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Speciation in a keystone plant genus is driven by elevation: a case study in New Guinean *Ficus*

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Abstract

Much of the world's insect and plant biodiversity is found in tropical and subtropical 'hotspots', which often include long elevational gradients. These gradients may function as 'diversity pumps' and contribute to both regional and local species richness. Climactic conditions on such gradients often change rapidly along short vertical distances and may result in local adaptation and high levels of population genetic structure in plants and insects. We investigated the population genetic structure of two species of *Ficus* (Moraceae) along a continuously forested elevational gradient in Papua New Guinea. This speciose plant genus is pollinated by tiny, species-specific and highly coevolved chalcid wasps (Agaonidae) and represented by at least 73 species at our study gradient. We present results from two species of *Ficus* sampled from six elevations between 200 m and 2700 m a.s.l. (almost the entire elevational range of the genus) and 10 polymorphic microsatellite loci. These results show that strong barriers to gene flow exist between 1200 m and 1700 m a.s.l. Whereas lowland populations are panmictic across distances over 70 km, montane populations can be disjunct over 4 km, despite continuous forest cover. We suggest that the limited gene flow between populations of these two species of montane *Ficus* may be driven by environmental limitations on pollinator or seed dispersal in combination with local adaptation of *Ficus* populations. Such a mechanism may have wider implications for plant and pollinator speciation across long and continuously forested elevational gradients if generalist insect pollinators and vertebrate seed dispersers also form populations based on elevation.

Introduction

Many of the world's biodiversity 'hotspots' include long tropical or subtropical elevational gradients (Myers *et al.*, 2000; Mittermeier *et al.*, 2004; Mutke & Barthlott, 2005). Rapidly changing environmental conditions along such elevational gradients can lead them to function as 'diversity pumps' which may contribute to the origin of a large proportion of the world's biodiversity (Robin *et al.*, 2010; Schultheis *et al.*, 2012; Toussaint *et al.*, 2014). Phylogeographic studies of insects indicate that the formation of species in parapatry, where species ranges abut but do not overlap (Gavrilets, 2004), in montane habitats, can create speciation 'cradles' that fuel lowland diversity (Hall, 2005). Studies of plant communities also reveal high levels of species turnover at mid-elevations in large, species-rich tropical families (e.g. Burger, 1995). Local adaptation (and the filtering of maladapted genotypes) and limitations to insect mediated gene flow are likely to be

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especially important in insect pollinated flowering plants (Reis *et al.*, 2015), which represent the majority of tropical forest floras. Given that over 75% of species in terrestrial ecosystems is involved in a plant based food web (Price, 2002), it is not unreasonable to suggest that understanding the mechanisms of speciation in plants along elevational gradients is an important research goal, especially given our rapidly changing climate.

Montane habitats surrounded by lowland forest (called 'sky islands') have a clear role in promoting peripatric speciation in the hyperdiverse Australasian region (Toussaint *et al.*, 2013), with species of montane origin feeding back into the overall lowland species pool. The region's highlands provide an excellent natural laboratory in which to investigate ongoing or incipient speciation. Within the wider region, Papua New Guinea (PNG) is recognized as being particularly biodiverse. Indeed, 5% of the world's animal and plant species are found in PNG (an area representing 0.5% of the world's total land mass), and two-thirds of these species are endemic. The country is also known for its dramatic and geologically active topography which may contribute considerably to its high levels of endemism and biodiversity. Many ecologically important plant genera have diversified considerably in PNG, acting as important host islands for insect herbivores (Weiblen *et al.*, 2001, 2006; Novotny *et al.*, 2010). One such genus is *Ficus* (Moraceae). This pantropical genus is extraordinarily species-rich, containing over 800 species, 157 of which occur in PNG (Berg & Corner, 2005; Cruaud *et al.*, 2012). In PNG, *Ficus* species are overrepresented amongst plant species with wide elevational ranges and represent one of the key genera in forest communities along elevational gradients (Novotny *et al.*, 2005). Pollination in *Ficus* is performed exclusively by wasps in the chalcid family Agaonidae (Wiebes, 1979). These tiny wasps are usually species-specific and can act as effective pollinators over tens to hundreds of kilometres (Nason *et al.*, 1996; Ahmed *et al.*, 2009), whereas seed dispersal is carried out by a wide range of vertebrates, including bats and birds (Shanahan *et al.*, 2001). As such, both fig pollen and seeds can be transported over large distances (by pollinating wasps and frugivores, respectively). However, little is known about gene flow in *Ficus* along ecological gradients, for instance within populations of *Ficus* species with wide elevational ranges. Whereas there are documented examples of lowland and highland varieties or subspecies in at least three sections of *Ficus* (Berg & Corner, 2005), several examples of extremely close relatives occupying lowland and highland habitats can be found within the Papuan species in section *Sycocarpus* (which is pollinated by wasps from the genus *Ceratosolen*). A section is an infrageneric clade of species and each *Ficus* section is often, but by no means exclusively, pollinated by one genus of wasp (Cook & Segar, 2010). The endemic Papuan species in section *Sycocarpus* have a relatively recent origin (around 15 MY) (Cruaud *et al.*, 2012) and are represented by several complexes, with some species still capable of hybridizing (Moe & Weiblen, 2012). We studied gene flow in two understorey species of *Ficus* from section *Sycocarpus*, *F. arfakensis* King and *F. hahliana* Diels, along a continuously forested elevational gradient from 200 m a.s.l. to the local elevational limit of the genus at 2700 m a.s.l. in Papua New Guinea's Central Range.

Given that wasp-mediated gene flow between populations of *Ficus* in lowland habitats can cover tens to hundreds of kilometres (Nason *et al.*, 1996; Ahmed *et al.*, 2009), we might expect to see a similar pattern in montane populations. This would be evidenced by panmixia in the populations of both species studied here. However, whereas forest cover can be continuous, environmental conditions vary dramatically across elevational gradients, and may lead to limitations on pollinator and/or seed dispersal and even local adaptation followed by phenotypic isolation. This would result in genetic structure corresponding to gradual or sudden climactic changes in vertical distance. We expect genetic diversity in *Ficus* to decrease with elevation. This is because lowland populations are connected to a large gene pool through long-distance wasp migration (Nason *et al.*, 1996), whereas highland allelic diversity would be a nested subset of lowland diversity if vertical transmission is limited (mountains acting as bottlenecks). Mechanistically, the above canopy winds that facilitate long-range dispersal of wasps in lowland habitats are likely to be a less effective method of pollinator dispersal to higher elevations. This may be especially true for understorey tree species (Harrison, 2003), like *F. arfakensis* and *F. hahliana*. Furthermore, major genetic bottlenecks may occur at climatic interfaces, for example at the 'cloud layer' (the site of near constant cloud immersion resulting from relief precipitation). These interfaces may limit gene flow between elevations and exacerbate the genetic disparity between adjacent populations, allowing population specific alleles to accumulate. We combined extensive surveys of local *Ficus* species and population genetic data to address the hypothesis that limitations to gene flow occur along our study gradient.

Materials and methods

Survey of local *Ficus* diversity

A detailed survey of all local *Ficus* species was carried out at six of seven study sites along an elevational gradient focused on Mt. Wilhelm in Papua New Guinea (excluding Degenumbu, see Table 1 and Fig. S1 for site locations). At each elevational study site, teams of researchers (led by L. Sam) and paraecologists tagged all *Ficus* trees having a d.b.h. (diameter at breast height)

Table 1 Names of sample sites, their elevation (m a.s.l), their GPS coordinates, distance in a straight line to the gradient site with the lowest elevation (distance to lowest elevation (DLE)), and sampled species. Mean syconial volume (cm^3) at each site is given for *Ficus hahliana*.

Site name	Elevation (m)	Latitude	Longitude	DLE (km)	Sampled *Ficus* species	*N* = 49	Syconia volume (cm^3) ± SE (*N*)	Sampled *Ficus* species	*N* = 58
Ohu	200	05°14′00″S	145°41′00″E	70	*F. hahliana*	4	NA	*F. arfakensis*	1
Kausi	200	05°44′33″S	145°20′01″E	0	*F. hahliana*	10	0.99 ± 0.03 (158)	*F. arfakensis*	14
Numba	700	05°44′14″S	145°16′12″E	7	*F. hahliana*	5	0.89 ± 0.02 (154)	*F. arfakensis*	15
Memeku	1200	05°43′18″S	145°16′17″E	7	*F. hahliana*	10	1.17 ± 0.03 (179)	*F. arfakensis*	13
Bananumbu	1700	05°45′21″S	145°14′11″E	11	*F. hahliana*	5	NA	*F. arfakensis*	5
Degenumbu	1700	05°45′45″S	145°11′55″E	15	*F. hahliana*	10	2.88 ± 0.44 (9)	*F. arfakensis*	10
Sinopass	2200	05°45′34″S	145°10′49″E	17	*F. hahliana*	5	4.48 ± 0.43 (30)		
Bruno Sawmill	2700	05°48′57″S	145°09′02″E	22	*F. hahliana*	5	5.45 ± 0.28 (60)		

>1 cm within ten 500 × 10 m transects; transects were located at least 200 m from each other. Each tree was identified to species level and given a unique tree identifier number. We summarized species turnover along the gradient by calculating the percentage dissimilarity for each elevation in comparison with the 200 m site, using the Chao-Sorensen distance based on abundance data.

Focal species, plant tissue collection and genotyping

The genus *Ficus* is very species-rich at this site, and after a detailed survey of local *Ficus* diversity, we selected two species with wide elevational ranges for our population genetic study. Our study species are also both endemic to PNG and form part of a recent radiation, such that any population genetic patterns found are more likely to have occurred *in situ*, rather than as a result of multiple long-distance colonizations. Indeed, PNG itself is relatively young (Toussaint *et al.*, 2014) with the Central Range likely to be between 5 and 10 MY old. *Ficus arfakensis* has a recorded range of up to 1600 m in elevation (Berg & Corner, 2005), and it is widespread in PNG. As with many members of section *Sycocarpus*, *F. arfakensis* grows as a small understorey tree and is often locally abundant in secondary forest (Berg & Corner, 2005). *Ficus hahliana,* described by Berg & Corner (2005) as a lowland species, is often found close to rivers throughout PNG. Morphologically, *F. hahliana* is easily confused with *F. bernaysii* King (up to 1800 m a.s.l.). Both species form a species complex including also the highland species *F. iodotricha* Diels (700–2900 m a.s.l.), and as such, we genotyped four individuals of the latter two species.

For clarity, we use the term *population* solely for inferred biological clusters, we use the term *site* exclusively for sampling sites (each comprising 10 transects) and we use the term *elevation* for combining sites with the same elevation along our gradient. All elevations are given in metres above sea level (a.s.l.). We refer to our main sampling location as the 'elevational gradient'. There are eight sampling sites in total (Table 1). One elevation (1700 m) was sampled across two sites. We had to relocate our 1700-m sampling site during the project but after our survey (Table 1) due to land ownership disputes at our original site. The Ohu site was located in Ohu village (145°41′ E, 5°14′ S) near Madang (around 70 km north-east of our elevational gradient). Hence, there are seven sites and six elevations along the gradient, and one site outside of the gradient. Although Ohu is a lowland site, it is not part of our elevational gradient; we therefore do not group it with Kausi (also 200 m) when *a priori* assumptions are needed. *Ficus arfakensis* is present at five sites and four elevations (Ohu and between 200 and 1700 m) and absent at the two highest elevations (2200 and 2700 m), and we sampled an average of 14 individual trees per elevation. *Ficus hahliana* is present at all eight sites (Ohu and between 200 and 2700 m), and we sampled an average of eight individual trees per elevation (Table 1).

We have no *a priori* information on what constitutes a population for species with such wide elevational ranges. We therefore sampled evenly spaced sites that likely represent discrete within population samples, with some expectation that populations will comprise multiple sites. This means that site sample size is usually smaller than population size (see Results). We initially aimed to sample at least one tree per transect, so that the major barriers to gene flow could be identified. We selected only male trees so that pollinating wasps could be subsequently collected and associated with a given host tree (Souto-Vilarós *et al.*, in prep). Our selective sampling criteria and the naturally low density of mature trees meant that we effectively sampled haphazardly across transects at each site. The distance between trees sampled at a given site therefore ranged from 20 m to 1 km but was always less than the distance between sites, so that sampling at each site was representative of the local population. GPS location and voucher specimens were collected for a subset of the trees.

We sampled leaf discs, which were only collected from male trees large enough to bear fruit, at least 20 m was left between individuals and clonal individuals were avoided. Leaf discs (collected using a cork borer of 2.4 cm in diameter) were dried in the field in ziplock plastic bags containing two table spoons of colour indicating silica gel, which was replaced when necessary. All samples were then stored at −20 °C until needed for analysis. We isolated DNA from one leaf disc per individual using Invisorb Spin Plant Mini Kits (STRATEC Molecular, Germany). Due to polyphenol and secondary metabolite carry-over through the spin column in some samples (in particular for *F. hahliana*), we also extracted DNA using a modified CTAB protocol (Doyle & Doyle, 1987) with an additional cleaning step through a silica spin column or agarose gel. This removed all traces of contaminants yielding highly concentrated and pure DNA as measured by both Qubit Fluorometer (Invitrogen, OR, USA) and NanoDrop (Thermo Scientific, Wilmington, DE, USA). The syconia of *F. hahliana* clearly vary in size with elevation, and this may influence both wasp entry and seed dispersal. We quantified this variation by collecting a total of 590 mature syconia across six sites (Table 1) and measured both height and width to the nearest 0.01 mm using vernier callipers. Volume (cm^3) was calculated using a standard cone volume formula: $V = \pi r^2(h/3)$.

To analyse population genetic structure, we selected 11 microsatellite loci previously published for the genus *Ficus* (Moe & Weiblen, 2011; Garcia *et al.*, 2012), which were amplified in three multiplex sets (Table 2). Each PCR was composed of 4 μL of Multiplex PCR Master Mix (QIAGEN Inc. Valencia, CA, USA), 0.2 μM of each primer, 1 μL Q-solution (QIAGEN) and approximately 20–50 ng of template DNA and filled with PCR H_2O to the total volume of 10 μL. Conditions for the PCRs were as follows: 15 min of 94 °C, followed by 35 cycles of 94 °C (30 s), 54 °C (90 s) and 72 °C (60 s), with final elongation at 60 °C for 30 min. Genotypes were scored using the software Genemapper 3.7 (Applied Biosystems). We calculated genetic diversity parameters using GenAlEx v 6.5 (Peakall & Smouse, 2006), that is the number of alleles per locus and the observed and expected heterozygosities.

Stepwise analysis of population structure

We used Bayesian inference to determine both the major barriers to gene flow within each species studied (e.g. estimating the minimum *k*) and the fine-scale relationships between individuals (distance-based clustering to place genotypes across a bifurcating tree). We used methods as implemented in two different software packages and explored several commonly used criteria for defining the number of populations. We then tested for panmixia between our inferred populations using AMOVA before testing the strength and significance of gene flow between elevations and populations using a series of pairwise F_{st} comparisons. For the Bayesian analysis and AMOVA, we used the full data set, including individuals from Ohu. We only calculated pairwise F_{st} values for elevations along the gradient because N was <5 for Ohu.

Bayesian analysis of overall population structure was performed to determine (i) the number of population clusters using both STRUCTURE v2.3.4 (Pritchard *et al.*, 2000) and BAPS v5.4 (Corander *et al.*, 2004) as well as the proportion of the sampled genome of an individual that came from any populations present using STRUCTURE and ii) the fine-scale hierarchical clustering of individuals using BAPS. In STRUCTURE, we used the admixture model with the default settings and a burn-in of 10 000 and 1 000 000 replicates, we did not use sampling location as a *prior* in the analysis. We estimated *k* (the number of allelic clusters in our data set) using Evanno's Δ*K* (Evanno *et al.*, 2005), using 10 replicates for each value of *k* between 1 and 6. We also report the mean of the estimated log probability Ln(*K*) which is sometimes used to estimate the true value of *k* and referred to as Ln P(D) in STRUCTURE (Pritchard *et al.*, 2000; Evanno *et al.*, 2005); Δ*K* gives the minimum level of population genetic structure and can sometimes underestimate the number of clusters present (Waples & Gaggiotti, 2006). We used STRUCTURE Harvester (Earl & von Holdt, 2012) to compare Δ*K* and CLUMPP v1.1.2 (Jakobsson & Rosenberg, 2007) (using the 'full search' algorithm) and Distruct v1.1 (Rosenberg, 2003) to summarize and plot the output. In BAPS, we grouped individuals using the 'clustering of groups of individuals' and 'clustering of individuals' to assign elevations (but treating Ohu as its own group) and individuals to clusters with *k* set to 100, the number of clusters (*k*) was determined using maximum likelihood. The relationships between the clusters of individuals were visualized by plotting neighbour-joining trees using Nei's distance. A species-level neighbour-joining tree was estimated for the *F. hahliana* complex.

To compare genetic variation within and between the major populations, we used analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992). We performed AMOVA of pairwise Hamming distances ('diss.dist' function) using the 'poppr.amova' function in the R package 'poppr' (Kamvar *et al.*, 2014). We tested the significance of genetic structure at each level (within individuals, within populations and between populations) using the 'randtest' function in the package 'ade4' (Dray & Dufour, 2007) with 999 permutations. Individuals were grouped to populations using the clusters derived by Δ*K* as the most conservative estimate of *k*, and this gave the same conclusions as using the BAPS elevation clusters (results not shown).

Pairwise F_{st} values between elevational sites and their significance levels (10 000 permutations) were calculated using software GenAlEx v 6.5 (Peakall & Smouse,

Table 2 Genetic diversity over 10 microsatellite loci in the two *Ficus* species studied. N = number of alleles; H_o = observed heterozygosity; and H_e = expected heterozygosity. Source studies: A: Garcia *et al.* (2012); B: Moe & Weiblen (2011).

			Ficus arfakensis			*Ficus hahliana*		
Locus name	Multiplex set	Source study	N	H_o	H_e	N	H_o	H_e
Micr2(CA)	1	A	16	0.60	0.89	6	0.23	0.60
Sur1(GA)	1	A	3	0.19	0.21	3	0.13	0.12
Car10(TG)	1	A	5	0.30	0.42	3	0.58	0.53
Sur2(AG)	2	A	1	0.00	0.00	4	0.40	0.36
Car11(CA)	2	A	10	0.53	0.80	9	0.59	0.76
Micr3(CT)	2	A	1	0.00	0.00	4	0.12	0.48
P211(GA)	3	B	5	0.33	0.67	8	0.61	0.72
B83(AG)	3	B	11	0.35	0.77	8	0.83	0.67
B47(GAA)	3	B	14	0.23	0.63	7	0.37	0.46
P215(ATGT)	3	B	13	0.51	0.89	10	0.74	0.79
Mean			9.63	0.38	0.66	6.20	0.46	0.55

2006). In addition, we calculated pairwise F_{st} (and its significance) between the lowland and highland populations revealed via the Bayesian STRUCTURE analysis (as defined by ΔK). We summarized the F_{st} values within and between each population and conducted more detailed analyses of population genetic parameters between the BAPS elevational clusters to describe the finer scale differences along the gradient, given that the mid-elevation populations of each species may represent contact zones which contained a number of private alleles. We used 'poppr' (Kamvar *et al.*, 2014) to calculate the number of private alleles in each population. Differences in pairwise F_{st} values between populations of both species from different elevations were visualized using parametric smoothing as implemented in the R package 'loess', and the smoothing parameter was selected using AIC.

We recognize that using only a limited number of individuals and loci can influence estimates of genetic distance and clustering inferences. We therefore tested the power of our data to detect nonhomogenous population structure using the software 'Powsim' v4.1 (Ryman & Palm, 2006). We stress that our main aim was quite simple, to detect nonhomogeneity and assign major genetic clusters. We tested the power of our data to detect the two major populations inferred using STRUCTURE at an F_{st} threshold of 0.025 for both species. We used the default MCMC chain settings but set N_e to 2000 and t to 100 to give the desired F_{st} threshold of 0.025. We used 100 replicates in each case. Power was assessed as the proportion of significances according to both the chi-square test and Fisher's exact test. We also estimated α (the chance of a type I error) by setting the F_{st} threshold to 0 and sampling directly from the base population.

Results

In our field surveys, we identified 12 880 individuals from 73 species, around 45% of the country's 157 *Ficus* species. The dissimilarity in the *Ficus* communities increased with elevation. Furthermore, there are strong elevational patterns in the distribution of multiple *Ficus* species with unique *Ficus* species found at almost all elevations (Fig. 1). In total, we genotyped 58 individuals of *F. arfakensis* and 49 individuals of *F. hahliana* for 11 microsatellite loci. In *F. arfakensis*, three loci (Car9, Micr3 and Sur2) were either monomorphic or failed to amplify, so that eight polymorphic loci were used for the analysis. In *F. hahliana*, locus Car9 was monomorphic, but the remaining 10 loci were polymorphic and were included in the analyses (Table 2).

We used STRUCTURE to estimate the number of populations and the proportion of each individual genome sampled that came from each population. For *F. arfakensis*, we identified two population clusters using ΔK (mean L(K) = −1143.8, ΔK = 83.1) and three population clusters using L(K) (mean L(K) = −1039.0, ΔK = 6.6) (Fig. 2). For *F. hahliana*, we identified two clusters of genotypes using both ΔK (mean L(K) = −896.3, ΔK = 2730.6) and L(K). For *F. arfakensis* in particular, it was difficult to rule out the existence of more than two clusters of genotypes given the conflicting results of ΔK and L(K). Given the nested structure of our data set and nonhomogenous gene flow (mid-elevation sites represent a mixture of lowland and highland alleles, but the highlands contain a subset of these), we consider the ΔK clusters to represent the major genetic divisions. Despite relatively modest sample sizes at each site, we recovered inferred populations with sizes of between 15 and 43 individuals. We used BAPS to cluster elevations into populations based on their genotypes, and indeed, we recovered four clusters for *F. arfakensis* (cluster 1: Ohu; cluster 2: 200 m and 700 m; cluster 3: 1200 m; and cluster 4: 1700 m) and three clusters for *F. hahliana* (cluster 1: Ohu, 200 m, 700 m, 1200 m; cluster 2: 1700 m; and cluster 3: 2200 m and 2700 m). Our final level of clustering addressed individual genotypes, and we showed a clear contact zone at 1200 m for *F. arfakensis*, with some

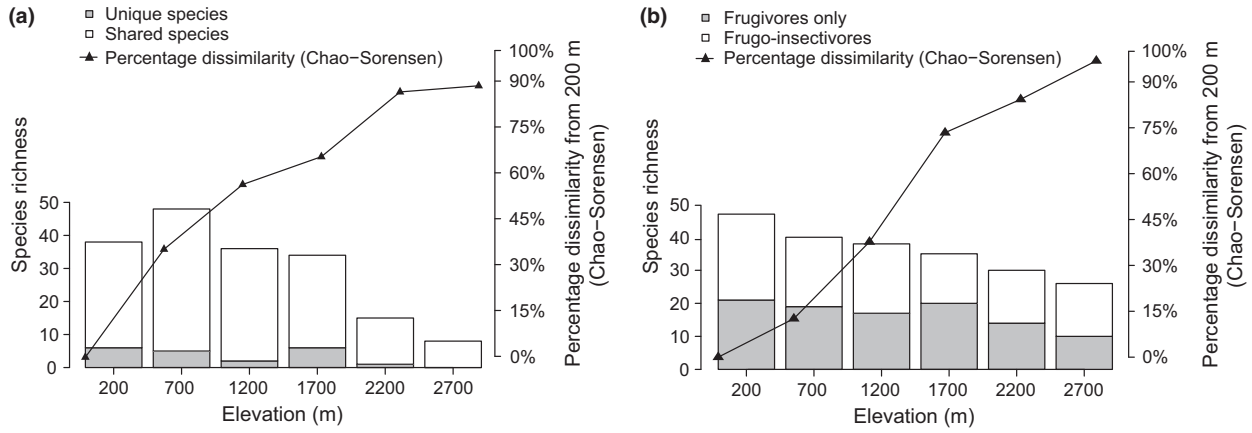

Fig. 1 The species richness of *Ficus* for each of six elevations (bars, left axis) and percentage dissimilarity in comparison with 200 m calculated using the Chao-Sorensen abundance-based distance (line, right axis). Bars are partitioned into species unique only to that elevation (grey) and species shared across more than one elevation (white) (a). The species richness of birds with at least a partially frugivorous diet for each elevation (bars, left axis) and percentage dissimilarity in comparison with 200 m calculated using the Chao-Sorensen abundance-based distance (line, right axis) (b). Bars are partitioned into bird species that are purely frugivorous (grey) and species that also eat insects (white) – data from point count surveys by Sam & Koane (2014).

individuals showing the strongest affinity to the 1700-m cluster, whereas others grouped with genotypes more common at 200 m and 700 m (Fig. 3).

We used AMOVA to test for panmixia across all individuals from each species and to test for hierarchical structure in genetic variation. We found significant between-population genetic structure in our data set and rejected the null hypothesis of panmixia for both species. This is evidenced by the fact that for both *F. arfakensis* and *F. hahliana*, the variance explained at the individual level was significantly less than the value obtained through permutation (Table 3). However, in both cases it explained a considerable amount of genetic variation (45% in *F. arfakensis* and 69% in *F. hahliana*). For both species, genetic variation between populations was much greater than within populations, suggesting that populations represent biologically meaningful groups with some limitations to gene flow between them. In *F. arfakensis*, genetic variation was significantly greater than under null expectations for both between and within populations (Table 3), but genetic variation between populations explained almost twice as much of the total variance (36%) than the variation within populations (19%). For *F. hahliana*, genetic variation between populations was large, whereas genetic variation within populations was very low (Table 3).

Pairwise F_{st} values between elevations along the gradient ranged from 0.03 to 0.36 in *F. arfakensis* and from 0.03 to 0.32 in *F. hahliana*. In *F. hahliana*, all pairwise F_{st} values between elevations were highly significant with the exception of between 200 m and 700 m and 700 m and 1200 m; for *F. arfakensis*, all pairwise F_{st} values between elevations were highly significant, with the exception of between 200 m and 700 m (Table 4). In general, the pairwise F_{st} values between elevations within lowland or highland populations (populations as defined by ΔK) were lower than F_{st} values between elevations from different populations. The mean pairwise F_{st} value within the three lowland elevations was 0.09 for *F. arfakensis*, and this value could not be calculated for the highlands which were represented by only one elevation. The mean pairwise F_{st} value was 0.04 within the three lowland elevations and 0.14 within the three highland elevations for *F. hahliana*. Whereas the F_{st} value between lowland and highland populations was 0.27 in *F. arfakensis* and 0.21 in *F. hahliana*, both between-population F_{st} values were highly significant (Table 4). This distinct reduction in gene flow at mid-elevations is visualized as a sharp increase in pairwise F_{st} values between 1200 m and 1700 m (Fig. 4). This is seen in both species studied here. It is notable that genetic diversity decreases with elevation. Although private alleles could be found for each population, they are more dominant in the lowlands, suggesting a bottleneck effect (Table 5). Our power analysis suggested that we employed a suitable number of loci and individuals to test our simple hypothesis of nonhomogeneity in both species. For *F. arfakensis*, the probability of detecting population differentiation at an F_{st} of 0.025 was 98% using the chi-square test and 97% using Fisher's exact test (α = 4% and 2%), and for *F. hahliana*, it was 93% using the chi-square test and 93% using Fisher's exact test (α = 4% and 4%). We confirmed our observation that the syconia of *F. hahliana* generally increase in size with elevation and form groups that overlap with the genetic clusters recovered, with the largest divide being between 1200 m and 1700 m (Table 1).

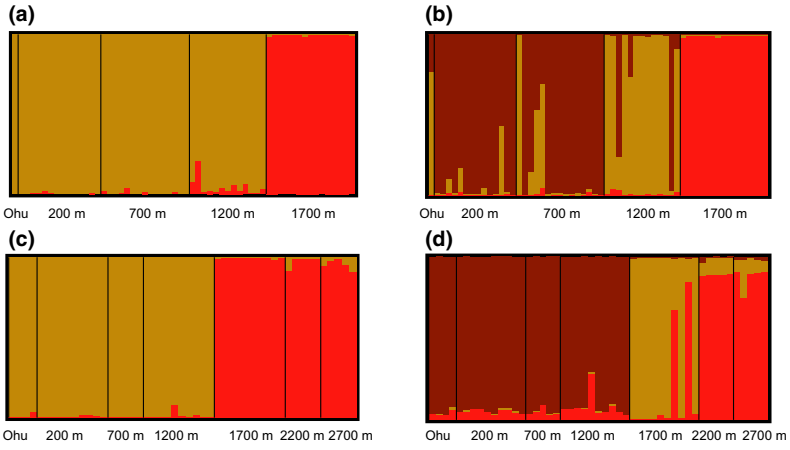

Fig. 2 The proportion of the sampled genome of each individual originating from each population as derived by ΔK using STRUCTURE: *Ficus hahliana* (k = 2) (a), *F. hahliana* (k = 3) (b), *Ficus arfakensis* (k = 2) (c) and *F. arfakensis* (k = 3) (d).

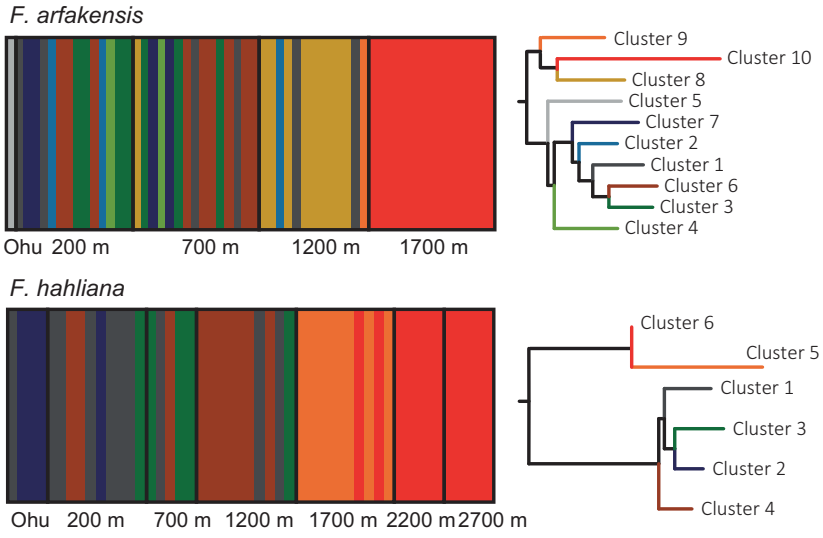

Fig. 3 Clusters resulting from the distribution of alleles amongst individuals for *Ficus arfakensis* (a) and *Ficus hahliana* (left-hand side) (b) and neighbour-joining trees estimated using Nei's distances coloured according to cluster (right-hand side).

Discussion

We demonstrate that strong barriers to gene flow exist between 1200 m and 1700 m for two species of *Ficus*. Our results show that distinct lowland and highland populations exist for *F. arfakensis* and *F. hahliana* growing along a continuously forested elevational gradient in Papua New Guinea. Indeed, most lowland (below 1200 m) individuals of *F. arfakensis* are more similar to those found 70 km away than to those from a population <4 km away but separated by 500 m in elevation. For *F. arfakensis,* at least, these populations are not likely to represent isolated genetic entities (a proportion of alleles are usually shared between peripatric populations along the entire gradient and three loci are invariable in this species). The 1200-m population contains alleles that are otherwise unique to both the lower and higher populations, often in the form of heterozygote individuals suggesting that this population is a contact zone. Genetic diversity also drops considerably at 1700 m, but allele frequency is consistent across two separate 1700-m sites, suggesting genuinely limited gene flow to this elevation has resulted in low genetic diversity. However, there are also a proportion of private lowland and highland alleles for each species and clear genetic and morphological distinctions between lowland and highland *F. hahliana*, which may represent a case of recent divergence into two species (likely to be sisters given our current sampling; Fig. S2). Indeed, we suggest that the highland populations should be referred to as *F.* cf *hahliana* form hereon until further work is conducted to clarify the taxonomic status of these distinct populations. The most obvious limitations of our work are the relatively low numbers of individuals and loci sampled. Furthermore, our sampling strategy includes only one elevational gradient. However, we detected relatively high levels of allelic diversity (see Table 1) amongst a relatively small number of

Table 3 Two-population nested analysis of molecular variance (AMOVA) based on (a) eight polymorphic loci for *Ficus arfakensis* and (b) 10 polymorphic loci for *Ficus hahliana*. *P*-value estimates are based on 999 permutations. d.f. = degrees of freedom; and MS = mean squared deviations. Populations based on ΔK from the STRUCTURE analysis.

AMOVA	d.f.	MS	Variation	% of Total variation	Phi	Direction	*P*-value
(a)							
Between populations	1	54.22	1.16	35.63	0.36	Greater	0.001
Between individuals within populations	56	2.72	0.63	19.39	0.30	Greater	0.001
Within individuals	58	1.46	1.46	44.98	0.55	Less	0.001
Total	115	2.53	3.25	100.00			
(b)							
Between populations	1	52.61	1.07	32.56	0.33	Greater	0.001
Between individuals within populations	47	2.14	−0.07	−1.99	−0.03	NS	0.754
Within individuals	49	2.27	2.27	69.44	0.31	Less	0.001
Total	97	2.73	3.27	100.00			

Table 4 Above: Pairwise F_{st} comparisons between elevational sites for *Ficus arfakensis* (left) and *Ficus hahliana* (right). Below: Pairwise F_{st} comparisons between clusters as derived by ΔK for *F. arfakensis* (left) and *F. hahliana* (right). In all cases, the diagonal is highlighted in bold text, and numbers below the diagonal give Fst values, whereas numbers above give significance.

Elevation	200	700	1200	1700	1700	200	700	1200	1700	2200	2700
200	**0.00**	0.55	<0.001	<0.001	<0.001	**0.00**	0.69	0.03	<0.001	0.001	<0.001
700	0.03	**0.00**	<0.001	<0.001	<0.001	0.03	**0.00**	0.16	0.001	0.008	0.009
1200	0.13	0.11	**0.00**	<0.001	<0.001	0.05	0.05	**0.00**	<0.001	0.001	0.001
1700	0.36	0.34	0.21	**0.00**	<0.001	0.29	0.30	0.32	**0.00**	<0.001	<0.001
2200	NA	NA	NA	NA	NA	0.24	0.24	0.23	0.12	**0.00**	0.014
2700	NA	NA	NA	NA	NA	0.21	0.20	0.18	0.18	0.11	**0.00**

Cluster	Lowlands	Highlands	Lowlands	Highlands
Lowlands	**0.00**	<0.001	**0.00**	<0.001
Highlands	0.27	**0.00**	0.21	**0.00**

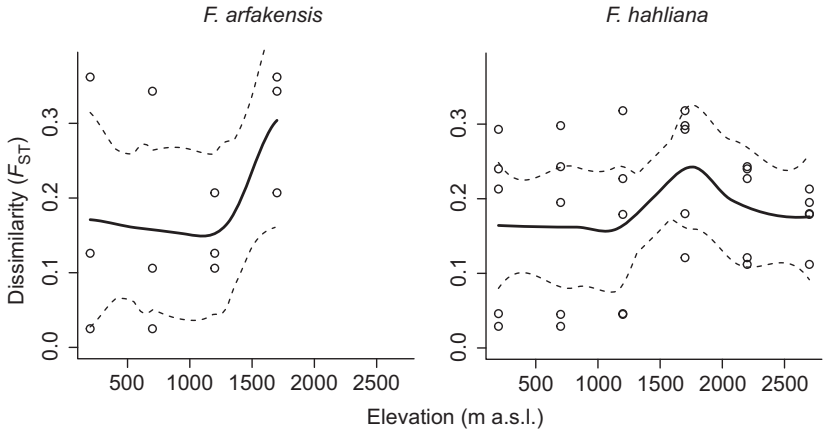

Fig. 4 Pairwise dissimilarity values between groups of individuals form all elevations for *Ficus arfakensis* and *Ficus hahliana*, based on F_{st} with curves and 95% confidence intervals fitted with loess smoothing.

individuals, which allows us to consider the results trustworthy, even with the use of a moderate number of loci (Kalinowski, 2002). This is supported by the results of our power analysis, which suggests that 8–10 polymorphic loci is enough to detect large genetic structure given the number of individuals and variability of the loci used. For more detailed analyses of fine-scale genetic structure and hybridization, we would suggest increasing the number of loci used, because a low number of loci may overestimate genetic distances

Table 5 Genetic diversity of the *Ficus* studied over the three elevational clusters as derived from the BAPS analysis (excluding Ohu). Na, mean number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; Pa, number of private alleles; %Pa, proportion of private alleles. *Ficus hahliana* (cluster 1: 200 m, 700 m, 1200 m; cluster 2: 1700 m; and cluster 3: 2200 m and 2700 m) and three clusters for *Ficus arfakensis* (cluster 1: 200 m and 700 m; cluster 2: 1200 m; and cluster 3: 1700 m).

	F. arfakensis					*F. hahliana*				
	Na	H_o	H_e	Pa	%Pa	Na	H_o	H_e	Pa	%Pa
Cluster 1	6.63	0.35	0.52	19	0.26	4.40	0.46	0.42	24	0.40
Cluster 2	6.13	0.51	0.64	15	0.19	3.10	0.43	0.40	7	0.11
Cluster 3	2.13	0.30	0.27	5	0.06	2.40	0.53	0.43	2	0.03

(Kalinowski, 2002). It would be very useful to include additional elevational gradients, but this would require a considerable amount of extra funding given the costs and practicality of working at one of the world's only fully forested elevational study sites.

Our findings suggest that there are at least two occurrences of limitations to gene flow at our study site. Furthermore, the dissimilarity of *Ficus* communities increases with elevational distance suggesting that elevation may limit the distribution of several other *Ficus* species. This is in contrast to two previous studies on the genetic structure of *Ficus* populations, which have demonstrated high levels of gene flow between sites at the same elevation (Nason *et al.*, 1996; Ahmed *et al.*, 2009). In the latter case, the dispersing wasp is congeneric with the pollinators of *F. arfakensis* (*Ceratosolen solitarius*, Weibes) and *F. hahliana* (*C. hooglandii*, Weibes). At the sites used for both previous studies, dispersing wasps face relatively constant temperatures and are likely to be aided by strong above canopy winds (Compton *et al.*, 2000; Harrison, 2003; Harrison & Rasplus, 2006). Both of these environmental conditions change with elevation. Although there is a predictable decrease in temperature, it is harder to generalize about wind strength and direction, which can vary according to aspect along tropical mountains (Beck *et al.*, 2008). To our knowledge, our study represents the first study of gene flow between *Ficus* populations along an elevational gradient, where environmental conditions change rapidly with vertical distance creating much less homogenous conditions for dispersing wasps than those found in lowland habitats. These apparent barriers to gene flow occur despite continuous forest cover, suggesting a strong abiotic limitation to biotic pollen and/or seed dispersal. It is likely that fig wasp dispersal is important in explaining the observed results. These tiny insects are particularly sensitive to changes in temperature (Jevanandam *et al.*, 2013) and may be unable to cross the 15 °C temperature gradient found between lower and upper elevations. This hypothesis is supported by the occurrence of two species of pollinator associated with *F. sur* Forssk. in West Africa that are also segregated by elevation. Whereas *F. sur* is pollinated by *Ceratosolen capensis* Grandi and *C. silvestrianus* Grandi in the lowlands, the pollinator in the highlands is *C. flabellatus* Grandi (Kerdelhué, 1997). It is possible that vertebrate seed dispersers also have limited ranges, with many endemic birds and mammals having restricted elevational ranges (Winter, 1997). Indeed, we see a strong turnover in bird community structure around 1200–1700 m along our gradient (Fig. 1), with distinct highland and lowland communities potentially limiting the vertical distance that seeds can be dispersed (Sam & Koane, 2014; Marki *et al.*, 2016). However, some degree of limited wasp dispersal is required in both scenarios because long-distance pollen dispersal can mask even highly limited seed dispersal.

Local adaptation in *Ficus* itself may also play a role in reducing gene flow, especially if this is linked to changes in fruit morphology that prevent maladaptation through the exchange of genetic material from higher or lower elevations. Indeed, both *F. arfakensis* and *F. hahliana* exhibit a degree of morphological variation along the gradient with respect to fig size, figs being larger at higher elevations (Table 1). Observations from other *Ficus* species demonstrate even more extreme morphological variation with elevation than species examined in this study. For example, *F. dammaropsis* Diels has cricket ball/baseball-sized fruits in the lowlands which are covered with open bracts; in contrast, highland populations have substantially larger fruits which are generally smoother and have the bracts closed. There are also well-documented instances of highland and lowland varieties or subspecies of *Ficus*; for example, *F. trichocerasa* subsp. *trichocerasa* Diels is found mainly up to elevations of 1400 m but grades slowly into subsp. *pleioclada* (Diels) C.C. Berg in higher elevations up to 2600 m (Berg, 2004). Furthermore, *F. wassa* Roxb. has a similarly large range (up to 3000 m) and is found as var. *nubigena* Diels in the highlands (1300–3000 m) (Berg & Corner, 2005). The main form grows as a tree up to 15 m and has red figs at maturity, and the highland variety has a scadent, scrambling habit, growing up to 3 m and bearing greyish white figs while ripening. Despite these apparently important ecological differences, neither variety can be separated on the mostly vegetative characters listed in Berg & Corner (2005). The extent to which this

variation is genetic or environmental is yet to be established in these species. Indeed, members of the genus *Ficus* can display high levels of phenotypic plasticity (Harrison, 2005). We suggest that additional detailed morphological studies are required across several of the species found at this site to assess the true degree of variation observed and that these should be conducted in conjunction with more detailed and wide-scale population genetic studies of both figs and their pollinating wasps.

It has long been recognized that species turnover (or beta diversity) along elevational gradients is usually high, whereas community level nestedness is low. However, peripatric species are often close relatives, suggesting that speciation is facilitated by local adaptation and decreased gene flow. Although our study addresses gene flow in a specialized pollination mutualism, we suggest that it may have wider implications for less specialized systems because any level of specialization in pollination or seed dispersal may lead to potential isolation. Furthermore, although insect herbivores (Craft *et al.*, 2010) and pollinators (Nason *et al.*, 1996; Ahmed *et al.*, 2009) of *Ficus* show low levels of population structure in lowland habitats, we have little understanding of how pollinator and insect herbivore populations are structured along elevational gradients, but turnover of species within genera appears likely for herbivores (Novotny *et al.*, 2005). *Ficus* species represent one of the key genera in forest communities, supporting extremely species-rich communities of herbivorous insects from several guilds (Novotny *et al.*, 2005). Being one of the most important plant genera for tropical frugivores, *Ficus* also provides an important food source for a broad variety of vertebrates with some of them being dependent on fig consumption (Shanahan *et al.*, 2001). Our data on the composition of *Ficus* communities suggest that multiple *Ficus* species have limited elevational ranges. Elevational barriers to gene flow may therefore be present in additional *Ficus* species not studied here. Divergence in *Ficus* populations and associated variation in their traits, fruit morphology and phenology are thus likely to have pronounced effects on numerous associated organisms. It would certainly be valuable to conduct further studies along tropical elevational gradients to investigate the population genetic structure of additional plant species and its correlation with associated communities of other organisms. We suggest that such an approach would be a useful step in understanding the processes of speciation in some of the world's most biodiverse hotspots.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Figure S1 A map of our sampling sites, contour lines are given every 100 m.
Figure S2 A neighbour-joining tree constructed using Nei's distances derived from a 'clustering of groups of individuals' analysis as implemented in BAPS (Corander *et al.*, 2004).
Appendix S1 Plate S1. *Ficus iodotricha* (2200 m a.sl., above) and *Ficus hahliana* (2700 m a.s.l., below).
Plate S2. *Ficus hahliana* 700 m a.s.l. above, 2200 m a.s.l. below.

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