in: PCR Technology: Current Innovations, H.G. Griffin and A. M. Griffin, eds.,
CRC Press, Boca Raton, FL. 1994

Chapter 21

THE USE OF PCR-RAPD ANALYSIS IN PLANT TAXONOMY AND EVOLUTION

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I. INTRODUCTION

Molecular markers such as restriction fragment length polymorphisms (RFLPs) and isozymes have been extensively used for genetic studies and plant identification.[1-3] Restriction fragments are codominantly and stably inherited in a Mendelian fashion, and they disclose unlimited polymorphic markers. The problem with RFLPs is that the procedure is time consuming, requires a large amount of DNA (2 to 10 μg) and suitable probes, and usually

0-8493-8674-8/94/$0.00+$.50

involves the use of radioisotopes. Isozyme analysis is limited by the small number of loci sampled by the technique.

The polymerase chain reaction (PCR) has facilitated genetic studies in plants and animals.[4,5] DNA fingerprinting, forensic analysis, genetic mapping, and phylogenetic studies have tremendously benefited from PCR. One variation of PCR is the random amplified polymorphic DNA (RAPD), which generates DNA fingerprints with a single synthetic oligonucleotide primer. The polymorphisms observed may result from point mutations, insertions, deletions, and inversions.[6] RAPDs are usually dominant markers and are inherited in a simple Mendelian fashion. In comparison with RFLP, the procedure is less expensive, faster, requires a smaller amount of DNA (0.5 to 50.0 ng), does not involve the use of radioisotopes, and requires less skill to operate. Because of these advantages, RAPDs have proven useful in genotype identification and gene mapping. Although details about RAPD analysis and use are presented by Yu and Pauls in Chapters 22 and 23, it is worth mentioning that Penner et al.[7] recently investigated reproducibility in RAPDs using the same target DNA and primers in different laboratories. They found most RAPD markers to be reproducible, with differences between PCR machines accounting for most of the variations. This review will focus on the use of RAPDs for taxonomic and evolutionary analysis.

II. ANALYSIS AT THE SUBGENERIC (SECTIONAL) LEVEL

Although almost all the research to date has focused on taxonomy and classification at the species level or below, one paper (Adams and Demeke[8]) has dealt with taxonomy at the sectional level (subgeneric) in *Juniperus*. The genus *Juniperus* L. is very diverse, with about 75 to 80 taxa worldwide, of which 41 taxa are found in the Western Hemisphere. On the basis of morphology, three sections (*Caryocedrus*, *Juniperus*, and *Sabina*) have been recognized.[9] The utility of RAPDs at different taxonomic levels was investigated in *Juniperus*.[8] Notice the separation of sections *Caryocedrus*, *Juniperus*, and *Sabina* (Figure 1) by the principal coordinate analysis (PCO). In this instance, RAPDs were found to be taxonomically useful at the subgeneric level. In addition, species in the two series, entire and serrate, in section *Sabina* were separated on the second principal coordinate (Figure 1). The established relationships based on morphological and terpenoid analysis were confirmed by the RAPD data. Adams and Demeke[8] found that some primers amplify DNA that is highly conserved and will thus help to generate polymorphisms at a high levels of classification whereas other primers amplify DNA that is highly variable and useful for classification and analyses at and below the species level. As more studies are done, it is expected that this observation will be tested and that primers may be classified in this manner and thus be more predictable concerning their utilization.

III. INTERSPECIFIC TAXONOMY AND CLASSIFICATION

RAPDs have been used in several taxonomic studies at the species level. Wilkie et al.[10] used 20 random primers (10-mers) to analyze *Allium* species (and cultivars). The results were in broad agreement with classical classifications. However, *A. roylei* was shown to be the closest relative of *A. cepa*, in contrast to results from previous work.

The evolution and genetics of *Brassica* species has been well documented. The different techniques used include RFLP,[11,12] chloroplast analysis,[13] morphological taxonomy and cytogenetics, [14,15] and analysis of isozymes and rDNA genes.[16] These studies have confirmed the classical U triangle relationship[17] among the diploid and amphidiploid *Brassica* taxa. Demeke et al.[18] examined the potential taxonomic use of RAPDs in *Brassica* species, *Sinapis* and

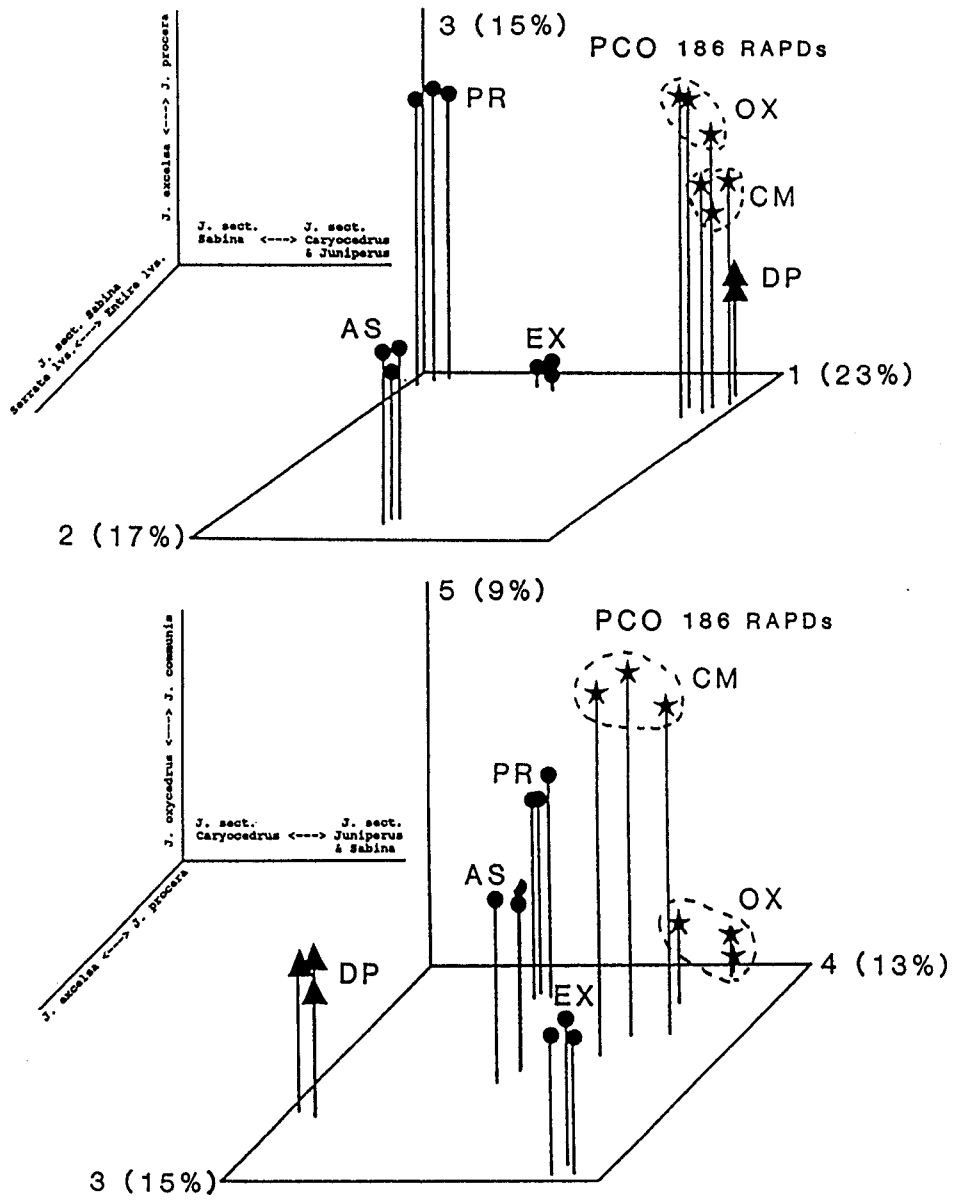

Figure 1. Ordination of *Juniperus* species using PCO3D, based on 186 RAPDs (RAPD bands). Notice (upper) the separation of sections *Sabina* and *Caryocedrus*/*Juniperus* on the first coordinate (23% of the variance) and section *Caryocedrus* from *Juniperus*/ *Sabina* on the fourth coordinate (lower, 13% of the variance). Each symbol represents a different individual of a taxon. (Adapted from Adams, R. P. and Demeke, T., *Taxon*, 42, 553, 1993, permission of the International Association for Plant Taxonomy from Adams and Demeke.)

Raphanus taxa. Principal coordinate analysis (PCO) of 284 RAPD bands showed the classical U triangle relationships among diploid and amphidiploid *Brassica* taxa (Figure 2). A minimum of ten primers with a total of 100 RAPD bands were needed to explain the genetic relationships. The use of only six primers resulted in a poorer classification. Cultivars of cabbage and cauliflower were also clearly distinguished by the RAPD analysis. Polymorphisms were also obtained among individual seedlings of *B. carinata* cv. *Dodola*. In this study RAPDs proved to be useful at taxonomic levels ranging from individuals to cultivars and species.

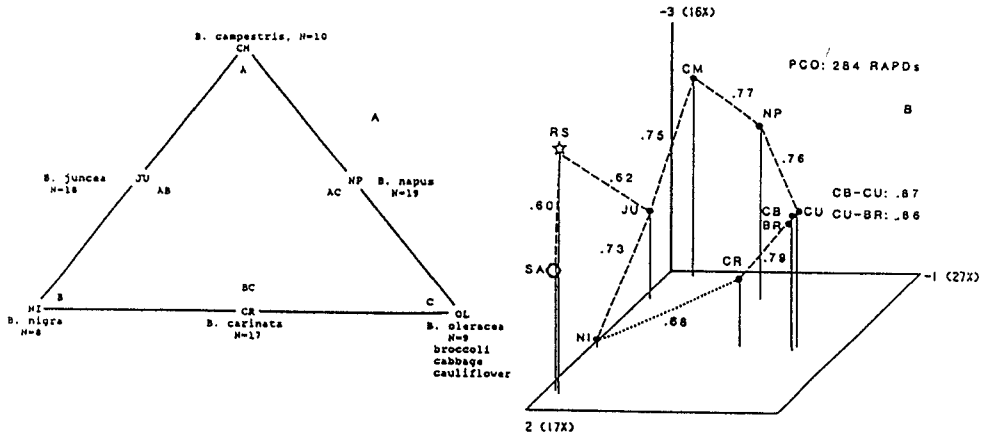

Figure 2. (A) Classically defined relationships among *Brassica* species (U triangle). (B) Results of PCO ordination using 284 RAPDs (markers). Notice the U triangle, with vertices of CM, OL (CU, CB, and BR), and NI, just as in the case of the classical system. Also note the intermediate nature of the amphidiploids, JU, CR, and NP. (From Demeke, T., Adams, R. P., and Chibbar, R., *Theor. Appl. Genet.*, 84, 990, 1992. With permission.)

Halward et al.[19] compared cultivated peanut with 29 wild diploid species of *Arachis* using ten primers. They found no variation in banding patterns among the cultivars but found variation among the wild species. PAUP and HyperRFLP programs were used to generate dendrograms showing genetic relationships among the diploid *Arachis* species.

Four *Stylosanthes* species were analyzed[20] using 22 RAPD primers. This resulted in 200 RAPD markers (RAPDs) that were used to generate clustering. Four main clusters were produced (Figure 3) in which all accessions of each species cluster together. Genetic variation appeared to be greater in *S. guianensis* and lesser in *S. hamata* (Figure 3). The phylogenetic results were in agreement with morphology, cytology, and enzyme electrophoresis.

IV. GEOGRAPHIC VARIATION AND INFRASPECIFIC TAXONOMY

Both RAPDs and leaf volatile terpenoids were used to compare junipers from Abha, Saudi Arabia, with *Juniperus excelsa* from Greece and *J. procera* from Addis Ababa, Ethiopia.[21] *Juniperus procera* Hochst. ex Endl. is the only species of the genus that grows naturally in the Southern Hemisphere. It has been postulated[22] that *J. procera* originated from *J. excelsa* in Asia Minor in the Mio-pliocene as *J. excelsa* expanded its range southward along the Western mountains of the Arabian Peninsula, thence across the Red Sea to Ethiopia and southward along East African rift mountains. Most taxonomic treatments call the juniper from the Saudi Arabian Peninsula *J. excelsa*.[22] The RAPD data clearly show (Figure 4) that the juniper at Abha, Saudi Arabia, is *J. procera*, even though the morphological data were inconclusive. In addition, the volatile leaf terpenoids show the same pattern as the RAPDs. Analyses using RAPDs are clearly shown to be of use in analyses of geographical variation questions.

Other studies using RAPDs for the analysis of geographic variation include that of *Microseris elegans*,[23] where 17 primers were used to generate 134 RAPDs from ten populations from throughout California. Brauner et al.[24] used 16 primers to analyze genetic diversity among populations of *Lactoris fernandeziana*, endemic to the island of Masatierra in the Juan Fernandez Archipelago. They found nearly all variants restricted to single populations.

The only paper depicting geographically contoured variation in RAPD similarities is that of Demeke and Adams[25] on the medicinal plant *Phytolacca dodecandra* in Africa. Geographical variation of *P. dodecandra* in Africa has been investigated in terms of morphology, leaf chemistry, and triterpene aglycones.[26-28] Use of PCO, followed by contour mapping of the

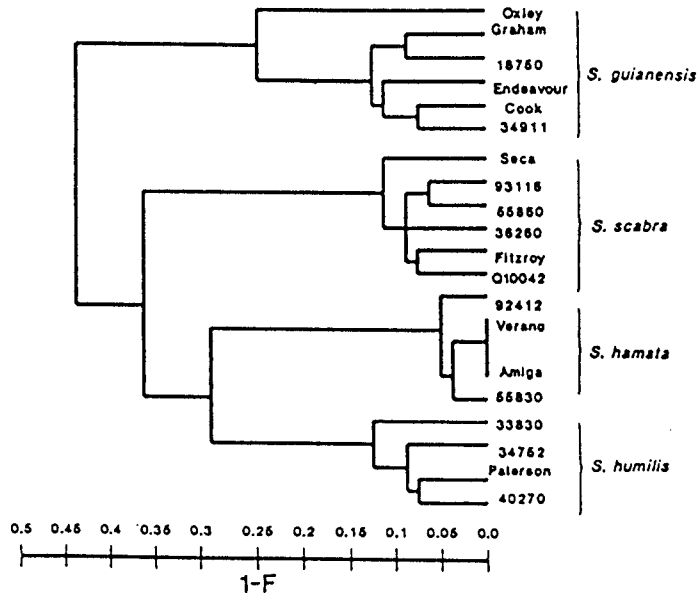

Figure 3. Clustering of four *Stylosanthes* species by UPGMA based on RAPDs data. Notice the clustering of all the accessions by species. (From Kazan, K., Manners, J. M., and Cameron, D. F., *Theor. Appl. Genet.*, 85, 882, 1993. With permission.)

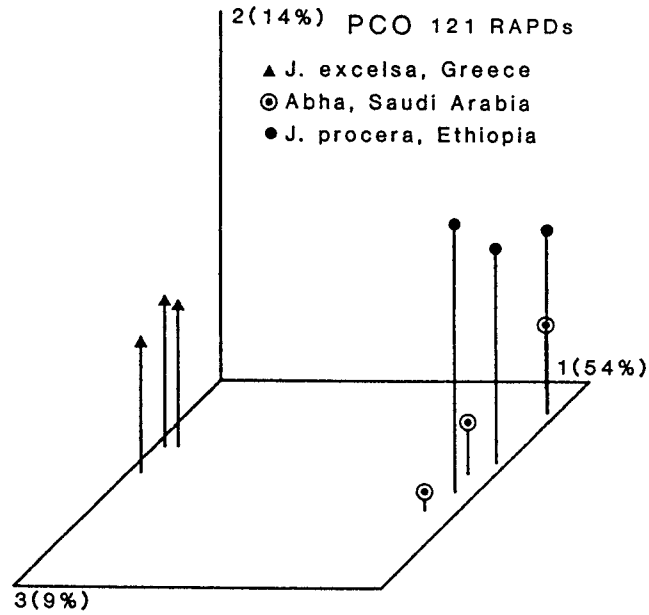

Figure 4. PCO ordination of *J. excelsa*, Greece, *J. procera*, Ethiopia, and junipers from *Saudi Arabia*, based on 121 RAPDs. The Saudi Arabian junipers clearly cluster with *J. procera* (axis 1, 54% of the variance among individuals). (From Adams, R. P., Demeke, T., and Abulfatih, H. A., *Theor. Appl. Genet.*, 87, 22, 1993. With permission.)

population scores on each axis revealed geographic patterns (Figure 5). Several of these trends were the same as those previously found in morphological and chemical data. Four major groups were discovered among *P. dodecandra* populations: Madagascar, Nigeria, Ethiopia, and Southern Africa. The studies suggest these four groups to be the center of focus for future *P. dodecandra* germplasm collection.

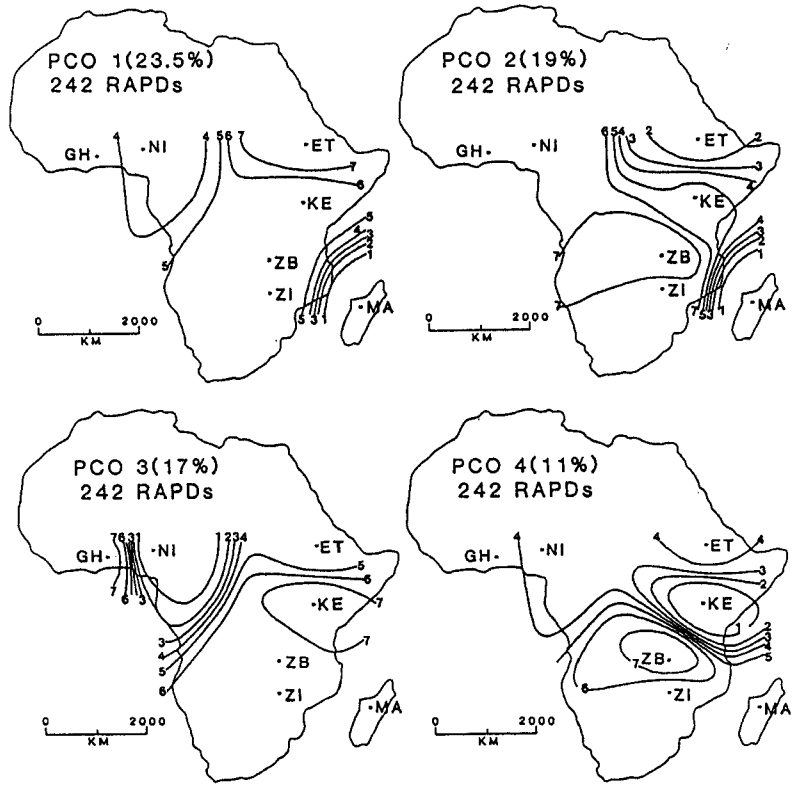

Figure 5. Geographical variation patterns in the RAPDs of *Phytolacca dodecandra*. The population coordinate scores on each principal coordinate (1 to 4) were used at Z values for contouring. (From Demeke, T. and Adams, R. P., in *Conservation of Plant Genes II: Utilization of Ancient and Moder2n DNA*, Adams, R. P., Miller, J. S., Golenberg, E. M., and Adams, J. E., Eds., Missouri Botanical Garden Press, St. Louis, 1994, chap. 11. With permission.)

Finally, it should be mentioned that RAPDs have also been used for the analysis of geographic variation in the fungus *Colletotirchum graminicola*.[29]

V. CULTIVAR CLASSIFICATION AND IDENTIFICATION

Cultivar classification, germplasm collection, and characterization are major undertakings in developing and developed countries. Characterization is usually done with morphological markers, which may not give an accurate picture because of environmental influences. RAPD analysis is a simple, fast, accurate, and relatively inexpensive tool to characterize germplasm collections. The procedure can have wide applicability in developing countries.

A study of the relationships among ten papaya (*Carica papaya* L.) cultivars was made with 11 decamers.[30] A dendrogram was constructed using 102 distinct DNA fragments (Figure 6). The seven Hawaiian types are grouped in the upper branch. Divisions within the Hawaiian types were mostly consistent with the known genetic background of the cultivars. The RAPD procedure was found to be fast, precise, and sensitive for genomic analysis.

Pedigree relationships in spring barley lines were examined by Tinker et al.[31]. Two- and six-row lines were separated by cluster analysis. Within the two- and six row groups, other qualitative similarities were observed. A linear relationship was found between kinship coefficients and genetic distance in the cluster analysis. It was concluded that RAPD markers could be used to gain information about genetic similarities or differences that are not evident from pedigree information.

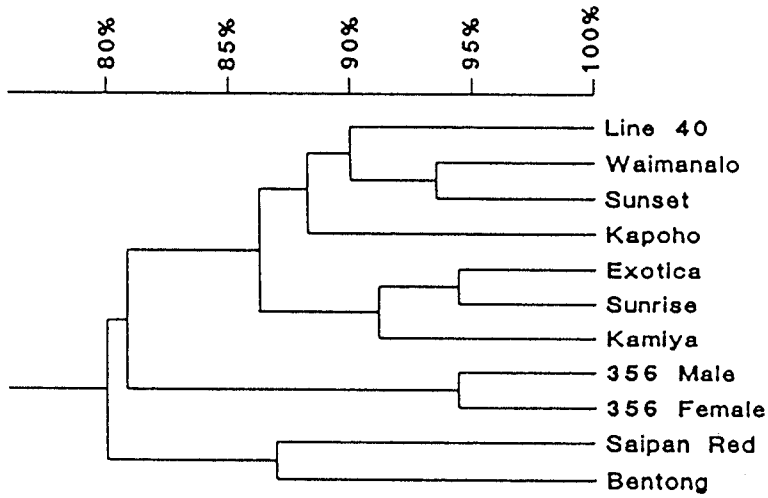

Figure 6. Phenogram of papaya cultivars based on *UPGMA* clustering of 102 RAPDs. The upper seven cultivars are the Hawaiian type and cluster together. Divisions within the Hawaiian branch were mostly consistent with known genetic backgrounds. (From Stiles, J. I., Lemme, C., S., Morshidi, M. B., and Marshardt, R., *Theor. Appl. Genet.*, 85, 697, 1993. With permission.)

Wheat cultivars generally have a low level of intervarietal polymorphisms. According to Gale et al.,[32] RFLP mapping in *Triticum aestivum* ($2n = 6x = 42$) has been hindered by low levels of intervarietal polymorphisms, complexities arising from polyploidy, and large genome size. RAPD analysis often generates more polymorphisms than RFLP procedure. Inter- and intraspecific diversity was found in wheat, barley, rye, and wheat-barley addition lines using conserved, semi-random, and random primers.[33] Vierling and Nguyen[34] investigated the genetic diversity of two diploid wheat species, *T. monococcum* and *T. urartu* ($2n = 2x = 14$) using random primers. A higher rate of polymorphisms were observed in *T. urartu* than in *T. monococcum*.

In order to visualize more markers in cereal crops with low levels of intervarietal polymorphisms such as wheat, denaturing gradient gel-electrophoresis (DGGE) might be used. DDGE has previously been used to analyze polymorphisms.[35,36] DNA fragments differing by single base pair substitutions are separated on denaturing gradient gels. More polymorphisms have been observed by running RAPD products on DGGE gel than on the normal agarose gel.[37] The DGGE method is also reported to generate many more polymorphisms than the RFLP procedure.

One of the problems in using RAPDs for identification of markers in wheat (e.g., disease resistance) is lack of reproducibility.[38] The problem is less pronounced in dicots. The high ploidy level of wheat may contribute to the complexity of the problem. Some primers are more unstable than others. Optimization of the PCR procedure is very important to get reasonably reproducible results. The development of reliable PCR-based markers such as SCARs (sequence characterized amplified regions), explained by Paran and Michelmore,[39] increases the use of RAPDs as genetic markers.

RAPDs were used to identify 16 rice accessions.[40] 28 decamers generated 244 different RAPD bands, and 116 of them were informative in that they differentiated one or more accessions. All accessions were uniquely distinguished by at least one RAPD band, and the accessions clustered into three ecospecies, i.e., *Japonica*, *Javanica*, and *Indica*, supporting previous morphological recognition of the three taxa. The differences among the species were demonstrated with at least 23 polymorphisms between *Japonica* and *Javanica*, 71 between *Japonica* and *Indica*, and 72 between *Indica* and *Javanica*. The results confirmed the previous isozyme and RFLP studies.[41,42] RAPDs are very useful in the identification of rice accessions and are much simpler to use than RFLP analysis.

Very low variability was detected among cultivated peanut cultivars (*Arachis hypogaea* L.) using RFLPs.[43] Peanut cultivars have a narrow genetic base. Genetic polymorphisms were studied in wild peanut plants and cultivated species by RFLP, RAPD, and four-cutter analysis of PCR-amplified fragments.[44] A high level of polymorphism was found among wild species, whereas little polymorphism was found in cultivated peanut in all cases. The utilization of wild germplasm for peanut breeding programs has been emphasized.

Other examples of cultivar and/or accession RAPD analyses include apples,[45] banana,[46] and celery.[47]

VI. GENETIC ANALYSES WITHIN CLONES

A. GENETIC LINKAGE

Tulsieram et al.[48] report on a unique application of RAPD technology for genetic linkage mapping of a single spruce tree using haploid DNA from megagametophyte tissue of individual seeds. Sixty-one segregating loci were analyzed for inheritance and linkage using RAPDs. This tool should be useful in the study of the evolution of linkage among genes.

B. ANALYSIS OF SOMACLONAL VARIATION

Isabel et al.[49] used RAPDs to evaluate the genetic integrity of somatic embyrogensis-derived cell lines of *Picea mariana* (black spruce). No variation was found in the clonal lines in the RAPDs, so the authors propose use of RAPDs to assess genetic stability and certify stability during somatic embryogenesis. This is an interesting application of DNA fingerprinting by RAPDs that may find wider usage in the nursery industry as well as in biotechnology.

VII. ANALYSES OF GENE FLOW BETWEEN INDIVIDUALS

Lewis and Snow[50] show that RAPDs can be of significant use to ecologists in studying mating systems and assigning paternity. The unambiguous assignment of paternity can be extremely valuable in understanding mating patterns of birds, for example. In plants, insect pollination might be more exactly studied because all the adjacent plants (potential pollen sources) could be fingerprinted by RAPDs and the dominant RAPD bands seen in the resulting seeds.

VIII. HYBRIDIZATION STUDIES

A. INTERGENERIC HYBRIDIZATION

Crawford et al.[51] used RAPDs to document the origin of an intergeneric hybrid (*Margyracaena skottsbergii*). One parent, *Acaena argentea* had 18 species-specific bands, the other parent, *M. digynus*, had 27 species-specific bands, and the putative intergeneric hybrid had all 45 bands present. They conclude that RAPDs are particularly attractive for the study of hybridization in rare species because they can provide numerous genetic markers while requiring minimal amounts of DNA.

B. INTERSPECIFIC HYBRIDIZATION AND INTROGRESSION

Arnold et al.,[52] in one of the earliest papers that used RAPDs, analyzed a classical case of hybridization and introgression in irises. They reported intermediate frequencies for RAPD markers in putative hybrid populations, corresponding to cpDNA markers. The study used only three primers and reported on only nine RAPD markers. Additional primers would have been desirable.

Waugh et al.[53] used six RAPD primers for the detection of introgression in the potato. The RAPD data showed that dihaploids, generated after interspecific pollination of a tetraploid

Solanum tuberosum cultivar by *S. phureja*, was the result of gene flow, not of parthenogenic origin. They concluded that RAPDs would be useful for the detection of gene introgression in both natural and cultivated plant populations.

In order to confirm the genetic basis of RAPD markers in conifers, Carlson et al.[54] examined inheritance of RAPDs in both Douglas fir and white spruce. The RAPD markers were found to be inherited as dominants, as originally shown for soybeans by Williams et al.[6] They concluded that RAPDs would be useful genetic markers.

IX. ANALYSES OF GENETIC DIVERSITY

A number of workers have used RAPDs for the analyses of genetic diversity. We have already mentioned the work on wheat by Vierling and Nguyen.[34] Mosseler et al.[55] examined populations of red pine, white spruce, and black spruce by RAPDs and found that disjunct Newfoundland populations of red pine were largely monomorphic for 69 RAPD bands and that they relate to genetic isolation from mainland populations. The RAPD data corresponded with genetic diversity estimates using isozymes for red pine, white spruce, and black spruce.

Genetic diversity in *Arachis* (peanut) germplasm was investigated by use of RAPDs.[56] Sixty 10-mer primers yielded 49 polymorphic loci between a cultivated *A. hypogaea* (TMV-3) and a synthetic amphidiploid. The researchers concluded that RAPDs offer a simple, yet efficient tool for the characterization of *Arachis* germplasm and for use in hybridization research.

Other examples of genetic analysis include alfalfa[57] and wheat.[38]

X. ANALYSES OF THE EVOLUTION OF DISEASE RESISTANCE

The evolution of disease resistance in plants is of great interest to crop scientists as well as botanists. Martin et al.[58] used 144 RAPD primers and obtained seven RAPD bands that were associated with *Pseudomonas* resistance in tomato. Four of these RAPDs were tightly linked to the bacterial-resistance *Pto* gene. This provides both an insight into the evolution of disease resistance and information that should be useful for plant breeding.

In lettuce, RAPD markers have been discovered that are linked to downy mildew resistance genes,[39,59] and bacterial blight has been analyzed by RAPDs.[60]

XI. STUDIES OF ANCIENT DNA

It is not our intention to give a detailed review of ancient DNA studies. However, several papers have already been published and others will follow quickly. PCR has already made a tremendous contribution to the study of archaeological and fossil specimens and will usher in the new field of molecular archaeology.

Nanogram quantities of DNA were extracted from plant tissues ranging in age from 22 to more than 45,000 years old,[61] showing the potential of obtaining usable DNA from ancient samples.

Brown et al.[62] used PCR to amplify a 246-bp DNA fragment from charred wheat seeds 700 to 3300 years old. They considered using RAPDs but are cautious that "jumping PCR"[63] might produce spurious bands. However, they do indicate plans to try RAPDs on their material in the future.

Rollo et al.[64] examined DNA from 1000-year-old maize kernels using PCR to amplify the *Mu* termini and the cytochrome *c* oxydase subunit I gene. Although, RAPDs were not used in the study, their application seems appropriate.

A 790-bp cpDNA fragment of rbcL from a 17 to 20 million year old *Magnolia* leaf compression fossil has been amplified by PCR and sequenced.[65] The fossil specimen clustered

with extant members of *Magnolia*. However, results from the study were later questioned, with the emergence of a model that predicted that DNA would be completely degraded after 4 million years.[66] In a later study, *Taxodium* material from the same fossil formation (a 1320 bp portion of the chloroplast rbcL gene from a Miocene *Taxodium* specimen from the Clarkia site) has been successfully amplified and sequenced,[67] validating the *Magnolia* study.

The study of genealogical relationships of extinct species and vanished populations has been reported.[68-70] Mitochondrial DNA from a 7000-year-old human brain were amplified by PCR and sequenced. The sequences showed that the individual belonged to a mitochondrial lineage that is rare in the Old World and not previously known to exist among native Americans.

This area of research will grow very rapidly, and PCR and PCR-RAPD will be increasingly applied to these problems.

XII. NUMERICAL ANALYSES OF RAPD DATA

Several numerical methods have been used to analyze RAPD data. In our work, we have coded the data both as presence/absence and intensity values (e.g., 0 to 6, with 0 being no band, and 6 being a very bright band). We found intensity values to be more informative than presence/absence data.[8,18] Similarity measures were computed using absolute character state differences (Manhattan metric) divided by the maximum observed value for that character over all taxa (= Gower metric, Gower;[71] Adams[72]). Principal coordinate analysis was performed according to Gower.[73] Principal coordinate analysis is ideally suited for use with large numbers of characters and numerous taxa. Each eigenroot extracts a separate component of variance among the taxa. This enables one to progressively examine the relationships, particularly in geographic trend analysis.[25] However, care should be taken in the interpretation of three-dimensional figures when the number of eigenroots extracted is large (see Adams and Demeke[8]). In our RAPD research, we obtained very convincing results for *Brassica*, *Juniperus*, and *Phytolacca dodecandra*, as previously noted. The results supported previous chemical and morphological studies, and the *Brassica* data also confirmed the previous molecular studies. In our experience, data from at least ten primers with a total of about 100 RAPD bands are needed to produce a stable classification. Using more primers may not be necessary, as little additional information was gained by increasing the number of primers from 11 to 17 in our *Brassica* study.[18] The computer software for PCO analysis, PCO3D, is available from R.P. Adams for distribution.

A number of similarity coefficients have been used for phenogram constructions in other studies: Jaccard's similarity coefficients;[74] index of genetic similarity;[75] Jaquard index;[76] and Rogers' distance.[77] Phenograms have been constructed by the *UPGMA* program[78] and parsimony analysis.[79] It might be noted that *UPGMA* uses the average similarity of an operational taxonomic unit (OTU) rather than the nearest neighbor to bring a new member into a group. It would seem that evolution would favor the fewest mutations and thus nearest-neighbor or single linkage clustering would be preferable over *UPGMA*. The programs used include *PAUP*, *PHYLIP*, *CLINCH*, *MaClade*, and *NT-SYS*. Some analyses have also been performed with the *SAS* program (SAS Institute).

XIII. CONCLUSION

Because of their technical ease and cost effectiveness, PCR-RAPDs will become commonplace (perhaps a cornerstone) in taxonomic and evolutionary studies. Good agreement has been achieved between RAPD analysis and morphology, chemical, and molecular (isozyme, RFLP) data. RAPDs will also have a wide applicability in germplasm collection and characterization programs. Analyses of ancient DNA will move toward the use of RAPDs in many

cases. However, fossil DNA will probably require sequence data due to problems of homology of RAPD bands with extremely ancient taxa. RAPD protocols will also be refined through time so that some of the current problems such as occasional lack of reproducibility will be overcome.

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