4.0 Discussion

4.1 Diversity and Distribution of Odonata in Selangor

The sampling sites in Selangor produced 54 species belonging to 9 families. Libellulidae (Suborder: Anisoptera) was the most abundant family of odonates in Selangor, followed by Chlorocyphidae > Euphaeidae > Calopterigidae > Coenagrionidae > Platycnemididae > Aeshnidae > Ptotoneuridae > Amphipterigidae. The family group Libellulidae is commonly well represented in previous work conducted by other workers. For example, a study that was done by Rehn (2003) reported that the two largest families were Libellulidae and Coenagrionidae, and were believed to be relatively recent and well-known.

These two families were dominating the unshaded habitats of stagnant water which included a species with the greatest migratory capacity, *Pantala flavescens*. Another study conducted locally by Norma-Rashid (2010), in Bachok Coast, Kelantan found that Libellulidae was the highest number of species which made up of 75% of the sampling while family Coenagrionidae was the second largest representative. Other results are also reported in other studies were in parallel with the finding in this finding where Libellulidae appeared to be the predominant family in diversity samplings (Lim & Furtado, 1975; Asahina, 1993; Hamalainen, 1994; Gupta *et al.*, 1995; Norma-Rashid, 1995a, 1995b, 1998, 1999; Norma-Rashid *et al.*, 2001; Subramanian *et al.*, 2008). The abundance of Libellulidae and Coenagrionidae in the present study might be also due to their shorter life cycles (Norma-Rashid *et al.*, 2001) and tolerant to the wide range of habitats (Gentry *et al.*, 1975; Samways, 1989).

Frequency distribution of species from 1298 known individuals that were collected in this work showed that *Euphaea ochracea* was the most abundant species followed by *Neurobasis chinensis* and *Neurothemis fluctuans. Euphaea ochracea* is known to exist in forest streams, river or near waterfall, and they mostly breed in running water (Lok & Orr, 2009). On the other hand, *Neurobasis chinensis* was found in moderate to swift flowing clear forest streams (Orr & Hamalainen, 2007) and widely distributed insect in South and South-East Asia (Hamalainen, 1992). However, *Neurothemis fluctuans,* are more widespread found in the open ponds (Norma-Rashid, 2010) and reported to be one of the most common species found in Malaysia (Lieftinck, 1954; Norma-Rashid *et al.,* 1996). Shelton & Edward (1983) explained that the common species have more individuals compared to the rare species due to their ability to survive in the existing environmental conditions.

In terms of number of individuals, the suborder Anisoptera recorded 701, while 597 from suborder Zygoptera. Anisopterans are more abundant as they are noted for their high dispersal ability (Batzer & Wissinger, 1996; Williams, 1997; Lawler, 2001; Kadoya *et al.,* 2004; Arulprakash & Gunathilagaraj, 2010) and their adaptability to the wide range of habitats (Hodgkin & Watson, 1958; Suhling *et al.,* 2004, 2005). Likewise, the less abundant of Zygopterans are probably due to their limited dispersal patterns (Weir, 1974) and intolerant to the undulating environment provided by the temporary water bodies (Williams, 1997; Kadoya *et al.,* 2004).

4.2 Preferred Habitats of Odonates in Selangor Areas

Ecological indicators can be demarcated as a taxon or community that reflects the biotic or abiotic state of an environment (Hodkinson & Jackson, 2005). Odonates are known to exploit a wide range of aquatic habitats and they are characterized as excellent habitat indicators due to their complex habitat requirements in specific species (Corbet, 1999). Identifying the habitat types based on species presence has potential applications in terms of choosing and assessing the species as indicators (Sato & Riddiford, 2007).

This study reveals that the highest number and species recorded across all the localities were for the site SS7 which contained 12 species with 159 numbers of individuals. In contrast, SS22 had the lowest count. The high species diversity in SS7 areas could be due to the presence of rivers and forest streams and may also be attributed to the availability of many different microhabitats for the species to thrive and divide themselves spatially, which is supported by the studies done by MacArthor (1965) who stated that high species abundance was related to diversification in ecosystems.

Correspondingly in this study, the habitat clusters are found to fit into the tropical lowland rainforest and the open areas (previously discussed). The suborder Anisoptera made up 412 individuals from the tropical lowland rainforests while 277 from the open areas. On the other hand, the suborder Zygoptera recorded 437 individuals from the tropical lowland rainforests and 148 from the open areas. A paired-samples t-test that was conducted to compare the distribution of odonates between those

two areas suggested that there is variability of number species and total of individuals in OP and TLR.

The species composition of Odonata in the tropical lowland rainforest (TLR) was higher compared to the open area (OA) due to the complexity of landscape provided by the TLR areas not only in terms of vegetation structure but also bottom substrate, water flow, canopy cover and other variable physical parameters. Hawking & New (1999) reported that the habitat structures affects the odonate community within an area and other factors such as physical-chemical parameters of the rivers, or availability of the food sources have impacts on the distribution of the odonates (Furse *et al.,* 1984; Askew, 1988).

A paired-samples t-test was conducted to compare the distribution of odonates in open areas (OP) and tropical lowland rainforest (TLR) study sites. There was a significant difference in the scores for OP (M = 8.49, SD = 14.62) and TLR (M = 16.94, SD = 24.47); t (50) = -2.00, p = 0.051. These suggest that there is variability of number of species and total of individuals in OP and TLR.

The biological indices indicate that species richness between the two categories of the study sites supported the findings. TLR demonstrated the higher value of richness index (R) with 7.26 compared to OA with only 4.46. This R index refers to the different species of Odonata in that certain area representing the observed species richness in an ecosystem. However, this R index is measure on its own, and it takes no account of the number of individuals of each species present. It gives as much weight to those species represented with few individuals as to those with higher individuals.

The diversity index, H index, was higher for TLR areas in contrast to the OP areas which was 3.22 and 2.83, respectively. This index offers information on community composition and structure rather than simply species richness, which also take into account the relative abundances of different species. The evenness index (E) values at TLR: 0.81 and OP: 0.71, which is a measure of the relative abundance or proportion of individuals among the species.

Many researchers believed that the indices of diversity could give better information implications about the environmental conditions under which the organism live than by considering individual sole taxon (Gaufin, 1973; Hawkes, 1979; Teles, 1994). The results from the current work found higher richness index (R) value in the tropical lowland rainforest compared to the open areas. Similarly, diversity indices (H') showed higher value in the tropical lowland rainforest than in the open areas.

One of the most important components of biodiversity would be the actual number of species defined as species richness, but according to Palmer (1995), it would be impossible to develop complete species lists for areas of more than a few hectares in size. The accuracy of index would depend on how well species were caught and identified (Cook, 2008).

On the other hand, chemical parameters such as acidity (pH) and dissolved oxygen (DO), and physical parameter such as water temperature can influence the composition, diversity and distribution of Odonata larvae in habitats (Corbet, 1999). In Selangor, for the tropical lowland rainforest (TLR) have a good pH value ranged compares to open areas (OA), which the value was towards acidic.

In general, according to Corbet (1999) the pH range of the water ranges was from 4 to 9. However, Pollard & Berril (1992) had found that dragonfly species can tolerate a pH range of 3.25 to 8.0. For examples, *Gomphid ophiogomphus* spp. can live in water pH 4.2, but the distribution of this species will be limited when the pH dropped to 3.4 ranges (Hellawell, 1986). *Coenagrionid erythromma najas*, *Corduliid cordulegaster boltonii* and *Somatochlora metallica* live well in the range of pH 4.6 to pH 6.4. Nevertheless this species can adapt and survive in high pH streams. Muller (1986) in the Corbet (1999) has documented the suitability of these four species live in the water alkaline with a pH of 8.

For the dissolved oxygen (DO), TLR had recorded the higher concentration, oppositely to the OA, which have the lowest concentration until reached 1.9 ppm. In aquatic ecosystems, oxygen availability is one of the limiting factors of the survival of insect larvae, such as Odonata (Gaufin *et al*., 1974; Corbet, 1999; Hoback & Stanley, 2001; Apodaca & Chapman, 2004). Besides, dissolved oxygen is a key requirement for life in aquatic habitats and become an important parameter in determining the quality of water (Hilsenhoff, 1988). According to Dodds (2002), waters of the swift current have a high content of dissolved oxygen rather than slow torrent river with a muddy bottom which contains a small amount of dissolved oxygen due to filled by sediment organic matter.

Besides DO and pH, the surface water temperature also influenced the distribution of odonates. But according to Macan (1974), temperature may not be a major factor controlling aquatic insect distribution. In TLR and OA, the mean of water temperature recorded was about the same. The water temperature can be effective

positive or negative depending on the degree of water at the area. In tropical regions and in the desert, water temperatures exceed 30 ^{o}C (Hart *et al*., 1990). For example in Africa, larvae *Platycypha caligata* (Chlorocyphidae) inhabited the river temperature of 35^{o}C while in North America, *Hetaerina americana* (Calopterygidae) was found at a temperature of 30^{o}C (Silsby, 2001). In Peninsular Malaysia, *Neurothemis tullia* (Libellulidae) can live on rice field water temperature which reached until 40^{o}C (Che Salmah, 1996) whereas in the Philippines, *Chlorocypha straeleni* (Chlorocyphidae) can live in river temperature 5^{o}C to 25^{o}C (Corbet, 1999).

However, the results of diversity and distribution of odonates in Selangor contribute as bioindicators for the biodiversity and environment in that state. This is because the possible disappearance of certain species in the area may implicate habitat destruction of which some of the causal factors as listed in the Malaysian Wetland Directory (1987) were: shifting cultivation, possible pollution, destruction of watershed, logging operations, errosional and siltation that could have occurred.

Moreover, the presence of the odonates is generally perceived to indicate a healthy ecosystem (Corbet, 1999) and this group has already been identified as 'flagships' within the field of conservation biology (Sahlén & Katarina, 2001). Several researchers reported the values of odonates as a source of indicator are: Carle, 1979; Moore, 1984; Schmidt, 1985; Castella, 1987; Clark & Samways, 1996).

In spite of this, the distribution and composition of insects such as Odonata in a community reveal changes from time to time following the change of environmental factors (Lenat, 1993). Global changes and principally climate warming are likely to

have various impacts on Odonata and this may lead to many Odonata species are extending their geographical area and also increase in species richness. Lately, it has become evident that many odonates of temperate regions are responding to global climate change by shifting in distribution and in phynology (Ott, 2001).

Though, the habitat structures can affect the suitability of an area for these odonates (Hawking & New, 1999) and other factors such as physical-chemical parameters of the rivers, or availability of the food sources may also lead to the distribution of the odonates (Furse *et al.,* 1984; Askew, 1988). Thus, the presence or absence of certain species of Odonata would mirror human activities surrounding the water habitats whether as positive or negative impacts (Rith-Najarian, 1998; Sahlén, 1999).

Hence, in this study, the richness of odonate community in Selangor was confined to those groups of species because of the suitability and adaptability of the species to their habitats. The understanding on ecology, habitat use and diversity of odonate communities of different land use types is very important in developing a wetland bio monitoring technique.

4.3 Analysis and Characterization the Potential of *ND1* Genes

4.3.1 PCR Amplification & PCR Purification

From all the samples collected and studied, there were 40 of the odonates were successfully amplified, purified and sequenced. Part of the failure could be caused by the poor quality of the samples or also could be caused by the inappropriate PCR techniques. Besides, other factors predicted to have influenced to those errors were included the unbound primers to the template DNA. This could be the case for the certain species of odonates in which the primer binding site is not homologous to others.

Poor quality of the samples collected may contribute to the difficulty in the extraction of DNA process and quality of the product obtained. In this study for some samples, the extractions were repeated several times in order to get the DNA, but unfortunately with the similar results. It was suspected that the difficulty in DNA extraction of certain samples was due to the lack of DNA of the samples or inappropriate preservation method.

At the initial stages of this study, the PCR amplification did not show satisfying results. The unspecific band may be due to random priming as proposed by Palumbi (1996) where for the organisms that with a large genome, there is likely to be a region that possibly will anneal with any primer. Thus, when the primer anneal at the unexpected site, it will produce unwanted products. To overcome this problem, the annealing temperature should be increase in order to increase the primer specificity.

In addition, to obtain good amplified products, several factors and conditions should be taking into consideration and need to be monitored throughout the procedure. Roux (1995) stated that the most important aspect for successful amplification is regarded the cycling condition particularly the annealing temperature of the primer. The formula to calculate the ideal melting temperature (T_m) for the primer-template pairing was as follows:

$$\mathbf{T_m = (G + C)\ 4 + (A + T)\ 2}$$

Obeying to the above formula, the annealing temperature in this study for both primers P850 (fw) and P851 (rev) were premeditated to be 64^o C and 56^o C each. Nevertheless, for determining the best melting temperature, other factors which include the individual buffer concentration and also the primer-template concentration need to be inspected too besides the nucleotide composition.

Henceforth, in this study, the primer design and optimization of annealing primer temperature were not done and tested. The used of 49^o C of annealing temperature and the primer design were followed on Rach *et al.* (2007). This was due to the primer design and the primer temperature provided was presumed to be well suited based on the good quality of amplified products obtained.

Instead, if the results were unsatisfactory, minor adjustment was made for the volume Mg^{2+}, *Taq* polymerase and also on the template concentration. Mg^{2+} is a cofactor in enzymatic catalysis of the synthesis reaction. Titrating the Mg^{2+} concentration has been a common method which this can often increase the yield and also reduce the unwanted product besides increase the reaction efficiency (Palumbi,

1996). In numerous times when the non-good quality of bands were obtained after the adjustments had been made, faulty in the DNA extraction, enzyme degradation (*Taq* polymerase) were suggested to be the reasons.

4.3.2 Sequence Analysis

Sequence analysis results are depend on the quality of the chromatograms obtained. The good result of chromatogram data should present a distinctive peak reading from the other of noise peak. The present of uncertainty area such as the overlapping peaks of the same height to the original sequence, the gap between the chromatogram peaks and also messy background would mean the sequence cannot be determined and have to be repeated. The troubleshooting of this problem, presence of the uncertainty area indicates that the sample could probably be contaminated in the purification process.

Out of 40 samples studied were successfully sequenced and the sequence reads obtained are in range of 533 bp to 574 bp. Owing to the unclear result at the fragments ends close to the primers, the sequences were trimmed at the beginning (forward primer) and at the end (reverse primer) of the sequences. According to Hillis *et al.*(1996), the length of reliable reads depends on the model of the sequencer, the quality of the template and also the details of the sequencing reaction. Thus, in order to build up an optimum and reliable sequence of the samples, the information of forward sequence and also reverse sequence should be compared and aligned.

From the results of base nucleotide composition, the total alignment length was 544 bp or 544 sites. There were 458 variable sites (84.2%) with remaining of 75 conserved sites (13.8%). The singleton sites totalled was 153 (28.1%) with 305 parsimony-informative sites (56.1%). The studied of 16S rRNA, $tRNA^{Leu}$ and partial of *ND1* region have low conserved sites with only 13.8%. Henceforth, according to Pashley & Li (1992), insect's mtDNA are more conserved and less susceptible for mutation could be applied for this study case.

One of the possibilities due to this case is that the odonates have existed since 345 million years ago and have evolved into many diverse species. They are also among the most ancient of winged insects, dating back well into the Permian (Grimaldi & Engel, 2005). Their recognizable progenitors date to the Carboniferous (360-290 million years ago) and are probably the most widely known extinct insects. Thus, the insect order alone encompassed varieties of species with distinct morphology characteristics and the high evolution processes could have correlated with the degree of mutation.

The nucleotide compositions from all the samples collected were T (45.7%), C (11.0%), A (27.0%) and G (16.3%). As expected, this region of mtDNA was observed to have a strong AT bias (72.7%), which is characteristic of insect mitochondrial DNA (Crozier & Crozier, 1993), and also seemed to fulfill the criteria of Stewart & Beckenbach (2005) which stated that the average of AT content in insects is 77%.

In addition, from the results, the GC content was 27.3%. According to Chandra *et al.* (2006), the approximate amount percentage of GC content can also be observed in other orders of insect mtDNA such as Orthoptera, Caleoptera and Diptera with around 20-29% which parallel with this result.

Genetic distance values can reveal the genetic differences between species. From the pairwise divergence dataset, the lowest intra-specific genetic distance value was zero (0.00) for the species of *Trithemis aurora, Euphaea ochracea, Aethriamanta gracilis, Neurobasis chinensis, Zygonyx iris* and *Trithemis festiva* that are all within the same species.

This implies that the studied of the mitochondria region has the potential to distinguish organism at its lowest taxa level (species) and the variation among the species population do exist. Therefore, such criteria make the partial of 16S rRNA, $tRNA^{Leu}$ and *ND1* region become a potential and impending 'barcoding' region for the subspecies population variation.

The highest genetic distance value (1.503) was observed between the *Aethriamanta gracilis* from suborder Anisoptera with *Bactrocera dorsalis* (Order: Diptera) that was the outgroup used in this study. While, the highest genetic distance within Odonata, Anisoptera – Zygoptera, was 0.338 which between the species of *Neurothemis fluctuans* and *Libellago lineata* and this indicated the distance between the two suborders of Odonata.

4.4 Phylogenetic Relationship Among the Taxa

This study has revealed a more comprehensive phylogeny of odonates species using mitochondrial gene-partial regions of *NADH dehydrogenase 1* (*ND1*) and 16S rDNA as well as full sequence of intervening $tRNA^{Leu}$ region, which in dragonflies, the *ND1* sequences known to be greatly informative at the different and diverse taxanomic levels (Hadrys *et al.,* 2006; Dijkstra *et al.,* 2007; Groeneveld *et al.,* 2007).

In order to establish a trustworthy character-based DNA barcodes system, the samples should be subjected to the use of an appropriate genetic marker (Rach *et al*., 2007). Some researchers had attempted to use wing venation to resolve the relationships among the odonate species (Carle, 1982; Trueman, 1996), with limited success.

Up to now, in DNA barcoding studies, the region of mitochondrial genome *Cytochrome oxidase* gene *subunit 1* (*CO1*) has been the choice of reference marker (Hebert *et al*., 2004; Kress *et al*., 2005; Bely & Weisblat, 2006; Hajibabaei *et al*., 2006; Smith *et al*., 2006; Witt *et al*., 2006), but some studies have faced problems with a distance-based and single locus approach (Vences *et al*., 2005; Gomez *et al*., 2007). Due to that, *ND1* sequences were used since in dragonflies it has been known to be highly informative at different taxonomic levels (Hadrys *et al*., 2006; Dijkstra *et al*., 2007; Groeneveld *et al.,* 2007).

From the analysis of the phylogenetic, it could be inferred that Odonata contains a paraphyletic Zygoptera and a monophyletic Anisoptera. The relationships among the Zygoptera and Anisoptera was also investigated by Saux *et al.* (2003), and the

relationships among the suborders were consistent with the relationships that were found in this study.

Tillyard (1935) and Fraser (1957) also found that Anisoptera are clearly a monophyletic group. This finding is supported by the venational characters and structures of the secondary sexual apparatus of the dragonflies.

With specimens covering 22 locations within Selangor, Malaysia, with some that included both male and female, and from a wide scale of odonate populations, genera and species, the phylogeny appears to facilitate the species identification of Odonata. Thus, the character based of DNA barcoding may be well suited to identify the genetic entities at different taxanomic levels.

Although the data for the total of base pairs that were sequenced seem to be small, but this is further supported by the bootstrap value of >50%, and especially strongly supported by the clade containing the suborder Anisoptera in both of the NJ and MP trees. The database will also be able to assist in the species identification of the larvae of odonates since the morphology based characters may be difficult and frequently not exceeding the family level with certain genera not forming monophyletic groups (Artiss *et al.*, 2001).

Among the identified species, *Trithemis aurora* (Burmeister, 1839) commonly known as Down drop wing (Norma-Rashid *et al*., 2008) appears to be polyphyletic. *Trithemis aurora* with the voucher number E7, H89 and C9 formed their own clade, while *Trithemis aurora* A5, A10, C17 and GU323085 (Gen Bank) were related as a sister group with *Trithemis festiva* (Rambur, 1842), the common name being Indigo

dropwing (Norma-Rashid *et al*., 2008) with confidence level 82% in the NJ analyses and 97% in the MP analyses.

However, this DNA barcodes do not initiate that these potential units are definitely new species due to the same morphologically identified. Moreover, the distinction was not due to the geographical isolation because all the species were collected in the same state, Selangor.

Thus, this polyphyly could possibly be due to the ancestral polymorphism or convergent evolution, or may be accounted as a cryptic species. Hence, integrated and cohesive taxonomic approaches are obligatory to comprehend this species discovery process (Rubinoff, 2006a, 2006b).