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Distribution and Diversity of *Libellulidae (Odonata: Anisoptera)* from Indo-Burma Biodiversity Hotspot Region and their Phylogenetic Organization

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ABSTRACT

Mizoram represents an important part of the Indo-Myanmar biodiversity hotspot situated in the southernmost part of Northeast India. Direct searching and observation method with opportunistic sample collection was employed to survey the distribution of dragonfly family *Libellulidae*. The distribution according to altitudinal range and forest types has been established and correlated with humidity, rainfall and temperature. Twenty eight species of Libellulids are recorded in the present study; of these ten species are new records from the Northeastern Indian region. Higher diversity was confined to the lower altitudes of the Tropical Wet Evergreen forests. The phylogenetic relationship among the Libellulids was established using two mitochondrial genes CO1 and ND1. The molecular phylogeny was inferred by using maximum parsimony, maximum likelihood and the Bayesian methods. Among the eighteen genera analyzed, *Trithemis, Neurothemis, Tramea* and *Orthetrum* were recovered as monophyletic. The nucleotide distance between *Tramea limbata* and *Tramea basilaris* was found to be lowest and the highest was found between *Potamarcha congener* and *Neurothemis tullia* in the combined CO1 and ND1 gene.

INTRODUCTION

According to the checklist of Odonata of India there are 87 species of *Libellulidae* under 39 genera [1]. Lahiri recorded eight species of Libellulids under seven genera from Mizoram [2]. Mitra recorded 18 species in 13 genera of Libellulids from Mizoram [3]. The Zoological Survey of India recorded 24 species in 15 genera from *Libellulidae* family [4].

Libellulidae are one of the diverse groups of Odonata with a controversial phylogeny, which have been resolved to a certain extent by molecular dataset [5,6]. Eleven monophyletic subdivisions of *Libellulidae* are tentatively recognized as subfamilies [7]. The mitochondrial gene Cytochrome *c* Oxidase 1 (CO1) can serve as the core of a global bio-identification system for all animal phyla [8]. NADH Dehydrogenase Oxidase Subunit 1 (ND1) sequence analysis in dragonfly studies revealed strong interspecific and intraspecific differences in the population structures of all species and has been shown to be highly informative at different taxonomic levels in dragonflies [9,10].

Mizoram is located at latitude 21°58' and 24°35' N; 92°15' and 93°29'E longitude covering an area of 21,081 sq. km. Mizoram represents an important part of the Indo-Myanmar biodiversity hotspot situated in the southernmost part of North-East India [11]. The forest can be classified into three broad types Tropical Wet Evergreen Forest, Tropical Semi Evergreen Forest and Mountain Sub Tropical Forest [12]. The genetic relationships of the *Libellulidae* species found in Mizoram are not known yet and analysis of the phylogenetic work within *Libellulidae* may provide a framework for understanding the relationship between taxa.

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Hence, the present study was carried out to understand the diversity and distributional pattern of *Libellulidae* in Mizoram and to study the phylogenetic relationship among the Libellulids using two mitochondrial gene markers CO1 and ND1.

4. MATERIALS AND METHODS

Sample Collection

Opportunistic sample collection of the Libellulids was used and target species or group of species were observed visually [13]. All the specimens were identified with the help of identification keys provided by Fraser, Prasad and Subramanian [14,15].

Diversity and Distribution Study

The altitudinal range in the study area was divided into 4 categories [<100 m; 100-500 m; 500-1000 m; and >1000 m above sea level (asl)] in the state of Mizoram, Northeast India **(Figure 1)**. The number of species sighted during the survey between 2010 and 2012 were counted in the sampling areas and the Libellulids were categorized based on their abundance in the different altitudinal range. The distribution of each species in all the four altitudinal ranges have been generated from the data collected from all sampling sites. Shannon and Simpson diversity indices were calculated as a measure of diversity in each altitudinal range. Berger-Parker dominance has also been evaluated for each altitudinal range. For the data analysis Biodiversity Pro software was used [16].

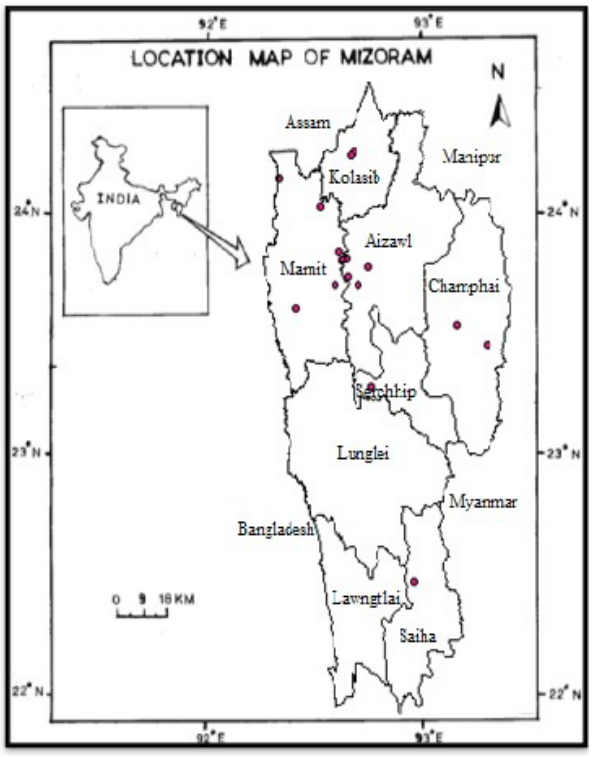

Figure 1. Mizoram map showing surveyed sites.

Sampling was done in altitudinal range below 100 m asl which was confined within Tropical Wet Evergreen Forest. In the Tropical Semi Evergreen Forest, the altitude ranged between 100 to 1000 m and the altitudinal ranges above 1000 m asl were found to be confined within Mountain Sub Tropical Forest. During the survey period (2010 - 2012), the monthly average rainfall, temperature and humidity in each forest types were recorded. The diversity of *Libellulidae* found in the three forests type was also generated using the same three diversity indices. The diversity of dragonfly was statistically correlated with the temperature, humidity, rainfall during the survey period.

Extraction of DNA and Sequence Analysis

Total genomic DNA was extracted from all individuals using modified protocol of [17]. The COI and ND1 gene were amplified using previously reported primer sequences [8,10]. The mitochondrial genes were amplified using PCR performed in a final volume of 25 µl. The PCR reaction mixtures contained 1X amplification buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTP, 0.2 pM each primer, 0.8 BSA and 0.5 U Taq DNA polymerase. The PCR thermal regime for amplification was 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 40 s at 43° - 58°C, 30 s at 72°C and a final elongation for 6 min at 72°C.

The PCR products were sequenced using Sanger's di-deoxy method (GCC Biotech, Kolkata). All the sequences were checked for contaminations using BLAST. Sequences were aligned and checked using Pairwise Sequence Alignment (EMBOSS-water, EBI) and FinchTV version 1.4.0 followed by manual adjustments. All the protein coding sequences were translated into amino acids and their ORFs checked [Sequence Manipulation Suite (Bioinformatics.org) and ORF Finder (NCBI)] [18].

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Phylogenetic Analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [19]. The sequences of CO1 and ND1 from each species were combined and aligned using ClustalW implemented in the program MEGA6 [19]. Relationships between individual taxa were assessed by maximum likelihood (ML) method with nucleotides distances (p-distance), transition/ trans version rate ratios and nucleotide diversity.

Maximum parsimony (MP) trees were obtained using PAUP* by heuristic search option with tree-bisection-reconnection (TBR) branch-swapping [20]. The number of bootstrap replicates was set at 100. Starting tree was obtained via stepwise addition and the number of trees held at each step during stepwise addition equals one. Steepest descent option was not in effect and the initial MaxTrees setting was 100 wherein gaps were treated as missing data. All characters were equally weighted, and zero length branches were collapsed to polytomies. Multistate taxa were interpreted as uncertainty, topological constraint was not enforced and the generated 50% consensus trees were saved.

Maximum likelihood (ML) analysis was performed with RaxML [21]. Phylip file was generated for RaxML analysis using ALTER Alignment Transformation Environment [22]. For ML analysis, the bootstrap was set at 100 and the model was set at GTR-GAMMAI.

Bayesian inference was performed with MrBayes v3.1.2 [23]. Bayesian posterior probabilities for the clades were obtained using Metropolis-coupled Markov chain Monte Carlo (MCMC) analysis as implemented in MrBayes. Trees were sampled every 100 generations and burn-in was assessed with Tracer v1.5 [24].

RESULTS

A total of 28 species under 18 genera Libellulids were found to be present in Mizoram and 10 species were record for the first time during the present study period of 2010 - 2012 **(Table 1)**. Shannon H' and Simpson's result showed that diversity was highest (1.315 and 21.474) below 100 m asl and in the altitudinal ranges between 100 - 500 m diversity was slightly higher (1.172 and 14.895) than 500 - 1000 m asl range (1.163 and 14.875). The least diversity (1.041 and 11.262) was found at an altitude above 1000 m asl. High dominance percentage (12.5) was found in the altitudinal range above 1000 m asl, in the altitudinal ranges between 100 - 500 m and 500 - 1000 m asl the species dominance percentage were 9.375 and 9.74 respectively and the lowest dominance percentage (8) was found below 100 m range **(Table 2)**.

Table 1. List of Taxa recorded for first time in Mizoram, Northeast India.

S No	Taxa name	Locality	District	Altitude
1.	*Aethriamanta brevipennis*	Lengpui MZU	Mamit Aizawl	100-500 100-500
2.	*Brachydiplax chalybea*	Buhchang	Kolasib	<100
3.	*Brachydiplax sobrina*	Buhchang	Kolasib	<100
4.	*Bradinopyga geminata*	Reiek MZU	Mamit Aizawl	100-500 100-500
5.	*Diplacodes nebulosa*	Buhchang	Kolasib	<100
6.	*Neurothemis tullia*	Buhchang Zawlnuam	Kolasib Mamit	<100 <100
7.	*Rhyothemis variegata*	Buhchang	Kolasib	<100
8.	*Tetrathemis platyptera*	MZU Lengpui Reiek Kolasib	Aizawl Aizawl Mamit Kolasib	100-500 100-500 100-500 <100
9.	*Tramea basilaris*	Kolasib	Kolasib	<100
10.	*Tramea limbata*	Lengpui	Aiza wl	100-500

Table 2. Diversity indices and Berger-Parker dominance among the different altitudinal zones.

Altitudinal Range in meter	Shannon H' Log Base 10.	Shannon Hmax Log Base 10.	Shannon J'	Simpsons Diversity (D)	Simpsons Diversity (1/D)	Berger-Parker Dominance (d)	Berger-Parker Dominance (1/d)	Berger-Parker Dominance (d%)
<100	1.315	0.966	0.966	0.047	21.474	0.08	12.5	8
100-500	1.172	1.23	0.952	0.067	14.895	0.094	10.667	9.375
500-1000	1.163	1.204	0.966	0.067	14.875	0.097	10.267	9.74
>1000	1.041	1.079	0.965	0.089	11.262	0.125	8	12.5

Altitudinal ranges below 100 m asl confined within Tropical Wet Evergreen Forest, was found to have the highest number of species diversity (1.32 in Shannon and 21.47 in Simpson), highest average humidity (78.18%) and highest average temperature (23.59°C) during the study period. Altitudinal ranges between 100 m and 1000 m asl confined within Tropical Semi Evergreen Forest was found to received highest average rainfall (337.42 mm) with an average temperature of 22.56°C and average humidity

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75.41% during the surveyed period, the diversity was 1.19 in Shannon and 14.64 in Simpson. The altitudinal ranges above 1000 m asl confined within Mountain Sub Tropical Forest was found to have the lowest species diversity (1.04 in Shannon and 11.26 in Simpson), lowest average temperature (20.64°C) and lowest average humidity (70.12%). In Tropical Wet Evergreen forest, the average rainfall received during the surveyed period was 238.81 mm and in the Mountain Sub Tropical Forest, it was 228.76 mm **(Table 3)**. The correlation between species richness, temperature and humidity was statistically significant but there was no significant correlation between species richness and rainfall.

Table 3. Diversity indices and physical factors in the three forest types during 2010-2012.

Forest type	Average Rainfall (mm)	Average Temperature (°C)	Average Humidity (%)	Shannon H' Log Base 10.	Simpsons Diversity (1/D)
Tropical Wet Evergreen	238.81	23.59	78.18	1.32	21.47
Tropical Semi Evergreen	337.42	22.56	75.41	1.19	14.64
Mountain Sub Tropical	228.76	20.64	70.12	1.04	11.26

CO1 and ND1 Data Analysis Using MEGA6

CO1 primers amplified approximately 750 bp long fragment of the mitochondrial genome of CO1 gene for all the 28 species of Libellulids identified in the present study. ND1 primer amplified approximately 580 bp long fragment of the mitochondrial genome, which includes fragments of 16S rRNA, the intervening tRNA leu region and the ND1 gene region. The sequences generated for the present study were submitted in GenBank **(Table 4)**.

Table 4. Voucher name and GenBank accession number given for the present study.

Name of species	Voucher Name	CO1	ND1
Acisoma paranorpoids	MZDF09	KC122228	KC197038
Aethriamanta brevipennis	MZDF18	KC287158	KC306707
Brachydiplax chalybea	MZDF19	KC287156	KC197041
Brachydiplax sobrina	MZDF20	KC287154	KC306708
Brachythemis contaminata	MZDF21	KC287157	KC197040
Bradinopyga geminata	MZDF01	JN817424	KC197039
Cratilla lineata	MZDF07	KC122226	KC197042
Crocothemis servilia	MZDF02	JN817425	KC197043
Diplacodes nebulosa	MZDF22	KC287155	KC197044
Diplacodes trivialis	MZDF25	KC287153	KC306710
Neurothemis intermedia	MZDF08	KC122227	KC197046
Neurothemis tullia	MZDF10	KC122229	KC197047
Neurothemis fulvia	MZDF04	JN817427	KC197045
Orthetrum pruinosum	MZDF17	KC122236	KC306711
Orthetrum sabina	MZDF15	KC122234	KC197048
Orthetrum triangulare	MZDF26	KC287152	KC306709
Orthetrum glaucum	MZDF13	KC122232	KC306712
Palpopleura sexmaculata	MZDF24	KP241936	KC306715
Pantala flavescens	MZDF03	JN817426	KC306714
Potamarcha congener	MZDF11	KC122230	KC306713
Ryothemis variegata	MZDF23	KC287151	KC197054
Tetrathemis platyptera	MZDF16	KC122235	KC306706
Tholymis tillarga	MZDF28	KJ499454	KC306716
Tramea basilaris	MZDF12	KC122231	KC197050
Tramea limbata	MZDF14	KC122233	KC197052
Trithemis aurora	MZDF05	JN817428	KC197049
Trithemis festiva	MZDF06	JN817429	KC197051
Trithemis pallidinervis	MZDF16	KJ499455	KC197053

The genetic distance using CO1 and ND1 data between the 28 species of Libellulids of Mizoram was generated. The nucleotide maximum distance (p-distance) between *Tramea limbata* and *Tramea basilaris* was found to be lowest at 0.07 and the highest p-distance was found between *Potamarcha congener* and *Neurothemis tullia* at 0.32 **(Table 5)**.

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Table 5. The ND1 genetic distance (p-distance) of the Libellulids of Mizoram.

Acisoma panorpoides																											
Aethriamanta brevipennis	0.20																										
Brachydiplax chalybea	0.15	0.20																									
Brachydiplax sobrina	0.20	0.20	0.19																								
Brachythemis contaminata	0.21	0.20	0.21	0.20																							
Bradinopyga geminata	0.18	0.17	0.18	0.19	0.21																						
Cratilla lineata	0.19	0.19	0.19	0.21	0.19	0.17																					
Crocothemis servilia	0.20	0.19	0.19	0.20	0.21	0.17	0.19																				
Diplacodes nebulosa	0.19	0.21	0.20	0.21	0.22	0.18	0.19	0.21																			
Diplacodes trivialis	0.20	0.23	0.23	0.22	0.22	0.19	0.20	0.20	0.22																		
Neurothemis fulvia	0.21	0.24	0.21	0.21	0.21	0.21	0.21	0.22	0.19	0.24																	
Neurothemis intermedia	0.20	0.23	0.19	0.20	0.21	0.19	0.19	0.19	0.20	0.24	0.14																
Neurothemis tullia	0.22	0.25	0.21	0.21	0.24	0.23	0.23	0.24	0.22	0.25	0.16	0.13															
Orthetrum pruinosum	0.17	0.18	0.17	0.20	0.19	0.18	0.14	0.17	0.18	0.22	0.20	0.19	0.22														
Orthetrum sabina	0.18	0.19	0.18	0.16	0.21	0.16	0.16	0.16	0.21	0.21	0.21	0.19	0.22	0.15													
Orthetrum triangulare	0.18	0.18	0.19	0.20	0.19	0.17	0.17	0.19	0.18	0.22	0.20	0.20	0.22	0.08	0.15												
Othetrum glaucum	0.16	0.17	0.18	0.19	0.19	0.16	0.16	0.18	0.18	0.21	0.21	0.19	0.22	0.11	0.13	0.10											
Palpopleura sexmaculata	0.19	0.22	0.20	0.22	0.23	0.17	0.19	0.19	0.20	0.22	0.20	0.20	0.22	0.18	0.18	0.19	0.19										
Pantala flavescens	0.17	0.20	0.19	0.20	0.20	0.21	0.17	0.18	0.22	0.23	0.22	0.19	0.22	0.17	0.18	0.18	0.18	0.18									
Potamarcha congener	0.25	0.24	0.25	0.25	0.25	0.24	0.23	0.22	0.26	0.27	0.30	0.27	0.32	0.23	0.23	0.22	0.21	0.25	0.25								
Rhyothemis variegata	0.17	0.18	0.18	0.19	0.20	0.17	0.17	0.18	0.20	0.20	0.21	0.19	0.24	0.15	0.16	0.17	0.14	0.18	0.17	0.23							
Tetrathemis platyptera	0.20	0.19	0.21	0.19	0.21	0.18	0.19	0.20	0.21	0.24	0.21	0.20	0.24	0.17	0.17	0.18	0.17	0.20	0.20	0.24	0.17						
Tholymis tillarga	0.20	0.18	0.20	0.21	0.20	0.17	0.20	0.20	0.21	0.22	0.22	0.20	0.23	0.18	0.17	0.19	0.18	0.21	0.21	0.26	0.17	0.19					
Tramea basilaris	0.18	0.20	0.21	0.19	0.22	0.17	0.20	0.19	0.21	0.21	0.23	0.21	0.24	0.20	0.20	0.19	0.18	0.22	0.20	0.25	0.19	0.21	0.22				
Tramea limbata	0.17	0.19	0.19	0.18	0.21	0.16	0.19	0.18	0.21	0.20	0.23	0.19	0.23	0.20	0.18	0.19	0.18	0.20	0.18	0.24	0.16	0.20	0.20	0.07			
Trithemis aurora	0.19	0.20	0.19	0.19	0.21	0.18	0.20	0.20	0.21	0.20	0.21	0.19	0.21	0.17	0.20	0.17	0.18	0.21	0.20	0.24	0.18	0.20	0.20	0.20	0.20		
Trithemis festiva	0.17	0.18	0.17	0.19	0.18	0.17	0.18	0.18	0.20	0.20	0.20	0.17	0.22	0.16	0.16	0.17	0.16	0.19	0.18	0.23	0.16	0.17	0.18	0.20	0.19	0.13	
Trithemis pallidinervis	0.17	0.19	0.20	0.18	0.20	0.16	0.19	0.20	0.18	0.22	0.22	0.20	0.23	0.17	0.16	0.17	0.15	0.20	0.18	0.24	0.16	0.10	0.18	0.20	0.17	0.19	0.16

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The estimated Transition/Transversion bias (R) is 1.005. Substitution pattern and rates were estimated under the General Time Reversible model (+G). A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories, [+G], parameter = 0.2603). The Maximum composite likelihood estimate of transitional substitution matrix between A/G = 13.7, T/C = 22.29, C/T = 8.65 and G/A = 8.66. The nucleotide frequencies are 27.72% (A), 39.45% (T), 15.30% (C), and 17.53% (G). Codon positions included were 1st, 2nd, 3rd and non-coding. There were a total of 947 positions in the final dataset and positions containing gaps and missing data were eliminated.

Phylogenetic Analysis among Libellulidae

The sequences of CO1 and ND1 were combined and in Parsimony analysis a total of 947 characters were included; 475 characters were constant and the number of parsimony informative characters was 367. Bayesian analysis for the combined gene set was set at 10 million generations resulted in trees. The effective sample size was 2057.8059 and LnL score was ☐10988.589. The first 20% trees were considered as the burn-in phase and discarded.

The phylogenetic analysis supported six major clades **(Figure 2)**. Clade 1 contained all the three *Trithemis*, Clade 2 included *A. brevipennis* and *P. congener* as sister clade; *B. contaminata* and *T. tillarga* as sister clade and a separate *D. trivialis*. Clade 3 included *D. nebulosa*, *P. sexmaculata* and *B. geminata* which formed a sister clade with *C. servillia* and clade 4 having all the four *Orthetrum* which formed a sister clade with *C. lineata*. Clade 5 has the two *Tramea* forming a sister clade with *R. variegata* and *P. flavesence* and clade 6 included the three *Neurothemis* which formed a sister clade with *A. paranorpoides* and *B. chalybea* and together they formed a sister clade with *B. sobrina* and *T. Platyptera.*

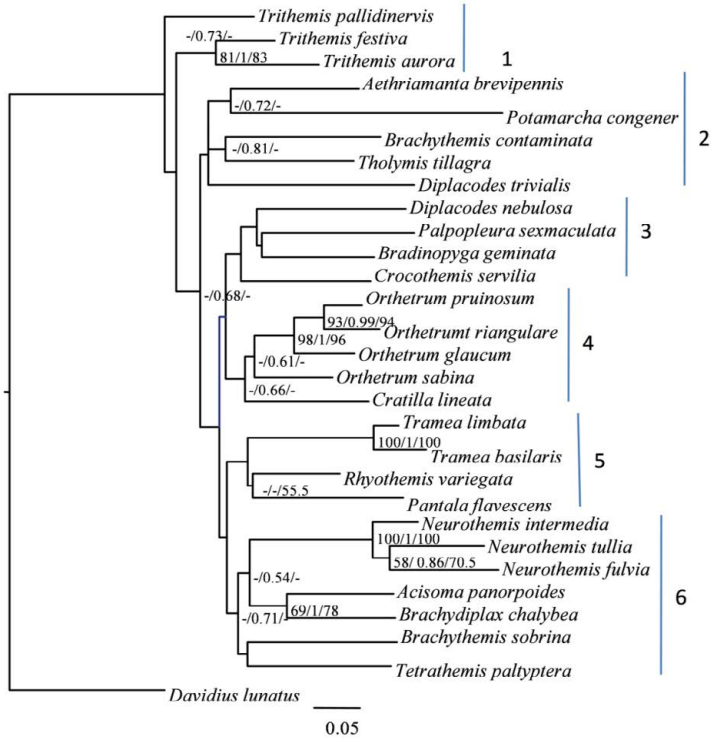

Figure 2. Maximum likelihood tree based on ND1 and CO1 sequence of *Libellulidae*. Clades with more than 50% support value in the order ML, Bayesian and MP are shown.

DISCUSSION

The present study included 28 species of the *Libellulidae* which is 34% of the *Libellulidae* found in India, of these ten species are new record from the area. Higher diversity was confined to the lower altitudes of the Tropical Wet Evergreen forests. A significant correlation between species richness with temperature and humidity was found but rainfall did not significantly affect the species richness.

The distributions of *Libellulidae* are widespread and are represented by more than 1000 species and attempt has been made to organize the *Libellulidae* into subfamilies using molecular and morphological characters. In the combined gene of CO1 and ND1, transitional substitution matrix between C/T was highest and the nucleotide frequencies were high in AT which is typical for arthropods [25]. The nucleotide maximum distance (p-distance) between *Tramea limbata* and *Tramea basilaris* was found to be lowest and but highest value was between *Potamarcha congener* and *Neurothemis tullia*.

Among the 18 genera of the Libellulide of Mizoram, six of them are represented by more than one species. The *Neurothemis* represented by three species and the *Tramea* represented by two species were recovered in one clade in all the analysis with high

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bootstrap value. *Trithemis* having three species were recovered in one clade in Bayesian analysis but the status of *T. pallidinervis* was not conclusive since the support value of Maximum Likelihood and Maximum Parsimony analysis was low. The *Orthetrum* represented by four species were also recovered as monophyly but the bootstrap value of *O. sabina* was low in ML and MP analysis and *C. lineata* was included in the clade with low bootstrap value. The *Brachydiplax* represented by two species were not recovered as monophyly in any of the analysis but remained in one group. The *Diplacodes* represented by two species were recovered as monophyletic only in Bayesian analysis. *A. paranorpoids* and *B. chalybea* seems to form a close relationship with good support value in all the analysis of the 11 subfamilies of *Libellulidae* recognized by Carle et al., nine subfamilies were included in the present study. *Zyxommatinae* represented by *Brachythemis* and *Tholymis* were in one clade, *Palpopleurinae* represented by six genera were found in two separate groups one group containing *Diplacodes, Palpopleura, Bradinopyga* and *Crocothemis*, another group containing *Neurothemis* and *Acisoma*. The *Libellulinae* represented by *Orthetrum* and *Cratilla* remained in one group but *Potamarcha* was in a different clade. *Pantalinae* represented by *Trithemis* and *Pantala* were not recovered in one clade [7,26].

CONCLUSION

The present study revealed the diversity and distributional patterns of the *Libellulidae* in Mizoram and the phylogenetic analysis from this area may contribute to the classification of the family *Libellulidae* into a smaller group and offered a better understanding of the diversity within the mitochondrial genes.

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