DISCUSSION
6.1. Simuliids species in Arunachal Pradesh:
In the present study 17 species (including 4 unidentified) species belonging to three sub genera were recorded from Arunachal Pradesh. In World Black flies Inventory Adler and Crosskey (2015) reported 12 Simulium species from Arunachal Pradesh. These species are - *S.(S) indicum, S. (S) himalayense, S. (S) rufibasis, S.(S) nitidithorax, S.(S) novolineatum, S.(S) pradyai, S. (S) barnesi, S. (S) decuplum, S. (G) darjeelingense, S.(M) nemorivagum, S. (N) aureohirtum* and *S.(S) grisescens*. The species which were not reported earlier from the Arunachal Pradesh are *S. (N). praelargum, S. (G). metatarsale, S. (S). hirtipanus* and *S. (S). griseifrons*, however these species have been reported from other parts of India (Puri, 1932c, d; Datta *et al.*, 1975). The other unidentified 4 species might be new a record from the country. Only a few larvae of these species were collected which were reared to pupa and adult.

In the present study *S. (S) barraudi* Puri was observed as dominant species (20.21%) in Arunachal Pradesh. In an earlier study, *S. (S) himalayense* was reported as the predominant (>90%) species (Das *et al.*, 1985). However in the present study *S. (S) himalayense* was found to be only 9.08% of the total population of the Simuliids.

6.2. Simuliids species in Meghalaya:
Simulium species reported earlier from Meghalaya are *S. (S) rufibasis, S. (N) aureohirtum, S. (S) grisescens, S. (S) dentatum* and *S. (S) indicum* (Puri, 1932c, e, h; Lewis, 1974). In the present study only three species of Simuliids, *S. (S) barraudi, S. (S) striatum* and *S. (S) himalayense* were recorded and these species were recorded for the first time in Meghalaya. This may be due to ecological and seasonal changes (Datta *et al.*, 1975). It was found that *S. (S) barraudi* was the most dominant species (54.7%) in the state.
6.3. Simuliids species in Sikkim and Darjeeling area:

In Sikkim and Darjeeling Simuliids were collected from 18 places. Out of 18 study area 13 were in Sikkim and rest in West Bengal (Darjeeling district). In the present study 8 Simuliid species including 4 unidentified species have been observed. The identified species are *S. (S) himalayense*, *S. (S) rufibasis*, *S. (S) barraudi* and *S. (G) metatarsale*. Twenty eight Simuliid species reported earlier from Sikkim and Darjeeling are *S. (G) tenuistylum*, *S. (G) metatarsale*, *S. (S) indicum*, *S. (S) dentatum*, *S. (S) rufibasis*, *S. (S) himalayense*, *S. (N) praelargum*, *S. (M) dattai*, *S. (N) rufithorax*, *S. (N) aureohirtum*, *S. (S) nodosum*, *S. (S) kapuri*, *S. (S) ramosum*, *S. (M) ghoomense*, *S. (M) dasguptai*, *S. (G) williei*, *S. (G) sachini*, *S. (G) darjeelingense*, *S. (S) singtamense*, *S. (S) nigrifacies*, *S. (S) biforaminiferum*, *S. (S) ashisi*, *S. (S) tenuitarsus*, *S. (N) purii*, *S. (N) gracile*, *S. (M) yuntaiense*, *S. (M) nemorivagum* and *S. (S) nitidithorax* (Adler and Crosskey, 2015; Datta, 1973, 1974, 1975; Datta and Dasgupta, 1973; Takaoka, Thapa and Willie, 2010). In the present study only 8 species of Simuliids could be recorded. This may be due to lesser number of study area covered or due to seasonal and ecological changes of species (Datta et al., 1975). It was found that *S. (S) himalayense* and *S. (S) barraudi* were the most predominant species, 27.6 and 26.3% respectively in this area.

Twenty Simuliid species were recorded from all the study areas from Arunachal Pradesh, Meghalaya, Sikkim and Darjeeling. Out of 20 species, 7 species remained unidentified due to non availability of the pupal and adult stages. The unidentified species were classified as two species under *Nevermannia* subgenus, one species under *Gomphostilbia* and four species under subgenus *Simulium*. 
6.4. Diversity of Simuliids in Arunachal Pradesh, Meghalaya, Sikkim and Darjeeling:

Arunachal Pradesh, Sikkim and Darjeeling of West Bengal come under the Eastern Himalayan mega biodiversity hot spot and Meghalaya comes under Indo-Burma biodiversity hotspot (Mayer et al., 2000). In Arunachal Pradesh, the Margalef Index \(D_{Mg} = 1.627\) and specific richness \(S = 17\) show the highest diversity which may be due to various reasons. First, Arunachal Pradesh is the most extensive area facilitating landscape heterogeneity, altitude from 105-3377m AMSL, covering eastern Himalayan sub alpine coniferous forest. These conditions favour colonization of species and increase the likelihood of presence of suitable substrate/host on which they may feed. Another aspect which favours the combination of species is the water quality with low level of eutrophy and the high level of discharge (Rivas Martinez, 2004).

In Meghalaya, the Margalef Index \(D_{Mg} = 0.315\) and specific richness \(S = 3\) exhibit the lowest species diversity. At the time of present study there was a dry season for 3-4 months and all water bodies including streams and rivers dried out. Pramual and Wongpakam (2010) also observed marked difference in the Simuliid community structure between the dry and rainy season and species richness was significantly higher in the rainy season when the rivers and streams are faster and deeper. Moreover sample size in the study was also less in comparison to Arunachal Pradesh. Human related changes and discharge of waste water reduces the abundance of non-anthropophic Simuliid species (McCreadie et al., 2005). Various anthropogenic factors like coal and cement industry were the major environment hazard in Meghalaya that may decrease the diversity of Simuliids in the State.

The Sikkim and Darjeeling show moderate biodiversity, Margalef Index \(D_{Mg} = 1.078\) and specific richness \(S = 9\). This is may be due to the variation in species ecology and seasonal
changes (Datta et al., 1975). Earlier study reported 28 species of Simuliids from this region (Datta, 1973, 1974, 1975; Datta and Dasgupta, 1973; Takaoka, Thapa and Willie, 2010).

6.5. **Vertical distribution and substrate preference of Simuliids:**

Generally, it is believed that the Simuliid species are found only at high altitude. In the present study Simuliids were recorded at altitude ranging from 105-3377m AMSL in Arunachal Pradesh, 99-1910m AMSL in Meghalaya and 354-2356m AMSL in Sikkim and Darjeeling area. In Arunachal Pradesh maximum number of Simuliid species (10-11 species) was recorded at the altitude ranging from 300-699m AMSL. This altitude could be a favourable condition for colonization of Simuliids in Arunachal Pradesh. But in Sikkim and Darjeeling, maximum four Simuliid species were recorded at the altitude of 1500-1899m AMSL. In Meghalaya two Simuliid species were recorded at the altitude of 500-899m AMSL but at higher altitude (1700-1899m) only one species was observed. Jedlika (2006) has described the altitudinal distribution of three species of European Simuliids viz. *Prosimulium latimucro* Enderlein, *Twininia hydroides* Novak and *Simulium (N) oligotuberculatum* Knoz. According to the study, these species were found distributed at the altitude from 900-2600m above sea level. In the present study it was found that *S. (S) barraudi* and *S. (S) rufibasis* had broad range of altitudinal distribution (262-1499m AMSL) and found attached to all type of substrate available in water bodies. These species were found attached to leaves, stones, twigs, wood and plastic sheets. Das et al., (1988) evaluated the presence for artificial substrates by placing them in Simuliid larval habitat and found that polyethylene strip was the most preferred substrate for Simuliid larvae followed by banana leaves. A study conducted by Uzoigwe et al., (2013) in Mada River, Nigeria found that *Simulium damnosum s.l.* preferred trailing leaves (46.3%) and submerged dead leaves (39.8%).
In the present study also it was observed that most of the Simuliid species preferred trailing and submerged leaves (Table 5.3, 5.4 and 5.5).

6.6. Physico-chemical properties of Simuliids breeding water:

In Arunachal Pradesh, 85% water bodies (river/stream) were found to be positive for Simuliids suggesting that river and streams of Arunachal Pradesh are highly conducive for Simuliid breeding ($S= 17; D_{Mg} = 1.627$). 56% water bodies in Meghalaya were found positive for Simuliid. In Sikkim and Darjeeling, 72% water bodies (river/streams) were observed positive for Simuliids habitat. These results suggest that Meghalaya is comparatively less suitable habitat for Simuliid ($S= 3; D_{Mg} = 0.315$).

The range of pH where Simulium was present and absent was 6.74-7.90 and 7.11-7.62 respectively in Arunachal Pradesh. In Meghalaya, the range of pH where *Simulium* was recorded and not recorded was 6.54-7.62 and 3.78-7.71 respectively. The pH range where *Simulium* was present and absent was 7.2-7.92 and 7.4-8.03 respectively in Sikkim and Darjeeling. Among the three study area the lowest pH was observed in Meghalaya. The maximum pH was observed in Sikkim and Darjeeling area where there was no Simuliid breeding. There are many factors that can affect pH in water bodies, both natural and man made. In Meghalaya, both the factors are responsible for pH alteration. The hills of Meghalaya are full of carbonate rocks, moreover a lot of mining work is also going on in full swing. These factors affect the pH of water and inversely affect the Simuliid and other aquatic insect population. Recent *in situ* water pH study on *Simulium damnosum* by Sam-Wobo *et al.* (2014) reported that range of pH for *Simulium* abundance is 7.3-8.1. Earlier it was reported that a pH from 5.4-7.4 is conducive for Simuliid population (Opoku, 2000). The pH and altitude are important factors which affect Simuliid species richness and species distribution (Landeiro *et al.*, 2009). McCreadie and Adler (2006)
observed that pH is among the variables that contribute most to ecoregion separation of Simuliidae.

Dissolved Oxygen (DO) is also one of major important factor for aquatic organism. The range of DO where Simulium was present and absent was 4.20-7.77mg/L and 4.52-7.00mg/L respectively in Arunachal Pradesh. In Meghalaya the range of DO where Simulium was recorded and not recorded was 4.26-6.73mg/L and 4.21-6.72mg/L respectively. The DO range where Simulium was recorded and not recorded was 4.61-6.92mg/L and 4.8-6.13mg/L respectively in Sikkim and Darjeeling. Amongst all the study area the Maximum DO (7.77mg/L) was recorded in Arunachal Pradesh and the lowest (4.21mg/L) in Meghalaya. The water bodies having low dissolved oxygen range below 5.0-6.5mg/L indicate a mild degree of pollution (Anbalagan et al., 2011). Rivers/Streams with high dissolved oxygen of between 6.5 - 7.2 mg/L support the growth and development of Simulium (Ikpeama et al., 2006). Adeleke et al. (2011) found the sites positive for Simulium larvae had higher mean values of dissolved oxygen than the negative sites, although this result did not correlate with abundance of adult flies. Higher oxygen level might support an increase in the adult fly population (Crosskey, 1990 and Opara et al., 2008).

The rivers of Arunachal Pradesh have high water flow due to high rainfall which support the breeding and distribution of Simuliids. The pre-adult stages of the species were found to occur on trailing leaves, twigs, stone surfaces, stems, etc., which were about 5 cm below the water surface. The abundance and distribution of larvae and pupae is influenced by rainfall which causes an increase in the water flow, nutritive status of the river and decolonising algae leading to an increase in larval density. The water flow rate, pH and turbidity were the limiting parameters in the distribution of the pre-adult flies. A variety of factors (water temperature, velocity, streambed, dissolved oxygen concentration and vegetation) acting in concert, influences
black fly richness in Amazonas (Hamada et al., 2002). In the present study only three species were observed and each river recorded only one species in a limited sampling time suggesting that the number of species increases with the collecting time and efforts. However, there are regions that naturally support fewer species and a large number of samples fail to record an increase in species richness. Further, water variables form distinct eco-regions contribute most in the Simuliids species separation in different eco-regions. The overall river health is important for the distribution and richness of the Simuliid species which have biotic interactions acting as the primary determinants of species diversity (Opoku, 2006; Gotelli and Colwell, 2001). In the present study, the Simuliid distribution was not associated with altitude, however in the earlier studies altitude has been reported as major component in species richness and distribution. Freshwater ecologist believe that in addition to the certain measurable water variables, there are other parameters which either singly or in combination with each other limit the proliferation of pre-mature stages of Simuliids in the breeding habitat. Therefore, prevalence of aquatic stages in the rivers acts as biological indicator of the river health which is often considered analogous with human health (Begon et al., 1996, 2006). In Meghalaya, no Simuliid specimen was recorded from the seven river sites, two were near the cement factory, three had excavation sites and remaining two were near the open field coal mines. These rivers were receiving high effluent discharge through developmental activities. The industrial effluent waste discharged into the river disturb the microhabitat turning it unfit for the Simuliid breeding and growth. McCreadie et al. (2005) has reported that human related activities and discharge of waste water reduce the abundance of a non-anthropophilic Simuliid species.

Present study provides new information on Simuliid species distribution and its association with breeding water parameters. Turbidity, water flow and pH are important water parameters
affecting the Simuliid species prevalence and density in freshwater rivers. The study indicates that each Simuliid species prefer different sets of physico-chemical variables in breeding habitat which are specific to that particular species. Therefore Simuliid species community as a whole cannot be considered as a suitable indicator of the streams water quality. In addition to describing Simuliids, the information provided herein will be useful for the conservation of aquatic ecology and biodiversity in this environmentally important state of India.

6.7. Molecular characterization of Simuliid species:

In this study the rDNA ITS2 sequences of Simuliids was found to vary in length from 257 in S. (S) grisescens to 287 bp in S. (G) metatarsale (1 and 2) and S.(S) himalayense which is similar to the ITS2 sequences of other Simuliids and mosquitoes such as S. (S) vittatum (259bp) (Miller, Crabtree and Savage, 1997), An. quadrimaculatus complex (287-329bp) (Cornel, Porter and Collins, 1996), An. maculipennis complex (280-312bp) (Marinucci et al., 1999; Proft, Maier and Kampen, 1999). It was observed that the ITS2 sequences of all Simuliids had a high AT content (70.4-82.97%). These percentages are similar to values observed in Simuliids and other insects such as S. (S) vittatum (77.6%) and Culicoides variipennis (74.5%) (Miller, Crabtree and Savage, 1997), Drosophila melanogaster (80%) (Tautz et al., 1988), Musca domestica (74.9%) (Schlotterer et al., 1994), and Phlebotomus perniciosus (75.9%) (Muccio et al., 2000). In contrast, a high GC content was found in other Anopheline and Culicine mosquito species such as An. dirus A (69%) (XU and Qu, 1997), Ae. albopictus (56.4%) (Wesson, Porter and Collins, 1992) and Cx. Pipiens (58%) (Severini et al., 1996).

Variation in the average evolutionary divergence between species group was observed in the study which was found to be highest in unidentified species group [0.371±0.042 (d±SE)] and
lowest in *Nevermannia* species group 0.168±0.026 (d±SE). These results indicate that a large frequency of intraspecific and interspecific variability exist in the ITS2 of Simuliid species. Molecular Phylogenetic analysis using the neighbor-joining and maximum parsimony method revealed that all the three clades are well resolved. The subgenus *Nevermannia* is closely related to the subgenus *Gomphostilbia* compared to subgenus *Simulium* as found in both the trees. This finding is consistent with the existing morphological and behavioural data. The adult females of the subgenera *Nevermannia* and *Gomphostilbia* are ornithophilic (taking blood from birds) with toothed claws adapted for movement through feathers (Adler, Currie and Wood, 2004). Moreover, the larvae of subgenera *Nevermannia* and *Gomphostilbia* have ventral tubercles (Takaoka and Davies, 1995). On the other hand, adult females of subgenus *Simulium* are more typically mammalophilic and some anthropophilic with toothless claws (Crosskey, 1990) and no ventral tubercles in larvae (Takaoka and Davies, 1995).

In the clade with the subgenus *Simulium*, the molecular data in both the trees indicate that the *multistriatum* group is more closely related to *striatum* group than to the *tuberosum* group. The morphological data also support this finding. The number of pupal gill filaments for species in the *multistriatum* group and *striatum* group are 8 and 10 respectively. In contrast, the *tuberosum* group has 6 pupal gill filaments.

The mitochondrial COI of four Simuliid species of three subgenera were analysed. The average evolutionary divergence over COI sequence pairs of the three subgenera was 0.17± 0.02. The nucleotide diversity per site (π=0.135) and genetic diversity estimated from segregating sites (Θs= 0.124) were found to be higher. Thaijaren et al. (2014) has observed high level of genetic diversity at the molecular level in COI gene of *S. aureohirtum*, Thailand. DNA barcoding analysis based on the mitochondrial DNA COI sequences suggested that *S. aureohirtum* is likely
to be a species complex because of the high intraspecific genetic divergence (maximum K2P distance of 7.24%) (Pramual and Adler, 2014). Neighbor-Joining (NJ) and UPGMA analysis revealed that two sequences of *S. (N) aureohirtum* from Thailand shows 100% bootstrap value with one sequence of *S. (N) aureohirtum* India.

The mitochondria COII gene analysis between two subgenera, *Nevermannia* and *Simulium* showed nucleotide diversity per site ($\pi = 0.316$) and genetic diversity estimated from segregating sites ($\Theta_D = 0.245$). The rates of different transitional and transversional substitutions were found between 7.70 - 36.9% and 1.51 – 3.09%, respectively in COII sequences of two subgenera, *Nevermannia* and *Simulium*. Thajaren et al. (2014) reported high level of nucleotide diversity in COII gene of *S. aureohirtum* (0.034) compared to other black fly species complexes in Thailand, such as the *Simulium siamense* complex (0.0057), *Simulium feuerborni* complex (0.025) and *Simulium angulistylum* complex (0.0249) (Pramual and Kuvangkadilok, 2012).

Black flies provide an excellent example where species level identification and complex finding can be enhanced by implementation of a DNA-based identification system.

### 6.8. Herbal essential oil as repellent against Simuliids:

The plant based repellents might play an important role in reducing the biting nuisance and infections by preventing the man-fly contact (Curtis, 1992; Fradin, 1998; Fradin and Day, 2002; Usip et al., 2006; Spero et al., 2008) and to preclude any adverse effect that could emanate from the use of synthetic repellents (Osimitz et al., 1995; Goodyer and Behrens, 1998). Many plant materials and oils have been tested as potential insect repellents (King, 1954; Tawatsin et al., 2001) and are available in the market either single or in combination with other. The results of present study exhibited that topical application of essential oil extracted from some plants significantly reduced the biting rate of Simuliids species. Earlier, extracts of *Aristolochia indica*,...
Cassia angustifolia, Diospyros melanoxylon, Dolichos biflorus, Gymnema sylvestre, Justicia procumbens, Mimosa pudica and Zingiber zerumbet have been used as mosquito repellent and produced good results (Kamaraj et al., 2010), Volatile oils of some plants have also been proved effective as repellent against blackflies (Usip et al., 2006).

In the present investigation essential oil of Homalomena aromatica, Vitex negundo and Ageratum conizoides at 10% concentration repelled the blackflies for >5 h during their peak biting hours indicating their topical application could provide effective repellency for 5 to 6 h. In another study N, N diethyl-meta-toluamide (DEET) and p-menthane-3, 8-diol–based repellents have produced 8-10 h protection time against mosquito species whereas 2-3 h protection was reported by plant based repellent (Fradin and Day, 2002; Trongtokit et al., 2005; Zahir et al., 2009; Kamaraj et al., 2010). However, in the study > 2 h and > 5 h repellency was achieved at 5% and 10% concentration respectively with some of the tested essential oils suggesting that essential oils extracted from Homalomena aromatica, Vitex negundo and Ageratum conizoides were very effective against black flies in northeast India. These findings are similar to those reported elsewhere (Bhuyan et al., 1974; Kumar et al., 1984; Das et al., 1985; Malhotra et al., 1986; Usip et al., 2006). Prevention of vector borne diseases lies in the use of protective wearing in parasite infested areas and applying efficient repellents on exposed body parts (Usip et al., 2006). Present findings are in agreement with Aisen et al., (2004) and Usip et al., (2006) where volatile oils have produced protection for more than 3 h. However the studies carried by Das et al., (1985) revealed that 20% dimethyl phthalate and DEET are capable of producing protection uptill 6 and 7 h respectively whereas both at 10% concentration could produce protection time > 4 h. The efficiency of a repellent depends on various factors such as age, sex, vector species, temperature, rainfall and wind velocity etc. (Fraddin, 1998; Golenda et al., 1999). These factors
singly or in combination may result in varied repellency of an extract or oil even in the same individual. Therefore, a given repellent may not protect all the users equally and variation may occur in the ability of repellent to protect different subjects (Fradin and Day, 2002). The repellency time achieved by an essential oil should be taken as an indication of its effectiveness and not as absolute value. Various reports on the use of plant based materials for the management and control of various pest and vectors of disease have been documented. Herbal repellents provide a safe, eco friendly and inexpensive means to protect individuals from vector biting (WHO, 1995). The bio-chemical substances in plant based repellent do not accumulate in the food chain like other synthetic chemical compounds which are the major cause of environmental pollution (Malau and James, 2008). The testing of new compounds as insect vector repellent is of immense value in the preview of development of resistance in insect vectors.