CHAPTER 6: CONCLUSION

6.1 Conclusion

Biochemical genetic markers are important in determining species boundaries where there is large morphological plasticity, such as in the algae. Varying degrees of success have been achieved by molecular genetics in taxonomic and systematic problems in algae (Manhart & McCourt, 1992). Molecular data are also expanding our view of diversity, not only in familiar taxa but also in newly revealed, previously unsampled taxa. Thus, development of alternative molecular markers, such as microsatellite markers in distinguishing different populations and across related species, is essential to select valuable strains of the species for cultivation. It is important to choose and breed *Gracilaria* species with high economic traits and use them for cultivation. Additional studies on gel strength, gel contents and growth rates among the different populations of *Gracilaria* species will provide valuable information regarding strain selection for cultivation. Hence, molecular genetic studies, particularly on the development of molecular markers, are essential to effectively select such strains. Further studies concerning developing more markers from different *Gracilaria* species or even across *Gracilaria* species will be beneficial in the differentiation of *Gracilaria* species.

6.2 Significant Observations in This Study

Based on our results, the research questions can be answered as followed:

(i) It is possible to mine microsatellite markers from the chloroplast genome and EST database of seaweeds downloaded from GenBank using available bioinformatics tools. We have mined eight cpSSRs with designed primer-pairs from the chloroplast

genome of *Gracilaria tenuistipitata*, ten EST-SSRs with designed primer-pairs from the EST database of *G. tenuistipitata* and 45 EST-SSRs with designed primer-pairs from the EST database of *Gracilaria* species (*G. changii*, *G. gracilis* and *Gp. lemaneiformis*). We have also mined seven EST-SSRs for *Kappaphycus* species and 30 EST-SSRs for brown seaweeds with designed primer-pairs.

(ii) In this study, we found a microsatellite marker (primer-pair P3) that was able to solve the confusion between three different *Gracilaria* species (*G. changii, G. fisheri and G. manilaensis*) that are morphologically indistinguishable. Besides, *Gracilaria changii* from Morib, Selangor, Malaysia showed unique allele that can distinguish *Gracilaria changii* from the other populations. The microsatellite markers (primer-pairs GT5 and GT8) developed from chloroplast genome of *G. tenuistipitata* were able to differentiate seaweed populations into two main geographical regions: (i) populations in the west coast of Peninsular Malaysia and (ii) populations facing the South China Sea. Besides, the population from Kelantan, Malaysia was grouped in a distinct clade. The microsatellite markers (primer-pairs GT12 and GT18) developed from EST database of *G. tenuistipitata* generated four different genotypes and were able to distinguish the populations from Kuah (Malaysia) and Pattani (Thailand) into two distinct clades. This showed that the microsatellite markers that we developed are suitable to differentiate between different populations and species of *Gracilaria*.

(iii) The SSRs mined from EST database of *Gracilaria tenuistipitata* showed higher polymorphisms in comparison to genomic SSR marker. The EST-SSRs were able to give four different genotypes while the cpSSRs only showed three different genotypes on the specimens tested. The numbers of SSR markers obtained from EST database (ten) were higher than the genomic SSR markers (eight) but more different kind of motifs (mono-, di-, and tri-nucleotide) were observed in genomic SSR markers.

(iv) The commonly used marker for DNA barcoding and genetic diversity study, *cox*1 gene showed higher variability in comparison to the microsatellite marker that we developed as they gave six haplotypes while only three genotypes were obtained using primer-pair P3.

Microsatellite markers are highly polymorphic, require low amount of DNA and highly transferable between species as shown in this study. In previous studies, most of the works done by the researchers were related to the development of microsatellite markers, their characterization and identification of polymorphism in various organisms. Nowadays, microsatellite markers have a large number of applications ranging from identifications of a single locus to the genome mapping. The microsatellite markers that we developed can be applied not only for intraspecific study but also in functional diversity study of *Gracilaria* species. The genic SSRs designed from the transcribed regions of the genes suggest that they might have a role in gene expression or function. For example, the variation in the number of GA or CT repeats in rice was correlated with amylose content (Bao et al., 2002). Besides, the microsatellite markers $(CCG)_n$ of some ribosomal protein genes of maize were believed to be involved in the regulation of fertilization (Dresselhaus et al., 1999).

Although sequencing may give a higher discriminating power for species delineation and show that amplification products of primers-pairs were due to polymorphisms, they always cost higher than the other molecular assays. We are searching for an alternative molecular marker that is cheaper, fast and have advantages for species identification due to the specific length produced by SSRs. The microsatellite markers that we developed can be used for phylogenetic studies among *Gracilaria* species as well as across different species in which more specific genetic variation can be identified. This is important in the applications of plant breeding and germplasm conservation and is suitable for non-taxonomists without going into detailed.

6.3 The Proposal for Future Studies

In recent years, a few researchers have used M13 strategy in molecular study using microsatellite markers which will significantly reduce the overall cost (Schuelke, 2000). This technique using one universal fluorescently labeled primer (M13 primer) that works together with the unlabeled microsatellite primer set (forward and reverse) in each PCR reaction. The M13 strategy has been set up in such a way that M13 primer and reverse primer are in excess to forward primer which is limited and this allows the forward M13-tailed primer initiate the PCR reaction with reverse primer. When the limited forward primer is depleted, the labeled primer takes the place of the limited forward primer in the remaining PCR cycles. Thus, one only needs to purchase one universal labeled primer instead of using specific labeled primer for different PCR reactions. However, they need to be tested thoroughly before applying to many DNA samples as some primer-pairs does not work after adding a common tail (Wang et al., 2009). This shows that microsatellites can be used as DNA markers for various applications with lower cost compared to other DNA finger print methods.

More microsatellite markers can be designed using the SSRs pipeline that we developed and this will be useful for the studies in other species especially those are commercially important. For example, we have mined the SSR markers for *Kappaphycus* as well as brown seaweeds (Phaeophyta) and tested on some of the samples. *Kappaphycus* are important for their carrageenan and is widely used in food industry due to the gelling, thickening and stabilizing properties. Few markers showed significant results that can differentiate between *Kappaphycus* and *Eucheuma* species and also *Kappaphycus striatum* from different localities. In brown seaweeds, only few specimens, e.g.: *Sargassum binderi* and *Sargassum bacularia* gave amplifications with very faint band. This may be due to the incomplete EST database found in GenBank as some transcripts that are low abundance in certain tissues sampled may not be

sequenced at all (Bouck & Vision, 2007). Although still in preliminary stage of experiment, the markers that we developed for *Kappaphycus* and brown seaweeds can be potentially used in wide range of studies (Appendix C1 and C2).

Nowadays, SNP markers are becoming the marker of choice because they are easily developed from sequence data and highly reproducible (Rafalski, 2002). These markers provide a foundation in understanding the plant functional genomics, and maybe find important application in breeding, agronomic practice and ecosystem research.

6.4 Appraisal

We have successfully mined the microsatellite markers from both chloroplast genome and EST database of *Gracilaria* species that were downloaded from GenBank and these SSR markers were proved to be suitable for the identification of *Gracilaria* species. However, there are limitations arising from our study. First, the numbers and polymorphisms of SSR markers obtained were rather low in comparison to other studies. This may due to the high stringency of parameters that we have used in the mining of primer-pairs in order to obtain useful primer set that will able to give amplifications. More SSR markers can be obtained by lowering the stringency for the designing of the primer-pairs but this will results in non-specific amplification. For example, if we were to lower the melting temperature (*Tm*) of the primer-pairs designed, stutter bands will be obtained as they will annealed to non-target sequences. Hence, the parameters used in the mining of primer-pairs for SSR markers can be modified in order to obtain more SSR markers but the quality has to be maintained.

Second, the numbers of populations for *Gracilaria* species used in our study were not enough to give an overview about the gene flow and geographic structuring

pattern as the polymorphisms obtained are low. Moreover, most the specimens collected from different localities shared the same genotypes even the distance is far apart from each other, for example *G. tenuistipitata* from Quy Kim, Hai Phong, Vietnam and Pattani Thailand using the chloroplast microsatellite marker (primer-pair GT5). This can be verified with wider area of samples collections from more populations and using higher amount of microsatellite markers as shown in the study by Provan and Maggs, 2012.

Indeed, there is no single perfect molecular marker can be found for every study that has different objectives but to compare the efficiency of different markers that is possible to be used in the analysis. For example we compared the variability between *cox*1 gene marker and microsatellite marker that we developed in the study of *Gracilaria changii* and results showed that *cox*1 gene is more variable as they give higher number of haplotypes than the microsatellite marker. The *cox*1 gene marker is more suitable in the genetic diversity study but the microsatellite marker that we developed was able to give two different genotypes for *G. changii* from Gua Tanah, Malacca and Sg. Merbok, Kedah while *cox*1 gene only showed single haplotype in both localities. This showed that *cox*1 gene marker is suitable for genetic diversity study of *G. changii* as a whole but microsatellite marker is variable for some *G. changii* from a single population. DNA sequencing is the most common method employed in most of the molecular study as they give higher discriminating power for species delineation. However, this molecular assay cost higher and time-consuming especially for a farmer that aimed to cultivate economically important seaweeds. Microsatellite marker is cheaper, fast and easy to conduct particularly for non-taxonomist in the species identification of certain seaweeds. Thus, we would suggest using the microsatellite markers that have been established before opt for going into details using DNA sequencing method.