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A conservation genetic study of *Rafflesia speciosa* (*Rafflesiaceae*): patterns of genetic diversity and differentiation within and between islands

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Key words

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Abstract *Rafflesia speciosa* is a threatened endo-holoparasitic species. It has several populations in the Central Panay Mountain Range (CPMR) of Panay island and a single population on Negros Island. Because *R. speciosa* is the only Philippine species of the genus that is not endemic to an individual island, it is a suitable species for improving our understanding of the factors underlying the high island endemism of Philippine *Rafflesia*. For this purpose and to inform the conservation management of *R. speciosa*, patterns of genetic diversity and differentiation were studied using 15 microsatellite loci and samples from nine populations. None of these populations shows evidence of inbreeding and *R. speciosa* has similar levels of heterozygosity as generally observed in outcrossing or perennial plant species. The results of AMOVA and Bayesian cluster analyses indicate that the Negros population is genetically differentiated from the CPMR populations. In addition, it has lower genetic diversity than similar-sized *R. speciosa* populations. These findings suggest that sea straits potentially provide significant reproductive barriers to *Rafflesia* species, and are perhaps responsible for their high island endemism. The general lack of genetic differentiation among the CPMR populations as suggested by the AMOVA, PCoA, and STRUCTURE results indicates recent gene flow among them and this finding improves our understanding of the geographical scale and context at which gene flow between *Rafflesia* populations occurs. Conservation efforts should be targeted towards avoiding further habitat degradation in the Negros population. We also recommend protective status for the entire CPMR and reforestation efforts to mitigate the severe habitat fragmentation, destruction, and degradation in this area.

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INTRODUCTION

Rafflesia R.Br. (*Rafflesiaceae*) is a Southeast Asian parasitic plant genus of c. 30 species (Nickrent 1997 onwards). Most of these are only known from individual islands in the Malesian archipelago (Nais 2001, Barcelona et al. 2009b, Bendiksby et al. 2010, David et al. 2011). This pattern of island endemism is perhaps most pronounced in the Philippines, where 12 of the 13 currently recognised species (Pelser et al. 2011 onwards, Galindon et al. 2016, Hidayati & Walck 2016) are only found on a single island (Pelser et al. 2017). Within these islands, several species have very narrow distributions and two are only known from a single population (i.e., *R. aurantia* Barcelona et al., *R. manillana* Teschem., Barcelona et al. 2009a, b, Pelser et al. 2017). Other Philippine *Rafflesia* species are more widespread within an island, but have relatively few and isolated populations. For example, eight species are only known from two or three populations (i.e., *R. baletei* Barcelona et al., *R. consueloae* Galindon et al., *R. leonardi* Barcelona & Pelser, *R. mixta* Barcelona et al., *R. verrucosa* Balete et al., and *R. schadenbergiana* Göpp. ex Hieron.; Balete et al. 2010, Barcelona et al. 2006, 2008, 2009b, 2011, 2014, Galindon et al. 2016). The rarity of *Rafflesia* species and their disjunct distribution makes them particularly vulnerable to extinction as a result of the on-going destruction and degradation of their habitat (Nais 2001, Barcelona et al. 2009b). The conversion of rainforests for agriculture, mining, and other uses has undoubtedly resulted in local extinction, a reduction of the size of remaining *Rafflesia* populations, and lower genetic connectivity among them (Pelser et al. 2017). The obligate endo-holoparasitic life style of these plants and their pronounced host specificity are factors that further increase extinction risk, because they cannot live in the absence of the few *Tetrastigma* (Miq.) Planch. (*Vitaceae*) species that are within their host range (Pelser et al. 2016).

Information about the host specificity, habitat preferences, and reproductive biology of *Rafflesia* species can assist their conservation management by revealing the environmental conditions that the populations need to remain viable, thereby providing a better understanding of the factors that explain their current distribution patterns. The results of a recent study of host specificity and host preference of Philippine *Rafflesia* species (Pelser et al. 2016) indicate that all six known host species have widespread distributions within the Philippines. *Tetrastigma* host plants are also more common and widespread than their parasites in other parts of the distribution area of *Rafflesia* (Nais 2001). This suggests that host ranges are not a limiting factor in the distribution of *Rafflesia*, although local differences in host presence and abundance might explain its distribution patterns at the population level (Pelser et al. 2016).

Although detailed research into the habitat requirements of individual *Rafflesia* species has, to our knowledge, not been carried out, it appears that they are not very habitat specific. Members of the genus are confined to tropical rainforest ecosystems, but generally grow in primary as well as secondary forests, have wide elevational ranges, and their hosts are found on various substrates (Nais 2001, Barcelona et al. 2009b, 2011). Habitat

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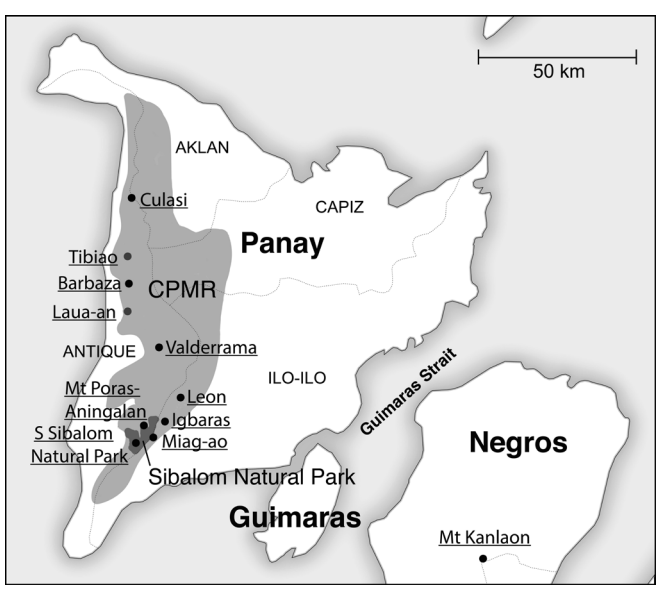
Fig. 1 Tissue of opened mature fruit of *Rafflesia speciosa* (Fig. 2d) showing an ant transporting a seed. Vertical arrow indicates a seed. Horizontal arrow indicates a seed that is being moved by an ant. — Scale bars = 1 mm.

preference is therefore an unlikely explanation for the narrow and disjunct distribution patterns of *Rafflesia* species and their high island endemism.

Instead, it is perhaps more likely that these distribution patterns are a result of poor seed dispersal beyond relatively small distances (Barkman et al. 2017) and the very low probability that *Rafflesia* seeds are deposited in sufficient proximity of a suitable host plant to result in infection. Very little is known about seed dispersal in *Rafflesia*. The morphology of their seeds suggests the presence of an elaiosome (Kuijt 1969, Pelser et al. 2013), indicating myrmecochory, and Pelser et al. (2013, present study) indeed observed ants transporting seeds away from *Rafflesia* fruits (Fig. 1). If ants are the primary seed dispersers, dispersal over large distances, particularly between islands, might be very infrequent. However, evidence of mammalian frugivory (Emmons et al. 1991, Bänziger 2004) suggests that ants might not be the exclusive dispersers of *Rafflesia* seeds and that seeds might be able to be more readily transported over greater distances (Pelser et al. 2017).

Population genetic data can provide useful context for understanding the factors that have shaped the distribution patterns of *Rafflesia* species, as well as for more directly informing conservation management by identifying populations that are of conservation interest, such as those showing unique genetic variation (i.e., private alleles), evidence of low genetic diversity and inbreeding, or poor genetic connectivity with other populations (Ellstrand & Elam 1993, Frankham 2005). Population genetic analyses of a microsatellite data set previously revealed low genetic connectivity between populations of the Philippine *R. lagascae* species complex that consists of *R. lagascae* s.str., Mt Labo *R. lagascae*, and *R. manillana* (Pelser et al. 2017). Furthermore, these analyses indicated that, despite their very close morphological similarity, the allopatric *R. lagascae* s.str. (Luzon Island) and *R. manillana* (Samar Island) are reproductively isolated. In addition, a genetically distinct but morphologically indistinct population of *R. lagascae* was identified from Mt Labo in the Bicol Peninsula of Luzon, which might represent an undescribed cryptic species. These findings suggest limited effective dispersal of pollen and seeds over larger distances and particularly between islands. Limited seed dispersal was also concluded for two *Rafflesia* species from Peninsular Malaysia and Thailand (*R. cantleyi* Solms) and Borneo (*R. tuan-mudae* Becc.) in a separate study (Barkman et al. 2017). The genetic patterns obtained for the *R. lagascae* species complex suggest that such limitations might subsequently result in allopatric speciation, potentially explaining the high island endemism of *Rafflesia*. The discovery of strong genetic differentiation between morphologically similar populations of the *R. lagascae* complex on different islands also raises the question of whether other currently recognised *Rafflesia* species with populations on different islands need to be more narrowly circumscribed (Pelser et al. 2017). *Rafflesia speciosa* Barcelona & Fernando (2002) is such a species and therefore a suitable candidate for further exploring patterns of genetic diversity of this genus in a geographical context.

Rafflesia speciosa is the only Philippine species in the genus that is not endemic to a single island within the archipelago. It is found at c. 350–1100 m a.s.l. in the Central Panay Mountain Range (CPMR) of Panay Island and on Mt Kanlaon in northern Negros Island (Map 1). The forests that are home to *R. speciosa* are rapidly being fragmented and degraded as a result of land conversion for cattle grazing and other farming practices, road construction, and housing (Fig. 2). At higher elevations, recreational housing, agritourism and ecotourism have recently decimated fragile ultramafic and limestone habitats such that



Map 1 Distribution of all currently known populations of *Rafflesia speciosa* (underlined). Faint dotted lines indicate provincial boundaries. Black dots show populations that were sampled for this study. Grey dots indicate two municipalities with unconfirmed reports of *R. speciosa*. The grey area shows the area of the Central Panay Mountain Range (CPMR). The darker grey area indicates Sibalom Natural Park.

Fig. 2 a–d. *Rafflesia speciosa*. a. Flower; b. flower in habitat; c. flower bud; d. opened fruit showing small orange-brown seeds. — e–h. Threats to the conservation of *R. speciosa* habitat. e. Mammal trap (Mt Kanlaon Natural Park); f. conversion into agricultural land (barangay Aningalan, San Remigio); g. slash-and-burn practices in Valderrama (smoke of fires in the background, burnt hill slope in the foreground) leave small *Rafflesia*-inhabited forest remnants amidst unproductive grassland; h. a landslide following deforestation (Valderrama).

now only small forest fragments remain. These and other activities have also been reported to negatively impact other species with which *R. speciosa* shares its habitat (e.g., Oliver 2014, Oliver et al. 1991, 1993, Hamann et al. 1999, Ferner et al. 2000, Gaulke 2010, Mould 2012, Sammler et al. 2012), thereby prompting appeals to declare the CPMR a protected area (e.g., Oliver et al. 1991, 1993). While Sibalom Natural Park (Panay) and Mt Kanlaon enjoy legal protection, even these areas are under the constant pressure of, among others, illegal firewood collecting, charcoal making, and hunting (Fig. 2). If *R. speciosa* shows similar patterns of low genetic connectivity as was observed in the *R. lagascae* complex, this species is potentially at risk of losing genetic diversity through inbreeding and genetic drift, and might be in need of urgent conservation management.

The objective of this study is to provide the first information about the patterns of genetic connectivity and diversity of *R. speciosa* with the primary aims of 1) improving our understanding of the factors contributing to the narrow distributions and high island endemism of *Rafflesia* species; and 2) informing the conservation management of *R. speciosa*. For this, we use population genetic analyses of a microsatellite data set in which specimens from nine populations are included.

MATERIALS & METHODS

Specimen sampling

Rafflesia speciosa is known from nine areas (Map 1). One of these is at the foothills of Mt Kanlaon in Negros and the other eight are found in the CPMR in Panay. Three of these are located in the municipalities of Igbaras, Leon, and Miag-ao in Iloilo province at the eastern side of the Range. The remaining five areas are in Antique Province at the western side of the divide. Within the wider Sibalom Natural Park area, *R. speciosa* sites are concentrated in two areas that are separated by the Tipuluan River. We here refer to the area north of the river as the Mt Poras-Aningalan area, which spans the barangays of Cabladan and Imparayan of Sibalom municipality and barangay Aningalan of San Remigio. The area south of the Tipuluan River is part of barangays Bad-as and Bululacao of Sibalom and referred to as the southern Sibalom Natural Park area. *Rafflesia speciosa* is further known from the municipalities of Barbaza, Culasi, and Valderrama. Anecdotal evidence suggests that *R. speciosa* is also present in Laua-an and Tibiao (Antique Province), but these reports remain to be confirmed. For the purpose of convenience, because of difficulties in circumscribing the actual limits of populations, we refer to these nine areas as individual *R. speciosa* populations.

Table 1 Voucher information for *Rafflesia speciosa* populations sampled.

Population	Voucher (herbarium)[1]
Culasi, Antique Prov.	*Barcelona 3801* (CANU, PNH)
Barbaza, Antique Prov.	*Barcelona 3791* (CANU, PNH)
Valderrama, Antique Prov.	*Barcelona 3769 with Pelser* (CANU, PNH)
Mt Poras-Aningalan, Sibalom & San Remigio, Antique Prov.	*Barcelona 3701 with Pelser* (SIU)
Southern Sibalom Natural Park, Sibalom, Antique Prov	*Barcelona 3990 with Pelser* (PNH)
Leon, Iloilo Prov.	No voucher
Igbaras, Iloilo Prov.	*Barcelona 4154 with Pelser* (PNH)
Miag-ao, Iloilo Prov.	*Barcelona 4133 with Pelser* (PNH)
Mt Kanlaon, Negros Occidental Prov.	*Barcelona 4013 with Pelser* (PNH)

[1] CANU = University of Canterbury Herbarium; PNH = Philippine National Herbarium; SIU = Southern Illinois University Herbarium.

Following the sampling strategy described by Pelser et al. (2017), tissue samples on silica gel were collected from small parts of two to 16 flower buds or flowers from one or more sites from each of the nine populations (Table 2). The number of samples per population is relatively low, because *Rafflesia* populations are often small and are sometimes even associated with just a single host plant (Pelser et al. 2017). However, because tissue samples were taken from all or nearly all host plants that were identified in these populations, the percentage of individuals sampled in each population is high. Regardless, our estimates of allele frequencies for small populations should be interpreted with care. Voucher specimens (Table 1) are deposited at CANU, PNH, and SIU.

DNA extraction and microsatellite genotyping

Using the DNeasy plant mini kit (Qiagen, Germantown, Maryland), total genomic DNA was extracted from tissue of a total of 105 *R. speciosa* flowers or flower buds. The results of a pilot study in which the amplification of 17 microsatellite markers developed for *R. lagascae* (Pelser et al. 2017) was tested (results not reported here) showed that 15 of these loci amplified well for *R. speciosa* (Man78, Man80, Man109, Man111, Man120, Man142, Man144, Man166, Man171, Man273, Man553, Man714, Man866, Man1134, Man1169), but that the loci Man788 and Man1193 frequently failed to yield unambiguous genotyping profiles. These 15 loci (see Pelser et al. 2017

Table 2 Genetic diversity and number of private alleles observed at 15 microsatellite loci for nine populations of *Rafflesia speciosa*. Number of *Rafflesia* samples (number of *Tetrastigma* host plants from which *Rafflesia* samples were obtained), percentage of polymorphic loci (P), allelic richness (Na), number of effective alleles (Ne), number of private alleles (Na(p)), observed heterozygosity (H_o), expected heterozygosity (H_e), unbiased expected heterozygosity (uH_e), and inbreeding coefficient (F_{IS}). SE = Standard Error. *Population with a significant deviation from Hardy-Weinberg proportions due to heterozygote deficits after B-Y correction (Narum 2006).

Population (# sites)	Protected area	# *Rafflesia* samples (# hosts)	P	Na (SE)	Ne (SE)	Na (p)	H_o (SE)	H_e (SE)	uH_e (SE)	F_{IS} (SE)
Culasi (5)	No protective status	7 (3)	93 %	4.13 (0.49)	3.18 (0.35)	0	0.65 (0.08)	0.60 (0.06)	0.65 (0.07)	-0.06 (0.05)
Barbaza (4)	No protective status	9 (6)	93 %	3.93 (0.44)	3.07 (0.33)	0	0.62 (0.08)	0.59 (0.06)	0.63 (0.06)	-0.01 (0.05)
Valderrama (10)	No protective status	16 (16)	100 %	5.67 (0.57)	3.15 (0.26)	5	0.58 (0.06)	0.63 (0.05)	0.65 (0.05)	0.06 (0.06)*
Mt Poras-Aningalan (6)	Sibalom Natural Park pro parte	14 (9)	93 %	5.47 (0.58)	3.38 (0.45)	6	0.63 (0.07)	0.61 (0.06)	0.63 (0.07)	-0.03 (0.04)
Southern Sibalom Natural Park (5)	Sibalom Natural Park	8 (5)	100 %	4.27 (0.43)	3.00 (0.28)	3	0.69 (0.06)	0.62 (0.04)	0.66 (0.04)	-0.12 (0.06)
Leon (1)	No protective status	2 (2)	87 %	2.27 (0.21)	2.10 (0.20)	0	0.60 (0.11)	0.46 (0.06)	0.63 (0.08)	-0.30 (0.17)
Igbaras (6)	No protective status	9 (9)	93 %	4.40 (0.63)	2.97 (0.49)	2	0.58 (0.08)	0.55 (0.07)	0.58 (0.07)	-0.05 (0.06)
Miag-ao (5)	No protective status	12 (11)	100 %	4.60 (0.43)	3.07 (0.33)	3	0.58 (0.06)	0.61 (0.05)	0.64 (0.05)	0.04 (0.05)
Mt Kanlaon (4)	Mt Kanlaon Natural Park	10 (5)	87 %	4.33 (0.47)	2.75 (0.28)	6	0.60 (0.09)	0.55 (0.07)	0.58 (0.07)	-0.07 (0.07)
Totals and means		87 (66)	94 %	4.34 (0.18)	2.96 (0.11)	3.57	0.61 (0.03)	0.58 (0.02)	0.63 (0.02)	-0.06 (0.03)

for primer and microsatellite information) were subsequently genotyped for all available *R. speciosa* samples. Multiplex PCR analyses using the Qiagen Type-it microsatellite PCR kit and up to four primer combinations per PCR sample followed an M13 protocol as described in Pelser et al. (2017). The PCR products were run on an ABI 3130xL Genetic Analyzer at the University of Canterbury. Geneious 6.1.7 (Biomatters Ltd, Auckland, New Zealand) was used to determine fragment lengths.

Data analyses

Tests for the presence of null alleles were performed with Microchecker v. 2.2.3 (Van Oosterhout et al. 2004; 95 % confidence interval; 1 000 repetitions). Only loci that were flagged as displaying null alleles across all populations were considered to be truly producing null alleles after a re-inspection of the associated genotyping electropherograms. GENEPOP v. 4.2 (Raymond & Rousset 1995) as implemented in GenePop on the web (http://genepop.curtin.edu.au/) was used for exact tests for deviations from Hardy-Weinberg equilibrium. Significance levels for this were adjusted for multiple tests using the B-Y method FDR at $p = 0.05$ (Narum 2006).

The percentage of polymorphic loci, allelic richness (mean number of alleles and number of effective alleles), observed (H_o) and expected (H_e) heterozygosity, unbiased expected heterozygosity (uH_e), and the inbreeding coefficient were calculated in GenAlEx v. 6.5 (Peakall & Smouse 2012) to determine the genetic diversity of each *R. speciosa* population. GenAlEx was also used to assess patterns of genetic differentiation among these populations by determining the number of private alleles of each population and by performing an analysis of molecular variance (AMOVA; F_{ST}, 999 permutations). Furthermore, a Principal Coordinate Analysis (PCoA) was carried out in GenAlEx, in which a covariance matrix of co-dominant genotypic pairwise distances between individual samples was used. Genetic differentiation was also studied by performing Bayesian model-based clustering analyses in STRUCTURE v. 2.3.4 (Pritchard et al. 2000, Falush et al. 2003, 2007) for which an admixture model and correlated allele frequencies were used. The clustering analyses were run with K values from 1–16 and with 20 iterations for each K value. Each analysis was run for 200 000 generations, including 100 000 generations that were discarded as burn-in. STRUCTURE HARVESTER (Earl & Von Holdt 2012) was used to select K using both the Evanno et al. (2005) method (K with the highest value of ΔK) and the method of Pritchard et al. (2000; K with the highest Pr(X|K)). The STRUCTURE results were summarised for the selected values of K in CLUMPAK v. 1.1 (Kopelman et al. 2015).

GenAlEx was used to perform Mantel tests (999 replicates) with the aim of testing the correlation between non-transformed geographic distance and co-dominant genotypic distance between individuals. These tests were repeated with a data set in which geographic distances were transformed using the natural logarithm.

RESULTS

The *R. speciosa* flowers or flower buds that were sampled for this study were obtained from 66 *Tetrastigma* host plants from nine populations. A total of 20 of these hosts belong to *T. harmandii* Planch. (30 %) and 45 to *T.* cf. *magnum* Merr. (68 %). One host plant could not be identified to species level. Of the 83 *Rafflesia* specimens for which the sex could be determined, 58 (70 %) were staminate and 25 (30 %) were pistillate. Because we sampled buds or flowers from nearly all infected host plants that we encountered in each population and we did not notice substantial differences in the proportion of infected host plants and those that did not show signs of infection, population sample sizes are assumed to be rough estimates of the relative sizes of *R. speciosa* populations (Table 2).

Genotyping analyses using 15 microsatellite loci showed that 18 of the 105 DNA samples had identical genotypes to those of other flowers or buds obtained from the same host plant. These 18 samples could potentially represent the same *Rafflesia* individual and were therefore excluded from the genetic analyses. A total of 85 of the remaining 87 samples were genetically unique. The two samples that were genetically identical to each other were obtained from different but nearby host plants at the same site of the same population.

The 15 microsatellite loci yielded 118 alleles. Across all populations, the number of alleles varied between 4 and 13 per locus, with a mean of 7.87. None of the loci showed evidence of producing null alleles. Only the Valderrama population deviated significantly from Hardy-Weinberg proportions owing to a deficiency of heterozygotes (Table 2). The observed mean heterozygosity ($H_o = 0.61$) was slightly higher or lower than two estimates of the mean expected heterozygosity ($H_e = 0.58$, $uH_e = 0.63$).

The Mt Poras-Aningalan, Southern Sibalom Natural Park, and Valderrama populations have the highest genetic diversity as measured by the percentage of polymorphic loci, allelic richness, number of effective alleles, and heterozygosity (Table 2). The populations in Culasi, and Miag-ao show somewhat less genetic diversity. The lowest levels of genetic diversity were found in Barbaza and Igbaras, but particularly in Leon and Mt Kanlaon. The inbreeding coefficients are low for all populations (F_{IS} = -0.30–0.06; Table 2).

The number of private alleles per population varies from 0 in Barbaza, Culasi, and Leon, to 6 in Mt Kanlaon and Mt Poras-Aningalan. The results of the AMOVA analysis indicate that the overall genetic differentiation within *R. speciosa* is statistically significant ($F'_{ST} = 0.11$, $p < 0.001$). A total of 4 % of the genetic variation is found among populations and 5 % among individuals. The AMOVA further shows statistically significant pairwise F_{ST} values (at P = 0.05 after the B-Y correction) between Mt Kanlaon and all other populations, except Leon (Table 3). A total of seven of the pairwise F_{ST} values between populations

Table 3 Pairwise F_{ST} values between populations of *R. speciosa*. *Significant pairwise comparisons after B-Y correction (Narum 2006).

	Valderrama	Barbaza	Culasi	Leon	Miag-ao	Igbaras	Mt Poras-Aningalan	Southern Sibalom Natural Park
Barbaza	0.013							
Culasi	0.000	0.017						
Leon	0.000	0.042	0.006					
Miag-ao	0.001	0.032*	0.001	0.000				
Igbaras	0.037*	0.039	0.015	0.000	0.036*			
Mt Poras-Aningalan	0.009	0.017	0.011	0.045	0.015	0.060*		
Southern Sibalom Natural Park	0.032	0.045	0.034	0.010	0.050*	0.046*	0.052*	
Mt Kanlaon	0.105*	0.114*	0.094*	0.076	0.084*	0.086*	0.097*	0.108*

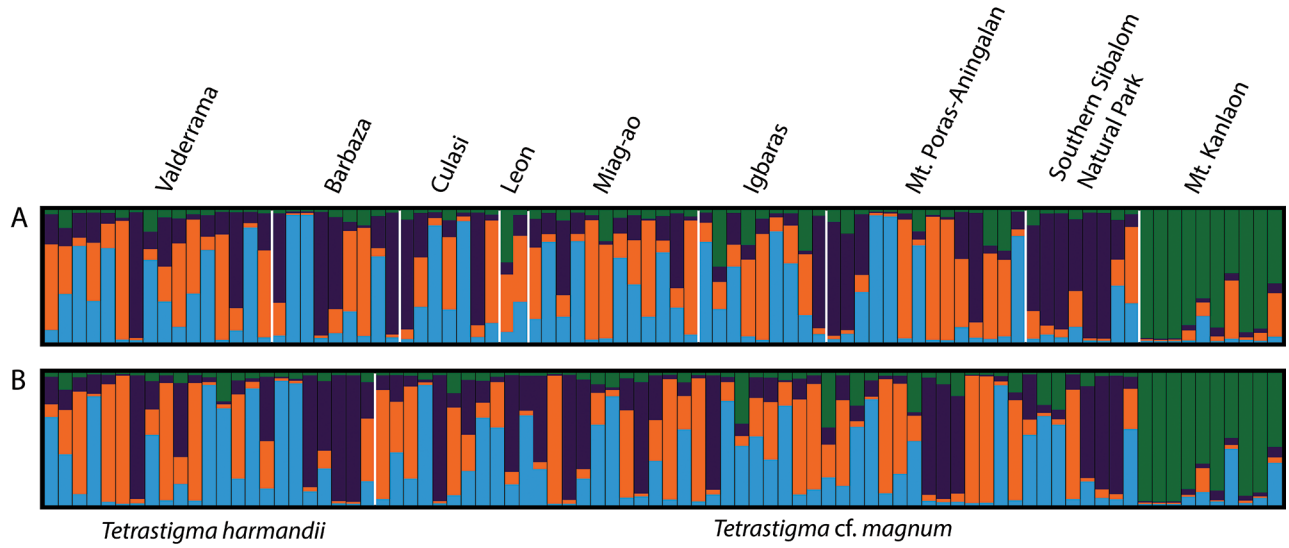


Fig. 3 K = 4 STRUCTURE results for *R. speciosa*. Each bar represents an individual plant and bar colours indicate the proportion of membership to each genetic cluster (*q* values). a. Individuals grouped by population; b. individuals grouped by *Tetrastigma* host species.

in Panay are significant as well. Analyses of the STRUCTURE data for K = 1–16 in STRUCTURE HARVESTER indicate the presence of four primary genetic clusters (K = 4; Fig. 3). One of these genetic clusters aligns well with the Mt Kanlaon population. The other genetic clusters do not align with individual populations or groups of populations in Panay. Most of these populations display a large diversity of admixture patterns at the level of individual plants (Fig. 3a). The four genetic clusters also do not align with the two *Tetrastigma* species that were identified as hosts for *R. speciosa* (Fig. 3b). The results of a PCoA of pairwise genetic distances show little genetic differentiation among populations (Fig. 4). The results of the Mantel tests do not reveal a statistically significant positive correlation at $p = 0.05$ between geographic and genetic distances between individuals.

DISCUSSION

Using data from 15 microsatellite loci, this study presents the first information about the patterns of genetic diversity and genetic differentiation of *R. speciosa*. Although this species is the second-most common species of Philippine *Rafflesia* after *R. lagascae* s.str. from Luzon Island in terms of the number of known populations (9 vs 13; Pelser et al. 2017), its total distribution area (CPMR in Panay and Mt Kanlaon in Negros) is relatively small (Map 1) and all populations are exposed to habitat degradation and fragmentation as a result of slash-and-burn agriculture and other destructive practices (Barcelona et al. 2009b; Fig. 2). Many of the World's tropical forests experience on-going degradation and fragmentation of their habitat (Turner & Corlett 1996, Laurance 1998, Haddad et al. 2015), which can result in populations that are smaller and more isolated from each other. Such populations are more prone to losing

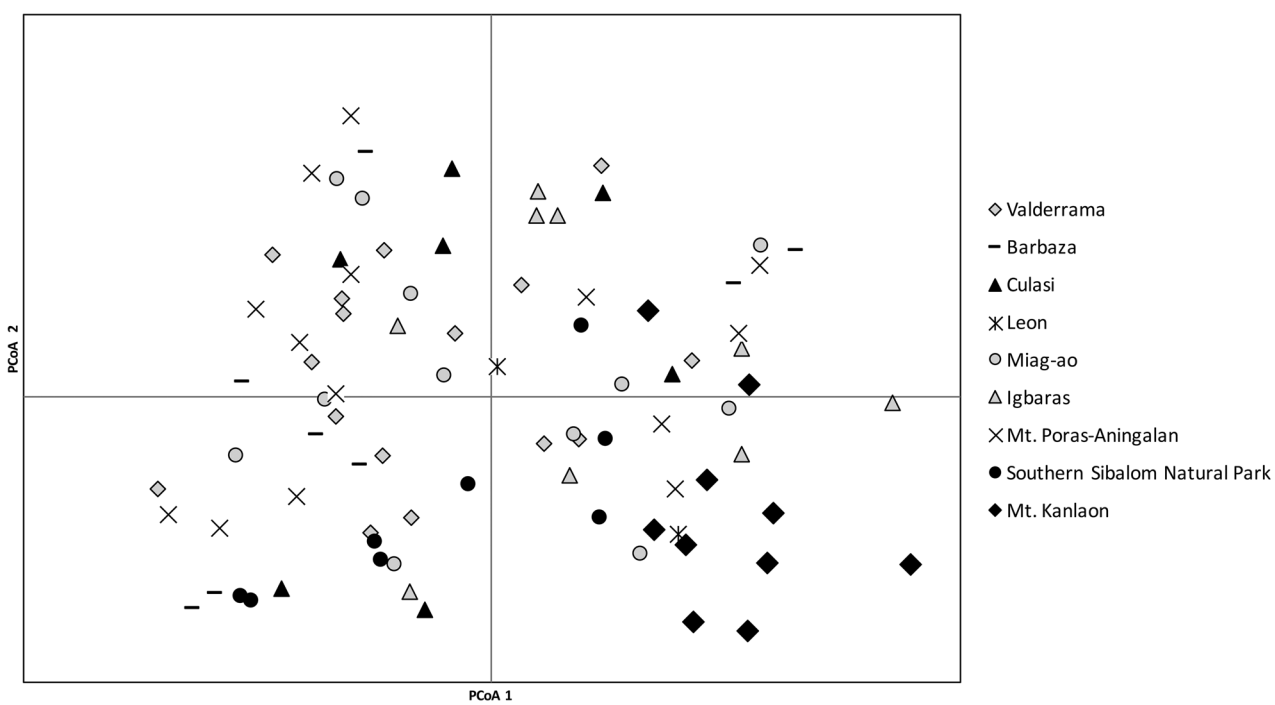


Fig. 4 The PCoA ordination plot of *R. speciosa* using a covariance matrix of co-dominant genotypic pairwise distances between individual samples with data standardization. The first and second PCoA axes explain 9.0 % and 7.2 % of the variation, respectively.

genetic diversity through genetic drift and inbreeding depression, increasing their extinction risk (Ellstrand & Elam 1993, Young et al. 1996, Lowe et al. 2005, Chávez-Pesqueira et al. 2014). However, the results of our study indicate that habitat degradation and fragmentation have not yet had a measurable detrimental effect on the genetic diversity of *R. speciosa* populations, because we did not observe evidence of inbreeding as measured by the inbreeding coefficient F_{IS} (Table 2). In addition, *R. speciosa* has very similar levels of heterozygosity (H_o = 0.61, H_e = 0.58, uH_e = 0.63) as the *R. lagascae* complex (H_o = 0.60, H_e = 0.53, uH_e = 0.64; Pelser et al. 2017). These values are comparable to those reported for outcrossing or perennial plant species in a meta-analysis of genetic diversity in plants (Nybom 2004). Under the assumption that our sample sizes are approximately proportional to relative population sizes, smaller populations of *R. speciosa*, particularly in Leon, appear to have somewhat lower levels of genetic diversity, as measured by the percentage of polymorphic loci and allelic richness, than larger populations (e.g., Valderrama). However, this pattern is much less pronounced than what was observed for the *R. lagascae* complex (Pelser et al. 2017). The only *R. speciosa* population known from Negros (Mt Kanlaon) deviates from this pattern by being relatively large, but showing among the lowest levels of genetic diversity when compared to populations from which similar numbers of samples were obtained (Table 2).

In addition to having relatively low genetic diversity, the results of our AMOVA (Table 3) and STRUCTURE (Fig. 3a) analyses indicate that the Mt Kanlaon population is genetically differentiated from the Panay populations of *R. speciosa*. The lack of a statistically significant positive correlation between geographic distances and genetic distances of *R. speciosa* individuals suggests that this might not be due to isolation by distance. Very similar patterns of genetic diversity and genetic differentiation were previously identified for *R. manillana* of the *R. lagascae* complex (Pelser et al. 2017). This species resembles the Mt Kanlaon population of *R. speciosa* in constituting a single population on a different island than where its most closely related populations are found. Whereas all other populations of the *R. lagascae* complex are found on Luzon Island, *R. manillana* is only known from a single population on the nearby island of Samar. Like the Mt Kanlaon population, *R. manillana* has relatively low genetic diversity and is genetically differentiated from populations on the other island, although these patterns are more pronounced in the *R. lagascae* complex than in *R. speciosa* (Pelser et al. 2017). These findings are congruent with the hypothesis that the sea straits that separate islands form significant barriers to gene flow for *Rafflesia* species. Although our results indicate that the Mt Kanlaon population of *R. speciosa* is genetically differentiated from populations on Panay, the lack of distinct clustering of Mt Kanlaon individuals in the PCoA plot (Fig. 4) and the admixture patterns of the Mt Kanlaon population (Fig. 3a) suggest that some gene flow between both islands has occurred. This possibly took place in the late Pleistocene, when the islands of Cebu, Guimaras, Masbate, Negros, and Panay were connected during times with lower sea levels (Negros-Panay Pleistocene Aggregate Island Complex; Brown et al. 2013).

We can, however, not exclude the possibility that the genetic differentiation between the Panay and Negros populations of *R. speciosa* is instead, or in part, due to the natural absence of suitable habitat between the CPMR and Mt Kanlaon. *Rafflesia speciosa* populations have only been found between c. 350 and 1100 m a.s.l., but all land between the CPMR and Mt Kanlaon (including the island of Guimaras, which is located in the Guimaras Strait between Panay and Negros; Map 1) is below 200 m and most of it well below 100 m. However, because other *Rafflesia* species have been found as low as 50 m a.s.l. as well as at higher elevations (e.g., *R. lagascae*) and because almost all forest in Panay and Guimaras at lower elevation has been cleared in historic times (Hamann et al. 1999, Ferner et al. 2000, Sammler et al. 2012), it is also possible that the disjunct distribution pattern of *R. speciosa* is more recent and a result of the anthropogenic destruction of its forest habitat. The latter explanation has been proposed to explain the genetic differentiation between Panay and Negros populations of two hornbill species, *Penelopides panini* and *Aceros waldeni* (Sammler et al. 2012).

Regardless of whether the genetic differentiation between the Panay and Negros populations is a result of a lack of gene flow across the Guimaras Strait or across unfavourable terrestrial habitats, the results of this study and that of Pelser et al. (2017) improve our understanding of the geographical context and scale at which geographic isolation manifests as genetic differentiation between *Rafflesia* populations. Further context for this is provided by the patterns of genetic differentiation among the Panay populations of *R. speciosa*. Although the presence of private alleles in some of these populations (Table 2) suggest that they are genetically differentiated, the lack of population-level genetic differentiation revealed by the STRUCTURE (Fig. 3) and PCoA (Fig. 4) analyses, the low percentage of genetic variation found among populations (4 %; AMOVA), and the absence of statistically significant pairwise F_{ST} values between most Panay populations (Table 3), suggests that they recently had relatively high genetic connectivity among them. This contrasts with the low genetic connectivity among populations of *R. lagascae* s.str. (Pelser et al. 2017). Considering that all Panay populations are associated with the CPMR, it is possible that the lack of genetic differentiation in Panay indicates a previously more continuous distribution of *R. speciosa* on the island. If gene flow between these populations is still on-going, the contrast between the patterns of genetic differentiation in *R. speciosa* and *R. lagascae* s.str. might also be explained by the generally smaller geographical distances between *R. speciosa* populations than between populations of *R. lagascae* s.str. These distances might be small enough for its seed dispersers to cross. However, since habitat destruction and fragmentation in the CPMR are quite severe (e.g., Oliver et al. 1991, 1993, Hamann et al. 1999, Ferner et al. 2000, Gaulke 2010, Mould 2012, Sammler et al. 2012, Oliver 2014), and because *Rafflesia* seeds are most likely dispersed by ants (Fig. 1) or mammals, it is perhaps more likely that gene flow across deforested terrain primarily happens by means of pollen dispersal by flies. *Rafflesia* pollen is dispersed by carrion flies (*Calliphoridae*), which are attracted to the rotting odour of *Rafflesia* flowers and the visual appearance of their large red flowers (Bänziger 1991, Nais 2001). These flies are long-lived and strong fliers, and can travel distances of 22 km in a few days (Bishopp & Laake 1921, Bänziger 1991). They have therefore been considered to be able to disperse *Rafflesia* pollen over larger distances (Bänziger 1991, Nais 2001). If the unconfirmed report of *R. speciosa* in Tibiao is correct, all populations in the CPMR are within 22 km of one or more of the other populations, so it is possible that pollen dispersal currently plays a more important role in sustaining gene flow between populations of *R. speciosa* than seed dispersal. This hypothesis, however, remains to be tested.

Rafflesia speciosa exclusively parasitizes two *Tetrastigma* species (*T. harmandii* and *T.* cf. *magnum*), although it is sympatric with four additional species (Pelser et al. 2016). When different populations of a parasite species evolve preferences for different species within their host range, this can result in genetically distinct groups within the parasitic species (i.e., host-race formation; De Vega et al. 2008, Norton & Carpenter 1998, Román et al. 2007, Pelser et al. 2016). These host races

might eventually evolve into different species (Amico & Nickrent 2009). The STRUCTURE results (Fig. 3b), however, fail to show that *R. speciosa* individuals growing on *T. harmandii* are genetically differentiated from those infecting *T.* cf. *magnum* plants and therefore demonstrate that host-race formation cannot explain the genetic patterns observed for *R. speciosa*.

In conclusion, our study resulted in a better understanding of the patterns of genetic diversity and differentiation of *R. speciosa* in the context of its current distribution. When compared to those of the *R. lagascae* complex (Pelser et al. 2017), our results indicate that although *Rafflesia* species are typically rare, have restricted and fragmented distributional ranges, show high island endemism, and have small population sizes, they may still retain similar levels of genetic diversity as outcrossing and perennial plant species. In addition, closely related populations on different islands are genetically differentiated from each other. This finding is compatible with the hypothesis that sea straits form significant barriers to gene flow in *Rafflesia*, although, in the case of *R. speciosa*, the genetic differentiation between Panay and Negros can also be explained by the current presence of unfavourable habitat between these two islands. Finally, our results contribute to a better understanding of patterns of genetic differentiation among *Rafflesia* populations in different geographical contexts, with *R. speciosa* showing stronger genetic connectivity among populations within a single mountain range, and *R. lagascae* displaying less genetic connectivity between generally more distant populations belonging to different mountains and mountain ranges (Pelser et al. 2017).

Our results show that particularly the *R. speciosa* populations in two protected areas (Sibalom Natural Park and Mt Kanlaon Natural Park) and Valderrama contain private alleles (Table 2). These areas are therefore of noted conservation significance as sources of unique genetic diversity. The Mt Kanlaon population, however, has relatively low genetic diversity and this together with its isolated location make it vulnerable to further reductions in genetic diversity as a result of inbreeding and genetic drift. It is located close to settlements and agricultural land and therefore exposed to human activities that result in habitat degradation. This previously included the conversion of forest into plantations of exotic tree species for forestry, but currently perhaps mostly illegal firewood collecting, charcoal making, logging, and hunting (Fig. 2). The most significant threats to the populations in Panay, including those in Sibalom Natural Park, are habitat destruction and fragmentation resulting from land conversion to farms, roads, and settlements, as well as wildfires that spread from burning nearby grasslands (Fig. 2). Cattle are also often allowed to graze inside the remaining patches of natural forest and this has resulted in severe habitat degradation. In some areas, grazing of understory plants appears to have negative impacts on natural forest regeneration. Particularly in Valderrama, deforestation on steep slopes prompted landslides that damaged nearby forest patches (Fig. 2). *Rafflesia speciosa* populations in Panay that enjoy partial legal protection currently still display genetic connectivity and do not show evidence of inbreeding. However, on-going habitat destruction, fragmentation, and degeneration are undoubtedly bringing this species closer to extinction, as is the case with other threatened species that share its natural habitat (e.g., Walden's Hornbill, the Visayan spotted deer, the Panay monitor, and the Visayan warty pig). In our opinion, this downward trend can only be halted if the entire CPMR receives effective legal protection and the loss of genetic connectivity between *R. speciosa* populations is prevented by creating corridors of native forest between the remaining forest fragments.

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REFERENCES

Amico GC, Nickrent DL. 2009. Population structure and phylogeography of the mistletoes Tristerix corymbosus and T. aphyllus (Loranthaceae) using chloroplast DNA sequence variation. American Journal of Botany 96: 1571–1580.

Balete DS, Pelser PB, Nickrent DL, et al. 2010. Rafflesia verrucosa (Rafflesiaceae), a new species of small-flowered Rafflesia from eastern Mindanao, Philippines. Phytotaxa 10: 49–57.

Bänziger H. 1991. Stench and fragrance: unique pollination lure of Thailand's largest flower, Rafflesia kerrii Meijer. Natural History Bulletin of the Siam Society 39: 19–52.

Bänziger H. 2004. Studies on hitherto unknown fruits and seeds of some Rafflesiaceae, and a method to manually pollinate their flowers for research and conservation. Linzer Biologische Beiträge 36: 1175–1198.

Barcelona JF, Cajano MO, Hadsall AS. 2006. Rafflesia baletei, another new Rafflesia (Rafflesiaceae) from the Philippines. Kew Bulletin 61: 231–237.

Barcelona JF, Co LL, Balete DS, et al. 2009a. Rafflesia aurantia (Rafflesiaceae): a new species from northern Luzon, Philippines. Gardens' Bulletin Singapore 61: 17–27.

Barcelona JF, Fernando ES. 2002. A new species of Rafflesia (Rafflesiaceae) from Panay Island, Philippines. Kew Bulletin 57: 647–651.

Barcelona JF, Fernando ES, Nickrent DL, et al. 2011. An amended description of Rafflesia leonardi and a revised key to Philippine Rafflesia (Rafflesiaceae). Phytotaxa 24: 11–18.

Barcelona JF, Manting MME, Arbolonio RB, et al. 2014. Rafflesia mixta (Rafflesiaceae), a new species from Surigao del Norte, Mindanao, Philippines. Phytotaxa 174: 272–278.

Barcelona JF, Pelser PB, Balete DS, et al. 2009b. Taxonomy, ecology, and conservation status of Philippine Rafflesia. Blumea 54: 77–93.

Barcelona JF, Pelser PB, Cabutaje EM, et al. 2008. Another new species of Rafflesia (Rafflesiaceae) from Luzon, Philippines: R. leonardi. Blumea 53: 223–228.

Barkman TJ, Klooster MR, Gaddis KD, et al. 2017. Reading between the vines: hosts as islands for extreme holoparasitic plants. American Journal of Botany 104: 1382–1389.

Bendiksby M, Schumacher T, Gussarova G, et al. 2010. Elucidating the evolutionary history of the Southeast Asian, holoparasitic, giant-flowered Rafflesiaceae: pliocene vicariance, morphological convergence and character displacement. Molecular Phylogenetics and Evolution 57: 620–633.

Bishopp FC, Laake EW. 1921. Dispersion of flies by flight. Journal of Agricultural Research 21: 729–766.

Brown RM, Siler CD, Oliveros CH, et al. 2013. Evolutionary processes of diversification in a model island archipelago. Annual Review of Ecology, Evolution, and Systematics 44: 411–435.

Chávez-Pesqueira M, Suárez-Montes P, Castillo G, et al. 2014. Habitat fragmentation threatens wild populations of Carica papaya (Caricaceae) in a lowland rainforest. American Journal of Botany 101: 1092–1101.

David M, De Jesus AC, Barcelona JF. 2011. Rafflesia of the Philippines: a story of adventure, appetite, and affinity. Energy Development Corporation (EDC), Manila.

De Vega C, Berjano R, Arista M, et al. 2008. Genetic races associated with the genera and sections of host species in the holoparasitic plant Cytinus (Cytinaceae) in the Western Mediterranean basin. New Phytologist 178: 875–887.

Earl DA, Von Holdt BM. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4: 359–361.

Ellstrand NC, Elam DR. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Review of Ecology and Systematics 24: 217–242.
Emmons LH, Nais J, Briun A. 1991. The fruit and consumers of Rafflesia keithii (Rafflesiaceae). Biotropica 23: 197–199.
Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software structure: a simulation study. Molecular Ecology 14: 2611–2620.
Falush D, Stephens M, Pritchard JK. 2003. Inference of population structure: extensions to linked loci and correlated allele frequencies. Genetics 164: 1567–1587.
Falush D, Stephens M, Pritchard JK. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. Molecular Ecology Notes 7: 574–578.
Ferner JW, Brown RM, Sison RV, et al. 2000. The amphibians and reptiles of Panay Island, Philippines. Asiatic Herpetological Research 9: 1–37.
Frankham R. 2005. Genetics and extinction. Biological Conservation 126: 131–140.
Galindon JMM, Ong PS, Fernando ES. 2016. Rafflesia consueloae (Rafflesiaceae), the smallest among giants: a new species from Luzon Island, Philippines. PhytoKeys 16: 37–46.
Gaulke M. 2010. Overview on the present knowledge on Varanus mabitang Gaulke and Curio, 2001, including new morphological and meristic data. Biawak 4: 50–58.
Haddad NM, Brudvig LA, Clobert J, et al. 2015. Habitat fragmentation and its lasting impact on Earth's ecosystems. Science Advances 1: e1500052.
Hamann A, Barbon EB, Curio E, et al. 1999. A botanical inventory of a submontane tropical rainforest on Negros Island, Philippines. Biodiversity & Conservation 8: 1017–1031.
Hidayati SN, Walck JL. 2016. A review of the biology of Rafflesia: what do we know and what's next? Buletin Kebun Raya 19: 67–78.
Kopelman NM, Mayzel J, Jakobsson M, et al. 2015. CLUMPAK: a program for identifying clustering modes and packaging population structure inferences across K. Molecular Ecology Resources 15: 1179–1191.
Kuijt J. 1969. The biology of parasitic flowering plants. University of California Press, Berkeley and Los Angeles.
Laurance WF. 1998. A crisis in the making: responses of Amazonian forests to land use and climate change. Trends in Ecology & Evolution 13: 411–415.
Lowe AJ, Boshier D, Ward M, et al. 2005. Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. Heredity 95: 255.
Mould A. 2012. Cave bats of the central west coast and southern section of the Northwest Panay Peninsula, Panay Island, the Philippines. Journal of Threatened Taxa 4: 2993–3028.
Nais J. 2001. Rafflesia of the world. Sabah Parks, Kota Kinabalu.
Narum SR. 2006. Beyond Bonferroni: less conservative analyses for conservation genetics. Conservation Genetics 7: 783–787.
Nickrent DLN. 1997 onwards. The parasitic plant connection (http://parasiticplants.siu.edu/, last accessed 3 Sept. 2017).
Norton DA, Carpenter MA. 1998. Mistletoes as parasites: host specificity and speciation. Trends in Ecology & Evolution 13: 101–105.
Nybom H. 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Molecular Ecology 13: 1143–1155.
Oliver WLR. 2014. The taxonomy, distribution and status of Philippine wild pigs. Journal of Mountain Ecology 3: 26–32.
Oliver WLR, Cox CR, Dolar LL. 1991. The Philippine spotted deer conservation project. Oryx 25: 199–205.
Oliver WLR, Cox CR, Gonzales PC, et al. 1993. Cloud rats in the Philippines: Preliminary report on distribution and status. Oryx 27: 41–48.
Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics 28: 2537–2539.
Pelser PB, Barcelona JF, Nickrent DL (eds). 2011 onwards. Co's digital Flora of the Philippines (http://www.philippineplants.org, last accessed 3 Sept. 2017).
Pelser PB, Nickrent DL, Barcelona JF. 2016. Untangling a vine and its parasite: host specificity of Philippine Rafflesia (Rafflesiaceae). Taxon 65: 739–758.
Pelser PB, Nickrent DL, Callado JRC, et al. 2013. Mt. Banahaw reveals: The resurrection and neotypification of the name Rafflesia lagascae (Rafflesiaceae) and clues to the dispersal of Rafflesia seeds. Phytotaxa 131: 35–40.
Pelser PB, Nickrent DL, Gemmill CEC, et al. 2017. Genetic diversity and structure in the Philippine Rafflesia lagascae complex (Rafflesiaceae) inform its taxonomic delimitation and conservation. Systematic Botany 42: 543–553.
Pritchard JK, Stephens M, Donnelly P. 2000. Inference of population structure using multilocus genotype data. Genetics 155: 945–959.
Raymond M, Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86: 248–249.
Román B, Satovic Z, Alfaro C, et al. 2007. Host differentiation in Orobanche foetida Poir. Flora 202: 201–208.
Sammler S, Ketmaier V, Havenstein K, et al. 2012. Mitochondrial control region I and microsatellite analyses of endangered Philippine hornbill species (Aves; Bucerotidae) detect gene flow between island populations and genetic diversity loss. BMC Evolutionary Biology 12: 203.
Turner IM, Corlett RT. 1996. The conservation value of small, isolated fragments of lowland tropical rain forest. Trends in Ecology & Evolution 11: 330–333.
Van Oosterhout C, Hutchinson WF, Wills DPM, et al. 2004. Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535–538.
Young A, Boyle T, Brown T. 1996. The population genetic consequences of habitat fragmentation for plants. Trends in Ecology & Evolution 11: 413–418.