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MICROSATELLITE MARKERS FOR POPULATION AND CONSERVATION GENETICS OF TROPICAL TREES[1]

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We have developed microsatellite or simple sequence repeat (SSR) genetic markers for the tropical tree *Pithecellobium elegans* (Mimosoideae). The frequency of this class of marker is estimated and the level and distribution of variability at these markers is assessed and contrasted to that found at isozyme markers in the same populations. The results indicate that SSR loci are powerful tools for the analysis of population structure and that, in these populations, they provide a means of accurately examining two important parameters in conservation biology, gene flow and paternity.

Key words: conservation biology; microsatellite markers; Mimosoideae; *Pithecellobium*; population genetics; tropical trees.

Levels of inbreeding, gene flow, and genetic differentiation in tropical rain forest trees are of central interest to tropical ecologists and conservation biologists. Models of evolution that explain species richness of trees in tropical rain forests are based on contrasting perceptions about the amount of inbreeding and genetic differentiation (Federov, 1966; Ashton, 1969; Bawa, 1992). In situ conservation of genetic resources in rapidly depleting rain forests also requires information about spatial organization of genetic variability and effective population sizes of canopy trees (National Research Council, 1991; Bawa, 1993). However, data on critical population genetic parameters such as genetic differentiation and inbreeding for some species and direct measurements of gene flow and effective population sizes for many species have been difficult to obtain because of the paucity of variable genetic markers revealed by traditional approaches based on allozymes (O'Malley and Bawa, 1987; Hall, Chase, and Bawa, 1994; Hall, Orrell, and Bawa, 1994; Hall, Walker, and Bawa, 1995).

Simple Sequence Repeats (SSRs) are powerful new genetic markers that are being used extensively in human forensic analyses and genetic mapping studies (Dietrich et al., 1992; Hearne, Ghosh, and Todd, 1992; Weissenbach et al., 1992). Their utility derives from their abundance, the simplicity of the assay, codominance, and high level of allelic diversity. As many as eight or nine alleles per locus have been identified in humans and mouse with an average of five (Dietrich et al., 1992; Hearne, Ghosh, and Todd, 1992; Weissenbach et al., 1992). In plants SSRs have been reported to be less frequent, though the sequence data of few species have been examined (Akkaya, Bhagwat, and Cregan, 1992; Lagercrantz, Ellegren, and Andersson, 1993; Morgante and Olivieri, 1993). These reports have investigated allelic variability in domesticated species with a general emphasis on the development of genetic maps or cultivar identification. Despite the potential usefulness of SSRs in population genetic studies (Bruford and Wayne, 1993; Queller, Strassmann, and Hughes, 1993), few reports have investigated the variability of SSRs in natural populations. Remarkably, 37 and 54 alleles have been detected at single loci in barley and pilot whales, respectively (Amos, Schlotterer, and Tautz, 1993; Saghai-Maroof et al., 1994). This enormous allelic diversity provides a means of accurately characterizing individuals in natural populations and monitoring gene transmission through generations and thus can accelerate progress in the estimation of genetic parameters that are of considerable interest to population and conservation biologists.

In this study of a tropical canopy tree species, *Pithecellobium elegans* (Mimosoideae) we have identified SSRs by screening genomic libraries, estimated the genomic frequency of $(TG)_n$ and $(TC)_n$ repeats, surveyed two populations for allelic diversity at five SSR loci, and screened open-pollinated progeny arrays to assess the inheritance of the markers and the number of contributing pollen parents in pods and bulk seed collections. The contrast between these data and those obtained with isozymes in earlier studies of the same populations shows the resolving power afforded by these genetic markers for population genetic and conservation studies.

MATERIALS AND METHODS

Plant material and DNA extractions—*Pithecellobium elegans* (Mimosoideae) is a large tropical tree, often reaching over 1 m in diameter and 40 m in height, in the Atlantic lowlands of Central America and Amazonia in Brazil. The trees occur at an average density of less than one reproductive individual per hectare. Leaf material for DNA analysis was collected from two populations in Costa Rica: La Selva (22 trees) and Cedral (30 trees). Both populations are located in the Atlantic wet evergreen forests of Costa Rica and are ≈21 km apart.

Leaf samples were frozen in liquid nitrogen and transported to the University of Massachusetts, Boston. Additional leaf and seed samples were collected from La Selva and surrounding areas in the spring of 1994. Seeds were germinated and grown for ≈6 wk in growth rooms at the University of Massachusetts. DNA was extracted from seedlings and frozen leaf tissue following CTAB procedures (Bernatzky and Tanksley, 1986).

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TABLE 1. Oligonucleotide primer sequences, repeat pattern, number of alleles and product length for five microsatellite loci.

Locus	Primer (5′ to 3′)	Clone repeat sequence	No. of alleles	Fragment sizes (bp)
Pel1	CGGTGATGCTCAAACTTCGT CCTTTCCTACCATTTTTGAA	(GA)4GGAGAA(GA)3A(GA)2	1	
Pel2	TAACGCAATCAGTTTATCAA CACACTATTTATGTTCAAGA	(GT)15(GA)11	5	115–135
Pel3	AGAGATGGACTGGAAACTTC CCTCCTTAGATTTCTTGTCT	(TC)14(TA)13	6	300–326
Pel5	TCTCTGCACACAGGAACCCTTTTGC CCCAGAAATAAGGCTCTTTTGCACA	(AAAG)6	5	190–200
Pel6	TCTTATACAATTACAAAAGAAAAGGTG CCTACCTCTCCTTTATATCTACTCTTT	(CT)12(CA)13(TA)2(CA)9	15	110–184

Library construction—One gram of fresh leaf material was used for DNA extractions. DNA was digested to completion with *Sau* 3a and electrophoresed in 2.5% NuSeive GTG Agarose (FMC) 1xTBE. Fragments between 350 bp and 750 bp were excised and purified with a Promega PCR Wizard kit. The vector Pgem 7z was phosphatased, and the genomic fragments were ligated into the *Bam*HI site. Ligation was transformed into XL 1-Blue MRF'(Stratagene) and grown on ampicillian and tetracycline agar plates. Tranformants were picked, streaked on 85-mm plates (100 per plate) and regrown at 37°C for 4–5 h.

For additional libraries, size-fractionated DNA was also cloned into M13 that had been digested with *Bam*HI and phosphatased. Transformations were performed as described bacteria were grown on tetracyclene plates (200–400 colonies per plate).

Screening of recombinants for repeat sequences—Recombinant bacteria were lifted with nitrocellulose filters. Filters were treated for 5 min in each of the following solutions, 10% SDS; 0.5 M NaOH, 1.5 M NaCl; and 0.5 M Tris (pH 8), 1.5 M NaCl and finally rinsed briefly in 2X SSC. Filters were baked at 80°C for 1 h. The oligonucleotides (10 rm) $(TC)_{15}$, $(CA)_{15}$ were 5′ end-labeled by using 5 μl of 10 μCi/μl of g ^{32}P ATP and 1 unit of T4 polynucleotide kinase. The labeled probe was purified through Bio-Rad spin columns. Filters were prehybridized overnight at 65°C in 6X SSC, 1% BSA, and 0.1% SDS to remove residual bacterial debris. Prehybridization solution was discarded and replaced with fresh solution. Both probes were added and hybridized at 65°C for 1 h, followed by an additional hour at 50°C. Filters were washed three times in 2X SSC at 55°C. Autoradiography was carried out at −80°C for 2–3 h.

Sequencing of positive clones—Positive clones were picked and grown overnight in standard LB medium. Plasmid DNA was extracted with Promega Magic Mini preps. Inserts were sequenced using Promega frnol system and ^{35}S labeling. Sequencing with the reverse as well as the forward primers (PUC/m13) was sometimes necessary to obtain both flanking regions of microsatellites.

Design of primers and resolution of SSR polymorphisms—PCR primers were designed with the aid of the program OLIGO (National Biosciences). Primers were synthesized by Operon Technologies (Alameda, CA). Conditions for PCR were 30 cycles of 94°C for 1 min, 50°C or 55°C anneal temperatures for 1 min and 72°C for 1 min. The SSRs were visualized on either acrylamide or agarose. Gels of 6% polyacrylimide, 7 M urea and 1 X TBE were used to resolve allelic variants. For these gels, 10 μl volume reactions were used, which included: 1 μl of 10 X buffer (Promega), 1 μl of 25 mM $MgCl_2$, 0.4 μl of primers (2.5 pM each), 0.4 μl dNTPs (5 mM A, G, T and 1.7 mM C), 0.1 μl Taq polymerase, 2.0 μl template DNA (10–15 ng/μl) and 0.1 μl of ^{32}P labeled dCTP (10 μCi per μl).

Ethidium bromide stained agarose gels, 1 x TBE, 3.5% MetaPhor (FMC Bioproducts, Rockland, ME) were used to score segregation of open-pollinated progeny arrays. Conditions for PCR were similar to those used for SCAR primers described elsewhere (Paran and Michelmore, 1992; Kesseli, Paran, and Michelmore, 1994).

RESULTS

Identification and characterization of SSRs—The genomic library of *P. elegans* was screened with two dinucleotide repeats: $(CA)_{15}$ and $(TC)_{15}$. Approximately 4,000 clones have been screened. To estimate the frequency of repeats, a subset of 1,200 plaques probed simultaneously with radiolabeled $(CA)_{15}$, and $(TC)_{15}$ was examined in detail. Eleven distinct clones were scored as postives. All clones sequenced (seven) from this group possessed either a TG/CA or AG/TC series ranging in length from 9 to 23 repeats. Assuming a mean insert size of 550 bp, $(TG/CA)_n$ or $(AG/TC)_n$ repeats occur approximately every 60 kb. In addition to the expected $(TG/CA)_n$ or $(AG/TC)_n$ repeats, several other repeat motifs were discovered in the sequenced clones. A separate tetranucleotide repeat, AGGG, was identified in one clone and many of the $(TG/CA)_n$ or $(AG/TC)_n$ repeats were part of larger compound patterns often including AT repeats.

Of the many clones that tested positive for repeats, primers were developed for five (Table 1). These primers were subsequently used to assess levels of variation in two populations of *P. elegans*. The relationship between repeat length and level of polymorphism discussed by others (Weber, 1990) appears to hold in our small sample of five SSRs. Primers for the clone with the largest compound repeat, 72 bp in clone Pel6, revealed a high of 15 alleles among the 52 individuals of the two populations (Fig. 1). For this locus the allelic variation spanned 74 bp with fragments ranging from 110 bp to 184 bp. Since we have not sequenced the various alleles, it is not known which portion of the compound motif is most often variable. The locus with the shortest repeat, *Pel1,* was monomorphic and the other three (e.g., *Pel2, Pel3* and *Pel5*) gave intermediate levels of variation (Table 1).

There appeared to be a patchy distribution of length polymorphisms for each locus. The repeat unit was two or four bases for these loci, but gaps in the distribution indicate that lengths have periodically increased, or decreased, by steps of greater than two or four bases (Fig. 2). The distribution of alleles at the *Pel6* locus is particularly patchy. There are two clustered peaks of alleles one at 121 bp and another at 135 and 137 bp. These peaks are followed by a gap of 16 bp devoid of alleles. Following this gap there is a fairly continuous distribution of fragment lengths from 155 bp to 171 bp that differ by 2

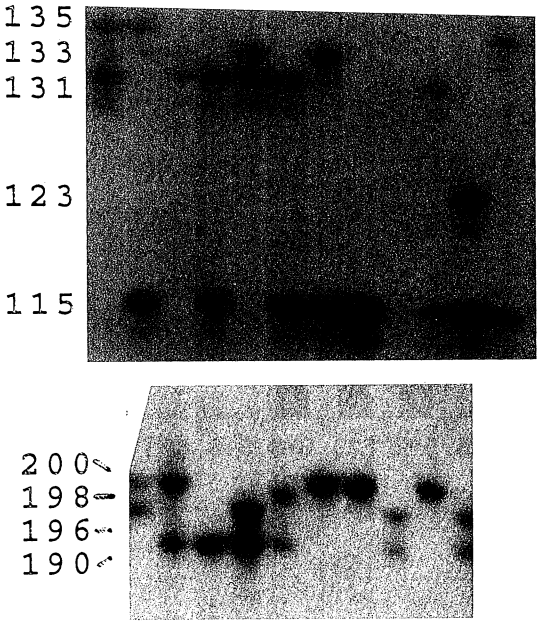

Fig. 1. Autoradiograph of a sample of the allelic variability detected for *Pel2* (top) and *Pel5* (bottom). Five alleles for *Pel2* and four alleles for *Pel5* are identified to the left of the figures.

bp. This pattern suggests that large deletion/insertion events have occurred to create nodes from which a series of small two-base changes are derived.

Inheritance of SSRs and number of paternal parents—The segregation patterns of three of the four highly polymorphic SSRs have each been examined on open-pollinated half-sib arrays. Two different classes of progeny arrays were examined. Bulk arrays are seeds collected from a variety of pods. Pod arrays are seeds collected from single pods. None of the three loci violate predicted Mendelian segregation patterns (Fig. 3) as all progeny possess at least one of the two maternal alleles (determined from separate analyses of the adult leaves). Although the most frequent maternal allele was as high as 0.67 in some of these small samples (Table 2), it appeared that segregation distortion was minimal (χ^2 tests, $P > 0.05$). Outcrossing rate estimates for *P. elegans* are near 1.0 (O'Malley and Bawa, 1987; Hall, Walker, and Bawa, 1995), but without knowledge of the pollen pool allele frequencies, which would require a larger sample than we have at this time, precise evaluation of distortion is not possible. Apparent distortion could be caused by pollen pools with one of the two maternal alleles in high frequency. For both bulk and pod arrays, we determined the number of different paternal alleles. At least two to four different fathers (3–7 paternal alleles) must have contributed to each bulk array. As few as one father (1–2 paternal alleles) contributed to the pod arrays.

Level of polymorphism at SSRs—For the five SSRs, we identified a total of 32 alleles among 52 individuals of two populations. One SSR, *Pel1,* appeared monomorphic and may not have been a good choice for a potential marker because of its short and interrupted pattern (Table 1). This locus will not be discussed further. Of the remaining 31 alleles, ten (six in Cedral and four in La Selva) were found in only one of the two populations sampled. These localized alleles were generally rare and found in 1–4 individuals of the population. Surprisingly, for two of these four loci, the two populations did not share the most common allele; a condition not found for any isozyme locus (Hall, Walker, and Bawa, 1995). Locus *Pel2* showed the largest population differentiation; allele 115 was the most frequent allele in Cedral (0.47) but the least frequent in La Selva (0.09).

The overall diversity detected by these few SSR loci greatly exceeds that identified by all other genetic markers previously studied in these populations. Table 3 shows several diversity statistics obtained for the SSR loci in the two populations of this study and compares these values to those obtained with six polymorphic isozyme markers. The latter data were extracted from the work of Hall, Walker, and Bawa (1995). All arbitrarily choosen SSR loci with six or more consecutive repeats were polymorphic (i.e., excluding *Pel1*). These four loci averaged more than double the effective number of alleles (n_e = 3.43) and expected heterozygosities or gene diversity (H_e = 0.65) of polymorphic isozyme loci (Table 3). Only one locus, *Pel3,* showed an allelic diversity pattern similar to isozymes with one allele in relatively high frequencies, 0.70 and 0.84, and the remaining alleles being generally rare. Even for this locus the number of alleles detected (six) in this small sample greatly exceeded that obtained in larger samples surveyed with isozymes.

Locus *Pel6,* which possessed the largest repeat, showed extraordinary variability and highlighted the extreme heterozygosities found at the SSR loci. In the Cedral population H_e was 0.81 and observed heterozygosity, H_o, was 0.97; there was only one homozygous individual in the sample of 30 trees. While the observed heterozygosity was high, the fixation index, F, was comparable with that found at other loci in this population. The fixation indices, which measure the deviations from Hardy–Weinberg expectations, were generally negative, indicating an excess of observed heterozygotes in the Cedral population. The value for locus *Pel6* (−0.19) resided at the mean for all SSR loci. In addition, these values were similar to those found at most isozyme loci in this population. One isozyme locus, *Idh,* showed a deficiency of heterozygotes, but the others showed excesses. For La Selva, the average fixation index for the two classes of markers was identical (−0.03) and not significantly different from zero, a fit to Hardy–Weinberg expectations.

The SSR loci would be powerful new tools if they could be used to fingerprint individuals and to detect rare events in population studies. To examine this we scored the number of genotypes revealed by the different loci. The numbers ranged from a low of four for *Pel3* in Cedral to a high of 17 for *Pel6* in Cedral. This last value is remarkable given that only 30 individuals were scored. Using the three most polymorphic loci (*Pel5, Pel2,* and *Pel6*) we constructed multilocus genotypes for all individuals in both populations. All individuals in La Selva were distinguished based on their three locus genotypes. In Cedral, 27 different genotypes were identified in 30 individuals (90%). For three genotypes there were two individuals each with a match

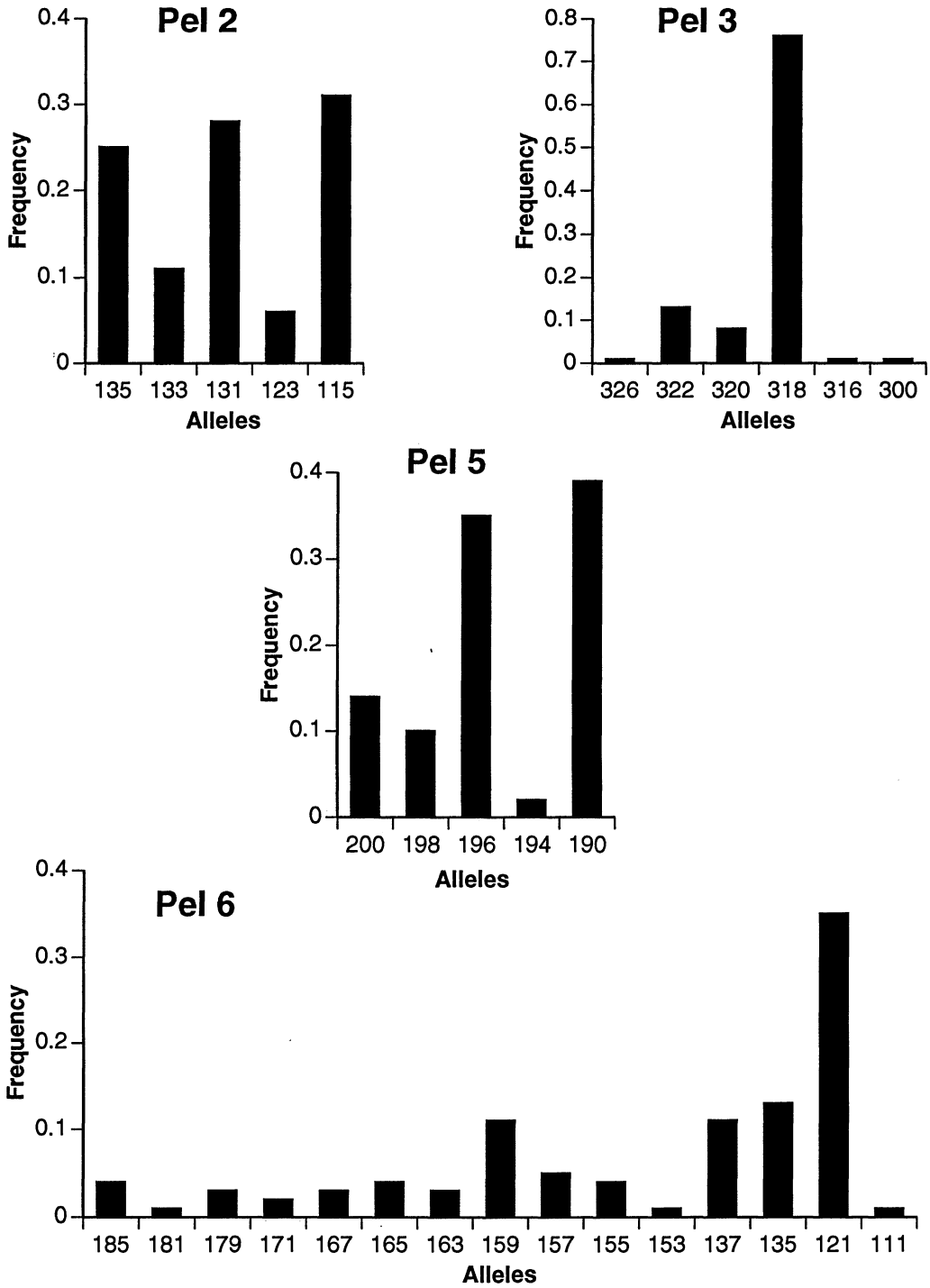

Fig. 2. Frequency of alleles for four SSR markers among the collection of *Pithecelobium elegans* individuals. The loci possess 5, 6, 5, and 15 alleles, respectively.

thus allowing 80% of the population to be uniquely identified. This level of resolution greatly exceeds that obtained with isozyme data. At most only 37% of a population could be uniquely identified with the six polymorphic isozyme loci.

Paternity exclusion probabilities were estimated (Weir, 1990) for the different loci from the full set of data (Table 4). Except for *Pel3,* which has an allelic distribution pattern similar to that found at many isozyme loci, the other SSRs show markedly higher values. The combined effect of all isozyme loci (0.62), assuming unlinked loci, is lower than the probability acheived with *Pel6* alone (0.69). The paternity exclusion probabilities for the SSRs combined was 0.94, a value that should make detection of

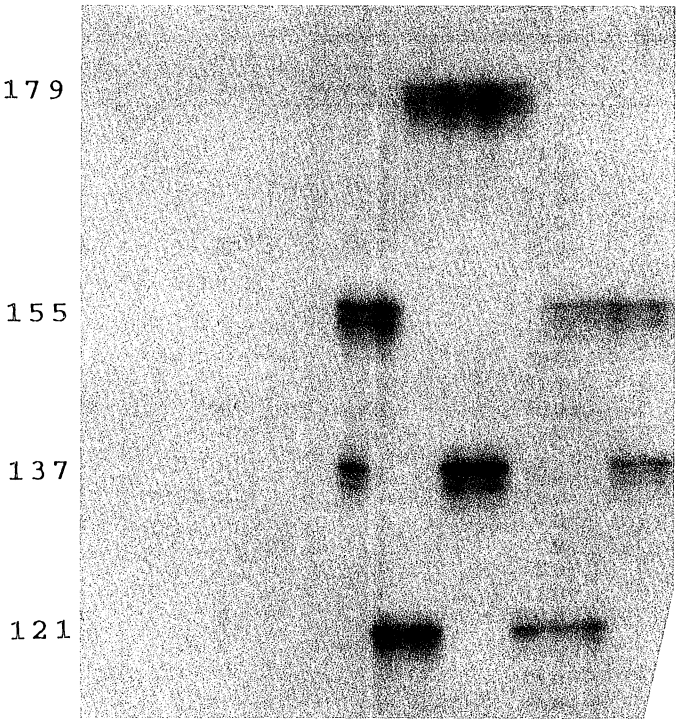

Fig. 3. Autoradiograph of allelic variation at *Pel6* in an open-pollinated progeny array obtained from a single pod. The two alleles (137 and 121) were contributed by the female parent and determined from a separate analysis of leaf samples. Only two paternal alleles are observed.

TABLE 2. Segregation of SSR loci in open-pollinated progeny arrays

Locus	Number of progeny/family	Frequency of common maternal allele[a]	Number of different paternal alleles
Bulk collections			
Pel5	19	0.55	3
Pel2	18	0.61	3
Pel6	28	0.64	7
Pod collections			
Pel6	15	0.67	1
Pel6	10	0.50	2

[a] Assuming genotypes identical to maternal parent had equal likelihood of receiving either of the two maternal alleles.

paternity within small fragments and gene flow among fragments of trees possible.

The data shown understates the relative advantages of SSR loci. The data presented for the SSR loci essentially represents a random selection of markers drawn from libraries of *P. elegans*. We had no apriori knowledge of variability levels. Excluding short repeats will undoubtedly increase the likelihood of polymorphisms. The isozyme data represents the results of an exhaustive search for all informative loci. The loci monomorphic in all populations studied in the earlier studies have been excluded so that the likelihood of any random isozyme being polymorphic and informative is much lower than indicated by the data given here (Table 3); expected heterozygosities of all isozyme loci are less than half those given for the polymorphic loci.

DISCUSSION

Several studies have estimated the frequency of SSR in plant genomes. These estimates have been based on either database surveys or direct searches. Regarding dinucleotide repeats it has been notable that TG/AC repeats are far less and AT/TA repeats far more frequent in plants than in mammals (Lagercrantz, Ellegren, and Andersson, 1993; Morgante and Olivieri, 1993). While the methodology varies considerably among studies that directly search for SSRs (length of repeat considered significant, length of probe, hybridization temperatures, vectors, etc.) the values range from 1 TG/AC per 26 kb to 1 per 350 kb (Condit and Hubbell, 1991; Lagercrantz, Ellegren, and Andersson, 1993; Morgante and Olivieri, 1993; Terauchi and Konuma, 1994). Our value (1/60 kb) falls easily within this range. In addition, all of our sequences showed repeat lengths of nine or greater, implying that a change in methodolgy (shortening the hybridization probe) may reveal a far greater frequency in *P. elegans* relative to other studies that are likely detecting shorter repeats. Since short repeats are less likely to be polymorphic (Weber, 1990), a strategy of enriching for higher repeat lengths by probing with longer repeats may prove valuable. We have not attempted to screen for $(AT)_n$ repeats, but many have been detected as part of compound SSRs or in adjacent regions of clones that carry TG/AC or TC/AG repeats. This implies that as in other plant taxa, $(AT)_n$ repeats are likely the most common dinucleotide repeat. The lower frequency of SSRs as a group in plants should not generally be a problem for workers searching genomic libraries; database searches are, of course, limited by the number of sequences available. The estimated number of SSRs still reaches into the tens of thousands in most plant genomes. Proper screening procedures for informative SSRs can eliminate excessive and expensive sequencing and primer synthesizing steps.

The true worth of SSRs for genome as well as population genetic analyses is high allelic diversity. In general, screenings of accessions of most populations have revealed eight or fewer alleles. The number of alleles do, however, vary depending upon the specific locus and the extent of the sampling. A recent study of worldwide human accessions revealed, on average, greater than ten alleles per locus (Bowcock et al., 1994). Rarely, a locus is detected that possess a remarkably high number of alleles. Saghai-Maroof et al. (1994) found 3, 3, 28, and 37 alleles at four loci in a screen of 207 worldwide accessions of barley. Amos, Schlotterer, and Tautz (1993) identified 3, 5, 6, 8, 8, and 54 alleles in a sample of 193 pilot whales. The significance of these occasional highly variable loci is not known, and detailed examinations of segregating progenies may be necessary to determine the stability of these loci.

Our studies have been limited to 52 individuals in two populations of *P. elegans* that cover ≈300 (La Selva) and 50 (Cedral) ha respectively. The allele numbers detected in this restricted sample (5, 5, 6, and 15) indicate both the informativeness of SSRs for intrapopulation analyses and a likely high diversity in the species as a whole. Our recovery of 15 alleles for locus *Pel6* in these two small

TABLE 3. A comparison of isozyme and simple sequence repeat (SSR) markers for two populations of *Pithecelobium elegans*.

Locus	n^a	n_e	H_{exp}	H_{obs}	F	No. obs. genotypes	% unique genotypes/ population	% total genotypes/ population
Cedral (N = 30)								
Pel2	5	3.15	0.68	0.77	−0.12	9		
Pel3	4	1.9	0.47	0.61	−0.3	4		
Pel5	5	2.74	0.64	0.73	−0.15	9		
Pel6	12	5.24	0.81	0.97	−0.19	17		
Mean	6.5	3.26	0.65	0.77	−0.19	9.75	80	90
La Selva (N = 22)								
Pel2	5	3.86	0.74	0.82	−0.1	10		
Pel3	5	1.4	0.29	0.31	−0.07	5		
Pel5	4	3.17	0.68	0.73	−0.06	7		
Pel6	12	5.94	0.83	0.73	0.125	16		
Mean	6.5	3.59	0.64	0.65	−0.03	9.5	100	100
Cedral (N = 42)								
Aat	2	1.05	0.04	0.05	−0.02	2		
Idh	3	1.58	0.37	0.21	0.422	4		
Mdh	1	1	0	0	0	1		
6pg	1	1	0	0	0	1		
Tpi	2	1.95	0.49	0.63	−0.29	3		
Ugpp	2	1.86	0.46	0.51	−0.11	3		
Mean	1.8	1.41	0.23	0.23	0.001	2.3	21	43
La Selva (N = 61)								
Aat	2	1.47	0.32	0.37	−0.17	3		
Idh	3	2.64	0.62	0.6	0.03	6		
Mdh	2	1.02	0.02	0.02	−0.01	2		
6pg	2	1.1	0.09	0.1	−0.05	2		
Tpi	2	1.92	0.48	0.45	0.06	3		
Ugpp	2	1.46	0.32	0.34	−0.06	3		
Mean	2.2	1.6	0.31	0.31	−0.03	3.2	37	61

[a] Notation within table: n = the number of alleles/locus; n_e = the effective number of alleles/locus; H_{exp} = the expected heterozygosity assuming Hardy–Weinberg equilibrium conditions; H_{obs} = the observed number of heterozygotes; F = the fixation index.

populations certainly rivals the highly variable loci identified in studies of barley and whales.

The four polymorphic SSR loci examined in this study provide more resolution and sensitivity for the estimation of population parameters than the combined effect of all isozyme markers previously detected in several detailed studies conducted over 5 yr. The effective number of alleles and expected heterozygosity (gene diversity) of our SSRs are generally more than double that of the isozyme loci (Table 1). From the extensive isozyme studies of these populations, only two rare alleles were private and thus restricted to single populations. Better than one-quarter of the SSR alleles identified in this study were found to be restricted and appear to be diagnostic, though more detailed samplings will be needed.

TABLE 4. Paternity exclusion probabilities for the isozyme and SSR markers of the Cedral and La Selva populations.

Locus	Exclusion probability
SSRs	
Pel2	0.55
Pel3	0.22
Pel5	0.47
Pel6	0.69
Overall	0.94
Isozymes	
Aat	0.10
Idh	0.28
Mdh	0.01
6pg	0.03
Tpi	0.21
Ugpp	0.22
Overall	0.62

The SSR loci behaved like neutral and stable genetic markers. The fixation indices of the SSR loci paralleled those found at isozyme loci. Also, the family data showed no evidence of instability through one generation. Assuming no selection at either isozyme or SSR loci and only the forces of drift and mutation, the great difference in allele numbers for these classes of loci can best be explained by differences in rates of mutation. The mutation rates of the SSR loci, based on these data, should be ≈5–20 fold greater than those of the isozyme loci. These higher rates are still likely very low and would not affect most population analyses. To empirically test this, the sample sizes used here must be increased.

These data show that the variability of SSR loci allows the accurate estimation of specific biolgical parameters not achievable with conventional markers. Components of breeding systems can be accurately estimated at the tree level and for individual fruits (pods). Muona, Moran, and Bell (1991) in a detailed study of another mimosoid tree, *Acacia melanoxylon,* examined hierarchical patterns of mating. They used 12 isozyme markers to estimate the frequency of multiple paternity in pods and showed multiple paternity to be low (17–33%); pods are likely pol-

linated by a polyad of 16 pollen grains from a single father. To obtain these estimates, values had to be adjusted to correct for the high frequency of cryptic multiple paternity events (>50%). From the adult allele frequencies (or seed for *pgdl* for which adult data was lacking) we estimated the paternity exclusion probabilities from the data of Muona, Moran, and Bell (1991). Assuming unlinked loci, the joint probability for the 12 loci was 0.83, less than the value we obtained for four SSR loci. Muona, Moran, and Bell (1991) clearly note the need for more variable loci, and our analyses of SSRs demonstrates that these markers will fulfill this need.

Our data also indicate that only one or a few fathers pollinate individual pods, while many may contribute to the progeny of a single fruiting tree. This provides important information regarding the pollination biology of this species. Moreover, because virtually each individual in a population can be identified, paternity and maternity of seedlings and saplings can be determined, gene flow can be accurately estimated, and effective population sizes determined. We are currently using SSRs to estimate these parameters in tropical trees to assess the effect of deforestation and forest fragmentation on natural populations.

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