

LIMITED SEED DISPERSAL MAY SHAPE GENETIC STRUCTURE OF *Hydnophytum formicarum* JACK. POPULATIONS IN MANGROVE ECOSYSTEM**

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ABSTRACT

Hydnophytum formicarum is an epiphytic plant, commonly distributed throughout Southeast Asia. However, its distribution is currently declining due to forest fragmentation and the subsequent habitat changes which may have also consequently affected the genetic structure of *H. formicarum* populations. Hence, this study aimed to understand the genetic variation and differentiation within and among populations of this species. Using Random Amplified Polymorphic DNA (RAPD) markers, the genetic variation and genetic differentiation among three populations were assessed in order to identify potential conservation management strategies for this species in the mangrove ecosystem in Malaysia. Ten highly reproducible primers were used in the population analysis, resulting in a total of 221 discernible fragments. Genetic variation among populations was high at 98% identified polymorphic fragments. AMOVA showed significant genetic differentiation among the populations ($\Phi_{PT} = 0.554$, $p \leq 0.001$), with pairwise genetic distances between the populations ranging from 0.495 - 0.589. PCoA clustering analysis separated the populations according to their geographical locations. The high genetic variation within a population, high genetic differentiation between populations and clear separation in the cluster analysis indicated a restricted seed dispersal of the species.

Keywords: AMOVA, epiphyte, myrmecophyte, RAPD, Setiu Wetlands

INTRODUCTION

Epiphytes are plants growing on other plants without directly harming their hosts (Zotz 2016). It is one major plant groups contributing largely to worldwide plant diversity. Vascular epiphytes alone constitute about 10% of overall global vascular floristic composition (Brown *et al.* 2015) and are distributed extensively across the world. Some epiphytic species have been recorded as myrmecophytes due to their association with ants (Chomicki & Renner 2015). Epiphyte studies have also revealed that these plants are particularly sensitive to environmental and habitat changes.

Hydnophytum formicarum Jack is one epiphyte of the Rubiaceae family that is identified as a

myrmecophyte due to its close association with ants as nesters and dispersers (Hosoishi *et al.* 2018). *Hydnophytum formicarum* is a species among 93 others in the genus *Hydnophytum*, under subtribe Hydnophytinae, which also considered as the world's most species-rich ant plant group (Chomicki & Renner 2015). This species is distributed natively in Southeast Asia (Huxley 1978). Notably, the species is widely distributed in the tropical region, and no record can be found on this species in the temperate region. This species favours coastal trees, mangroves and lowland swamp savannahs and hill savannahs (Huxley 1978). Giesen *et al.* (2006) have described this species as a mangrove-associated species as its abundance is prominent in this ecosystem. In Singapore, *H. formicarum* has been listed as critically endangered, with few in numbers currently present and no other surviving myrmecophytic epiphytes of the Rubiaceae family (Lok & Tan 2009).

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The association of Rubiaceae epiphytes including *Hydnophytum* with their ant occupants is called symbiosis or mutualism (Huxley 1980). Notably, ants of the *Iridomyrmex* spp. and *Crematogaster* spp. were found dwelling in the epiphytes domatia (Huxley 1980). Ant's occupation on the epiphytes were highly observed in parts of the plants including the stem, leaf or root of domatia (Chomicki & Renner 2015). Ants also act as dispersers for their host epiphytes. For instance, the ant species *Philidris nagasau* (Dolichoderinae) was doing an obligate farming of some epiphytes from genus *Squamellaria* in Fiji (Chomicki & Renner 2016). However, the epiphytes and ants mutualism can also be affected when other changes occur such as habitat shift and also the morphological changes of the host (Chomicki & Renner 2017). Habitat deterioration and fragmentation has affected the distribution and genetic structure of the epiphyte species *Crepis triasii* (Asteraceae) in the Mediterranean Islands (Mayol *et al.* 2012) and *Hedyotis chrysotricha* (Rubiaceae) in China (Yuan *et al.* 2012). Along the east coast of Peninsular Malaysia, aggressive coastal development has affected the coastline (Muslim *et al.* 2011; Ahmad *et al.* 2014). In that same region, in the state of Terengganu, an abundance of *H. formicarum* has been observed in the coastal islands with high proclivity to be affected by the aforementioned changes. Hence, this fragmentation threat has directed the assessment of the genetic structure of *H. formicarum* on island populations along the coasts.

Studies on the genetic structure of plant's island population have discovered that their spread is in accordance with the species' geographical distribution (Oiki *et al.* 2001; Zhang *et al.* 2018). However, when there is intermixing of genetic groups, dispersers were found to normally play their roles (Godoy & Jordano 2001; Schidegger *et al.* 2012). Geographical barriers and isolation by distance (IBD) of populations separated by bodies of water or oceanic barriers have been thoroughly studied (Dias *et al.* 2016; Levy *et al.* 2016) as these factors will eventually contribute to species' gene flow (Lee & Thomas 2011). One study on the genetic structure of plant populations in an island ecosystem (Hufford *et al.* 2013) (discovered a strong genetic differentiation within and among

island populations and apparent IBD between islands. A high variation within island populations and significant genetic differentiation among populations were also observed in the epiphytic orchids (Mallet *et al.* 2014). Similarly, the geographical isolation has limited the seed and pollen dispersal of *Banksia arborea* in terrestrial islands (Nistelberger *et al.* 2015). As island populations are relatively smaller in comparison to mainland populations, the genetic variation in island populations should be less (Frankham 1996), a theory that was substantiated by Hufford *et al.* (2013) and Laukkanen *et al.* (2014).

Since no study using molecular markers has yet been conducted for *H. formicarum*, this study aimed to investigate the genetic variation and differentiation within and among populations for this species as these will provide basic information for management plans focusing in the conservation of this species. Specifically, this study will determine and compare the genetic variation of this species in Pulau Telaga Tujuh, Pulau Layat and Pulau Redang populations.

MATERIALS AND METHODS

Plant Sampling

Leaves were sampled from 21 individual plants (one leaves per individual), of the 7 individual plants from each of the three different island mangrove forests in Terengganu on the east coast of Peninsular Malaysia. Pulau Telaga Tujuh (PT) and Pulau Layat (PL) are nearby small mangrove islands, while Pulau Redang (PR) is a tourism island with a fragmented mangrove ecosystem located in the district of Kuala Terengganu, approximately 30 km away from Setiu Wetlands (Fig. 1). Each *H. formicarum* leaf was collected from different phorophytes. Leaves were preserved in zip-lock bags with silica gel and then frozen in -20 °C conditions prior to genetic material extraction.

Molecular Analysis

DNA was extracted from the frozen leaf samples using modified CTAB method (Doyle & Doyle 1990) after leaf grinding into lyophilized form using liquid nitrogen. The DNA concentration was estimated using

BioDrop (Denville Scientific) spectrophotometer. Ten out of 20 screened universal RAPD (Random Amplified Polymorphic DNA) primers by Operon Technologies were chosen in this study (Table 1). The reproducibility of the selected primers was ensured by replicating the amplification process during optimization.

Genomic DNA was amplified in a reaction volume of 20 μ L consisting of template DNA, 1X reaction buffer (Promega), 2 mM MgCl₂ (Promega), 0.1 mg/mL BSA (Promega), 0.25 mM dNTPs (Promega), 0.25 μ M of each primer (OPA, 1st Base) and 0.5 units of Taq polymerase (Promega). The DNA was amplified using thermocycler (Applied Biosystems) at 30 and 45 cycles for 180 sec of initial denaturation and then 30 sec of denaturation at 95 °C, 30 sec annealing temperature at 32.6 °C to 37.7 °C, 60 sec of extension and then 420 sec of final extension at 72 °C. The PCR products were electrophoresed in 1.5% Agarose gel and TBE

buffer and ran for 90 min at 60 V. The gel was then stained using Diamond® Nucleic Acid dye (Promega) and was finally visualized and photographed using the Bio Rad gel documentation system.

Data Analyses

The amplified fragments were scored as 1 if present and 0 if absent and then assembled into a data matrix. The Principal Coordinate Analysis (PCoA) cluster analysis based on genetic distance was performed using PAST 3 software (Hammer *et al.* 2001). Analysis of the molecular variance (AMOVA) (Excoffier *et al.* 1992) was used to partition the total genetic diversity between and among populations. The population genetic differentiation indicator, pairwise Φ_{PT} of AMOVA, was also calculated. AMOVA and pairwise Φ_{PT} were both performed using GENALEX V6.1 (Peakall & Smouse 2006).

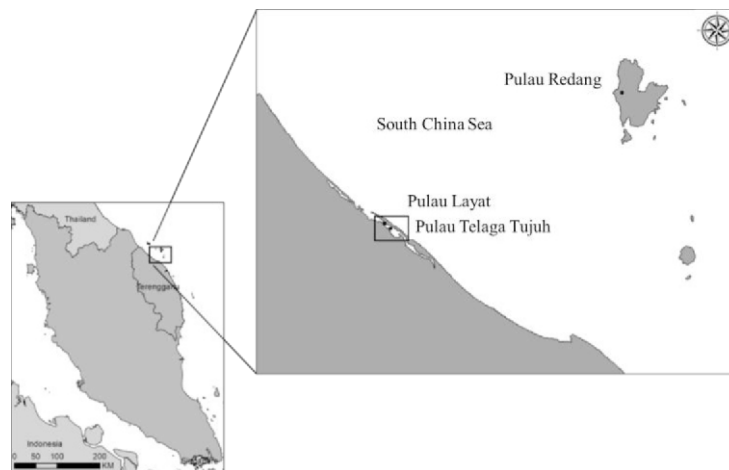


Figure 1 Map of the *Hydnophytum formicarum* populations in Terengganu

Table 1 Primer names and sequences chosen for PCR

No.	Primer name	Primer sequence 5' to 3'	Nucleotide length	C+G content (%)
1	OPA-01	CAGGCCCTTC	10-mer	70.0
2	OPA-03	AGTCAGCCAC	10-mer	60.0
3	OPA-05	AGGGGTCTTG	10-mer	60.0
4	OPA-07	GAAACGGGTG	10-mer	60.0
5	OPA-09	GGGTAACGCC	10-mer	70.0
6	OPA-11	CAATCGCCGT	10-mer	60.0
5	OPA-13	CAGCACCCAC	10-mer	70.0
8	OPA-15	TTCCGAACCC	10-mer	60.0
9	OPA-17	GACCGCTTGT	10-mer	60.0
10	OPA-19	CAAACGTCGG	10-mer	60.0

RESULTS AND DISCUSSION

Genetic Variation

A total of 221 discernible fragments (Fig. 2) from the 21 individual plants of *H. formicarum* were produced at an average of 22.1 fragments per primer. The amplified fragment sizes ranged from 150 to 2500 base pairs (bp).

Using the primer OPA17, the fragments showed 98% polymorphism, with Pulau Telaga Tujuh scoring the highest percentage (89.5%), followed by Pulau Redang (78.11%) and Pulau Layat (60.11%) (Table 2) indicating that Pulau Telaga Tujuh has the highest genetic variation since a high percentage of polymorphism in a population means a high genetic variation (Oiki *et al.* 2001; Boneh *et al.* 2003). Moreover, genetic variation is positively correlated with population size (Frankham 1996). This high genetic variation was also found within the island population of epiphytic orchids (Mallet *et al.* 2014). The Principal Coordinate Analysis (PCoA) further showed that the individuals from the three different populations were genetically different (Fig. 3).

Furthermore, the RAPD marker confirmed the genetic variation among the plants' population (Monaghan & Halloran 1996). The percentage of polymorphism in Pulau Redang, an island located at sea, was 18% higher than Pulau Layat even though Pulau Layat was located just meters away from Pulau Telaga Tujuh, which had the highest polymorphism percentage. The relatively low genetic variation in Pulau Redang compared to Pulau Telaga Tujuh is possibly due to its oceanic location,

which may have limited the gene flow from the mainland resources (Maki 2001). Islands that are closer to mainland, like Pulau Telaga Tujuh are more likely to act as allelic sink (Curto *et al.* 2017). Presumably, Pulau Layat is a newly established population, considering its low genetic variation and possibly genetic diversity (Hagen & Hamrick 1998). Since the lowest polymorphism percentage was in this population, it is considered as newly founded (Ouborg *et al.* 1999). However, in this study, no further investigation was done to validate the reasons for the low genetic variation in Pulau Layat.

The PCoA cluster analysis exhibited that the individuals were grouped in accordance with the populations where the samples were collected. The results from this study suggest that gene flow between populations was highly restricted. Geographical barriers that have formed may have impeded the dispersal of seeds, ultimately preventing the gene flow from occurring in a wider region (Monaghan & Halloran 1996). However, the epiphytic ferns of Hawaiian Islands were generally not genetically distinct between island populations, possibly due to high interisland gene flow (Ranker 1992). In contrast, the epiphytic orchids sampled from island populations in Puerto Rico, Dominican Republic and Cuba, had patterns of genetic variation more similar within the islands than with populations of other islands (Ackerman & Ward 1999). A study on the *Jumellea* epiphytic orchid also showed that the genetic variation between two islands populations differed significantly (Blambert *et al.* 2016).

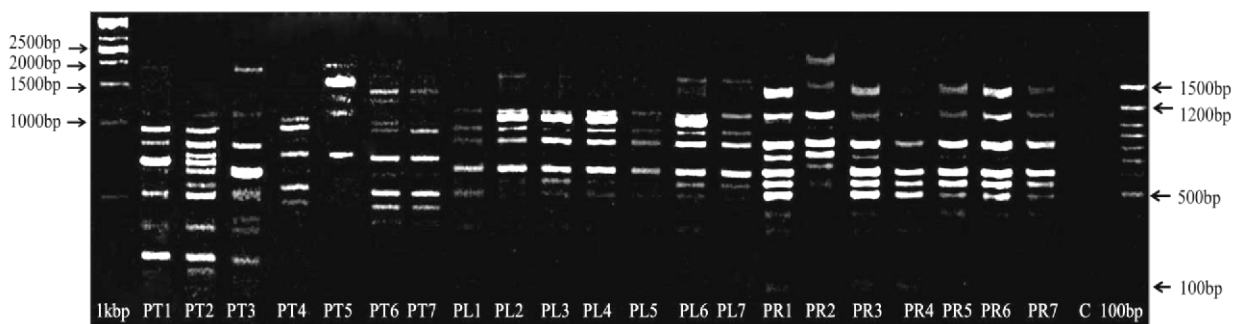


Figure 2 Photograph of Diamond® stained agarose gel of RAPD fragments using primer OPA 17 DNA samples from Pulau Telaga Tujuh (PT), Pulau Layat (PL) and Pulau Redang (PR)

Note: Lanes 1 kbp and 100 bp are the markers, while lane C is the control.

Table 2 Genetic variability estimates for populations of *H. formicarum* from RAPD analysis

No.	Code	Locality	N	n	No. of loci	No. of polymorphic loci	% of polymorphism
1.	PT	Pulau Telaga Tujuh	> 3000	7	137	123	89.05
2.	PL	Pulau Layat	> 100	7	101	65	60.11
3.	PR	Pulau Redang	7	7	121	99	78.11

Notes: N = estimated population size; n = number of sampled individual plants of *H. formicarum*.

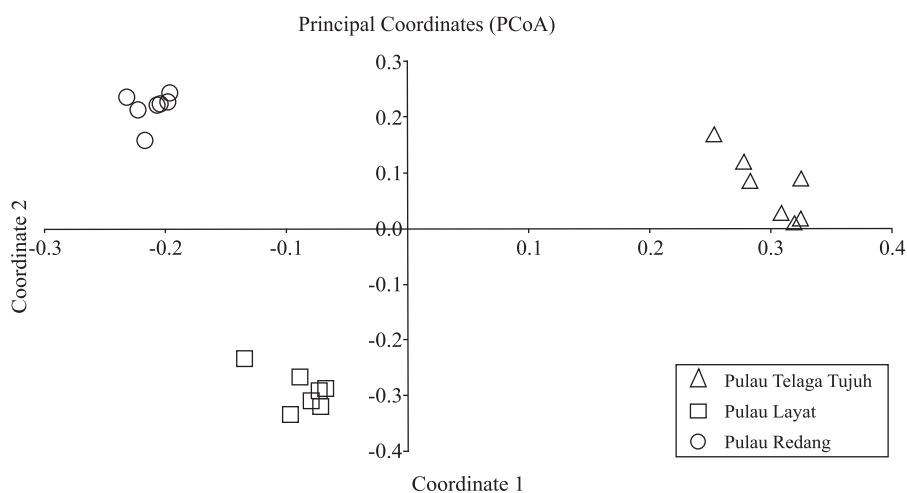


Figure 3 PCoA plot based on RAPD variations amongst populations

Genetic Differentiation between Populations

The analysis of molecular variance (AMOVA) performed on the three different populations showed that the percentage of variations amongst populations (55%) were higher than the percentage of variation within populations (45%) with $\Phi_{PT} = 0.554$ ($p = 0.001$; 999 permutations) (Table 3).

Based on the pairwise Φ_{PT} statistics (modified version of Wright's F), the highest variation was observed between population Pulau Layat and Pulau Redang (0.589), followed by that between Pulau Layat and Pulau Telaga Tujuh (0.584) and finally that between Pulau Telaga Tujuh and Pulau Redang (0.495) (Table 4). Greater Φ_{PT} values indicated greater variation.

Table 3 Analysis of molecular variance (AMOVA) within and among the three populations of *H. formicarum*

Source of variation	df	SS	MS	Est. var.	%	p
Among populations	2	387.52	193.76	24.82	55%	0.001
Within populations	18	359.42	19.96	19.96	45%	0.001
Total	20	746.94		44.78	100%	

Table 4 Pairwise population Φ_{PT} values (Φ_{PT})

Pulau Telaga Tujuh	Pulau Layat	Pulau Redang	
0.000	0.584	0.495	Pulau Telaga Tujuh
0.584	0.000	0.589	Pulau Layat
0.495	0.589	0.000	Pulau Redang

AMOVA values signified high genetic differentiations, both between and within populations, with Φ_{PT} values higher than 0.25. Genetic differentiations also existed among Indian epiphytic orchids (Nagananda *et al.* 2012). Similarly, a study on epiphytic Bromeliads *Vriesea scalaris* and *Vriesea simplex*, showed high genetic differentiations between and within populations of *V. scalaris* (Neri *et al.* 2017). The high genetic differentiation between populations and clear separation in cluster analysis indicated a restricted seed dispersal of *H. formicarum* (Nieder *et al.* 2000; Mucciarelli *et al.* 2014), and this restricted seed dispersal could lead to fine scale genetic differentiation and restricted gene flow (Theim *et al.* 2014). In our study, the geographical distance and isolation may have affected the gene flow (Ouborg *et al.* 1999; Rousset 2000). However, a low genetic differentiation among three different island populations of *Cephalanthera rubra* (Brzosko & Wróblewska 2003) was possibly due to short distances between the islands. Hence, no barrier for gene flow existed, as the populations probably originated from the same source. The low genetic differentiation among populations of the hemi-epiphytic species *Ficus pumila* L. in southern China, including several islands, suggested the success of pollinators in dispersal (Liu *et al.* 2015).

The relationship between *Hydnophytum formicarum* and ants is considered a facultative mutualistic type, where ants take charge of protecting the plants from phytophagous animals and other abiotic and biotic threats, while plants provide them with a food source, nesting space or sometimes both (Heil 2010). Once the plant is disturbed, the ants will swarm and fend off the threats. Many ant plant species have adapted their caudexes or tubers and provided shelter and protection for the ant colony associated with them (Heil 2010; Richards & Coley 2012; González-Teuber *et al.* 2014).

This mutualism has limited the seed dispersal of *H. formicarum* almost exclusively to ants as dispersers. The pattern of seed dispersal indeed can shape the genetic structure and composition of plant populations (Hamrick *et al.* 1993). On top of the geographical barrier and the isolation separating the populations, conceivably, ants were unable to cross the water between islands, over time limiting the seed dispersal of

H. formicarum. Higher genetic variation found within populations suggests that long distance dispersal by migrating birds has contributed to the variation (Zarek 2009). However, even if some flying birds were able to consume the fruit of *H. formicarum* (Lok & Tan 2009), the number and frequency of such incidents are still low because of the ants that protect the species, making it difficult for the birds to feed on the species' fruit.

The relationship between *H. moseleyanum* and *Philidris* ants however, proved that ants' behavior has resulted in the population structure changes of *H. moseleyanum* (Maeyama & Matsumoto 2000). Territorial *Philidris* ants which aggressively attack ants from different colony could possibly result in the dispersal of *H. moseleyanum* seeds restricted only to a colony. A similar phenomenon could have happened in the restricted seed dispersals of *H. formicarum* in this study and may have eventually shaped its genetic structure. Seeds dispersal by ants generated a local spatial genetic structure (Peakall & Beattie 1995). In other studies, the genetic structure of some plants were also found to be promoted by ants (Yao 2010; Pérez-Collazos *et al.* 2015).

CONCLUSION

Seed dispersal was limited due to geographical barriers, yet the role of ants as agents of dispersal, shed light on the changes of *H. formicarum*'s genetic structure and its high abundance in three mangrove forests. Seeds movement by ants have structured the genetic population of *H. formicarum* in these 3 islands. The limitation of ants as being agents of dispersal, if not halted, curbed the gene flow occurrence among populations. This study served as the first step in evaluating conservation strategies for this species and in assisting conservation efforts particularly, on the genetic variation of the *H. formicarum* populations.

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