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Characterization of genetic identities and relationships of *Brassica oleracea* L. via a random amplified polymorphic DNA assay

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Summary. Effective conservation and the use of plant genetic resources are essential for future agricultural progress. Critical to this conservation effort is the development of genetic markers which not only distinguish individuals and accessions but also reflect the inherent variation and genetic relationships among collection holdings. We have examined the applicability of the random amplified polymorphic DNA (RAPD) assay for quick, cost-effective, and reliable use in addressing these needs in relation to collection organization and management. Twenty-five decamer oligonucleotide primers were screened individually with a test array composed of individuals representing a range of genetic relationships in *Brassica oleracea* L. (vegetable and forage cole crops). Over 140 reproducible, polymorphic fragments were generated for study. Each individual of the test array exhibited a unique molecular genotype and composites specific for accessions and botanical varieties could be established. An analysis of similarity based on amplified DNA fragments reflected the known genetic relationships among the selected entries. These results demonstrated that RAPD markers can be of great value in gene bank management for purposes of identification, measurement of variation, and establishment of genetic similarity at the intraspecific level.

Key words: DNA typing – Genetic similarity – Genetic structure – Genetic resource conservation – Vegetable and forage cole crops

Introduction

The use and value of plant genetic resources to past, present, and future crop agriculture has been recognized by scientists and society alike (Shands and Weisner 1991 a, b; Oldfield 1989). Progress in plant breeding, genetics, and molecular biology, combined with an enhanced concern for both ex-situ and in-situ preservation of biodiversity, has greatly increased the responsibility and visibility of those charged with long-term conservation and the use of plant genetic resources (Kresovich 1992). Unfortunately, curators and affiliated researchers are routinely challenged by chronic shortages of funding and by collections that are growing at rates faster than they can be effectively maintained, characterized, evaluated, and utilized (Kresovich and McFerson 1992).

Part of the solution to this challenge may lie in the use of powerful, yet relatively simple and inexpensive, molecular techniques (Avise 1989; Erlich et al. 1991) to generate information to better organize the useful genetic variation present within a collection. These molecular techniques, closely linked with classical approaches, might be employed for purposes of (1) identification, (2) measurement of the genetic variation, (3) establishment of genetic relationships, and (4) correlation of markers with desirable traits for introgression.

Recent research (Welsh and McClelland 1990, 1991; Williams et al. 1990, 1992) has demonstrated various scientific, operational, and budgetary advantages for use of the RAPD assay for genome mapping. These same advantages also appear valid for characterization of the genetic variation of crops for effective conservation and use. The objective of this research was to investigate the applicability of the RAPD assay (Williams et al. 1990, 1992) for purposes of identification and assessment of genetic structure and relationships within a crop species. A test array of *Brassica oleracea* L. (vegetable and forage cole crops) (IBPGR 1991) was established as the experimental material for this research because of its tremendous phenotypic diversity, varied end use, and the com-

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plexity associated with its ex-situ maintenance (large plant size, vernalization requirement, allogamous breeding system, and limited seed viability).

Materials and methods

Plant materials

The test array included 22 *B. oleracea* entries representing ten of the 14 recognized botanical varieties as well as a range of genetic relatedness among entries (Table 1). Two individuals of an accession of *Brassica rapa* L. were utilized as a reference and for comparisons between species. The array allowed for comparisons of individuals representing a known pedigree among individuals within accessions, among accessions within botanical varieties, and species, of the genus. Both elite and wild materials were included. These entries are also being characterized for genetic and agronomic/horticultural traits via complementary phenotypic, biochemical, and molecular approaches.

DNA extraction

Plant genomic DNA samples were extracted from young leaves following the CTAB method of Williams et al. (1992). Young leaf tissue (1.0 g) was ground in liquid nitrogen and mixed with 1.0 ml of CTAB extraction buffer and 0.4 ml of chloroform. The sample was heated to 55 °C for 10 min, centrifuged for 5 min, and the resulting supernatant was recovered and mixed with 1.2 vol of isopropanol. The nucleic acid precipitate was recovered by centrifugation, washed with 1.0 ml of 70% ethanol, dried, and dissolved in a solution of 10 m*M* Tris-HCl, pH 7.5, and 0.1 m*M* EDTA. The DNA concentration of each sample was estimated by comparing band intensities with known standards of lambda DNA on an agarose gel.

RAPD assay

Twenty-five decamer oligonucleotides (custom synthesized by Operon Technologies, Inc., Alameda, Calif., USA) were utilized for amplification of random DNA sequences of *B. oleracea* and *B. rapa* (Table 2). The nucleotide sequence of each primer was randomly generated with the sole specifications of a 50–70% GC content and no palindromes of greater than 4 bp. (Williams et al. 1990). The protocol reported by Williams et al. (1990) was followed with some modifications. Numerous adjustments were examined in order to optimize the RAPD assay to achieve the necessary reproducibility, resolution, cost-effectiveness, and speed (Fig. 1). Key modifications of, or additions to the RAPD assay included: (1) increased stringency of temperature (38 °C) during the annealing stage of amplification cycle, (2) increased annealing time, and (3) reduced gelatin concentration (0.001%) of the reaction buffer.

Reaction mixtures (25 µl) contained 10 m*M* Tris-HCl (pH 8.3); 50 m*M* KCl; 2.0 m*M* $MgCl_2$; 0.001% gelatin; 0.1 m*M* each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J., USA); 0.2 µ*M* of a single decamer primer; approximately 25 ng of genomic DNA; and 0.5 unit of DNA *Thermus aquaticus* (*Taq*) polymerase (Perkin Elmer Cetus, Norwalk, Conn., USA) overlayed with one drop of mineral oil. Reaction mixtures were incubated in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, Conn., USA) programmed for 45 cycles of 1 min at 94 °C, 5 min at 38 °C, with a 3-min ramp to 72 °C, and 2 min at 72 °C. Negative controls, the reaction mixture without genomic DNA, were run with each amplification. Amplification products were analyzed by gel electrophoresis in 1.4% agarose in 1 × TBE. Molecular sizes of the amplification products were estimated by utilizing a 1-kb DNA ladder (BRL, Bethesda, Md., USA). The gels were stained with ethidium bromide and photographed with black and white film #667 (Polaroid, Cambridge, Mass, USA) under UV light.

Table 1. List of individuals included among *Brassica* test array

Botanical variety	Cultivar[a]
capitata (cabbage)	'Reed Inbred A'
capitata	'Reed Inbred C'
capitata	'Reed A × C' (F_1 hybrid)
capitata	'Market Prize' (F_1 hybrid)
capitata	'Jersey Wakefield'
capitata	'Golden Acre'
capitata	'Wisconsin Golden Acre' (Plant #1)
capitata	'Wisconsin Golden Acre' (Plant #2)
botrytis (cauliflower)	'Extra Early Snowball'
botrytis	'All Year Round'
italica (broccoli)	'Packman' (F_1 hybrid)
italica	'Texas 107'
gemmifera (Brussels sprouts)	'Long Island Improved Catskill'
gongylodes (kohlrabi)	'White Vienna'
acephala (kale)	'Westland Winter'
ramosa (thousand head kale)	'New Zealand Thousand Head'
medullosa (marrow stem kale)	'Tall Marrowstem'
medullosa	'Giganta'
costata (Portuguese cabbage and kale)	'Couve Nabica'
costata	'Couve Galega Frisada' (Plant #1)
costata	'Couve Galega Frisada' (Plant #2)
costata	'Couve Poda'
Brassica rapa L. *rapifera* (turnip)	'Purple Top White Globe' (Plant #1)
Brassica rapa L. *rapifera*	'Purple Top White Globe' (Plant #2)

[a] For access to, or additional information about, specific entries, please contact J. R. McFerson at address noted previously

Genetic marker nomenclature

The amplified products useful for discrimination of genetic identities and relationships were identified by their associated primer number and the approximate size of the amplified fragment in base pairs (Table 2).

Statistical analysis

Results of the RAPD assay represented a consensus of four replicates (across amplifications and electrophoretic runs). Inconsistent areas of amplified activity were not included in the analysis. Therefore, we possibly omitted additionally useful information to ensure reliability of the assay. Data generated from detection of polymorphic fragments were analyzed employing the equation (Nei and Li 1979):

$$\text{Similarity} = (2 \cdot N_{ab})/(N_a + N_b)$$

Table 2. List of decamer oligonucleotides utilized as random primers, their sequences, and associated polymorphic fragments amplified among the *Brassica* test array

Primer identification	Sequence (5′ to 3′)	Number of polymorphic fragments	Fragment size range (bp)
1	CCCGATCCAC	4	510–1,460
2	CGACCTTTCA	4	710–1,010
3	ACAAGTACGG	3	1,310–2,140
4	AAGAGCCCGT	6	540–1,910
5	TGCCGAATTC	4	920–2,660
6	CGAACTAGAC	9	310–2,160
7	GGCTTAACAC	5	400–1,920
8	AAGACTGTCC	3	2,130–2,830
9	GCGGAAATAG	4	520–2,110
10	GTTTCCGCCC	2	1,010–1,640
11	CCGCAGCCAA	9	710–1,570
12	CCGATCTAGA	4	580–1,290
13	GCCCATACCA	4	650–2,500
14	CTACCAGAAC	6	750–2,090
15	GAGGACAAAG	6	540–1,930
16	AACGCGCAAC	5	620–1,210
17	GACGACTATC	5	530–1,120
18	GCGATCCCCA	5	780–1,860
19	GTCAACGAAG	9	630–2,040
20	AGCCAGTTTC	5	720–1,720
21	CGCATAGGTT	10	550–2,730
22	GGGGTTGACC	9	510–1,940
23	ACTTGCATCC	8	570–2,520
24	GTGGATGCGA	7	470–1,860
25	CCCGTCAGCA	7	540–2,370
Total among array (*B. oleracea* and *B. rapa*)		143	310–2,830
Total among *B. oleracea*		117	

when,

N_{ab} = number of shared fragments between individuals 'a' and 'b',
N_a = number of scored fragments of individual 'a', and
N_b = number of scored fragments of individual 'b'.

We chose this method of computation over other general similarity indices because of the increased weighting of fragment matches versus that of non-matches.

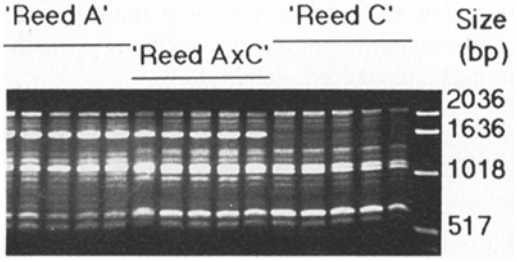

Fig. 1. Example of RAPD assay reproducibility based on modified protocols. Genomic DNA of five individual plants of 'Reed Inbred A' (*lanes 1–5*), 'Reed Hybrid A × C' (*lanes 6–10*), and 'Reed Inbred C' (*lanes 11–15*) amplified with primer #1. Note inheritance of fragment at approximately 1,600 bp (contributed from maternal parent to offspring)

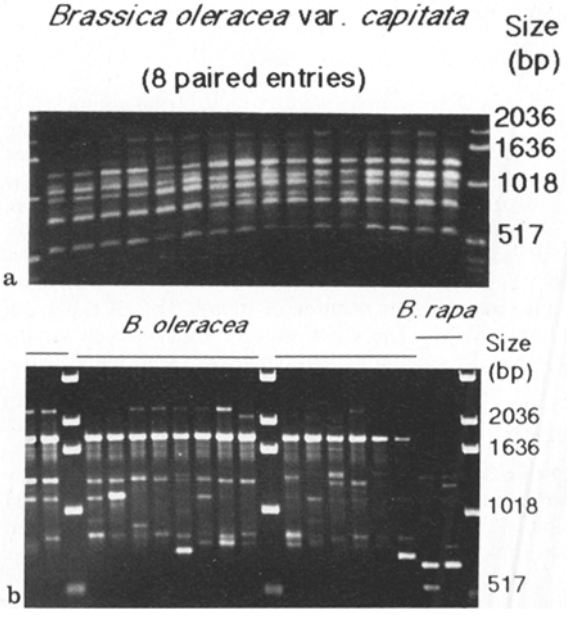

Fig. 2 a, b. Examples of ranges of molecular variation found in the *B. oleracea* test array. **a** Limited variation encountered among eight entries (duplicated lanes) of *B. oleracea* var. *capitata* assayed with primer #15. **b** Greater variation encountered among entries of *B. oleracea* and *B. rapa* assayed with primer #25

Results and discussion

The characteristic fragments generated by the array of 25 decamer oligonucleotides employed as single, arbitrary primers are summarized in Table 2. The number of polymorphic fragments varied per primer (2–10) as did the size range of fragments (310–2,830 bp). The minimum size difference detected between any two polymorphic fragments generated by a primer was approximately 20 bp. The amplified DNA reflected the genomic complexity of the plant species, the composition and size of the primer, and the experimental resolution and reproducibility (Fig. 2). Reproducibility was most critical in ultimate inclusion for analysis.

Overall, approximately 140 polymorphic fragments were generated among entries of the *Brassica* test array. An additional 50+ fragments were monomorphic across all of the primers. Invariant fragments ranged from 0–4 per primer. These findings contrast with those reported by Halward et al. (1991 a, b) in studies of *Arachis hypogaea* L. The greater degree of intraspecific variation detected in our research via the RAPD assay may well reflect differences in the pollination and breeding systems of the two taxa, the amount of domestication and crop

improvement, the number and types of individuals sampled per taxa, the number of primers screened, and/or technique optimization.

The number of primers used in the assay was important from two perspectives. First, the analysis of identities and relationships could either be uninformative or biased due to the use of a limited number of primers. For example, primer #16 generated five polymorphic fragments; however, four of these were useful only for discrimination of the two individuals of 'Purple Top White Globe' (*B. rapa*) from all the *B. oleracea* entries. Second, the increasing number of primers utilized greatly increases the overall cost of the analysis.

The empirical evidence of this research suggested that the genetic relationships among entries of the *Brassica* test array varied minimally after approximately 10–12 primers and/or 50–60 fragments were scored. Species could clearly be discriminated with as few as one well selected primer and/or 5–25 randomly chosen fragments. Botanical varieties formed groups following the analysis of 5–8 primers and/or 30–40 fragments. For finer resolution among accessions within a botanical variety, approximately 15 primers and/or 60–75 fragments were sufficient. These associations were usually maintained regardless of which specific primers were utilized.

Numerous unique markers were detected (Fig. 3). Because the *Brassica* test array was of limited size, more information on a greater number of individuals would be needed to satisfactorily corroborate the specificity of the fragments. However, the following information is presented heuristically to demonstrate what may be obtained for discrimination purposes. Ten fragments (#1-510, #1-600, #3-1,490, #15-1,930, #17-530, #17-650, #18-780, #18-1,100, #21-2,730, and #25-540) were unique to ten different individuals, and one fragment (#10-1,010) was unique to two individuals representing 'Couve Galega Frisada'. Three fragments (#15-540, #20-1,380, and #22-1,940) were unique to *B. oleracea* var. *capitata*. In addition, one characteristic fragment was generated specifically by entries of *B. oleracea* var. *italica* (#8-2,830) and *B. oleracea* var. *medullosa* (#19-1,300). Between *B. oleracea* and *B. rapa*, 26 fragments were useful for discrimination (data on specific fragments not shown). Therefore, the value of the RAPD assay proved two-fold, i.e., genetic similarities complemented with distinct fragment identification.

Each entry of the *Brassica* test array had a unique composite molecular genotype. Therefore, it is likely that a random decamer oligonucleotide primer screen may be utilized as a form of DNA typing for individual and accession identification (Fig. 4). Depending on the relatedness and number of the individuals to be distinguished, a different number of arbitrary and/or specific primers may be needed to document differences. We speculate that this type of reproducible, high resolution molecular

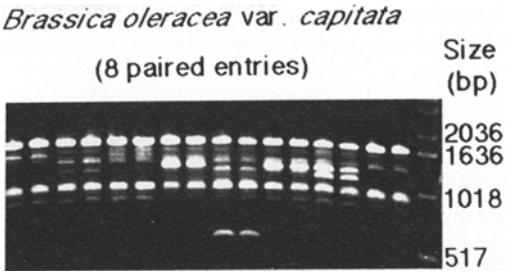

Fig. 3. Shared and unique polymorphic fragments among eight entries (duplicated lanes) of *B. oleracea* var. *capitata* generated by primer #1. Note unique fragment in 'Jersey Wakefield' (*lanes 10–11*) at approximately 510 bp

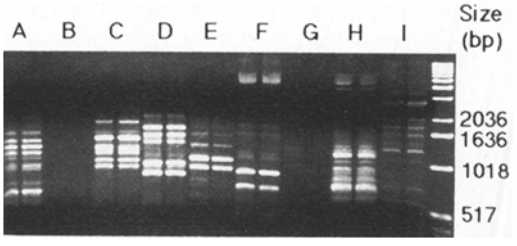

Fig. 4. *Brassica* DNA typing via decamer oligonucleotide primer screen. Results of the amplification of nine primers (designated as 'A–I' and duplicated) with genomic DNA from 'Reed Hybrid A × C'. Primers in *lanes 4–5* and *14–15* amplified extremely faint or no products

information will be applied to (1) the determination of cultivar purity, and (2) the establishment of property rights (plant variety protection and patent).

The genetic similarities among closely related individuals of *B. oleracea* var. *capitata* are summarized in Table 3a. Contrasts among 'Reed Inbred A', 'Reed Inbred C', and 'Reed Hybrid AxC' suggested that the RAPD assay can be employed for pedigree analysis and possibly for evaluations of crop improvement strategies. For example, the maternal parent ('Reed Inbred A') shared seven more fragments (about 50% of the polymorphic fragments discriminating 'Reed Inbred A' and 'Reed Inbred C') with the offspring than did the male progenitor ('Reed Inbred C'). Data suggested that the RAPD markers may be correlated with desirable traits associated with the maternal phenotype. It has also been suggested that bias toward the maternal parent may be expected with RAPD markers because of the excess of plastid genomes present in the amplification reaction. However, results of a preliminary study of genome mixing and competition due to concentration and complexity (Williams et al. 1990) differ with this premise. Nonetheless, RAPD markers may be readily employed for the fine discrimination required in pedigree analysis and inheritance studies (Fig. 1).

Many accessions in plant gene banks have similar names, origins, and historical backgrounds; the concern

Table 3. Genetic similarities among individuals representing a range of relationships of *B. oleracea* var. *capitata* (cabbage) and *costata* (Portuguese cabbage and kale). Results are based on 117 fragments generated by 25 decamer primers

Entry	*B. oleracea* var. *capitata*							
	'Reed Inbred A'	'Reed Inbred C'	'Reed Hybrid A × C'	'Market Prize'	'Jersey Wakefield'	'Golden Acre'	'Wisconsin Golden Acre' (Plant #1)	'Wisconsin Golden Acre' (Plant #2)
'Reed Inbred A'	–							
'Reed Inbred C'	0.892	–						
'Reed Hybrid A × C'	0.938	0.896	–					
'Market Prize'	0.889	0.864	0.877	–				
'Jersey Wakefield'	0.794	0.831	0.829	0.812	–			
Golden Acre'	0.831	0.824	0.881	0.848	0.817	–		
'Wisconsin Golden Acre' (Plant #1)	0.899	0.859	0.887	0.840	0.837	0.889	–	
'Wisconsin Golden Acre' (Plant #2)	0.899	0.859	0.902	0.840	0.823	0.889	0.940	–

Entry	*B. oleracea* var. *costata*			
	'Couve Nabica'	'Couve Galega Frisada' (Plant #1)	'Couve Galega Frisada' (Plant #2)	'Couve Poda'
'Couve Nabica'	–			
'Couve Galega Frisada' (Plant #1)	0.662	–		
'Couve Galega Frisada' (Plant #2)	0.652	0.824	–	
'Couve Poda'	0.657	0.782	0.762	–

of a curator is to understand how genetically similar or different these entries are. Among the *B. oleracea* var. *capitata* array, three entries (representing two accessions) have been identified as 'Golden Acre'. Results revealed more dissimilarity between the two accessions than between the two individuals of 'Wisconsin Golden Acre' (0.889 and 0.940, respectively). Linked with other genotypic, phenotypic, and historical data, this information could be used in structuring collections and establishing maintenance, regeneration, and evaluation priorities.

The most dissimilar accession of *B. oleracea* var. *capitata* was 'Jersey Wakefield', a historically important open-pollinated European cultivar (Fig. 3). This finding was in accord with what one might expect based on breeding and historical records.

B. oleracea var. *costata* represents landraces more heterogeneous than the highly inbred entries of *B. oleracea* var. *capitata*. This diverse background was reflected in the lower genetic similarities among *B. oleracea* var. *costata* entries (Table 3b). The greatest similarity was detected between the two individuals of 'Couve Galega Frisada' and most likely was to be expected based on sibship. The array of *B. oleracea* var. *costata* also included the most genetically dissimilar *B. oleracea*, 'Couve Nabica'.

The RAPD assay was useful in aggregating the botanical varieties of *B. oleracea*, thereby reflecting identity by descent and corroborating classical horticultural taxonomy. With the exception of *B. oleracea* var. *costata*, all individuals representing a botanical variety grouped most closely together (Table 4). The entries of *B. oleracea* var. *costata* were about as genetically similar to each other as they were to any other entry of *B. oleracea*. Therefore, the RAPD assay, through providing a random sampling of the genome, yields detailed information similar to the operationally more difficult, time-consuming, and costly RFLP assay employing a number of single-copy probes (Figdore et al. 1988; Slocum et al. 1990; Song et al. 1988, 1990).

In the most genetically distant comparisons, the results imply that the RAPD assay could be useful in discriminating *Brassica* species (Table 4). Because of the size and organization of the test array, no hypothesis could be proposed regarding either the evolutionary rela-

Table 4. Genetic similarity ranges among entries representing botanical varieties of *B. oleracea* and *B. rapa*. Results are based on 143 fragments generated by 25 decamer primers

Botanical variety (number of entries)	Similarity
Among entries representing botanical varieties of *B. oleracea*	
capitata (cabbage) (n=8)	0.794–0.940
botrytis (cauliflower) (n=2)	0.883
italica (broccoli) (n=2)	0.880
medullosa (marrow stem kale) (n=2)	0.846
costata (Portuguese cabbage and kale) (n=4)	0.652–0.824
Between botanical varieties of *B. oleracea* and entries of *B. rapa*	
capitata (cabbage) (n=8)	0.224–0.281
botrytis (cauliflower) (n=2)	0.257–0.268
italica (broccoli) (n=2)	0.180–0.240
gemmifera (Brussels sprouts) (n=1)	0.303–0.311
gongylodes (kohlrabi) (n=1)	0.271–0.281
acephala (kale) (n=1)	0.220–0.232
ramosa (thousand-head kale) (n=1)	0.193–0.222
medullosa (marrow stem kale) (n=2)	0.231–0.283
costata (Portuguese cabbage and kale) (n=4)	0.224–0.472

tionships among botanical varieties of *B. oleracea* or between *B. oleracea* and *B. rapa*. Nonetheless, our findings support the previous work of Quiros et al. (1991) suggesting that, when merged with cytogenetic analysis, the RAPD assay could be useful in discriminating among closely related species.

Conclusions

This research provides evidence that markers generated via the RAPD assay can provide practical information for the management of genetic resource collections. Technologies which make use of DNA amplification may revolutionize our approaches to, and our understanding of, plant genetic resource conservation and use. Methods employing random and semi-random priming for DNA amplification (Welsh and McClelland 1990, 1991; Williams et al. 1990, 1992; Caetano-Anolles et al. 1991 a, b; Weining and Langridge 1991) can aid in the development and maintenance of higher quality collections. Conversely, our reliance on a gross number count as an estimator of quality (Cohen et al. 1991) may be diminished and the need for core subsets may be obviated because cost-effective and convenient means will exist to characterize and evaluate entire collections. In particular, these molecular techniques may be applied to resolve specific needs such as determination of genetic identity and representation, partitioning of variation, monitoring of seed regeneration, targeting of useful genes, etc. Continued close linkage to progress in plant breeding theories and practices is also essential.

In the longer-term, researchers may screen collections directly for useful genes employing diagnostic methods such as the ligase chain reaction (LCR) (Nickerson et al. 1990; Barany 1991). For this to become a reality, we must be able to cost-effectively extract and store high-quality genomic DNA, improve our understanding of gene and genome organization and regulation, locate and sequence useful genes, and develop automated systems to support these efforts. All of these avenues are vigorously being pursued by various groups worldwide.

Management and use of collections will improve significantly as we develop a better understanding of the genetic foundation of the crops being conserved and exploited. Tools of molecular biology should provide new opportunities for more effective management of gene banks in the 21st century.

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