Finding Polymorphism in *R. apiculata* and *R. mucronata* using Microsatellites

Mangrove forests are unique and vital ecosystems that are situated in low-energy intertidal zones. They provide many ecosystem services such as harboring a range of species, slowing erosion, causing sedimentation, and absorbing wave energy from storms (Ewel *et al.* 1998). In Singapore, many populations of mangroves have been separated either naturally by location (island vs. mainland) or by human activities. Compared to other locations in Thailand and Malaysia, Singapore’s mangroves occur as very fragmented populations (Chou *et al.*, 1994). This raises many ecological concerns about the future sustainability of these populations, including the reduction of diversity. One of the tools often used to evaluate genetic diversity are microsatellites, which are highly polymorphic sections of DNA usually found in the non-coding region of the genome. Microsatellite markers for *R. stylosa* developed by Islam *et al.* in 2004 can be used on two common Singaporean species, *R. apiculata* and *R. mucronata*. Using these markers, the level of genetic diversity in these two species can be evaluated.

DNA extraction was conducted using three different methods—one protocol with DNAzol (Invitrogen Corporation, California) and two modified CTAB protocols from Islam *et al.* (2006) and Takayama *et al.* (2008). After trial-and-error testing, it was shown that *R. apiculata* is best extracted with a modified DNAzol method (procedure from Invitrogen Manual) and *R. mucronata* with a modified CTAB method by Doyle and
Doyle (1987). These methods required many tests and refinements in order to make them work effectively. However, with enough persistence, an adequate protocol was found. After obtaining good quality DNA extracts, polymorphism tests were conducted. PCR was performed with a an S10000 Thermal Cycler by Bio Rad, and *R. Stylosa* markers *Rhst* 1, 2, 11, 13, and 15 (Islam *et. al.*, 2004). The volume of the amplification solution was 20 µL and contained between 40 to 60 ng DNA solution, 2 µl *Taq* buffer (Fermentas, Canada), 2 U Taq Polymerase (Fermentas, Canada), 0.4 µM dNTP, 0.4 mM Mg$^{2+}$ 0.4 µL of both primers. The PCR protocol used was 60 s at 94ºC followed by 30 cycles of 94ºC for 30 s, 60 s at the primer annealing temperature, and 72ºC for 60 s, after which was 74ºC for 4 min. This procedure also had many complications. After completing tests for the optimization of the concentration of Mg$^{2+}$, DNA template, and *Taq*, as well as type of *Taq*, the PCR reaction was much more effective.

The final stage in the project was performing fragment analysis of the amplified fragments. It was limited largely by time, as well as a problem with sizing data. Sizing data to fragment ratio was often too low, resulting in the GeneMapper software not being able to size samples. Using diluted samples and a longer hot-start time allowed for the sizing data to be read. After this problem was fixed, there was too little time to complete the full fragment analysis. However, the results of the *R. mucronata* samples tested shows that heterozygosity is 6.7%. This heterozygosity is low compared to other regions, but because of the incomplete test, it is not known at this point if this percentage is accurate.

While all of the sections of this project had their individual challenges, there were two serious overarching issues. The first is a general lack of consistency in the results
achieved. Throughout the research, procedures that would work effectively once would not always do so. Even within a specific procedure, there were often inconsistent results. For instance, an extraction using one species with the same chemicals and protocol might yield several different levels of success. While this problem did lead to more research on other methods that are better and is also common with lab work, it is also a mark of human error and should happen much more infrequently. The other serious problem is the lack of planning and forward thinking. Improperly planned tests that left out variables or tested more than one at once also left us repeating tests in order to determine the root cause or solution of problems. This issue could be easily fixed by spending a small amount of time planning on a weekly basis, which would allow the research to run more smoothly and encounter fewer technical problems.

There are many avenues of future work that can come from this research. The completion of a full fragment analysis on both *R. apiculata* and *R. mucronata* is a logical first step. Afterwards, studies on the topics of genetic diversity, habitat fragmentation and its effects on pollinators, spacial genetic structure (SGS), and inbreeding levels can all be conducted.