first and second position of codons. The A+T bias was pronounced in general for this region for all codon positions.

The transition/transversion (Ts/Tv) ratio is significant in deducing the magnitude and direction of natural selection. The transitions (75.42%) and transversions (24.58%) depicts ratio of 3:1 (Table 2) and transition/transversion (Ts/Tv) bias of COI (R) is 1.67. The ratio greater than one indicates the positive or Darwinian selection and less than 1 implies purifying selection and neutral selection indicated by ratio equal to one. The current transitions/transversions value of 1.67 signifies the presence of positive genetic divergence in *Bombus haemorrhoidalis* of Western Himalaya. The current results suggested divergence between the *Bombus haemorrhoidalis* of Western Himalaya.

Phylogenetic analysis of mitochondrial CO1 of *Bombus haemorrhoidalis* samples

The phylogenetic relationship among *Bombus haemorrhoidalis* of the present study obtained through Neighbor-Joining (NJ), were shown in Figure 3. Phylogenetic relationship showed that among the seven samples sequences obtained from the present study area, the sample of Darlaghat and Potterhill were found to be closely related to each other. Sample of Alsindi and Dhar show homology with each other and close to the sample of Kotla Panjola and Chandol. Naldehra sample found to be distinct from other samples. Distance matrix clearly signifies the vast difference among the sampled species proves that there is genetic diversity between them (Figure 3).

Discussion

Form this study, we were able to estimate genetic diversity and to compare this with each other species collected from different areas on *Punica granatum* (wild pomegranate) in the Western Himalaya. This approach allowed us to conclude that phylogenetic relationship among the seven samples sequences obtained from the present study areas, the sample of Darlaghat and Potterhill were found to be closely related to each other. Sample of Alsindi and Dhar show homology with each other and close to the sample of Kotla Panjola and Chandol. Naldehra sample found to be distinct from other samples. Distance matrix clearly signifies the vast difference among the sampled species proves that there is genetic diversity between them.

According to numerous research [19-23], bumblebee species with declining population typically having lower genetic diversity level than stable bumblebee species. This phenomenon clearly indicate the decrease in genetic variety through time [21,22] as a result of one or more potential drivers of bee decline such as intensifying agriculture, emerging diseases and changing climates that result in smaller population sizes [24-27].

Studies using historical populations, however, discovered a comparable difference in genetic variation between declining and stable bumblebee species [28-31]. These studies also detected no significant decline in genetic diversity over a century in Belgium, possibly because dispersal counteracted drift effects. However, studies using historical populations found a similar difference in genetic variation between declining and stable bumblebee species [28-31], detecting no major drop in genetic diversity over one century in Belgium, possibly due to dispersal countering drift effects [31].

The latter finding suggests that there is no correlation between species abundance and genetic diversity, at least among populations in Belgium. This runs counter to the generally accepted idea that suggests that when a species is locally plentiful, its huge population should exhibit greater genetic diversity than that seen in areas where the species is less common [13,14,32]. This may not always be the case, though, for social insects like bumblebees [33].

As a result, our findings showed that determining a population's health state locally, particularly over a longer period of time, cannot be done just by counting the number of bumblebees present. Additionally, because bumblebee species with lower genetic diversity are less fit [23,34,35]. It appears that evaluating genetic diversity factors can provide an accurate estimate of how vulnerable a particular population or bumblebee species is to future population decrease. Genetic diversity has been proved to be important for the fitness of species because harmful mutations can be counterbalanced by high levels of heterozygozity [36-39]. Our study suggested that mitochondrial COI genes are well suited for determining genetic difference within species and there is need of extensive sampling and further characterization of genetic diversity with different mitochondrial genes to lessen the risk of extinction of species and ecosystems.

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