CHAPTER 2

LITERATURE REVIEW

2.1 Diversity of algae

Biodiversity is the variety of life forms and it also refers to variation at all levels of biological organization. Three aspects of biodiversity are essential to preserve ecological systems and functions. First, genetic diversity is a measure of the variety of genes within an individual species. Second, species diversity, describes the number of different kinds of organisms within individual community or ecosystem. Third, ecosystem diversity compasses the scale of physical conditions different from populations to niches and habitats (Gaston and Spicer, 2004). The diversity is influenced by many environmental factors. The most influencial factors are habitat characteristics and ecological complexity. High diversity of organisms reflects the complexity of ecosystem, while the decrease in diversity will obviously affect ecosystem change (Baimai, 2003).

Algae are the primary producers in the oceans and seas, the area that covers 71% of the earth surface. Algae also occur in freshwater lakes, ponds and streams as well as on and in soil, rocks, ice, snow, plants and animals. In total, 40% of global photosynthesis is contributed by algae (Andersen, 1992). The biggest group of photoautotrophic organisms with chlorophyll *a* and unicellular reproductive structure is algae, which also plays a very important role in aquatic ecosystems. There are

approximately 8 up to 19 divisions with more than 128,204 species on the list of recorded species of algae in the world (Guiry and Guiry, 2011). The size of algae ranged from microscopic to macroscopic. They can be separated by their habitats into 2 groups: floating algae as phytoplankton and benthic algae which are attached to substrates.

2.2 *Pediastrum* spp. study

2.2.1 Classification of *Pediastrum* spp. (Meyen, 1829)

Pediastrum spp. are green algae belonging to:

Division Chlorophyta

Class Chlorophyceae

Order Sphaeropleales

Family Hydrodictyaceae

Genus *Pediastrum*

Pediastrum composes of two Greek words: "pedion" means flat or plain and "astrum" means star which refers to the more or less radiate process from the colony. *Pediastrum* spp. are non-motile coenobial (fixed number of cells). The dominant characteristics include the disc shape or stellate coenobia. The cells in the coenobia are usually arranged in a single layer, forming a flat circular plate. Occasionally, large coenobia have cells arranged in two layers at the center. There is one chloroplast per cell, parietal and massive containing one pyrenoid, which sometimes multiplies in older cell, particularly in the large adult coenobia (Parra, 1979). The cells are joined one to another but without any physiological connections through cell walls (plasmatic connection, pore, etc.) Cells are morphologically species specific

depending to some extent on the form of coenobia (with or without holes, regular holes, size of holes). The outer margin of peripheral cells in coenobia is usually modified and elongated into one to 4 lobes and process of the species characteristic shape (the number and morphology of these process are also species specific). The number of cells in a coenobium ranges mainly from 4 to 32 and exceptionally reaches 64-128 and even 512 (2^9) cells (Komarek and Jankovska, 2001)

Cell wall of *Pediastrum* is composed of thick inner layer of cellulose derivatives and the outer layer is composed of sporopolenin combined with silicon oxide which makes them highly resistant to decay (Atkinson *et al.,* 1972). The intensity of surface sculptures depends on the environmental factors and developmental stage to a certain degree but the type is stable and species specific (Hegewald and Yamagishi, 1994). Various modifications are characteristic of different taxa (Parra, 1979).

The types of cell wall sculptures together with morphological modifications in the coenobial form are diagnostic features for different taxa at the species or variety levels. They can therefore be used as serious markers for taxonomic identification of various species in palynological analyses. The basic sculpture types are granular or net like and occur in various modifications and intensities. At the ends of process and also near the connecting walls between cells in several species, special rosettes, sometime bristles arise under special environmental conditions. Rosettes occur probably only in some species with granular cell walls (Hegewald and Schnepf, 1984).

Pediastrum have both sexual and asexaul reproduction. The sexual phase of the life cycle has been observed very rarely. Asexual reproduction of this genus is

called "Autocolony Formation". The process is started by production of a number of biflagellate zoospores, i.e. motile spores having two flagella, from a parent cell. These zoospores are generated by repeated mitotic division without cytokinesis, therefore, at this stage, the parent cell is multinucleate. After that, cytokinetic process for each nucleus takes place concomitantly and several mononuclear daughter cells transform into biflagellate zoospores within a vesicle derived from the innermost layer of a parent cell wall. Subsequently, the zoospores aggregate and arrange themselves into a flat circular colonial form (Chitchai, 2003). During this time, motile spores will lose their flagella and adopt a round shape. The transformation process from round cells to flat butterfly-shaped cells like those of *Pediastrum duplex* will take some time. In other species, cell shape may differ depending on the species. An emergence of a new daughter colony will make a parent cell expire. This can clearly be seen by the appearance of empty cell and its cleft (Bold and Wynne, 1985) in Figure 1.
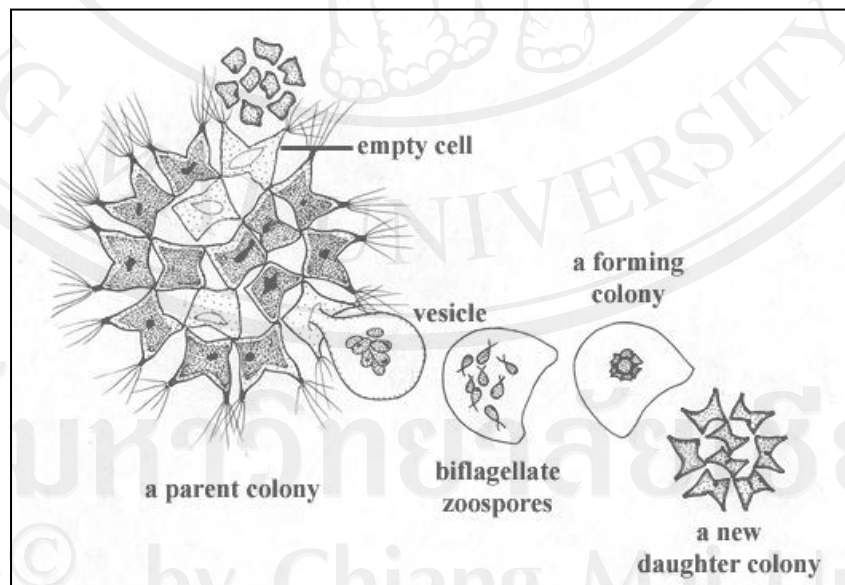

Figure 1 Asexual reproduction of *Pediastrum*

Source: http://www.microscopy-uk.org.uk/mag/indexmag.html

All cells in the coenobium are able to produce the same type of daughter coenobia. The number of daughter cells depends, to a certain degree (in specific limits in various species) on environmental factors. The cells in one coenobium are ripen almost simultaneously or successively in short time intervals. The enveloping plasmic vesicle disappears after the formation of the daughter coenobium. This whole process is completed in approximately 30 minutes. The first nuclear division starts again soon after the formation of a daughter coenobium (Bold and Wynne, 1985).

In the sexual process (homothallic isogamy) e.g. in *P. boryanum* (Esser, 1976) in Figure 2. Two flagellated isogametes arise in mother cells in the same way as zoospores. However, they are smaller in size. The isogametes from one and the same cell are able also to copulate. They join by apical ends and form a thick walled hypnozygote, which produces numerous endogeneously differentiated motile zooids (meiospores). Zygotes are able to survive a long lasting phase of dormancy. Meiospores change into immotile haploid polyhedral, from which zoospores are produced forming characteristic coenobia, as from vegetative cells. Isogamtes (and exceptionally also zoospores) change sometimes into haploid zygotes (Davis, 1967).

All these data explain the uniformity of daughter cells arising from one *Pediastrum* mother cell. Their final morphology (with or without processi) is influenced only by the position in coenobiam. Zoospores are oriented by their apical ends into the coenobial center during the formation of zoospores (Komarek, 1989). The position of zoospores in a new coenobium is not predetermined, and all the zooids are of the same quality and originally uniform, that means that the morphological transformation of zoospores into internal or marginal cells (with processi) and their final morphology are influenced exclusively by their position in

the developing daughter coenobium. Processi are formed during the change of zoospores into vegetative cells, the cell walls are synthesized during the last period of the formation of coenobium (Davis, 1967).
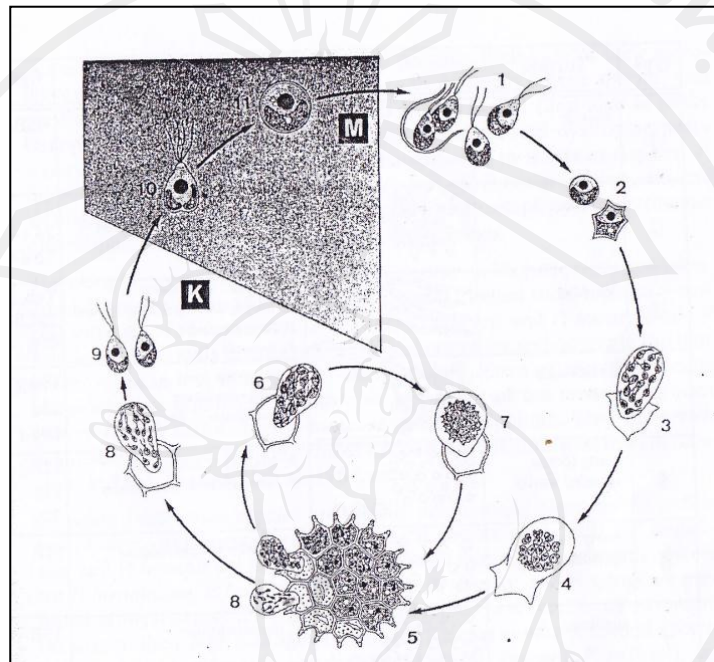
Figure 2 Sexual process in *Pediastrum boryanum*

    Source: Esser (1976)

The major taxonomic characteristics for identification of the species of *Pediastrum* are, (i) the forms and characteristics of coenobia (outline and holes), (ii) the number of cells in coenobia (distinct limits in various species) (iii) the morphology of the cells (particularly of marginal cells with characteristic lobe, incisions and processi) and (iv) the surface sculpture of the cell wall (Parra, 1979; Wu, 1987; Komarek and Jankovska, 2001).

2.2.2 Ecology and Distribution of *Pediastrum* spp.

Genus *Pediastrum* is only found in freshwater. Reports on *Pediastrum* spp. from brackish and salty water are very rare (Parra, 1979). They are abundant in

planktonic of permanent or semi-permanent lakes, pools, ponds and ditches. They are typical constituent of the plankton in meso-eutrophic and eutrophic waters with high nutrient content, especially, nitrogen and phosphorus (Xu *et al*., 2004). They prefer standing water and avoid flowing or running water of streams and ravines. The coenobia are free-floating. They usually float on the surface of water alone or with other colonial forms and aquatic plants. They sink to bottom of the pond when water level is low. In oligotrophic water, these taxa are often confined to the littoral zone associated with aquatic macrophytes and Sphagnum moss (Komarek and Jankovska, 2001; John *et al.,* 2002). Livingstone *et al*. (1958) found that in Alaska, *Pediastrum* are most abundant in very shallow lakes, which would lend further support to the existence of a non-planktonic life form of *Pediastrum* in some environmental settings. Some species are cosmopolitan, while others are more restricted in their distribution.

Komarek and Jankovska (2001) recorded 24 species of *Pediastrum* spp. but only four species, *P. boryanum, P. duplex, P. simplex and P. tetras* have been found all over the world. While *P. simplex* is commonly distributed in tropical countries and occurs in the temperate zone with less frequency in warm seasons, three other species are dominant in eutrophic reservoirs of the temperate zone. However, their distribution is more or less cosmopolitan (Jarnefelt *et al*., 1964). Common species are usually ecologically classified as indicators of oligo-to β-mesosaprobic waters (Sulek 1996); *P. angulosum* and *P. braunii* are dominant in oligosaprobic and oligotrophic to mesotrophic waters, but *P. biradiatum, P. boryanum, P. duplex, P. simplex*, and *P. tetras* are even found in α- mesosaprobic. The indication value, however, is questionable because of their high ecological tolerance (Komarek and Jankovska, 2001). On the contrary, several species occur only in ecologically restricted biotopes

(*P. braunii* and *P. privum*) and their diagnostic use for recent biotopes is without sense. However, all species can be used to characterize biotopes by palaeoecological reconstruction (peaty deposits, acidic or alkaline swamps, lake sediments, lowland and warm stagnant water bodies, high-mountain cold and oligotrophic lakes, etc.). The geographical distribution of the species is mainly determined by two factors: global distribution responds to temperature, whereas regional distribution to water quality e.g. some species such as *P. kawraiskyi* are restricted to cold and acidic waters (Komarek, 1989).

The characteristic morphology of various *Pediastrum* species diminishes the number of taxonomic misinterpretations and permits the satisfactory evaluation of their recent distribution. Other taxa belong to a wide range of various ecologically and geographically different types such as very narrowly delimited endemites (*P. marvillense, P. boryanum var campanulatum, P. asymmetricum,* the unconfirmed *P. tricuspidatum* from Central Africa) species known only from delimited tropical (*P. argentiniense*) or northern temperate regions (*P. privum*, *P. alternans* and *P. sculptatum*) and pantropical (*P. longecornutum*) or temperate species (*P.integrum*) (Table 1).

The whole genus *Pediastrum*, in spite of its wide distribution, has a decreasing evolutionary trend. Several species have the character of relicts and occur in biotopes that are endangered in the present world (*P. kawraiskyi, P.integrum, P.sculptatum, P. privum* and *P. braunii*). All these species are very rare and occur only in few localities, but many of them were commonly distributed in the Late Glacial and Holocene (Komarek and Jankovska, 2001).

Table 1 Geographical distribution of *Pediastrum*

Distribution	Species	Geographic locality
Endemic species	*P. marvillense*	Kerguelen Islands
Cosmopolitan (ecologically delimited)	*P. braunii*	High mountain peaty bogs
Delimited areas of distribution	*P. privum*	circumpolar
	P.integrum	boreo-alpine
	P. kawraiskyi	boreo-alpine
	P. obtusum	tropical and subtropical
	P. longecornutum	pantropical
	P. argentiniense	neotropical
Cosmopolitan species	*P. simplex*	(only few varieties)
	P. duplex	
	P. boryanum	
	P. tetras	

Source: Komarek and Jankovska (2001)

2.2.3 General study of *Pediastrum*

The first study of *Pediastrum* spp. was by Meyen in 1829 who found three species namely *P. simplex*, *P. duplex*, and *P. biradiatum* (Wu, 1987).

The first study of *Pediastrum boryanum* was by Meneghini in 1840 found more than 50 taxa, which are now identified as varieties and forms of *P. boryanum.* They had been reported by many investigators.

Parra (1979) treated more than 50 taxa of *Pediastrum* which had been reported by many investigators as varieties and forms of the species *P. boryanum.* Four varieties and one form of *P. boryanum* were reported in Korea.

Wu (1987) introduced the ultrastructure of the cell wall as an important character in *Pediastrum* taxonomy as well as observing the cell wall ornamentation and the process of coenobia formation.

Chang and Mi (1997) studied the of taxonomy of *Pediastrum boryanum* Meneghini in Korea and found 10 strains which were classified into three varieties of *P. boryanum* i.e. *P. boryanum* var. *horyanum, P. boryanum* var. *brevicorne* which were reported for the first time in Korea, and *P. boryanum* var. *longicorne.* The shape of the peripheral cell and the length of the process on the peripheral cell along with the cell wall ultrastrucmre were the imporlant characters for identification of the species of *Pediasrum boryanum.*

A review of the green algae in the genus *Pediastrum* was published by Komárek and Jankovská (2001). A total of 24 species i.e. *P. simplex* Meyen, *P. asymmetricum, P. tricuspidatum*, *P. musteri*, *P. patagonicum*, *P. kawraiskyi*, *P. orientale*, *P. integrum*, *P. boryanum*, *P. subgranulatum*, *P. marvillense*, *P. sculptatum*, *P. duplex*, P. *alternans*, *P.angulosum*, *P. orbitale*, *P. argentiniense*, *P. privum*, *P. obtussum*, *P. tetras, P. longecornutum, P. biradiatrum, P. braunii* and *P. taylori* had been identified based on morphological characters.

In 2004, Pasztaleniec and Poniewozik studied phytoplankton in Sumin Lake. 15 taxa of *Pediastrum* were found. Common species were *P. boryanum, P. tetras, P. duplex and p. simplex* including rare species such as *P. integrum* and *P. kawraiskyi.*

In 2010, Kowalskal and Wołowski studied *Pediastrum privum* in Lake Małe Zmarłe (Tuchola Forest) using light microscopy (LM). Micrographs, together with data on its ecology was recorded. The population of *P. privum* was composed of four-celled and single eight-celled coenobia, similar to other natural populations.

Rai and Misra (2012) studied the taxonomy and diversity of Genus *Pediastrum* Meyen in East Nepal. Twenty taxa of *Pediasturm* were reported i.e. *P. angulosum, P. biradiatum, P. boryanum, P. boryanum var. longicorne, P. duplex, P.*

duplex var. brachilobum, P. duplex var. clathratum, P. duplex var. gracillium, P. duplex var. subgranulatum, P. duplex var. reticulatum, P. duplex var. rugulosum, P. integrum, P. obtusum, P. ovatum, P. simplex, P. simplex var. duodenarium, P. tetras, P. tetras var. apiculatum, P. tetras var. excisum and *P. tetras var. tetraodon.*

The cultivation of *Pediastrum* was studied by Rojo *et al*. (2009) on the growth of *P. tetras*, after inoculated in fresh medium from a stock culture. The growth did not show a lag phase (or the lag phase was less than three days). Furthermore, three days after being inoculated, the population increased fourfold. The maximum density was 20 times more cells than at the beginning when the culture was 12 days. Afterward the culture was relatively decline. During a period of at least ten days after this decline, the population entered a stationary phase.

Lee *et al*. (2009) reported the composition of *P. duplex* that it contained 46.3% protein, 30.4 % carbohydrate, 2.4% lipid, 15.2% ash and 6.1% moisture. Protein is therefore the major component close to that of *Chlorella* sp. (35–60%) and *Spirulina* sp. (46–75%) (Becker, 2007). It provides highest protein than other natural food and can be used as food supplement in human and animal feed. Moreover, the potential antioxidant activities of enzymatic digests from *P. duplex* and *Dactylococcopsis fascicularis* were also studied by using 5 carbohydrases: viscozyme, celluclast, AMG, termamyl and ultraflo; 5 proteases: protamex, kojizyme, neutrase, flavourzyme and alcalase. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity of all enzymatic digests from *P. duplex* was relatively higher than those from *D. fascicularis*. Especially, ultraflo, flavourzyme and neutrase digests of *P. duplex* exhibited strong scavenging activity (above 90%) and termamyl (60.6%) digest from *P. duplex* possessed the highest effects on hydrogen peroxide scavenging. These data

suggested that enzymatic digests of the fresh water microalgae, *P. duplex* and *D. fascicularis* might be valuable sources of antioxidant which can be applied in food and pharmaceutical industries.

2.2.4 Study on *Pediastrum* in Thailand

Pediastrum in Thailand has been investigated by foreign scientists for a hundred years, according to a checklist of the algae in Thailand compiled from 53 publications (Lewmanomont *et al.,* 1995). Total lists were 161 genera, 1,001 species, 287 varieties and 63 forms that included freshwater algae and marine algae. A total of 10 species, 38 taxa of *Pediastrum* spp. have been recorded.

The first record of *Pediastrum* in Thailand was reported by West and West (1902). The material collected by the joint Thai-Japanese Biological Expedition to South East Asia was identified by Hirano (1967). The following noteworthy species were suggested by Hirano (1967) i.e. *P. clathratum var. radians* (Lemmermann) Bachmann and *P. duplex* Meyen. Boraphet Lake, the biggest lake in Thailand was examined by Hirano (1975) who found *P. biradiatum* var. *longicornutum* Gutwinski, *P. duplex* Meyen, *P. duplex* var. *cohaerens* (Bohlin) Ergashev, *P. duplex* var. *gracilimum* West. & G.S. West, *P. duplex* var. *subgranulatum* Raciborski amd *P. simplex* Meyen. Peerapornpisal (1996) studied the phytoplankton seasonality and limnology of three reservoirs in the Huai Hong Khrai Royal Development Study Centre in Chiang Mai. As much as 127 species were found including 8 species of *Pediastrum*; *P. duplex* Meyen, *P. duplex* var. *gracilimum* West. & G.S. West, *P. duplex* var. *subgranulatum* Raciborski, *P. longicornutum* (Gutwinski) A. Comas, *P. simplex* var. *biwaense* Fukushima, *P. simplex* var. *echinulatum* Wittrock, *P. simplex* var. *simplex* Meyen and *P. tetras* (Ehrenberg) Ralfs.

2.3 Molecular study of *Pediastrum*

The molecular analyses can reveal the extensive diversity of known and especially novel groups in natural samples (Caron *et al*., 2004). A number of studies using molecular phylogenetics to test morphological species within the green algae has grown up (McManus and Lewis, 2005). There is increasing evidence of hidden diversity among morphologically similar taxa and evidence of phenotypic plasticity (Lewis and Flechtner, 2004). Recent studies of the Class Chlorophyceae using morphological and ultrastructural data in a noncladistic approach, and phylogenetic analyses based on 18S ribosomal DNA (rDNA) sequence data, have supported the close relationship of *Pediastrum* (Buchheim *et al.,* 2001). 18S ribosomal RNA genes are the most widely used nuclear sequences for phylogeny reconstruction at higher taxonomic levels in plants. However, due to a conservative rate of evolution, 18S rDNA alone sometimes provides too few phylogenetically informative characters to resolve relationships adequately. Previous studies using partial sequences have suggested the potential of 26S or large-subunit (LSU) rDNA for phylogeny retrieval at taxonomic levels comparable to those investigated with 18S rDNA. Since the advent of comparative DNA sequencing in plants and algae, the chloroplast gene rbcL has been the primary molecular marker used for phylogenetic inference at higher taxonomic levels. Its utility in these taxonomic levels has been well established (Soltis and Soltis 1997). Nonetheless, phylogenetic hypotheses based on a single gene or character may not represent true organismal relationships. Molecular systematists therefore seek additional genes for phylogeny reconstruction to test rbcL-based topologies, to obtain additional resolution and to elucidate relationships at a variety of taxonomic levels (Thompson, 1988).

McManus and Lewis (2005) reported that molecular systematic of the Family Hydrodictyaceae revealed *P. duplex* to be far more diverse than previously thought based on morphology. In the same year, Buchheim *et al.* (2005) studied phylogeny of Family Hydrodictyaceae by optimal trees from maximum likelihood (ML) analysis of combination between 18S, 26S, and ITS-2 rDNA. *P. boryyanum* (Heg 1976-16), *P. boryyanum* v. *cornutum* (UTEX 470) and *P. kawraiskyi* (SEG 35.81) were transferred to the new Genus *Pseudopediastrum, P. simplex* (UTEX 1601) was transferred to the new genus *Monactinus, P. biradiatum* (UTEX 37) was transferred to the new genus *Parapediastrum* and *P. tetras* (UTEX 38), *P. tetras* (UTEX 84), *P. tetras* (SEG 37.81) and *P. privum* (SEG 36.81) were transferred to the new genus *Stauridium* (Buchheim *et al.,* 2005). In 2011, McManus and Lewis studied molecular phylogenetic of Family Hydrodictyaceae with the emphasis on *P. duplex* by the 26S and rbcL. Data were analyzed using maximum-likelihood (ML) and Bayesian phylogenetic methods. The results supported previous indications that *P. duplex* Meyen 1829 morphotype is nonmonophyletic and resolved some previously ambiguous relationships recovered in earlier phylogenetic estimations using fewer isolates. These new data allowed testing of the recent taxonomic revisions of the family that split *Pediastrum* into five genera. The data were well supported by the work of Buchheim *et al.* (2005) in the Genus *Stauridium* and *Monactinus*, while the others were not supported i.e. genus *Pediastrum, Pseudopediastrum* and *Parapediastrum.*

2.4 Phylogenetic analysis

Phylogenetic trees construction has become increasingly popular in many fields of biology. Their inclusion reflects the growing recognition of tree as a tool for understanding biological processes (Hall, 2011). Phylogenetic tree is used to study the evolution of gene and proteins, the phylogenetic relationship of organisms, clarifying the taxonomic position of an organism, determining the origin of organelles (mitochondria and chloroplasts) and to organize the knowledge of biodiversity (Ludwig *et al*., 1998).

Phylogenetic tree is a simple structure composed of nodes and branches. There are two types of nodes: **external nodes** are located at the tips of the tree and represent the taxa (or in the case of sequence data and the molecular sequences) that exist and can be actually examined; **internal nodes** represent ancestral taxa, whose properties can only be inferred from the existing taxa. Nodes are connected by branches, the lines whose lengths represent the amount of genetic change occurred between an ancestral node and its descesdant (Hall, 2011).

2.4.1 Method for constructing phylogenies

There are two primary approaches to tree construction: algorithmic and tree-searching. The algorithmic approach uses algorithm to construct a tree from the data. The tree-searching method involves many trees and then uses some criterion to decide which is the best of the tree (Ludwig *et al*., 1998).

The algorithmic approach has two advantages, it is fast, and it yields only a single tree from any given data set. The two most popular algorithmic methods are Neighbor Joining and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA). All the other current used approaches are tree-searching method: such as

Parsimony, Maximum Likelihood and Bayesian analysis search for the tree that best meets some optimal criterion by evaluating individual tree (Hall, 2011).

2.4.2 Distance methods

Distance-matrix method of phylogenetic analysis are explicit and trustable on the measure of the "genetic distance" between the sequences being classified, and therefore the method requires multiple sequence alignment (MSA) as an input. Distance method converts the aligned sequences into a distance matrix of pair-wise differences (distances) between the sequences. Distance is often defined as the portion of mismatches at aligned positions (Mount, 2004). Distance method attempts to construct an all-to-all matrix from the sequence query set describing the distance between each sequence pair. This constructed phylogenetic tree will place close related sequences under the same interior node and distances always underestimate the actual amount of change in the lineages. The main disadvantage of distance-matrix method is its inability to efficiently use information about local high-variation regions that appear across multiple sub-trees. The two most popular distance methods, UPGMA and Neighbor Joining, are both algorithmic methods i.e., they use a specific series of calculations to estimate a tree (Felsenstein, 2004).

2.4.2.1 Unweighted Pair-Group Method with Arithmetic Mean (UPGMA)

UPGMA is an example of a clustering method. The program first finds the pair of taxa with the smallest distance between them and defines the branching between them as half of that distance-in effect, placing a node at the end of the branch. It then combines the two taxa into a "cluster" and rewrites the matrix with the distance from the cluster to each of the remaining taxa (Hall, 2011). The number of

entries in the matrix is now reduced by one. The process is repeated on the new matrix and reiterated until the matrix consists of a single entry. That set of matrices is then used to build up the tree by starting at the root and moving out to the first two nodes represented by the last two clusters. UPGMA has built into it an assumption that the tree is additive and that it is ultrametric i.e., that all taxa are equally distant from a root, an assumption that is likely to be incorrect (Nei and Kumar, 2000).

2.4.2.2 Neighbor Joining (NJ)

NJ is similar to UPGMA in that it manipulates a distance matrix, reducing it in size at each step, then reconstructs the tree from that series of matrices. It differs in that NJ does not construct clusters but directly calculates distances to internal nodes. From the original matrix, NJ first calculates for each taxon its net divergence from all other taxa as the sum of the individual distances from the taxon. It then uses that net divergence to calculate a corrected distance matrix. NJ then finds the pair of taxa with the lowest corrected distance and calculates the distance from each of those taxa to the node that joins them (the distances from the two taxa to the node need not be identical). A new matrix is then created in which the new node is substituted for those two taxa. NJ does not assume that all taxa are equidistant from a root (Hall, 2011).

2.4.3 Character-based method

Character-based method includes Maximum Parsimony, Maximum Likelihood and Bayesian Inference. All these methods use multiple alignments directly by comparing characters within each column (each site) in the alignment (Hall, 2011).

2.4.3.1 Maximum Parsimony (MP)

MP or minimum change is based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data of protein or nucleotide sequences in the alignment. Parsimony or minimum change is the criterion for choosing the best tree. That number is the score for the tree and the tree or trees with the lowest score are the most parsimony tree (Tamura *et al.,* 2011). For protein or nucleotide sequences, the data are the aligned sequences. Each site in the alignment is a character and each character can have different states in different taxa. Not all characters are useful in constructing a parsimony tree (Nei and Kumar, 2000).

2.4.3.2 Maximum Likelihood (ML)

ML is a powerful statistical method that seeks the tree that makes the data most likely. ML tries to infer an evolutionary tree by finding that tree which maximizes the probability of observing the data (Yang, 1997). For sequence, the data are the alignment of nucleotide or amino acid. The advantage of the ML method is that the likelihood of the resulting tree is known (Tamura *et al.,* 2011).

2.4.3.3 Bayesian Inference (BI)

BI is a recent variant of ML. BI like ML is a powerful and well caped method for estimating phylogenetic tree. It differs from ML in that BI seeks the tree that is most likely given the data and the chosen substitution model, whereas ML seeks the tree that make the data the most likely. Like ML, BI uses log likelihood as a criterion for choosing among possible tree (Mau *et al.*, 1999).