PRELIMINARY GENETIC CHARACTERIZATION OF SELECTED MANGROVE SPECIES IN SRI LANKA

R. K. Sahan Bimsara*, S. R. Weerakoon and K. A. Roshan S. Perera
Department of Botany, The Open University of Sri Lanka, Sri Lanka

Mangroves are woody plants that thrive at the interface of land and sea, inhabiting harsh environmental conditions such as high temperature, strong wind, high salinity, extreme tide, and anaerobic muddy conditions. They show specialized adaptations, morphologically as well as physiologically for survival in this environment. Mangroves do not form a single phylogenetic group; instead, they have evolved independently and exhibit convergent evolution rather than shared descent. Currently, there are 21 species of mangroves remaining genetically unclassified. Therefore, this study attempted to classify selected Sri Lankan mangrove species using *rbcL* and *matK* gene markers. Specimens from six species (*Rhizophora mucronata*, *Lumnitzera racemosa*, *Rhizophora apiculata*, *Avicennia officinalis*, *Lumnitzera littorea* and *Avicennia marina*) were collected from the West Coast of the country covering wet, dry, and intermediate climatic zones. Dried specimens were submitted to the National Herbarium for further morphological identification while DNA was extracted from each leaf sample using modified CTAB method and Biospin genomic extraction kit. Although over 15 extractions were performed, most yielded low DNA concentrations and poor purity. However, Biospin kit extraction produced significantly better results with clear three genomic bands visualized of each six species through the Biospin method. PCR amplification using *rbcL* and *matK* primers confirmed higher efficiency with DNA extracted using the Biospin method. In this study, one species, *Avicennia officinalis* produced clear genomic bands with both extraction methods and both *rbcL* and *matK* amplification. However, other five species did not yield reliable bands with both methods. The secondary metabolites and high salt concentration of these species would have affected efficient DNA extraction. Sodium ions may bind to DNA complicating DNA extraction from mangrove plants. Interestingly, *Avicennia officinalis* which grows only in low-salinity environments showed better DNA yield, possibly due to reduced salt interference. This study pioneered research of its kind on mangroves in Sri Lanka and will be baseline research for optimizing DNA extraction protocols for mangroves and explore a variety of primers in future studies on genetic diversity of mangroves in Sri Lanka.

Keywords: genetic diversity, mangroves, molecular markers, Sri Lanka

*C*orresponding author:bimsarasahan@gmail.com*

PRELIMINARY GENETIC CHARACTERIZATION OF SELECTED MANGROVE SPECIES IN SRI LANKA

R. K. Sahan Bimsara*, S. R. Weerakoon and K. A. Roshan S. Perera
Department of Botany, The Open University of Sri Lanka, Sri Lanka

INTRODUCTION

Mangrove species are distributed along the coastal area (19,758 ha) in Sr Lanka, and of these, 38.6 % of mangroves are distributed in the North Province while 34.5 % of mangroves are distributed in the Eastern Province and 14 % of mangroves are distributed in the North Western Province (Arulnayagam et al., 2021). Some mangrove species are dominated at specific areas; *Scyphiphora hydrophylla* is distributed only at Puttalam Lagoon and Kala Oya. *Xylocarpus moluccensis* is found at the Dutch Bay. *Sonneratia alba* is found at the Malwathu Oya. *Nypa fruticans* is distributed at the upstream area of wet zone (IUCN, 2012). *Avicennia marina* is the most abundant and comprised of 1/3 of mangroves in Sri Lanka and a low presence of *Avicennia marina* is distributed in the intermediate zone. Lagoons with high salinity in the upper of the East Coastal area are comprised of *Avicennia marina* with scattered *Rhizophora mucronata*. These two species are dominated at the Viduthalathvu area near Mannar, and not in Pambala Lagoon due to the low salinity level. *Avicennia officinalis* is found in low salinity areas when compared to *A. marina*, and therefore, *A. officinalis* is distributed at Pambala (Amarasinghe et al., 2002). The second most abundance species in the dry zone is *Lumnitzera racemosa* and *Excoecaria agallocha* (Amarasinghe & Perera, 2017). Some species are rare such as *Lumnitzera littorea*, *Bruguiera cylindrica*, *Ceriops decandra*, *Sonneratia alba* and *Xylocarpus granatum* (Kariyawasam, 2015). *L. racemosa* is shown luxury growth at the Kala Oya and Chillaw Lagoons. *R. apiculata* is formed monospecific band at Batticaloa Lagoon but absent in the Urani and Potuvil Lagoons. Also, *Ceriops tagal* is dominated at the Rekawa Lagoon. *Rhizophora* species are distributed near the lagoon/estuary, then followed by *Lumnitzera* and *Avicennia* species. Distribution of mangroves are influenced by temperature, precipitation, fresh water runoff, sedimentation rate, salinity, nutrients input, and ocean circulation pattern (De Silva & Amarasinghe, 2010). Also, high tidal range occurs in the West Coast and low tidal range occur at the East Coast; then, low tidal caused low rise level and resulted in the formation of sand bank at the mouth of lagoon/estuary disrupting the connection with the sea. These current patterns and sea level rise have influenced the distribution of mangroves in Sri Lanka (Amarasinghe & Perera, 2017).

Mangroves are distributed as the layers of coastal area and then formed the zonation of the mangrove ecosystem around Sri Lanka. Mangrove formation is not closely related phylogenetics groups but they have independently evolved unrelated to each other. Also, they have independently developed features and functions for survival in the tidal condition and then showing convergence instead of shared decent. Currently, there are 21 species of mangroves available in Sri Lanka which need to determine their genetic variability. The objectives of the study are to determine the genetic diversity among six selected mangrove species, *R. mucronata*, *L. racemosa*, *R. apiculata*, *A. officinalis*, *L. littorea*, and *A. marina* using *rbcL* and *matK* gene markers and to optimize DNA extraction protocols for these mangrove species.

METHODOLOGY

Live specimens of *R. apiculata*, *R. mucronata*, *A. marina*, *A. officinalis*, *L. racemosa*, and *L. littorea*. Three (03) replicates of each species in a same area were collected from the West Coast covering the wet, dry, and intermediate climatic zones. Dried specimens were submitted to the National Herbarium, Peradeniya for morphological identification. DNA was isolated from young leaves of mangrove species according to the Healey et al. (2014) with some modifications. DNA extraction was done by grinding young mangrove leaves with pre-heated CTAB extraction buffer [2% CTAB, 100 mM Tris- HCl, 2.5 mM EDTA, 1.5 mM NaCl, 1% PVP], followed by 1-hour incubation of samples at 65 ^{o} C. Samples were centrifuged at 13,000 g for 10 minutes. Supernatant was transferred into a new tube, an equal volume of chloroform: iso amylalcohol (24:1) was added and centrifuged at 13,000 g for 10 minutes. This step was repeated three times. The upper aqueous layer was transferred to a new tube and equal volume of cold (20 ^{o} C) isopropanol and 4 M NaCl was added. Samples were incubated at 1 hour at -20 and centrifuged at 16,000 rpm for 15 minutes at 4 ^{o} C. DNA pellets were washed with 70% ethanol followed by 100% ethanol. Air dried DNA pellets were re-suspended in Nuclease free water and stored at -20 ^{o} C for future usage. Alternative DNA extraction was done using the plant genomic extraction kit. Plant tissues were ground under the liquid nitrogen. Tissues were transferred up to 100 mg to 1.5 ml or 2.0 ml microcentrifuge tube. 450 µL of LP buffer was added and mixed thoroughly, then incubated at 65 ^{o} C for 15 minutes and removed the tube from 65 ^{o} C. Incubation was extended when the samples were difficult to lysis. 150 µL of DA buffer was added and mixed thoroughly and vortex for 1 minute. Centrifuged the lysate at 12000 × g for 5 minutes. The supernatant was transferred to a new 1.5 ml tube. After 1.5 volume of P binding buffer was added. Mixed thoroughly and mixture was transferred to the spin column, then centrifuged at 12000 × g for 1 minute, discarded flow through. Sample volume was more than 750 µL, simply loaded and spun again. 500 µL of G binding buffer was added into the spin column. Centrifuged at 12000 × g for 30 seconds. 600 µL of washing buffer was added to the spin column. Centrifuged 12000 × g for 30 seconds. Repeated the step.

Centrifuged for an additional 1 minute at 12000 × g and transferred the spin column to a sterile 1.5 ml microcentrifuge tube. 100 µL of Elution buffer was added and incubated at room temperature for 2 minutes; then, centrifuged at 12000 × g for 1 minute. The buffer in the microcentrifuge tube contained the DNA. A volume of 3 µL genomic DNA from each mangrove species was mixed with the 2 µl of loading dye and the mixture was loaded into 0.8% agarose gel.The electrophoresis was carried out for 45 minutes at 80 V and it was visualized using a gel visualizer system (Khan et al., 2009). The quality and quantity of the undiluted and ×100 diluted genomic DNA samples were measured by Nano-drop method (Nakayama et al., 2016; Simbolo et al., 2013).

PCR were carried out according to the protocol of Gori et al. (2019). The primer pairs of rbcL_F1 (5‟ - ATG TCA CCA CAA ACA GAG ACT AAA GC- 3‟) and rbcL_R (5‟ - GAA ACG GTC TCT CCAACG CAT -3‟) were used as forward and reverse primers, respectively. The reaction mixture was mixed spinning at 6,500 rpm for 1 minute. The amplification was carried out in a thermal cycler (primus 96). Also, PCR were carried out using *matK* primers according to the protocol of Gori et al. (2019). PCR were performed using ~50 ng of genomic DNA in a 15 µl of reaction PCR cocktail containing 7.5 µl of 2X Promega Gotaq master mix (2X Gotaq reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3 mM $MgCl_2$), 0.5 µl of each 5 µM primers, and 5.5 µl of deionized water. The primer pairs of KIM_3F (5‟ - CGT ACA GTA CTT TTG TGT TTA CGA G- 3‟) and 15 KIM_1R (5‟ - ACC CAG TCC ATC TGG AAA TCT TGG TTC -3‟) were used as forward and reverse primers, respectively. The reaction mixture was mixed spinning at 6,500 rpm for 1 minute. A volume of 7.0 µL of amplified products were mixed with 2 µl of loading dye and loaded into 1.5% agarose gel to visualize the amplified DNA product. A volume of 4.5 µl of 100 base pair (bp) ladder was used as a marker. The electrophoresis was carried out for 1 hour and 15 minutes at 80 V and visualized using a gel documentation system (Wu et al., 2017). In DNA spot test, each six DNA spot were placed separately into the agarose gel; then observed through the UV light using Gel.doc.

RESULTS AND DISCUSSION

A comparison of the DNA concentration differences between CTAB and Biospin Plant genomic kit extraction method is given in Figure 1.

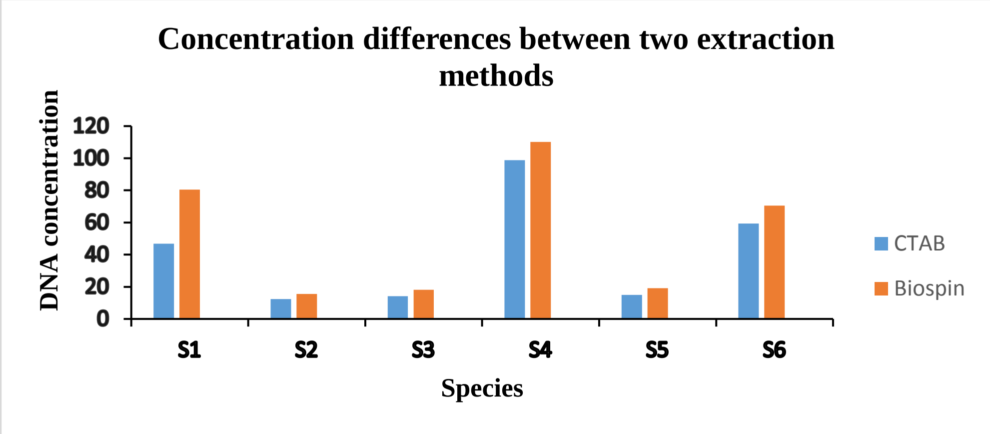

Figure 1: Differences of DNA concentration between two types of DNA extraction method

According to the DNA concentrations of each six species, *Avicennia officinalis* was shown proper values with their purities. If samples have greater values than 1.8, those are RNA contaminated while the samples that have shown lower values than 1.8 are contaminated by proteins. Mangrove species were shown specialized adaptations against the harsh environmental conditions, which comprised of a large number of secondary metabolites that were inhibited the DNA extraction and decreased the purity values. S4 species was distributed at low salinity areas that was a major factor and salinity may have influenced the DNA concentrations. As a result, S4 was shown with clear DNA bands while high DNA concentration and clear PCR products were shown with *matK* and *rbcL* primers (Figures 2 and 3).

CONCLUSIONS

In this study, *Avicennia officinalis* was shown clear genomic bands from CTAB and Biospin kit method and were clearly visualized through the *rbcL* and *matK* in amplification. However, other five species (*Rhizophora mucronata*, *Lumnitzera racemosa*, *Rhizophora apiculata*, *Lumnitzera littorea*, and *Avicennia marina*) were not shown clear band *via* both methods. The secondary metabolites and high salt concentration of these species would have affected efficient DNA extraction. Sodium ions may have combined with negative charge hydrogen ions and dissolved making DNA extraction difficult due to high concentration of salt in mangrove plants. However, *Avicennia officinalis* which showed better results is distributed only in low salinity areas, which cannot survive in high salinity areas. This study pioneered research of its kind on mangroves in Sri Lanka and will be baseline research for optimizing DNA extraction protocols for mangroves and exploring a variety of primers in future studies on genetic diversity of mangroves in Sri Lanka.

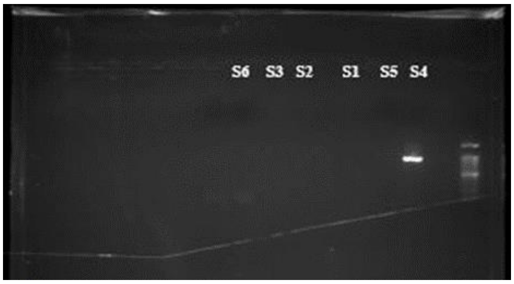

Figure 2: Visualized PCR product using matK

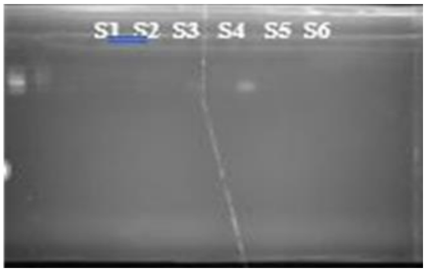

Figure 3: Visualized PCR product using rbcL

(S1*: Rhizophora mucronata*, S2: *Lumnitzera racemosa*, S3: *Rhizophora apiculata*, S4: *Avicennia officinalis,* S5: *Lumnitzera littorea*, S6: *Avicennia marina*)

REFERENCES

Abeysinghe, P. D., Triest, L., De Greef, B., Koedam, N., & Hettiarachi, S. (2000). Genetic and geographic variation of the mangrove tree Bruguiera in Sri Lanka. Aquatic Botany, 67(2), 131-141.

Alongi, D. M. (2012). Carbon sequestration in mangrove forests. Carbon management, 3(3), 313-322. https://doi.org/10.4155/cmt.12.20

Amarasinghe, M. D., & Perera, K. A. R. S. (2017). Historical biogeography of Sri Lankan mangroves. Ceylon Journal of Science, 46(5).

Arulnayagam, A., Khim, J. S., & Park, J. (2021). Floral and faunal diversity in Sri Lankan mangrove forests: a systematic review. Sustainability, 13(17), 9487.https://doi.org/10.3390/su13179487

Ball, M. C. (1996). Comparative ecophysiology of mangrove forest and tropical lowland moist rainforest. In Tropical Forest plant ecophysiology (pp. 461-496). Boston, MA: Springer US.

Gori, M., Pecchioli, S., Giordani, E., Saeedi, M. A., Wafa, F. H., & Biricolti, S. (2019). Barcoding assessment of the Citrus species cultivated in eastern Afghanistan. Advances in Horticultural Science, 33(3), 403-408.

Healey, A., Furtado, A., Cooper, T. and Henry, R. J. (2014) „Protocol: a simple method for extracting next-generation sequencing quality genomic from recalcitrant plant species‟, PLANT METHODS. Available at: http://www.plantmethods.com/content/10/1/21

Jayatissa, L. P. (2012). Present status of mangroves in Sri Lanka. The National Red List, 197 199. Kathiresan, K., & Bingham, B. L. (2001). Biology of mangroves and mangrove ecosystems.

Kauffman, J. B., Heider, C., Cole, T. G., Dwire, K. A., & Donato, D. C. (2011). Ecosystem carbon stocks of Micronesian mangrove forests. Wetlands, 31, 343-352.

Khan, E. U., Fu, X. Z., Wang, J., Fan, Q. J., Huang, X. S., Zhang, G. N., & Liu, J. H. (2009).

Ministry of Environment (2012). The National Red List 2012 of Sri Lanka; Conservation Status of the Fauna and Flora. Ministry of Environment, Colombo, Sri Lanka. viii + 476 pp.

Nakayama, Y., Yamaguchi, H., Einaga, N., & Esumi, M. (2016). Pitfalls of DNA quantification using DNA-binding fluorescent dyes and suggested solutions. PloS one, 11(3), e0150528.

Parani, M., Lakshmi, M., Elango, S., Ram, N., Anuratha, C. S., & Parida, A. (1997). Molecular phylogeny of mangroves II. Intra-and inter-specific variation in Avicennia revealed by RAPD and RFLP markers. Genome, 40(4), 487-495.

Ranawana, K. B. (2017). Mangroves of Sri Lanka. Publication of Seacology-Sudeesa Mangrove Museum, 1(1), 25-28

Regeneration and characterization of plants derived from leaf in vitro culture of two sweet orange (Citrus sinensis (L.) Osbeck) cultivars. Scientia Horticulturae, 120(1), 70-76.

Scholander, P. F., Hammel, H. T., Hemmingsen, E. A., & Garey, W. (1962). Salt balance in mangroves. Plant Physiology, 37(6), 722

Simbolo, M., Gottardi, M., Corbo, V., Fassan, M., Mafficini, A., Malpeli, G., ... & Scarpa, A. (2013). DNA qualification workflow for next generation sequencing of histopathological samples. PloS one, 8(6), e62692.

Wu, L. R., Chen, S. X., Wu, Y., Patel, A. A., & Zhang, D. Y. (2017). Multiplexed enrichment of rare DNA variants via sequence-selective and temperature-robust amplification. Nature biomedical engineering, 1(9), 714-72