Using Randomly Amplified Polymorphic DNA (RAPD) Markers to Identify *Annona* Cultivars

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Abstract. The native American genus *Annona* contains many species that are cultivated for their edible fruit, including the custard apple (*A. reticulata* L.), soursop (*A. muricata* L.), cherimoya (*A. cherimola* L.), sugar apple (*A. squamosa* L.), and interspecific hybrids, the atemoyas. RAPD analysis of *A. cherimola*. ‘Campa’ and ‘Jete,’ *A. squamosa* ‘Lessard,’ and the atemoyas ‘Ubranitzki,’ ‘Malali,’ and ‘Kaspi’ resulted in very distinctive patterns, indicating that RAPD markers, may be an efficient method of fingerprinting genotypes within and between *Annona* species. All 15 primers used generated repeatable, polymorphic patterns. An F, population of ‘Jete’ x ‘Lessard’ was analyzed to determine the inheritance of the RAPD banding patterns. Fifty-two polymorphic loci were identified, which segregated in an expected Mendelian fashion.

*Annona* contains more than 50 species and interspecific hybrids, many of which are cultivated in tropical and subtropical America for their edible fruit. The genus has been divided into five groups and 14 sections based on morphological characters (Safford, 1914). Cherimoya (*A. cherimola*) originated in the highlands of Ecuador and Peru (Zeven and Zhukovsky, 1975). Cultivars of this early-maturing species have a superior flavor and are cold-tolerant ‘Jete’ and ‘Campa’ are two Spanish cultivars representative of this species. Sugar apple (*A. squamosa*) is native to the Central American lowlands and the West Indies, and is suited to tropical climates. The fruit is considered inferior to cherimoya, but is exceptionally hardy, productive, fruit quality, and postharvest and shipping qualities could be very rewarding, as evidenced by the performance of the atemoya under the subtropical, low elevations of southern Florida (Sturrock, 1959).

Genetic variation between cherimoya cultivars has been studied using isozyme markers (Ellstrand and Lee, 1987; Lee and Ellstrand, 1987; Pascual et al., 1993); however, isozyme analysis is limited by the relatively small number of loci. Recently, RAPD, a polymerase-chain reaction technology using 10-mer primers, has been introduced (Welsh and McClelland, 1990; Williams et al., 1990). RAPD markers offer the potential of generating large numbers of markers representing a random sample of the genome, thereby presenting an advantage over isozyme markers. Our objective was to estimate variation between *A. cherimola*, *A. squamosa*, and interspecific atemoya hybrids using RAPD markers. Also, we determined the inheritance of these markers within an F, family of the interspecific cross *A. squamosa* ‘Jete’ x *A. cherimola* ‘Lessard’ to ascertain the efficacy of such markers for genetic analysis of *Annona*.

Materials and Methods

Plant materials. *Annona cherimola* ‘Jete’ and ‘Campa,’ *A. squamosa* ‘Lessard,’ and the atemoya ‘Kaspi’ are located at the Experimental Farm of the Faculty of Agriculture, Hebrew University, Rehovot, Israel. The atemoyas ‘Ubranitzki’ and ‘Malali’ were collected from the Warburg Acclimatization Garden in Rehovot. The family used in this study, located at the U.S. Dept. of Agriculture National Clonal Germ Plasm Repository in Miami, includes 38 F, individuals from the interspecific cross ‘Jete’ x ‘Lessard.’

**DNA extraction.** Genomic DNA was extracted according to the protocol of Dellaporta et al. (1983) with modifications. Young leaves were ground in a mortar and pestle with 10 ml g of fresh weight (FW) extraction buffer (100 mM Tris-HCl pH 8.0, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) 2-mercaptoethanol; 0.1 M diethyldithiocarbamic acid (DEDTC); 2% (w/v) polyvinylpyrrolidone (PVPP)). The homogenate was filtered through four layers of cheesecloth. Sodium dodecyl

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Table 1. RAPD Primers used to analyze genetic variation between *Annona cherimola* 'Jete' and 'Campa,' *A. squamosa* 'Lessard,' and the atemoyas 'Ubranitzki,' 'Malali,' and 'Kaspi.' Percent polymorphism reflects the number of total bands from each primer that distinguish at least one cultivar.

| Primer | No. bands | No. polymorphic bands | Percent polymorphism |
|--------|-----------|-----------------------|---------------------|
| A3     | 6         | 6                     | 100                 |
| A14    | 3         | 3                     | 100                 |
| A16    | 3         | 3                     | 100                 |
| A17    | 2         | 2                     | 100                 |
| A18    | 12        | 12                    | 100                 |
| A20    | 5         | 5                     | 100                 |
| B11    | 5         | 5                     | 100                 |
| B18    | 6         | 6                     | 100                 |
| C11    | 10        | 9                     | 90                  |
| C19    | 3         | 3                     | 100                 |
| D8     | 7         | 7                     | 100                 |
| D11    | 15        | 14                    | 93                  |
| E3     | 4         | 1                     | 25                  |
| E7     | 5         | 5                     | 100                 |
| H20    | 6         | 5                     | 83                  |
| Total  | 92        | 86                    | 93.5                |

Sulfate (SDS) was added to a final concentration of 1.25%, and the solution was incubated at 65°C for 10 min. Potassium acetate was added to a final concentration of 1.2 M. The suspension was allowed to stand for 20 min in an ice water bath, then samples were centrifuged at 25,000 × g, 4°C, for 20 min. After filtration through two layers of Miracloth, the nucleic acids were precipitated from the supernatant with cold isopropanol. Resuspended pellets were treated with RNase A, and DNA concentrations were determined spectrophotometrically.

Amplification. Genomic DNA was amplified using 15 different RAPD primers (Operon Technologies; Alameda, Calif.) (Table 1). The reaction included 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% (v/v) Triton X-100, 2.5 mM MgCl₂, 0.1 mM each dATP, dCTP, dTTP, and dGTP, 0.02 U/µl Taq DNA polymerase (Promega; Madison, Wis.), 0.3 µM primer, and 0.4 ng·µl⁻¹ genomic DNA, in a final volume of 25 µl. Reactions were either amplified immediately or stored at 20°C for up to 1 week. Amplifications were performed in a DNA thermal cycler (model 480; Perkin Elmer, Norwalk, Conn.) preheated to 94°C. Cycling parameters were 45 cycles of 94°C, 1 min; 37°C, 1 min; and 72°C, 2 min. PCR products were separated by electrophoresis on a 1.4% agarose gel in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), and visualized by ethidium bromide staining.

Results

While more than 30 Operon primers were used initially, 15 gave optimal results, generating a total of 107 bands in *A. cherimola* 'Jete' and 'Campa,' *A. squamosa* 'Lessard,' and the atemoyas 'Ubranitzki,' 'Malali,' and 'Kaspi' (Fig. 1). Fifteen of these bands, all but two of which were light in intensity, were repeatable; therefore a total of 92 bands, or loci, were scored. All 15 primers produced at least one variable locus in all cultivars; all but four primers were 100% polymorphic (Table 2).

Percent bands shared between pairwise combinations of species and cultivars are shown in Table 3. Band sharing between cultivars showed that a high identity (83.7%) exists between *A. cherimola* 'Jete' and 'Campa.' Good identity was also found within the atemoya cultivars (65.2% to 79.3%) and between *A. squamosa* 'Lessard' and the atemoyas (67.4% to 71.7%). The least amount of genetic identity was found between *A. cherimola* cultivars and *A. squamosa* (26.1% and 27.2%). Identity between the two *A. cherimola* cultivars and the three atemoyas was average (34.8% to 41.3%).

Fig. 1. Phenotypic variation in RAPD patterns between six *Annona* cultivars of two species and interspecific hybrids, amplified with RAPD primers A18, C11, H20, A20, B11, and D8. Lane 1: *A. cherimola* 'Jete'; lane 2: *A. cherimola* 'Campa'; lane 3: *A. squamosa* 'Lessard'; lane 4: atemoya 'Ubranitzki'; lane 5: atemoya 'Kaspi'; lane 6: atemoya 'Malali.' Size markers are 100 bp ladder (Gibco BRL, Gaithersburg, Md.). PCR products were electrophoresed on 1.4% agarose in 0.5× TBE and stained with EtBr.

Table 2. Scheme used to determine expected segregation ratios the F₁ progeny of *Annona cherimola* 'Jete' x *A. squamosa* 'Lessard.' 1 = band present; 0 = band absent.

| Parental phenotypes (genotypes) | Jete | Lessard | Expected segregation in F₁ |
|--------------------------------|------|---------|---------------------------|
| 1 (+/+), 1 (+/+)               | All 1|         |
| 1 (+/+), 1 (+/-)               | All 1|         |
| 1 (+/-), 1 (+/+)               | All 1|         |
| 1 (+/+), 0 (+/-)               | 3:1  |         |
| 1 (+/-), 0 (+/-)               | 1:1  |         |
| 0 (+/-), 1 (+/+), 1 (+/-)      | All 1|         |
| 0 (+/-), 0 (+/-), 1 (+/-)      | 1:1  |         |

Table 2. Scheme used to determine expected segregation ratios the F₁ progeny of *Annona cherimola* 'Jete' x *A. squamosa* 'Lessard.' 1 = band present; 0 = band absent.
Table 4. Number of RAPD loci identified in the F₁ progeny from Annona cherimola 'Jete' x A. squamosa 'Lessard', amplified with 15 Operon primers. Expectations are based on parental phenotypes.

| Locus       | Jete | Lessard | Observed | Expected | χ²  |
|-------------|------|---------|----------|----------|-----|
| A3-1050     | 0    | 1       | 20:18    | 19:19    | 0.105 |
| A3-1080     | 1    | 0       | 18:20    | 19:19    | 0.105 |
| A3-1200     | 0    | 1       | 21:17    | 19:19    | 0.421 |
| A16-1450    | 1    | 0       | 23:15    | 19:19    | 1.684 |
| A17-950     | 0    | 1       | 13:25    | 19:19    | 3.789 |
| A17-1100    | 1    | 0       | 22:16    | 19:19    | 0.947 |
| A18-700     | 1    | 0       | 31:5     | 27:9     | 2.370 |
| A18-920     | 1    | 0       | 14:24    | 19:19    | 2.632 |
| C11-490     | 1    | 0       | 15:23    | 19:19    | 1.684 |
| C11-670     | 0    | 1       | 23:15    | 19:19    | 2.189 |
| C11-840     | 1    | 0       | 21:17    | 19:19    | 0.421 |
| C11-910     | 0    | 1       | 23:15    | 19:19    | 1.684 |
| C11-1270    | 1    | 0       | 20:18    | 19:19    | 0.105 |
| D8-1450     | 1    | 0       | 16:22    | 19:19    | 0.947 |
| D11-480     | 1    | 0       | 20:18    | 19:19    | 0.243 |
| D11-1650    | 1    | 0       | 14:24    | 19:19    | 2.632 |
| E3-480      | 1    | 0       | 16:22    | 19:19    | 0.974 |
| F7-690      | 1    | 0       | 25:13    | 19:19    | 3.789 |
| F7-700      | 0    | 1       | 22:16    | 19:19    | 0.947 |
| F7-750      | 0    | 1       | 14:24    | 19:19    | 2.632 |
| F7-760      | 1    | 0       | 24:14    | 19:19    | 2.632 |
| F7-1050     | 1    | 0       | 15:23    | 19:19    | 1.684 |

In addition, 30 loci were observed which were present in one parent only and in all of the F₁ progeny, as expected.

Eighty-three repeatable loci were identified in the F₁ progeny, of which six were monomorphic (Table 4). Fifty-two of the polymorphic loci (67.5%) segregated as expected in the F₁, as determined by chi-square goodness-of-fit tests (Tables 4 and 5; Fig. 2). Twenty-five loci, or 32.5%, had irregular ratios. Of 52 loci for which parental genotypes could be ascertained, 'Jete' was 30.8% heterozygous, while 'Lessard' was 13.5% heterozygous (Table 6). The proportion of "positive" (present) and "negative" (absent) alleles was about 40% and 60%, for 'Jete' and 'Lessard,' respectively.

Discussion

Pascual et al. (1993) distinguished all but two of seven Spanish cherimoya cultivars with 13 isozyme loci; 10 of these loci were identical between 'Jete' and 'Campa.' Our band-sharing results also show a high homology between 'Jete' and 'Campa,' the A. cherimola cultivars (Table 3). Since a single RAPD primer may generate several loci (ranging from 2-15 bands in this study), 10 of the fifteen primers, used individually, could be used to distinguish these two closely related cultivars.

For five loci, ratios of 37:1 or 36:2 were observed, while no segregation (38:0) was expected. These were determined to be close enough to expected for inclusion into the normally segregating group. It is possible that two or more co-migrating loci exist, which would alter expected ratios as demonstrated in cacao (Ronning et al., 1995). Since the sample size of 38 is too small to test this hypothesis, it is assumed that structural, or nongenetic changes at these sites prevented amplification.

Due to the flowering cycles and patterns of A. cherimola and A. squamosa, spontaneous hybrids will form naturally and inevitably when they are planted in close proximity (Gazit and Eisenstein, 1985). However, the ecological and climatic requirements of the two species are so different as to form a geographical barrier between them in nature. Indeed, A. squamosa 'Lessard' shared only one-fourth of the bands with either A. cherimola cultivar (Table 3).

Although RAPD markers are dominantly inherited, expected segregation ratios in an F₁ can be inferred, in some cases, from parental phenotypes. This approach has been useful in studying conifers (Carlson et al., 1991) and cacao (Ronning et al., 1995), perennial species with long generations times in which obtaining segregating F₁ progeny from inbred parents is difficult or impossible. As shown in Table 2, expected segregation ratios in an F₁ can be unambiguously determined from parental phenotypes in all but three cases. For our data, six loci were monomorphic; parental genotypes and hence progeny ratios could not be definitively assigned. Fifty-two loci, however, exhibited standard Mendelian segregation ratios that were used both to determine expected F₁ ratios and to estimate heterozygosity of the two parents.

When the RAPD genotypes of the parents are extrapolated from the observed segregation ratios in the F₁, it was found that 'Jete' is 69.2% homozygous for these 52 RAPD loci, and 'Lessard' is 86.5% homozygous, even though allele frequencies between the two cultivars do not differ (Table 6). These results are to be expected; A. squamosa cultivars are often propagated by seeds, since they reproduce true to type (Gazit and Eisenstein, 1985). Since the atemoyas are the progeny of A. cherimola x A. squamosa hybridizations, it is interesting to note that the identity between 'Lessard' and the atemoyas is much higher than that between A. cherimola and the atemoyas (averaging 69.9% and 37.9%, respectively). The presence of higher amounts of A. squamosa 'Lessard' is much higher than that between A. cherimola cultivars and atemoyas is much higher than that between A. cherimola and the atemoyas (averaging 69.9% and 37.9%, respectively).
Fig. 2. Inheritance of RAPD banding patterns produced by primers D8 (A) and E7 (B) in the progeny of the interspecific cross Annona cherimola 'Jete' × A. squamosa 'Lessard', and in two selfed progeny of 'Jete' and 'Lessard.' PCR products were separated on 1.4% agarose in 5× TBE, and stained with EtBr. Lanes 1, 13, 25, 26, 38, and 50: 100 bp ladder; lanes 2 and 3: 'Jete' selfs; lanes 4 and 5: 'Lessard' selfs; lane 6: 'Jete'; lane 7: 'Lessard'; lanes 8-12, 14-24, 27-37, and 39-49: F1 progeny.

Table 6. Proportion of heterozygotes and homozygotes, and allele frequencies, in Annona cherimola 'Jete' and A. squamosa 'Lessard', as calculated from 52 polymorphic RAPD loci. 1 = allele present; 0 = allele absent.

|         | Jete | Lessard |
|---------|------|---------|
| Percent homozygous | 69.2 | 86.5 |
| Percent heterozygous | 30.8 | 13.5 |
| Percent (1) alleles | 40.4 | 39.4 |
| Percent (0) alleles | 59.6 | 60.6 |

‘Campa’ and the atemoyas, and 2 between the atemoyas and ‘Jete’. However, 25 bands were found to be unique to ‘Lessard’ and at least one of the atemoya cultivars.

Using RAPD markers, it should be possible to discern these relationships and determine likely parentage. This first work with Annona indicates that RAPDs will be a useful tool in identifying Annona cultivars, and may be valuable for genetic and breeding studies as well.

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