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# MOLECULAR MARKERS: AN INTRICATE TOOL FOR NEW INSIGHTS IN MANGROVE GENETICS

**Sunil Kumar Sahu and *Kandasamy Kathiresan**

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences,
Annamalai University, Parangipettai, Tamil Nadu - 608 502, India
*Corresponding author: Email: kathirsum@rediffmail.com, Tel: +91-9442068003; Fax no. +91-4144238080

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**ABSTRACT:**
Molecular phylogenetics has become an imperative tool for genetic studies and has enticed considerable scientific attention during the last few decades. Mangroves are the characteristic complex plant communities of tropical and sub-tropical sheltered coastlines which have been the subject of taxonomic, floristic, physiological, and ecological studies. Undeniably, to investigate population structure and phylogenetic relationships molecular techniques have been extensively applied to mangroves and these techniques has paved opportunities to take mangrove research in new directions and to address vague issues in mangrove studies. But the availability of plethora of molecular markers, allozymes or DNA (mitochondrial DNA or nuclear DNA such as microsatellites or RAPDs/RFLPs/AFLPs) and the phylogenetic software leads many geneticists in dilemma to select the most appropriate marker or software for their respective study. In this review we present how the phylogenetic studies have provided new insights into population genetics, conservation issues and molecular taxonomy in different mangrove taxa by the use of molecular techniques. Moreover an outline on the most commonly used molecular markers are also presented with a critical review on the application, advantages, and limitations of major classes of these markers in mangrove research.

**Keywords:** *Mangroves; molecular markers; phylogenetics; biodiversity*

## [I] INTRODUCTION

Mangrove plants are a heterogeneous group of plants, specially adapted to withstand high salt concentrations and low soil aeration in tropical and subtropical coasts [1]. Mangroves are economically important in having the value up to $3.5 million per square km per year through a combination of coastal defenses, tourism and farming [2]. Approximately one-fourth of the world's tropical coastline is dominated by mangroves and they extend over 15.2 million ha world-wide [3]. The most extensive and luxurious mangroves extend across the Indo-Pacific regions where they are best developed in the delta system of major rivers. Mangroves also penetrate some temperate zones, but there is a rapid decrease in the number of species with increasing latitude [4]. The mangroves and their associated organisms constitute the 'mangrove forest community' or 'mangal'. The mangal and its associated environmental factors constitute the 'mangrove ecosystem'. The mangrove forests are often called as 'tidal forests', 'coastal woodlands' or 'oceanic rainforests' [1, 5, 6]. Mangroves are among the 'most threatened major environments on earth', having 35% of the global loss by anthropogenic activities [7]. Because of the adaptive characteristics, the ecology of mangroves has been extensively studied [8]. However, molecular

aspects of mangroves are only little studied. The genetic diversity has an impact on the higher levels of biodiversity [9]. Studies of genetic variability and interspecific relationships among the mangrove species using molecular markers have been used to accurately quantify the extent of genetic diversity among the species [10]. Unlike morphological markers, molecular markers are not prone to environmental influences and accurately portray the genetic relationships between plant groups [11]. The markers can also be used to select priority areas for conservation and provide vital information to improve strategies for genetic conservation [12]. The lack of genetic markers has prevented an in-depth understanding of population genetics and evolution of mangrove plants. Studies on genetically differentiated organisms depend largely upon the results of molecular genetic analyses. Molecular markers like AFLP and/or RAPD have been used to study the inter- and intra-specific variations in some mangrove species [13, 14, 15, 16]. A number of attempts have been made to study the inter- and intra-generic relations among them [17, 18] using RAPD alone or with other markers. Sequences of the internal transcribed spacer (ITS) regions of nrDNA and the chloroplast gene *matK* have been used for phylogenetic studies at the inter and intrageneric levels in many angiosperm families [19, 20], but conflicts between nrDNA and cpDNA phylogenies have been reported in some groups [21, 22, 23]. The recent advancement in sequencing technologies has taken phylogenetic analysis to a new height. Phylogenies have permeated nearly every branch of biology, and the availability of plethora of phylogenetic methods and software packages may seem daunting to an experimental biologist [24]. DNA sequencing has emerged as one of the most utilized molecular approaches for inferring phylogenies, especially utilizing chloroplast genes [25]. About 90% of the mangrove forest cover are present in developing countries but are nearing extinction in 26 countries. Long term survival of mangroves is at great risk due to fragmentation of the mangroves. It is possible that the ecosystem services offered by mangroves may be totally lost within 100 years [26]. The mangrove habitats continue to disappear globally at a rate of 0.66% per year during the 2000–2005 periods [3]. Mangroves habitat loss has put at least 40% of the animal species which are restricted to mangrove habitat globally at an elevated risk of extinction. Considering fundamental roles attributed to genetic diversity in evolutionary processes and in the present biodiversity, understanding genetic structures of mangroves is important both for evolutionary and conservation biology. The knowledge of genetic diversity within and among populations is important for conservation management [27]. To ensure the optimal conservation of the mangrove forests in any region, it is essential to explore the genetic diversity of the remnant populations [28]. Population genetic studies of mangroves are also essential for evaluating afforestation, domestication and breeding programs. It is in this context; this review aims to present how the phylogenetic studies in mangroves have provided new insights into population genetics, conservation issues and molecular taxonomy of mangroves.

## [II] ORIGIN AND EVOLUTION OF MANGROVES

The word ‘mangrove’ dates its origin as 1613 and it is derived from Portuguese word ‘mangue’ and the English word ‘grove’. However, mangrove plants do not exhibit very primitive plant characteristics. It is believed that the first appearance of mangroves as early as 80 million years ago, possibly arising just after the first angiosperms [29]. *Avicennia* and *Rhizophora* were probably the first genera to evolve, appearing near the end of the Cretaceous period [30]. The origin of mangrove is still under debate. It is not clear whether the origin and spread of mangroves are from the Malaysian peninsular and spread to a region between Australia and Papua New Guinea, and or between Malaysia and Northern Australia. The process of continental drift appears to have played a pivotal role in determining both the diversity and type of mangroves found today, as well as their current global distributions. Evidence

for an adaptive radiation in the mangrove habitat comes from the age and location of fossils, and from phylogenetic reconstruction of ancestral habitats. The oldest fossil evidence for a modern mangrove genus (the palm Nypa) is from the Late Cretaceous, but most groups arose during the Palaeocene and diverse mangrove forests were established by the Early Eocene [31, 32]. The mangrove plants consist of about 70 species worldwide, comprising trees, shrubs and ferns that belong to 21 family-level lineages each independently derived from non-mangrove ancestors [31, 33]. They provide an impressive instance of trait evolution and a combination of diverse morphological and physiological adaptations. There has been much interest in the historical biogeography of mangroves, in particular attempting to explain why their diversity is strongly focused near the center of the Indo-West Pacific (IWP) region. This has been called the 'mangrove biodiversity anomaly', [31, 33] although it is not anomalous in the context of the similar pattern displayed by many marine animal groups. Molecular phylogenetic evidence suggests that most mangrove lineages originated in the Old World [33] and diversified within the IWP [34, 35], although there are no molecular phylogenetic studies at species-level of entire mangrove clades, even for the more species rich and widespread groups such as Avicenniaceae and Rhizophoraceae. Of the 40 species of true mangroves, 30 species are known to occur in the Indian sub-continent. The phylogenetic relationships among and within the tribes, however, are in need of detailed studies [8]. The distribution and independent origin of several mangrove lineages within angiosperms is illustrated in Fig.1.

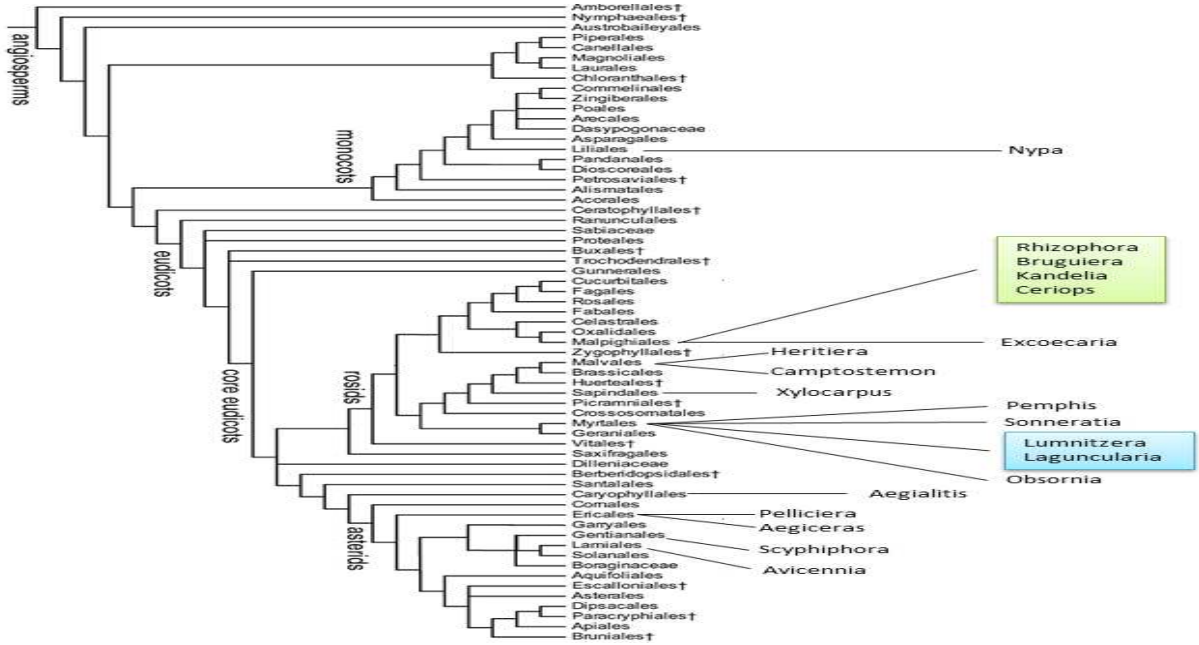


Fig.1. The phylogenetic distribution and independent origin of several mangrove lineages within angiosperms. Independent mangrove lineages are shown in the extreme right. Monophyletic groups are represented in box. Ordinal classification of angiosperms is adapted from Angiosperm Phylogeny Group (APG-III, 2009) [102] and Schwarzbach and Ricklefs [103].

## [III] BIODIVERSITY OF MANGROVES

Biodiversity can be defined at genetic, species and community levels of biological organization. Even though genetic diversity is at the lowest hierarchy, without genetic diversity, a population cannot evolve and adapt to environmental changes. Diversity of mangroves is low as compared to that of tropical rainforests. Mangrove habitats include diversified habitats, such as core forests, litter-forest floors, mudflats, water bodies (rivers, bays, intertidal creeks, channels and backwaters), adjacent coral reefs and seagrass ecosystems [6]. These diversified habitats support

a wide variety of flora and fauna of marine, freshwater and terrestrial species and make the mangrove system biologically diverse. There are 65 species of mangroves globally, of which about 40 species exist in Southeast Asia, 15 in Africa and 10 occur in the Americas. Mangrove do harbours unique microbial diversity but there is paucity of information regarding the association of rhizobacteria with mangroves [36]. Although, mangroves and their components have been studied extensively but as far as their biodiversity is concerned it still remains poorly understood [37].

## [IV] DISTRIBUTION OF MANGROVES

Mangrove forests are distributed in the intertidal region between the sea and the land in the tropical and subtropical regions of the world approximately between 30° N and 30° S latitude [38]. Northern extensions of this limit occur in Japan (31°22' N) and Bermuda (32°20' N); southern extensions are in New Zealand (38°03' S), Australia (38°45' S) and on the east coast of South Africa (32°59' S) [39]. Mangroves find it difficult to colonize the coastal zone with waves of high energy and hence they normally establish themselves in sheltered shorelines [1]. The distributions of mangroves are very complex. There are approximately one-third fewer taxa in the Atlantic East Pacific (AEP) [34], even though there is an equivalent area of mangrove habitat in each region [39]. Despite important studies and review articles on the systematics, biogeography and evolution of mangroves [14, 31, 40, 41] progress is slow. For instance, we still know little about the so-called 'centres' of mangrove evolution and we are still unclear about how or whether these relate to centres of extant diversity. However, a dearth of primary data has meant that alternatives have not been properly explored. Climatic factors such as temperature and moisture affect mangrove distribution [29, 42]. World-wide, species diversity, height and biomass are the lowest in the northern and southern extremes and increase toward the tropics. The largest extent of mangroves is found in Asia (42%) followed by Africa (20%), North and Central America (15%), Oceania (12%) and South America (11%) [3]. The Indo-Malaysian region has 48 mangrove species [34], the highest species diversity anywhere in the world. Although the exact reasons for species diversity can be debated, the conservation and sustainable management of this diversity are critical. Important questions remain unreciprocated, like why is the diversity of mangrove taxa greater in the Indo West Pacific (IWP) than in the Atlantic East Pacific (AEP). Distributions of mangroves are complicated. In the AEP, the American continents currently separate East-Pacific and West-Atlantic coastlines. Similarly, the Atlantic separates East-America from West-Africa and the ocean distance between (comparable with the East-Pacific barrier) suggests there is little, if any, genetic exchange between these sub-regions currently (Fig. 2). Current distribution patterns in the AEP, therefore, cannot be explained by existing dispersal routes and geological conditions. There is an urgent need to promote greater understanding by collecting detailed new observations and appropriate data.

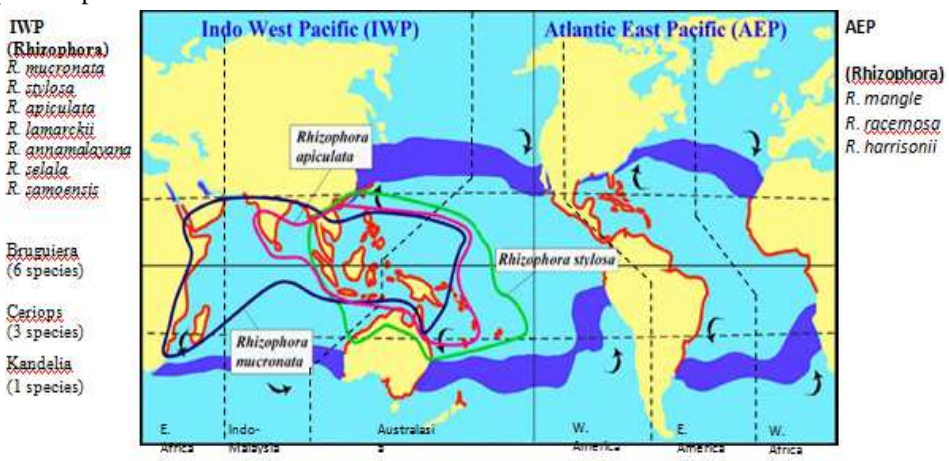


**Fig. 2.** World distributions of the Indo West Pacific Rhizophora species [104]. There are 17 taxa in the IWP and three taxa in AEP. Six dispersal barriers include

two land barriers and variously effective water barriers [105]. The red marked coastlines indicates the distribution of all mangroves. (Note: The east coast of Africa and Americas has more extent of mangroves than their west coasts and this pattern can be attributed to the warmer currents tending towards Polar region in the east coast and colder currents from Polar region in the west coast).

## [V] MANGROVES DNA EXTRACTION - CONSTRAINTS AND REMEDIES

One prerequisite to reliable molecular biology work is the isolation of pure, intact and high quality DNA. DNA based methods are highly specific, reproductive and sensitive and characterized by high discriminatory power, rapid processing time with low costs. However, the methods are strongly limited by the presence of inhibitors in plant tissues [43]. Mangroves contain high levels of polysaccharides, polyphenols, pigments and other secondary metabolites which make DNA unusable for downstream work in molecular biology research [44, 45]. Polysaccharides and polyphenols are particularly abundant in mangrove leaves and are not completely removed during classical extraction protocols; they remain as contaminants in the DNA preparations. Polysaccharides make DNA viscous, glue like and non-amplifiable in the PCR reaction by inhibiting the activity of restriction enzymes and Taq DNA polymerase [46]. The oxidized forms of polyphenols covalently bind to DNA, giving a brown colour and reduce their maintenance time, making it useless for molecular studies [47]. Polysaccharides do interfere with biological enzymes such as restriction endonucleases and ligases, resulting in unsuccessful amplification [45]. In order to overcome the above said constraints, a number of methods for DNA isolation from plants containing high levels of secondary metabolites have been developed [43, 45, 46, 48, 49, 50]. Polyvinylpolypyrrolidone (PVPP) or its water-soluble counterpart polyvinylpyrrolidone (PVP) is used to extract genomic DNA from polyphenol-rich plants [51, 52]. PVP forms complex with latex lactones, actucin and other phenolics and they accumulate at the interface between the organic and the aqueous phases by centrifugation after addition of chloroform. Similarly, Cetyl Trimethyl ammonium bromide (CTAB) binds to fructans and other polysaccharides and forms complexes that are removed during subsequent chloroform extraction [45, 53]. Recently, a rapid CTAB method has been developed for extracting DNA especially from mangroves and salt marsh species without using liquid nitrogen and phenols [54].

## [VI] MOLECULAR TECHNIQUES

With the advent of the PCR (polymerase chain reaction) technologies, an ample of techniques are available for identifying genetic differences between organisms. There are mainly three types of markers namely morphological, biochemical and DNA based molecular markers. These DNA based markers are further differentiated into non-PCR-based (RFLP) and PCR-based markers (RAPD, AFLP, microsatellites, etc.). The choice of technique depends upon the material being studied and the nature of the questions being addressed. Table 1 shows a comparative detailed analysis of major molecular tools with their advantages and disadvantages.

### 6.1 Biochemical marker

#### *Allozyme*

Allozymes have been widely used to address phylogenetic relationships and to estimate population parameters such as gene flow, local differentiation and migration pathways. Allozyme phenotypes also have been described in several mangrove genera including *Avicennia Bruguiera, Kandelia*, *Rhizophora* and *Sonneratia* [55, 56]. However, none of these studies provided more powerful means of interpreting the gene flow among populations and the breeding systems of mangrove species. Allozyme markers have also been used to quantify gene flow and infer dispersal within and between the populations of *Avicennia* [34]. They demonstrate that gaps in geographical distribution represent significant barriers to gene flow, revealing a limited long-distance dispersal capacity in *Avicennia.* Critical studies on the genetic structure of *Ceriops*, have shown that *Ceriops tagal* var. *tagal* and *C. tagal* var. *australis* are completely reproductively isolated [57].

Allozymes and intersimple sequence repeats have also been used to study the reproductive biology and genetic diversity of *Aegiceras corniculatum* [58]. These studies indicate that molecular markers may be extremely useful to solve the problems related to population genetics where normal taxonomical markers are not able to throw light. Even though, proteins are used for molecular taxonomic analyses, applications involving the protein are far fewer than those with DNA markers due to meager level of protein polymorphism. Proteins are also often differentially expressed both in space (tissue specificity) and time (developmental regulation), which limit availability of the proteins. Also, proteins are difficult to store in non-denatured condition than DNA under field conditions.

6.2. **Molecular markers**

**6.2.1. Non-PCR-based techniques**

***Restriction Fragment Length Polymorphism (RFLP)***

RFLP is a non-PCR-based indirect method which is used as a major tool to identify the genetic diversity within and between species [59]. Polymorphism is detected where there is an addition or loss of restriction site, or if a length polymorphism exists between two restriction endonuclease sites due to insertion or deletion of nucleotide sequences. RFLP has been used to study the genomic relations among 24 mangroves and its associates [14, 15]. These studies provide molecular data favouring the separation of *Avicennia* species from the Verbenaceae to create a monotypic family the Avicenniaceae. Using RFLP and RAPD based markers it has been demonstrated that considerable intra-population and inter-population genetic variations exist in *Excoecaria agallocha,* and that lack of genetic variation is not the reason for the morphological uniformity observed across the range of the species [60]. They have not found any marker for differentiating sex of the plant. The mitochondrial genome is known to have evolved at a slower rate and the evolution rates differ between lineages over long evolutionary time periods [61].

**6.2.2. PCR-based techniques**

***Random Amplified Polymorphic DNA (RAPD)***

RAPD is another extensively utilized indirect molecular approach for genotypic differentiation and molecular taxonomy among different mangrove species [62]. This method is based on polymerase chain reaction (PCR) where short sequences of DNA fragments are randomly amplified using decamer oligonucleotide sequences. Recently, genetic variability among male populations of *Excoecaria agallocha* has been studied by using DNA markers [63]. Mangroves do have inter and intra-generic relations, as proved by RAPD [17]. The RAPD markers alone or along with other molecular markers have extensively been used to study genetic variations in mangrove species such as, *Avicennia, Excoecaria, Acanthus* and *Bruguiera*. RAPD markers have been helpful to study the parentage analysis in *Rhizophora* hybrid [13]. Two non-mangrove species of Rhizophoraceae has been successfully separated from nine mangrove species using RAPD markers along with AFLP (amplified fragment length polymorphism) markers [16]. Similar result does not hold true when genomic relations among 31 species have been studied using RAPD and AFLP markers. RAPD markers together with cytological evidence provide an effective tool to access the interspecific genetic polymorphism in mangrove species, to solve the taxonomic problems and to design their conservation strategy [64]. Using RAPD markers, eight ecotypes of *Xylocarpus granatum* has been distinguished in an Indian mangrove forest (Bhitarkanika) which is one among the two mangrove genetic paradises in the world [65]. Mangrove species display greater variation within their population than across [13, 14, 66, 67]. High levels of polymorphism observed in mangrove species such as *Avicennia marina* [13] and *Excoecaria agallocha* [66] may also be indicative of the high microsite variation within a given population and in contrast to the high levels of inter-specific variation, the levels of intra-specific variation are very low. However, RAPD is often criticized for its lack of reproducibility [68].

***Amplified Polymorphic Length Polymorphism (AFLP)***

AFLP is a PCR-based approach to DNA fingerprinting [69] which provides high levels of resolution to delineate complex genetic structures. These markers have a very high diversity index, resulting in a limited number of primer combinations required to screen a whole genome and is applied to develop a system for the fingerprinting [70]. In contrast to the restriction fragment length polymorphism (RFLP), the AFLP technique generates an unlimited number of DNA fragments from nanogram amounts of genomic DNA. AFLP markers have been used to study the genomic relations in three species of *Heritiera* and the results have revealed a clear-cut segregation of true mangrove species *H. fomes* from mangrove associate *H. littoralis* and also land race species *H. macrophylla* [16]. Similar result has also been reported in the Rhizophoraceae group where two non-mangrove species are separated out from the rest nine mangrove species [16]. The reliability of the RFLP technique is combined with the power of the PCR technique in AFLP. In comparison to the random amplified polymorphic DNA (RAPD) approach, the AFLP technique uses more stringent conditions which give better reproducibility. Furthermore, this technique is quantitative and AFLP can be used as co-dominant markers [71].

***Microsatellites***

Microsatellites, also known as simple sequence repeats (SSRs), are an important class of markers because of their abundance and hyper-variability. The regions flanking the microsatellites are generally conserved and PCR primers relative to the flanking regions are used to amplify SSR-containing DNA fragments. The length of the amplified fragment varies according to the number of repeated residues. Although this marker possesses extremely high levels of polymorphism, it has come very late into usage for understanding the genetic variations of mangroves [72, 73, 74, 75]. These codominant and highly informative markers have been used in population genetic studies in many plants, including mangroves [72, 76, 77]. Polymorphism is higher with SSRs than with other markers in many studies related to a variety of plant species [78, 79]. Among dominant markers, ISSRs (inter-simple sequence repeats) are superior to RAPDs in terms of higher polymorphism and reliability [80]. SSRs have been used in population genetic studies in mangroves [58, 81, 82]. Cross-priming studies has revealed polymorphic nature of all the loci in *Kandelia candel* and *Rhizophora stylosa* and proved that the microsatellite markers are ideal for population studies [74]. Recently, genetic relationship in three species of *Rhizophora* (*R. annamalayana, R. apiculata* and *R. mucronata*) have been studied by using microsatellite marker. This study reveals that *R. annamalayana* and *R. mucronata* are genetically much closer (0.006) than *R. apiculata* (0.008) [83]. The optimization of PCR conditions is a matter of necessity for cross-amplification tests. Despite their extremely fast rates of repeats, evolution of many microsatellite loci is quite conservative in their flanking regions and hence they can persist for long evolutionary time and they are much unchanged. Due to this, primers developed for a species from the flanking regions of a microsatellite locus can be used to amplify the same locus in other related species. Generally, the designing of microsatellite primers, specific to new species is expensive and time consuming, but the cross priming can be alternative and attractive option as it is cheap and fast.

**[VII] DNA BARCODING OF MANGROVES**

Mangroves are highly productive, but extremely sensitive and fragile [1]. Lack of adequate genetic studies impairs conservation practices of mangrove genetic resources. To date, traditional taxonomy relies mostly on diagnostic morphological characters, requiring expert knowledge to identify specimens. In this regard, DNA barcoding has proved to be a useful alternative method for rapid global biodiversity assessment, providing an accurate identification system for living organisms[84]. Thus, there is a demanding need for taxonomic expertise in a broad range of taxa. DNA barcoding has proved to be a powerful alternative method to traditional

morphological approaches, which allows to complement the identification techniques for living organisms. The two-locus combination of *rbcL* + *matK* has been recommended as the plant barcode [85, 86] and has been approved by Consortium for the Barcode of Life (CBOL) in 2009. Therefore in this context we have discussed about the two proposed standard plant barcode genes for their possible application in mangrove DNA barcoding.

### 7.1. *matK* (Maturase kinase) gene

The *matK* gene formerly known as *orf*K is emerging as a valuable gene to study mangrove molecular systematics and evolution, because of its reasonable size, high substitution rate, evenly distributed codon position variation, low transition and transversion ratio, and the easiness of amplification due to its two flanking coding *trn*K exons [87]. The 1500 bp *matK* gene is named according to its possible maturase function and its location within the *trn*K gene encoding the $tRNA^{Lys}$ (UUU). The gene also exhibits a relatively high proportion of transversions, with the transition/transversion ratio (ti/tv) approaching unity [88]. Recent studies have substantiated the use of *matK* gene for resolving phylogenetic relationships in a broad taxonomic range [87, 88]. Although substitution rates in plastid DNA are generally low compared to nuclear DNA, and thus may not have high resolution for population level studies, the substitution rate of the *matK* gene is among the highest in plastid genes [89]. On the one hand, slow evolving coding regions (e.g. genes for photosynthesis, ribosomal subunits, etc.) have a high likelihood of alignability across a wide set of land plant lineages, but often fail to resolve rapidly radiated lineages [90]. On the other hand, non-coding and fast evolving coding markers are more troublesome in terms of primary homology assessment and thus less frequently they are used. However, once the rather time consuming alignment has been performed, fast evolving regions, especially *matK* not only tend to provide the highest phylogenetic structure, they also offer the desired phylogenetic information [91, 92]. In order to use the *matK* gene sequence at various taxonomic levels, more detailed information on its pattern of variation, phylogenetic signal distribution, and transition and transversion ratios will be of immense help.

### 7.2. Ribulose-bisphosphate carboxylase large subunit (*rbcL*) gene

*rbcL* gene, located on the chloroplast genome, is an appropriate choice for inference of phylogenetic relationships at higher taxonomic levels [93]. It is a 1350 bp gene which is one of the most widely and best-investigated chloroplast DNA genes by means of molecular techniques, and is known to exhibit extensive variation above the species level [94]. The origin of angiosperms, relationship of dicotyledons and monocotyledons, and phylogenetic reconstruction at class, order, family and genus levels has been studied using the *rbcL* gene sequence data [93]. The chloroplast genes have a very slow rate of nuclear substitution thereby having a slower rate of evolution than the nuclear genes [95]. In a molecular phylogenetic analysis of Myrtales based on *rbcL* sequence data, two mangrove genera of Rhizophoraceae namely *Bruguiera* and *Rhizophora* were positioned in the genera outside Myrtales, instead forming a common clade with Euphorbiaceae, Humiriaceae, and Malpighiaceae in a “rosid” clade [96]. The cpDNA of some mangrove and mangrove-associate species have been investigated by PCR amplification of two specific regions, *trn*S-*psb*C and *rbcL*, followed by restriction digestion with a four base restriction enzyme, *Hae* III [15]. Analysis of polymorphism in the restriction fragments (PCR-RFLP) has revealed 18 classes of restriction banding pattern in *trn*S-*psb*C region, which provides molecular evidence for diversity in the mangrove floral component above-species level. The molecular phylogeny of Rhizophoraceae based on *rbcL g*ene sequences supports monophyly of Rhizophoraceae [97]. Similarly, the tribal and generic relationships within Rhizophoraceae have been evaluated with a combination of six molecular data sets (*rbcL*, *atpB-rbcL* intergenic spacer, *trnL-trnF* intergenic pacer, ITS1, ITS2, and 5.8S) along with morphological data sets [98]. This work suggests

that how DNA sequence data can resolve systematic relationships that are difficult to infer from analysis of morphological data alone due to extensive homoplasy. The study also rejects previously suggested close relationships of the Rhizophoraceae with Celastraceae or Elaeocarpaceae and identifies the Erythroxylaceae as sister group to the Rhizophoraceae. Molecular phylogenetic analysis of mangroves for evolution studies of vivipary and salt secretion has been studied by analysing and sequencing the 18S rRNA, *rbcL*, and matR genes of large and representative samples across mangroves [99]. This work suggests the multiple origins for both vivipary and salt secretion in mangroves.

## [VIII] MOLECULAR PHYLOGENETIC SOFTWARE

Biological diversity of mangroves needs to be organized and catalogued effectively and efficiently. Molecular phylogenetics applies a combination of molecular and statistical techniques to infer evolutionary relationships among organisms or genes. Recent advances in sequence analyses methods and software have revolutionized the phylogenetics studies. Mangrove geneticists across the globe routinely employ molecular phylogenetic methods in their research. Phylogeny is an evolutionary tree that shows how species are related to each other. Table 2 describes a compilation of some most commonly used free and commercial phylogenetics software for constructing phylogenetic trees, and Table 3 presents a concise list of online software and applications used for phylogenetic tree visualization

## IX] CONCLUSION

The present work overviews about the molecular tools that are widely used in plants especially mangroves. Allozyme was used for many years as the standard tool in genetic studies of mangroves, but in the recent years, it has been replaced by DNA markers. A major drawback for the methods relying on unspecific primers and produce multilocus band patterns (i.e. RAPD, AFLP and ISSR), is that the investigated loci are biallelic (a band is present or absent). It is also difficult to distinguish heterozygotes from homozygotes based on band intensity. Mangroves are fast disappearing globally resulting in loss of genetic diversity and ecosystem functions [3]. The loss of mangrove species will have devastating economic and environmental consequences especially in those areas with low mangrove diversity. Across the globe, mangrove species found in estuarine areas are most threatened because they are most frequently cleared for development of aquaculture and agriculture [100]. It is necessary to collate comprehensive species-specific and site-specific information for the mangroves. In absence of which, it is difficult for the identification and implementation of conservation priorities. To overcome these losses, conservation and sustainable management of mangrove resources, is a major priority in the coastal areas of many countries. Population genetic studies of mangroves are, therefore, aimed at providing the information needed for restoration and conservation of genetic resources. Thus, to develop a simple, rapid and economical DNA extraction method from plants especially from mangroves is of dire need. Extensive studies on mangrove population genetics will be useful for designing conservation and breeding programs. Such studies also will help in identification of sites with high genetic diversity leading to establishment of in-situ mangrove genetic resource centers for preservation and protection of fast disappearing mangrove species. The genetic diversity of few mangrove species cannot be increased by merely enlarging their population size through artificial breeding. Artificial introduction must be done carefully, because the genetic divergence may also result in genetic pollution [101]. The risk of genetic pollution must be considered if individuals from populations with a relatively high level of genetic diversity (e.g., Thailand population) are introduced into populations with low-level genetic diversity (e.g., populations in China) for the purpose of enhancing genetic diversity. The most commonly used standardized mangrove DNA extraction methods that use toxic and hazardous chemicals such as phenol and chloroform require special

equipment to minimize exposure and limit their usage. Although, commercial DNA extraction kits do exist and are convenient and usually safe, but their high cost is a limiting factor. A future challenge for DNA barcoding in plants is to increase the efforts for identifications of unique species. Specific primers for the potential barcode locus of *matK* and *rbcL* should be developed to speed up the process of DNA barcoding in mangroves. Cytological and molecular phylogenetic research has mainly focused on the analysis of genomes of different plant taxa, but the detailed studies on the cytological, cytochemical and molecular aspects of mangroves are lacking. However, chromosome and cytophotometric studies are difficult in the mangrove species due to the accumulation of a high amount of secondary metabolites and their relatively small chromosomes. DNA sequencing has emerged as one of the most utilized of the molecular approaches for inferring phylogenies because of the direct comparison of the nucleotide sequences and the relative ease of interpreting the sequence information. Molecular characterization can play a role in uncovering the history, and estimating the diversity, distinctiveness and population structure. Awareness of the level of genetic diversity and the proper management of genetic resources are important issues in modern scenario. New markers deriving from DNA technologies are valuable tools to study genetic variability for conservation purposes. In the near future, the advent of genomics will give an impressive tool for assessment of genetic resources. We need not only the willingness to conserve genetic diversity, but also the knowledge of how to do so, in addition to the ability to turn that knowledge into action.

**[X] Perspectives**

Genetic diversity or variation and its measurement have vital importance in interpretation, understanding and management of populations and individuals. The use of molecular tools and markers in plant studies has been increased during the last decade and such data are now being extensively applied to study mangroves. The surge in the application of molecular information to systematic and evolutionary questions has resulted in significant contributions to both plant and animal systematics and in the emergence of molecular systematics. In the past the ability to discriminate between varieties was heavily dependent on morphological traits. Lately, DNA markers have been employed as a promising method of finger printing. In the future, molecular methods will become accessible to a larger number of researchers and will certainly play a vital role in mangrove plant research. Characterization of the types, patterns, and rates of nucleotide divergence in a gene prior to its utilization in systematic and evolutionary studies may frequently be an important prerequisite for generating robust and reliable phylogenies. Geneticists desire distinct, uniform, stable and superior strains of mangrove plants to implement conservation strategies. We foresee a large impact especially for conservation and management issues where molecular tools can provide information about the population genetics, which in turn will help efforts to conserve and protect genetic variation. More emphasis should be given on sampling more taxa throughout the range worldwide, especially different inland genera. Many plant molecular biology techniques are toiling, costly and time consuming. This is particularly true for DNA extraction techniques. Reduction or elimination of toxic chemicals such as phenol and chloroform needs to be reduced during the process of DNA extraction and the overall extraction of intact and unsheared DNA is of utmost importance. Not only mangroves, molecular identification and diversity assessment of the flora and fauna, especially the microorganisms should also be encouraged. Sequences of more conserved genes should be employed for the molecular phylogenetic analyses. To solve the taxonomic disputes for species such as *Acanthus*, natural hybrids of *Rhizophora* and *Sonneratia* species by using potential and most suitable molecular marker is the need of the hour. Future work should be focused on gathering more *matK* and *rbcL* sequences of mangroves for improved primer development, as well as to

examine PCR amplification and sequencing quality. Apart from phylogenetic studies, scientists have long been intrigued by the remarkable ability of mangroves in salinity tolerance but this specific area of research is still at infancy. Excavating the novel genes from mangroves could possibly help to develop abiotic stress resistant plants. Most importantly molecular marker techniques should be applied to construct a physical map of the mangrove genome which will ultimately help in determining the association of the genetic distance to the physical distance between mangrove taxa.

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### Glossary

**Molecular marker**: The DNA sequence used to 'mark' or track a particular location (locus) on a particular chromosome, i.e. marker gene.

**Biodiversity**: The number and variety of organisms found within a specified geographic region.

**Phylogeny**: Evolutionary tree that shows how different species are related to each other.

**Molecular phylogeny**: phylogeny based on molecular data (DNA sequence)

**Continental drift**: The movement of the Earth's surface relative to each other by drifting across the ocean bed.

**Intertidal**: The region between the high tide and the low tide mark.

**Polymerase chain reaction (PCR)**: A technique for amplifying DNA sequences *in-vitro* by separating the DNA into two strands and incubating it with oligonucleotide primers and DNA polymerase.

**Allozymes**: The non-denatured proteins with different net charges and allelic forms

**DNA barcoding**: It is a taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species

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**Table 1**. Characteristics of molecular marker techniques used in mangrove genetic research

| Characteristic | Allozymes | RAPD | AFLP | ISSR[c] | Microsatellite (SSR) | cpDNA sequences |
|---|---|---|---|---|---|---|
| Level of polymorphism[a] | Low | Medium | Medium | Medium | High | Medium |
| Loci[d] | Single | Multi | Multi | Single | Single | Single |
| Dominance[b] | Codominant | Dominant | Dominant | Dominant | Codominant | Haplotypic[C] |
| Sequence information needed[a] | No | No | No | No | Yes | Yes |
| Non-invasive sampling[b] | No | Yes | Yes | Yes | Yes | Yes |
| Start-up costs[a] | Medium[c] | Low | High[c] | Low | High | High |
| Development cost[a] | Low | Low | Medium | Low | High | Medium |
| Development time[b] | None | Limited | Limited | Limited | High[c] | Medium[c] |
| Reproducibility[a] | Medium/high | Low | Medium | Medium | High | High |
| Integration between labs[c] | Medium | Low | Medium | Medium | High | High |
| Allelic richness[c] | ++ | + | + | + | +++ | ++ |
| Heterozygosity[c] | +++ | + | + | + | +++ | (++) pop level |
| Gene flow[a] | +++ | (+) | (+) | (+) | +++ | ++ |
| Inbreeding[c] | +++ | — | — | — | +++ | — |
| Individual genotyping[a] | (+) | (+) | + | (+) | +++ | — |
| Population differentiation[a] | +++ | ++ | ++ | ++ | ++ | ++ |
| Hybridization[a] | ++ | ++ | ++ | ++ | + | ++ |
| Polyploidy[a] | +++ | — | — | — | + | (+)[c] |
| Phylogeography[a] | — | — | (+)[c] | — | (+)[c] | +++ |
| Phylogeny | (+) | — | — | — | (+)[c] | +++ |
| PCR[d] | No (*protein) | Yes | Yes | Yes | Yes | Yes |
| Overall variation[d] | Low | High | High | High | High | High |

**(+++) excellent; (++) good; (+) moderate; (+) has been used; (—) unlikely to be used or useless;**

**[a] Adapted from Lowe et al. (2004) [106]**

**[b] Adapted from Frankham et al. (2002) [107]**

**[c] Adapted from Triest (2008) [108]**

**[d] Added in this review**

**Table 2.** List of most widely used software for phylogenetic studies

| Software | Description | Methods | Link | Reference |
|---|---|---|---|---|
| **BAli-Phy** | Simultaneous Bayesian inference of alignment and phylogeny | Bayesian inference, alignment as well as tree search. | http://www.biomath.ucla.edu/msuchard/bali-phy/ | [109] |
| **BayesTraits** | Analyses trait evolution among groups of species for which a phylogeny or sample of phylogenies is available | Trait analysis | http://www.evolution.rdg.ac.uk/BayesTraits.html | [110] |
| **BEAST** | Bayesian Evolutionary Analysis Sampling Trees | Bayesian inference, relaxed molecular clock, demographic history | http://beast.bio.ed.ac.uk | [111] |
| **ClustalW** | Progressive multiple sequence alignment | Distance matrix/nearest neighbor | a)http://www.ebi.ac.uk/Tools/msa/clustalw2/<br>b)http://www.ch.embnet.org/software/ClustalW.html | [112] |
| **Geneious*** | Geneious provides sophisticated genome and proteome research tools | Neighbor-joining, UPGMA, MrBayes plugin, PHYML plugin | http://www.geneious.com/web/geneious/download-geneious | [111] |
| **MEGA** | Molecular Evolutionary Genetics Analysis | Distance, Parsimony and Maximum Composite Likelihood Methods | http://www.megasoftware.net/ | [114] |
| **MrBayes** | Posterior probability estimation | Bayesian inference | http://mrbayes.net | [115] |
| **PhyML** | A fast program for searching the maximum likelihood trees using nucleotide or protein sequence data | Maximum likelihood and Bayesian inference | http://www.atgc-montpellier.fr/phyml/binaries.php | [116] |
| **PAUP*** | Phylogenetic analysis using parsimony | Maximum parsimony, distance matrix, maximum likelihood | http://www.sinauer.com/ detail.php?id=8060 | [117] |
| **PHYLIP** | Phylogenetic inference package | Maximum parsimony, distance matrix, maximum likelihood | http://evolution.gs.washington.edu/phylip.html | [118] |
| **TNT** | Tree analysis using new technology | Fast parsimony program intended for very large data sets | http://www.zmuc.dk/public/ phylogeny/TNT | [119] |

**Note:** The listed software are compatible to run on Windows, Mac OSX and Unix or Linux platforms. All

the software are freely downloadable, except Geneious* and PAUP*.

**Table 3**. A compiled list of online software and applications used for phylogenetic tree visualization

| Name | Description | Link | Reference |
|---|---|---|---|
| **iTOL - interactive Tree Of Life**[a] | Annotate trees with various types of data and export to various graphical formats | http://itol.embl.de/ | [120] |
| **TreeVector**[a] | Java based scalable, interactive, produces dynamic SVG or PNG output | http://supfam.cs.bris.ac.uk/TreeVector/ | [121] |
| **PhyloExplorer**[a] | Facilitate assessment and management of phylogenetic tree collections. Identify related trees in the TreeBASE database | http://www.ncbi.orthomam.univ-montp2.fr/ | [122] |
| **PhyloWidget**[a] | View, edit, and publish phylogenetic trees online; interfaces with databases | http://www.phylowidget.org/ | [123] |
| **TreeViz**[a] | Java based tree viewer with the facility of treemaps | http://www.randelshofer.ch/treeviz/ | - |
| **BayesTrees**[b] | Display, analyze and manipulate samples of trees, in particular Bayesian samples | http://www.evolution.rdg.ac.uk/BayesTrees.html | - |
| **FigTree**[b] | Modern treeviewer with coloring and collapsing | http://tree.bio.ed.ac.uk/software/figtree/ | - |
| **Multi Dendrograms**[b] | Calculate and plot phylogenetic trees. Hierarchical Clustering of real data | http://deim.urv.cat/~sgomez/multidendrograms.php | [124] |
| **NJplot**[b] | Rooting of the unrooted tree. Graphical extension of any package of phylogenetic programs | http://pbil.univ-lyon1.fr/software/njplot.html | - |
| **TreeView**[b] | Classic treeviewing software that is very highly cited | http://taxonomy.zoology.gla.ac.uk/rod/treeview.html | [125] |

[a] Online software
b Applications (downloadable)
* The listed software and applications are free and compatible to run on any platforms.