RESEARCH ARTICLE

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Reading between the vines: Hosts as islands for extreme holoparasitic plants[1]

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PREMISE OF THE STUDY: Partitioning of population genetic variation in plants may be affected by numerous factors including life history and dispersal characteristics. In parasitic plants, interactions with host populations may be an additional factor influencing partitioning. To test for hierarchical population genetic patterns related to obligate endoparasitism, we studied three species of Rafflesiaceae, which grow as extremely reduced endophytes infecting *Tetrastigma* vines in Southeast Asia.

METHODS: Microsatellite markers were developed and multilocus genotypes were determined for *Rafflesia cantleyi*, *Rafflesia tuan-mudae*, and *Sapria himalayana* and each of their *Tetrastigma* hosts. Relatedness among parasite individuals was estimated, and AMOVAs were used to determine levels of population genetic subdivision.

KEY RESULTS: Microsatellite genotypes for 340 paired parasite and host samples revealed that host vines were infected by numerous Rafflesiaceae individuals that may spread for up to 14 m within stem tissues. Surprisingly, Rafflesiaceae parasites within a given host are significantly more closely related to each other than individuals of the same species in other host individuals. The pattern of hierarchical population genetic subdivision we detected across species is likely due to limited seed dispersal with reinfection of natal host vines.

CONCLUSIONS: These findings demonstrate common population genetic patterns between animal and plant parasites, potentially indicating advantages of close relatives infecting hosts. This study also has important conservation implications for Rafflesiaceae since our data suggest that destruction of a single infected host vine could result in large genetic losses.

KEY WORDS hierarchical population genetic structure; host–parasite interactions; infrapopulation; parasitic plants; *Rafflesia*; Rafflesiaceae; *Sapria*; *Tetrastigma*

Parasites are found in every community on Earth and are represented in all major branches of the tree of life, from prokaryotes and unicellular eukaryotes to multicellular animals, fungi, and plants (Combes, 2001). They are typically viewed as pests by humans but, at the same time, provide greatly underappreciated ecosystem services in the regulation of community structure (Hatcher and Dunn, 2011; Dougherty et al., 2016). Comparative studies across the phylogenetic diversity of parasites have revealed broad evolutionary trends: a tendency to evolve reduced body plans, specialized feeding structures, extreme host specificity and internal growth (Kuijt, 1969; Poulin, 2006). In spite of these clear cases of convergent traits at the species level, it remains unknown to what extent parasitic plant and animal parasite populations, in particular, exhibit similar genetic patterns.

One important evolutionary ecological finding from studies of animal parasites is that they exist as infrapopulations (all individuals infecting a single host) that may be genetically subdivided from those found in/on other host individuals due to restricted dispersal. These infrapopulations are collectively organized into larger,

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genetically differentiated component populations (all parasite individuals within a given geographic region) (Criscione et al., 2005, 2011; Prugnolle et al., 2005). This genetic structuring may be accompanied by inbreeding among individuals within a host (Criscione et al., 2005) and lead to metapopulation dynamics whereby significant genetic loss occurs if dispersal to, and colonization of, new hosts does not outpace infected host death (Harrison, 1991; Hanski and Simberloff, 1997). The hypothesis that parasitic plants also form genetically subdivided infrapopulations is tenable, but remains untested. Yet, since parasitic plants produce flowers outside of their host for sexual reproduction, unlike internal animal parasites, even occasional pollen dispersal could reduce inbreeding and limit genetic subdivision among infra- and component populations.

Rafflesiaceae provides an ideal system in which to examine the potential for hierarchical genetic subdivision between infra- and component populations. This endoparasitic and highly host-specific lineage includes only three genera, *Rafflesia*, *Sapria*, and *Rhizanthes*, and is renowned for producing the largest flowers in the world (Bendiksby et al., 2010). The species lack true leaves, stems and roots and grow completely embedded within their sole hosts, species of *Tetrastigma* vines (Vitaceae), which they rely upon for carbohydrates, mineral nutrients, and water (Nais, 2001; Barkman et al., 2004; Davis and Wurdack, 2004). Since all Rafflesiaceae are obligate endoparasites, it is impossible to determine how many distinct individuals grow embedded within a single host vine by sight alone, even though multiple flowers may be observed (Fig. 1A, B) (Davis et al., 2007; Barkman et al., 2008). Thus, basic aspects of Rafflesiaceae life history have remained unknown since the discovery of *R. arnoldii* R. Brown more than 180 years ago including infrapopulation and component population size, individual-level reproductive allocation, and even breeding system. It has long been assumed that most Rafflesiaceae species are dioecious since flowers are unisexual. However, both male and female flowers have been observed from a single host vine, which makes it possible that these species could be monoecious (Nais, 2001). Below, we present multilocus genotype data for flowers/buds of *Rafflesia cantleyi* Solms-Laubach, *R. tuan-mudae* Beccari, and *Sapria himalayana* Griff. and their associated host tissues to reveal, for the first time, fundamental life history characteristics of these parasitic populations. Additionally, we estimate genetic parameters that elucidate patterns in the distribution of parasite genotypes, which bear directly on conservation strategies and management efforts for these rare species.

MATERIALS AND METHODS

Sampling strategy—Parasite samples were obtained from two *Rafflesia* species and one *Sapria* species growing on three different host vine species from six populations throughout Southeast Asian rainforests of Peninsular Malaysia, Borneo, and Thailand (Fig. 2; online Appendix S1 [see Supplemental Data with this article]). We carefully collected nonessential external reproductive material to limit damage to developing flowers. In all cases, we exhaustively sampled from all parasite flowers/buds visible from each host vine. A total of 340 parasite samples were collected (Appendix S2). Gender was determined in 85 open flowers, naturally aborted buds, and through the selective, careful dissection of a few viable buds. Due to the endophytic nature of these parasite species, the corresponding number of unique individuals producing the 340 flower samples was unknown to us before genotyping. For nearly each Rafflesiaceae sample obtained, we collected a corresponding sample of its host: *Tetrastigma rafflesiae* in the case of *R. cantleyi*, *T. diepenhorstii* in the case of *R. tuan-mudae*, and *T. cruciatum* in the case of *Sapria himalayana* ($N = 338$ in total; Appendix S2) (Mokhtar et al., 2016; Wan Zakaria et al., 2016). Although host stem ramets were discernable, it was often unknown whether nearby ramets were part of the same genet or not because they may be produced from extensive unobservable, but linked, underground systems (Fig. 1C). Therefore, this sampling method was critical because it allowed us to determine which Rafflesiaceae parasite samples occupied the same host. At the outset of our study, we had the goal of assessing host vine population genetic structure; yet, after genotyping all host samples it became clear that most host populations were composed of few individuals (<7) (Appendix S2). As a result, the genotypes of *Tetrastigma* samples were only used to distinguish host vine stems and roots from one another at each site.

Microsatellite development—We used a nonradioactive approach to microsatellite development (Glenn and Schable, 2005) involving DNA extraction from dried bract tissue of *R. cantleyi* and *Sapria himalyana* and stem tissue of *Tetrastigma rafflesiae* using the DNeasy Plant Mini Kit (Qiagen, Valencia, California [CA], USA). We digested genomic DNA using the RsaI restriction enzyme and then enriched restriction fragments with biotinylated oligos using PCR. Fragments were recovered with magnetic Dynabeads (Invitrogen, Carlsbad, CA, USA), ligated into plasmids and inserted into competent bacterial cells using the TOPA-TA cloning kit (Invitrogen). We selected more than 192 colonies per taxon and amplified each using PCR to screen for inserts of 500–1100 bp. We then sequenced inserts and screened for the presence of microsatellites using an online tandem repeat finder (http://biophp.org). Primers were developed using Primer3 (v. 0.4.0) software (Misener and Krawetz, 2000).

We used fluorescently labeled forward and unlabeled reverse primers in multiplex reactions for genotyping analyses (6FAM, VIC, PET, NED). All PCR reactions were performed in 10 μL volumes using the Qiagen Multiplex kit as follows: 5 μL Multiplex PCR Master Mix, 1 × primer mix, 0.2 μL DNA and 3.8 μL dH_2O. We selected multiplexing primer groups based on shared annealing temperature and primer compatibility, with one to five primer pairs used per sample. Samples were separated on a 3730xl sequencer (Applied Biosystems, Foster City, CA, USA) at the Cornell University BioResource Center (Ithaca, New York, USA) with the LIZ 500 internal size standard, and conducted fragment analysis with Genemapper v. 4.0 software (Applied Biosystems). All polymorphic microsatellite loci amplified consistently. Moreover, none of the loci developed for *Tetrastigma* yielded amplicons for *Rafflesia* or *Sapria*, and vice-versa, suggesting that there was no laboratory or field contamination.

Genotyping and allele calls—Analyses of *Rafflesia* and *Sapria* electropherograms revealed a diploid state, with all loci scored as codominant, poly allelic (Appendices S3, S4). Because *Tetrastigma* is purportedly tetraploid (Shetty and Raman, 1960) and microsatellite loci developed for this study revealed a tetraploid state, we scored electropherogram peaks as dominant and diallelic (present/absent) (Appendix S5). When attempting to score anomalous or low amplitude peaks, we reran DNA samples and rescored electropherograms to confirm or refute the presence of a peak. We treated all unique multilocus genotypes as distinct individuals. In addition, we

also used Geneclone to verify our determination of distinct clonal groups (Arnaud-Haond and Belkhir, 2006). For cases in which Geneclone suggested possible electropherogram scoring errors, we double-checked electropherograms to ensure accurate identification of multilocus genotypes.

Data analyses—Duplicate parasite genotypes were excluded for all analyses using Genclone (Arnaud-Haond and Belkhir, 2006). We chose to perform population genetic analyses on the geographically defined populations shown in Fig. 2 except for *R. cantleyi*. In this species, individuals from the UG and TA sites were analyzed together because they comprise few individuals and the program STRUCTURE v. 2.3.4 (Pritchard et al., 2000) indicated they are clustered (Appendix S6).

To examine genetic diversity, we calculated alleles per polymorphic locus (A_p), observed heterozygosity (H_O) and expected heterozygosity (H_E) for all Rafflesiaceae samples (Appendix S7). We assessed deviations of loci from Hardy–Weinberg equilibrium (HWE) using exact tests (Markov chain parameters, 5000 dememorisation steps, 500 batches, 5000 iterations per batch) in the program GENEPOP (v. 1.2) (Rousset, 2008) and sequential Bonferroni corrections for multiple comparisons. No significant deviations from HWE were detected in loci of *R. cantleyi* using exact tests ($P > 0.05$). However, we did detect deviations from HWE in four loci across the two sites of *R. tuan-mudae* (Raff15 and Raff22 [population GG]; Raff19 and Raff21 [population WF]) and in three loci from the single population of *S. himalayana* (Sap3, Sap5, and Sap15). Linkage disequilibrium (LD) was not detected in *S. himalayana* but was for two locus pairs each in *R. cantleyi* (Raff11 and Raff20 in population TA; Raff15 and Raff26B in populations BM and UG) and *R. tuan-mudae* (Raff10 and Raff11 in population WF; Raff11 and Raff26B in population GG). Because there was no evidence of consistent LD between locus pairs across the sampling sites of each species, we included all loci in further analyses. In addition, we performed analyses to test for the presence of null alleles using ML-Relate (Kalinowski and Taper, 2006). Although some loci were inferred to have null alleles, there are alternative biological explanations for homozygote excess (e.g., Wahlund effect) and since many of our primers amplified across different *Rafflesia* species, we do not believe that null alleles were particularly abundant in the determined genotypes. Nonetheless, we incorporated null allele frequency estimates for relatedness analyses (see below).

To calculate *F* statistics, we used the program GENALEX 6.5 (Peakall and Smouse, 2006). For the two *Rafflesia* species, each of which were collected from multiple sites, we performed a hierarchical AMOVA to estimate the genetic structure among sites (F_{CT}), among vines within sites (F_{SC}), and among all vines (F_{ST}). This notation is comparable to other recent studies in parasites and facilitates comparisons with them (Cooper et al., 2003; Andras and Ebert, 2013). For *S. himalayana*, we performed a standard AMOVA to determine genetic structure among all vines (F_{ST}) from the single sampling site. For both *Rafflesia* and *Sapria*, we calculated the inbreeding coefficient (F_{IS}) within the hierarchical AMOVA in GenAlEx, which is equal to the estimated variation among individuals/(estimated variation among individuals + estimated variation within individuals). Significance was determined using 9999 nonparametric permutations.

To determine the relatedness of individual parasites within and between vines, we calculated maximum likelihood estimates of relatedness (*R*) for all pairs of *R cantleyi*, *R. tuan-mudae*, and *S. himalayana* genotypes using ML-Relate (Kalinowski et al., 2006). For those loci potentially exhibiting null alleles, we incorporated their inferred frequencies to obtain maximum likelihood estimates of relatedness (Kalinowski and Taper, 2006).

Statistical significance of mean differences in flower number per parasite genotype and parasite relatedness within vs. among host vines was determined using a two-tailed Student's *t* test.

RESULTS

Inside the black box—We found definitive evidence that numerous Rafflesiaceae parasite individuals infect a single vine (Fig. 1D, E). The 340 parasite floral tissues genotyped using nine or 10 microsatellite loci along with 338 paired *Tetrastigma* host stem samples genotyped with 10–12 loci revealed 129 unique multilocus genotypes of *Rafflesia cantleyi*, *R. tuan-mudae*, and *Sapria himalayana* from 33 genetically defined host vines (Appendices S2–S5). Quantification of the infection intensities carried by a host vine (the infrapopulation census size) varied widely because some individuals appear to support only a single parasite infection (genotype), while others, minimally contained 13 (mean = 3.9; Appendices S2, S8). The component population census sizes ranged from 10–45 parasites infecting as few as two, or as many as 14, host vines (Appendix S2).

Reproductive allocation varied widely among parasite individuals with most producing one flower, while others invested in more than 20 (mean = 2.6) (Appendix S9). Comparison of the mean flower number produced per parasite genotype indicated that the large-flowered (44–92 cm diameter) *R. tuan-mudae* individuals produce significantly fewer flowers than the smaller-flowered *R. cantleyi* (30–55 cm diameter) ($t = -2.99$, df = 83, $P < 0.05$) and *Sapria* (10–20 cm diameter) individuals ($t = -2.17$, df = 84, $P < 0.05$).

For parasites that produced more than one flower, we sought to infer how far individuals ramify throughout the host vine. The paired host and parasite genetic data indicate that, on average, *Rafflesia* infections extend 4 m, whereas those of *Sapria* ramify for 1.6 m (Appendix S2). The two largest floral displays were produced by *R. cantleyi* individuals both of which produced 25 buds and/or flowers with a minimum distance of infection extending 2.7 m in one and 7.7 m in the other. On the other hand, one individual parasite from the WF population of *R. tuan-mudae* produced only two flowers but had the most extensive distance (14.7 m) separating them (Appendix S2).

For 15 parasite individuals that produced more than one flower for which gender could be determined, in no case did a female and male flower have the same genotype (Appendix S10). Thus, our multilocus genotypes failed to reject the hypothesis that these Rafflesiaceae species are dioecious.

Parasite population genetic structuring within host islands—Remarkably, although many hosts grow less than 100 m apart (Fig. 2), parasites from different vines appear genetically differentiated from one another because significant subdivision was detected among infrapopulations of *R. cantleyi*, *R. tuan-mudae*, and *Sapria* (Table 1). Not unexpectedly, given the high level of infrapopulation differentiation, there is also evidence for significant genetic subdivision among component populations of both *R. cantleyi* and *R. tuan-mudae* that are ca. 1–100 km apart (Table 1, Fig. 2). The apparent infrapopulation differentiation is not due to the inclusion of multiple clones of the same parasite in the analyses because we were

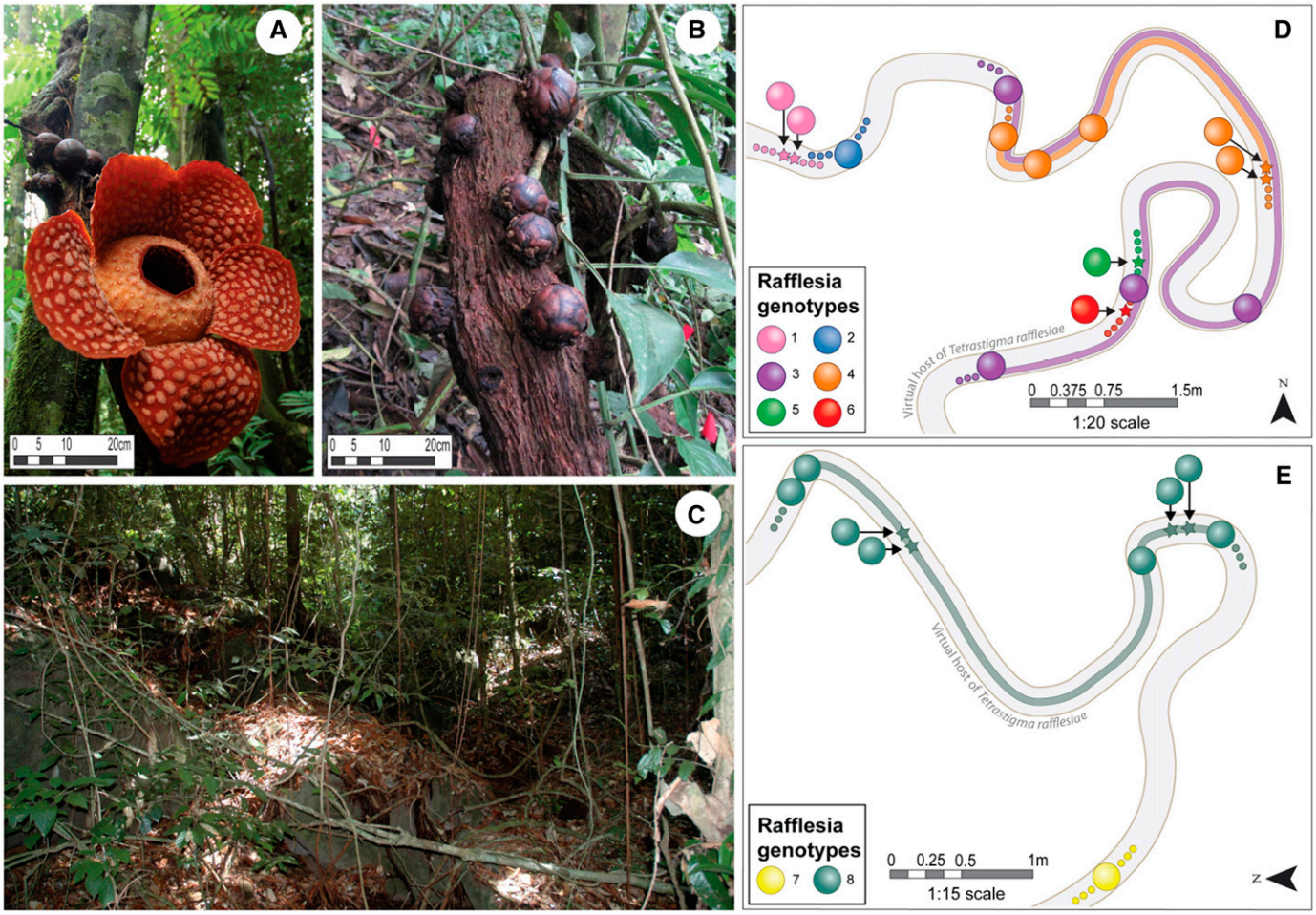

FIGURE 1 *Rafflesia cantleyi* habit and organization within host vines. (A) Open flower of *R. cantleyi* and additional unopened buds on host vine, *Tetrastigma rafflesiae*, which extends 10 m into the forest canopy. (B) Infected *T. rafflesiae* host vine with several emergent parasite flower buds. Whether each bud is produced by the same parasite is unknowable without genotypic information. (C) Peninsular Malaysia rainforest habitat of *Tetrastigma rafflesiae* and its parasite *R. cantleyi*. (D) Spatially reconstructed virtual host vine ("UG1") from Ulu Geroh, Perak, Malaysia infected with six individuals of *R. cantleyi* as identified from multilocus genotypes. Flowers/buds with identical genotypes are represented by the same color and solid line linking them. Dotted lines indicate that the distance that the parasite extends beyond the sampled bud is currently unknown. (E) Spatially reconstructed virtual host vine ("UG2") from Ulu Geroh, Perak, Malaysia infected with two individuals of *R. cantleyi* as identified from multilocus genotypes.

careful to exclude such genotypes (see Methods). Instead, the nonrandom distribution of genotypes into infrapopulations is due to the fact that individuals infecting the same vine are highly related to one another, on average (many are inferred to be parent–offspring or full- or half-sibs [average pairwise R = 0.26–0.41]) (Fig. 3). In sharp contrast to this finding, individuals that infect different vines are mostly unrelated, especially within *Rafflesia* (average pairwise R = 0.09–0.18) (Fig. 3). For example, for *R. cantleyi*, population UG2 is separated from population UG 1 by 40 m (Figs. 1D, 1E, 2); yet, average pairwise relatedness is 0.41 among the six parasite individuals in the former and 0.31 among the two parasites infecting the latter. In contrast, average pairwise relatedness among individuals found in UG1 and UG2 is 0.08 (Fig. 1D, E). This difference in parasite relatedness between hosts is significant in all three species, *R. cantleyi* (t = 7.89, df = 435, $P < 0.05$), *R. tuan-mudae* (t =14.8, df = 771, $P < 0.05$), and *Sapria* (t = 4.58, df = 989, $P < 0.05$) (Fig. 3). Although average relatedness within a single host vine is high, it should be noted that there is a wide range of relatedness within vines (0-0.7).

Small infrapopulations show little evidence of inbreeding—The discovery that multiple parasite individuals of different genders may occur within single vines sets up a possibility for inbreeding among close relatives (Fig. 3). Yet, in spite of the close proximity of highly related *Rafflesia cantleyi* and *Sapria* individuals within a single host vine and small component population census sizes (Appendix S2), there appears to be little evidence for inbreeding in either *R. cantleyi* or *Sapria* which show F_{IS} values of 0.031 and –0.178, respectively (Table 1). These F_{IS} estimates are not statistically significant and indicate there is no heterzygote deficiency in either species (Table 1). In contrast, a statistically significant signature of heterozygote deficiency was detected for *R. tuan-mudae* for which an F_{IS} estimate of 0.101 was obtained (Table 1).

DISCUSSION

Previously, it was unknown whether more than a single Rafflesiaceae individual infects an individual host vine since it is impossible

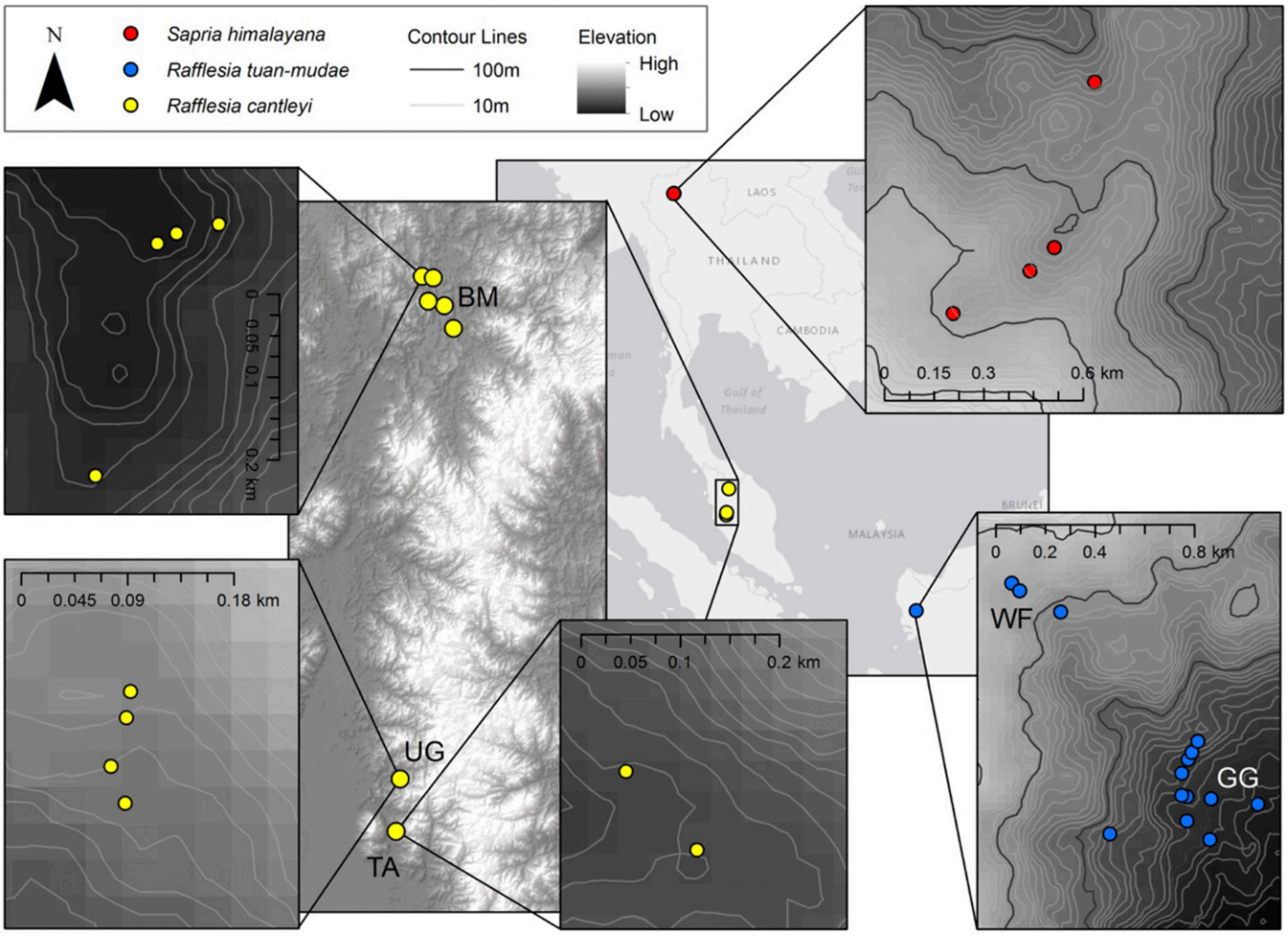

FIGURE 2 Geographic locations for sampling of *Rafflesia cantleyi* from three populations in peninsular Malaysia, *Rafflesia tuan-mudae* from two populations in Borneo and *Sapria himalayana* from one population in Thailand.

to determine whether multiple flowers or buds are connected within host tissues by sight alone (Fig. 1A, B). Additionally, host vines may form tangled masses of multiple localized stems making it unclear if they are all part of a single, large individual connected underground or multiple intertwined but genetically distinct individuals (Fig. 1C). This complicated biological system thus required the paired sampling of host with parasite buds, coupled with multilocus genotypes of each to definitively provide estimates of infection intensity, reproductive allocation, and spatial extent of infection (Appendices S2, S8, S9). Nevertheless, some of the life history estimates we provide are likely minimal values. For instance, since there may have been nonreproductive individuals that we could not sample due to phenological variation, the actual infection intensity per host is likely higher. Likewise, the spatial extent of infections are likely greater than what we inferred since parasite individuals may spread greater than the distance measured between the two most distant observable buds/flowers per parasite genotype. It remains unknown to what extent host physiological condition may also affect infection intensity and flower number. Continued sampling from these host vines, especially across a single season, may improve these estimates and could even reveal (1) the direction and rate of parasite growth, (2) parasite partitioning within host tissues, (3) temporal variation in parasite reproduction, and (4) the longevity of infections. Anecdotal evidence suggests that hosts may be infected for decades but whether that is due to one or a few long-lived parasite individuals or repeated infections is unclear. Finally, although we have concluded that *Rafflesia* and *Sapria* are dioecious, it should be noted that sex expression is labile within

TABLE 1. Hierarchical AMOVA estimates for *Rafflesia cantleyi*, *R. tuan-mudae*, and *Sapria himalayana*. Infrapopulations are defined as all parasite genotypes within a single host vine (see Fig. 1). Component populations are defined as all parasite genotypes within a geographically defined site (see Fig. 2). Note that there was only one component population for *Sapria* thus F_{ST} corresponds to subdivision among the four vines at that single site (*P < 0.05, **P < 0.01, ***P < 0.001).

Statistic	*R. cantleyi*	*R. tuan-mudae*	*S. himalayana*
Among geographic sites (component populations) (F_{CT})	0.055***	0.026*	
Among vines/hosts (infrapopulations) within a site (F_{SC})	0.138***	0.199***	
Among all vines/hosts (infrapopulations) from all sites (F_{ST})	0.185***	0.220***	0.072**
Inbreeding coefficient (F_{IS})	0.031	0.101**	−0.178

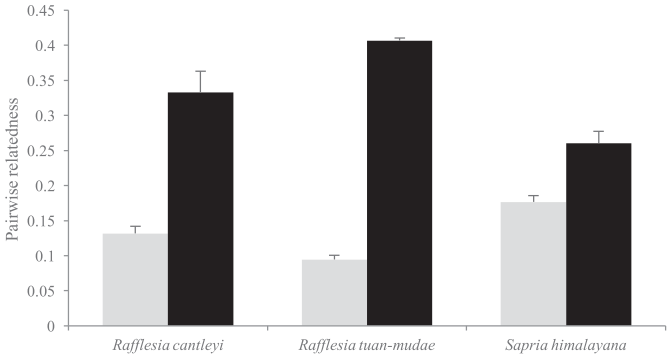

FIGURE 3 Average pairwise within-host relatedness (black) and between-host relatedness (gray) plus standard error for three Rafflesiaceae species. For all three species, individual parasites infecting the same vine are significantly more highly related to each other than those parasites infecting different host vines ($P < 0.05$ in all three cases).

this family since one species of *Rhizanthes*, although typically unisexual, may produce bisexual flowers (Banziger et al., 2007). Since environmental conditions are also known to affect sex expression in some systems (Glawe and de Jong, 2005), sampling over time and tracking of gender is needed.

This is the first time parasitic plant populations have been shown to be genetically subdivided into infrapopulations of close relatives defined by their host's distribution like some trematodes and lice (Sire et al., 2001; Koop et al., 2014). Infrapopulation genetic structuring exists in Rafflesiaceae populations in spite of the potential for pollinators and frugivores to widely disperse pollen and seeds, respectively. The division of parasites into infrapopulations defined by host boundaries likely results from reinfection of the natal host vine by groups of seeds from fruits produced over one or more generations. Such conditions are probable in Rafflesiaceae because they have large, immobile, woody, berry-like fruits that may produce up to 270,000 tiny seeds embedded within a pulp (Nais, 2001). Such a pattern of relatedness is also compatible with the possibility of clumped dispersal from a single fruit since the multiple tiny seeds could be carried en masse by a frugivore, or possibly seed-dispersing ants (Pelser et al., 2013), to new hosts. The pattern of genetic subdivision of parasites by host islands also undoubtedly reflects, at least in part, the distribution of parasitizable host vines. Few attempts have been made to determine what proportion of host vine species in a given area are infected vs. uninfected, but neighboring vines with and without obvious Rafflesiaceae infections have been reported (Nais, 2001).

The fact that we observed similar patterns of genetic differentiation among hosts in *Rafflesia* and *Sapria*, which diverged more than 60 million years ago (Bendiksby et al., 2010), suggests a highly conserved life history strategy that may be advantageous. Our genetic structure findings (Table 1) may be explained by the production of large woody fruits (~15 cm diameter) that are likely dispersal limited, virtually guaranteeing some degree of reinfection of natal *Tetrastigma* hosts. Additionally, if host–parasite genetic compatibilities limit new establishment, then reinfection by sib-ships that are genetically similar would be favored over dispersing to new hosts. These findings may also support theoretical predictions of kin selection whereby virulence may be reduced when related parasites infect a single host (Chao et al., 2000; Buckling and Brockhurst, 2008). On the other hand, if the large immobile fruits have been retained over time due to structural constraints associated with the production of their immense flowers, then the patterns we have documented could be nonadaptive in *Rafflesia* and *Sapria*. Unfortunately, distinguishing between these various hypotheses is difficult and at a minimum would require manipulative experimentation on a scale that is not currently feasible for *Rafflesia* or *Sapria* (Wicaksono et al., 2016).

While the pattern of close relatedness within vines could be a consequence of biased sampling of parasites from a single host, this is not the case since all buds that were visible on a given vine or site were collected. In addition, we recognize the possibility that the small sample size of parasites per host may lead to imprecise estimates of differentiation, yet this is an unavoidable consequence of the biology of this host–parasite system. Still, studies in two other cryptic, highly host-specific lineages of Cytinaceae provide limited comparative data indicating that the fine-scale differentiation we report for Rafflesiaceae may be associated with endoparasitism more broadly. In *Bdallophyton*, high genetic similarity was found for adjacent parasite inflorescences within the same host, as well as some adjacent hosts, although overall no significant isolation by distance was detected (García-Franco et al., 1998). In *Cytinus*, only a single parasite was sampled per host, but geographically adjacent populations appear to be most closely related for a given host lineage (Vega et al., 2008). The findings of our study may also be relatable to highly host-specific exoparasites; for instance, postglaciation migration of beechdrops (*Epifagus virginiana*; Orobanchaceae) does not appear to have tracked host range expansion and instead is dependent upon host population density (Tsai and Manos, 2010). This pattern would be expected in the case of a high degree of natal host reinfection and limited dispersal by *Epifagus* propagules each generation.

The genetic subdivision of parasites into infrapopulations has been shown to result in highly inbred populations of some animal parasites (Criscione et al., 2005; Van den Broeck et al., 2014). Nevertheless, unlike animal endoparasites, these plant species reproduce outside of the host, with carrion fly pollinators transporting pollen among flowers on different hosts due to both visual and olfactory attractants (Beaman et al., 1988). This may explain the lack of inbreeding we observed in *R. cantleyi* and *Sapria himalayana*, as even occasional pollen exchange among hosts would reduce inbreeding, while infection of the same host by sibling seeds or close relatives would still lead to the strong infrapopulation genetic subdivision we have reported. In addition, although we observed that some hosts had parasite flowers of both sexes with a high average relatedness (Fig. 3), simultaneously blooming flowers of different sexes from a single host vine is rare in these dioecious species (Beaman et al., 1988; Nais, 2001). It also remains possible that an incompatibility system may act in this family, such that shared alleles at the *S*-locus would prevent mating among closely related males and females (Charlesworth et al., 2005). In contrast to *R. cantleyi* and *Sapria himalayana*, *R. tuan-mudae* populations did exhibit a genetic signature of inbreeding. Such differences could be explained by different breeding systems, especially since autogamy has been reported in the family (Nais, 2001).

Collectively, our genetic data suggest that populations of Rafflesiaceae operate as metapopulations. In particular, we found low overall diversity in terms of allelic richness (Appendix S7). This feature is consistent with populations that have undergone numerous founder events and diversity loss due to local extinctions. Since multiple parasites occur within a vine, there is opportunity for

entire infrapopulation/subpopulation (deme-level) extinction should a single host die, which could result in the loss of substantial genetic diversity from the component populations (Appendix S2). The limited seed dispersal indicated by our results suggests a precarious relationship between extinction and recolonization that could lead to regional population loss should host mortality rise, or distance increase, among potential hosts. This discovery raises a substantial "red flag" to conservation biologists because these plants occur in small populations that are notoriously rare.

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