Genetic and clonal diversity of the endemic ant-plant *Humboldtia brunonis* (Fabaceae) in the Western Ghats of India

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Humboldtia brunonis (Fabaceae, Caesalpinioideae) is a dominant self-incompatible ant-plant or myrmecophyte, growing as an understorey tree in high-density patches. It is endemic to the biodiversity hotspot of the southern Western Ghats of India and, besides ants, harbours many endemic invertebrate taxa, such as bees that pollinate it as well as arboreal earthworms, within swollen hollow stem internodes called domatia. Using inter simple sequence repeat (ISSR) markers, three geographically separated populations were found to be multiclonal, characterized by high levels of clonal diversity. Values for the Simpson diversity index ranged between 0.764 and 0.964, and for Fager's evenness index between 0.00 and 0.036 for neighbourhoods within populations. This myrmecophyte was found to combine sexual recruitment (66.7%) and clonal production (33.3%) as methods of reproduction. Moderate amounts of genetic diversity at the species level were observed, with 52.63% polymorphism, and moderate values of Shannon's diversity index (0.1895) as well as of Nei's gene diversity (0.1186). In each population, observed genotypic diversity was significantly lower than expected, indicating significant genetic structure. Neighbour-joining trees demonstrated that Agumbe, which is the most northern population examined and geographically twice as far away from the other two populations, grouped separately and with larger bootstrap support from a larger cluster consisting of the Sampaji and Solaikolli populations, which are closer to each other geographically. Some neighbourhoods within each population showed spatial genetic structure even at small spatial scales of <5 m. A combination of clonality and short-distance pollen movement by small pollinating bees (*Braunsapis puangensis*) coupled with primary ballistic seed dispersal, and possible secondary seed dispersal by rodents, may contribute to spatial genetic structure at such small scales. The clonality of *H. brunonis* may be a factor that contributes to its dominance in Western Ghat forests where it supports a rich diversity of invertebrate fauna.

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1. Introduction

Many plant species are capable of reproducing both sexually and asexually. Sexual reproduction could lead to the production of novel genotypes while clonal regeneration (through root sprouts in woody plants) produces offspring which are genetically identical to each other and to the maternal parent (Ellstrand and Roose 1987). Thus, the demographic balance between sexually produced seedlings and clonal recruitment is likely to have a marked effect on the genetic structure of populations (Chung and Epperson 1999). In self-incompatible species with limited seed and pollen dispersal, clusters of genetically related or identical plants will limit the number of locally available mates (Eckert and Barrett 1993) and thus influence mating opportunities (Charpentier 2002). In such obligate or preferentially outcrossing species that also exhibit clonality, successful pollination resulting in the production of new genotypes will become less frequent (Eckert 2002; Frankham *et al.* 2002) and could also lead to biparental inbreeding, which is mating between closely related individuals, e.g. mating between closely related genets in a clonal species (Zhao *et al.* 2009).

Keywords. Caesalpinioideae; clonality; Detarieae; myrmecophyte; inter simple sequence repeats; spatial genetic structure

Abbreviations used: AFLP, amplified fragment length polymorphism; BS, bootstrap support; ISSR, inter simple sequence repeat; PPL, percentage of polymorphic loci; RAPD, random amplified polymorphic DNA; SGS, spatial genetic structure; TBE, Tris-boric acid-EDTA

This may account for reductions in seed set and progeny fitness when neighbouring plants are intercrossed, and may restrict available genetic variability (Levin 1984) that will also, in addition, be affected by clone size (Chung *et al.* 2004). Since the long-term survival and future evolution of any plant species depends on existing genetic variation, any factor contributing to the loss of genetic variation may have deleterious effects on species fitness and may threaten the survival of populations (Reed and Frankham 2003; Reisch *et al.* 2003).

Humboldtia brunonis Wallich is an ant-plant or myrmecophyte endemic to the wet evergreen forests of the biodiversity hotspot of the southern Western Ghats of India (Pascal 1988). It is a dominant understorey tree species which, in association with two dominant canopy trees, serves to characterize two Western Ghat forest types. In one type, the co-dominants are *Dipterocarpus indicus* and *Poeciloneuron indicum*, while in the other type the co-dominants are *Dipterocarpus indicus* and *Kingiodendron pinnatum* (Ramesh and Pascal 1997). *Humboldtia brunonis* has low natural fruit set (2.3%; Shenoy and Borges 2008) which is usually indicative of low pollination efficiency and/or resource limitation (Stephenson 1981; Bawa and Webb 1984; Larson and Barrett 2000; Wilcock and Neiland 2002; Ashman *et al.* 2004; Wesselingh 2007). The precise reasons for the low fruit set in this species are not known, although many tropical trees, including those in the Fabaceae, are recorded to set few fruit (e.g. 1% fruit set in *Caesalpinia eriostachys*; Bawa and Webb 1984). However, hand cross-pollination experiments carried out on *H. brunonis*, in which excess pollen was deposited on stigmas, also resulted in poor fruit set (5.8% for cross-pollinations performed in the morning and 6.8% for cross-pollinations performed in the evening; Shenoy and Borges 2008). *Humboldtia brunonis* is pollinated by small allodapine bees and has ballistic seed dispersal in which seeds scatter around the parent plant (Shenoy and Borges 2008), suggesting gene flow over short distances. Furthermore, visible root connections between trees observed in the study areas indicated the possibility of clonal reproduction through root sprouts which, if severed, could grow as independent individuals. Therefore, genetic relatedness of individuals due to restricted pollen and seed dispersal as well as clonality was hypothesized to be one possible reason for the low fruit set in the hand-pollination experiments, since pollen was often collected from neighbouring trees in these experiments (Shenoy 2008). We hypothesized that natural populations of the dominant understorey tree *H. brunonis*, which occurs in high-density populations in Western Ghat forests, may consist of a limited number of genets with a variable number of ramets, resulting in localized spatial genetic structure in this species. However, no information is available regarding the clonal and genetic diversity status of natural populations of this species. Characterizing the genetic diversity of this species is therefore important for the conservation of this endemic ant-plant and, thereby, the continued existence of the wide array of invertebrate fauna inhabiting its stem domatia (Rickson *et al.* 2003; Gaume *et al*. 2005a, b, 2006; Shenoy and Borges 2008; *see* Materials and methods section for more details).

Numerous molecular techniques have been used to investigate clonal and genetic diversity in plants (Widen *et al.* 1994). Although per locus information is lower when compared to co-dominant DNA markers (e.g. isozymes, microsatellites), dominant DNA markers such as random amplified polymorphic DNAs (RAPDs), inter simple sequence repeats (ISSRs) and amplified fragment length polymorphisms (AFLPs) have been successfully employed to reveal clonal structure and genetic diversity in plant populations (Bachmann 1994; Deshpande *et al.* 2001; Ci *et al.* 2008; Li and Dong 2009). All these dominant markers require no prior sequence information. In addition, the sequences that markers such as ISSRs target are abundant throughout eukaryotic genomes, and they evolve rapidly. Consequently, ISSRs may be used to reveal a large number of polymorphic fragments at multiple loci simultaneously (Wolfe and Liston 1998). Using ISSR markers in the present study, we addressed the following questions:

1. What is the clonal genetic structure of *H. brunonis* populations?
2. What is the genetic diversity at the population and species level in *H. brunonis*?
3. Could fine-scale spatial genetic structure play a significant role in the reduced seed set observed in this self-incompatible species?

2. Materials and methods

2.1 *Natural history of the plant*

Humboldtia Vahl (Fabaceae, Caesalpinioideae) is a small legume tree genus with only six species, all confined to the biodiversity hotspot of the southern Western Ghats of India except *H. laurifolia*, which is also found in Sri Lanka (Sanjappa 1986). Three of the species in this genus are myrmecophytes: *H. brunonis*, *H. decurrens* and *H. laurifolia*. *Humboldtia brunonis* is unique among known ant-plants in exhibiting polymorphism in a myrmecophytic traits wherein trees with or without caulinary domatia (swollen hollow stem internodes which house a large diversity of invertebrates including numerous ants and arboreal earthworms) coexist in the same population (Gaume *et al.* 2006; Shenoy 2008); it is hence referred to as a semi-myrmecophyte (Gaume *et al.* 2005a, b). Flowers are bisexual and occur in a racemose inflorescence that blooms in an acropetal fashion. Flowering lasts for a long period (December–April). The major pollinator of this self-incompatible species is the small

allodapine bee *Braunsapis puangensis*, which is resident within the plant's domatia (Shenoy and Borges 2008). The heavy non-fleshy seeds undergo ballistic dispersal and possible secondary dispersal by rodents.

2.2 *Plant sampling*

Samples of *H. brunonis* leaves were collected from three sites that span the range of the species from north to south across the Western Ghats, viz. Agumbe Reserve Forest (altitude 633 m, 13°31'N, 75°5'E), Sampaji Reserve Forest (altitude 665 m, 12°29'N, 75°35'E) and Solaikolli in Brahmagiri Wildlife Sanctuary (altitude 651 m, 12°4'N, 75°49'E) (figure 1). Agumbe is separated from Sampaji by about 128 km while Sampaji and Solaikolli are separated by about 50 km. A one hectare plot was established in each of the three sites. To investigate clonal structure and the possibility of biparental inbreeding (mating between closely related genets) at close distances, three patches of *H. brunonis* trees were selected randomly within each plot. Each patch comprised one focal *H. brunonis* tree and ten surrounding trees separated by a minimum distance of one metre in most cases. In some of these patches, root connections were visible between trees. DNA samples extracted from these physically linked trees were treated as positive controls to demonstrate the efficacy of the ISSR method in identifying known ramets of a particular genet. Fresh leaves were therefore harvested from a total of 33 plants (three randomly selected focal trees in a one hectare area and 10 nearest neighbours for each focal tree) in each of the three populations, and were used for analysis of clonal and genetic diversity at the population level. Each set of 11 samples thus generated constituted a neighbourhood, and three neighbourhoods were examined for each population. Data from all 99 samples were pooled to estimate genetic diversity at the species level. Leaf materials were stored in zip-lock plastic bags containing silica gel for fast drying. The dried leaf samples were kept at room temperature until DNA was extracted.

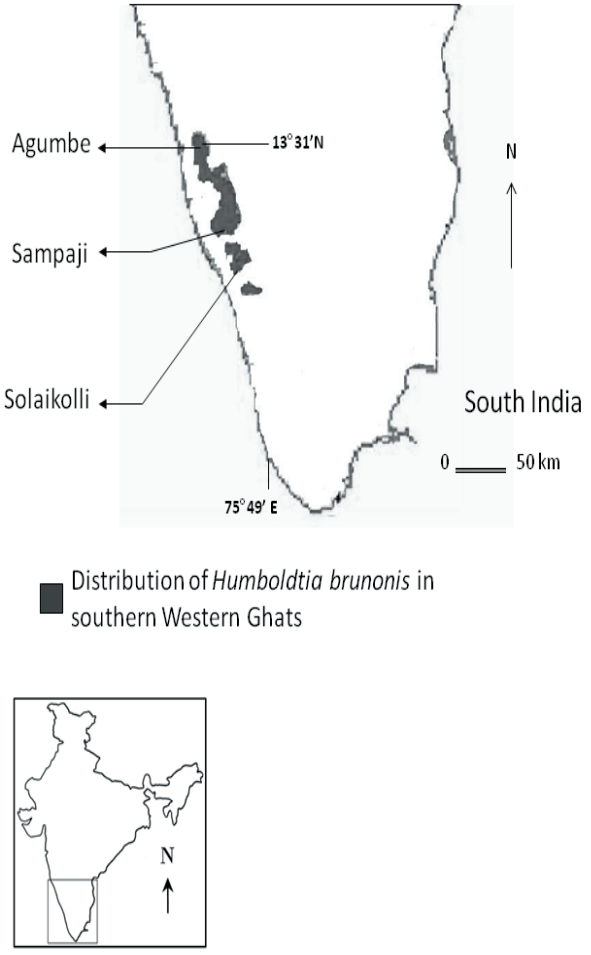

Figure 1. Locations of the three studied natural populations of *H. brunonis* in the Indian Western Ghats.

2.3 *Total DNA extraction*

Total genomic DNA was extracted from 75 mg of silica-dried leaves using the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., *www.qiagen.com*). Leaf specimens were powdered in liquid nitrogen with a mortar and pestle, and then treated for the isolation of DNA according to the manufacturer's protocol. DNA was quantified by comparison with a known concentration of lambda DNA (Genei, Bangalore) following electrophoresis on 1.5% agarose gels.

2.4 *ISSR PCR amplification*

PCR reactions were carried out in a volume of 20 μl containing 2 μl of 0.2 mM of each dNTP (New England Biolabs, UK), 2.5 μl of 10x Taq buffer, 2 μl of 1.5 mM $MgCl_2$, 3 picomoles of ISSR primer, 5 U of Taq DNA polymerase (Genei, Bangalore) and 50 ng of DNA template. Amplification of genomic DNA was done on a gradient PCR machine (Eppendorf, Hamburg) and commenced with initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, annealing for 1 min 30 s and extension at 72°C for 1 min, with a final extension at 72°C for 4 min. Twelve ISSR primers from Biotechnology Laboratory, University of British Columbia (UBC set no. 9), were initially screened for amplification, annealing temperatures were standardized, and ten primers that produced clear and reproducible fragments were chosen for the final analysis (table 1).

Table 1. List of inter simple sequence repeat (ISSR) primers used in this study*

ISSR primers	Sequences	Annealing temperatures (^{0}C)
814	5′ (CT)8TG 3′	50
830	5′ (TG)8G 3′	53.1
834	5′ (AG)8(CT)T 3′	49
840	5′ (YA)8(AG)C 3′	Poor resolution
841	5′ (GA)8(CT)C 3′	50.1
880	5′ G(GA)2GG(AG)2(GA)2 3′	51.1
844A	5′ (CT)8AC3′	49.3
844B	5′ (CT)8GC3′	50.5
17898A	5′ (CA)6AC3′	45.3
17898B	5′ (CA)6GT3′	39.1
17899A	5′ (CA)6AG3′	39.1
Issr4	5′ (ACT)(AGC)(ACT)(CA)7 3′	Poor resolution

*Biotechnology Laboratory, University of British Columbia (UBC Set number 9)

Amplification products were resolved electrophoretically on 2% (w/v) agarose gels run at 60 v for 2 h with a 100 bp DNA ladder (Genei, Bangalore) in 1x Tris-boric acid-EDTA (TBE), visualized by staining with ethidium bromide and photographed under ultraviolet light by a gel documentation and analysis system (Alpha Innotech Corporation Gel-Doc System, USA).

2.5 *Data analyses*

Since ISSR markers are dominant, each band represents the phenotype at a single biallelic locus. Amplified fragments for the ten ISSR primers were scored as present (1) or absent (0) for each sample (figure 2). The binary data matrix generated using ten ISSR primers was analysed at three levels (i.e. neighbourhood, population and species). Genetic diversity was analysed at the population and species level. Parameters used for measuring genetic diversity were the percentage of polymorphic loci (*PPL*), Nei's gene diversity (*h*) (Nei 1978) and Shannon's genetic diversity index (*I*) (Lewontin 1972) using POPGENE 1.31 (Yeh *et al.* 1997). Nei's gene diversity was calculated from the frequency of recessive phenotypes under an assumption of Hardy–Weinberg equilibrium (Nei 1978) which is warranted in this case since *H. brunonis* is self-incompatible (Shenoy and Borges 2008). Observed genotypic diversity (G_o) was calculated using the software GenoDiv.py (Jesse *et al.* 2009). Bayesian approaches were used to obtain accurate estimates of allele frequencies for dominant markers using the software AFLP-SURV's Method 4 (Zhivotovsky 1999; Vekemans 2002). Critical values of expected genotypic diversity (G_e) were calculated and a one-tailed test for the null hypothesis of no clonal structure was conducted using GenoDivDist.py (Jesse *et al.* 2009). To further investigate genetic relationships, the binary ISSR data were analysed using the neighbour-joining distance method (Saitou and Nei 1987) with 1000 bootstrap replicates in PHYLIP 3.5c (Phylogeny Inference Package: Felsenstein 1993).

Within each population, samples were considered separately at the neighbourhood level and together at the population level for four clonal diversity parameters (Ellstrand and Roose 1987): (1) number of genotypes (*G*), (2) clonal diversity (*G/N*), where *G* is the number of genets and *N* is the total number of individuals (ramets) sampled. This index will range from 0 when the clonal diversity is maximum (one clone representing the entire population) to 1 for maximal genotypic diversity, when every sampled unit is a new genet (Pleasants and Wendel 1989; Chung and Epperson 1999). *G*/*N* is therefore inversely proportional to clone size and can also be used as a measure of sexual recruitment; i.e. 1–(*G*/*N*) will indicate the amount of clonal recruitment in a population. (3) Simpson's diversity index corrected for finite sample size (Pielou 1969), $D = 1 - [\sum n_i (n_i - 1)/N(N-1)]$, where n_i is the number of samples of the i^{th} genotype; the value of *D* ranges from 0 to 1; the greater the value, the greater the diversity, and (4) genotypic evenness index (Fager 1972), $E = (D - D_{min})/(D_{max} - D_{min})$, where $D_{min} = (G-1)(2N-G)/N(N-1)$ and $D_{max} = (G-1)N/G(N-1)$. *E* can range from 0 for a population dominated by a single genotype to 1 for a population in which all genotypes are represented by the same number of ramets.

Spatial autocorrelation analysis was performed on the basis of genetic similarity/dissimilarity in the ISSR banding patterns using the SGS software (Degen 2000; Degen *et al.* 2001). The effects of possible clonal structure was evaluated by Tanimoto's genetic distances (D_G) (Degen 2000) on 11 samples each of three neighbourhood sets in all three populations versus restricted datasets constructed with a single ramet per genet to exclude clones in each

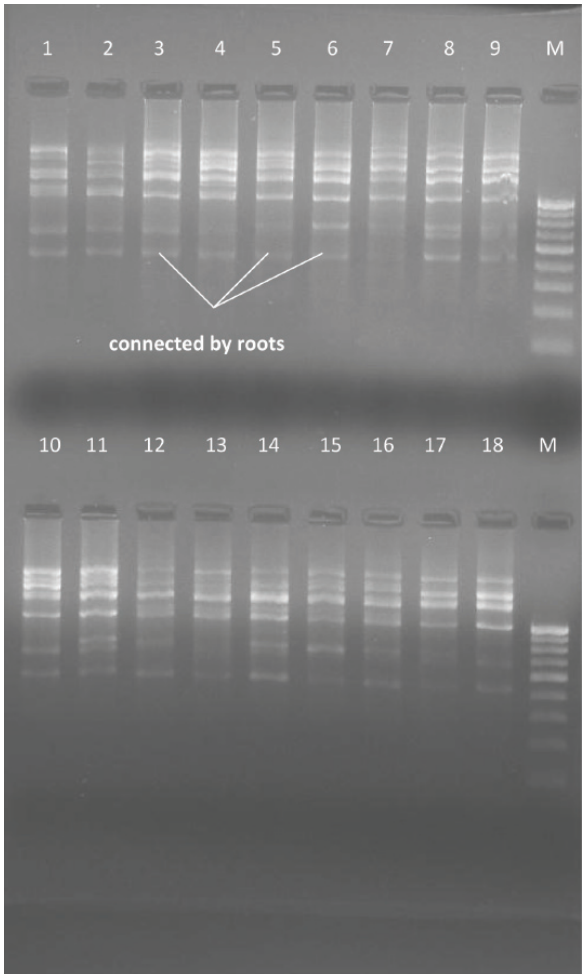

Figure 2. Representative inter simple sequence repeat (ISSR) profiles (samples from Solaikolli with primer 17898 B) showing profiles of plants with root connections, i.e. positive controls

Lane 1–11: samples of Solaikolli neighbourhood 1 (1–11 samples); Lane 12–18: samples of Solaikolli neighbourhood 2 (1–7 samples); M = 100bp ladder.

neighbourhood (Chung *et al.* 2004). To assess statistical significance, 95% confidence intervals were generated from Monte Carlo permutations (1000 replications) (Degen *et al.* 2001). Spatial autocorrelation was visually examined using autocorrelograms (distograms) that plotted D_G values (observed and expected) and the 95% confidence intervals as a function of geographical distance. We also examined the relationship between physical and genetic distances within neighbourhoods of all three populations (for sample sets including and excluding clones as used for the SGS software analyses) using Mantel tests by employing the software package zt (Bonnet and Peer 2002). Significant autocorrelation after excluding clones would indicate non-clonal contribution to genetic structure, e.g. resulting from biparental inbreeding.

3. Results

3.1 *Genetic and clonal diversity*

A total of 76 reproducible bands were found across the 99 individuals at the species level using 10 ISSR primers. Of the 76 loci thus surveyed, 40 were polymorphic (52.63%) at the species level. At the population level, the total numbers of loci resolved were 74, 72 and 74 with corresponding levels of polymorphism of 25%, 10% and 25% for the Agumbe, Sampaji and Solaikolli populations, respectively. The number of loci resolved ranged from 68 to 73 for the nine neighbourhoods drawn from the three different populations, and their sampled neighbourhoods yielded 4–17% polymorphism (table 2).

Within populations, ISSR genetic diversity was considerably lower than at the species level. Even lower values were obtained for all the diversity parameters at the level of neighbourhoods within the different populations (table 2), thus indicating genetic differentiation at population and neighbourhood levels. Nei's gene diversity value (*h*) ranged from 0.0214 to 0.1005 at the neighbourhood level, and from 0.049 to 0.120 at the population level. At the species level, the gene diversity index (*h*) was 0.119. Shannon's diversity index (*I*) ranged from 0.0318 to 0.1443 for the neighbourhoods within populations and from 0.073 to 0.159 at the population level. The highest value for Shannon's diversity index (*I*) was at the species level (0.1895). Among the three populations, Sampaji had low values for all the diversity parameters both at the neighbourhood and population levels; those for Agumbe and Solaikolli were approximately at the same level. Observed genotypic diversities (G_o) were lower than the critical value of expected heterozygosites (G_e) estimated through Bayesian simulation in all the three populations (table 3), indicating significant genetic structure given the available marker variability.

The clonal diversity and evenness of distribution of genotypes at neighbourhood and population levels are summarized in table 4. Within neighbourhoods, the number of genotypes (*G*) varied from 5 to 9 and clonal diversity (*G/N*) ranged from 0.45 to 0.82. The genotypes of the sampled trees with visible root connections were clearly identified as clones. The values for Simpson's diversity index (*D*) ranged from 0.764 to 0.964 and of Fager's evenness index (*E*) from 0.00 to 0.036. The third neighbourhood from Agumbe had the lowest number of genotypes (*G*), clonal diversity (*G/N*) and Simpson's diversity index (*D*). At the population level, out of the 33 individuals so analysed in each population, the highest number of genotypes (*G*) was in Solaikolli (24), followed by Sampaji (23) and Agumbe (19). *G/N* varied from 0.58 in Agumbe to 0.70 in Sampaji and 0.73 in Solaikolli. At the species level, the average *G/N* value was 0.667. Since *G/N* is inversely proportional to the clone size,

Table 2. Genetic diversity parameters at neighbourhood, population and species levels in *H. brunonis*

		Total loci	Polymorphic loci (PL)	Per cent polymorphic loci (PPL)	Nei's gene diversity index (h)	Shannon's diversity index (I)
Agumbe: Neighbourhood	1	73	14	19.2	0.0702	0.1053
	2	73	17	23.3	0.1005	0.1443
	3	72	9	12.5	0.0303	0.0500
Solaikolli: Neighbourhood	1	72	13	18.1	0.0670	0.0999
	2	72	10	13.9	0.0542	0.0795
	3	73	11	15.1	0.0480	0.0739
Sampaji: Neighbourhood	1	71	8	11.3	0.0419	0.0625
	2	70	6	8.6	0.0293	0.0445
	3	68	4	5.9	0.0214	0.0318
Agumbe population		74	25	33.78	0.1202	0.1590
Solaikolli population		74	25	33.78	0.0850	0.1364
Sampaji population		72	10	13.89	0.0487	0.0728
Species		76	40	52.63	0.1186	0.1895

Table 3. Observed (G_o) and critical expected genotypic diversity (G_e) at population levels

Population	Total samples (N)	Observed genotypic diversity (G_o)	Critical expected genotypic diversity (G_e)	Probability (P)
Agumbe	33	14.918	33.00	<0.05
Solaikolli	33	20.547	33.00	<0.05
Sampaji	33	18.458	27.923	<0.05

Table 4. Clonal diversity parameters of nine neighbourhoods and three populations of *H. brunonis*

		Total samples (N)	Number of genotypes (G)	Clonal diversity (G/N)	Simpson's diversity index (D)	Fager's evenness index (E)
Agumbe: Neighbourhood	1	11	7	0.64	0.909	0.010
	2	11	7	0.64	0.909	0.010
	3	11	5	0.45	0.764	0.036
Solaikolli: Neighbourhood	1	11	8	0.73	0.927	0.002
	2	11	8	0.73	0.891	0.000
	3	11	9	0.82	0.964	0.000
Sampaji: Neighbourhood	1	11	9	0.82	0.964	0.000
	2	11	7	0.64	0.909	0.010
	3	11	8	0.73	0.946	0.004
Agumbe population		33	19	0.58	0.960	0.926
Solaikolli population		33	24	0.73	0.890	0.890
Sampaji population		33	23	0.70	0.957	0.805
Species		99	66	0.667	0.936	0.874

it can be considered as an estimate of sexual recruitment. Accordingly, the average amount of clonality (clonal recruitment) was found to be 0.333 (1–0.667). The clonal diversity index (D) (0.96–0.98) and evenness index (E) (0.88–0.92) were almost similar in the three populations.

3.2 *Large- and fine-scale spatial genetic structure*

The neighbour-joining tree revealed three major clusters, each of which consisted of individuals of only one population (figure 3). There was no cross-grouping of individuals

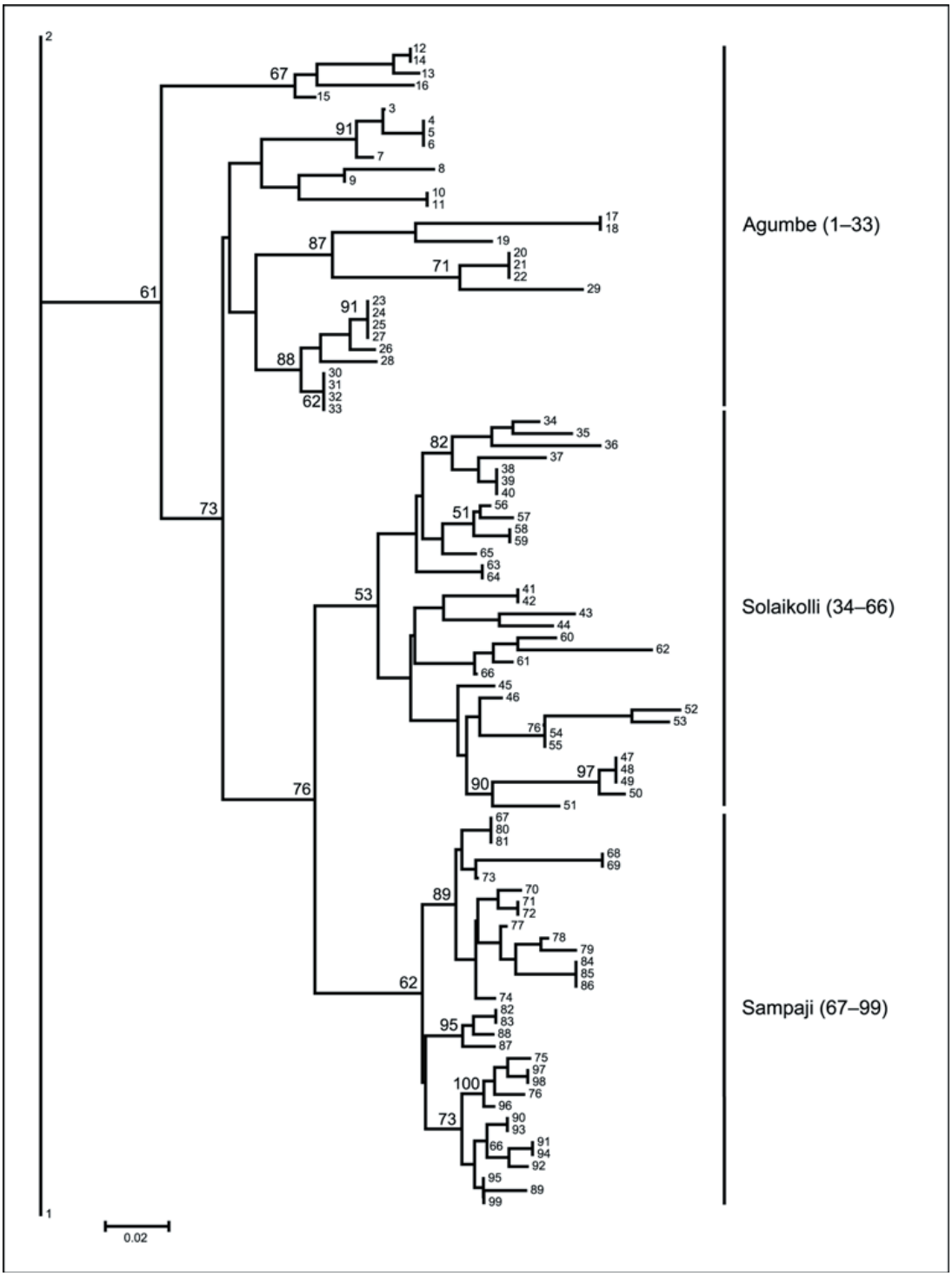

Figure 3. Neighbour-joining tree showing genetic grouping among the 99 sampled individuals of *H. brunonis*. Values at nodes are bootstrap values (>50) after 1000 resampling events. The scale bar indicates two changes per 100 positions.

among the three populations in the tree generated from the 99 individuals. The Agumbe population was reasonably distinct (bootstrap support 76%) but the Solaikolli and Sampaji populations were not very well supported (bootstrap percentages of 53% and 62%, respectively). Thus, at least one sampled population was more genetically distinct

Table 5. Pair-wise physical distances among clonal and non-clonal genotypes (m)

		N (pairs)	Mean	SD	Min	Max
Agumbe: Neighbourhood 1	Clones	5	1.88	0.88	1.10	3.25
2		5	1.43	0.66	0.80	2.15
3		12	2.14	2.03	0.29	6.20
Agumbe: Neighbourhood 1	Non-clones	50	2.62	1.20	0.70	4.85
2		50	3.25	1.33	0.20	6.50
3		43	2.54	1.41	0.40	6.80
Agumbe: Median (clones): 1.36; (non-clones): 2.75; W* = 915.5, $P<0.01$						
Solaikolli: Neighbourhood 1	Clones	4	1.35	0.91	0.20	2.30
2		7	1.64	0.75	0.80	3.05
3		2	1.18	0.64	0.73	1.63
Solaikolli: Neighbourhood 1	Non-clones	51	2.55	1.22	0.20	5.20
2		48	2.70	1.33	0.27	5.30
3		53	2.83	1.25	0.08	5.80
Solaikolli: Median (clones): 1.50; (non-clones): 2.72; W = 425.0, $P<0.001$						
Sampaji: Neighbourhood 1	Clones	2	1.78	0.60	1.35	2.20
2		5	1.40	0.69	0.90	2.50
3		4	2.29	0.89	1.35	3.10
Sampaji: Neighbourhood 1	Non-clones	53	3.92	1.77	0.57	8.75
2		50	2.99	1.37	0.40	5.48
3		51	2.13	0.85	0.50	3.98
Sampaji: Median (clones): 1.65; (non-clones): 2.85; W = 420.5, $P<0.01$						
Species Median (clones): 1.46; (non-clones): 2.75; W = 5327.5, $P<0.0001$						

* Mann–Whitney *U* test statistic obtained using the software package STATISTICA

than the other two populations examined, indicating its geographical isolation. There was also evidence for genetic grouping at the local scale (i.e. at the neighbourhood) since individuals within neighbourhoods were grouped together in the dendrogram with high bootstrap support (BS), e.g. individuals of neighbourhoods 1 and 2 in Agumbe (sample numbers 3–7 [91% BS] and 23–26 [91% BS], respectively; figure 3), neighbourhoods 1 and 2 in Solaikolli (sample numbers 34–40 [82% BS] and 47–51 [90% BS], respectively; figure 3), and neighbourhood 2 in Sampaji (sample numbers 82–87 [95% BS]; figure 3). These results are indicative of local gene flow. The maximum distance between any two individuals in a neighbourhood was 5.5 m in Agumbe, 8.75 m in Sampaji and 5.05 m in Solaikolli (table 5). The pair-wise physical distance between members of clones was significantly smaller than that between non-clonal individuals in all populations (table 5).

Spatial genetic structure analysis using the SGS software revealed significant fine-scale genetic structuring in the Agumbe and Solaikolli populations. Significant positive spatial autocorrelation was observed in neighbourhoods 2 (<2 m) in Agumbe and in neighbourhoods 2 and 3 (<3 m) in Solaikolli (figure 4; only distograms with significant results shown). Using the SGS software, no significant spatial autocorrelation was observed when clones were excluded in any of the populations (results not shown). Using Mantel tests (two-tailed) and datasets inclusive of clones, positive autocorrelations were also obtained for neighbourhood 2 in Agumbe ($r = 0.53$, $P = 0.0004$), neighbourhood 2 in Sampaji ($r = 0.33$, $P = 0.006$) and neighbourhood 2 in Solaikolli ($r = 0.62$, $P = 0.002$). Analysis excluding clones and using Mantel tests retained significant correlations in these same neighbourhoods (Agumbe: $r = 0.63$, $P = 0.002$; Sampaji: $r = 0.47$, $P = 0.004$; Solaikolli: $r = 0.77$, $P = 0.002$). Significant autocorrelation was observed in some neighbourhoods only after clones were excluded (Agumbe neighbourhood 1 [$r = 0.38$, $P = 0.002$], and Solaikolli neighbourhood 1 [$r = 0.450$, $P = 0.004$]). The SGS software and Mantel tests gave similar results in some cases (neighbourhoods 2 in Agumbe and Solaikolli using data inclusive of clones). These neighbourhoods were also the ones in which significant spatial autocorrelation was found without clones using the Mantel tests.

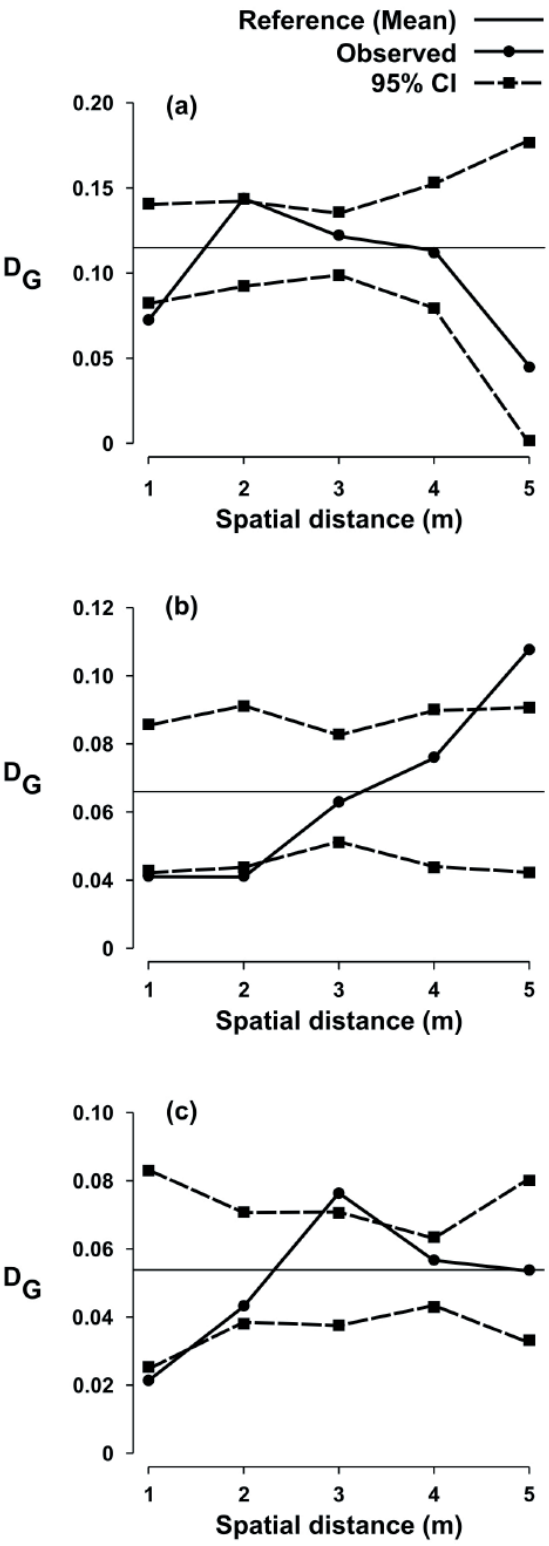

Figure 4. Inter simple sequence repeat (ISSR) distograms (including clones) generated using the SGS software for **(a)** Agumbe neighbourhood 2, **(b)** Solaikolli neighbourhood 2, and **(c)** Solaikolli neighbourhood 3 (CI indicates 95% confidence limits).

4. Discussion

All three natural populations of *H. brunonis* were found to be multiclonal, made up of different numbers of genets. The limited number of genotypes observed in all the populations (table 4) provides an indication of extensive asexual reproduction and this pattern is characteristic of plant species that show clonal reproduction (Ellstrand and Roose 1987). Furthermore, in all the populations, the observed genotypic diversity was significantly lower than the expected genotypic diversity (table 3), which again indicates a significant amount of clonal recruitment (Stoddart and Taylor 1988). Most genets consisted of a relatively small number of ramets (<5) at the spatial distances sampled. Clonal genetic structure is, however, dependent on the pattern of population founding. If seedling recruitment occurs only at the population establishment stage (initial seedling recruitment), populations are expected to consist of large numbers of a few genets (Eriksson 1997). *Humboldtia brunonis* appears to combine clonal recruitment (33.3%) and sexual regeneration (66.7%) as methods of reproduction estimated from *G/N* values. Mean values of clonal diversity (*G/N* = 0.667, *D* = 0.969, and *E* = 0.90) were considerably higher across the distributional range of this species than those reported for other clonal species (Ellstrand and Roose 1987; Widen *et al.* 1994). Similar high levels of clonal diversity were also reported for a tropical member of the Fabaceae, *Pueraria lobata* (Pappert *et al.* 2000).

The ISSR data revealed moderate amounts of genetic diversity at the species level (*PPL* = 52.63%, *I* = 0.1895, and *h* = 0.1186) and relatively lower genetic diversity at the population level (*PPL* = 10–25%, *I* = 0.0728–0.1590, and *h* = 0.0487–0.1023) (table 2). Similar levels of diversity, as obtained in the present study, at species and population levels, have also been reported for other members of the Fabaceae, e.g. *Trifolium stoloniferum* (Crawford *et al.* 1998), *Pueraria lobata* (Pappert *et al.* 2000), *Astragalus oniciformis* (Alexander *et al.* 2004), *Astragalus submitis* (Zarre *et al.* 2004) and also in some endemic or narrowly distributed plants (Ellstrand and Elam 1993; Luo *et al.* 2005). High levels of diversity at the species level and comparatively low levels of diversity at the population level can be explained by factors such as genetic isolation, speciation from a more widespread species, breeding system, somatic mutation and founder effects (Zawako *et al.* 1994). Some of these factors such as genetic isolation may be particularly germane to *H. brunonis*, which is largely restricted to mid-elevation forests in the Western Ghats and exhibits a patchy distribution (Ramesh and Pascal 1997; Gimaret-Carpentier *et al.* 2003). The proportion of polymorphism at the species level in *H. brunonis* (52.6%) was not very different from the mean values reported for dicots (44.8%), narrowly distributed species (45.1%) and asexual/sexual reproducers

(43.8%) (Hamrick and Godt 1989). The gene diversity value at the species level (0.1186) was similar to the mean value (0.123) reported by Hamrick and Godt (1989) for 56 plant taxa with both sexual and asexual modes of reproduction, and lower than the mean value reported for facultatively asexual species (0.36) (Loveless and Hamrick 1984). Thus, despite its endemism and clonality, genetic diversity in *H. brunonis* is comparable with other non-endemic and non-clonal species (using the allozyme data of Hamrick and Godt [1989] as reference).

Agumbe, which is the most northern population examined and geographically twice as far away from the other two populations, grouped separately and with larger bootstrap support from a larger cluster consisting of Sampaji and Solaikolli, which are closer to each other geographically (figure 3). This indicates that genetic distance correlates with geographical distance in this species, suggesting locally restricted gene flow. Furthermore, clusters of genetically grouped individuals within neighbourhoods in each of these three populations also indicated local and restricted gene flow. We had also hypothesized that the poor seed set found in our previous study of *H. brunonis* (Shenoy and Borges 2008) was the consequence of fine-scale genetic structuring and possible biparental inbreeding among related genets. Our present investigation provides possible evidence to substantiate this hypothesis. The significant spatial correlation observed in some neighbourhood sets using the SGS software disappeared when clones were excluded, indicating a significant contribution of clonal regeneration to genetic structure in these neighbourhoods (Ceplitis 2001; Kalisz *et al.* 2001). In neighbourhoods 2 in Agumbe, Solaikolli and Sampaji, significant spatial autocorrelation, as determined by Mantel tests, persisted when clones were removed; this may be due to restricted seed/pollen dispersal resulting from biparental inbreeding among related genets (Nason and Ellstrand 1995). Even though we had low sample sizes in each neighbourhood, the correspondence between the results from the SGS analysis and Mantel tests, in some neighbourhoods, is indicative of robust phenomena. Therefore, considerable clonal regeneration coupled with low amounts of restricted seed/pollen dispersal (due to localized mating among genetically related members) resulted in spatial genetic structuring within neighbourhoods.

Because *H. brunonis* is self-incompatible, pollen movements within short distances from related trees is likely to reduce fruit and seed set (Charpentier *et al.* 2000; Charpentier 2002; Reisch *et al.* 2007). The pollinators of *H. brunonis* are mostly small *Braunsapis* bees (not more than 0.5 cm in body length) that reside in the domatia of these ant-plants (Shenoy and Borges 2008). These bees are likely to collect and redistribute pollen within small areas around their nest sites, and this may contribute to limited and local pollen flow, and to the low natural as well as experimental fruit set values that we obtained (Shenoy and Borges 2008). The seeds of *H. brunonis* are heavy, without any fleshy pulp, and have primarily ballistic dispersal; they may also undergo secondary dispersal by rodents, as found among several other caesalpinioid rainforest trees with heavy diaspores in the Neotropics (Forget 1990; Hardy *et al.* 2006). Since fine-scale spatial genetic structure was observed in some of the sampled neighbourhood sets excluding clones, it is evident that restricted pollen and/or seed movement among related gamets must be occurring at such small spatial scales (<3 m). Since *H. brunonis* is also often found clustered along monsoon streams, it is possible that its heavy seeds also undergo dispersal via such seasonally active water courses. This may constitute longer gene movement events, and may contribute to population structure at larger scales; however, this has not been investigated.

Local biparental inbreeding (although at larger scales of between 30 and 60 m) was reported for another clustered caesalpinioid rainforest tree in French Guiana (Dutech *et al.* 2002); this species *Vouacapoua americana* was also found to be pollinated by small bees (Dutech *et al.* 2002) and dispersed by rodents (Forget 1990). Spatial genetic structuring caused by limited pollen dispersal (27–53 m) and resulting in biparental inbreeding at slightly larger scales was also found in the bird-pollinated *Symphonia globulifera* (Degen *et al.* 2004) and in fragmented populations of the bat-pollinated *Caryocar brasiliense* (Collevatti *et al.* 2001). Contrariwise, other rainforest species pollinated by diverse insects and dispersed by birds and rodents showed no evidence for biparental inbreeding (Cloutier *et al.* 2007). Therefore, the particularities of each pollination and seed dispersal system must be determined in order to make predictions about the spatial genetic structure that may be expected, and most tropical rainforest tree species do show evidence for restricted gene flow and biparental inbreeding, albeit at different scales (Dick *et al.* 2008).

The fact that, in the present study, local neighbourhoods, randomly selected within a 1 ha area (100 m x 100 m) in each population, showed clusters of related individuals, is indicative of the phenomenon of predominantly locally restricted gene flow. Low levels or the absence of gene flow, and consequently high genetic differentiation, have also been reported in many endemic species with restricted dispersal (Hamrick and Loveless 1986; Ellstrand and Elam 1993; Li *et al.* 2002; Cao *et al.* 2006) as found in this case. Furthermore, if *H. brunonis* historically occupied Pleistocene/Quarternary refugia in the Western Ghats, as has been suggested for several endemic Western Ghats species (Mani 1974; Gimaret-Carpentier *et al*. 2003), then the biology of restricted dispersal and the resultant population structure of this species may be a relict of its past history.

We observed root connections between several trees in *H. brunonis* populations during our study. Why should

H. brunonis propagate clonally, and could this contribute to its high-density dominant populations? Besides large-scale disturbances, understorey trees in tropical forests frequently suffer physical damage, and the tendency for vegetative sprouting after physical damage has also been noted in such tree species (Paciorek *et al.* 2000). Root sprouts always show rapid growth rates as they can use the pre-existing root system and photosynthates produced by the original tree trunk (Kaufmann 1991). Furthermore, high potential for root sprouting has been observed in many leguminous trees, and vegetative regeneration through root sprouting has been speculated to be the reason for dominance in these leguminous trees (Rodrigues *et al.* 2004). Richards (1996) also speculated that the frequency of vegetative sprouting in a tropical forest is related to difficulties in seedling establishment in the stand and is, therefore, reported to be more prolific in understorey species due to their greater rate of mechanical damage from falling stems. Seedling establishment rates of *H. brunonis* are unknown. However, vegetative regeneration through root sprouting might be a strategy to compensate for poor seedling establishment. Many of the dominant forest trees in diverse forest types are reported to have a vegetative regeneration strategy in addition to a sexual mode of reproduction (Connell and Lowman 1989). Therefore, this strategy of vegetative regeneration is probably what contributes to the dominance of *H. brunonis*.

Our study has thus shown that the dominant, understorey, leguminous tree species *H. brunonis* combines sexual recruitment and clonal regeneration as mechanisms of regeneration. All three examined natural populations were composed of a number of different genets (i.e. they were multiclonal) and were characterized by high levels of clonal diversity. A moderate level of genetic variation was observed within populations while a higher amount of diversity was observed at the species level. Fine-scale genetic structure at the neighbourhood level was evident in self-incompatible *H. brunonis* populations either due to clonal regeneration or biparental inbreeding among related genets (consequent to localized seed/pollen dispersal). As we had hypothesized, poor fruit set observed in our previous study (Shenoy and Borges 2008) can be very well explained by the fine-scale genetic structuring at the local scale. In such species, the ability to reproduce asexually may provide necessary reproductive assurance, which may be important for the species to survive (Chung *et al.* 2004). From a conservation perspective, multiclonal growth may enable *H. brunonis* and its associated invertebrate fauna to persist for a longer time in restricted populations and thus reduce the risk of extinction. Since very little is known about seed viability and seedling recruitment, future studies on these aspects would give us better insights into the population dynamics and genetic structure of the species, and enable predictions about population survival in the face of increasing fragmentation of its habitat.

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