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Molecular characterization of dwarf honey bees, *Apis florea* f. and *Apis andreniformis* smith from north east Himalaya

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Abstract
Exploration and molecular characterization of dwarf honey bees from five physiographic zones of north east Himalaya has been carried out in the Department of Entomology, Assam Agricultural University during the period 2014-2018. Two species of dwarf honey bees *viz.*, *Apis florea* F. and *A. andreniformis* Smith have been identified during the investigation. The cluster analysis for genetic diversity of dwarf honey bees revealed that the lowest similarity value was found between *Apis anderniformis* from Nagaon and *A. florea* from Pasighat (5.4%) and the highest similarity value was found between *A. florea* from Basar and Pasighat (82.0%). The dendrogram constructed for dwarf honey bees based on data obtained from the DNA fragment amplified by cluster analysis separated the *Apis florea* and *A. anderniformis* into two main clusters (A and B). Based on molecular characterization it had been found that there are two distinct morphoclusters within the physiographic zones forming hill races and plains races which could be concluded with maximum genetic similarity.

Keywords: Molecular characterization, dwarf honey bees, north east Himalaya, cluster analysis, cluster analysis

Introduction
Bees are one of the most wonderful organisms on earth. They are considered as effective ecosystem service provider and integral part of terrestrial ecosystem. Almost all the angiosperms require bees to pollinate thereby enhance crop productivity. There are almost 17,000 bee species which have been recognized and these species have been placed in 425 genera, 22 subfamilies, 6 families [1]. On the basis of morphological characters, Ruttner classified honey bees into 4 species; the European honeybees, *Apis mellif* era L., 1758; the eastern honey bees, *A. cerana* F., 1798; the giant honey bee, *A. dorsata* F., 1793; and the dwarf honey bee, *A. florea* F., 1787 [2]. The dwarf honey bee, *Apis florea* was first identified by Danish zoologist John Christian Fabricius in 1787. *Apis andreniformis*, black dwarf honey bee is most closely related to *Apis florea* with which it is commonly seen in sympatric distribution throughout southeast Asia [3]. Together they comprise the subgenus *Micrapis*, and are the most primitive of the living species of *Apis*. The workers of *Apis florea* are generally reddish-brown (rufous) in appearance while *A. andreniformis* is generally darker and the first abdomen segment is completely black [4]. The bees plays a major role in the field of agriculture (pollination) and medicine (production of important substances like: honey, royal jelly, bee wax, propolis, pollen and bee venom). North eastern Himalaya is highly diversified ecosystems inhabited by a variety of bee species. The varied ecological conditions with diversified flora have provided favorable habitat for this bee species. They share common foraging niche in human inhabited ecosystem, agricultural lands and shrubby vegetation. So, there is a vast scope for expansion of bee keeping. The area under protected cultivation of horticultural crops is gradually expanding, particularly in response to their export potential, for which new technological innovations would be required. However, crops grown under such conditions have specific requirements, particularly in terms of pollination. Therefore, the role of specific pollinators has become more important these days.

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

Extensive exploration for dwarf honey bees species 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. Each physiographic zone had been sub-divided into different locations based on Global Positioning System (Table 1). Bee samples were collected from plants, while foraging for nectar or pollen, with the help of sweeping hand net made up of nylon cloth and some samples were collected from the bee colony. The collection was based mainly on random sampling methods, covering different forest and agro-ecosystems. The samples were preserved in 75% ethanol to prevent any kind of deformation.

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: Isoamylalcohol (24°.1). The mix was stored at -20°C for overnight and had been centrifuged at 14,000 rpm for 15 minutes. Supernatant was discarded gently without dropping the pellet. 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 is 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. The power of this technique is that for each heating and cooling cycle, the amount of DNA is doubled, such that after only 20 cycles the yield of PCR product is approximately one million copies from each starting target DNA molecule. The basic reaction is set up in a micro centrifuge tube and placed in a thermo cycler machine which is programmable to carry out the phases of the Polymeric chain reaction (PCR) cycle automatically. 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. The molecular weight of each fragment was designated by a subscript to the name of the primer. Data was scored on the basis of presence or absence of the amplified particular DNA fragment products. During the band scoring, only intense bands were scored and faint bands and bands with smeared background were avoided. If a product was present in a certain genotype, it was designated as '1' and if absent; it was designated as '0'. Only the specific PCR products showing consistency in the successive amplifications were selected to minimize the possibility of mis-scoring markers. 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.

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

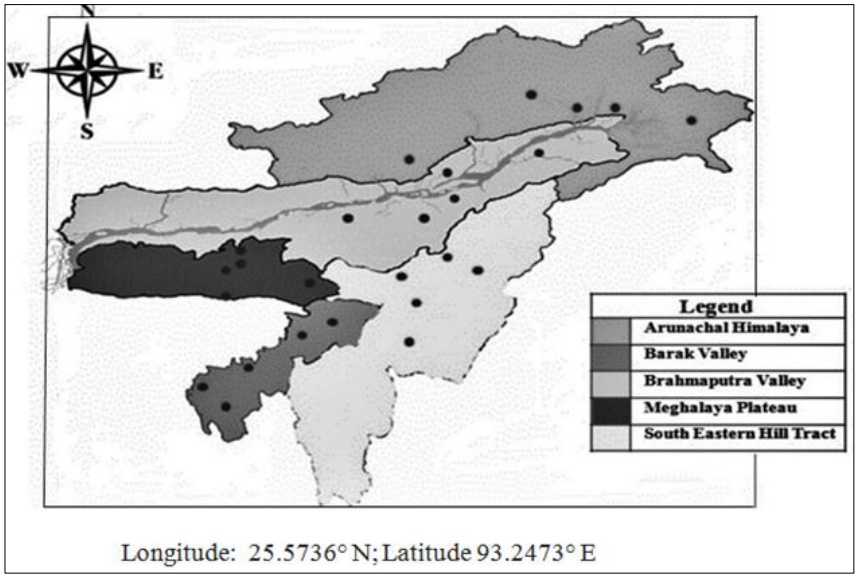


Fig 1: Map showing different Physiographic zones of North east India.

Table 2: Primer sequences used for PCR amplification in dwarf honey bees.

SSR Marker	Position	Primers (5'-3')
UN198	F	AAAGAATGTCGCGATCGATG
	R	GAAGCTCGGATCGGATCG
AT113	F	GACCGAGACGAGCCAGTTTG
	R	GCACCACGAGGTCTTCCCGT
K0412	F	ACGTTCCCTTTACGGACCTC
	R	AAGCCACGGTTGATACCGA
BI023	F	GGAGGGGACGACATTCCC
	R	TCCCTTCGCGAGACGACA
K1420B	F	CCTGGATAGAAGTTGGTTCCG
	R	TTACGCAGACTATGCACGCA
UN10BT	F	TCGATCCTACCTTCATAAGAAACG
	R	CGAGGAGAAGTCGTTGCAGA
SV257	F	ATGAGCATAACGGAACACGC
	R	GCGTATATCTGTGAATGTGCCTC
BI018	F	AGTCGACGAAATTTCTTGCTTC
	R	CACGAGAGTCGACGAGTAGCC
UN121	F	GACGTAAGCGTATATACCTGGCA
	R	CATGCGCTTAAGTACATGCATAC
K1601	F	TCGATCCATCGGTCGTCC
	R	GTGCACCCACGTTACGGTAC

Results and discussions

During the present investigation, 2(two) species of dwarf honey bees *viz., Apis florea* F. and *A. andreniformis* Smith had been identified. *Apis florea* had been recorded form all the 25 locations of five zones while *A. andreniformis*, a sister species of *A. florea* had been recorded only from five locations *viz*. Jorhat, Golaghat, Nagaon, Silchar and Karimganj. Molecular characterization of *Apis florea* and *A. anderniformis* were 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, total 10 SSR markers were used which are presented in Table 2. Similarity matrix for Jaccard's Coefficient based on SSR banding of *Apis florea* from 25 locations and *A. anderniformis* from 6 locations is presented in Table 3. The similarity value ranged from 0.054 to 0.820 *i.e.* 5.4% to 82.0%. The lowest similarity value was found between *A. anderniformis* from Nagaon *A. florea* from Pasighat (5.4%) and the highest similarity value was found between *A. florea* from Basar and Pasighat (82.0%). Yogesh and Khan stated that the genetic similarities matrix for *A. mellifera* ranged from 37.5% to 86.1% [5]. The highest genetic similarity was found to be 86.10% whereas lowest was found to be 37.50%. Smith studied the mitochondrial genome of *A. florea* collected from India and northern Thailand and *A. anderniformis* collected from peninsular Malaysia, Borneo and found that they were genetically distinct showing 5-7% sequence divergence [6]. Rattanawannee *et al.* conducted a phylogeny and genetic variation study using 32 Thailand and 5 Malaysian colonies of *A. anderniformis*. They analysed the cytochrome oxidase sub unit b [7].

Cluster analysis

The dendrogram had been constructed for dwarf honey bee population based on data obtained from the DNA fragment amplified by SSR markers (Fig. 2). The dendrogram readily separated the *Apis florea* and *A. anderniformis* into two main clusters (A and B). Cluster A consist of two sub-clusters A_1 and A_2. The sub-cluster A_1 included *A. anderniformis* from Silchar, Karimgang and Umiam while sub-cluster A_2 included *A. anderniformis* from Jorhat, Golaghat and Nagaon. The cluster B consist of two sub-clusters B_1 and B_2. Sub-clusters B_1 comprised with two sub clusters B_{1a} and B_{1b}. The sub cluster B_{1a} included *A. florea* from Sohra, Sanmer, Haflong,

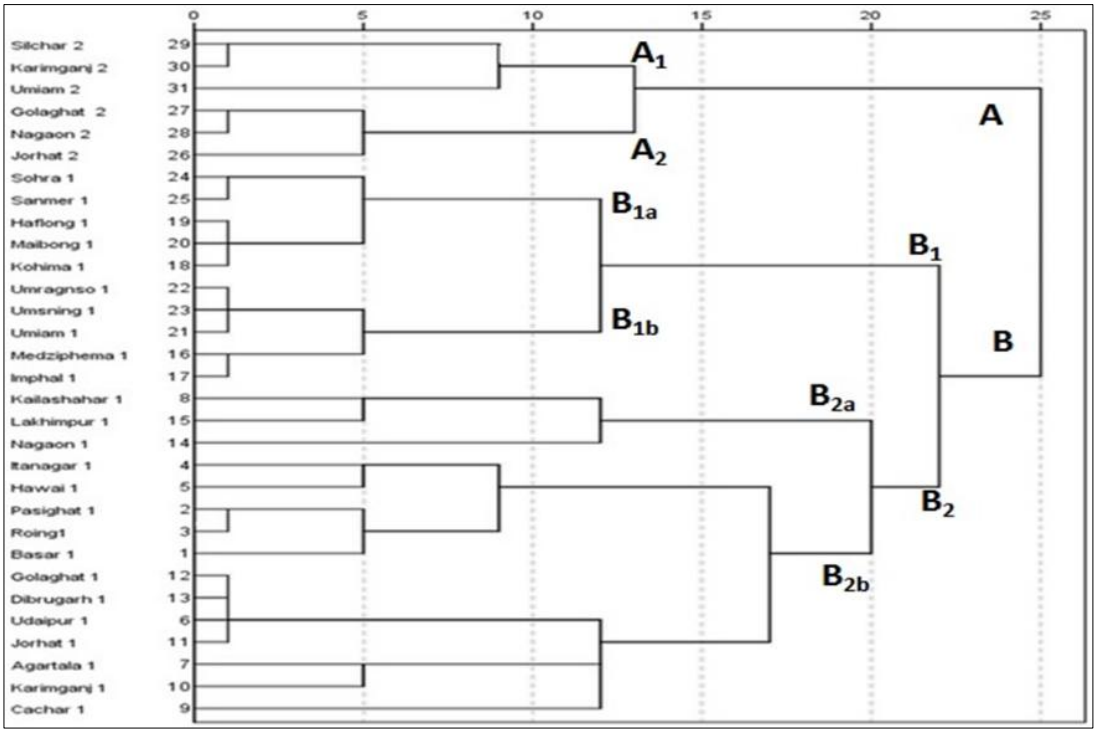
Maibang and Kohima. The sub cluster B_{1b} includes geographic races from Umrangso, Umsning, Umiam, Medziphema and Imphal. The sub cluster B_2 consisted of two sub-clusters B_{2a} and B_{2b}. The sub-cluster B_{2a} includes *A. florea* from Kailashahar, Lakhimpur and Nagaon while sub-cluster B_{2b} is again divided into two groups. The first group consisted of *A. florea* from Itanagar, Hawai, Pasighat, Roing and Basar anf the secong group included Golaghat, Dibrugarh, Udaipur, Jorhat, Agartala, Karimganj and Cachar. The genetic differentiation test of honey bee populations from Balearic Islands clustered into two groups: Gimnesias (Mallorca and Menorca) and Pitiusas (Ibiza and Formentera), which agrees with the biogeography of the study site [8]. Similar results reported when studying genetic variation between mid-hill and plain region of India [5].

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	29	30	31
1	1.000																														
2	0.820	1.000																													
3	0.812	0.808	1.000																												
4	0.612	0.764	0.764	1.000																											
5	0.356	0.524	0.524	0.764	1.000																										
6	0.102	0.327	0.327	0.250	0.218	1.000																									
7	0.802	0.524	0.524	0.218	0.480	0.218	1.000																								
8	0.120	0.218	0.218	0.140	0.218	0.500	0.218	1.000																							
9	0.408	0.218	0.218	0.123	0.218	0.500	0.655	0.200	1.000																						
10	0.612	0.218	0.218	0.250	0.327	0.375	0.764	0.134	0.500	1.000																					
11	0.102	0.327	0.327	0.250	0.218	0.567	0.218	0.500	0.500	0.375	1.000																				
12	0.102	0.327	0.327	0.250	0.218	0.569	0.218	0.500	0.500	0.375	0.789	1.000																			
13	0.102	0.327	0.327	0.250	0.218	0.784	0.218	0.500	0.500	0.375	0.345	0.670	1.000																		
14	0.250	0.089	0.089	0.102	0.356	0.102	0.535	0.408	0.134	0.408	0.102	0.102	0.102	1.000																	
15	0.167	0.089	0.089	0.102	0.356	0.612	0.089	0.816	0.145	0.102	0.612	0.612	0.612	0.583	1.000																
16	0.089	0.086	0.148	0.327	0.429	0.327	0.348	0.655	0.218	0.218	0.327	0.327	0.327	0.089	0.535	1.000															
17	0.089	0.458	0.428	0.327	0.429	0.327	0.448	0.655	0.218	0.218	0.327	0.327	0.327	0.089	0.535	0.567	1.000														
18	0.089	0.148	0.428	0.218	0.148	0.327	0.429	0.218	0.218	0.327	0.327	0.327	0.327	0.356	0.089	0.524	0.524	1.000													
19	0.089	0.248	0.448	0.218	0.148	0.327	0.429	0.218	0.218	0.327	0.327	0.327	0.327	0.356	0.089	0.524	0.524	0.463	1.000												
20	0.089	0.148	0.148	0.218	0.348	0.327	0.429	0.218	0.218	0.327	0.327	0.327	0.327	0.356	0.089	0.524	0.524	0.802	0.712	1.000											
21	0.250	0.089	0.089	0.408	0.289	0.102	0.189	0.408	0.408	0.102	0.102	0.102	0.102	0.167	0.250	0.802	0.802	0.356	0.356	0.356	1.000										
22	0.250	0.085	0.182	0.408	0.089	0.102	0.089	0.408	0.408	0.102	0.102	0.102	0.102	0.167	0.250	0.802	0.802	0.356	0.356	0.356	0.456	1.000									
23	0.250	0.083	0.089	0.408	0.089	0.102	0.089	0.408	0.408	0.102	0.102	0.102	0.102	0.167	0.250	0.802	0.802	0.356	0.356	0.356	0.459	0.675	1.000								
24	0.250	0.085	0.089	0.102	0.356	0.102	0.535	0.356	0.450	0.408	0.102	0.102	0.102	0.583	0.167	0.356	0.356	0.802	0.802	0.802	0.583	0.583	0.583	1.000							
25	0.250	0.084	0.088	0.102	0.356	0.102	0.535	0.098	0.560	0.408	0.102	0.102	0.102	0.583	0.167	0.356	0.356	0.802	0.802	0.802	0.583	0.583	0.583	0.490	1.000						
26	0.816	0.066	0.655	0.500	0.218	0.259	0.655	0.200	0.200	0.500	0.340	0.212	0.231	0.408	0.345	0.218	0.218	0.218	0.218	0.218	0.234	0.256	0.345	0.234	0.459	1.000					
27	0.667	0.074	0.535	0.408	0.535	0.408	0.535	0.467	0.408	0.408	0.408	0.408	0.408	0.167	0.250	0.089	0.089	0.089	0.089	0.089	0.250	0.250	0.250	0.250	0.250	0.816	1.000				
28	0.667	0.054	0.535	0.408	0.535	0.408	0.535	0.489	0.408	0.408	0.408	0.408	0.408	0.167	0.250	0.089	0.089	0.089	0.089	0.089	0.250	0.250	0.250	0.250	0.250	0.816	0.690	1.000			
29	0.250	0.089	0.089	0.408	0.535	0.408	0.089	0.408	0.376	0.102	0.408	0.408	0.408	0.250	0.667	0.356	0.356	0.089	0.089	0.089	0.167	0.167	0.167	0.250	0.250	0.408	0.583	0.583	1.000		
30	0.250	0.089	0.089	0.408	0.535	0.408	0.089	0.408	0.450	0.102	0.408	0.408	0.408	0.250	0.667	0.356	0.356	0.089	0.089	0.089	0.167	0.167	0.167	0.250	0.250	0.408	0.583	0.583	0.789	1.000	
31	0.167	0.356	0.356	0.612	0.356	0.408	0.089	0.515	0.408	0.408	0.408	0.408	0.408	0.167	0.167	0.089	0.089	0.356	0.356	0.356	0.250	0.250	0.250	0.167	0.167	0.408	0.250	0.250	0.667	0.667	1.000

A.f.= Apis florea; *A.a= Apis anderniformis*)

1:Basar (*A.f*) 2:Pasighat (*A.f*) 3:Roing (*A.f*) 4:Itanagar (*A.f*) 5:Hawai (*A.f*) 6:Udaipur (*A.f*) 7:Agartala (*A.f*) 8:Kailashahar (*A.f*) 9:Cachar (*A.f*) 10:Karimganj (*A.f*) 11:Jorhat (*A.f*) 12:Golaghat (*A.f*) 13:Dibrugarh (*A.f*) 14:Nagaon (*A.f*) 15:Lakhimpur (*A.f*) 16:Medziphema (*A.f*) 17:Imphal (*A.f*) 18:Kohima (*A.f*) 19:Haflong (*A.f*) 20:Maibong (*A.f*) 21:Umiam (*A.f*) 22:Umragnso (*A.f*) 23:Umsning (*A.f*) 24:Sohra (*A.f*) 25:Sanmer (*A.f*) 26:Jorhat (*A.a*) 27:Golaghat (*A.a*) 28:Nagaon (*A.a*) 29:Silchar (*A.a*) 30:Karimganj (*A.a*) 31:Umiam (*A.a*)



1: *Apis florea*; **2:** *Apis anderniformis*

Fig 2: Dendrogram showing linkage based on DNA fragment amplified by SSR markers across dwarf honey bee population from different physiographic zones of North east India

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