Discrimination among Cultivars of Cabbage Using Randomly Amplified Polymorphic DNA Markers

Rogério L. Cansian

Laboratory of Biotechnology, Department of Biological Sciences, URI, Av. 7 de Setembro, 1621, Erechim, CEP 99700-000, Rio Grande do Sul, Brazil

Sergio Echeverrigaray¹

Institute of Biotechnology, University of Caxias do Sul, C.P. 1352, Caxias do Sul, CEP 95001-970, Rio Grande do Sul, Brazil

Additional index words. *Brassica oleracea*, polymerase chain reaction, cultivar identification, genetic diversity, molecular markers

Abstract. Randomly amplified polymorphic DNA (RAPD) markers were used to discriminate among 16 commercial cultivars of cabbage (*Brassica oleracea* L. Capitata Group). A set of 18 decamer primers was selected from 100 random sequences and used to characterize cultivars and to evaluate distances. The selected primers produced 105 (54%) polymorphic bands ranging in size from 100 and 2500 base pairs, out of a total of 195 bands, which allowed for discrimination of all cultivars. Similarity indices between cultivars were computed from RAPD data, and ranged from 0.72 to 0.87 with an average of 0.82. Unweighted pair-group method with arithmetic average (UPGMA) cluster analysis revealed two groups, one formed by two cultivars recommended for summer cropping, and the other by 14 cultivars. This large group was additionally divided into two subgroups. RAPD analysis provides a quick and reliable alternative for the identification of cabbage cultivars and for determination of the relationships among them.

Cabbage is one of the most important commercial crops of *Brassica oleracea*. Many open-pollinated cultivars and F₁ hybrids are commercialized around the world. In southern Brazil, a typical subtropical region, cultivars recommended for both tropical and temperate regions are commercialized, and new cultivars are continuously being released. Traditional cultivar identification in cabbage, as in other crops, is based on a laborious evaluation of phenological and morphological characteristics. Morphological identification is difficult because visual traits, especially quantitative traits, can vary with environment. Furthermore, many cultivars are morphologically similar because of the convergent improvement toward an ideal phenotype, making their identification more difficult. As an alternative, seed proteins, isozymes, fatty acids, and other biochemical markers have been used for the identification of cultivars and phenetic analysis in several *Brassica* species (Arús et al., 1982; White and Law, 1991; Wills et al., 1979). The main drawback of such biochemical markers is that their expression can also be influenced by environmental conditions.

Received for publication 20 Apr. 1999. Accepted for publication 9 Dec. 1999. This study was supported by a grant from the Univ. of Caxias do Sul and the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS). The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

¹To whom reprint requests should be addressed. E-mail address: selaguna@ucs.tche.br

In recent years, DNA-based molecular markers have been used for cultivar identification in many plant species (Gepts, 1993). Studies of *B. oleracea* using DNA-based markers, including restriction fragment length polymorphisms (RFLPs) and RAPDs, have provided information regarding genetic relationships between *B. oleracea* and other species of the same genus (Demeke et al., 1992; Ren et al., 1995) and the diversity that exists within *B. oleracea* germplasm pools (dos Santos et al., 1994; Farnham, 1996; Kresovich et al., 1992; Nienhuis et al., 1993). Genebank management is particularly troublesome in *Brassica* species because of variation in life cycles, growth form, reproductive habit, and pollination systems that demand expensive seed regeneration protocols on a recurring basis.

The identification of plant cultivars has become increasingly important with the requirement of Plant Breeders Rights (PBR) to demonstrate distinctiveness, uniformity, and stability (Plant Varieties Journal, 1991) for each new cultivar. In this context, DNA-based markers have been used as cultivar and genotype fingerprinting tools in *Brassica* (Hu and Quiros, 1991; Kresovich et al., 1992; Mailer et al., 1994). Despite the economic importance of cabbage, limited work has been reported on the identification and evaluation of genetic relationships between cultivars or germplasm bank entries for this crop. Most of the studies using molecular markers in *Brassica* have included only two to eight cabbage entries (Farnham, 1996; Kresovich et al., 1992; Nienhuis et al., 1993; Song et al., 1988). A molecular characterization of 24 French cab-

bage cultivars (Margale et al., 1995) showed that RAPD data gave valuable information for the analysis of genetic variability and the management of germplasm banks. However, we know of no study on the molecular characterization of commercial cabbage cultivars currently grown in Brazil. The objectives of this research were to identify the most important commercial cultivars of cabbage used in southern Brazil, and to determine the relationships among them.

Material and Methods

Sixteen commercial cultivars of cabbage were used for analysis (Table 1). Although this is a limited number of individual lines, it is a thorough representation of the commercial cabbage crop in South Brazil. These 16 cultivars represent >95% of all the cabbage seed market, and almost 100% of the cultivated area. 'Early Jersey Wakefield', 'Fuyutoyo', and 'Louco de Verão' account for >70% of the production in South Brazil (data supplied by the Secretary of Agriculture of Rio Grande do Sul State).

Fifty seeds of each entry were sown, one seed per cell in a 1 soil : 1 sand : 1 vermiculite mixture, in 120-cell seedling trays. Seedlings were grown in a greenhouse from April to May 1998, at which time leaves were sampled. Equal amounts (0.5 g) of leaf tissue from each of 10 plants were combined to make composite leaf samples of each entry. The composite samples were initially evaluated by comparing the amplification products of five independent mixtures of five, 10, 20, and 30 plants of the open-pollinated cultivars Early Jersey Wakefield and Early Round Dutch. In all cases, 10 plants were enough to ensure the same amplification patterns among replications. The composite samples were frozen in liquid nitrogen, ground, and mixed with a mortar and pestle, and immediately used for DNA extraction or stored at -20 °C.

DNA was extracted from 1.5 g of leaf powder using the hexadecyltri-methylammonium bromide (CTAB) protocol described by Doyle and Doyle (1987), and triplicate extractions were made for each entry. The DNA pellets were dissolved in 200 µL TE (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, disodium salt (EDTA), pH 8.0), and DNA content of all samples was measured at 260 nm using a spectrophotometer. To check for degradation of the DNA, the samples were loaded into 0.8% agarose gels in 1× TBE (tris/borate/EDTA, pH 8.0), submitted to electrophoresis, stained with ethidium bromide, and observed under ultraviolet (UV) illumination. The DNA used for the amplifications was of high molecular weight with little degradation and free of RNA as indicated by UV spectra and gel electrophoresis.

Of the 100 primers of kits A, B, W, X, and Y, obtained from Operon DNA Technologies (Alameda, Calif.), 18 decamer oligonucleotides that showed consistent banding patterns and amplification were chosen for use. The polymerase chain reaction DNA amplification protocol was a variation of that reported

Table 1. Type of line, source, and origin of cabbage cultivars compared by RAPD analysis.

Entry	Type of line	Seed Origin	Commercialized in Brazil by	Season	Head characteristics
Midori	Hybrid	Japan	Tokita Seed Co.	All year	Flat
Early Jersey Wakefield	OP Cultivar	Brazil	Isla Seed Co.	Summer	Heart shape
Louco de Verão	OP Cultivar	Brazil	Isla Seed Co.	Summer	Round
Saikô	Hybrid	Japan	Agroflora Seeds	Winter	Round
Scarlet O'Hara	Hybrid	Japan	Tokita Seed Co.	Winter	Red, small, heart shape
4-Estações	OP Cultivar	USA	Feltrin Seed Co.	All year	Flat
Fuyutoyo	Hybrid	Japan	Sakata Seed Co.	All year	Flat
Kenzan	Hybrid	Japan	Kyowa Seed Co.	Winter/spring	Flat, early hybrid
Mammoth Red Rock	OP Cultivar	USA	Isla Seed Co.	All year	Red, small, round
Astrus	Hybrid	Brazil	Asgrow Seed Co.	All year	Flat
Brunswick	OP Cultivar	USA	Isla Seed Co.	All year	Flat
Early Round Dutch	OP Cultivar	USA	Isla Seed Co.	All year	Round, early variety
Black Head	OP Cultivar	USA	Agroflora Seeds	All year	Red, round
Japanese	Hybrid	Japan	Isla Seed Co.	Winter/spring	Flat
Early Flat Dutch	OP Cultivar	USA	Isla Seed Co.	All year	Flat
Shinsei	Hybrid	Japan	Tohoku Seed Co.	Winter/spring	Flat

by Williams et al. (1990). Reactions were performed in a 25- μ L volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 0.25% Triton-X-100, 1.25 mM of each dATP, dCTP, dTTP, and dGTP (Pharmacia LKB Biotechnology), 30 ng of single decamer primer, 60 to 80 ng of genomic DNA, and 1.5 units of Taq DNA polymerase (Pharmacia LKB Biotechnology). DNA amplification was performed using a thermal cycler (model PTC100; MJ Research, Watertown, Mass.). The thermal cycle used was 94 °C for 1 min; then 45 cycles of 94 °C (1 min), 35 °C (1 min), and 72 °C (2 min), and finally 72 °C for 3 min. A negative control including all components except genomic DNA was included in all thermal cycle runs.

Following amplification, the RAPD products (20 μ L) were loaded in 1.5% agarose gels in 1 $\times$ TBE buffer and separated by electrophoresis. After electrophoresis, the amplification products were stained with ethidium bromide and photographed under UV light. The sizes of amplification products were determined by comparison with Lambda DNA digested with *Eco*RI and *Hind*III restriction enzymes.

Bands were scored as present (1) or absent (0). Only bands that were between 100 and 2500 base pairs (bp) and that appeared consistently in three independent runs were rated. Bands that were not well defined were not included in the data set as these were assumed to be unreliable markers. Band-sharing analysis (Jaccard's coefficient) was conducted. Dendrograms based on UPGMA were generated to estimate relationships among cabbage populations using NTSYS-pc (Rohlf, 1992).

Results and Discussion

Initially, the 100-decamer sequences were tested against Louco de Verão and Fuyutoyo. These cultivars were chosen because they exhibited the greatest differences in pattern with the two primers arbitrarily tested (OPA-01 and OPA-02) in a first amplification. Eighteen primers were selected for the identification of cultivars and evaluation of their relationships, based on the total number of bands, the number of polymorphisms, and their reproduc-

ibility in three independent amplifications.

Using the 18 selected primers, 195 RAPD bands were scored, ranging in size from 100 and 2500 bp (Table 2). Between four and 18 bands were scored per primer, which is within the range previously reported in *Brassica* (Farnham, 1996; Hu and Quiros, 1991; Kresovich et al., 1992; Thorman et al., 1994). Of the total bands scored in this study, 105, or 54%, were polymorphic. This percentage of polymorphic bands is lower than that reported in the above-cited studies, which was $\approx$ 70%. This difference can be attributed to the genotypes evaluated, the primers used, and to the selection of scorable bands in the present study (Ferreira and Grattapaglia, 1996).

An example of the patterns obtained by the RAPD analysis of cabbage cultivars is shown in Fig. 1. Although there was a high number of shared bands between cultivars, the 16 cultivars included in this study could be differentiated by combining the information supplied by several reactions. The smallest difference, 21 bands, was found between '4-Estações' and 'Early Round Dutch', and the largest difference, 44 bands, was between 'Kenzan' and 'Louco de Verão'. At least six primers were necessary to uniquely characterize all the

cultivars. Although the number of primers needed to distinguish among cultivars in other botanical varieties of *B. oleracea* (Hu and Quiros, 1991) and other plant species has been variable (Ferreira and Grattapaglia, 1996), the present results are within the expected range (three to eight primers) for the identification of 16 cultivars.

The ability to differentiate all tested cultivars by RAPD bands suggests that this technique could be practical for the identification of cabbage cultivars, as it is for the identification of other *Brassica*, like broccoli, cauliflower (*B. oleracea* L. Botrytis Group) (Hu and Quiros, 1991), rapeseed (*B. napus* L.) (Mailer et al., 1994), and collard (*B. oleracea* L. Acaphala Group) cultivars (Farnham, 1996), as well as phenotypically similar accessions of cabbage (Phippen et al., 1997). Moreover, the quick and inexpensive RAPD technique provides a level of resolution equivalent to RFLP for the identification of cultivars within *Brassica oleracea* (dos Santos et al., 1994; Thorman et al., 1994). Despite the differences between the genetic relationships obtained with RAPDs and RFLPs analysis (Thorman et al., 1994), both techniques give useful information for breeding programs.

Table 2. List of decamer oligonucleotides utilized as random primers, their sequence, the total number of fragments amplified, and polymorphic fragments amplified among the cultivars.

Primer identification	Sequence (5' to 3')	No. of amplified fragments	No. of polymorphic fragments
OPA-01	CAGGCCCTTC	6	4
OPA-02	TGCCGAGCTG	11	3
OPB-11	GTAGACCCGT	14	10
OPB-12	CCTTGACGCA	6	1
OPB-20	GGACCCTTAC	4	3
OPW-03	GTCCGGAGTG	12	8
OPW-05	GGCGGATAAG	16	10
OPW-06	AGGCCCGATG	15	9
OPW-07	CTGGACGTCA	7	1
OPW-08	GACTGCCTCT	18	12
OPW-10	TCGCATCCCT	12	6
OPW-13	CACAGCGACA	10	6
OPW-15	ACACCGGAAC	16	8
OPX-01	CTGGGCACGA	12	4
OPX-06	ACGCCAGAGG	11	5
OPX-15	CAGACAAGCC	10	5
OPX-18	GACTAGGTGG	5	1
OPY-08	AGGCAGAGCA	10	9
Total		195	105

The similarity of the cultivars, estimated by Jaccard's coefficient, is depicted in the UPGMA dendrogram (Fig. 2). The 16 cultivars of cabbage fell into two clusters at the 0.73 level of similarity. One was formed by two cultivars, 'Louco de Verão', originated in a Brazilian breeding program, and 'Early Jersey Wakefield', a historically important European cultivar. The other cluster can be separated into two subclusters at the 0.77 level of similarity, one formed by five cultivars, and the other by the remaining nine. Comparing morphological characteristics and groupings depicted by UPGMA, some coincidences can be observed. The two cultivars that form the first group are recommended for summer planting in Brazil, whereas all the cultivars within the large group are recommended for winter and spring planting. 'Brunswick' and 'Early Flat Dutch' are two old open-pollinated (OP) European cultivars with characteristic flat heads and large leaves clustered together. The three red cabbage cultivars fell within the same group, two of them within the same subgroup. However, no correlations were observed between quantitative characters, such as head volume, head weight, and productivity (Fracaro et al., 1999), and the groups formed by the phenetic analysis of RAPD data.

Similarity indices among cabbage cultivars ranged from 0.72 to 0.87 with a mean of 0.82. Similar values were obtained by Kresovich et al. (1992) in studying the relationships among eight cabbage cultivars. However, 0.87 is a high value when compared with those obtained by Farnham (1996) among collar entries and Mailer et al. (1994) among rapeseed cultivars. Although all the cultivars and hybrids used in this study had different origins, some shared parentage can be expected as the same germplasm may have been used in different breeding programs. Several reports, based on morphological and molecular data, demonstrated that *Brassica oleracea* Capitata Group has a large genetic base, as compared with broccoli, cauliflower, and other botanical varieties of *B. oleracea*.

We have shown that RAPD markers can be used for the identification of commercial cabbage cultivars. The application of this technique may alleviate some of the confusion of cultivar identity associated with morphological characteristics and multiple cultivar registrations. These results also indicate that RAPDs can be used to determine the relationships among cultivars, and provide important information for cabbage breeding programs.

Literature Cited

- Arús, P., S.D. Tanksley, T.J. Orton, and R.A. Jones. 1982. Electrophoretic variation as a tool for determining seed purity and for breeding hybrid varieties of *Brassica oleracea*. *Euphytica* 31:417-428.
- Demeke, T., R.P. Adams, and R. Chibbar. 1992. Potential taxonomic use of randomly amplified polymorphic DNA (RAPD): A case study in *Brassica*. *Theor. Appl. Genet.* 84:990-994.
- dos Santos, J.B., J. Nienhuis, P. Skroch, J. Tivang, and M.K. Slocum. 1994. Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L.

Fig. 1. Amplification products for 16 cabbage cultivars generated using (A) primer OPA-02 and (B) OPY-08. From left to right: 'Midori', 'Early Jersey Wakefield', 'Louco de Verão', 'Saikô', 'Scarlet O'Hara', '4-Estações', 'Fuyutoyo', 'Kenzan', 'Lambda EcoRI/HindIII molecular marker', 'Mammoth Red Rock', 'Astrus', 'Brunswick', 'Early Round Dutch', 'Black Head', 'Japanese', 'Early Flat Dutch', and 'Shinsei'.

Fig. 2. Unweighted pair-group method with arithmetic average (UPGMA) dendrogram showing the phenetic similarity among 16 cabbage cultivars as determined by the analysis of 195 RAPD markers. Scale indicates Jaccard's coefficient of similarity.

- genotypes. *Theor. Appl. Genet.* 87:909–915.
- Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bul.* 19:11–15.
- Farnham, M.W. 1996. Genetic variation among and within United States collard cultivars and landraces as determined by randomly amplified polymorphic DNA markers. *J. Amer. Soc. Hort. Sci.* 121:374–379.
- Ferreira, M.E. and D. Grattapaglia. 1996. Introdução ao uso de marcadores moleculares em análise genética. 2nd ed. EMBRAPA-CENARGEM, Brasília, D.F., Brazil.
- Fracaro, F., M. Sartori, E. Bizzani, E. Grelman, and S. Echeverrigaray. 1999. Comportamento agrônomo de cultivares e híbridos de repolho na região nordeste do Rio Grande do Sul. *Ciência Rural* 52:465–468.
- Gepts, P. 1993. The use of molecular and biochemical markers in crop evolution studies. *Evol. Biol.* 27:51–94.
- Hu, J. and C.F. Quiros. 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. *Plant Cell Rpt.* 10:505–511.
- Kresovich, S., J.G.K. Williams, J.R. McFerson, E.J. Routman, and B.A. Schaal. 1992. Characterization of genetic identities and relationships of *Brassica oleracea* L. via random amplified polymorphic DNA assay. *Theor. Appl. Genet.* 85:190–196.
- Mailer, R.J., R. Searth, and B. Fristensky. 1994. Discrimination among cultivars of rapeseed (*Brassica napus* L.) using DNA polymorphisms amplified from arbitrary primers. *Theor. Appl. Genet.* 87:697–704.
- Margale, E., Y. Herve, J. Hu, and C.F. Quiros. 1995. Determination of genetic variability by RAPD markers in cauliflower, cabbage and kale local cultivars from France. *Gen. Res. Crop Evol.* 42:281–289.
- Nienhuis, J., M.K. Slocum, D.A. DeVos, and R. Muren. 1993. Genetic similarity among *Brassica oleracea* L. genotypes as measured by restriction fragment length polymorphism. *J. Amer. Soc. Hort. Sci.* 120:548–555.
- Plant Varieties Journal. 1991. Plant Breeders Rights Office, Ottawa, Ontario, Canada.
- Phippen, W.B., S. Kresovich, F.G. Candelas, and J.R. McFerson. 1997. Molecular characterization can quantify and partition variation among genebank holdings: A case study with phenotypically similar accessions of *Brassica oleracea* var. *capitata* L. (cabbage) 'Golden Acre'. *Theor. Appl. Genet.* 94:227–234.
- Ren, J., J.R. McFerson, R. Li, S. Kresovich, and W.F. Lamboy. 1995. Identities and relationships among Chinese vegetable brassicas as determined by random amplified polymorphic DNA markers. *J. Amer. Soc. Hort. Sci.* 120:548–555.
- Rohlf, F.J. 1992. NTSYS-PC: Numerical taxonomy and multivariate analysis system. Exeter Software, New York.
- Song, K.M., T.C. Osborn, and P.H. Williams. 1988. *Brassica* taxonomy based on restriction fragment length polymorphisms (RFLPs). 1. Genome evolution of diploid and amphidiploid species. *Theor. Appl. Genet.* 75:784–794.
- Thorman, C.E., M.E. Ferreira, L.E.A. Camargo, J.G. Tivang, and T.C. Osborn. 1994. Comparison of RFLP and RAPD markers in estimating genetic relationships within and among cruciferous species. *Theor. Appl. Genet.* 88:973–980.
- White, J. and J.R. Law. 1991. Differentiation between varieties of oilseed rape (*Brassica napus* L.) on the basis of the fatty acid composition of the oil. *Plant Var. and Seeds* 4:125–132.
- Wilde, J., R. Waugh, and W. Powell. 1992. Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. *Theor. Appl. Genet.* 83:871–877.
- Williams, J.G.K., A.R. Kudelik, K.J. Livak, and J.A. Rafalski. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:7213–7218.
- Wills, A.B., S.K. Fyfe, and E.M. Wiseman. 1979. Testing F₁ hybrids of *Brassica oleracea* for sibs by seed isoenzyme analysis. *Ann. Appl. Biol.* 91:263–270.