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Genetics of Brazil nut (*Bertholletia excelsa* Humb. & Bonpl.: Lecythidaceae)

1. Genetic variation in natural populations

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Summary. We provide an estimate of genetic variation within and between two populations of *Bertholletia excelsa* (Brazil nut), a large canopy tree found in the rain forests of South America. Average heterozygosity is 0.190, and 54.3% of the sampled loci are polymorphic. The population structure deviates significantly from Hardy-Weinberg expectations for *Fest2* and *Pgm2* ($F = 0.405$ and 0.443, respectively) in one population, and highly significantly ($F = -0.341$) for *Gdh* in the other population. Although allele frequencies of the two populations differ significantly for *Aat2*, *Est5*, *Mdh1*, and *Mdh2B*, Nei's coefficient of gene differentiation (G_{st}) indicates that the between-population component (D_{st}) of genic diversity represents only 3.75% of the size of the within-population component (H_s). The implications of these findings in terms of conservation genetics are that much of the genetic diversity of this species may be preserved within one or a few populations. However, such populations must be very large because it appears that the large amount of genetic variation in Brazil nut populations is maintained by extensive gene flow and bonds of mating over a large area. The genetic architecture of *Bertholletia excelsa* is similar to that expected for an extensively diploidized paleopolyploid species.

Key words: Genetic diversity – Population genetics – *Bertholletia excelsa* – Brazil nut – Isozyme analysis

Introduction

Population genetics of tropical forest trees remains unexplored despite a mounting interest in the evolution and population biology of tropical organisms. Our knowledge of genetic variation within and between populations is scant for the temperate zone angiospermous trees as well. Thus, comparisons of genetic diversity among different life forms often involves short-lived angiosperms and long-lived gymnosperms (Hamrick et al. 1979). In contrast to the temperate zone conifers that typically grow in high densities and have their pollen and seed dispersed by wind, many tropical forest trees occur in low population densities (e.g., one mature individual per hectare, Clark and Clark 1986) and have their pollen and seed dispersed by a wide variety of animals. The unique demographic and reproductive features of tropical rain forest trees may generate novel and diversified population structures, but virtually nothing is known about the amount and patterns of genetic variation in such organisms. This is particularly ironic because progress in two areas of contemporary interest, speciation in tropical forests and conservation of genetic resources, is contingent on information about genetic diversity in natural populations.

Here we provide an estimate of the genetic variation within and between populations of *Bertholletia excelsa* Humb. & Bonpl. (Brazil nut), a large canopy tree in the rain forests of South America. Our study was designed to answer the following questions: (1) what is the amount of variation within populations as measured by the average level of heterozygosity and the proportion of polymorphic loci, and (2) what is the extent of genetic differentiation between populations? In addition, we sought to analyze the mating system to estimate the amount of outcrossing and to eventually determine the role of pollen flow and mating system in the maintenance and regulation of genetic variation. The results of the mating system study are presented in a subsequent paper (O'Malley et al. 1988).

We studied genetic variation (and mating system) by using allozymes as genetic markers. Although the potential of allozymes to investigate genetic problems has been

demonstrated in many rain forest tree species (Gan et al. 1977; Hamrick and Loveless 1986), we provide the first description of the genetic architecture of a particular rain forest tree species, including genetic models for allozyme inheritance. Furthermore, we present the first estimate of genetic diversity based on established genetic polymorphisms for a South American rain forest tree species.

Materials and methods

Bertholletia excelsa is a large canopy tree distributed in the lowland tropical forests of South America. The tree bears large, complex flowers that are pollinated by large bees (Prance 1985). The fruits are dropped on the ground where the woody fruits are gnawed by agoutis, who eat or cache the seeds (Prance and Mori 1978).

We sampled two populations from Brazil: population A from Acre, Mocambo (10°45'S, 68°10'W) and population C from Careiro-Porto Velho Road southeast of Manaus (3°23'S, 59°50'W). Approximately 100 seeds per tree (from several fruits) were collected from 28–30 trees in both populations. Seeds collected from the fruits on the ground represent open-pollinated half-sib families.

Seeds were soaked in water for 3 days before the distal and proximal ends of the hard seed coat were clipped off. Seeds were then treated in 3% benlate for 3 min, dried for 3–4 h, and then sown in sterilized coarse sand on growth benches in plant growth rooms of the Department of Biology, University of Massachusetts/Boston. Illumination was under high pressure sodium and mercury lamps (400 watt) for 14 h light and 10 h dark. Temperature was controlled at 25 °C. Germinating seeds were subjected to intermittant overhead mist.

Seedcoats were removed in preparation for electrophoresis. Enzymes were extracted from 20 seeds per family according to the methods of Mitton et al. (1979), except that the extraction buffer was supplemented with 1% bovine albumin. Starch gel electrophoresis methods followed Conkle et al. (1982) and Marty et al. (1984), as indicated in Table 1.

As progeny tests were not possible, and the families sampled (seed progeny from single maternal parents) were open pollinated, genetic interpretations are based on evaluations of enzyme polymorphisms from natural populations. Each putative locus exhibits an enzyme polymorphism consistent with a simple genetic model, except for monomorphic loci (which are based on invariant band regions). Also, the banding patterns interpreted genetically are consistent with those observed in other well-documented investigations where genetic interpretations have been corroborated by genetic analysis. For enzymes possessing multiple isozymes, the most anodal isozyme is arbitrarily designated number "1", with the remaining isozymes numbered sequentially. Allozymes are superfixed numbers in a similar fashion. Putatively duplicated isozymes are assigned the same number, followed by a letter postfix, "A" being more anodal than "B".

After a preliminary survey (one seed per family per population) to identify potentially polymorphic isozymes, 20 progeny per family wee screened for the isozymes *Aat, Est, Fest, Gdh, G3pdh, Lap, Mdh, Pgi,* and *Pgm* in order to resolve allozyme frequencies in the two populations, and in order to assess genetic assets of the sample for a mating system analysis (O'Malley et al. 1988).

Table 1. Buffer and enzyme combinations

Enzymes[a, b]	E.C. ref.	Gel-electrode buffer system/power
EST	3.1.1.1	B^a approx 50 mA/5 h
PGM	2.7.5.1	
MDH	1.1.1.37	
AAT	2.6.11	A^a approx 75 mA/5 h
PGI	5.3.1.9	
GDH	1.4.1.3	
LAP	3.4.11.1	
FEST	3.1.1.1	
MDH	1.1.1.37	$D^a = AC^b$ approx 50 mA/5 h
G3PDH	1.2.1.12	

[a] Conkle et al. 1982
[b] Marty et al. 1984

Results

Genetic interpretations

Interpretations of enzyme phenotypes are based on patterns of variability in two populations of 28 and 30 open-pollinated families, respectively. These interpretations are presented schematically in Fig. 1 and are consistent with analogous systems in other plant species for which more rigorous genetic evidence is available. Putative isozymes are listed in Table 1.

Aspartate aminotransferase. Two staining regions are apparent for this enzyme, the faster of which is invariant for a single band. The slower region appears to contain a single diallelic locus for a dimeric enzyme, with both single-banded homozygote phenotypes and triple-banded heterozygotes observed (Fig. 1). Interlocus heterodimers are not apparent.

Colorimetric esterase. Only *a*-esterases were detected in these populations. Two allozyme loci were restricted to mature leaf tissue *(Est2, Est3),* and one invariant locus *(Est1)* and two polymorphic isozymes were restricted to seedlings *(Est4, Est5).* All polymorphic loci have banding patterns consistent with a simple monomeric model, although the rare $Est4^c$ allele has not been observed in homozygous condition.

Fluorescent esterase. Three putative loci have been detected for this dimeric enzyme, none of which formed active inter-locus heterodimers. The fastest staining regions exhibit one invariant band interpreted to be a monomorphic locus, *Fest1. Fest2* and *Fest3* are both polymorphic, overlap in their mobilities, and exhibit classic dimeric enzyme phenotypes. Only *Fest1* and *Fest2* are expressed consistently in seed or sapling foliage. At least four alleles differing in mobility have been detected for *Fest2.*

a-Galactosidase. Galactosidase exhibits two invariant bands. These are provisionally interpreted as two monomorphic loci, although we cannot exclude the possibility that one locus might be responsible for both bands.

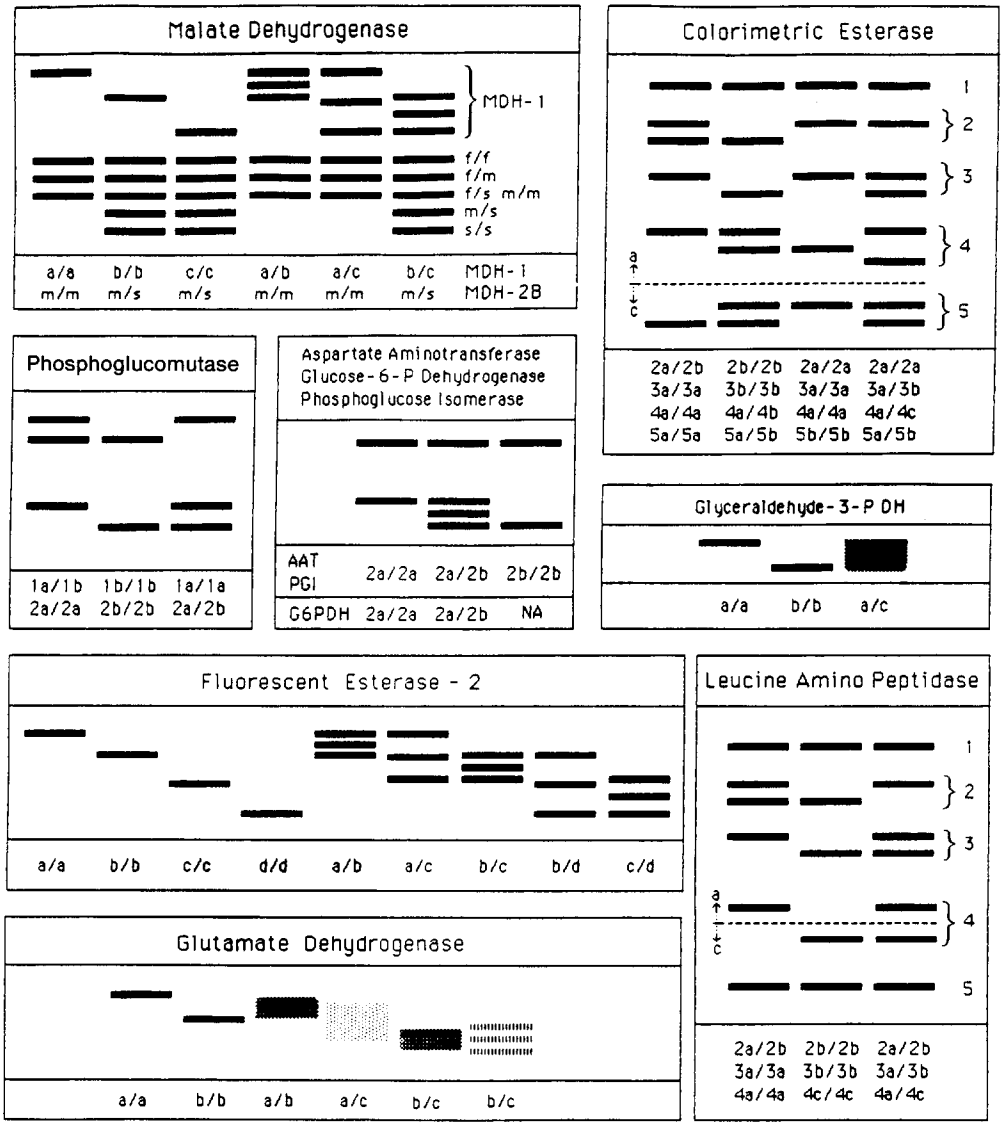

Fig. 1. Genetic models of allozyme inheritance in *Bertholletia excelsa*

Glutamate dehydrogenase. Only one gene coding for this enzyme has been detected in *Bertholletia,* although diploid flowering plant species commonly exhibit two loci (Moran and Bell 1983). This gene appears to be polymorphic and triallelic in both populations. These GDH phenotypes are typical of many plant species, with heterozygous phenotypes consisting of intermediate mobility staining areas and homozygotes having distinct individual bands. However, several individuals have provided clear three-banded heterozygous phenotypes, suggesting that the enzyme is dimeric.

Glyceraldehyde-3-phosphate dehydrogenase. Only one putatively polymorphic locus is apparent for this enzyme. Heterozygotes exhibit an unresolved staining region intermediate between the two electromorphs interpreted as homozygote bands.

Glucose-6-phosphate dehydrogenase. Two staining regions are observed for glucose-6-phosphate dehydrogenase. The faster consists of an invariant band which we infer to be a monomorphic *G6pdh1.* The slower region exhibits two apparently dimeric phenotypes: triple-banded heterozygotes and single, fast banded homozygotes for diallelic *G6pdh2.*

Leucine aminopeptidase. Five inconsistent staining regions were detected, each of which provided clear banding. Both the most anodal and the most cathodal regions were invariant for single bands, interpreted as *Lap1* and *Lap5,* respectively. The remaining two variable loci appear to be monomeric and diallelic.

Malate dehydrogenase. Bertholletia's MDH banding patterns are complex. However, three staining regions are apparent. The least anodal region appears to contain a

monomeric polymorphic locus *(Mdh3)*, which is difficult to interpret because of its lighter staining and close proximity to the origin. The intermediate staining region appears to be coded by two duplicate genes that form inter-locus heterodimers, one monomorphic *(Mdh2A)* for a fast mobility variant and the other one polymorphic *(Mdh2B)* for different alleles (medium and slow mobility variants). We did not observe a three-banded individual with the putative genotype *2Af/2Af, 2Bs/2Bs,* but only one or so are expected in such a small sample. The fastest anodal staining region appears to be coded by a single multiallelic locus for a dimeric isoenzyme, *Mdh1*. There are no inter-genic heterodimers apparent among the three isozymes.

Phosphoglucose isomerase. Two staining regions are apparent. We interpret the faster region conservatively as one monomorphic locus with poor resolution. However, clearly resolved banding patterns for this region contain five clear phenotypes: the three expected for a diallelic locus coding for a monomeric enzyme, plus two two-banded phenotypes with one light and one dark staining band having the same mobilities as the bands in the other phenotypes. The latter two phenotypes may represent homozygotes with ghost banding, however resolution is inconsistent, and a monomeric enzyme is not expected (though not impossible). A single diallelic locus coding for a dimeric enzyme is responsible for the slow region *(Pgi2)*. In other plants, this locus is commonly very variable, whereas *Bertholletia excelsa's Pgi2* exhibits a very low polymorphism level.

Phosphoglucomutase. Two diallelic loci for monomeric enzymes have been detected for phosphoglucomutase. Only the slower of these *(Pgm2)* is consistently observed.

Shikimic dehydrogenase. Shikimic dehydrogenase exhibits two invariant bands. These are provisionally interpreted as two monomorphic loci. In most plants in which it has been studied, shikimic dehydrogenase is usually coded for by only a single locus and when monomorphic, exhibits only a single invariant band.

Triosephosphate isomerase. Two staining regions are observed. The most anodal region is invariant for a single band and is interpreted tentatively as a single monomorphic locus *(Tpi1)*. The slower anodal region is invariant for a three-banded pattern that we interpret as fixed inter-locus heterozygosity for two duplicated genes *(Tpi2A* and *Tpi2B)*.

Genetic variability

Sampled genotypes, allele frequencies, and polymorphic indices for nine consistently scoreable polymorphic loci are presented for both populations in Table 2. The unweighted average heterozygosity is 0.190, and 54.3% of the sampled loci are polymorphic. Population structure deviates significantly from Hardy-Weinberg expectations for *Fest2* and *Pgm2* in the Acre population, and highly significantly for *Gdh* in the Careiro population. In the Acre population, both loci deviate in the direction of excess homozygosity, to an equivalent extent ($F = 0.405$ and 0.443, respectively). In contrast, the Careirò population exhibits an excess of *Gdh* heterozygotes.

Comparison of the two populations indicates significantly different allele frequencies for *Aat2, Est5, Mdh1,* and *Mdh2B* (Table 4). Based on the eight polymorphic loci in Table 3, for which allele frequencies are available for both populations, Nei's standard genetic distance is

Table 2. Enzyme variability in an Amazonian population (A) of *Bertholletia excelsa*

Enzyme	Isozyme	n	n_e
Aspartate aminotransferase	*Aat1*	1	1.000
	Aat2	2	1.882
Colorimetric esterase	*Est1*	1	1.000
	Est2	2	NA
	Est3	2	NA
	Est4	3	NA
	Est5	2	1.528 [1.105]
Fluorescent esterase	*Fest1*	1	1.000
	Fest2	1	1.000
	Fest3	2 [3]	1.960 [2.124]
	Fest4	2	NA
α-D-galactosidase	*a-Gal*	1	1.000
	a-Gal	1	1.000
Glutamate dehydrogenase	*Gdh*	3	2.923 [2.836]
Glyeraldehyde-3-PDH	*G3pdh*	2	NA
Glucose-6-*P* dehydrogenase	*G6pdh1*	1	1.000
	G6pdh2	2	NA
Leucine amino peptidase	*Lap1*	1	1.000
	Lap2	2	NA
	Lap3	2	NA
	Lap4	2	1.507 [1.105]
	Lap5	1	1.000
Malate dehydrogenase	*Mdh1*	3	2.330
	Mdh2A	1	1.000
	Mdh2B	2	1.342
	Mdh3	2	NA
Phosphoglucose isomerase	*Pgi1*	1	1.000
	Pgi2	2 [1]	1.152 [1.000]
Phosphoglucomutase	*Pgm1*	2	NA
	Pgm2	2	1.812 [1.991]
Shikimic dehydrogenase	*Skdh-1*	1	1.000
	Skdh-2	1	1.000
Triosephosphase isomerase	*Tpi1*	1	1.000
	Tpi2A	1	1.000
	Tpi2B	1	1.000

n = number of allozymes; the effective number of alleles is $(n_e) = 1/(f_1 + f_2 + \ldots f_i)$, where f_i is the frequency of the i^{th} allele; bracketed values refer to population C

Table 3. Allozyme frequencies for nine loci in two populations of *Bertholletia excelsa*

Locus	Genotypes								f (1)	f (2)	f (3)	PI [F]
	P	N	11	12	22	13	33	23				
Aat2	A	27	3	15	9	0	0	0	0.38±0.07	0.63±0.07		0.484
	C	27	16	9	0	0	0	0	0.76±0.06	0.24±0.06		0.373
Est5	A	27	17	8	2	0	0	0	0.78±0.06	0.22±0.06		0.091
	C	30	27	3	0	0	0	0	0.95±0.03	0.05±0.03		0.097
Fest2	A	28	8	8	12	0	0	0	0.43±0.07	0.57±0.06		0.489* [0.405]
	C	30	8	11	10	0	1	0	0.45±0.07	0.52±0.06	0.03±0.02	0.519
Gdh	A	27	6	2	3	8	1	7	0.41±0.06	0.32±0.05	0.28±0.06	0.669
	C	30	3	2	0	12	1	12	0.33±0.06	0.43±0.06	0.23±0.05	0.656** [−0.341]
Lap^4	A	28	17	10	1	0	0	0	0.79±0.06	0.21±0.06		0.342
	C	30	27	3	0	0	0	0	0.95±0.03	0.05±0.03		0.097
Mdh1	A	30	1	5	6	1	0	17	0.13±0.04	0.30±0.06	0.56±0.06	0.395
	C	28	0	0	6	0	11	12	0.37±0.07	0.63±0.07		0.475
Mdh2B	A	30	21	9	0	0	0	0	0.85±0.05	0.15±0.05		0.259
Pgi2	A	30	30	0	0	0	0	0	1.0	–		–
	C	28	24	4	0	0	0	0	0.93±0.03	0.07±0.03		0.134
Pgm2	A	28	15	7	6	0	0	0	0.66±0.06	0.34±0.06		0.456* [0.443]
	C	27	12	9	6	0	0	0	0.59±0.07	0.41±0.07		0.493

Populations (P): A = Acre, C = Careiro Puorto Velho; N = population sample size; f(i) = frequency of the ith allele ± its standard error; PI = Polymorphic Index; deviations from Hardy-Weinberg expectation are indicated as * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$) and are accompanied by estimates of Wright's Fixation Index [F]

Table 4. χ^2 tests of between-population variability

Locus	χ^2
Aat2	20.04**
Mdh1	9.36**
Est5	7.38**
Gdh	1.70 ns
Pgm2	0.29 ns
Fest2	0.05 ns

** $P<0.01$; ns = non-significant

D = 0.049, a value within the range expected for conspecific populations (Nei 1976). Nei's coefficient of gene differentiation (G_{st}) indicates that the between-population component (D_{st}) of genic diversity is only 3.75% as large as the within-population component (H_s).

Discussion

The unweighted average heterozygosity of these populations is similar to that for conifers and temperate angiospermous trees, however the fraction of polymorphic loci is somewhat lower (Hamrick et al. 1979).

Preliminary data for other tropical tree species also indicate high genetic variation, though many species seem to possess low levels of genetic polymorphisms (Hamrick and Loveless 1986). There are pitfalls in comparing levels of heterozygosity, even among populations of the same species, unless the same enzymes are assayed for each taxon (Simon and Archie 1985). Thus, unless the procedure are standardized, any comparison among species must remain tentative.

The high level of variation within the populations of *Bertholletia excelsa* is not surprising in the light of the high outcrossing rate revealed by mating system analysis (O'Malley et al. 1988). What is surprising is that there seems to be very little genetic differentiation between populations even though for particular loci the two show significant differences in allele frequencies. However, there is some evidence that some Brazil nut stands may have been planted by Amerindians (Mori and Prance 1988). The possibility exists that the two sampled populations have been recently derived from a common ancestral population (S.A. Mori, personal communication).

There is evidence for a lack of genetic differentiation among populations over a smaller scale in some other tropical tree species in Panama (Hamrick, in Hubbel and Foster 1983). On the other hand, on the basis of asynchronous flowering, biased sex ratios, and restricted mating due to limited gene dispersal in several species, Bawa (1976) suggests that populations of tropical rain forest trees may be more inbred than their temperate zone counterparts and may, therefore, exhibit greater genetic differentiation. It is difficult to make general statements on the basis of one species. Considering the diverse patterns of dispersion and a range of pollen and seed dispersal mechanisms, one would expect diversified population structures in tropical rain forest trees.

Our results have interesting implications for conservation genetics of Brazil nut. If our finding of extreme variation within a population and very little differentiation between population is substantiated by further work, then much of the genetic diversity may be preserved within one or a few populations. However, such populations must be very large because it appears that the large amount of genetic variation in Brazil nut populations is maintained by extensive gene flow and bonds of mating over a large area. Even so, pollen and seed vector habitat requirements may be more restrictive (Mori and Prance 1988), and the differentiation of natural populations may exceed that of the two populations reported herein.

The genetic architecture of *Bertholletia excelsa* is similar to that expected for an extensively diploidized paleopolyploid species. It is not surprising that *Bertholletia* may be paleopolyploid because its haploid chromosome number is relatively high (n=17; Mangenot and Mangenot 1962; Kowal et al. 1977; Prance and Mori 1979). The putatively duplicated genes *Mdh2A/Mdh2B* and *Tpi2A/Tpi2B* represent the strongest evidence of paleopolyploidy in this species, although gene duplications can result from other processes. SKDH also exhibits two isozymes in this species, although most species exhibit only one. Two isozymes are inferred for *a*-GAL, but no information is available about their homology. There are also multiple isozymes observed for a number of nonspecific enzymes (EST, FEST, LAP), but the homologies of these isozymes are also uncertain. No inter-locus heterodimers were observed among FEST or AAT isozymes, which suggests that they are either not homologous, or they are very ancient gene duplications. All remaining specific enzymes with multiple isozymes can be explained more parsimoniously as compartmentalized isozymes descended from ancient protoeukaryote and endosymbiont genomes (Weeden 1983).

The results presented here are crude and based on small sample sizes, but they provide the first estimate of genetic diversity of an ecologically and commercially important tropical rain forest tree species. More important, the putative genetic markers documented here open the way for more sophisticated genetic analysis of the Brazil nut and other tropical rain forest tree species, as shown by an accompanying paper on the mating system of *Bertholletia excelsa*. In future studies, we expect to utilize these genetic markers to study microevolution and population structure in the Brazil nut.

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