2.0 RESEARCH METHODOLOGY

2.1 Sampling Sites

In this study, field samplings were carried out in targeted area of Selangor state to reflect representative samples from Selangor, including the federal territories of Kuala Lumpur (3° 8’ 8.52” N 101° 41’ 16.8” E) and Putrajaya (2° 55’ 00” N 101° 40’ 00” E). The sampling sites were categorized into two main types:

(1) open areas (OA) - these covered distinctive habitats utilized by dragonflies such as at the stagnant waters which included ponds, swampy areas, marshy areas, paddy field or drains

(2) tropical lowland rainforest (TLR) – these covered typical habitats for odonates for instance flowing waters which included streams, rivers and tributaries.

The sampling sites were selected based on the presence of microhabitats preferred by odonates and also specific hydrological characteristics such as the velocity and also the light intensity. The specific locations are shown in Table 3 and the descriptions for each site were listed in Table 3.1, while Figure 5 shows the mapping sites:

Table 3: Collection sites of odonates in Selangor, Malaysia.

	LOCALITIES	GPS READING
SS1	**Templer Park Forest Reserve, Rawang, Selangor**	03°29’ 872” N 101°61’ 746” E
SS2	**Ulu Gombak forest area, Selangor**	03°18’ 619” N 101°73’ 230” E
SS3	**Ulu Kali, Batang Kali, Selangor**	03°43’ 526” N 101°65’ 917” E

Table 3: Continued

SS4	**Sungai Tekali, Hulu Langat, Selangor**	03°10' 703" N 101°85' 805" E
SS5	**Taman Kemensah, Ampang, Kuala Lumpur**	03°21' 416" N 101°75' 997" E
SS6	**Ulu Yam, Batang Kali, Selangor**	03°43' 173" N 101°65' 667" E
SS7	**RimbaIlmu, Universiti Malaya, Kuala Lumpur**	03°13' 131" N 101°65' 727" E
SS8	**Taman Tasik Shah Alam, Selangor**	03°07' 293" N 101°51' 384" E
SS9	**Morib, Banting, Selangor**	02°75' 453" N 101°44' 811" E
SS10	**Bukit Gasing, Petaling Jaya, Selangor**	03°09' 657" N 101°65' 702" E
SS11	**Kuala Selangor, Selangor**	03°33' 497" N 101°25' 802" E
SS12	**Sekinchan, Selangor**	03°50' 981" N 101°10' 398" E
SS13	**TanjongKarang, Selangor**	03°42' 419" N 101°18'454" E
SS14	**Sungai Congkak Forest Reserve, Hulu Langat, Selangor**	03°20' 925" N 101°82' 595" E
SS15	**Sungai Gabai, Hulu Langat, Selangor**	03°20' 976" N 101°86' 523" E
SS16	**Felda Sungai Tengi, Kuala KubuBaru, Selangor**	03° 34' 00" N 101° 39' 00" E
SS17	**Taman Putra Perdana, Putrajaya**	02° 55' 00" N 101° 40' 00" E
SS18	**Kampung Baharu Sungai Pelek, Sepang, Selangor**	02° 39' 00" N101° 43' 00" E
SS19	**Kampung Sungai Burong, SabakBernam, Selangor**	Data Not Available
SS20	**Pulau Tengah, Selangor**	02° 56'283" N 101° 15' 256" E
SS21	**PulauPintuGedung, Selangor**	02° 56' 358" N 101° 15' 515" E
SS22	**PulauKlang VGR, Selangor**	03° 36' 558" N 101° 20' 166" E

Table 3.1: The descriptions of each sampling site together with the hydrochemistry readings.

SAMPLING SITE	DESCRIPTION OF HABITATS	TYPE OF HABITATS	DO	pH	°C
SS1	Provided varieties of substrates which consist of stones, rocky, cobbles and sandy. Water surface was covered by canopies of trees and shrubs. Slow and fast flowing water.	Tropical lowland rainforest	115.9% 8.51ppm	6.87	31.7
SS2	An open area in the forest, pond with more sunlight, and bordered by varieties vegetation.	Open area	125.6% 10.53ppm	5.99	24.1
SS3	The flow was relatively fast and has clear water. The river is largely shaded and with some sunny spot.	Tropical lowland rainforest	109.5% 8.50ppm	7.09	33.8
SS4	The river was in forested area with fast-flowing and clear water. Rich of substrates like stone and big rocky.	Tropical lowland rainforest	123.6% 10.50ppm	7.35	29.7
SS5	A small intermittent stream with varieties substrate like sandy and muddy and have more sun and vegetation.	Open area	100.4% 8.27ppm	5.99	26.3
SS6	River in forested area. The flow was relatively fast and with clear water. Consist of stones, rocky and sandy.	Tropical lowland rainforest	110.4% 8.49ppm	6.76	30.2
SS7	Largely shaded area and have clear shallow water. Have some sunny spot and vegetation.	Tropical lowland rainforest	117.3% 8.66ppm	6.04	35.2
SS8	Pond, stagnant waters, and totally exposed to the sunlight. Open area.	Open area	-	-	-
SS9	Swampy habitat with stagnant to slow flowing water. Area was exposed to the sunlight.	Open area	-	-	-

Table 3.1: Continued.

SS10	River in forested area. Shallow water with sands and cobbles. The water ran slowly and passing diversified habitats along river.	Tropical lowland rainforest	118.6% 8.69ppm	7.09	30.1
SS11	Irrigation channels area around the paddy fields. Muddy areas with totally exposed to the sunlight. Open area.	Open area	45.7% 2.70ppm	6.02	32.5
SS12	Irrigation channels area around the paddy fields. Muddy areas with totally exposed to the sunlight. Open area.	Open area	45.9% 2.74ppm	5.75	32.7
SS13	Irrigation channels area around the paddy fields. Muddy areas with totally exposed to the sunlight. Open area.	Open area	45.1% 2.62ppm	4.80	35.2
SS14	Provided varieties of substrates which consist of stones, rocky, cobbles and sandy. Water surface was covered by canopies of trees and shrubs. Slow and fast flowing water.	Tropical lowland rainforest	116.2% 8.18ppm	7.12	24.1
SS15	Fast-flowing water in the forest. Have clear water and consist of stones, rocky and cobbles as well as sandy.	Tropical lowland rainforest	105.4% 7.13ppm	7.47	24.5
SS16	Swamps in forest. The water was stagnant to slow flowing and slightly shaded with some sunny spot.	Tropical lowland rainforest	-	-	-
SS17	Open area. The water was stagnant, pond and totally exposed to the sunlight.	Open area	-	-	-

Table 3.1: Continued.

SS18	Small running waters in the forest. The channel was narrow and slow-flowing.	Tropical lowland rainforest	-	-	-
SS19	The rivers bordered by the degraded forest, swampy habitat. Slightly exposed to the sunlight.	Tropical lowland rainforest	-	-	-
SS20	Swamps in island and exposed to the sunlight. The water was slow flowing and muddy.	Open area	23.80% 1.90ppm	6.46	28.4
SS21	The channel was with slow flowing water and very muddy. Exposed to sunlight bordered by vegetation.	Open area	56.60% 4.34ppm	6.56	30.3
SS22	A swampy habitat with small running waters in the island. Have more sun and vegetation.	Open area	46.10% 3.48ppm	6.49	29.8

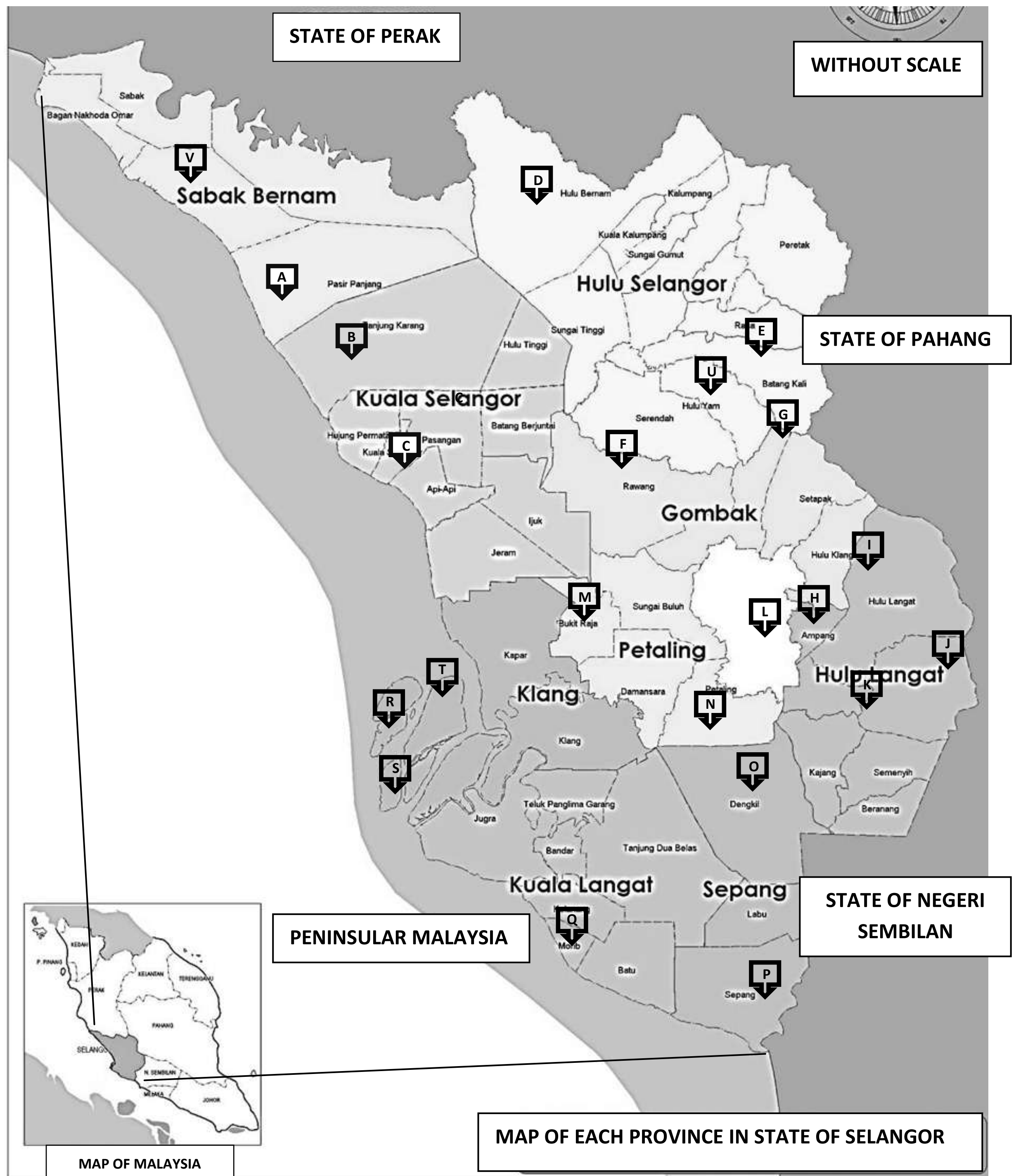

Figure 5: Map of sampling sites.

A:Sekinchan, **B:**TanjongKarang, **C:** Kuala Selangor, **D:** Felda Sungai Tengi, Kuala KubuBaru, **E:** Ulu Kali, Batang Kali, **F:** Templer Park, Rawang, **G:** Ulu Gombak, **H:** Taman Kemensah, Ampang, **I:** Sungai Congkak, Hulu Langat, **J:** Sungai Gabai, Hulu Langat, **K:** Sungai Tekali, Hulu Langat, **L:**RimbaIlmu, Universiti Malaya, **M:** Taman Tasik, Shah Alam, **N:** Bukit Gasing, Petaling Jaya, **O:** Taman Putra Perdana, Putrajaya, **P:** Kampung Baharu Sungai Pelek, Sepang, **Q:**Morib, Banting, **R:** Pulau Tengah, **S:** PulauPintuGedung, **T:** Pulau Klang VGR, **U:** Ulu Yam, Batang Kali, **V:** Kampung Sungai Burong, SabakBernam.

2.2 Sampling Methods

Methods for sampling and preservation of Odonata were based on Orr (2004) and Borror & White (1970). Odonata were caught with light and strong insect net (Appendix 2) throughout the study areas. The samplings were done during month of January 2010 until March 2011 on hot sunny days between 10:00 and 15:00 hr. The long handle net about 25 cm diameter with an open-mesh net with little air resistance so that it can be swung rapidly in order to catch the sample.

This method requires a certain amount of speed and skill because the net should be swung at correct angles. The Odonata were grasped by their body and stunned by pinching the thorax after they was removed from the net. Subsequently, the caught specimens were placed in triangle envelope with the wings folded together above the body.

Data on collection and information such as locality, date, time and collector's name were written on the surface of the envelope. The microhabitats frequented by odonates were recorded at every site where odonates were sampled. In general, only one specimen was kept in each envelope to avoid damage to the specimen. However, for pairs caught in tandem they were placed in the same envelope to assist in identification.

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2.3 Preservation Method

Acetone has become the choice method for killing adult odonates and for assisting in the rapid desiccation necessary for good preservation. Acetone dissolves the fats and absorbs the water from specimens, and prevents the specimens from rotting (Mark, 1999). The dragonflies were soaked in the acetone for about 8-12 hours, depending on size and damselflies for 4 hours.

However in this study, specimens were not immersed in acetone. It is because acetone may destroy or damage the DNA, rendering specimens preserved in acetone was unsuitable for molecular biological research. Thus, the adult specimens were left in the envelopes for a day so that they can void their intestinal contents. Three legs from the right side were removed for DNA extraction purpose (will be discuss later). Some specimens were pinned and preserved for display purposes.

The specimen's wings were spread on pinning board for best results and in pinned specimens, all the legs were arranged so as not to obscure the genitalia located on the second abdominal segment of males (Appendix 3), while some specimens were kept remain in the envelope. All the specimens were thoroughly dried in the oven at 35° C overnight before storage, which were later deposited (Appendix 4) and catalogued in the collection, Museum of Zoology, University of Malaya and given specific catalogue numbers (Appendix 5).

2.4 Phylogenetic Study

2.4.1 DNA Extraction and PCR Amplification

DNA was extracted according to a modified standard protocol (Hadrys *et al.,*1992). Prior to the DNA extraction, the water bath was heated with a temperature of 56°C. For most of the time, sampling and DNA extraction were done on the same day to retain and ensure the quality of DNA.

As initial step, the legs of odonates were detached; normally three legs from the right side of the body were taken and placed in a 1.5ml microcentrifuge tube. The tissue samples were freeze-dried with liquid nitrogen for better homogenization. The legs were grounded using plastic pestles into powder form in order to decrease the lysis time.

Subsequently, 180µl of Buffer ATL was added into the tube and homogenized together with the tissue using an electronic homogenizer. Before placing the tube in the water bath, 20µl of Proteinase K was added into the mixtures which were then mixed by vortexing. The mixtures were incubated in a 56ºC shaking water bath with 220rpm. Lysis usually would be completed in 1 to 3 hours but also depending on the type of tissues processed. In this study, the samples were left inside the water bath for an overnight or at least 3 hours.

After 3 hours or more, all tubes were briefly centrifuged to remove drops from the lid. 8µl of RNaseA (50mg/ ml) was added into each sample to remove RNA and then mixed by pulse-vortexing for about 15 seconds, then followed by brief centrifuging. Before proceeding to next step, all samples were incubated for 2 minutes

at room temperature, then centrifuged again before adding 200µl of Buffer AL into each samples.

The mixtures were then mixed another time by pulse-vortexing for about 15 seconds and incubated in the heat block for 10 minutes with a temperature of 70º C. Formation of white precipitate would occur on addition of Buffer AL, and in most cases would dissolve during incubation at 70ºC.

For the following step, right after the incubation, all the samples were centrifuged to remove drops from inside the lid, and then continued with adding 200µl ethanol (96-100%) to the samples. The samples were then mixed again with pulse-vortexing and were briefly centrifuged.

The samples, Buffer AL, and the ethanol were mixed thoroughly to yield a homogeneous solution. Then, the mixtures were loaded into a QIAamp spin column with its 2ml collection tube excluded the precipitate and centrifuged at 6000 x g (8000 rpm) in order to reduce noise for 1 minute. The tubes containing the filtrate were discarded and placed the QIAamp Spin Column in a clean collection tube provided.

Subsequently, 500µl of Buffer AW1 which was diluted with 100% ethanol was added into the QIAamp Spin column without wetting the rim and centrifuged at 6000 x g (8000 rpm) for 1 minute. Once again the tube containing the filtrate was discarded and replaced with a clean 2ml collection tube. The same step was repeated with the Buffer AW2 diluted with 100% ethanol and centrifuged for 3 minutes at full speed (20 000 x g; 14 000 rpm) and the collection tube containing filtrate discarded and were placed in the QIAamp Spin Column in a new 2ml collection tube which was later centrifuged for 1 minute at the same speed.

Subsequently, the QIAamp Spin Column was placed in a new 1.5 ml microcentrifuge tube and added with 200µl Buffer AE. All the tubes were incubated at room temperature for 5 minutes and then centrifuged at 6000 x g (8000 rpm) for 1 minute. The step was repeated for the second elution but incubated for 10 minutes at the room temperature. Finally the QIAamp Spin Columns were discarded and the filtrates in the 1.5 ml microcentrifuge tubes were kept as DNA products.

As for PCR amplification, master mix solution was first prepared with the correct amount depending on the desired numbers of samples. All PCR were performed with the specific primers alt*ND1* (fw) 5' TTC AAA CCG GTG TAA GCC AGG 3' and alt*ND1* (rev) 5' TAG AAT TAG AAG ATC AAC CAG C 3' amplified an approximatel y 580 bp long fragment of the mitochondrial genome (Rach *et al.,* 2007) which included 16S rRNA, intervening $tRNA^{Leu}$ and *NADH dehydrogenase 1* region. Later, the DNA products were loaded into each tube. A control was also prepared in order to ensure the amplified samples were truly purified and free from any contamination.

The ideal composition of the mixture after optimization are shown in table 4.

Table 4: Composition proportion of PCR master mix.

	Stock Concentration	Working Concentration	Volume (µl)
Sterilized distilled water (dH_2O)	-	-	24.6
Buffer (10xB)	10x	1x	5
dNTPs	5 mM	200 mM each	4
ND1 Fw Primer	10 mM	0.4 mM	3
ND1 Rev Primer	10 mM	0.4 mM	3
***Taq* DNA polymerase**	5 U/µl	2 U	0.4
DNA	20 ng/µl	100 ng	10
Total Volume (µl)	**-**	**-**	**50**

The PCR amplication was performed using PTC-100^{TM} Programmable Thermal Controller and PTC-200^{TM} Thermal Controller (MJ Research) with the annealing temperature of 49° C for primer *NADH dehydrogenase 1*. The temperature regime for this amplification was performed based on the protocol provided by Rach *et al.*(2007). The temperature profile as in Table 4.1.

Table 4.1 : Temperature profile for PCR amplification (Rach *et al.,* 2007).

PCR Step	Temperature (^{0}C)	Time (minutes)
1st (pre-denaturation)	95	2
2nd (denaturation)	95	30 sec x 30 cycles
3rd (annealing)	49	30 sec
4th (elongation)	72	1
5th	Repeat 2nd to 4th steps for 34 cycles	
6th (final elongation)	72	6
7th END		

After all the previous steps were carried out, a gel was prepared in order to view the fragments of each sample. This step was called gel electrophoresis. Firstly, the gel was prepared by mixing 1x TBE Buffer Sollution (Tris-base, Boric acid, EDTA) to agarose gel (100ml of TBE for every 1g of agarose).

Subseqently, the mixture was heated in a microwave oven until boiled. Before adding the ethidium bromide (EtBr), the mixture was cooled, then immediately poured onto a gel caster along with its comb intact. It was left for about 20 minutes to solidify inside the caster, then the gel with the cassete were transfered into the electrophorosis tank which was fully immersed with 1x TBE solution.

Before loading the PCR products and the control into the gel, the PCR products were mixed with 6x loading dye with the ratio of 5µl PCR products : 1µl loading dye. Additionally, 100bp ladder was loaded into one of the hole in order to check the correct size of the fragment obtained. At this stage, the gel was ready to electrophorese with 120V of power supply for about 30 minutes and was viewed under UV light after completion of electrophoresis.

2.4.2 Purification of PCR Products

The purification protocol for amplificationthe products was performed using QIAquick PCR Purification Kit Protocol (QIAGEN, Germany) to purify single-stranded DNA fragments from PCR and other enzymatic reactions. The fragments were purified from primers, nucleotides, polymerase, and salt using QIAquick spin columns in a microcentrifuge.

For the initial step, the amplified products from the PCR tubes were transfered into 1.5 ml microcentrifuge tube. Then, Buffer PBI was added to each tube with the volumn ratio 5 Buffer PBI : 1 PCR products and the color of the mixtures should be similar to Buffer PBI without the PCR sample.

All the mixtures were then transfered into QIAquick spin columns with a 2ml collection tube and centrifuged for 1 minute. After 1 minute, the flow-through was discarded and placed back into the QIAquick spin column into the same tube to be reused. Subsequently, 750µl of 35% guanidine hydrochloride was added into each tube and again centrifuged for 1 minute.

The flow-through was discarded and placed in the same tube. Next, 750µl of Buffer PE (diluted with absolute Ethanol) was loaded into each QIAquick spin column and centrifuged for 1 minute. The filtrate was then discarded and placed the spin column back into the tube and centrifuged the column for additional 1 minute for a complete removal of the Buffer PE added previously.

After this step, the QIAquick spin column was placed in a clean 1.5 ml microcentrifuge tube as a storage of purified products. Then, 30µl of Buffer EB (10mM

Tris.Cl, pH 8.5) was loaded to the centre of the QIAquick membrane to elute the DNA and left for 10 minutes. For final step, the tubes were centrifuged for 1 minute, then the columns were discarded and the tubes were kept as purified samples. The purified samples were then viewed by agarose gel electrophoresis and the samples were sent for sequencing.

2.4.3 Sequencing and Analysing

All the purified samples were sent for sequencing and the results were read and edited. Forward and reverse strands were assembled and edited using Chromas version 2.31. All 833 sequences was trimmed to ~527 bp of an unambiguously alignable core region. The combined data sets were then aligned by using Clustal W (Thompson *et al.,* 1994) to build a phylogenetic tree via neighbor joining (NJ) algorithms and maximum parsmony (MP) analyses.

Besides, the step was further proceeded using Kimura-2-parameter to build a neighbor joining tree with the bootstrap replicate of n=5000. The analysis of genetic distance, DNA sequence variation and nucleotide composition were performed using Molecular Evolutionary Genetics Analysis (MEGA) version 4.0.2, (Tamura *et al.,* 2007).

2.5 Statistical Analysis

Shannon Wiener Index was used to measure the diversity of dragonflies and damselflies that were collected from all sampling sites.

Species Diversity: H'

This index indicates the degree of species composition per unit area. The higher value of H', the greater diversity and supposedly the cleaner the environment it is. (Ludwig & Reynolds, 1988; Metcalfe, 1989).

$$H' = -\sum [(n_i / N) \ln (n_i / N)]$$

Where; H' = Shannon Wiener Index

N = Total individuals of population sampled

n_i = Total individuals belonging to the species *i* spesiec

Richness Index: R

This richness index that has been used was Margalef's Index (R). The index indicates the number of species in a sample or the abundance or the species per unit area. (Ludwig & Reynolds, 1988; Metcalfe, 1989).

$$R = S - 1 / \ln (N)$$

Where; R = Margalef richness Index

S = Total of species

N = Total of individuals sampled

Evenness Index: E

This index indicates the homogeneity or pattern of the distribution of species in relation to the other species in a sampled per unit area.(Ludwig & Reynolds, 1988; Metcalfe, 1989).

E = H’ / H’ max

Where; E = Evenness Index

H’ = Shannon-Wiener diversity Index

H’ max = Diversity Index observed to a maximum diversity

The distribution of odonate species from all study sites was calculated using software SPSS 20.0 (Statistical Package for Social Science version 20.0). This analysis was done using one way Analysis of Variance (ANOVA). While the abundance and environmental factors among the study sites was performed using paired samples T-test and descriptive analysis.

Additionally, several softwares were used for data analyses of the DNA sequences which were Chromas version 2.31 and Molecular Evolutionary Genetics Analysis (MEGA) version 4.0.2 (Tamura *et al.,* 2007).