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Extreme ecological and phenotypic differences in the tropical tree chicozapote (*Manilkara zapota* (L.) P. Royen) are not matched by genetic divergence: a random amplified polymorphic DNA (RAPD) analysis

H. J. HEATON, R. WHITKUS and A. GÓMEZ-POMPA
Department of Botany and Plant Sciences, University of California, Riverside, CA, USA

Abstract

The chicozapote (*Manilkara zapota*) is a tropical fruit tree that occurs in two morphologically distinct populations in the Yucatan peninsula of Mexico. Forest populations consist of tall, straight trees, while swamp populations have a short, shrub-like growth form. Swamp populations also have smaller leaves, fruit and seeds. We performed a random amplified polymorphic DNA (RAPD) analysis on four different populations of chicozapote to test if there was a genetic component to this variation. The populations differed in respect to habitat type (swamp vs. forest) and geographical location (east vs. west). We surveyed 80 random primers, nine of which revealed interpopulation band differences (28 band differences in total). Unweighted pair group method analysis (UPGMA) and neighbour-joining dendrograms showed no separation of individuals between the different populations. Analysis of the RAPD data showed no significant differences between swamp and forest populations ($P > 0.1$). The lack of genetic differentiation suggests a failure to find a correlation between the RAPD loci and adaptive traits. The observed morphological differences between the swamp and forest populations of chicozapote may either be that gene flow has prevented a build-up of neutral marker differences or a plastic response to differences in habitat.

Keywords: chicozapote, *Manilkara zapota*, population differentiation, RAPD

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Introduction

The chicozapote is an increasingly important fruit tree crop in India, Mexico and other tropical regions of the world. Over 23 000 ha of land in India is devoted to production of the fruit, a 10-fold increase from just 20 years ago (Chadha 1992). The tree thrives on a wide variety of soils and water regimes, and is resistant to salinity, drought, insects and pathogens. In the Yucatan peninsula of Mexico, morphologically distinct, native populations are found in forests and periodically inundated swamps. The populations differ noticeably in plant height, architecture, fruit size and seed size (Heaton, personal observation). Mature trees in swamp populations are smaller in size than their forest counterparts (10 m vs. 50 m) and have smaller leaves, fruits and seeds. Plants in swamp populations have a shrub-like growth form, with branching occurring very close to the ground. In contrast, trees in forest populations have an upright, straight growth form with an unbranched main trunk. The swamp habitat is characterized by an open canopy, seasonal inundation and thin or no soil. Forest sites are characterized by dense canopies, little or no inundation and moderate amounts of soil. Swampy regions occur in much of the Yucatan peninsula, and are inadequate for most traditional agricultural uses (corn, beans and squash). Given its ability to thrive in swampy conditions, the chicozapote could become a valuable crop plant in these areas.

The chicozapote is a variable, outcrossing species, which has historically led to many subdivisions of the original species as named by Linnaeus. In Pennington's 1990 monograph on the Sapotaceae, 57 synonyms were listed for *Manilkara zapota*. Most of the synonyms were based on minor variation in floral characters that was described by Pennington as being within the normal

Correspondence: Richard Whitkus, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA. Fax: +01-909-787-4437; E-mail: whitkus@moe.ucr.edu

range of an outcrossing species. Based upon the observed morphological variation between swamp and forest populations in the Yucatan peninsula, the populations may in fact be genetically differentiated ecotypes (Turesson 1922a,b) or may be expressing phenotypic plasticity in response to the different environments. To date, no studies on the population genetics of *M. zapota* have been published, and we therefore attempted to learn more about this important plant by examining its genetic variation.

This study addresses the following question: Is there a genetic correlate to the extreme morphological differentiation observed between swamp and forest populations of *M. zapota*? If this correlate is found, it opens the possibility of introducing swamp survival traits into agricultural varieties, allowing the fruit to be cultivated in swampy areas. In order to test this question, genetic variation was analysed using random amplified polymorphic DNA (RAPDs) (Williams *et al*. 1990). The use of RAPDs has many advantages for detecting genetic variation (Williams *et al*. 1990; Hadrys *et al*. 1992) amongst which are: relatively low cost; no radioactivity; a low sample DNA requirement; and the high frequency of polymorphic bands revealed. Another advantage is that it is not necessary to carry cumbersome containers of dry ice or liquid nitrogen for sample tissue preservation, a significant benefit given the remote locations of this study. It was possible to desiccate the samples using fine-grained silica gel, which preserved the DNA for several weeks – long enough to return the samples to the laboratory.

Previous investigations have proven the utility of RAPDs to investigate intraspecific genetic variation. This technique has been used to identify closely related cultivars of alfalfa (*Medicago sativa*) (Yu & Pauls 1993) and oilseed rape (*Brassica napus*) (Lee *et al*. 1996). It has also been used to discover relationships among cultivars of cocoa (*Theobroma cacao*) (Wilde *et al*. 1992) and alstroemeria (*Alstroemeria* spp.) (Dubouzet *et al*. 1997). Schierenbeck *et al*. (1997) used RAPDs to characterize diversity in tropical tree species along an elevation gradient, and Lashermes *et al*. (1996) investigated diversity with RAPDs in cultivated and wild coffee (*Coffea arabica*). To quickly evaluate genetic variation among populations, the technique of bulked segregant analysis was used, as described by Michelmore *et al*. (1991). In this technique, DNA from each individual in a population is combined into a single sample (bulk). Primers can then rapidly be screened for variation without screening each individual for every primer.

Materials and methods

Study sites and sample collection

Four populations of chicozapote were chosen for the study. Two were located in the El Eden Ecological Reserve, a privately owned reserve 20 km west of Cancun, Mexico, in the northeast Yucatan peninsula. The first of these (site 1) was a forest population, and the second (site 2) a swamp population ≈ 2–3 km from the forest. The third and fourth populations were located at Chunchucmil and Hocaba, Mexico, in the northwest Yucatan peninsula near the capital city of Merida. The Chunchucmil population (site 3) was located in an inundated swamp, and the Hocaba population (site 4) consisted of cultivated trees (of the forest phenotype) in the home gardens of town residents.

The selection of these four sites allowed us to study variation in habitat type (i.e. swamp vs. forest vs. garden), as well as geographical variation (El Eden, east vs. Chunchucmil and Hocaba, west). At each of the two El Eden sites (forest and swamp), samples were collected from 30 individuals along a 2-km stretch of trail. At Chunchucmil, samples were taken from 10 individuals along a prehistoric roadway. At Hocaba, samples were taken from 10 individuals in six different gardens. Approximately 2 g of leaves was collected from each individual and placed into plastic bags containing fine-grained silica gel. The silica gel rapidly desiccated the leaf samples and preserved the DNA until the samples could be processed in the laboratory.

Genomic DNA extraction

Genomic DNA was isolated from leaves with a modified CTAB extraction protocol (Saghai-Maroof *et al*. 1984). For each sample, 0.4 g of desiccated leaf material was ground to a fine powder with a mortar and pestle in liquid nitrogen. While the powder was still frozen, 2 mL of CTAB buffer (100 mmol/L Tris-HCl (pH 8), 2% w/v mixed alkyltrimethyl-ammonium bromide, 1.4 mol/L NaCl, 20 mmol/L EDTA (pH 8), 4% w/v PVP-40, 0.1% w/v ascorbic acid, 0.1% w/v diethyldithiocarbamic acid (DIECA) and 1% v/v 2-mercaptoethanol) was added and the resulting mixture was ground for 30 s. The mixture was transferred to 12-mL polypropylene tubes, with the remaining steps following the protocol described in Saghai-Maroof *et al*. (1984). The resulting DNA pellets were transferred to microcentrifuge tubes and suspended in 50 μL of TE (10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA, pH 8), followed by the addition of 1 μL of RNase (1 mg/mL) and incubation at 38 °C for 1 h. Protein was removed by extracting once with phenol–chloroform and once with chloroform–octanol (24:1). Total genomic DNA was precipitated with ice-cold ethanol and 0.3 M sodium acetate. Pellets were washed with 70% ethanol, air dried and dissolved in 50 μL of TE. Sample DNA concentrations were measured with a Hoefer TKO100 Mini-Fluorometer, following the manufacturer's instructions. Prior to use for RAPD analysis, samples were diluted to a DNA concentration of 5 ng/μL.

RAPD generation

RAPD amplification reactions (25 µl final volume) contained 25 ng DNA, 100 µM each of dATP, dCTP, dGTP and dTTP, 200 µM 10 bp primer (Operon Technologies Inc.), 1× reaction buffer, 2 mM $MgCl_2$ and 0.65 units of *Taq* DNA polymerase (Gibco BRL). Each reaction was overlaid with 30 µL of mineral oil to prevent evaporation. For enzymatic amplification, samples were subjected to 45 cycles of: 1 min at 94 °C, 1 min at 38 °C, 30 s at 54 °C, and 2 min at 72 °C, with a final extension of 72 °C for 13 min, in a MJ Research Inc. model PTC-100 thermocycler. Fragments generated by amplification were separated (according to size) on 2% agarose gels, stained with ethidium bromide, and visualized and photographed under UV light.

Bulking of DNA and screening of bulks

To create the DNA bulks for each population, 5 µL of 5 ng/µL DNA from every individual was combined into a single microcentrifuge tube. The contents of the tubes were briefly mixed and held at 4 °C overnight to allow the DNA to evenly disperse throughout the solution. Bulked DNAs were amplified with 80 random primers (Table 1) to identify primers that revealed interpopulation variation. When a primer showed a polymorphism between samples, DNA samples of every individual in each of the four populations were amplified, using that primer, in order to score population variation.

Data analysis

Individuals were scored for polymorphic loci revealed by the bulk analysis as either present (**a**) or absent (**t**), allowing the data to be imported into the MEGA program package (Kumar *et al*. 1993). Pairwise distances among all individuals were calculated as the proportion of shared **a** scores and **t** scores subtracted from 1 (equivalent to Manhattan distance, Sneath & Sokal 1973), and used to construct unweighted pair group method analysis (UPGMA) and neighbour-joining (Saitou & Nei 1987) dendrograms. Trees were constructed using all individuals (sites 1–4) to determine whether individuals would sort into geographical region, habitat, or site. A second set of trees were produced with individuals from El Eden populations only (sites 1 and 2) to determine whether they would sort into their respective habitats.

A significance test of the degree of differentiation between the El Eden swamp and forest populations was performed using the procedure of Raymond & Rousset (1995a). The test consisted of a 2×2 contingency table for each of the 28 RAPD bands with the number of presences and absences in paired cells for each site. Using a Markov chain method implemented in the STRUC program of GENEPOP (Raymond & Rousset 1995b), an unbiased estimate of the Fisher's exact test on an $R \times C$ contingency table was performed to test the null hypothesis of no differentiation between the two sites. The significance values obtained for individual RAPD bands were combined using Fisher's combined probability test (Fisher 1932) to give a one-tailed χ^2-value of overall differentiation with $2n$ degrees of freedom.

Results

A total of 28 interpopulation band differences were found with the 80 primers surveyed from the bulked DNA samples of the four populations (primer: number of bands; A19: 1; B09: 2; C10: 2; C14: 5; D12: 1; D16: 5; D20: 1; G5: 7; G12: 4). The observation of bands across all individual samples for each primer did not suggest codominant banding patterns. As RAPD bands are typically inherited as dominant Mendelian loci (Williams *et al*. 1990), and the RAPD patterns in this study appeared to concur with these findings, the banding patterns were putatively interpreted as dominant loci.

Although RAPD primers were chosen on the basis of interpopulation variation from the screening of bulked DNAs, scoring of individuals within populations revealed few fixed differences. Most bands showed polymorphisms

Table 1 Primers used to survey population bulk samples for random amplified polymorphic DNA

Operon primer set	Primers tested
A	02, 04, 05, 07, 08, 09, 10, 11, 12, 13, 17, 18, **19,** 20
B	01, 02, 03, 04, 05, 06, 07, 08, **09,** 11, 12, 13, 14, 17, 19, 20
C	02, 03, 07, 09, **10,** 13, **14,** 16, 17, 18, 20
D	01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, **12,** 13, 14, 15, **16,** 17, 18, 19, **20**
E	02, 06, 08, 09
F	01, 03, 04, 06, 07, 08
G	02, 03, 04, **05,** 10, 11, **12,** 20
Z	15

Primers that provided polymorphisms are in bold.

for presence/absence within populations. A total of six RAPD bands were fixed in one or two sites, and these were fixed absences (site: primer; 3: D12; 4: D20, G05, G12; 3 and 4: C14, G05).

All of the trees showed that individuals could not be separated into geographical region (west vs. east Yucatan), habitat (swamp vs. forest vs. garden) or site, based on the 28 RAPD bands (Fig. 1). The neighbour-joining tree showed that terminal branches tended to be longer than basal branches, suggesting that most of the RAPD distance in the data set exists between individuals rather than between groups. This observation is supported by the result of the test of differentiation. The El Eden swamp and forest populations were not significantly differentiated ($\chi^2 = 68.35$, $P > 0.1$, d.f. = 56) on the basis of the 28 RAPD loci.

Discussion

The lack of separation in the dendrograms reflects a lack of genetic differentiation, as measured by RAPDs, at the population level. Based on the loci examined in this study, variation among populations is not greater than among individuals. This is a common phenomenon in outcrossing woody plants such as the chicozapote. Other researchers have found that populations of outcrossing woody plants typically display greater genetic variation (allozymes) within populations than between popula-

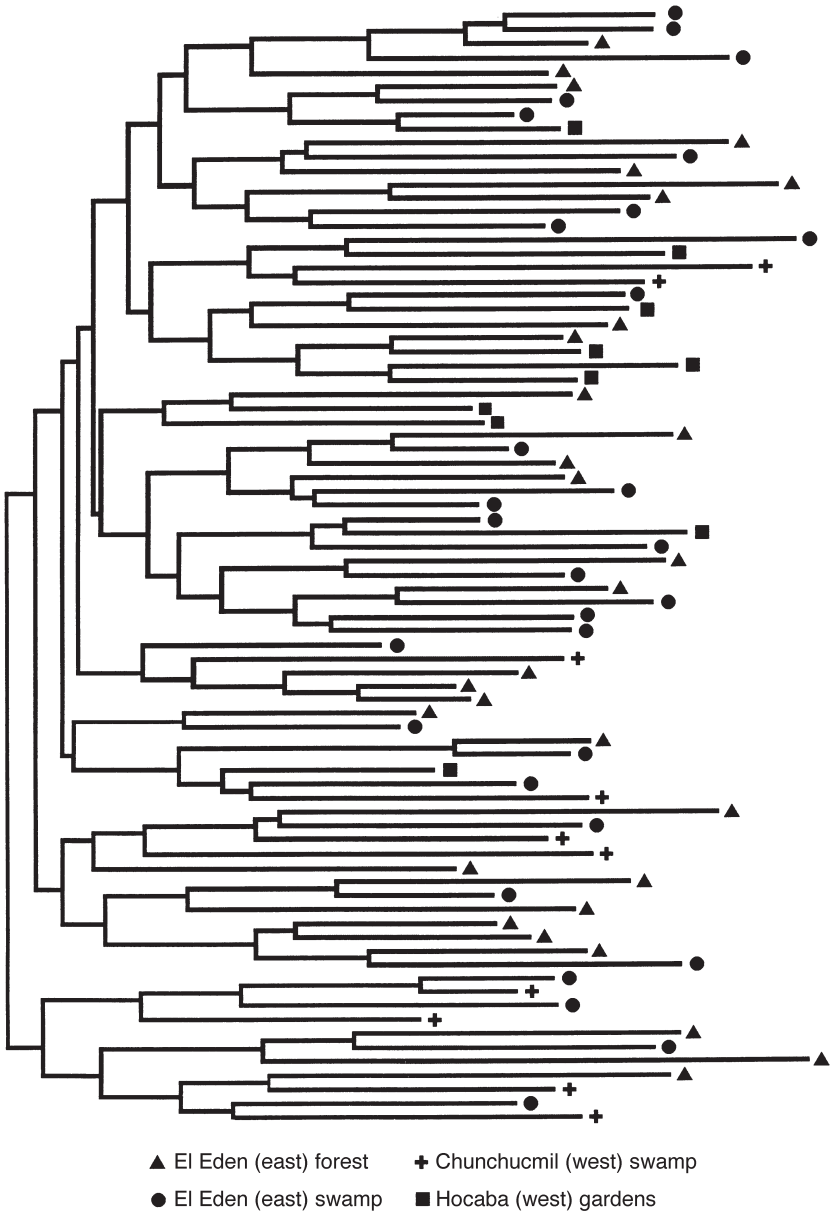

Fig. 1 Neighbour-joining tree of four study populations.

tions (Hamrick & Godt 1996). Erstad (1996) studied vegetative and generative characters in ecotypes of *Ribes rubrum* in Norway and found greater variation within populations than between populations in outcrossing woody species. Yeh *et al*. (1995) found similar results in a RAPD study of aspen trees (*Populus tremuloides*). In a study of four tropical tree species in Costa Rica, Schierenbeck *et al*. (1997) found that the species exhibited a high level of genetic variation (RAPD) between individuals, but found no population genetic differences along an elevation gradient. They hypothesized that tropical trees, in general, have large gene pools, possibly as a result of effectively widespread pollen and seed dispersal. Based on the studies referred to above, this statement could also be extended to many species of temperate trees. Chase *et al*. (1996) found evidence of long-distance gene flow with simple sequence repeats (SSR) in the moth-pollinated tropical tree *Pithecellobium elegans*. *Manilkara zapota* is believed to be pollinated by bats (Pennington 1990, 1991), and the large, sweet fruits are dispersed by birds and other large animals (Klimstra & Dooley 1990). This long-distance pollination and seed dispersal could explain why there is no observed genetic structure at the population level because gene flow tends to homogenize population genetic structure.

Another possibility is that the primers used in this study failed to find the essential genetic differences between the populations. The use of bulked DNA samples in the initial survey reduced the effort required to identify interpopulation polymorphisms and would accentuate overall genetic differences among populations. However, only six RAPD bands remained as fixed differences in the survey of individuals. One possibility for this finding is that the bulk DNA survey identified primers if they showed one band difference among populations, but multiple polymorphic bands were scored for each primer in the individual survey. A second reason is that a fixed difference among populations would be declared in the bulk DNA survey, even though the population contained individuals that lacked the band. These individuals would not contribute to the RAPD phenotype in the bulked DNA sample. Thus, the use of bulk DNA samples facilitates, but does not guarantee, the finding of genetic differences among natural populations.

Another disadvantage of using RAPDs is that they sample a relatively small portion of the genome and therefore the actual genetic differences between the swamp and forest populations of the species in this study may have been missed. Many of the polymorphisms revealed by this technique are likely to be neutral markers that are not correlated or linked to differences in phenotype or adaptation to habitat. Other studies involving genetic markers and morphological differences have found that there is often no correlation between phenotypic differences between populations and variation in genetic markers. Borghetti *et al*. (1993) found that variation in physical characters did not always correspond to variation in isozymes in a study of *Fagus sylvatica*. Venable *et al*. (1998) studied seed morphology and isozymes in *Heterosperma pinnatum* and concluded that the genetic distance between the study populations was not correlated with their morphology or geographical separation. Different genetic markers, such as restriction fragment length polymorphisms (RFLPs) and isozymes, may not correlate with each other or with phenotypic variation in populations (Beer *et al*. 1993). To address the lack of differentiation, additional primer sets may be screened to cover a greater proportion of the genome; alternative markers, such as isozymes, can be analysed; or common garden and reciprocal transplant experiments may be conducted to reveal differences between the populations that were not revealed with the markers employed in this study.

An alternative explanation for the lack of observed genetic differences between the swamp and forest populations of *M. zapota* is that the morphological differences are a result of phenotypic plasticity. Phenotypic plasticity has been found in several species of tropical and temperate trees for many traits, usually in response to changes in climate. In a study of seven European tree species, Kramer (1995) found phenotypic plasticity in response to temperature and photoperiod changes, Gray (1990) described plasticity in leaf and fruit characteristics of mulberry (*Morus* spp.), and Kitajima *et al*. (1997) found that the leaves of several species of tropical canopy trees showed seasonal variation in size and stomatal conductance. It is possible that the phenotypic differences observed between the swamp and forest populations are plastic responses to differences in habitat. We observed distinct differences in water and light levels between the forest and swamp habitats, as well as differences in the type and quantity of soil. The chicozapote can grow in various habitats around the world, from India to Mexico, and has shown sufficient variation in form to receive 57 synonyms since the initial description of the species (Pennington 1990). The wide natural distribution of chicozapote throughout southern Mexico and Central America, and the broad range of habitats in which the tree is found, support the idea that plasticity operates in this species.

Future studies on this plant should include reciprocal transplants of swamp and forest individuals, as well as common gardens of plants grown from seed. These additional experiments would give direct evidence of a presence or lack of a genetic correlate to the observed morphological variation between these populations. In addition, germplasm collections need to be made of the different forms of *M. zapota*, including the forest and swamp types, as well as of the different cultivated varieties. In India, which has the highest cultivation of

chicozapote, new varieties only arise by mutation because all trees are propagated vegetatively (Chadha 1992). A controlled breeding programme would be very useful for stabilizing traits of interest and developing new varieties of chicozapote. Finally, agricultural studies should be conducted with the different morphological types in the swamps and other marginal habitats of the Yucatan peninsula to determine the feasibility of chicozapote as a crop plant in these areas.

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