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Genetic variation among cultivated selections of mamey sapote (*Pouteria* spp. [Sapotaceae])

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

GENETIC VARIATION AMONG CULTIVATED SELECTIONS OF
MAMEY SAPOTE (*POUTERLA* SPP. [SAPOTACEAE])

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

by

Susan Carrara

2004

To: Dean R. Bruce Dunlap
College of Arts and Sciences

This thesis, written by Susan Carrara, and entitled Genetic Variation Among Cultivated Selections of Mamey Sapote (*Pouteria* spp. [Sapotaceae]), having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Richard Campbell

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Date of Defense: May 24, 2004

The thesis of Susan Carrara is approved.

Dean R. Bruce Dunlap
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Florida International University, 2004

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ABSTRACT OF THE THESIS

GENETIC VARIATION AMONG CULTIVATED SELECTIONS OF

MAMEY SAPOTE (*POUTERIA SPP.* [SAPOTACEAE])

by

Susan Carrara

Florida International University, 2004

Miami, Florida

Professor Javier Francisco-Ortega, Major Professor

Mamey sapote [*Pouteria* spp., Sapotaceae] is a tree fruit of economic and cultural importance in Central America, Mexico, and the Caribbean islands. It contributes greatly to local economies, habitats, and human nutrition. This study is among the first to analyze genetic variability among cultivated selections of mamey sapote. The Amplified Fragment Length Polymorphism (AFLP) molecular technique was used to estimate levels of genetic diversity and similarity between individual specimens in the germplasm collections of Fairchild Tropical Botanic Garden and University of Florida. The study found overall low levels of genetic diversity within these collections. However, higher relative levels of genetic diversity were found in a group of selections from northern Costa Rica and Nicaragua. It is anticipated that future plant collection in that region will capture greater genetic diversity among cultivated types. In addition, 'Pantin' selections were used to investigate the level of variation within supposedly identical selections. This baseline information can be applied to the management and expansion of the germplasm collections by identifying duplicate selections and homonyms and by locating geographical areas for future collection.

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MAMEY SAPOTE BIOLOGY AND GENETIC VARIATION

Knowledge of and access to the full range of a crop's genetic resources is vitally important to the continuing development of agriculture. The genetic resources of a crop refer to the complete range of traits found in modern cultivars, wild relatives and traditional varieties (FAO 1997). Genetic diversity, defined as the total number of different alleles present in a species, is an important component of a plant's genetic resources. Factors ranging from land clearing to changing national and international markets threaten the genetic diversity of many crops. International initiatives have been undertaken to conserve the genetic diversity of the world's crops through the development of *ex-situ* germplasm collections and on-site conservation. However, the distribution and scale of a crop's genetic diversity must be understood before conservation through germplasm collection can be undertaken. Comprehensive genetic information allows curators of living collections to optimize the genetic diversity in their collections, provide characterized sources for the breeding of superior cultivars, facilitate conservation initiatives, and ultimately furnish a wider selection of plants to growers.

Mamey sapote (*Pouteria* spp.) is a regionally important crop in Central America, the Caribbean, and South Florida. Fairchild Tropical Botanic Garden (FTBG) holds one of the most representative and genetically diverse collections in the United States of America. Yet, a greater range of morphological traits can be observed in mamey sapote's native or cultivated range than exist in the FTBG germplasm collection (R. J. Campbell, FTBG, personal communication). In addition, changing ideas about the plant's taxonomy

have the potential to greatly enlarge the range of traits that should be represented in such a collection.

Mamey sapote is commonly grown in home gardens and small orchards throughout the Caribbean basin where it can be an important cash crop; in some communities, 80% of home gardeners grow mamey sapote (Rico-Gray et al. 1990). In Florida, approximately 300 acres are under commercial cultivation, for a total value of about \$3.6 million per year (Mossler and Nesheim 2001). While the majority of production is in the New World, mamey sapote is also cultivated in Asia. Mamey sapote is an exotic addition to home gardens in the Philippines (Coronel 2002), and Australia produces approximately 500 metric tons of mamey sapote a year for export to Asia, primarily Japan (Australian Trade Commission 2004).

Mamey sapote produces a fruit with sweet creamy flesh with a range of sizes, colors (red, orange, pink, and salmon), sugar contents, and ripening characteristics. Fruit weight ranges from 300 g to 1,500 g, with a mature tree producing up to 500 fruit per season (Balerdi et al. 1996). In Florida, trees may grow to about 12 m in height, while in tropical regions they grow up to 40 m (Balerdi et al. 1996, Pennington 1990). Mamey sapote selections vary in their tolerance to cold. While leaves of some trees may turn red and begin to drop at approximately 10°C, others retain their leaves and coloring throughout the South Florida winter.

Mamey sapote grows well in lowland tropical and seasonally dry climates at elevations between sea level and 1,000 m (Pennington 1991). In Central America, mamey sapote cultivation is concentrated on the Caribbean coast of Honduras and Guatemala, and in a strip between the mountains and the west coast of Central America at

appropriate elevations (Pool 2001). Cultivated mamey sapote may be found at higher elevations, with reductions in production and vigor. Although not native to the West Indies, mamey sapote has been introduced to many of the Caribbean islands. Mamey sapote achieved cultural importance in Cuba, having been incorporated into place names, songs, and folklore. It is also cultivated in Puerto Rico, the Dominican Republic, Haiti, and Trinidad (Morera 1992).

Several biological and social factors have the potential to influence the genetic diversity of mamey sapote and its distribution. These factors include the existence of three closely related species, the potential for genetic separation of different populations, the cultivation history of the species, and propagation – both human and natural. Each of these factors will be explored below.

The species *Pouteria sapota* (Jacq.) H. E. Moore & Stearn produces the fruit most commonly called mamey sapote. Botanical classification of the genus *Pouteria* has been problematic, resulting in a list of 21 synonyms for *P. sapota*. Other than *P. sapota*, the scientific names most commonly seen in the literature are *Calocarpum mammosum* (L.), *Calocarpum sapota* (Jaquin) and *Pouteria mammosa* (L.) (Pennington 1990).

Two species closely related to *P. sapota*, *Pouteria viridis* (Pittier) Cronquist and *P. fossicola* Cronquist, also produce a fruit that some people call mamey sapote. The three species can be distinguished by comparing a combination of morphological characteristics (Table 1) (Pennington 1990). Culturally, the fruits of all three species are treated in the same manner. However, sometimes the fruits are distinguished linguistically through the local use of common names, such as ‘Injerto’ for *P. viridis* in Guatemala (Standley and Williams 1967). In addition, many specimens have

morphological characters intermediate to *P. viridis* and *P. sapota*, particularly their fruit skin color and texture.

There appears to be geographic separation among the species. *Pouteria sapota*'s native range is ambiguous because of the species' long cultivation and trade by pre-Columbian people (Pennington 1990). However, Pennington (1990) identified its native range as tropical wet and semi-arid habitats at altitudes from 0 to 800 m above sea level in Guatemala, Honduras, El Salvador, Belize and Nicaragua, and southern Mexico. *Pouteria viridis* shares the same geographical range as *P. sapota* but tends to grow at a higher altitude (1000-1500 m). *Pouteria fossicola* is distributed further south than *P. viridis* and *P. sapota* with a range extending from Nicaragua through Costa Rica to Panama between 0 and 800 m above sea level (Pennington 1990).

Pouteria sapota, *P. viridis*, and *P. fossicola* are monoecious. The majority of flowers appear to be perfect but staminate flowers often have a non-functional gynoecium, while the stamens of pistillate flowers are often reduced to staminodes (Pennington 1990). Davenport and O'Neal (2001) surveyed flowering phenology of five selections, finding a high rate of floral abscission, illustrating a technical barrier to studies of mamey sapote floral biology. There have been no studies regarding the relative proportions of male and female flowers or the potential for cross-pollination within a species or among the species. These aspects of a species' pollination biology have important implications for its patterns of genetic diversity.

The area where mamey sapote collections were made can be divided into two biogeographic regions with the potential for population differentiation: the Caribbean coast and the Pacific coast (Figure 1). The Caribbean coast has a pronounced dry season

and limestone substrate, while the Pacific coast offers greater variation in climate, altitude, and substrate. In addition, fruit trees in the Yucatán were specifically targeted for destruction during the Spanish colonization (Landa 1978).

Cultivation history also impacted the distribution of genetic diversity of this species. Few specifics are known about pre-Colombian cultivation of mamey sapote, but it was clearly cultivated throughout the Central American isthmus and southern Mexico. In northern Guatemala and the Yucatán peninsula mamey sapote was an important component of the diet for the pre-Colombian Mayans. The mamey sapote seed was an important source of oil because fat was limiting in Mayan diets (Lentz 1999). For the Itzaj Maya of the Petén, mamey sapote was a staple on par with yucca (*Manihot esculenta*), name (*Dioscorea* sp.), and camote (*Ipomoea batatas*) (Altran and Ucan Ek 1999). Horticulturally-improved mamey sapote trees are found in association with sites of present human activity or near abandoned homes and archaeological features. Mamey sapote was part of a suite of species that Mayans planted in forest gardens around water holes (Gómez-Pompa et al. 1987). When Spanish explorers arrived in Central America, they found a thriving trade in fruit, grown in orchards and transported along the Caribbean coast of Central America in canoes (Jones 1982). In addition, it can be inferred that pre-Colombian trade brought mamey sapote to Cuba, as “mamey” was the Arawak name for the fruit that the Mayans called “ha’az” (Marcus 1982). Currently, the tree is a valued component of the home garden in many regions. It is frequently present in home garden surveys in Campeche, Yucatán, Guatemala, Nicaragua and Nicoya, Costa Rica (Barrera 1981, Gillespie et al. 1993, Rico-Gray et al. 1990, Niembro Rocas and Sánchez 1994). Because of this history of cultivation, geographical barriers that might otherwise

have fostered divergence would be less meaningful to a species whose fruits (and therefore seeds) were transported across barriers by humans.

Knowledge of predominant propagation methods is critical to understanding the genetic diversity present in a cultivated species. For mamey sapote, seedlings require at least seven years after germination to bear fruit, while grafted plants can bear fruit in as little as two years (R. J. Campbell, FTBG, personal communication). Propagation by grafting has been common in South Florida for the last 30 years. Anecdotal evidence indicates that grafting of mamey sapote has long been practiced in parts of Costa Rica and Nicaragua, with the existence of large, old grafted trees. However, much of the small orchard and home garden production in Central America at present is by trees grown from seed (R. J. Campbell, FTBG, personal communication). Although seedling-based selection has been in process for centuries, this process has not yet produced cultivated plants that can be clearly differentiated from wild types, in contrast to other domesticates such as tomato or maize (Hawkes 1983).

Few published works have dealt with the genetic diversity of *P. sapota* or other members of the Sapotaceae. Azurdia et al. (1997) found seven polymorphic isozymes and were able to differentiate between 37 *P. sapota* and *P. viridis* individuals.

THE AMPLIFIED FRAGMENT LENGTH POLYMORPHISM PROCEDURE

Amplified Fragment Length Polymorphism (AFLP) analysis is a molecular technique useful in analyzing genetic variation below the species level (Vos et al. 1995). The more similar two individuals are, the more AFLP markers they share. This technique is based on a positive relationship between genetic similarity and the profile of genomic

DNA fragments generated by restriction digestion with endonuclease enzymes. It can be used to make inferences about relationships if the study sample includes family groupings. In the AFLP procedure, DNA is digested with a pair of restriction enzymes and the resulting fragments are replicated in polymerase chain reaction (PCR) using selective primers. Next, an array of different selective primer combinations is used to generate a large number of polymorphic fragments (Mueller and Wolfenbarger 1999). The number of fragments per primer pair and the number of polymorphic fragments used in analysis varies by species, for example, from 68 markers per primer pair with 87% polymorphism (Monte-Corvo et al. 2000) to 144 markers per primer pair with 26.6% polymorphism (Hurtado et al. 2002). As yet, no standard method exists for ascertaining when enough markers have been identified to differentiate among study specimens (Mueller and Wolfenbarger 1999).

AFLP data has a number of applications, from assessing genetic diversity in germplasm collections to studying population genetics among clonally reproducing species (Douhovnikoff and Dodd 2003). Cervera et al. (1998) used AFLP analysis on a collection of 67 grape accessions to eliminate duplicates and identify homonyms. Hurtado et al. (2002) used AFLP markers to identify relatedness among a group of 16 apricot cultivars as part of a breeding program. Winfield et al. (1998) studied the diversity of 146 *Populus nigra* subsp. *betulifolia* individuals in England to identify population structure prior to a reintroduction program.

In comparison to other molecular techniques, AFLP is popular for germplasm collection analysis because many markers can be produced quickly and a relatively small amount of DNA is required (± 500 ng). Serious limitations balance these advantages and

restrict the application of resulting data. Although AFLP markers are considered repeatable, studies have found repeatability ranging from 97.8% (Hansen et al. 1999) to 100% (Jones et al. 1997). Studies of clonally reproducing plants have indicated that the AFLP procedure itself has a certain degree of error in scoring identical specimens, with similarity values for identical genotypes ranging from 95% to 100% (Winfield et al. 1998). Finally, analysis cannot detect the difference between homozygous dominant and heterozygous markers because of the marker's dominant nature, limiting the use of AFLP in studies of inheritance (Mueller and Wolfenbarger 1999).

AFLP markers may have an advantage over microsatellites (SSR) in resolving relationships between closely related individuals, especially when clones are included in the sample group. It has been suggested that AFLP had greater power to identify clones than SSRs due to the large number of markers that can be generated (Patzak 2001). In spite of this, investigators found that AFLP markers tend to indicate greater similarity between samples than SSRs, perhaps because of the dominant nature of the AFLP marker (Heckenberger et al. 2003).

CHAPTER 2

GENETIC VARIATION AMONG CULTIVATED SELECTIONS OF MAMEY SAPOTE (*POUTERIA* SPP., SAPOTACEAE)

INTRODUCTION

Knowledge of and access to the full range of a crop's genetic resources is vitally important to the continuing development of agriculture. The genetic resources of a crop refer to the complete range of traits found in modern cultivars, wild relatives and traditional varieties (FAO 1997). Genetic diversity, the total number of different alleles present in a species, is an important component of a plant's genetic resources. Factors ranging from land clearing to changing national and international markets threaten the genetic diversity of many crops. However, the distribution and scale of a crop's genetic diversity must be understood before conservation through germplasm collection can be undertaken. Comprehensive genetic information allows curators of living collections to optimize the genetic diversity in their collections, provide characterized sources for breeding superior cultivars, facilitate conservation initiatives, and ultimately furnish a wider selection of plants to growers.

Mamey sapote (*Pouteria* spp.) is a regionally important crop in Central America and the Caribbean, including South Florida. Fairchild Tropical Botanic Garden (FTBG) holds one of the most representative and genetically diverse collections of mamey sapote in the United States of America. Yet a greater range of morphological traits can be observed in mamey sapote's native or cultivated range than exist in the germplasm collection (R. J. Campbell, FTBG, personal communication). In addition, changing ideas

about its taxonomy have the potential to greatly enlarge the range of traits that should be represented in such a collection.

Mamey sapote contributes to local economies, habitats, and human nutrition, and makes an economic contribution to many Mesoamerican households. It is commonly grown in home gardens and small orchards in Costa Rica, Cuba, Dominican Republic, El Salvador, Guatemala, Honduras, Nicaragua, Mexico, Puerto Rico, South Florida, and Trinidad for home use as well as local and export markets.

Mamey sapote produces a fruit with sweet creamy flesh with a range of sizes, colors (red, orange, pink, and salmon), sugar contents, and ripening characteristics. Fruit weight ranges from 300 g to 1,500 g, with a mature tree producing up to 500 fruit per season (Balerdi et al. 1996). In Florida, trees may grow to about 12 m in height, while in tropical regions they grow up to 40 m (Balerdi et al. 1996, Pennington 1990).

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morphological characteristics (Table 1) (Pennington 1990). In addition, many specimens have morphological characters intermediate among the three species, most notably in fruit skin color and texture.

The area where mamey sapote collections were made can be divided into two biogeographic regions with the potential for population differentiation: the Caribbean coast and the Pacific coast (Figure 1). The Caribbean coast has a pronounced dry season and limestone substrate, while the Pacific coast offers greater variation in climate, altitude, and substrate. Distribution of genetic diversity may also differ because fruit trees in the Yucatán were specifically targeted for destruction during the Spanish colonization (Landa 1978), while there is no record of this occurring in the Pacific region.

Cultivation history also impacted the distribution of genetic diversity of this species. Few specifics are known about Pre-Colombian cultivation of mamey sapote, but mamey sapote was clearly cultivated throughout the Central American isthmus and southern Mexico. Horticulturally improved mamey sapote trees are found in association with sites of present human activity and near abandoned homes and archaeological features (Gómez-Pompa et al. 1987). Currently, the tree is a valued component of the home garden in many regions (Barrera 1981, Gillespie et al. 1993). Due to this history of cultivation, geographical barriers that might otherwise have fostered divergence would be less meaningful to a species whose fruit (and therefore seeds) were transported across barriers by humans.

Little published work has dealt with the genetic diversity of *P. sapota* or other members of the Sapotaceae. Azurdia et al. (1997) found seven polymorphic isozymes and were able to differentiate between 37 *P. sapota* and *P. viridis* individuals.

RESEARCH QUESTIONS

Mamey sapote selections held by FTBG and the University of Florida's Tropical Research and Education Center (TREC) were analyzed using the Amplified Fragment Length Polymorphism (AFLP) technique to identify the quantity and distribution of genetic variation. We hoped to identify misnomers in the collections by assessing similarity among clones and to identify geographic areas for future collection by analyzing the relationship between genetic similarity and geographic distribution.

Assessment of similarity among clones

This study aimed to identify a range of similarity coefficients for clones attributable to experimental error by sampling selections that were supposedly genetically identical. We hypothesized that the range would be 0.95 to 1, similar to that found by other studies (Winfield et al. 1998). By applying this range of similarity index values to the entire dataset, we aimed to identify instances of error in plant collection labeling.

Relationship between genetic similarity and geographic distribution

Many mamey sapote selections in this study can be grouped into two main categories based on their region of collection (Figure 1). These categories are the Caribbean coast of Central America (the Yucatán Peninsula, Guatemalan Petén, and Belize), and the Pacific coast of Central America (Guatemala, El Salvador, Nicaragua and northern Costa Rica). We hypothesized that mamey sapote selections from the Yucatán peninsula would be highly similar due their morphological similarity and

because of the land clearing occurred there only 500 years ago. In addition, we predicted that selections from the Pacific coast population would have higher levels of variation within the population than do selections from the Caribbean coast because there are three species present in that region that are considered mamey sapote. By examining these two hypotheses, the objective was to identify geographical areas for future collection.

MATERIALS AND METHODS

Plant material

A total of 73 mamey sapote DNA samples were analyzed from two germplasm collections and one commercial nursery (Table 2). Twenty nine different selections from FTBG's mamey sapote collection were studied, including one *P. viridis* selection. The FTBG collection is located on the property of the United States Department of Agriculture Subtropical Horticultural Research Station at Chapman Field, Miami, FL, USA (USDA-ARS-SHRS). Fourteen unique selections from TREC were included, as well as multiple individuals of the selections 'Pantin' (n=6), 'Piloto' (n=5) and 'Lara' (n=3). The study also included thirteen 'Pantin' samples from Greenland Nursery in Homestead, Florida.

All samples in this study were collected as selections of *P. sapota* with the exception of *P. viridis* 'Whitman'. Selection names are indicated by single quotes. Some selection names include a number, in which case the number is included in quotes. When multiple samples were taken of the same selection, they are differentiated numerically by a number placed outside of the quotes.

DNA extraction

Laboratory procedures were carried out in the USDA-ARS-SHRS Plant Sciences Laboratory. DNA was isolated using the ethanol-precipitation based Epicentre MasterPure™ Plant Leaf DNA Purification Kit (Epicentre, Madison, WI, USA).

Molecular weight of approximately 40 DNA extractions was estimated by electrophoresis across a 1% agarose gel with results consistently indicating the presence of high molecular weight DNA. The AFLP procedure depends on the ability of the restriction enzymes *EcoR*1 and *Mse*1 to digest DNA, which can be affected by the DNA extraction procedure. For this reason, six DNA samples were digested in reactions containing both enzymes in order to test the suitability of the DNA produced by the Epicentre kit. Electrophoresis across 1% agarose gel showed that the DNA of each of the six samples was successfully digested.

AFLP procedure

AFLP markers were generated using Applied Biosystem's AFLP™ Ligation/Preselective Amplification Module. Restriction/ligation and preselective amplification were performed following the manufacturer's protocols (Applied Biosystems 2000). The only modification was that reaction volumes for pre-selective and selective amplification were reduced by one half.

A total of 38 primer combinations were screened across eight selections. Twenty combinations were found to produce a satisfactory number of fragments for all selections, visually determined by comparison with primer combinations that produced few or no

fragments (Table 3). After primer screening, all samples were analyzed across nine primer pairs.

The resulting fragments were separated by capillary electrophoresis on an automated DNA sequencer (ABI 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Fragment size was calculated from an internal dye standard (Rox 500, Applied Biosystems) using the local Southern size calling method. Fragment size was recorded using the program GeneScan version 3.7 (Applied Biosystems), and fragment sizes were analyzed with Genotyper version 3.7 (Applied Biosystems).

Marker selection

From the multitude of fragments produced, AFLP markers were selected based on their consistency over two AFLP replicates of each sample. Fragment sizes identified by Genotyper were manually verified. Mismatches between replicates were recorded as missing data, for a total of 1.4% missing data over a total of 104 markers.

Data analysis

Pairwise similarity between samples was estimated using Nei and Li's (1979) similarity coefficient, also known as the Dice coefficient (Swofford et al. 1996) using NTSYS (Exeter Software, Setauket, NY). This statistic does not consider the shared absence of a marker to be a similarity, an important consideration for AFLP analysis.

Dendrograms were produced using the unweighted pair group method of analysis (UPGMA) (Sneath and Sokal 1973). Confidence levels were placed on the dendrograms using 5000 bootstrap replications with the program WinBoot (Yap and Nelson, 1996).

Finally, groups identified after principle component analysis (PCA) using SPSS (SPSS Inc., Chicago, IL, USA) were compared to those formed through the process described above.

In order to reduce the potential for unconscious bias, samples were assigned random numbers after DNA extraction. After the AFLP fingerprint was finalized, the random numbers were restored to selection names.

RESULTS AND DISCUSSION

General remarks

This study amplified a total of 73 DNA extractions of 41 different selections across nine primer combinations. Sixty eight individuals were sampled, and multiple DNA extractions were made from three individuals. Five samples ('Alejos' B, 'Lara' 3, 'Piloto' 1, 'Piloto' 3, and 'Copan' B) were discarded due to low quality of the DNA extraction or AFLP template. A total of 104 markers were scored (Table 4). Marker profiles of the two replicates for most samples matched well, although approximately 1.5% of markers were ambiguous and listed as missing data. Pairwise similarity values ranged from 0.404 to 1.000, with an average of 0.900, median of 0.922 and mode of 0.927.

A preliminary dendrogram was constructed in order to observe trends. A large cluster including many of the 'Pantin' samples dominated the top of the dendrogram. These samples exhibited high similarity values, although the values were not as close to unity as expected for clones (Figure 2).

The selection most distinct from the others was ‘Cartagena’, a selection grown in cultivation in Cartagena, Colombia and collected in a Colombian natural area, well outside the presumed native range of *P. sapota* or its close relatives. ‘Cartagena’ could be a different *Pouteria* species, although it was morphologically similar to the *P. sapota* complex and was graft compatible.

Bootstrap analysis did not statistically support the groups obtained after cluster analysis. While similarity of isolated pairs or clusters was strongly supported, it is only among the most diverse selections that dendrogram branches received 50% to 100% support (Figure 3).

Principal component analysis (PCA) agreed with the UPGMA-based cluster analysis. The first three axes summarized 20.6, 7.5 and 6.3 % of the data set’s variability, respectively. A scatterplot of the first three principal components shows a tight cluster that includes the majority of the study selections, with only the selections found at the base of the dendrogram discernable from the cluster (Figure 4). PCA was repeated after removing these outliers and the majority of ‘Pantin’ selections, revealing a tight cluster of 16 selections (Figure 5), a grouping that was neither evident in the UPGMA dendrogram nor supported by bootstrap analysis (Figure 3).

Assessment of similarity among clones

Twenty ‘Pantin’ individuals were analyzed to assess genetic variation attributable to the AFLP procedure and to verify the identity of plants sold as ‘Pantin’ in South Florida, USA. Because selections are clonally propagated, they should be genetically identical. Initial analysis of the ‘Pantin’ cluster revealed that the majority of individual

selections clustered together as expected, although there were four distinct outliers.

Taken together, the 20 'Pantin' selections analyzed had similarity values ranging from 0.713 to 1.000. 'Pantin' 3, 4, 11, 16, and 19 were visually determined to be separate from the 'Pantin' cluster based on Figure 2, and were removed under the assumption that they had been mislabeled. The range of similarity values among the remaining 'Pantin' samples was 0.899 to 1.000, a wider range than has been reported for clones. This pattern is more indicative of half-siblings or self-pollinated plants, revealing the possibility that horticulturists have taken seeds from 'Pantin' trees, giving resultant plants the name of the parent. Various researchers have identified expected ranges of variation of similarity coefficients in AFLP profiles for identical samples, from 0.96 to 1.0 (Winfield et al. 1998), 0.972 to 0.990 (Douhovnikoff and Dodd 2003) and 0.95 to 0.985 (Huys et al. 1996, in Winfield et al. 1998), attributable to experimental error.

Because the range of variation in the 'Pantin' samples was larger than expected, an additional experiment was carried out in order to define a range of similarity values that could be expected from multiple samples of the same individual. Three DNA extractions of two 'Pantin' individuals ('Pantin' 1 and 'Pantin' 20) for a total of six extractions were compared in order to partition the variation due to experimental error and that due to genetic variation. 'Pantin' 1 and 'Pantin' 20 were chosen because they represent the largest distance between 'Pantin' samples that were part of the 'Pantin' cluster as indicated by preliminary analysis. The DNA extraction and evaluation, as well as the AFLP procedure and marker analysis, were carried out as described in the materials and methods section. However, one sample of each individual was discarded due to failure of the AFLP procedure. In order to increase the sample size of the test, the

samples 'Pantin' 1a and 20a from the original AFLP reaction were included in the analysis, resulting in a final data set of three samples of each individual. Because the AFLP procedure is considered repeatable, combining AFLP data generated during different runs should be valid.

The Analysis of Molecular Variance (AMOVA) test was carried out on the 'Pantin' test data using the program GenAlEx (Peakall and Smouse 2001). The results of AMOVA analysis showed that all of the variation was due to the AFLP procedure, and none could be attributed to genetic variation (Table 5). When assuming the AFLP procedure is repeatable, the range of uncertainty in similarity values is 0.907 (Table 6).

A closer look at the similarity values and cluster analysis of the 'Pantin' subset show that 'Pantin' 1a and 'Pantin' 20a, which were produced first, are more similar to each other than to 'Pantin' 1b, 1c, 20b, and 20c (Figure 6). This suggests a difference between the two runs.

Eliminating the assumption that the AFLP procedure is repeatable permits the removal of 'Pantin' 1a and 20a from the analysis. When AMOVA and similarity values are computed only on the newly extracted samples, the results show that 14% of variability can be attributed to genetic differences (Table 5). Finally, the actual degree of similarity of sample pairs with similarity index values above 0.962 is uncertain (Table 6).

These results indicate that the two AFLP runs cannot be directly compared, although the data sets are based on identical AFLP markers. This indication contrasts with previous studies finding AFLP studies to be highly repeatable (Heckenberger et al. 2003). The uncertainty level in this study is comparable to that found by some researchers, yet lower than others. For instance, Fahleson et al. (2003) found three

markers that did not match in their set of 154 markers (1.3%) for five different individuals that were each replicated three times. In comparison, 'Pantin' 1 had non-matching markers for four out of 104 (3.8%), while 'Pantin' 20 had 14 out of 104 (13.5%).

The failure of AFLP repeatability in the present study does not prevent the interpretation of the results. While analysis of the present data set is valid, this failure does eliminate the possibility for future studies to build upon this one because results from present and future experiments cannot be directly compared. We hypothesize that this failure was due to the quality of the DNA extracted. A number of different DNA extraction procedures and tissue types and preparations were attempted before choosing the Epicentre Kit, which was the only one that yielded any high molecular weight DNA. It is likely that extraction was problematic due to high levels of secondary compounds in the plant tissue. If these compounds are not removed or neutralized, they can inhibit the restriction enzyme's action, the reaction on which the AFLP is based.

Hence, when comparing samples tested during the same AFLP run, genetically identical samples have similarity values ranging from 0.962 to 0.978 (Table 6). Using these values as a guide, it appears that some members of the 'Pantin' group have similarity coefficients that fall in the range consistent with clones. Several 'Pantin' individuals (numbers 1a, 3, 4, 11, 16, and 19) fall outside this cluster. 'Pantin' 1a, 3, and 4 came from TREC, while the remaining selections were from Greenland Nursery. Of these, only 'Pantin' 1a has a similarity coefficient placing it close to the main cluster of 'Pantin'. 'Pantin' 3, 4, and 19 are notable in their distance from the main 'Pantin' cluster,

and are most likely mislabeled. ‘Pantin’ 4 is one of the most distinct selections according to the AFLP analysis, although its morphology does not seem to corroborate this result.

A greater level of diversity is present within the ‘Pantin’ selections tested than would be expected among genetically identical plants, indicating that a variety of different genotypes are available commercially under the ‘Pantin’ name. This can be explained by mislabeling of graft scions and nursery trees, the use of multiple trees for graft scions, somatic mutation, giving seedlings the parent’s selection name, death of the scion and subsequent survival of the rootstock. Because mamey sapote selections are difficult to distinguish based on morphological traits, mistakes in plant identification are particularly difficult for growers to recognize and correct. In addition, mamey sapote selections do not currently have strong recognition among the public, which also leads to a failure to identify and correct mistakes.

Using the range of similarity coefficients of expected clones outlined above, the dendrogram indicates the presence of several groupings of individuals that may be genetically identical (Figure 7). While this study is unable to conclude that particular individuals are genetically identical, these results show the genetic variation in the germplasm collection is represented by a few individuals.

Conversely, several individuals presumed to be genetically identical were shown to be different. ‘Lara’ 1 and ‘Lara’ 2 were not close on the dendrogram, and had a pairwise similarity coefficient of 0.846. ‘Piloto’ 2 and ‘Piloto’ 5 fell together in the cluster analysis, but their similarity coefficient was 0.889. ‘Piloto’ 4 was distant to the other ‘Piloto’ selections. (‘Piloto’ 1 and 3, as well as ‘Lara’ 3 were eliminated from analysis due to poor quality of the DNA extraction or AFLP template). That neither the

‘Lara’ nor the ‘Piloto’ selections are genetically identical raises several issues. Firstly, the problems faced by the nursery grower in maintaining the identity of individual trees are compounded in the germplasm collection because the collection may be old and information about the collection must be transferred through the records of multiple curators. Also, a greater number of different genotypes are present in a germplasm collection compared to the typical nursery, increasing the opportunities for plants to become mislabeled. In addition, curators have access only to collection information provided by the collector, which may not be adequate to meet curators’ needs. Finally, these results raise issues regarding replication for other types of studies (whether morphological, physiological, or phenological) that require replication of the genetic individual. Such studies would have difficulty if they relied on the current selection identification.

Some of the plants that fell within the error range of the AFLP procedure and could be considered clones can be distinguished by morphological traits. For instance, ‘Celso 2’ and ‘Lopez 2’ could be considered clones, but their fruit size, shape and color are different. These may be genetically distinct, and the similarity value is due to error in the AFLP process. Alternatively, morphology is due not only to genotype, but also to a combination of genetic composition, phenotypic plasticity, rootstock influences, and phenological variation, any or all of which may be reflected in morphology rather than genetic differences.

Relation between genetic similarity and geographic distribution

Loose grouping of selections by region of sample collection was observed in UPGMA cluster analysis but did not have bootstrap support. There is no distinct grouping of selections based on their region of collection. The majority of selections collected from the Caribbean region and Cuba showed a high level of similarity. In contrast, those selections collected from the Pacific region appear more distributed across the dendrogram, including some with a high level of similarity to those in the Caribbean region group (Figure 8), an observation supported by PCA (Figure 9). This overlapping could explain the lack of bootstrap support for groups obtained by cluster analysis. The spread of selections collected from the Pacific region across the PCA scatterplot in Figure 9 and the UPGMA dendrogram in Figure 8 indicates that the selections from this region are more dissimilar to each other and therefore represent greater genetic diversity. The AMOVA comparing the Caribbean and Pacific regions attributed 13% of variation differences to between the regions (Table 7), which is not a great amount.

Selection '2002-165 A', which originated from a seed collected in a fruit market in northern Costa Rica, is one of the more distinct selections. It was collected because the fruit had morphological characteristics intermediate between *P. sapota* and *P. fossicola* consisting of a green skin with brown scurf on the nose. Because these selections from northern Costa Rica and Nicaragua appear to be more dissimilar from each other than do selections from any other location, future collecting in this region would increase the genetic diversity present in the FTBG collection more than would collecting from the Yucatán.

While study data suggests that Central America, Costa Rica and Nicaragua in particular, would be most productive for future collecting, the lack of bootstrap support for clusters defining regions prevents the identification of regions of origin for selections that were collected outside mamey sapote's native range. This could be because centuries of human cultivation and trade have reduced any potential for geographical separation between mamey sapote growing in the Caribbean coast and those from the Pacific Coast.

CONCLUSIONS

This study identified 104 AFLP markers for use in fingerprinting mamey sapote. By applying these markers to selections from two germplasm collections and a commercial nursery, a set of suggestions for germplasm collection management was developed.

The AFLP procedure was found not to be repeatable for the mamey sapote DNA extractions in this study, indicating that future AFLP fingerprints cannot be directly compared to those developed in this study, although the AFLP primer combinations and markers could be used. These results mandate a cautious interpretation of AFLP data derived from plants with high levels of secondary compounds. A systematic investigation of AFLP repeatability involving replicated laboratories and technicians, as well as plant species with low and high levels of polyphenolics would be instructive.

The level of genetic variation among trees labeled 'Pantin' suggests that a number of different genotypes are being sold under that name. Because it is difficult to detect mislabeling on the basis of morphological characteristics, molecular analysis is necessary to detect these problems. In addition, common commercial nursery practices do not

prioritize the maintenance of named cultivars through promotion or care taken during propagation and labeling. For the sake of building a strong germplasm collection, it is advisable to propagate from the original ‘Pantin’ to keep an accurate source for this important commercial South Florida cultivar.

At present, the FTBG germplasm collection holds the major commercial cultivars in South Florida as well as a large number of individuals, mainly from the Yucatán peninsula, that appear genetically similar. AFLP analysis indicates that selections made in Southern Nicaragua and Costa Rica have greatly expanded the range of genetic diversity represented in the collection.

Table 1. Morphological characters used to distinguish *Pouteria sapota*, *P. viridis*, and *P. fossicola* (Pennington 1990). Geographic and altitudinal ranges are also included.

	<i>P. sapota</i>	<i>P. viridis</i>	<i>P. fossicola</i>
Buds	Long pubescent or villous	Adpressed, puberulous, pale	Dense pubescent, brown
Leaf base	Long tapering	Not long tapered	Not long tapered
Secondary venation	20-25 pairs	13-20 pairs	13-20 pairs
Lamina	Glabrous	Glabrous	Pubescent with erect 2 branched hairs
Corolla length	0.7-1 cm	1-1.3 cm	1-1.2 cm
Anther length	1.5-2 mm	1.5-2 mm	2-3 mm
Fruit	Rough brown skin	Smooth or lenticellate, grey-brown skin	Smooth or lenticellate, green skin.
Geographic Range	Southern Mexico to Northern Panama	Guatemala, Honduras, El Salvador	Southern Nicaragua through Panama
Altitude	0 – 1,000 m	1,000 – 1,500 m	0 – 1,000 m

Table 2. Mamey sapote selections analyzed with AFLP markers. Population group was only assigned to selections collected from the Pacific or Caribbean regions. Collection location was not known for all selections.

Sample	Number of DNA extractions	Germplasm Location	Population Group	Collection Location	Notes
2002-165 A	1	FTBG	Pacific	Nicaragua/Costa Rica	Intermediate fruit characteristics Selected for rootstock (consistently high seed number).
'9998'	1	TREC			
'Alejos'	1	FTBG	Caribbean	Campeche, Mexico	Red, sweet fruit, little fiber
'AREC 3'	1	TREC	Not applicable	Isle of Pines, Cuba	Selected for use as rootstock
'Buena Vista'	1	FTBG	Caribbean	Belize	Large fruit
'Cartagena'	1	FTBG	Not applicable	Cartagena, Colombia	Deep red, fibrous fruit
'Casillo #1'	1	TREC			
'Celso 2'	1	FTBG	Caribbean	Campeche, Mexico	Seedling of 'Alejos'
'Celso 3'	1	FTBG	Caribbean	Campeche, Mexico	Seedling of 'Alejos'
'Cepeda Especial'	1	FTBG	Caribbean	Campeche, Mexico	Synonym of 'Adelantado 2'
'Chenox'	1	FTBG	Caribbean	Belize	
'Copan'	1	TREC	Not applicable	Cuba	Came from Gary Zill
'Danny'	1	FTBG	Caribbean	Mexico or Belize	
'Don Vicente'	1	FTBG	Caribbean	Campeche, Mexico	
'Felipe Larguito'	1	FTBG	Caribbean	Campeche, Mexico	Fruits in March (Campeche) Hard exoderm, resistant to fruit flies, fruits in May in Campeche
'Felipe Mayo'	1	FTBG	Caribbean	Campeche, Mexico	
'Florida'	1	TREC			
'Gilberto'	1	FTBG	Pacific	Orotina, Costa Rica	Large fruit
Guatemala Seed	1	TREC	Pacific	Guatemala	
'Hawaii 1'	1	FTBG	Not applicable	Hawaii	
'Jamaica'	1	TREC			
'Lara' 1	1	TREC			Collected by Julian Lara
'Lara' 2	1	TREC			Collected by Julian Lara
'Lara' 3	1	TREC			Collected by Julian Lara

Table 2. Continued

Sample	Number of DNA extractions	Germplasm Location	Population Group	Source	Notes
'Lobo'	1	FTBG	Pacific	Jesus Maria, C. R.	Small fruit and seed. Some green.
'Lopez 1'	1	FTBG	Not applicable	FL or Cuba	Collected by Pedro Lopez
'Lopez 2'	1	FTBG	Not applicable	FL or Cuba	Collected by Pedro Lopez
'Lorito'	1	FTBG	Caribbean	Campeche, Mexico	Small fruit, strong red
'Magaña' 1	1	TREC	Pacific	El Salvador	Large fruit
'Magaña' 2	1	TREC	Pacific	El Salvador	Reputed scion of original
'Marin'	1	FTBG	Caribbean	Campeche, Mexico	Large fruit, red, little fiber
'Mayapan'	2	TREC	Not applicable	Isle of Pines, Cuba	
'Morales'	1	FTBG	Not applicable	FL	Collected by Dr. Morlaes
<i>P. viridis</i> 'Whitman'	1	FTBG	Pacific		
'Pace'	1	FTBG			
'Pantin' 1	3	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 2	1	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 3	1	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 4	1	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 5	1	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 6	1	TREC	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 7	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 8	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 9	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 10	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 11	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 12	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 13	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 14	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 15	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 16	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar

Table 2. Continued

Sample	Number of DNA extractions	Germplasm Location	Population Group	Source	Notes
'Pantin' 17	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 18	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 19	1	Greenland Nursery	Not applicable	Cuba	S. Florida commercial cultivar
'Pantin' 20	3	FTBG	Not applicable	Cuba	S. Florida commercial cultivar
'Piloto' 1	1	TREC			Collected by Julian Lara
'Piloto' 2	1	TREC			Collected by Julian Lara
'Piloto' 3	1	TREC			Collected by Julian Lara
'Piloto' 4	1	TREC			Collected by Julian Lara
'Piloto' 5	1	TREC			Collected by Julian Lara
'San Verde'	1	FTBG	Pacific	Jesus Maria, C. R.	Green skinned fruit
'Santo Domingo'	1	FTBG	Pacific	Omotepe, Nicaragua	Remnant tree, 200+ years
'Tazumal'	1	TREC	Pacific	El Salvador	
'Tichaná'	1	FTBG	Pacific	Omotepe, Nicaragua	Tree 100+ years old
'Tobias'	1	FTBG	Pacific	Geronimo, Costa Rica	Giant, old remnant tree
'Vidal'	1	FTBG	Caribbean	Campeche, Mexico	Possibly 'Vidal'
'Vidal Redondo'	1	FTBG	Caribbean	Campeche, Mexico	Round, red fruit
'Viejo'	1	TREC	Pacific	El Salvador	

Table 3. Assessment of AFLP primer combination efficacy on mamey sapote. Proportion of monomorphic markers was not evaluated due to the small number of samples screened.

		<i>Mse</i> I Primers							
		CTA	CTG	CAC	CAA	CTT	CTC	CAG	
<i>Eco</i> RI Primers	ACG Joe	Good	Good	Good	Good	Good	X	X	
	ACA Fam	Good	Good	Good	Good	X	X	X	
	AGC Ned	X	X	X	X	–	–	–	
	ACC Ned	Good	Good	X	Good	Good	Good	Good	
	AAC Ned	Good	Good	X	X	Good	X	X	
	AAG Joe	X	Good	–	Good	X	X	X	

Key: Good: Satisfactory amplification with mamey sapote DNA.
X: Poor amplification with mamey sapote DNA.
–: Primer combination not assessed with mamey sapote DNA.

Table 4. Summary of markers scored for each primer combination included in this study of cultivated selections of mamey sapote.

Primer Combination	Markers Scored	Markers: frequency >0.95	Markers: frequency <0.05
<i>EcoR</i> I ACG Joe + <i>Mse</i> I CTA	14	6	2
<i>EcoR</i> I ACG Joe + <i>Mse</i> I CTG	9	4	0
<i>EcoR</i> I ACG Joe + <i>Mse</i> I CAA	7	3	0
<i>EcoR</i> I ACA Fam + <i>Mse</i> I CTA	19	0	1
<i>EcoR</i> I ACA Fam + <i>Mse</i> I CTG	6	0	1
<i>EcoR</i> I ACA Fam + <i>Mse</i> I CAC	17	2	1
<i>EcoR</i> I AAG Joe + <i>Mse</i> I CTG	13	8	1
<i>EcoR</i> I ACC Ned + <i>Mse</i> I CTA	13	5	1
<i>EcoR</i> I ACC Ned + <i>Mse</i> I CTG	6	1	0
Total	104	29	7

Table 5. Analysis of Molecular Variance (AMOVA) for 'Pantin' replicates.

Source of Variation	df	Variance Component	Percentage
Three samples of each 'Pantin'			
Between Individuals	1	0	0%
Within Individuals	4	7	100%
Two samples of each 'Pantin'			
Between Individuals	1	0.75	14%
Within Individuals	2	4.5	86%

Table 6. Similarity matrix of Dice coefficients for ‘Pantin’ replicates. Here, 0.907 is the lowest similarity value expected among samples known to be genetically identical when the AFLP with mamey sapote is considered repeatable. However, that value is 0.962 when the AFLP is not considered to be repeatable.

		'Pantin'					
		1a	1b	1c	20a	20b	20c
'Pantin'	1a	1					
	1b	0.907	1				
	1c	0.919	0.977	1			
	20a	0.972	0.94	0.927	1		
	20b	0.899	0.971	0.959	0.921	1	
	20c	0.905	0.975	0.949	0.941	0.962	1

Table 7. Analysis of Molecular Variance (AMOVA) for Caribbean and Pacific regions.

Source of Variation	df	Variance Component	Percentage
Caribbean versus Pacific Regions			
Between Region	1	1.244	13%
Within Regions	27	8.216	87%
Control: Same Individuals Distributed Randomly into Regions			
Between Regions	1	0.167	2%
Within Regions	27	8.773	98%

Figure 1. Map of the cultivated distribution of mamey sapote. Regions from which selections were collected are superimposed.

Figure 2. UPGMA dendrogram for cultivated selections of mamey sapote computed using Dice's similarity index based on 104 AFLP markers identified for use with mamey sapote.

Figure 3. Bootstrap values above 50% for the UPGMA dendrogram.

Figure 4. Principal component analysis for 65 mamey sapote selections based on 104 AFLP markers. This plot of the three first principal components contains 34.4% of the data set's total variability.

Figure 5. Principal component analysis of 43 cultivated selections of mamey sapote based on 104 AFLP markers. The majority of 'Pantin' selections and outliers have been removed.

Figure 6. UPGMA dendrogram of 'Pantin' selections used to estimate the uncertainty associated with the AFLP procedure. *Pouteria viridis* 'Whitman' is included for reference.

Figure 7. UPGMA dendrogram for cultivated selections of mamey sapote including approximate ranges of uncertainty for the identity of genetically identical individuals. Light shading indicates range of uncertainty when the AFLP is considered repeatable (0.907 - 1). Dark shading indicates range of uncertainty when the AFLP is not considered repeatable (0.962 - 1).

Figure 8. UPGMA dendrogram for cultivated selections of mamey sapote with highlighted regions of collection.

Figure 9. Principal component analysis of 43 selections of mamey sapote. The majority of the 'Pantin' selections have been removed, as have the outliers. Collection regions are indicated.

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