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Phenotypic diversity and taxonomic relationship of *Rhizophora* species based on morphological markers

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Abstract

The phenotypic diversity and taxonomic relatedness of Indo-West Pacific (IWP) *Rhizophora* species in Pagapas Bay, Calatagan, Batangas, Philippines was investigated based on morphometric markers. Comparative analysis of characterization data revealed the occurrence of *R. apiculata*, *R. mucronata*, *R. stylosa* and hybrid *R.* × *lamarckii* in overlapping population. At field condition, the *Rhizophora* species were effectively distinguished by leaf, floral and hypocotyl attributes. Analysis of 40 morphometric parameters using standardized Shannon-Weaver Diversity Index (H') showed computed average H' value of 0.77 indicating high intraspecific phenotypic diversity among the *Rhizophora* species. Cluster analysis of qualitative traits using UPGMA SAHN Simple Matching Coefficient revealed that at coefficient 0.76, *R. mucronata* and *R. stylosa* showed 100% similarity while analysis of quantitative traits using Euclidean Distance Coefficient at coefficient 7.12, *R. mucronata* showed 100% similarity with *R.* × *lamarckii*. In both dendrograms, *R.* × *lamarckii* clustered with *R. mucronata* and *R. stylosa* while *R. apiculata* formed a separate distinct group. Hence, this study claims that either *R. mucronata* or *R. stylosa* is the possible parental species of *R.* × *lamarckii*. Interestingly, however, *R.* × *lamarckii* exhibit intermediate morphometric features of *R. apiculata* and *R. mucronata*. Having adapted to the same environment through time the expressed morphological traits are mainly genotypic effects, hence, are distinctive, uniform and stable at species level. Reliance to morphometric markers remain useful in species identification, diversity assessment and taxonomic studies.

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Introduction

Mangrove species diversity, distribution and population structure is variable at different spatial (global, regional, and local) and temporal scale. Across its geographical range, each species has developed specific adaptive survival mechanisms in response to prevailing environmental and climatic conditions (Alongi, 2008; Clough, 2013; Ceron-Souza *et al.*, 2014). The Philippines is one of the richest in the world in terms of mangrove genetic resources, containing about 80-85 % of the total number of species recorded worldwide (Primavera, 2000). However, with the large destruction and degradation of mangroves much of the original genetic pool has been lost and with indiscriminate harvesting and planting of only a number of selected species the genetic resources have been diluted and mixed up.

Rhizophora was recorded to be the oldest mangrove taxa which exhibit high degree of speciation (Wee *et al.*, 2014). The genus *Rhizophora* is pantropical and comprised of 6 to 8 species and 3 to 4 natural hybrids (Duke, 2006). The taxonomy of these mangrove taxa has not been completely established despite the advances in genetics and molecular biology. Further, studies are often limited by the wide geographical distribution of *Rhizophora* species across the tropical and warm subtropical regions and the presence of sibling species and natural hybrids. Specifically, the taxonomic relationships of the endemic IWP *Rhizophora* species namely, *R. apiculata* Bl., the reported sister species *R. mucronata* var. *stylosa* (Grif.) Salvoza and *R. stylosa* Grif., and the putative hybrid *R.* × *lamarckii* Montr. remain controversial (Duke, 2010). These species exhibit highly plastic growth habit across the intertidal zones and overlapping morphological features making identification at intraspecific level more complicated and challenging. Therefore, there is an obvious lack of knowledge about the extent of phenotypic diversity and taxonomic affinity of this important plant group.

Despite several attempts to determine the taxonomic affinities of IWP *Rhizophora* species using molecular markers, however at field conditions, species identification primarily depend on distinctive visual traits. Though molecular markers are said to be highly stable, there are claims of fluctuating amplification results and lack of reproducibility of banding patterns which are attributed to wide geographical coverage of sampling, different polymerase chain reaction (PCR) conditions and different band-scoring criteria (Triest, 2008). Similarly, for a given population or distinct breeding group, phenotypic diversity and taxonomic relationship using morphological keys can only be assessed by eliminating the potential effect of environmental heterogeneity. Hence, morphological characterization data could only be validated on sites where these species are present in overlapping population adapted to prevailing climatic and environmental condition for a considerable time period.

This study aims to identify morphological markers and to assess the phenotypic diversity and taxonomic relationship based on of *Rhizophora* species present in primary growth mangrove stand in Pagapas Bay, Calatagan, Batangas Philippines.

Materials and methods

Study site

The study was conducted in mangrove forest bordering Pagapas Bay, Calatagan, Batangas Province, Philippines. Bounded by latitudes 13°45' to 13°55' N and longitudes 120°35' to 120°45' E, Calatagan, Batangas has a type I climate with annual average temperature of 27°C. The dry season generally starts from November and continuous to April while the rainy season, which is usually accompanied by typhoons and monsoons, begins in May and ends in October. Pagapas Bay has generally muddy substrate but there are some small areas with sandy flats. The tide range is high, and at low tides the mudflats extend outward about 900 m. The fringing mangrove vegetation is dominated by dense primary growth of *Avicennia-Rhizophora* stand. The population dynamics of *Rhizophora* species in this area has been preserved through assisted natural regeneration and conservation approach.

Plant materials and sampling techniques

Rhizophora individuals with approximate height of 18-20m and D_{130} [tree diameter measured at 130cm above the ground (Brokaw and Thompson, 2000)] of 25-30cm thriving within in the demarcated 2-ha sampling site along Pagapas Bay, Calatagan, Batangas were used in the study. Individuals were initially grouped based on similarities on leaf and floral morphological traits. Members of each group were tagged with numbered metal tags. For the succeeding field surveys, 20 representative individuals were considered for morphological characterization, evaluation and monitoring.

In situ morphological characterization

In situ morphological characterization was carried out for two years to cover the entire reproductive cycle, that is, from flower initiation to propagule maturity of *Rhizophora* species and to document morphological expressions as influenced by changing climatic factors. Ten healthy flowering shoots and propagules located at the mid-canopy layer (at the angle of about 20-30^{o} with reference to the main trunk) were randomly collected from each tree. All the variations in the stipules, colleters, leaves, inflorescence, flower and propagule were recorded and documented. Few prominent characters shared by all taxa were also considered.

The descriptive attributes were classified into two broad categories: qualitative and quantitative. Qualitative parameters include the shape, color and texture. The variations in shape and margin were illustrated by line drawings, and the differences in color were defined using the Royal Horticultural Society (RHS) Color Chart V. Surface texture, like in the case of leaves, sepals and petals, were rated based on visual observations on the degree of waxy covering and pubescence (hairy index). For the petals, the extent of hairiness was classified as "smooth", "moderately hairy" and "hairy" based on hair density, hair length and hair distribution. For the hypocotyl surface texture, the degree of smoothness or roughness was based on wart and lenticel density.

Quantitative attributes, on the other hand, include countable data such as "number of flowers per inflorescence" (classified as discrete data) and measurable data such as "leaf width, length, and thickness" (classified as continuous scale) which cover the full range of variation from one extreme to the other. All morphometric measurements were taken using vernier caliper (Shanghai, China); scales or classes were established based on actual generated data set for each parameter.

Data analysis

Phenotypic diversity analysis. Characterization data were scored following the standard procedure published by Bioversity International (2007). The parametric scores were subjected to diversity analysis using the standardized Shannon-Weaver Diversity Index (H') to assess the extent of phenotypic variations present among the *Rhizophora* individuals evaluated. The individuals are said to exhibit either "high diversity", "moderate diversity" or "low diversity" for a particular parameter if H' value is ≥ 0.68, 0.34-0.67, or ≤ 0.33, respectively.

Cluster analysis

The dendrogram showing taxonomic relationship of the *Rhizophora* individuals based on morphological markers was generated using Unweighted Pair Group Arithmetic Mean (UPGMA) clustering method via Sequential Agglomerative Hierchial Combinatory Strategy (SAHN) module in the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) ver 2.10 by Applied Biostatistics Inc. (Rohlf, 2002). Similarity coefficient for qualitative traits was computed using Simple Matching Coefficient and for quantitative traits using Euclidean Distance Coefficient.

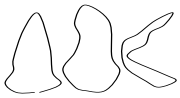
Results and discussion

Morphological characterization

Comparative analysis of the morphological characterization data with the descriptions cited by Primavera *et al.* (2004) revealed the presence of the four *Rhizophora* species namely, *R. apiculata*, *R.*

mucronata, *R. stylosa* and the putative natural hybrid *R.* × *lamarckii* in Pagapas Bay, Calatagan, Batangas, Philippines. This is the first to report the occurrence of all these identified *Rhizophora* species in overlapping population in just a single location. Given such condition, the effects of environment on the expression of the observed phenotypic variations between the *Rhizophora* individuals growing in sympatry were eliminated. Hence, the defined morphological markers which effectively discriminate each species are products of genotypic expression (Table 1).

Table 1. Distinctive morphometric markers used to discriminate the four *Rhizophora* species in Pagapas Bay, Calatagan, Batangas, Philippines.

Parameter	*R. apiculata*	*R.* × *lamarckii*	*R. mucronata*	*R. stylosa*
Leaf orientation (entire leafy cluster located at the mid-canopy region)	Horizontal	Horizontal to upward	Horizontal to upward	Upward
Leaf shape and margin (3rd fully expanded leaf from the shoot tip)	Elliptic/flat	Broad elliptic/wavy	Broad elliptic/wavy	Obovate/ revolute
Leaf color (3rd fully expanded leaf from the shoot tip)	Dark green	Green	Green	Light green-yellow
Size (Length:Width) cm (3rd fully expanded leaf from the shoot tip)	14:5	17:10	17-9	9:5
Stipule color	Dark purple	Red to dark purple	Light green to creamy white	Green
Note: Red stipules are also observed in *R. mucronata* and *R. stylosa* especially during hot summer months.				
Colleter aggregated shape	Rectangular band/ 3-4 rows	Rectangular band/ 6-8 rows	Rectangular band/ 4-6 rows	Rectangular band/ 4-6 rows
Colleter color	Dark yellow	Creamy white	Creamy white	Creamy white
Colleter exudate color	Clear, transparent	Milky white	Milky white	Milky white
Immature bud color	Light yellow	Dark pink to red	Light green	Light green
Inflorescence	2-flowered (rarely 3)	2 or 4-flowered (rarely 3)	5- to 8-flowered	7- to 22-flowered
Petal shape and pubescence (at anthesis)	Lanceolate/ glabrous	Navicular/ slightly hairy	Navivular/ moderately hairy	Navicular/ hairy
Stamen shape				
Number of stamen	12	14-16	8	8
Style, stigma and ovary structure (at anthesis)				
Fruit shape		Sterile		
Cotyledonary collar color (at maturity)	Red	Sterile	Yellow	Dark yellow
Note: Red cotyledonary collar are also observed in *R. mucronata* and *R. stylosa* especially during hot summer months				
Hypocotyl color (at maturity)	Dark green	Sterile	Light green	Light green
Hypocotyl texture (at maturity)	Slightly warty (with lenticels)	Sterile	Warty (with lenticels)	Moderately warty (with lenticels)
Hypocotyl length (at maturity)	25-35	Sterile	65-90	30-40
Hypocotyl apex	Blunt	Sterile	Pointed, sharp	Pointed, sharp

Under field condition, each *Rhizophora* species exhibit distinctive leaf, stipule, colleters and inflorescence and floral morphometric features. *R.* × *lamarckii* showed intermediate features of *R. apiculata* and *R. mucronata* such as leaf size, number of flowers per inflorescence, petal shape and pubescence, number of stamens, and style, stigma and ovary structure. This observation parallels the molecular studies using probe-enzyme combination wherein *R.* × *lamarckii* showed similar patterns as that of *R. apiculata* (Parani *et al.*, 1997; 1998; 2000). Further, results of such studies indicated that *R.* × *lamarckii* has intermediate profile between *R. apiculata* and *R. mucronata* while *R. stylosa* remain distinct.

Table 2. Standard Shannon-Weaver diversity index (H') values of qualitative and quantitative traits recorded in 20 *Rhizophora* individuals evaluated in Pagapas Bay, Calatagan, Batangas, Philippines.

	Qualitative traits	H'		Quantitative traits	H'
	Vegetative parameter			*Vegetative parameter*	
1	Stipule color	0.84	1	Stipule length	0.63
2	Colleter color	0.84	2	Number of colleter series	0.95
3	Colleter aggregated shape	0.78	3	Number or colleters per band	0.56
4	Colleter exudate color	0.81	4	Colleter length	0.59
5	Leaf orientation in space	1.00	5	Leaf length	0.48
6	Leaf margin	0.95	6	Leaf width	0.57
7	Leaf shape	0.95		*Reproductive parameters*	
8	Leaf color	0.91	7	Peduncle length	0.78
	Reproductive parameter		8	Number of floral buds per inflorescence	0.78
9	Inflorescence type	0.81	9	Number of bifurcation	0.78
10	Bud color at emergence	0.81	10	Bud length at maturity	0.95
11	Petal shape	0.81	11	Calyx diameter	0.41
12	Petal pubescence	0.78	12	Sepal length	0.41
13	Style, stigma and ovary structure	0.87	13	Corolla diameter	0.41
14	Stigma color	0.78	14	Petal length	0.41
15	Stamen shape	0.78	15	Style length	0.87
16	Fruit shape	0.89	16	ovary height	0.87
17	Cotyledonary collar color	0.85	17	Number of stamens	0.78
18	Hypocotyl color	0.89	18	Fruit length	0.89
19	Hypocotyl surface texture	0.85	19	Hypocotyl length	0.89
20	Hypocotyl apex	0.81	20	Hypocotyl diameter	0.89
	Average	0.85		Average	0.70
	General average	0.77			

Phenotypic diversity

Analysis of scores of 40 morphological traits using standardized Shannon-Weaver Diversity Index (H') revealed that the *Rhizophora* individuals exhibit high diversity (H'= 0.78-1.0) in all the 20 qualitative traits and moderate to high diversity (H'=0.41-0.95) for quantitative traits (Table 2). These indicate that the entries are well distributed among the defined morphological states. The general average H' value of 0.77 implies that there is a high phenotypic diversity among *Rhizophora* individuals examined.

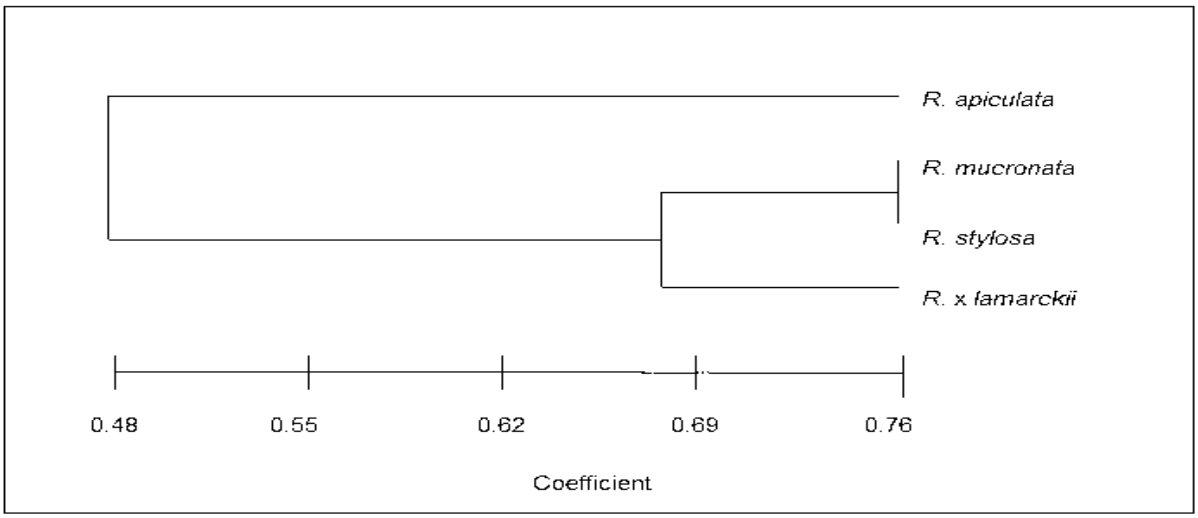


Fig. 1 A. Dendrogram generated based on the analysis of qualitative morphological traits of *Rhizophora* species using Simple Matching Coefficient by UPGMA SANH.

Taxonomic relationship

The dendrogram generated using morphological markers based on UPGMA SAHN clustering method separated the 20 *Rhizophora* individuals at intraspecific level showing that there is no variation between individuals belonging to the same species. Analysis of scores for qualitative traits using Simple Matching Coefficient revealed that *Rhizophora* individuals formed two clusters: cluster I comprised of *R. apiculata* individuals and cluster II comprised of *R. mucronata*, *R. stylosa* and *R.* × *lamarckii* at coefficient 0.62 (Fig. 1A). At coefficient 0.69 cluster II was divided in two groups with *R. mucronata* clustering with *R. stylosa* confirming the sibling status of these two species (Tomlinson, 1986; Duke, 2006), while *R.* × *lamarckii* formed a separate distinct group. *R. mucronata* and *R. stylosa* shared overlapping qualitative traits particularly those pertaining to color, however, they are effectively separated based on leaf, floral and hypocotyl structural attributes like orientation, margin, shape, and texture. The *R.* × *lamarckii* individuals examined are sterile and showed intermediate qualitative features of *R. apiculata* and *R. mucronata* confirming its hybrid status.

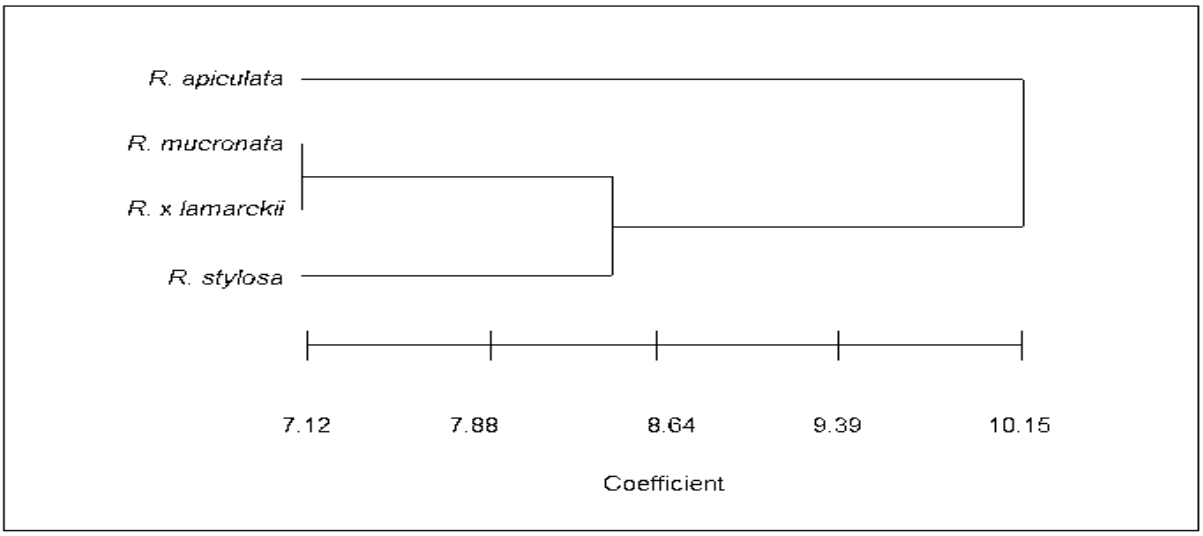


Fig. 1 B. Dendrogram generated based on the analysis of quantitative morphological traits of *Rhizophora* species using Euclidean Distance Coefficient by UPGMA SANH.

For quantitative traits using Euclidean Distance Coefficient at coefficient 8.64 similar grouping as for qualitative traits was obtained. However, *R.* × *lamarckii* clustered with *R. mucronata* at coefficient 7.12 indicating 100% similarity on most quantitative parameters (Fig. 1B). This finding is comparable with the report of Yao (1998) that *R.* × *lamarckii* individuals sighted in the Philippines show high resemblance with *R. mucronata*. Further, Yao (1998) mentioned that up close *R.* × *lamarckii* belies its genetic make-up showing little of the visible characteristics of *R. stylosa*, instead it looks more like a cross between *R. apiculata* and *R. mucronata*.

The above results conform with the findings of Parani *et al.* (1997; 2000) that *R. mucronata* and *R.* × *lamarckii* shared common ribulose-bisphosphate gene carboxylase gene (*rbc*L) banding pattern indicating *R. mucronata* as its female parent while disputing the earlier claim of Ng *et al.* (2013, 2014) that *R.* × *lamarckii* was a natural cross between *R. apiculata* and *R. stylosa* as revealed by chloroplast deoxyribonucleic acid (cpDNA) locus analysis.

Conclusion

At field condition, *Rhizophora* individuals are easily identified at species level based on stipule, colleters, leaf, inflorescence, flower, fruit and hypocotyl morphological attributes. *Rhizophora* species exhibit high phenotypic diversity indicating the distinctiveness of each species at intraspecific level and the sterile status of the natural hybrid *R.* × *lamarckii*. *R.* × *lamarckii* shows intermediate morphological characteristics of *R. apiculata* and *R. mucronata*. Cluster analysis revealed the close relationship *R. mucronata* and *R.* × *lamarckii* and the distinctiveness of *R. stylosa* from its sister species *R. mucronata*. Combined results of phenotypic diversity and cluster analysis are congruent with earlier findings using molecular approach that *R. apiculata* and *R. mucronata* are the potential parental species of the rare *R.* × *lamarckii* and not *R. stylosa*. The occurrence of all these four IWP *Rhizophora* species in overlapping population opens greater opportunity for further scientific investigations. Moreover, the above results provide concrete evidence for the urgent need of legal protection and conservation of this *Rhizophora* species-rich mangrove forest in Pagapas Bay, Calatagan, Batangas, Philippines.

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