Microsatellite analysis of *Rafflesia cantleyi* from the Rafflesia Conservation and Interpretive Centre, Bersia Timur and Gerik Forest Reserve, Perak, Malaysia

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Abstract. Located at Bersia Timur, the Rafflesia Conservation and Interpretive Centre (RCIC) was initiated by the Forest Research Institute Malaysia (FRIM) and FELDA to boost research and conservation efforts of the iconic Rafflesia species in the state of Perak. It also plays a role in supporting the economy of local communities by promoting local eco-tourism, providing trainings to local tour-guides and involving them in conserving the Rafflesia population. As part of the R & D and conservation effort, genetic diversity assessment of *Rafflesia cantleyi* from RCIC and Gerik Forest Reserve (FR) was carried out. Bract samples from a total of eight and 35 *R. cantleyi* individuals of different flowering/anthesis stages were collected from RCIC and Gerik FR, respectively. We used nine polymorphic microsatellite markers for genotyping. Despite the small sample size (~75% lesser compared with Gerik FR), the total number of alleles observed in RCIC is > 50% than that of Gerik FR, i.e., 33 compared to 57. The genetic diversity measure in terms of observed ($H_o$) and expected ($H_e$) heterozygosities from both sites are comparable (RCIC: $H_o = 0.5298$, $H_e = 0.5347$; Gerik FR: $H_o = 0.5145$, $H_e = 0.5868$). Our findings suggest that the *R. cantleyi* population in Gerik FR is a suitable source for future translocation activities.

1. Introduction

*Rafflesia* is a genus of holoparasitic flowering plants of the family Rafflesiaceae. The genus is small but interesting as some *Rafflesia* spp. are renowned for producing the largest single flowers in the world, growing up to a meter in diameter [1]. These extraordinary flora lack true leaves, stems and roots, they depend completely on their sole host plants, *Tetrastigma* vines (Vitaceae) for water and nutrients. *Rafflesia* spp. can only be found in the tropical rainforest of Southeast Asia. In Malaysia, there are currently 12 described *Rafflesia* species, seven in Peninsular Malaysia and five in Sabah and Sarawak [2].

The Rafflesia Conservation and Interpretive Centre (RCIC), with an area of 223.6 ha, is located at Bersia Timur, Gerik, Perak, Malaysia. It was initiated by the Forest Research Institute Malaysia (FRIM) and Federal Land Development Authority (FELDA) back in 2015, following the discovery of a *Rafflesia* population along Sungai Lebey in FELDA Bersia Timur. The initial objective of setting up the center was to boost R & D and conservation efforts of the iconic *Rafflesia* species in the state of Perak. A few years later, the idea came to fruition with funding from the 11th Malaysia Plan, under the Program: Research and Conservation of *Rafflesia* Populations in the Northern Region of Peninsular Malaysia. Within RCIC, there are eco-tourism zone, *in-situ* and *ex-situ* conservation zones. At
present, equipped with basic infrastructure and research facilities, RCIC also plays a role in supporting the economy of local communities by promoting local eco-tourism pertaining to Rafflesia sighting, providing trainings to local tour-guides, and involving them in conserving the Rafflesia population.

There are two Rafflesia species in RCIC, R. cantleyi Solms and R. azlanii Latiff & Wong, the latter being scarce. As part of the R & D and conservation effort, genetic diversity assessment of R. cantleyi from RCIC and Gerik Forest Reserve (FR) was carried out to investigate their genetic relatedness, which will facilitate future genetic restoration work.

2. Materials and Methods

2.1 Sampling

In 2019, Rafflesia cantleyi samples were collected from RCIC and Gerik Forest Reserve (FR), Perak. Non-destructive sampling was carried out. Bract tissues from a total of eight and 35 R. cantleyi individuals of different flowering/ anthesis stages were collected from RCIC and Gerik FR, respectively. The samples collected were kept in silica gels prior to DNA extraction with modified CTAB method [3].

2.2 Microsatellite genotyping

Nine microsatellite markers were used in genotyping the 43 R. cantleyi samples (Table 1). Multiplex PCRs were conducted in 8 µL reaction mixture, with ~10 ng of template DNA, 0.3 µM of each forward and reverse primer and 1x master mix of Type-it Multiplex PCR Kit (QIAGEN GmbH). PCR was run in GeneAmp PCR System 9700 (Applied Biosystems) using the following program: initial denaturing step at 95°C for 5 min, 55 cycles of 95°C (30 sec), 60°C (90 sec) and 72°C (30 sec), and a final extension of 30 min at 60°C. Subsequently, fragment analyses were carried out using an ABI 3130xl Genetic Analyzer with ROX 400 (Applied Biosystems) as the internal size standard.

2.3 Data analysis

Genotyping was performed using GeneMarker v2.6.4 (SoftGenetics). Genotypic data was analysed using GDA version 1.0 [6]. Cluster analysis at the individual level was performed using PowerMarker version 3.25 [7], adopting shared allele method [8], which is based on the proportion of shared alleles.

3. Results and Discussion

As reported by Barkman et al. (2017) [4], the microsatellite peak patterns of R. cantleyi revealed a diploid state. While the markers Raff 11 and Raff15 were developed for R. cantleyi [4], six of the nine microsatellite markers used in this study were developed for R. lagascae and R. manillana (Man109 - Man1169) [5]. The latter were transferable in R. cantleyi. Cross-amplification of microsatellite markers in closely related species has been demonstrated in numerous studies [9]. The majority of the microsatellite markers developed in R. lagascae have also been proven applicable in R. speciosa [10]. Although Barkman et al. (2017) reported no amplification for Raff12 [4], we were able to obtain PCR products for this locus, with observed alleles of 216, 220 and 246bp. The corresponding allele size range reported for 42 R. tuan-mudae was 208 – 224bp [4].

There has been a reduction in the R. cantleyi population size in RCIC due to trampling by elephants prior to the installation of electric fence. Nevertheless, despite the small sample size (~75% lesser compared with Gerik FR), the total number of alleles observed in RCIC is more than 50% of that of Gerik FR, i.e. 33 vs 57 (Table 2). In fact, two alleles (364bp and 383bp) at locus Man109 were found exclusively in RCIC. The genetic diversity measures in terms of observed (H_o) and expected (H_e) heterozygosities from both sites are comparable (RCIC: H_o = 0.5298, H_e = 0.5347; Gerik FR: H_o = 0.5145, H_e = 0.5868) (Table 2). This reflects that the R. cantleyi population in RCIC is not depauperate in genetic diversity compared with Gerik FR, the adjacent natural population, which is good for in situ conservation.
Table 1. Primer sequences (5′ – 3′) of the nine microsatellite markers used in this study.

| Locus Name | Repeat motif | Forward & Reserve Primer Sequences | Reference |
|------------|--------------|------------------------------------|-----------|
| Raff11     | AC           | F: CGAGCATCAACATCATATCC            | Barkman et al. 2017 [4] |
|            |              | R: GCATGCAAGGTGCTTGTGTAT           |           |
| Raff12     | GA           | F: GAGAAAGTGAGCGTGAGTTGA           | Barkman et al. 2017 [4] |
|            |              | R: TGCCCTACCAATAAAAACTGG           |           |
| Raff15     | TC           | F: ACCTATGGTCCTTGGAGTGGG           | Barkman et al. 2017 [4] |
|            |              | R: CAGCAACAGCAAAAAGAAGG            |           |
| Man109     | AC           | F: ACGTAGTCCATCCATTGAAGG           | Pelser et al. 2017 [5] |
|            |              | R: ACCTAGCCACAGCTTC               |           |
| Man120     | AG           | F: GTGTATCTTCGCTGCTTTCAC           | Pelser et al. 2017 [5] |
|            |              | R: GTGTATCAACAGAGCAG              |           |
| Man171     | AAT          | F: GCCCGCCTTCACCATTAATC            | Pelser et al. 2017 [5] |
|            |              | R: AGAAGCGAGGTGAAACTGCTC           |           |
| Man273     | AC           | F: GCGTGGTTCATTCATGGAGG           | Pelser et al. 2017 [5] |
|            |              | R: AACTCAGGCCCTTCCTC               |           |
| Man714     | AC           | F: GTGCCTGCTAATGTGCTGCACACCCTT    | Pelser et al. 2017 [5] |
|            |              | R: CATAGGCTCTGACACCTTTGC          |           |
| Man1169    | AC           | F: CTTTGTCGAGTAAAGGCTAGTC         | Pelser et al. 2017 [5] |
|            |              | R: ACCTCAACTTCAATGCAGTC           |           |

Table 2. The genetic diversity parameters of the *Rafflesia cantleyi* populations at RCIC and Gerik FR.

| Population | Sample Size | Observed Heterozygosity ($H_o$) | Expected Heterozygosity ($H_e$) | Total no. of alleles | Mean no. of alleles per locus |
|------------|-------------|---------------------------------|---------------------------------|----------------------|-----------------------------|
| RCIC       | 8           | 0.5298                          | 0.5347                          | 33                   | 3.67                        |
| Gerik FR   | 35          | 0.5145                          | 0.5868                          | 57                   | 6.33                        |

The neighbour-joining tree based on DAS, shared allele distance [8] showed that the *R. cantleyi* population at FELDA Bersia Timur is not genetically distinct from those at Gerik FR, in that the eight *R. cantleyi* individuals from RCIC were not clustered under one branch in the dendrogram (Figure 1). This indicates that there is no prominent genetic differentiation between these two populations. Hence, our findings suggest that the *R. cantleyi* population in Gerik FR is a suitable source for future translocation activities, in other words, future introduction of *R. cantleyi* individuals from Gerik FR to RCIC for enrichment will not disrupt the existing population genetic structure.
Figure 1. Neighbour-joining tree based on shared allele distance [8] showing the relationship among the Rafflesia cantleyi samples. The eight individuals from the Rafflesia Conservation and Interpretive Centre (RCIC) did not form a cluster on their own (branches in red), indicating no prominent genetic differentiation between the RCIC and Gerik FR populations. Note: PPIR is the abbreviation for RCIC in Bahasa Malaysia, Pusat Pemuliharaan dan Interpretif Rafflesia.

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