Int.J.Curr.Microbiol.App.Sci (2020) *9*(5): 2010-2016

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 9 Number 5 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.905.227

Molecular Characterization of Bumble Bees Species from North East Himalayas

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Keywords

Bumble bee, Molecular characterization, North east Himalayas, Dendrogram

Article Info

Accepted:
15 April 2020
Available Online:
10 May 2020

A B S T R A C T

Molecular characterization of bumble bees has been carried out in the Department of Entomology, Assam Agricultural University during the period 2015-2018. During the investigation, 5 (Five) species of bumble bees *viz. Bombus orientalis* Smith, *B. buccinatoris* Smith, *B. tunicatus* Smith, *B. haemorrhoidalis* Smith and *B. miniatus* Bingham have been recorded. Similarity matrix for Jaccard's Coefficient based on SSR banding of 5 bumble bee species ranged from 0.062 to 0.921 *i.e.* 6.2% to 92.1%. The dendrogram readily separated the bumble bee species into two main clusters (A and B). The cluster A includes *Bombus orientalis* and *B. buccinatori* while cluster B includes *B. tunicatus, B. haemorrhoidalis* and *B. miniatus.*

Introduction

Bumble bees (genus *Bombus*) are large, colorful, ubiquitous pollinators found throughout the holarctic, oriental, and neotropical regions of the world, especially in alpine and arctic zones. The genus *Bombus*, comprises over two hundred and fifty (250) known species of bumblebees present on global basis (Williams *et al.*, 2008). Bingham (1897) included 24 species of *Bombus* in Fauna of British India that included records from India and the neighboring countries; such as Myanmar, Bhutan, Nepal, Sri Lanka etc. The generic name *Bombus*, assigned by Latreille in 1802, is derived from the Latin word for a buzzing or humming sound. Bumblebees rank among the most abundant

and conspicuous of flower visitors in alpine, temperate and arctic environment of the northern hemisphere. They are called primitively eusocial and are associated with the high lands and play a key role in the functioning of agricultural ecosystems as pollinators of crops, orchards and wild flowers.

Thus, they serve as important pollinators, especially in alpine environments and early in the flowering season (Kevan and Baker, 1983; Yu *et al.,* 2012). Bumble bees (*Bombus* spp.) can be used in greenhouses for pollination services, because they are very efficient pollinators that can be handled with great ease. Bumble bees exceed other pollinator species in pollination efficiency, due to their behavioural particularity of "buzz pollination" (Buchmann, 1985).

In molecular study, the relationships among organisms or genes are studied by comparing homologues of DNA or protein sequences. Dissimilarities among the sequences indicate genetic divergence as a result of molecular evolution during the course of time. . By comparing homologous molecules from different organisms it is possible to establish their degree of similarity thereby establishing or revealing a hierarchy of relationship a phylogenetic tree. Both the classical morphology based methods and molecular analysis based methods are of importance as the basic bio-molecular framework of all organisms are similar and morphology of an organism is actually the manifestations of its genome, proteome and transcriptome profiles.

Materials and Methods

Sample collection

North east Himalaya is located at 25.5736^{o} N latitude and 93.2473^{o} E longitude in Indian sub-continent. Extensive exploration for bumble bees have been carried out in 5 (five) physiographic zones of north east Himalaya *viz*. Arunachal Himalaya, Barak valley, Brahmaputra valley, Meghalaya Plateau and South eastern hill tract during 2014-18.

Each physiographic zone had been sub-divided into different locations based on Global Positioning System (Table 1). Bee samples were collected based on random sampling methods, covering different forest and agro-ecosystems. The samples were preserved in 75% ethanol to prevent any kind of deformation.

Molecular analysis

DNA extraction was done from the thorax of the bee. The homogenous mix was prepared after grinding the thorax with CTAB buffer and 25 µl of 10 g/ml proteinase K were added.

The homogenous mix had been centrifuged at 14,000 rpm for 15 minutes at 4°C and then 500µl of upper phase transferred to another sterile tube, mixed with equal volume of chloroform: Isoamyl alcohol (24°.1). The mix was stored at -20°C for overnight and had been centrifuged at 14,000 rpm for 15 minutes.

Isolated DNA was quantified by using Nanodrop 200UV- visible spectrophotometer and electrophosed on 0.85 agarose gel. The DNA amplification was achieved by use of synthetic oligonucleotides termed primers that are forward and reverse primer (Table 2).

DNA polymerase was then used to carry out the synthesis of a complementary strand of DNA in the 5´ to 3´ direction of sense and antisense strands using the oligonucleotides primer. PCR reactions require typically three phases: firstly the DNA template has to be denatured (92-94°C); secondly the primers have to be annealed (40-65°C) and thirdly

DNA polymerase extends the annealed primers along the single-stranded template (72°C). The molecular weight of PCR products, obtained for each marker was designated, based on a ladder of known molecular weight. Data was scored on the basis of presence or absence of the amplified particular DNA fragment products.

Statistical analysis

An agglomerative method of clustering genotype was employed utilizing the Unweighted Pair Group Method with Arithmetic averages (UPGMA). The relationship between species was presented graphically in the form of dendrogram and matrix.

Results and Discussion

Molecular characterization of bumble bees had been carried out to study the variations at molecular level as well as to obtain molecular markers that can distinguish the species from one another. In the present study, 20 SSR markers were used of which 14 showed banding patters (Table 3). Similarity matrix for Jaccard's Coefficient based on SSR banding of 5 bumble bee species ranged from 0.062 to 0.921 *i.e.* 6.2% to 92.1%. The lowest similarity value was found between *Bombus haemorrhoidalis* from Maibang and *B. orientalis* from Pasighat (6.2%) and the highest similarity value was found *B. orientalis* from Basar and Pasighat (92.1%).

Table.1 Physiographic zones of North East India showing locations

Physiographic Zone	Location	Latitude	Longitude	Elevation
Arunachal Himalaya	Pasighat	28.07°N	95.33°E	155 m
	Roing	28.14° N	95.84° E	390 m
	Basar	27.98° N	94.66° E	578 m
	Itanagar	27.1°N	93.62°E	750 m
	Hawai	27°53 N	96°48 E	1296 m
Barak valley	Agartala	23.83° N	91.26° E	12 m
	Karimganj	24.86° N	92.36° E	13m
	Udaipur	23.53°N	91.48°E	22 m
	Kailashahar	24.33° N	92.02° E	24 m
	Cachar	24.78° N	92.86° E	25m
Brahmaputra Valley	Jorhat	26.75°N	94.20° E	93m
	North Lakhimpur	27.24°N	94.11°E	96m
	Golaghat	26.59°N	93.75° E	98m
	Nagaon	26.57°N	93.00°E	70m
	Dibrugarh	27.47°N	94.92°E	110m
Meghalaya plateau	Umragnso	25.51°N	92.73° E	640m
	Umsning	25.75°N	91.89° E	782m
	Umiam	25.67°N	91.89° E	946m
	Sohra	25.28°N	91.73° E	1484m
	Sanmer	25.55N	91.84° E	1726m
South Eastern Hill Tract	Maibang	25.30° N	93.13° E	355m
	Medziphema	25.76°N	93.87° E	456m
	Imphal	24.81° N	93.90° E	786 m
	Haflong	25.16°N	93.01° E	966m
	Kohima	25.66°N	94.11° E	1445m

Table.2 Primer sequences used for PCR amplification in bumble bees

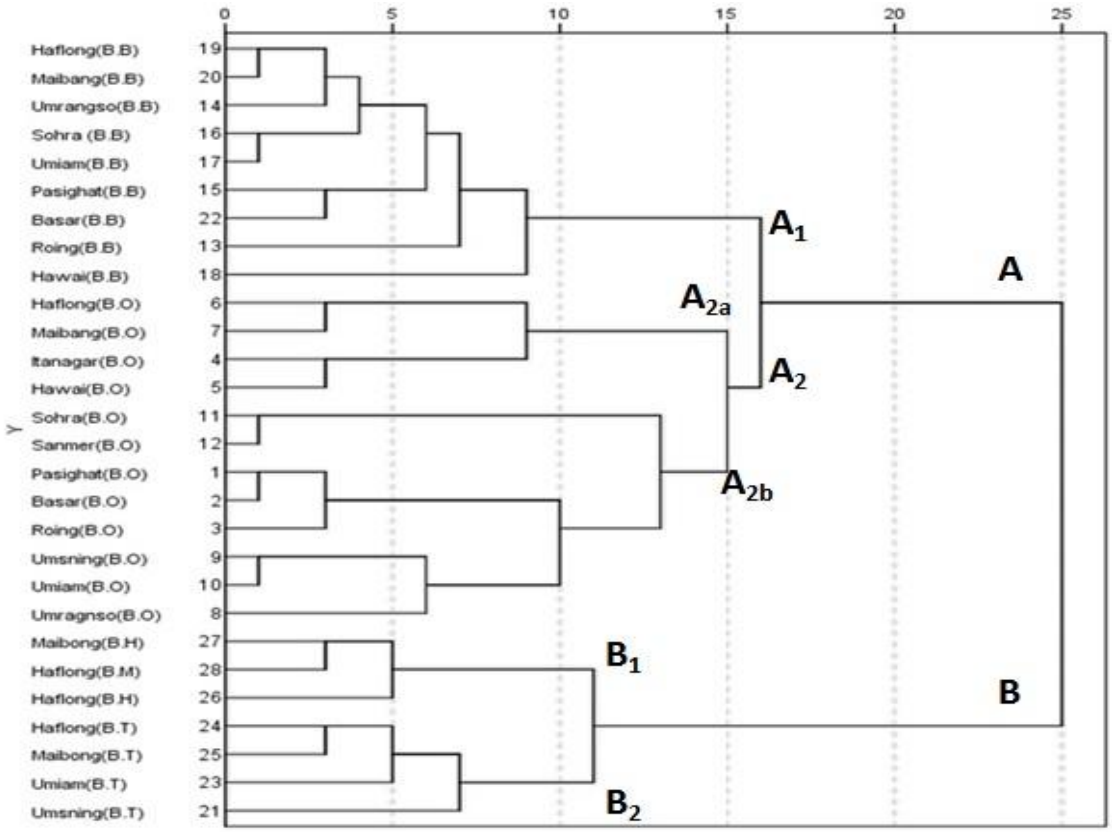
Locus	Position	Primers (5′ – 3′)
BT01	F	CCGATCTGTGAGAATGACAGTATCG
	R	CGTGTTTCGATTAGCAAAGCTACG
BT02	F	AGGAACCGAGCGATAGAACCAC
	R	GCTTTGCCTTTCCATCTTGCTG
BT04	F	GAGAGAGATCGAATGGTGAGAGC
	R	TGAGCACGTTCTTTCGTTCAC
BT05	F	TTTCCTATGCCGAACGTCACC
	R	CCCAGATAAAAGACCGCCTCTAGTC
BT08	F	AGAACCTCCGTATCCCTTCG
	R	AGCCTACCCAGTGCTGAAAC
BT10	F	TCTTGCTATCCACCACCCGC
	R	GGACAGAAGCATAGACGCACCG
BT16	F	CAGCCAAAAAATCAGTGGAGTGC
	R	TCTTCCTCTGTTTCTCGTTCACG
BT17	F	GCGGATGCACGATATAAAATG
	R	TCTTTCTCTCCTTCTTCCCATC
BT20	F	TTCCACAGCGTTTTCTTAAGTC
	R	ATGGACGGCGAGATCGTGAG
BT23	F	GCAACAGAAAATCGTCGGTAGTG
	R	GCGGCAATAAAGCAATCGG
BT24	F	TCTTTCCGTTTTCCCCCTG
	R	CACCCACTTACATACATACACGCTC
BT28	F	TTGCTGACGTTGCTGTGACTGAGG
	R	TCCTCTGTGTGTTCTCTTACTTGGC
BL01	F	GCGTCGAGAAACTATCTAGGAGAG
	R	CGAAGATTCCCAAAACTGCG
BL02	F	GAACAGTGAGAGCGAGGAACAGAG
	R	TTGCCACGTATATCCGAGCGAACC
BL05	F	CGAAAATCAGGGGTGACAAAC
	R	CCTTTCTGTTTATAGTTCGTCCG
BL08	F	ATGTTGCAGCACCTTCGTGG
	R	AATTAAAGGCGTGCGCTCGC
BL11	F	AAGGGTACGAAATGCGCGAG
	R	TGACGAGTGCGGCCTTTTTC
BL13	F	CGAATGTTGGGATTTTCGTG
	R	GCGAGTACGTGTACGTGTTCTATG
BTERN01	F	CGTGTTTAGGGTACTGGTGGTC
	R	GGAGCAAGAGGGCTAGACAAAAG
BTERN02	F	TTTCCACCCTTCACGCATACAC
	R	GATTTTATCCTCCGACCGTTCC

Table.3 Similarity matrix of Jaccard's coefficient of dwarf bees from different physiographic zones

	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
1	1.000																											
2	0.921	1.000																										
3	0.866	0.866	1.000																									
4	0.429	0.429	0.577	1.000																								
5	0.577	0.577	0.708	0.866	1.000																							
6	0.143	0.143	0.289	0.714	0.577	1.000																						
7	0.289	0.289	0.167	0.577	0.458	0.866	1.000																					
8	0.577	0.577	0.458	0.289	0.458	0.289	0.417	1.000																				
9	0.577	0.577	0.458	0.234	0.167	0.615	0.125	0.708	1.000																			
10	0.577	0.577	0.458	0.282	0.167	0.615	0.125	0.708	0.781	1.000																		
11	0.289	0.289	0.458	0.289	0.458	0.602	0.167	0.125	0.417	0.417	1.000																	
12	0.289	0.289	0.458	0.289	0.458	0.602	0.167	0.125	0.417	0.417	0.874	1.000																
13	0.174	0.174	0.101	0.174	0.101	0.174	0.251	0.251	0.251	0.251	0.251	0.251	1.000															
14	0.167	0.522	0.091	0.090	0.091	0.316	0.411	0.291	0.391	0.791	0.391	0.234	0.826	1.000														
15	0.149	0.149	0.343	0.149	0.343	0.149	0.258	0.258	0.258	0.258	0.258	0.258	0.701	0.849	1.000													
16	0.289	0.289	0.167	0.289	0.167	0.289	0.417	0.125	0.125	0.125	0.125	0.125	0.603	0.730	0.559	1.000												
17	0.289	0.289	0.167	0.289	0.167	0.289	0.417	0.125	0.125	0.125	0.125	0.125	0.603	0.730	0.559	0.678	1.000											
18	0.577	0.577	0.417	0.289	0.417	0.289	0.458	0.167	0.167	0.167	0.167	0.167	0.452	0.548	0.645	0.750	0.750	1.000										
19	0.149	0.149	0.430	0.149	0.430	0.447	0.559	0.258	0.258	0.258	0.643	0.493	0.701	0.849	0.689	0.861	0.861	0.645	1.000									
20	0.149	0.149	0.430	0.149	0.043	0.447	0.559	0.258	0.258	0.258	0.143	0.438	0.701	0.849	0.689	0.861	0.861	0.645	0.764	1.000								
21	0.814	0.450	0.125	0.289	0.125	0.190	0.125	0.167	0.125	0.125	0.125	0.125	0.251	0.411	0.559	0.125	0.125	0.458	0.258	0.258	1.000							
22	0.289	0.289	0.167	0.190	0.167	0.289	0.417	0.417	0.417	0.417	0.125	0.125	0.603	0.730	0.861	0.708	0.708	0.750	0.861	0.861	0.417	1.000						
23	0.410	0.520	0.091	0.316	0.091	0.097	0.391	0.491	0.291	0.191	0.491	0.291	0.440	0.650	0.849	0.411	0.411	0.548	0.519	0.519	0.730	0.730	1.000					
24	0.749	0.149	0.143	0.149	0.343	0.149	0.258	0.258	0.258	0.258	0.443	0.340	0.337	0.519	0.689	0.559	0.559	0.645	0.689	0.689	0.559	0.861	0.849	1.000				
25	0.130	0.312	0.125	0.289	0.125	0.560	0.125	0.125	0.417	0.417	0.125	0.125	0.251	0.411	0.559	0.417	0.417	0.458	0.559	0.559	0.708	0.708	0.730	0.861	1.000			
26	0.316	0.316	0.228	0.510	0.228	0.316	0.411	0.411	0.411	0.411	0.411	0.411	0.440	0.650	0.849	0.411	0.411	0.548	0.519	0.519	0.411	0.730	0.650	0.519	0.411	1.000		
27	0.062	0.630	0.167	0.620	0.167	0.289	0.125	0.125	0.417	0.417	0.708	0.708	0.251	0.411	0.559	0.125	0.125	0.167	0.258	0.258	0.417	0.417	0.411	0.258	0.417	0.730	1.000	
28	0.149	0.149	0.344	0.149	0.344	0.447	0.258	0.258	0.258	0.258	0.559	0.559	0.337	0.519	0.689	0.258	0.258	0.344	0.378	0.378	0.258	0.559	0.519	0.378	0.258	0.849	0.861	1.000

(BO=*Bombus orientalis;* BB=*B. buccinatoris;* BT=*B. tunicatus;* BH=*B. haemorrhoidalis* and BB=*B. miniatus*)

1:Pasighat(B.O)	5:Hawai(B.O)	9:Umsning(B.O)	13:Roing(B.B)	17:Umiam(B.B)	21:Umsning(B.T)	25:Maibong(B.T)
2:Basar(B.O)	6:Haflong(B.O)	10:Umiam(B.O)	14:Umrangso(B.B)	18:Hawai(B.B)	22:Basar(B.B)	26:Haflong(B.H)
3:Roing(B.O)	7:Maibang(B.O)	11:Sohra(B.O)	15:Pasighat(B.B)	19:Haflong(B.B)	23:Umiam(B.T)	27:Maibong(B.H)
4:Itanagar(B.O)	8:Umragnso(B.O)	12:Sanmer(B.O)	16:Sohra (B.B)	20:Maibang(B.B)	24:Haflong(B.T)	28:Haflong(B.M)



(BO=*Bombus orientalis;* BB=*B. buccinatoris;* BT=*B. tunicatus;* BH=*B. haemorrhoidalis* and BB=*B. miniatus*)

Fig.1 Dendrogram showing linkage based on DNA fragment amplified by SSR markers across bumble bee population from different physiographic zones of north east Himalaya

Similarity matrix for Jaccard's Coefficient based on SSR banding of 5 bumble bee species ranged from 0.062 to 0.921 *i.e.* 6.2% to 92.1% (Table 3). The lowest similarity value was found between *Bombus haemorrhoidalis* from Maibang and *B. orientalis* from Pasighat (6.2%) and the highest similarity value was found *B. orientalis* from Basar and Pasighat (92.1 Estoup *et. al*. (1996) have conducted the studies on *Bombus terrestris* by using microsatellites and found that there is a high level of intrapopulational polymorphism with tested microsatellite. The dendrogram readily separated the bumble bee species into two main clusters (A and B) (Fig. 1). The cluster A includes *Bombus orientalis* and *B. buccinatori* while cluster B includes *B. tunicatus, B. haemorrhoidalis* and *B. miniatus*. The Cluster A consists of two sub-clusters A_1 and A_2. The sub-cluster A_1 includes *B. buccinatori* from Haflong, Maibang, Umrangso, Sohra, Umiam, Pasighat, Basar, Roing and Hawai. The sub-cluster A_2 is consisting of two sub clusters A_{2a} and A_{2b}.

The sub cluster A_{2a} includes *B. orientalis* from Haflong, Maibang, Itanagar and Hawai while the sub cluster A_{2b} again divided into two groups. The first group includes *B. orientalis* from Sohra and Sanmer while second group includes Pasighat, Basar, Roing, Umsning, Umiam and Umrangso. The cluster B consist of two sub-clusters B_1 and B_2. The sub cluster B_1 *B. haemorrhoidalis* from Haflong and Maibang and *B. miniatus* from Haflong while sub cluster B_2 includes *B. tunicatus* from Haflong, Maibang, Umiam and Umsning. Funk *et al.*, (2006) report the

details and characteristics of a total 44 novel microsatellite loci for *Bombus* spp. Most of them were highly polymorphic to *B. terrestris* and a high degree of polymorphism was also found where these primers have been tested in 10 other bumble bee species. The results obtained from this study suggest that there is considerable level of genetic diversity among bumble bee species from north east Himalaya.

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How to cite this article:

Ritu Ranjan Taye, Ataur Rahman, Priyabrata Sen, Shimantini Borkataki and Manha Bathari. 2020. Molecular Characterization of Bumble Bees Species from North East Himalayas. *Int.J.Curr.Microbiol.App.Sci.* 9(05): 2010-2016. doi: https://doi.org/10.20546/ijcmas.2020.905.227