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Genetic diversity of *Castanopsis acuminatissima* (Bl.) A. DC. in northern Thailand and the selection of seed trees for forest restoration

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Abstract. *Castanopsis acuminatissima* (Bl.) A. DC. is one of a number of 'framework species' which are being planted to restore seasonally dry tropical forests in northern Thailand. This study describes the level of microsatellite variation within and among three populations of this species in three National Parks in northern Thailand: Doi Suthep-Pui, Doi Inthanon and Jae Sawn, using published primers developed for *Castanopsis cuspidata* var. *sieboldii* Nakai. The five microsatellite loci employed in this study detected a total of 54 alleles ($n = 72$). The informativeness of the microsatellite loci varied from six to 18 alleles, with an average of 10.8 alleles found over all loci. The mean observed heterozygosities in the three populations showed no significant deviations from Hardy-Weinberg expectations. The vast majority of genetic diversity was contained within the populations, with no significant differentiation between them ($F_{ST} = 0.006$). Algorithms were designed to capture microsatellite diversity, and the rationale for using microsatellite markers to inform genetic conservation is discussed. The implications for seed collection of *C. acuminatissima* for forest restoration are also discussed.

Introduction

Forest restoration measures are necessary to counter the continued disappearance of forests in tropical Asia, which is estimated at 1.1% annually (1990–95) (FAO 1997). The 'Framework Species Method' was developed within the Queensland Wet Tropics World Heritage Site (Goosem and Tucker 1995; Lamb et al. 1997; Tucker and Murphy 1997), and has since been applied to the seasonally dry forests of northern Thailand (Blakesley et al. 2002; Kerby et al. 2000). This method involves planting mixtures of 20–30 native tree species to provide a framework for re-establishing biodiversity. Tree species are selected for their fast growth, spreading canopy (for suppression of weed growth), ease of propagation and provision of resources for wildlife (particularly fruit) at an early age. The planted trees re-establish basic forest structure and functioning, and frugivores attracted by such resources disperse the seeds of additional non-planted tree species into the planted sites, thus accelerating the return of biodiversity.

An important issue which should be addressed by any forest restoration programme is the selection of parental plant material. Genetic variation in a founding population is critical, particularly if restored areas are not within the range of wild pollen sources. The Convention on Biological Biodiversity (Rio de Janeiro, Brazil 1992) emphasised the importance of maintaining intraspecific genetic diversity and evolutionary potential. The collection of plant material from a few individuals can result in low effective population size, severe inbreeding depression and a decrease in the adaptive evolutionary potential of the population (Barrett and Kohn 1991). Molecular techniques provide a valuable tool for measuring the genetic diversity of trees, thus contributing to decisions to enable better genetic management for forest restoration.

Microsatellites, which consist of tandemly reiterated, short DNA sequence motifs are highly informative, PCR-based molecular markers. They are considered to be neutral DNA markers (Nauta and Weissing 1996) which should therefore be used with caution in conservation genetics (Hedrick 2001). Whilst microsatellites have been used to assess genetic variation in tropical tree species in threatened forests and forest fragments (Aldrich et al. 1998), there have been no reports of their use to contribute to the selection of parent trees with a broad genetic base from which to collect seed for replanting to restore natural forest.

Nursery and field trials in deforested sites within Doi Suthep-Pui National Park identified *Castanopsis acuminatissima* (Bl.) A. DC. (Fagaceae) as meeting all the criteria of a framework species (Elliott et al. 2002; Kerby et al. 2000). It is distributed through north-eastern India, Indo-China, Malaysia, northern Sumatra, western Java, Sulawesi, New Guinea, New Britain and Taiwan. It occurs in evergreen and deciduous forest, evergreen forest, and evergreen and pine forest, at elevations of 760–2100 m asl (Kerby et al. 2000). It is also used by local people as wood for medium to heavy construction, furniture, plywood, veneer and firewood. Cut branches are used to cultivate mushrooms and the bark is a laxative, which is chewed with betel nut. However, no studies addressing the issue of conservation of genetic diversity have been carried out on the selection of seed trees of *C. acuminatissima* for forest restoration. Seven microsatellite primers have been isolated and characterised from *Castanopsis cuspidata* var. *sieboldii* Nakai (Ueno et al. 2000), but none of these have been tested for cross-species amplification in other *Castanopsis* species. The main aims of this study were: to investigate the conservation of seven microsatellite loci identified in *C. cuspidata* (Ueno et al. 2000); to assess genetic diversity within and between three national parks in the north of Thailand; and to consider how this genetic information might contribute to the selection of seed trees for forest restoration.

Materials and methods

Source of plant material

Plant material was collected from three sites in northern Thailand. Doi Suthep-Pui

Table 1. Summary of primer pair characteristics and heterozygosity of five microsatellite loci studied over all populations

Locus‡	Repeat motif	Annealing temp (°C)	Size range of products (bp)	No. of alleles	Mean A†	H_E	H_O*
Ccu16H15	$(TC)_{16}$	60	115–139	10	8.00	0.743	0.750
Ccu17F15	$(TC)_{34}$	52	89–103	6	4.00	0.566	0.329***
Ccu28H18	$(CT)_{26}$	65	100–136	9	6.33	0.683	0.286***
Ccu33H25	$(TG)_{11}(TC)_{15}$	67	186–236	18	12.67	0.769	0.729
Ccu5F45	$(CT)_{40}$	45	292–320	11	8.33	0.821	0.493***

‡ (Ueno et al. 2000) † Mean A = mean no. alleles per locus per population * significant departure from Hardy-Weinberg equilibrium (*** $P < 0.001$).

National Park (18°43′ – 19°08′N, 98°48′ – 98°58′E), Doi Inthanon National Park (18°24′ – 18°41′N, 98°21′ – 98°38′E) and Jae Sawn National Park (18°30′ – 19°05′N, 99°25′ – 99°30′E). Young leaves of *Castanopsis acuminatissima* were collected in September 2000 from 25 trees in Doi Inthanon National Park, 22 trees in Jae Sawn National Park and 25 trees in Doi Suthep-Pui National Park. Trees sampled were at least 100 m apart.

Microsatellite analysis

Total genomic DNA was extracted from fresh leaves (0.2 g) of *C. acuminatissima* using the hexadecyltrimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980), with the following modifications. 800 μl CTAB buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris (pH 8.0), 2% of PVP 40 (polyvinylpolypyrrolidone; mol wt. 40000) and 1% β-mercaptoethanol) pre-heated to 65 °C, was added to 0.2 g frozen, ground material, mixed and incubated at 65 °C for three minutes. The samples were extracted with chloroform:isoamyl alcohol (24:1) and the DNA precipitated in 500 μl cold isopropanol. The DNA pellet was washed in 500 μl 70% ethanol, air dried and resuspended in 50 μl TE (10 mM Tri-HCl, 0.1 mM EDTA, pH 8.0) containing 10 $\mu g.\mu l^{-1}$ RNAse. Incubation at 37 °C for 20 min removed RNA prior to storage at 4 °C, or longer term storage at −20 °C. The DNA was quantified against lamda standards on agarose gels stained with ethidium bromide.

Seven microsatellite primers reported to identify a high degree of polymorphism in *Castanopsis cuspidata* var. *sieboldii* Nakai (Ueno et al. 2000) were selected for the amplification reactions (Table 1). DNA extracted from 72 individuals was screened with these primers. PCR amplification was performed in a 12.5 μl reaction mix containing 10 ng of DNA, 45 mM Tris-HCl pH 8.0, 12 mM ammonium sulphate, 4.5 mM $MgCl_2$, 4 μM EDTA, 0.114 ng BSA, 0.0005% β-mercaptoethanol, 0.2 mM of each dNTP, 0.2 um forward primer labelled with $\gamma^{33}P$, 0.2 um reverse primer and 0.025 units of Taq polymerase (Gibco-BRL). The following conditions were used: an initial denaturation step at 94 °C for 3 min, followed by 35

cycles of 94 °C for 30 s, annealing temperature (see Table 1) for 1 min, 72 °C for 1 min 30 s. A final extension at 72 °C for 8 min was included.

One volume of 80% formamide containing 0.5% bromophenol blue and xylene cyanol was added to the PCR products. The microsatellites were denatured at 90 °C for 3 min and separated on a 6% denaturing sequencing gel in 1 × TBE buffer at a constant 65 W for 2.5 hours (Sambrook et al. 1989). The gel was dried on Whatman 3 MM paper and the microsatellites visualised following autoradiography (Kodak Biomax MR). The size of each fragment was estimated against a 10 bp ladder (Gibco-BRL).

Genetic diversity

The mean observed heterozygosity (H_O, direct count estimate) and Hardy-Weinberg expected heterozygosity (H_E), inbreeding coefficients (F_{IS}, F_{IT} & F_{ST}) (Wright 1965) as well as the Rho correlation coefficient (R_{ST}) were computed using GENEPOP computer program (version 3.3, Raymond and Rousset 1995) for each locus and averaged over all loci. A bilateral test for departure from Hardy-Weinberg equilibrium was performed again using GENEPOP. The expected frequency of null alleles was estimated using Identity 1.0 (Wagner and Sefc 1999) and an exact binomial test was carried out to estimate the goodness of fit of observed frequencies of putative null alleles to expected frequencies. The distribution of alleles in the different populations was classified in two frequency classes as suggested by Marshall and Brown (1975).

Two algorithms were designed to select seed trees based on their individual genotype (algorithm I), and to randomly select seed trees from a population of unknown genetic makeup (algorithm II). Both algorithms were implemented in GENSTAT computer program (version 5.2, Payne and other Genstat Committee members 2000). Algorithm I was designed to find the minimum size selection, which would contain 100% of allele diversity, considering all of the seed trees, and including a decision rule to keep or remove each item in turn. An item was removed from the list if its removal did not imply any loss of alleles. To ensure a minimum sample size at the same time, the status of genotypes concentrating the rarest alleles was checked first. Algorithm II makes a selection without preference from a population of trees assuming the same relative allele frequencies as in the original data set. The population is taken as infinite so that the selection of individuals did not affect the allelic frequency in the population. This algorithm was designed to provide expected diversity as a function of the selection sample size.

Results

Genetic diversity

All seven primer pairs from *Castanopsis cuspidata* amplified polymorphic loci, although two, Ccu22F30 and Ccu9T20 were not included in the study due to

Table 2. Measure of microsatellite DNA genetic diveristy in the three populations

	Doi Suthep-Pui	Jae Sawn	Doi Inthanon	Total
No. of trees	25	22	25	72
Allele number over 5 loci	41	37	41	54
Unique alleles	5	3	6	14
Alleles common to all populations	25	25	25	25
Mean no. of alleles per loci	8.2	7.4	8.2	10.8
H_E	0.675	0.740	0.731	0.716
H_O	0.537**	0.537***	0.483***	0.519***
F_{IS}	0.205	0.274	0.340	0.276

(**$P < 0.01$; ***$P < 0.001$) significant departure from Hardy-Weinberg equilibrium.

Table 3. Distribution of 54 alleles in allele frequency classes in the three populations, and combined population

Allele frequency class	Number of alleles (% of total number of alleles)			
	Doi Suthep-Pui	Jae Sawn	Doi Inthanon	Combined
Total no. alleles	41	37	41	54
Common ($P \geq 0.05$)	21 (0.512)	19 (0.514)	20 (0.488)	19 (0.352)
Rare ($P < 0.05$)	20 (0.488)	18 (0.486)	21 (0.512)	35 (0.648)

complex banding patterns. No more than two clearly discernible alleles were amplified for each tree/primer pair combination. The five loci were found to be highly polymorphic, with six to 18 alleles detected. A total of 54 alleles were identified among the 72 trees, representing the three populations. The observed heterozygosity (H_O) for each locus ranged from 0.286 to 0.750 (Table 1). The observed heterozygosities significantly deviate from Hardy-Weinberg expectations for locus Ccu17F15, Ccu28H18 and Ccu5F45 (heterozygote deficiency, $P < 0.001$). Based on Wright's F_{IS} values (Table 4) the deviations are due to an excess of homozygotes (positive F_{IS} values). The influence of the presence of null alleles had to be tested where deviation from HW equilibrium was observed (3 loci out of 5). Using Identity 1.0 (Wagner and Sefc 1999) the frequency of null alleles for loci Ccu17F15, Ccu28H18 and Ccu5F45 were 0.15, 0.23 and 0.17 respectively. Expected frequencies for the same loci were respectively 0.00, 0.17 and 0.20. An exact binomial test showed that the observed deviation of heterozygotes from Hardy-Weinberg expectations could be explained by putative null alleles.

The three populations exhibited similar degrees of allelic richness, ranging from 37 alleles in Jae Sawn to 41 alleles in Doi Inthanon and Doi Suthep-Pui (Table 2). The mean number of alleles per locus was also similar between the three populations (Table 3). For each locus, the number of unique alleles was two for Ccu16H15, two for Ccu17F15, two for Ccu28H18, six for Ccu33H25 and two for Ccu5F45. In total, six of the alleles present in the Doi Inthanon population were unique to that population, in comparison to five in Doi Suthep-Pui and three in Jae Sawn.

The distribution of the alleles when classified as rare or common showed similar distributions between the three populations and were almost equally distributed

Table 4. Estimates of the parameters F_{IS}, F_{IT}, F_{ST} and R_{ST}

Locus	F_{IS}	F_{IT}	F_{ST}	R_{ST}
Ccu16H15	−0.005	−0.012	−0.006	−0.017
Ccu17F15	0.4278	0.415	−0.022	−0.026
Ccu28H18	0.579	0.583	0.010	−0.033
Ccu33H25	0.028	0.064	0.038	0.002
Ccu5F45	0.401	0.399	−0.003	−0.012
All	0.276	0.280	0.006	−0.018

between the common class and the rare class (Table 3). All of the trees had a unique combination of alleles, and consequently could be distinguished from each other.

For each population, over all the loci, the expected heterozygosity (H_E) was significantly higher ($P < 0.01$) than the observed heterozygosity (H_O) leading to positive inbreeding coefficients (F_{IS}). For each population, the observed heterozygosity ranged from 0.483 to 0.537, with an average heterozygosity of 0.519 (Table 2), indicating considerable genetic diversity within the populations. The levels of inbreeding for each population ranged from 0.205 to 0.340, with an average inbreeding coefficient of 0.276 (low level of inbreeding) (Table 2). Over the three populations, the F-statistic analysis showed positive levels of F_{IS}, F_{IT}, and F_{ST} (0.276, 0.280 and 0.006 respectively) (Table 4). The overall F_{ST} for the three populations was 0.006 (not significantly greater than zero), indicating that only 0.6% of the variation was attributable to differentiation among the populations. Several estimated F_{ST} values were negative (Table 4), which might be expected when the parameter to estimate is zero. R_{ST} values ranged from -0.033 to 0.026, with the overall value for the three populations of −0.018 (Table 2), confirming that there is no significant genetic differentiation among the populations.

Selection of seed trees to capture microsatellite allelic diversity

Applying Algorithm I, a minimum of 24 trees captured all of the alleles in the combined sample of 72 trees. Algorithm II was employed to predict the number of trees which would capture a given percentage of genetic diversity of a random sample of trees, assuming the whole set of seed trees would describe a true infinite population. A predictive curve was plotted for the overall population (Figure 1). Twenty-four trees for example, sampled blind would capture approximately 60% of microsatellite allelic diversity, compared with 100% under algorithm I assumptions (Figure 1). The lack of individual genetic information on parent trees evaluated using algorithm II compared to those evaluated using algorithm I, leads to a 40% decrease of the expected allelic diversity.

Discussion

This paper represents the first report of the use of molecular markers to examine levels of genetic diversity in *Castanopsis acuminatissima*. The five microsatellite

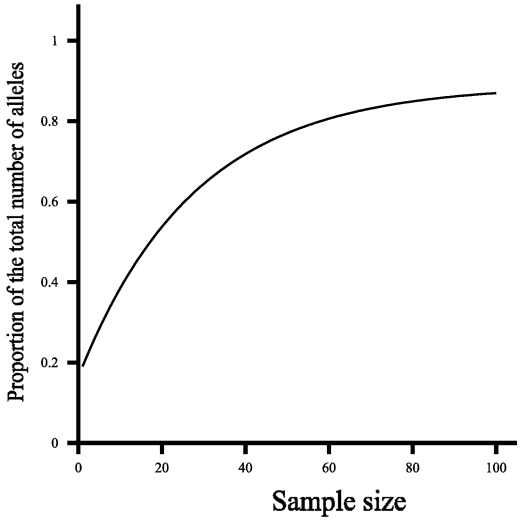

Figure 1. Selection of parent trees from a population with the same relative allele frequencies as the combined *C. acuminatissima* population sampled from three National Parks, using algorithm II. Expected proportion of the total number of alleles for sample sizes from 1 to 100 are shown. Each sample element was drawn with replacement assuming an infinite size population. Empirical distributions were obtained from 500 samples for each size and each site. Expected proportions displayed on the figure are 5th percentiles of these distributions.

loci employed in this study detected a total of 54 alleles ($n = 72$). The informativeness of the microsatellite loci varied from six to 18 alleles, with an average of 10.8 alleles found over all loci. Ueno et al. (2000) detected a total of 70 alleles in *C. cuspidata* with these five primers, with an average of 14.0 alleles found over the five loci, and 13.9 over seven loci ($n = 32$). For both *Castanopsis* species, this is relatively high, and accords with the mean number of alleles per locus in other tropical tree species, for example 8.75 alleles found over four loci in *Neobalanocarpus heimii* (King) Ashton (Dipterocarpaceae) ($n = 30$) (Konuma et al. 2000), 9.7 alleles found over four loci in *Swietenia humilis* Zucc. (Meliaceae) ($n = 88$) (White and Powell 1997) and 8.2 alleles found over five loci in *Prunus cerasoides* D. Don (Rosaceae) ($n = 82$) (Pakkad et al. 2003).

Our work also demonstrates how useful heterologous amplification using published primers from closely related species can be in allowing some genetic analysis to be undertaken to support a practical forest restoration programme. Published primers avoid the time and costs associated with generating and sequencing a new microsatellite library. This accords with other studies on tropical trees, for example our own work with *Prunus cerasoides* utilises primers developed with several temperate *Prunus* species Pakkad et al. (in press) and Khadari et al. (2001) reported that *Ficus carica* microsatellites gave amplification products in 17 other *Ficus* species in 86% of the cases.

The range of heterozygosity is quite broad, which suggests that overall diversity

values, as a measure of observed heterozygosities, are dependant on which loci are selected. There were significant deviations from Hardy-Weinberg expectations at three out of five loci. With *Swietenia humilis*, White and Powell (1997) also reported that some individual loci exhibited significant deviations from Hardy-Weinberg expectations, which were due to an excess of homozygotes. No significant deviations from Hardy-Weinberg expectations were found for the mean observed heterozygosities in the three populations. The levels of heterozygosity (H_O) detected in the present study for each population, over all loci indicate that each had a high level of genetic variation. Based on Wright's *F*-values, the populations appeared to be outbreeding, with some inbreeding. However, if there was a considerable amount of inbreeding, it might be expected to be evident across all of the loci, which was not the case here. It is possible that the deviations from Hardy-Weinberg which we observed with three loci were due to the presence of null alleles, as indicated by the putative null allele frequencies. However, the heterozygote deficiency could also be due to the population structure and consequently an examination of larger natural populations, or the inclusion of more microsatellite loci will be necessary to address this issue.

The value of F_{ST} over the three populations was 0.006, indicating that the vast majority of genetic diversity was contained within the populations, with no differentiation between the three populations. This accords with Hamrick and Godt (1990) who generalised that in outcrossing species, the majority of genetic diversity resides within populations, rather than between populations (F_{ST}), as would be the case with a selfing species. No similar studies of genetic diversity have been undertaken with other *Castanopsis* spp using microsatellites.

We believe that the relatively simple study of the genetic diversity of *C. acuminatissima* presented here enables a more informed selection of seed trees in our forest restoration programmes. Firstly, the F_{ST} value indicates that there is no differentiation between the three populations, hence seed may be collected and moved between them. There are large areas of degraded forestland outside the National Parks, which are devoid of local sources of seed. Seed could be supplied to these areas from one or more of our study sites. Secondly, the data suggests a large amount of genetic diversity in *C. acuminatissima* because of the high number of rare microsatellite alleles. It is important that, in a forest restoration programme such as ours, some consideration is given to the conservation of genetic diversity. Seed collection is a crucial element, and yet there is no clear advice to help seed collectors select seed trees from tracts of intact forest for use in small scale forest restoration programmes. Our data suggests that seed should be collected from as many trees as possible, certainly within, or close to the FAO recommendation of 25–50 individuals per population (FAO Forest Resources Division 1995).

The question also arises; can microsatellite diversity be used more directly to select individual seed trees? It has been frequently stated that genetic conservation should be concerned with the conservation of 'adaptive genetic diversity' (Ennos et al. 2000; Hedrick 2001; FAO 2001). Evaluation of allelic diversity of adaptive genes necessitates expensive technologies comprising expressed sequence tag sequencing, QTL and candidate gene mapping, followed by the determination of

allelic diversity for these candidate genes (FAO 2001). These are clearly impractical for small scale forest restoration programmes. Microsatellites are considered by many to be ''absolutely'' neutral markers (e.g. Schlötterer and Wiehe (1999)). In contrast, there is increasing evidence which indicates that microsatellites are functionally important in regulating genome size, DNA structure, gene expression, human neurological disorders and cancers (see review, Kashi and Soller (1999), Li et al. (2002)). Numerous lines of evidence demonstrate that SSR genomic distribution is *nonrandom* across protein coding, untranslated and untranscribed regions (Li et al. 2002). Furthermore, it has been proposed that microsatellites may be a major source of adaptively meaningful genetic diversity facilitating Darwinian evolutionary adaptation (e.g. Kashi et al. 1997; Kashi and Soller 1999; King and Soller 1999; Marcotte et al. 1999; Moxon and Wills 1999; Li et al. 2000a, 2000b, 2002; Wren et al. 2000). There are examples of a high correlation between microsatellite and adaptive (MHC) loci in Gila topminnow (*Poeciliposis o. occidentalis*) (Hedrick et al. 2001) and bighorn sheep (*Ovis canadensis*) (Gutierrez-Espeleta et al. 2001).

Consequently we believe that microsatellite data can 'inform' a genetic conservation programme 'at this time', in the absence of more sophisticated genetic data, through the selection of individuals to capture microsatellite allelic diversity. We have applied an algorithm (I) to the combined *C. acuminatissima* sample of 72 trees, and found that 24 identified trees capture all of the microsatellite allelic diversity. Using a second algorithm (II), we were able to show that a sample of 24 trees selected 'blind' from the forests would be predicted to capture 60% of diversity. In view of the rarity of many alleles, future work could include the analysis of more trees, or the modification of the algorithms to capture alleles which occur more frequently in the population.

The genetic information reported in this paper will clearly influence the selection of *C. acuminatissima* seed trees for practical forest restoration in these three locations and their surrounds. Molecular studies should ideally be carried out in conjunction with morphological and phenotypic studies (Brown 1978). When selecting parent seed trees for forest restoration sites, it is important to also consider phenotypic characters, fitness and growth traits. For example, survival and growth rates in the field show adaption to the site, and are important for management and effectiveness of the restoration process. As a continuing part of this research programme, seeds from parent trees sampled are being trialled in our nursery and in field plots. We will compare the genetic data reported here with seedling performance data. This will enable a more robust, practical procedure for identifying parent seed trees to supply seedlings for local restoration programmes.

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