



E-ISSN: 2320-7078

P-ISSN: 2349-6800

JEZS 2020; 8(1): 1273-1280

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Received: 07-11-2019

Accepted: 09-12-2019

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Morphometric and molecular characterization of Eastern honey bee, *Apis cerana* F. populations in the Northeast Himalayas

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Abstract

Morphometric and molecular characterization of honeybees from five different physiographic zones of northeast India viz. Arunachal Himalaya, Brahmaputra valley, Barak valley, Meghalaya plateau and South-Eastern hill tract have been carried out in the Department of Entomology and Agricultural Biotechnology, Assam Agricultural University during the period 2011-2015. Morphometric analysis of 1551 individual *Apis cerana* workers from 50 locations revealed that, bees from Arunachal Himalaya has the largest body length (9.58 ± 0.03 mm), and the smallest was found in Barak valley (8.23 ± 0.01 mm), followed by South-eastern hill tract (9.18 ± 0.01 mm), Meghalaya plateau (9.04 ± 0.01 mm) and Brahmaputra valley (8.62 ± 0.02 mm). Cluster analysis of *Apis cerana* showed that maximum euclidean distance was found between Rangia and Hailakandi (11.73) and minimum distance had been observed between Nongpoh and Umiam (1.55). The genetic similarity between Basar and Itanagar of *A. cerana* was found to be maximum (81.8 per cent) while minimum (14.7 per cent) was recorded between Katlicherra and Roing. Based on morphometric and molecular analysis, two distinct morphoclusters of *Apis cerana* have been identified i.e., Plains and Hill races. Morphocluster designation is related to physiographic differences which create a partial temporal reproductive isolation associated with altitudes.

Keywords: *Apis cerana*, honey bees, Morphoclusters, North East Himalayas, India

1. Introduction

The eastern honey bee, *Apis cerana* is widely distributed across the geographic ranges of Asia from Afghanistan to Japan and Southeast Asia to the Wallace line. Four distinct groups were organised on the basis of multivariate analysis of morphometric characters which also categorised larger "hill" race and smaller "plain" race of *Apis cerana* in India [13]. Morphometric studies are critical for both identification and studying the variation in and within the populations of a species. Morphometric studies in combination with molecular studies are needed to produce stable evolutionary relationships. The morphoclusters separation is related to physiographic differences which create a partial temporal reproductive isolation associated with altitude [4]. The northern Himalayas region surrounded by the hills of Arunachal Himalaya to the plains of Brahmaputra valley reveals interesting morphological variation in different elevations. The morphometric variation to the recent morphometric analysis of *Apis cerana* populations in the Southern Himalayan region showed the decrease in size with decreasing altitude [4, 7]. This necessitates for extensive analysis of honey bee size altitude relationship, correlations between morphocluster distribution, physiography, and the nature of morphometric and molecular variance domains. The study is aimed at addressing the issue of morphometric and molecular variation of *Apis cerana* population in the northeast Himalayas of India and also it can be used to screen the population of honey bees from different locations for useful biological traits such as disease resistance, high honey yielding and good pollinator strains.

2. Materials and Methods

2.1 Honey bees

Eastern honey bee, *Apis cerana* 1551 workers were collected from five physiographic zones of northeast India, viz., Arunachal Himalaya, Brahmaputra valley, Barak valley, Meghalaya plateau and South eastern hill tract (Fig.1). Bee specimens foraging on different crop plants or from colonies were collected for each location (Table 1). The collected samples were killed

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with the help of carbon tetrachloride and later temporarily preserved in 75 per cent ethanol [11]. Of these, ten bees from each locality were taken for morphometry study. The important morphometric characters were chosen from the bees of the world [5]. A total of 32 numbers of characters were taken. Bees preserved in ethanol were dissected first to separate the tagmata. Later on head, wings, hind leg, tergites, sternites and wax plates were dissected out for making morphometric measurements. For molecular characterization specimens were preserved in 70 per cent ethanol and kept in a freezer (-20 °C) for DNA extraction. From each individual about 100mg insect tissues were taken for DNA extraction and subsequent amplification. The total genomic DNA was isolated from individual bees by a CTAB extraction method [15]. Isolated DNA was quantified using Nano Drop 2000 UV-Visible spectrophotometer and electrophoresed on 0.8% agarose gel.

2.2 Measurements and analysis

Thirty-two important morphological characters based on five physiographic zones were analysed (Table 2). The significant difference, mean, standard deviation and critical difference was computed for each morphometric character from each zone. An agglomerative method of clustering morphometric 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. The molecular weight of PCR products, obtained for each marker was designated, based on a ladder of known molecular weight and subscript to the name of the primer. Data were scored on the basis of presence or absence of the amplified particular DNA fragment primer. The intense bands were scored while the faint 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. Polymorphism information content (PIC) was evaluated [18].

3. Results

Thirty-two morphometric characteristics of *Apis cerana* collected from five physiographic zones have been studied. Morphometry data were statistically analysed and significant or non-significant differences have been worked out. Body length of *Apis cerana* was found to be the highest in Arunachal Himalaya (9.58 ± 0.03 mm) which was followed by South Eastern hill tract (9.18 ± 0.01 mm), Meghalaya plateau (9.04 ± 0.01 mm), Brahmaputra Valley (8.62 ± 0.02 mm) and the lowest was found in Barak Valley (8.23 ± 0.01 mm) as presented in Table 3. Highest head length was found in South Eastern hill tract (2.54 ± 0.07 mm) followed by Brahmaputra Valley (2.73 ± 0.04 mm), Arunachal Himalaya (2.71 ± 0.05 mm), Meghalaya plateau (2.66 ± 0.08 mm) and Barak Valley (2.54 ± 0.01 mm). Maximum head width was found in South Eastern hill tract (3.27 ± 0.13 mm) followed by Meghalaya plateau (3.22 ± 0.02 mm), Brahmaputra Valley (3.19 ± 0.04 mm), Barak Valley (3.16 ± 0.02 mm) and Arunachal Himalaya (3.06 ± 0.08 mm), respectively. Highest ratio of head length and width was found in Arunachal Himalaya (0.89 ± 0.03) followed by Brahmaputra Valley (0.85 ± 0.01), South Eastern hill tract (0.84 ± 0.13), Meghalaya plateau (0.83 ± 0.02) and Barak Valley (0.81 ± 0.01). The bees from Brahmaputra Valley were found to have the highest

mandible length (1.13 ± 0.01 mm) which was followed by South Eastern hill tract (1.11 ± 0.02 mm), Arunachal Himalaya (0.10 ± 0.01 mm), Meghalaya plateau (0.11 ± 0.01 mm) and Barak Valley (1.10 ± 0.02 mm). The bees from Brahmaputra Valley were found to have maximum mandible width (0.49 ± 0.13 mm) which was followed by South Eastern hill tract (0.49 ± 0.04 mm), Meghalaya plateau (0.49 ± 0.01 mm), Arunachal Himalaya (0.47 ± 0.01 mm), and Barak Valley (0.46 ± 0.01 mm), respectively. The ratio of mandible length and width was the highest in Meghalaya plateau (2.28 ± 0.06), and the lowest was found in Brahmaputra Valley (2.30 ± 0.07). The specimens from Brahmaputra Valley (7.43 ± 0.06) were found to have the highest fore wing length and the lowest were found in Barak Valley (6.91 ± 0.01). Similarly, fore wing width was significantly different between Meghalaya plateau (2.51 ± 0.01 mm) and Barak Valley (2.30 ± 0.01). The ratio of forewing length and width was the highest in Barak Valley (3.30 ± 0.01) whereas the lowest was found in Brahmaputra Valley (2.92 ± 0.06). Cubital index was found to be the highest from Arunachal Himalaya (5.79 ± 0.35) and the lowest was from South Eastern hill tract (3.46 ± 0.26). The meta-tarsus length was found to be the highest from Arunachal Himalaya (1.88 ± 0.07 mm) and the lowest was from Barak Valley (1.74 ± 0.01 mm). The 3rd sternite width found to be maximum in Brahmaputra Valley (3.60 ± 0.06 mm) and minimum from Barak Valley (3.41 ± 0.01 mm). The maximum ratio of sternite length and width was found from Barak Valley (0.60 ± 0.01) and minimum was found in Brahmaputra Valley (0.48 ± 0.01). Morphometric distance is based on five physiographic zones with all pair-wise combination are presented in Table 4. The cluster analysis of *Apis cerana* from different physiographic zones for 32 (thirty-two) morphometric characters showed that the maximum euclidean distance is in between Rangia and Hailakandi (11.731) and minimum distance has been observed between Nongpoh and Umiam (1.553). The cluster analysis of the *Apis cerana* based on value of morphological traits was performed by UPGMA method and a dendrogram was constructed and shown in Fig. 2. The dendrogram divided into five main clusters A, B, C, D and cluster E. The first main cluster A is produced at a morphometric distance of about 7.44 which comprised of two sub-clusters I A₁ and A₂ and A₁ was again divided into sub-cluster II as A_{1A} which comprised of two different populations A_{1A}, A over the A₂ was again sub divided into two sub-clusters A_{2A} and A_{2B}. The first cluster A included population from two sub-clusters A₁ and A₂, the sub-cluster A₁ included population from Arunachal Himalaya and second sub-cluster A₂ included population from five locations of South Eastern hill tract. The second main cluster B is formed at a morphometric distance of 7.83 with two sub-clusters B₁ and B₂ which included six races, first sub-cluster B_{1A} and B_{1B} which included the population from South Eastern Hill tract. The cluster C at a distance of 8.228 consisted of two sub-cluster C₁ and C₂ which included all the population from Brahmaputra valley zone and has similar traits such as body length, distance between two ocelli, mandible length, metatarsus length. The cluster D at a distance of 9.016 consist of two sub-cluster into D₁ and D₂ which included the population from Medziphema and Dimapur with a similar trait such as length of antenna, fore wing, hind wing width and metatarsus length and D₂ which included Imphal, Wokha, Rusoma have similar traits such as head length, fore wing width, hind wing length, hind wing width, sternum length and width and wax plate length and width. The cluster E was sub-divided into E₁ and E₂ at a

distance of 9.40, the sub-cluster E₁ included Silchar, Karimganj, Arunachal, Kumbhirgram which consisted of similar traits such as body length, mandible length, ratio of mandible length and width, fore wing length and width. Ratio of sternite length and width, wax plate length and subcluster E₂ at a distance 9.40 included Badarpur, Algapur, Katlicherra, Hailakandi and Lala which have similar traits such as mandible length, mandible width, distance between two ocelli, fore wing length and fore wing width and ratio of 3rdsternite length and width.

The molecular analysis with single sequence repeat (SSR), with data obtained from the DNA fragment amplified by markers 7AT113, 5704, AP226, AT191, B1269, B123555, K1420B, SV299, AT113, SV124, UN112, UN181, K0754, UN063, UN373, SV066, KO908, UN354T, K1420B and UN004 indicated that all the 50 geographic populations were monophyletic origin. The honey bee genotypes revealed that there were four major gene clusters formed breaking at 0.29 Jaccard's coefficient of similarity (Table 5 and Fig 3). The dendrogram readily separated the *Apis cerana* species into four main clusters (A, B, C and D). Cluster A was sub-clustered into A₁ and A₂ and A₁ was further sub-clustered into A_{1A} and A_{1B}. Cluster A_{1A} was again further divided into A_{1Aa} and A_{1Ab} which included similar populations of Naharlagun, Pasighat, Basar, Namsai, Rangia, Barpeta, Nalbari, Jagiroad, Bhalukpong, Nongpoh, Umiam, Upper Shillong and A_{1Ab} included the similar population from Bomdila and North Lakhimpur. Cluster A_{1B} was further sub-clustered into A_{1Ba} which included similar population from Biswanath, Bongaigaon, Nagaon, Umsning, Doldoli, Bokajan, Wokha, Hamren and A_{1Bb} include only the population from Byrnihat. Cluster A₂ included similar population from Imphal and Rusoma. Cluster B comprised of geographic population from Arunachal Himalaya, Barak valley and Meghalaya plateau which comprised of two sub-clusters B₁ and B₂. Itanagar and Barapani races were included under sub-cluster B₁ due to their similarity. B₂ further comprised of two sub-clusters B_{2A} and B_{2B}. Hailakandi, Sanmer, Mawklot, Sohra, Katlicherra races were included under sub-cluster B_{2A} because of their similarity. Badarpur, Algapur and Nongumlong were included in B_{2B} sub-cluster due to their similarity. Cluster C divided into two sub cluster C₁ and C₂. Tezu, Diphu, Medziphema, Dima Hasao, Dimapur races were included in subcluster C₁ and subcluster C₂ was further divided into two clusters C_{2A} and C_{2B}, cluster C_{2A} included the races from Anjaw, Nilam Bazaar and Sivasagar with genetic similarity. C_{2B} included Silchar, Arunachal, Kumbhirgram, Karimganj with high genetic similarity. Cluster D which included similar races from Roing and Jorhat

4. Discussion

The complex structure of *Apis cerana* populations were analysed through, morphometric and molecular tools in the Northeast Himalayan region and is facilitated by dendrograms (Fig. 2 and 3) The cluster analysis of *Apis cerana* for five physiographic zones revealed that there are two geographic races viz. Hill races (cluster A, B and D) and plain races (cluster C and E) as they tend to cluster morphologically. The dendrogram divides into five main clusters A, B, C, D and E. (Fig. 2). The first main cluster A is produced at a

morphometric distance of about 7.44 which comprise of two sub-clusters I and II. The cluster A include morphocluster from Arunachal Himalaya zone and southern hill tract. The second main cluster B is formed at morphometric distance of 7.83 with two sub clusters B₁ and B₂, which include populations from South eastern Hill tract. The cluster C at a distance of 8.228 consists of two sub-cluster C₁ and C₂ which include population from Brahmaputra valley. The cluster D at a distance of 9.016 and cluster E at a distance of 9.4 include populations partly from South eastern hill tract and from Meghalaya plateau (fig. 2). Similarly, three identified biometric groups in the North eastern Himalayan region: 1. Manipuri bees from Nagaland, Manipur and Mizoram; 2. Brahmaputra valley and also from Southern Assam and Meghalaya and 3. Himalayan bees from Sikkim, West Bengal, Northern and Western Assam and Arunachal Pradesh [14]. Multivariate analysis of 55 morphometric characters pertaining to forewing, hindwing, tongue, abdomen and antenna on the samples of *Apis cerana* from twenty localities in Himachal Pradesh, Jammu and Kashmir states of Northwest Himalayas and found the trend of decreasing size with declining of elevation [6]. Morphocluster related to physiographic zones which created partial temporal reproductive isolation associated with altitude [4]. Significant differences in population structure between geographical regions having gradient from higher to lower altitudes [9].

Molecular characterization by using Single Sequence Repeat (SSR) markers reveal that all the bee population belong to a monophyletic origin. Four major bee genotypes are formed breaking at 0.29 Jaccard's coefficient similarity (Table 5). The dendrogram (Fig. 3) readily separated the *Apis cerana* populations in four main clusters viz., A,B,C,D. The findings are in agreement with the populations of Balearic Islands genetically clustered into two groups: Gimnesis and Pitiusas as biogeography study [10]. The genetic structure was compared within an *Apis mellifera* colony using derived paternal DNA microsatellites with the genetic structure of the local honeybee population and found that the genetic diversity within the colony could provide a good estimate of that of the local honeybee population [3]. Similarity matrix for Jaccard's Coefficient based on SSR banding of the *Apis cerana* population revealed that the genetic similarity between Basar and Itanagar of *Apis cerana* has been recorded to be maximum 81.8 per cent while the minimum 14.7 per cent was recorded between Katlicherra and Roing. The genetic similarity matrix for *Apis mellifera* ranged from 37.5 per cent to 86.1 per cent and genetic variation of mid hill and plain region of India [16]. The highest genetic similarity was found to be 0.861 whereas the lowest was found to be 0.375. The population structure and inbreeding in a rare and declining bumble bee, *Bombus muscorum* reported that a segregated population showed low genetic diversity but high Jaccard's co-efficient showed high genetic similarity in bee population [2]. The genetic study of south Indian *Apis cerana* bees, recorded mitochondrial DNA variations in *Apis cerana* populations of Tamil Nadu and Karnataka [1] Both from morphometric and molecular analysis, two distinct morphoclusters of *Apis cerana* have been emerged as plains and hills races from northeast India.

Table 1: Physiographic zones of North East India showing locations

Physiographic Zones of North East India	Locations	Coordinates	Altitude
Arunachal Himalaya	Naharlagun (L1)	27°10'N 93°70'E	200m
	Pasighat (L2)	28°4'N, 95°19'E	153m
	Basar (L3)	27°59'N 94°40'E	578m
	Namsai (L4)	27°40'N, 95°52'E	131m
	Itanagar(L5)	27°6'N, 93°37'E	750m
	Bhalukpong (L6)	27°0'N, 92°38'E	213m
	Tezu(L7)	27°55'N, 96°10'E	185m
	Bomdila (L8)	27°15'N, 92°24'E	2,217m
	Anjaw (L9)	28°14'N, 95°84'E	1,296m
	Roing (L10)	28°32'N 94°68'E	1,968m
Brahmaputra valley	Jorhat (L11)	26°45'N, 94°13'E	116m
	Sivasagar(L12)	26°58'N, 94°37'E	95m
	Biswanath (L13)	26°43'N, 93°9'E	84m
	Lakhimpur (L14)	26°48'N 93°42'E	147m
	Nagaon (L15)	26°21'N, 92°40'E	70.5m
	Jagiroad(L16)	26°12'N, 92°24'E	63.82m
	Raha(L17)	26°14'N, 92°32'E	62m
	Nalbari (L18)	26°26'N, 91°26'E	42m
	Gossaigaon (L19)	26°25'N, 89°59'E	50.8m
	Rangia(L20)	26°28'N, 91°37'E	39m
South Eastern hill tract	Diphu (L21)	25°83'N - 93°43'E	197m
	Medziphema (L32)	25°76'N, 93°80'E	456m
	Dima Hasao (L23)	25°33'N, 92°10'E	513m
	Dimapur (L24)	25°55'N, 93°43'E	145m
	Imphal (L25)	24°49'N, 93°57'E	786m
	Doldoli (L26)	25°49'N, 93°25'E	197m
	Bokajan (L27)	26°02'N, 93°78'E	138m
	Hamren (L28)	25°92'N, 92°60'E	480m
	Rusoma (L29)	25°70'N, 94°10'E	154m
	Wokha (L30)	24°49'N, 93°57'E	1,313m
Barak valley	Silchar (L31)	24°49'N, 92°48'E	22m
	Khaspur (L32)	21°43'N, 92°71'E	26m
	Kumbhirgram (L33)	24°82'N, 92°31'E	107m
	Karimganj (L34)	24°87'N, 92°35'E	13m
	Nilambazaar (L35)	24°67'N, 92°56'E	14m
	Badarpur (L36)	24°85'N, 92°33'E	16m
	Hailakandi (L37)	24°75'N, 92°76'E	21m
	Algapur (L38)	24°52'N, 92°21'E	25m
	Katlicherra (L39)	24°85'N, 92°38'E	40m
	Lala (L40)	24°75'N, 92°76'E	21m
Meghalaya plateau	Barapani (L41)	25°54'N, 91°53'E	887m
	Nongpoh (L42)	25°54'N, 91°52'E	485m
	Umiam (L43)	25°39'N, 91°53'E	946.2m
	Upper Shillong (L44)	25°34'N, 91°52'E	1,496m
	Byrnihat (L45)	26°3'N, 91°52'E	67.58m
	Umsning (L46)	25°74'N, 91°89'E	782m
	Sanmer (L47)	25°72'N, 91°52'E	1726.7m
	Sohra (L48)	25°64'N, 91°885'E	1484m
	Mawklot (L49)	25°49'N, 91°54'E	350m
	Nongumlong (L50)	25°31'N, 91°44'E	1569m

Table 2: Morphometric characteristics for *Apis cerana*

Characters with Abbreviation in mm	
Body length (BL)	Ratio of Hind wing (HWL:HWW)
Head length (HL)	Tibia length (TL)
Head width (HW)	Metatarsus length (MTL)
Ratio of Head (HL:HW)	Metatarsus width (MTW)
Distance between two dorsal ocelli (DBO)	Ratio of Metatarsus (MTL:MTW)
Dorsal ocelli ocular distance (DOOD)	4 th Tergite length (TGL)
Antennal length (AL)	3 rd sternite length (SL)
Proboscis length (PL)	3 rd sternite width (SW)
Mandibles length (ML)	Ratio of sternite (SL:SW)
Mandibles width (MW)	Lateral width of 4 th tomentum (TOM)
Ratio of Mandibles (ML:MW)	Wax Plate length (WPL)
Forewing length (FWL)	Wax Plate width (WPW)
Forewing width (FWW)	Ratio of Wax Plate (WPL:WPW)
Ratio of Forewing (FWL:FWW)	Cubital Index (CuA: CuB)
Hind wing length (HWL)	Hamuli number (HAM)
Hind wing width (HWW)	Femur length (FL)

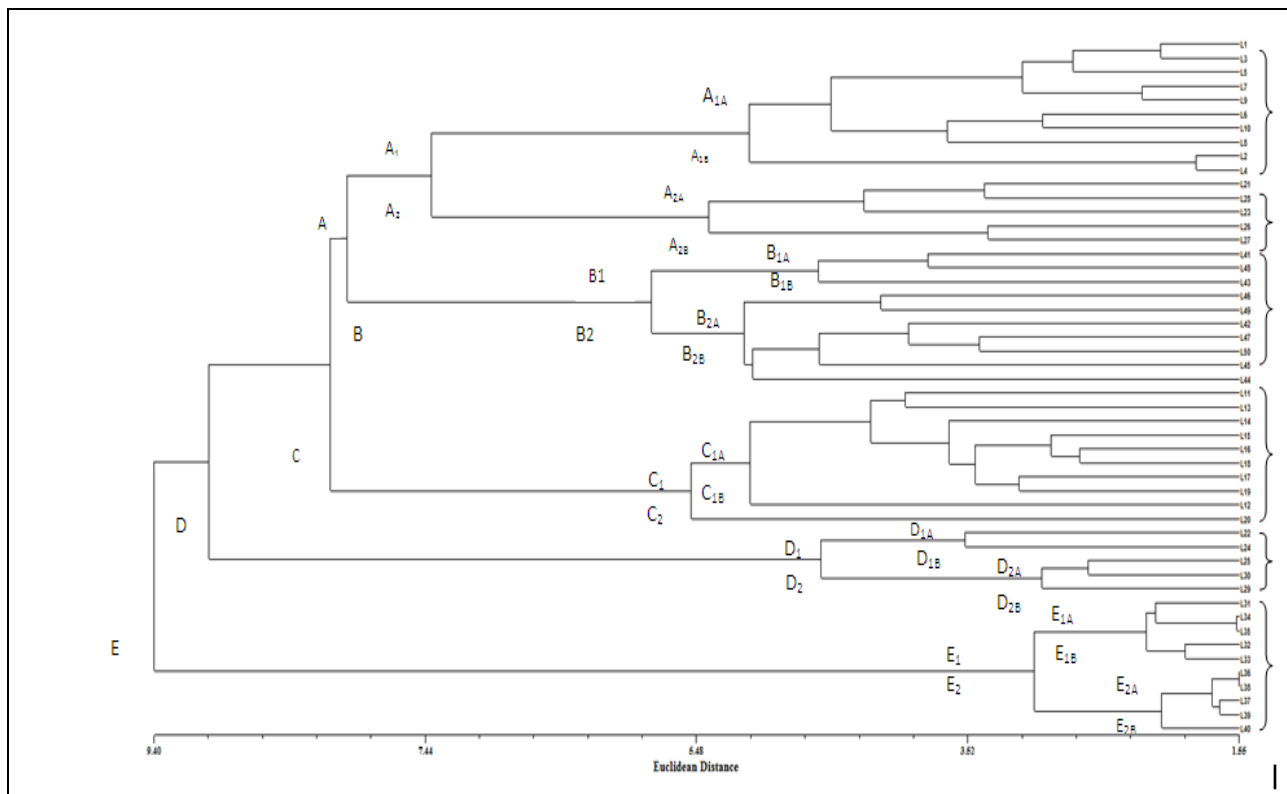


Fig 2: Euclidean distance matrix of *Apis cerana* from five different physiographic zones. L1-L10 Arunachal Himalaya; L11-20 Brahmaputra valley; L21-30 South eastern Hill tract; L31-L40 Barak Valley; L41-L50 Meghalaya plateau

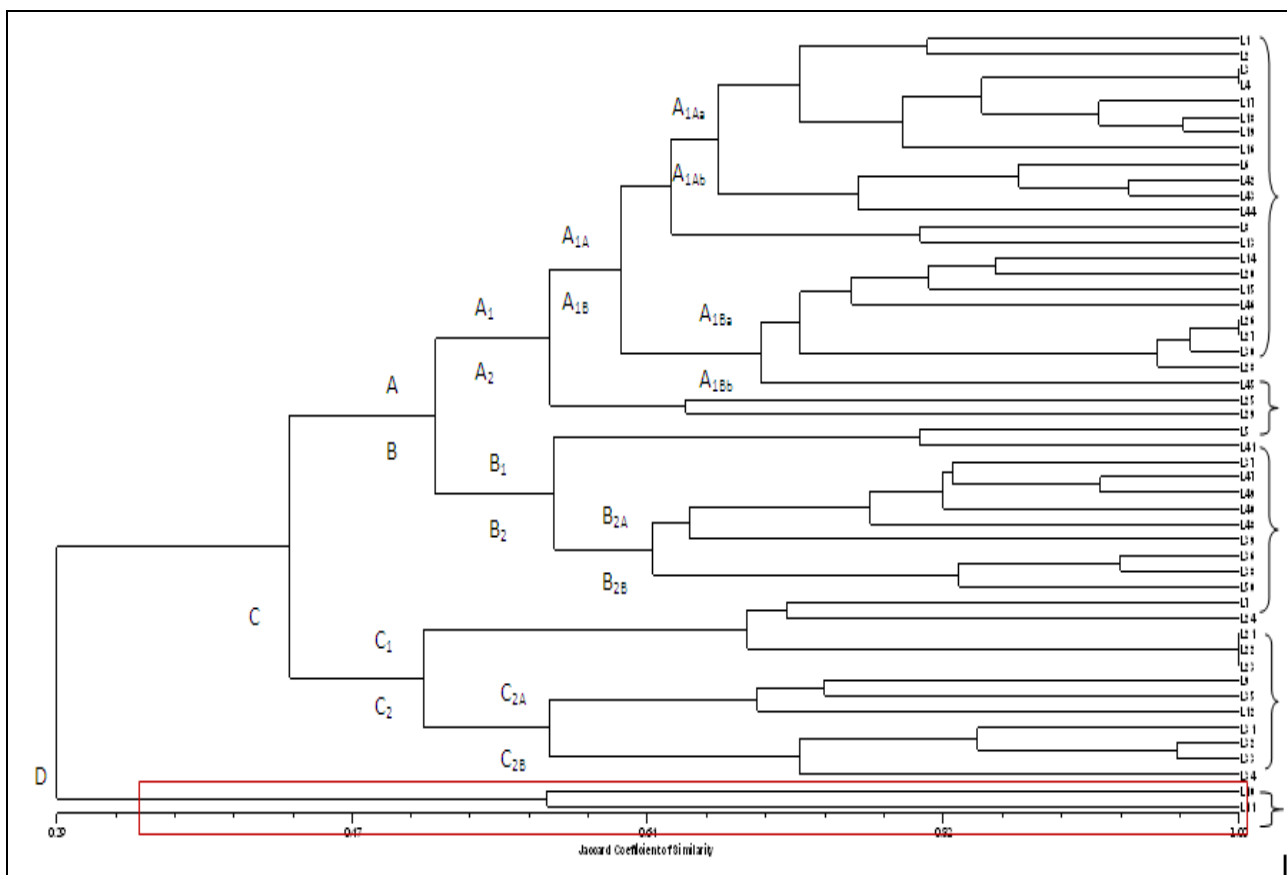


Fig 3: Jaccard's coefficient similarity of *Apis cerana* from different physiographic zones L1-L10Arunachal Himalaya; L11-20 Brahmaputra valley; L21-L30 Southeastern Hill Tract; L31-L40-Barak Valley; L41-L50 Meghalaya Plateau

5. Conclusion

The study has been carried out on Indian honey bee *Apis cerana* F. from five physiographic zones of NE India using both morphometry and molecular analysis. Morphometric

study of *Apis cerana* from five physiographic zones revealed that, *A. cerana* from Arunachal Himalaya had larger average body length ($9.577 \pm 0.03 \text{ mm}$) and smallest body length was found in Barak valley ($8.235 \pm 0.01 \text{ mm}$), followed by south

eastern hill tract ($9.178 \pm 0.01\text{mm}$) and Meghalaya Plateau ($9.040 \pm 0.01\text{mm}$) and Brahmaputra valley ($8.621 \pm 0.02\text{mm}$). Cluster analysis of *Apis cerana* reveals that maximum euclidean distance is in between Rangia and Hailakandi (11.731) and minimum distance has been observed between Nongpoh and Umiam (1.553). The genetic similarity between Basar and Itanagar of *A. cerana* has been recorded to be maximum 81.8 per cent minimum between Katlicherra and Roing 14.7 per cent. This study plays a vital role in establishing the genetic similarity and diversity of the indigenous species of *Apis cerana* in the region of northeast Himalayas.

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