CHAPTER III

*DROSOPHILA HEGDII* (DIPTERA: DROSOPHILIDAE), A NEW SPECIES FROM NAGALAND: ITS MOLECULAR PHYLOGENY
INTRODUCTION:

North-Eastern region of Indian subcontinent (this region includes eight hill states, namely Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura) with its diverse climatic conditions, variable altitudes, deep valleys, luxuriant flora, running streams and moist surroundings is one of the richest repositories of biodiversity in the world. Hence it provides an ideal location for the colonization of several *Drosophila* species (Singh and Gupta 1977; Dwivedi and Gupta 1979; Gupta and Singh 1979; Singh and Gupta 1980; Singh 1987; Yenisetti *et al.* 2002 and Achumi *et al.* 2011, 2013).

Nagaland is one of the sub-Himalayan hilly states of biodiversically rich north eastern part of the country. However very little work was done to understand *Drosophila* diversity in this part of the country. A preliminary survey on Drosophilids of Dimapur, Medziphema, and Kohima of Nagaland state was conducted (Singh 1987). Yenisetti *et al.* (2002) published a report on Drosophilids of Mokokchung town. In spite of existence of potential possibility, till today no new *Drosophila* species were reported from this sub-Himalayan hilly state bordering Indo-Burma region.

Survey of *Drosophila* was undertaken in Lumami (Lumami is a hamlet in Zunheboto district of Nagaland and is head quarters of the Nagaland University) which is situated at 94°.28’E Longitude and 26°.33’ N Latitude, having an altitude of 940 m above sea level. In Lumami temperature ranges from 8°C to 30°C and average annual rainfall is about 200 cm. The torrential monsoon rain is an integral feature of the weather in this place. Heavy rainfall during the monsoon favours the growth of thick forest, fruit bearing trees, providing favorable natural habitats for the colonization by the members of the genus *Drosophila*. In the present study 16 species were collected, belonging to 4 subgenera (*Sophophora, Drosophila, Dorsilopha* and *Scaptodrosophila*). Three species were unidentified. Of the three unidentified, one was recognized as a new species basing on morphological markers such as head, thorax, wings, legs, abdomen of male and female; internal characters such as periphallic organ, phallic organ, egg guide, egg and pupa
(Achumi et al. 2011). It is named after Prof. S.N. Hegde (retired) of Mysore University, Karnataka, India (Prof. Hegde made significant contribution to cytotaxonomy and genetics of Indian Drosophilids) as *Drosophila hegdii*.

“DNA barcoding” is identified as a promising tool not only for rapid identification of known species, that is “species identification,” but also for discovery and delimitation of species, that is, “species discovery” or “DNA taxonomy” (Hebert et al. 2003a,b; Jinbo et al. 2011). Simon (1991) observed that when focusing on very closely related species, one should select rapidly evolving regions, for example, mitochondrial genes as markers. Mitochondrial cytochrome c oxidase subunit I (*COI*) gene has been widely used as DNA barcoding for “species identification”: its 648 base pair fragment is the standard marker in the Barcode of Life Project (Hebert et al. 2003a, b). In the present study by employing mitochondrial cytochrome c oxidase subunit I marker, effort was made to establish the independent species status of *Drosophila hegdii*. Confirmation of new species status and understanding its molecular phylogeny was done with the help of “DNA barcoding.” Molecular analysis confirms the observations made through morphological markers that *Drosophila hegdii* is a new species.
MATERIALS AND METHODS:

*Drosophila* collections were made by following two methods: 1) Bottle trapping method and 2) Net sweeping method (detailed procedure of these methods was explained in chapter I).

The flies were then brought to the laboratory, isolated and sex was identified. The males were directly used for identification of species basing on morphological charterers such as presence or absence of the sex comb; if present the number of sex comb rows and teeth in each row and characteristics of genital plate. Individual females were kept in separate food vials and isofemale lines were generated. The males of the F1 progeny of these gravid females were used for species identification.

Categorization of the collected *Drosophila* flies were made to respective taxonomic groups by employing the parameters as suggested by Bock (1971), Patterson and Stone (1952), Sturtevant (1921) and Throckmorton (1962). The most important parameters employed to identify the species are the morphological features like colour and size of imagoes, number and nature of aristal branches, nature and arrangement of genital arch, nature and number of acrostichal hairs, length of the wings and its indices, the internal characters of the adults, the shape and number of egg filaments, pupal characters, pupal spiracles and behavior were also taken into consideration for species identification.

**Genomic DNA extraction and estimation:**

Genomic DNA was extracted from single adult male fly. The fly was freezed on ice and homogenized using crusher in extraction buffer (5% sucrose; 80mM NaCl; 100mM Tris, pH 8.5; 0.5% SDS; 50mM EDTA). The contents were mixed well and kept on ice, incubated at 65°C for 30 minutes and immediately chilled on ice. 8M potassium acetate was added and placed on ice after mixing well. Pellet was precipitated by centrifugation for 10 minutes at 10000 rpm at 4°C. The supernatant was transferred to a clean tube and DNA was precipitated with chilled ethanol. The tube was then centrifuged for 10 minutes at 10000rpm at 4°C to pellet DNA. The pellet was washed with 70% ethanol and dried. The pellet was re-suspended in double distilled water and kept for overnight at 55°C-60°C.
The DNA obtained was stored at 4°C until use. The quality of genomic DNA was checked with the help of A260/A280 ratio.

Calculation: 1.0 OD at 260 mm corresponds to 50 µg/ml of double-stranded DNA

dsDNA concentration= 50 µg x OD_{260} x dilution factor.

The primer sequences:

Set I
F: 5’-GCT CAA CAAATCATAAAGATATTGGC-3’
R: 5’-TAAACT TTA GCG TGA CCA AAA AAT CA-3’

Set II
F: 5’-ATTGAA CCA ATC ATA AGG ATA TTG C-3’
R: 5’-TAAACT TGT GGA TGT CAAAAATCG-3’

(Set I and Set II were mixed in 1:1 ratio)

The DNA extracted was amplified with PCR using above mentioned oligos. The target regions were amplified on a thermal Cycler (Bio-Rad). The PCR amplification was carried out with the following conditions:

94°C for 2 minutes

94°C for 30 seconds

40°C for 40 seconds

72°C for 1 minute

94°C for 30 seconds

45°C for 40 seconds

72°C for 1 minute

72°C for 10 minutes

4°C for α
PCR products were extracted from agarose gel and cleaned using DNA Gel Extraction Kit (Qiagen). The amplicons were purified by precipitation with isopropanol and then subjected to sequencing reaction using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) following the recommended protocol. The sequences were analyzed on the 3100-Genetic Analyzer (Applied Biosystems).

**Phylogenetic analysis:**

DNA sequences were edited and aligned using ClustalW (Figure 5). ClustalW is a widely used system for aligning any number of homologous nucleotide or protein sequences. For multi-sequence alignments, ClustalW uses progressive alignment methods. In these, the most similar sequences, that is, those with the best alignment score are aligned first. Then progressively more distant groups of sequences are aligned until a global alignment is obtained. This heuristic approach is necessary because finding the global optimal solution is prohibitive in both memory and time requirements. ClustalW performs very well in practice. The algorithm starts by computing a rough distance matrix between each pair of sequences based on pairwise sequence alignment scores. These scores are computed using the pairwise alignment parameters for DNA and protein sequences. Next, the algorithm uses the neighbor-joining method with midpoint rooting to create a guide tree, which is used to generate a global alignment. The guide tree serves as a rough template for clades that tend to share insertion and deletion features. This generally provides a close-to-optimal result, especially when the data set contains sequences with varied degrees of divergence, so the guide tree is less sensitive to noise.

Phylogenetic trees and molecular evolutionary analyses were performed using MEGA 5 (Tamura et al. 2011) (Neighbor-Joining Tree (NJ) method with bootstrap test (1000 replicates) using the Kimura 2-parameter model, with gaps treated by pairwise deletion. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.05102 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) was shown next to the branches (Felsenstein 1985). The tree was drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were
computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 519 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.
OBSERVATIONS:
Description of morphological markers/characteristics of *D. hegdii*:

**Head:**
Arista with 4 branches above, 3 below, plus the terminal fork. Antenna dark, basal segment of the antenna bears a pair of dark bristles. Vibrissae with two anterior and two posterior bristles. In between the anterior and posterior bristles are 10-12 small bristles. Palp with a large and many small bristles. Anterior orbital proclinate, median orbital half the size of anterior orbital, posterior equal to anterior. Anterior verticles direct inward, posterior convergent. Ocular triangle with a pair of dark bristles, eyes red.

**Thorax:**
Mid to dark brown, acrosticals in 8 regular rows, dorsocentrals convergent, anterior dorsocentrals are shorter than the posterior- approximately 2/3rd the length of the posterior, anterior scutellar convergent, posterior scutellar convergent and crossed. Both anterior and posterior scutellars are of equal length. Two humerals, upper humerals half the length of the lower, posterior allars longer than anterior. Notopleurals and stenopleurals are of equal length. Notopleural and supra allars are of equal length. There are about 2-3 smaller bristles along the anterior and posterior stenopleurals. Halters translucent.

**Wings:**
Transparent, wing length of male is 97 mm and female is 100 mm. Wing indices are calculated following the formula of Okada (1956) and presented in table 1.

**Legs: [Figure 2(1)]**
Sex comb present in male on first and second tarsal segment. First tarsal consists of about 25-27 teeth and second tarsal consists of 16 teeth. Teeth are uniform and slightly curved.
Abdomen of male and female:
First four tergites of male are shiny yellow-brown with broad dark apical band; last two segments are completely black. Tergites of female are shiny dark brown with broad darker apical bands.

Internal characters:
Female reproductive parts consist of ovarioles with 5-6 ventral receptacles that are transparent with 2-3 coils, spermetheca roundish colourless, [Figure 2(4)]. Male testis is short showing 2-3 coils and light yellow in colour, paragonia spherical transparent [Figure 2(3)].

Periphallic organ: [Figure 2(2)]
Ependrium broad, dorsally and laterally. Primary and secondary claspers present, primary claspers with a lateral row of about 5 teeth and a ventral medial cluster of teeth one elongated, toe with 3-4 bristles; secondary claspers oval, partially separated from anal plate with 3 black teeth, two are prominent and one is rudiment and about 1-8 small bristles along the ventral lateral and dorsal borders. Cerci rounded on the outer side and slightly curved on inner side and with about 25 long and short bristles.

Phallic organ: [Figure 2(5)]
Adeagus and anterior gonopophysis not fused. Anterior gonopophysis protrude dorsally. Novasternum with prominent median convexity of variable thickness bearing a pair of spines, ventral fragma broad and concave, basal apodeme is thick and short.

Egg guide: [Figure 2(6)]
Brown in colour with about 9-10 marginal and 1-2 discal teeth at the tip, teeth are dark in colour.
Egg: [Figure 2(7)]
White in colour with two filaments present at the anterior.

Pupa: [Figure 2(8)]
Yellow with 9-10 spiracle filaments. At the posterior end there are 3 pairs of projections—one pair is lateral, second pair is ventral and third pair is dorsal.

Holotype-Male: India, Nagaland, Lumami, 14.xi.11 Coll. Bovito Achumi and Sarat Chandra Yenisetti, Deposited in the Drosophila vivarium of Department of Zoology, University of Mysore, Manasagangtori, Mysore- 5700 006, India.
Allotype- Female: Same as above.
Paratype- 5♂♂ and 5♀♀, India, Nagaland, Lumami; Coll. Bovito Achumi and Sarat Chandra yenisetti.

DNA barcoding of Drosophila hegdiii:
Phylogenetic relationship of the unidentified species was analyzed using mitochondrial cytochrome c oxidase I (COI) DNA sequence. The nucleotide sequences of cytochrome c oxidase subunit of Drosophila hegdiii were submitted to GenBank (NCBI: National Centre for Biotechnology Information, Bethesda, USA). GenBank Accession nos of the new species: JX492316 and JX492317. The new species COI sequence data was compared with those of D. jambulina (GenBank accession No. AY737610.1); D. vulcana (courtesy: Dr. Maxi Polihronakis Richmand, Drosophila species stock center, University of California, San Diego, USA) and D. melanogaster (GenBank accession No. AF200846). DNA sequences were edited and analyzed using MEGA 5 (Tamura et al. 2011). Phylogenetic trees and molecular evolutionary analyses were performed by the Neighbor-Joining (NJ) method with bootstrap test (1000 replicates) using the Kimura 2-parameter model, with gaps treated by pairwise deletion. The tree was drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed
for each sequence pair. There were a total of 898 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

A phylogenetic tree was constructed by applying the method of Neighbor-Joining Tree (NJ) (Figure 6) for *Drosophila hegdii*, *Drosophila jambulina*, *Drosophila vulcana* and *Drosophila melanogaster*. According to Tamura *et al.* (2004) *Melanogaster* and *Montium* species groups diverged from one another 41.3 Mya ago. The tree indicates *D. hegdii* and *D. jambulina* belong to the same cluster with strong boot strap support of 86. The ancestor of *D. vulcana* and *D. hegdii* clade was estimated to have appeared about 0.02296 Mya, the divergent between *D. jambulina* and *D. hegdii* was estimated to be 0.02223 Mya.

The number of base differences per site between sequences was shown in Table 2. The evolutionary divergences between *D. hegdii* and *D. jambulina* were 0.11, *D. hegdii* and *D. vulcana* was 0.057 and *D. hegdii* and *D. melanogaster* was 0.88. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (10000 replicates). The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 898 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Maximum Likelihood Estimate of Substitution Matrix was shown in Table 3. Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model. The nucleotide frequencies are A = 29.43%, T/U = 39.88%, C = 14.11%, and G = 16.57%.

Estimates of base composition bias difference between sequences were shown in Table 4. The difference in base composition bias per site was shown according to Kumar and Gadagkar (2001). It was observed that even when the substitution patterns are homogeneous among lineages, the compositional distance will correlate with the number of differences between sequences. The analysis involved 4 nucleotide sequences. Codon
positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 519 positions in the final dataset.
Table 1. Wing indices of *D. hegdii* (Mean value of 10 flies)

<table>
<thead>
<tr>
<th></th>
<th>Costal index</th>
<th>4V index</th>
<th>4C index</th>
<th>5X index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>2.69</td>
<td>1.15</td>
<td>2.55</td>
<td>2.62</td>
</tr>
<tr>
<td>Female</td>
<td>2.81</td>
<td>1.1</td>
<td>2.75</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Table 2. Estimates of Evolutionary Divergence between CO I sequence of *D. melanogaster*, *D. jambulina*, *D. vulcana* and *D. hegdii*

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Drosophila hegdii</em></td>
<td></td>
<td>0.004323728</td>
<td>0.010135592</td>
<td>0.011800173</td>
</tr>
<tr>
<td>2. <em>Drosophila jambulina</em></td>
<td>0.011560694</td>
<td></td>
<td>0.009643225</td>
<td>0.011796287</td>
</tr>
<tr>
<td>3. <em>Drosophila vulcana</em></td>
<td>0.057803468</td>
<td>0.050096339</td>
<td></td>
<td>0.013121109</td>
</tr>
<tr>
<td>4. <em>Drosophila melanogaster</em></td>
<td>0.088631985</td>
<td>0.088631985</td>
<td>0.113680154</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Maximum Likelihood Estimate of Substitution Matrix with reference to *D. hegdii, D. jambulina, D. vulcana and D. melanogaster*

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T/U</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>10.98</td>
<td>3.88</td>
<td>6.63</td>
</tr>
<tr>
<td>T/U</td>
<td>8.10</td>
<td>-</td>
<td>6.94</td>
<td>4.56</td>
</tr>
<tr>
<td>C</td>
<td>8.10</td>
<td>19.62</td>
<td>-</td>
<td>4.56</td>
</tr>
<tr>
<td>G</td>
<td>11.77</td>
<td>10.98</td>
<td>3.88</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4. Estimates of Base Composition Bias Difference between CO I sequence of *D. melanogaster*, *D. jambulina*, *D. vulcana* and *D. hegdii*

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Drosophila hegdii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. <em>Drosophila jambulina</em></td>
<td>0.013487476</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. <em>Drosophila vulcana</em></td>
<td>0.109826590</td>
<td>0.129094412</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. <em>Drosophila melanogaster</em></td>
<td>0.025048170</td>
<td>0.025048170</td>
<td>0.050096339</td>
<td></td>
</tr>
</tbody>
</table>
a) Female  

b) Male  

**Figure 1.** Female and male *D. hegdi*
Figure 2. Morphological and anatomical characteristics of *Drosophila hegdii*. (1) S.C sex comb in first & second tarsal segment; (2) Periphallic Organ: E-Epandrium; P-Primary Claspers; S-Secondary Clasper; C-Anal Cercus; (3) Male reproductive system: T-Testis; V-Vasa deferentia; A-Paragonia; D-Anterior ejaculatory duct; S-Sperm pump; (4) Female reproductive system: O-Ovaries; D-Oviduct; D'-Common oviducts; S-Spermathecae; P-Paravaria; V-Vagina; (5) Phallic Organ: E-Edeagus; A-Anterior gonopophysis; S-Spines; N-Novasternum; O-Basal apodeme; V-Ventral fragma; (6) Egg guide; (7) Egg; (8) Pupa
DNA extraction and quantification

Amplify COI using PCR

Gel check the PCR product

Sequence the PCR product

Analysis using Mega 5.1

Figure 3. DNA Barcoding and Molecular Phylogeny of *Drosophila hegddii*
Figure 4. CO I sequence of *Drosophila hegdii*
Figure 5. Sequence alignment (using ClustalW) of *D. hegdii*, *D. jambulina*, *D. vulcana* and *D. melanogaster*
Figure 6. NJ tree inferred from the concatenated sequences of *D. melanogaster*, *D. jambulina*, *D. vulcana* and *D. hegdii*. It is estimated that *Melanogaster* and *Montium* species groups were diverged at 41.3 Mya (Time scale was given in Mya), which is considered as calibration point (Tamura *et al.* 2004). MEGA 5 was used for constructing the NJ tree (1000 replications; model: Kimura 2-parameter; gaps: treated by pairwise deletion)
DISCUSSION:

Taxonomic status/Phylogeny of *Drosophila hegdii* basing on morphological and molecular markers:

The nature of the banding pattern of the abdominal tergites, the presence of 2 egg filaments and the puparia warrant *Drosophila hegdii* inclusion in the subgenus *Sophophora*. The presence of long ventral receptacle, coiled testis, convergent scutellars and two pairs of malphigian tubules qualify its inclusion in the *melanogaster* species group (Patterson and Stone 1952). Further the presence of sex comb extending beyond the tips of the tarsal joint, the presence of primary claspers and secondary claspers with curved black teeth permit its inclusion in the *montium* sub group (Bock and Wheeler 1972).

Basing on the morphological markers, unidentified species is recognised as a new species-*Drosophila hegdii* (Achumi et al. 2011). *Drosophila hegdii* resembles *D. vulcana* and *D. jambulina* in the general colouration of the body, but differed in other morphological characters such as the number of teeth in sex-combs, the nature of arrangement of teeth in the sex comb, the prominent teeth, sex comb extending beyond the tips of the tarsal joints, the prominent teeth in the secondary claspers, number of rows of acrostical hairs, wing indices, periphallic and phallic organ. In addition the new species differed from other known species of *montium* sub group in characters such as the number of teeth in sex comb, and abdominal banding pattern.

DNA barcoding is a taxonomic method that uses a short genetic marker in an organism’s DNA to identify it as belonging to a particular species (Hebert 2003a). It differs from molecular phylogeny in that the main goal is not to determine patterns of relationship but to identify an unknown sample in terms of a pre-existing classification (Kress et al. 2005). The most commonly used barcode region, for animals, at least, is a segment of approximately 600 base pairs of the mitochondrial gene cytochrome oxidase I (*COI*). DNA sequences are obtained for above mentioned marker and these sequences are compared to a DNA database to determine to which species or other taxonomic unit the specimen belongs. DNA barcoding is, in one form or another, widely used in conservation
genetics and molecular ecology (Duminil et al. 2006; Rubinoff 2006; Ward et al. 2008) but is also used in a number of other areas including forensic applications (Dawnay et al. 2007) and ancient DNA studies (Willerslev et al. 2007). It has often been associated with methods for delineating and defining species based on DNA evidence (Floyed et al. 2002; Hebert et al. 2003a; Remigio and Hebert 2003).

A final taxonomic system for the animal kingdom will probably include at least 10 million species partitioned among more than a million genera. Given such high diversity, there is a growing realization that it is critical to seek technological assistance for its initial description and its subsequent recognition (Godfray 2002; Blaxter 2003). Recent investigations have suggested the feasibility of creating identification systems reliant on the analysis of sequence diversity in small segments of DNA (Tautz et al. 2003). Hebert et al. (2003a) proposed a DNA barcoding system for animal life that is based upon sequence diversity in cytochrome c oxidase subunit I (COI). They established that diversity in the amino acid sequences coded by the 5’ section of this mitochondrial gene was sufficient to reliably place species into higher taxonomic categories (from phyla to orders). They also found that diversity in nucleotide sequences of the same gene region regularly permitted the inequity of closely allied species of lepidopterans, a group with modest rates of molecular evolution and high species diversity. As such, these insects provided a challenging test for the ability of COI diversity to resolve species boundaries (Hebert et al. 2003b).

In present study the extents of COI divergence for Drosophila hegdii, Drosophila jambulina, Drosophila vulcana and Drosophila melanogaster was examined by using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length = 0.05102 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) was shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. DNA barcoding based on standard markers (sequence) fragment of COI inferred phylogenetic tree based on the concatenated sequences of the four species support
the monophyly of the melanogaster complex. Melanogaster and Montium species groups diverged from one another 41.3 Mya ago (Tamura et al. 2004).

D. jambulina D. vulcana and D. hegdii belong to montium species subgroup of the melanogaster species group, and D. melanogaster belong to melanogaster species subgroup of the melanogaster species group. The tree indicates D. hegdii and D. jambulina belonging to the same cluster with strong bootstrap support of 86. The ancestor of D. vulcana and D. hegdii clade was estimated to have appeared about 0.02296 Mya, the divergent between D. jambulina and D. hegdii was estimated to be 0.02223 Mya.

The number of base differences per site between sequences is shown in Table 2. The evolutionary divergence between D. hegdii and D. jambulina was 0.11, D. hegdii and D. vulcana was 0.057 and D. hegdii and D. melanogaster was 0.088. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (10000 replicates). The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 898 positions in the final dataset.

Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (Table 3). Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in italics. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100, the nucleotide frequencies are A = 29.43%, T/U = 39.88%, C = 14.11%, and G = 16.57%. The maximum Log likelihood for this computation was -1027.464. The analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 519 positions in the final dataset.
The base composition different between sequences per site showed *D. jambulina-* *D. hegdii* was 0.013, *D. vulcana-D. hegdii* was 0.109 and *D. melanogaster-* *D. hegdii* was 0.025.

Thus speciation order seems to be similar with the morphological differentiation among the three species by a diagnostic morphological characters such as the number of teeth in sex-combs, the nature of arrangement of teeth in the sex comb, the prominent teeth, sex comb extending beyond the tips of the tarsal joints, the prominent teeth in the secondary claspers, number of rows of acrostichal hairs, wing indices, periphallic and phallic organ. In addition the new species differed from other known species of *montium* subgroup in characters such as the number of teeth in sex comb, and abdominal banding pattern. The nature of the banding pattern of the abdominal tergites, the presence of 2 egg filaments and the puparia warrant its inclusion in the subgenus *Sophophora*. The presence of long ventral receptacle, coiled testis, convergent scutellars and two pairs of malphigian tubules qualify its inclusion in the *melanogaster* species group (Patterson and Stone 1952). Further the presence of sex comb extending beyond the tips of the tarsal joint, the presence of primary claspers and secondary claspers with curved black teeth permit its inclusion in the *montium* subgroup (Bock and Wheeler 1972). Molecular analysis confirms the observation made through morphological markers that *Drosophila hegdii* is a new species.