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Original articles

Inheritance of isozyme and RFLP markers in *Brassica campestris* and comparison with *B. oleracea*

J. M. McGrath* and C. F. Quiros

Department of Vegetable Crops, University of California, Davis, CA 95616, USA

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Summary. Using primarily cDNA restriction fragment length polymorphism markers (RFLPs) previously located to *Brassica oleracea* (cabbage, 2n = 18) chromosomes, we initiated a comparative RFLP map in an F_2 population of *B. campestris* (turnip × mock pak-choi, 2n = 20). As with *B. oleracea,* the genome of *B. campestris* showed extensive gene duplication, and the majority of detected duplicated loci were unlinked. Only 6 of the 49 identified loci were represented as a single copy, and 3 of these 6 were clustered on a single linkage group showing a distorted segregation ratio. Comparison with *B. oleracea* indicates this synteny is conserved between species. Two other linkage groups also appeared syntenic between *B. oleracea* and *B. campestris.* One single copy locus appears to have changed synteny between *B. oleracea* and *B. campestris.* These observations suggest that *B. oleracea* and *B. campestris* share a common ancestor, but that chromosome repatterning has occurred during or after speciation. Within *B. campestris,* 5 loci appeared duplicated in one parent or the other, and 2 of these were linked. Differentiation through subspecies-specific duplication or deletion events is suggested as one mechansim for the evolution of numerous morphotypes within each of these species.

Key words: Gene synteny – Restriction fragment length polymorphism – Crucifers – Genome evolution

Introduction

Species within the genus *Brassica* (Cruciferae) provide numerous oilseed, vegetable and fodder crops. World-wide, the diploid species *B. oleracea* (n = 9) and *B. campestris* (syn. *rapa,* n = 10) and their amphidiploid *B. napus* (n = 2 × = 19) have the greatest economic impact. The diploid species share a parallel range of uses and morphotypes: crop types of *B. oleracea* include cabbage, broccoli, cauliflower, kale, Brussels sprouts, kohlrabi; *B. campestris* contributes turnip, turnip-rape, pak-choi, Chinese cabbage, sarson, toria, broccoli raab and others. The close evolutionary proximity of *B. oleracea* and *B. campestris* is supported by many studies, such as chromosome pairing in inter-specific hybrids (Prakash and Hinata 1980; Attia and Röbbelen 1986), analyses of isozymes (Coulthart and Denford 1982), plastid (Palmer et al. 1983, Palmer 1988) and nuclear (Song et al. 1990) DNA restriction patterns, and haploid DNA contents of less than one picogram (Verma and Rees 1974).

Testing hypotheses of *Brassica* nuclear genome evolution has been hampered by the lack of genetic maps in *Brassica.* For example, it has been suggested that these diploid *Brassica* species are balanced secondary polyploids with a base chromosome number of six (reviewed in Prakash and Hinata 1980). Recently, the genetic organization of *B. oleracea* has been characterized through the inheritance of restriction fragment length polymorphisms (RFLP) using either random genomic fragments (Slocum et al. 1990) or cDNA clones (McGrath et al. 1990). In both cases, extensive duplication of genetic markers supports the notion that *B. oleracea* is a secondary polyploid. The pattern of duplicated loci suggests that chromosome rearrangement has accompanied chromosome number changes. To compare the level of gene duplication and the conservation of gene synteny (literally 'same strand') between *Brassica* species, we have utilized a set of RFLP markers located previously to *B. oleracea* chromosomes (McGrath et al. 1990) to analyze their inheritance in *B. campestris.* Inheritance and link-

* Present address and to whom correspondence should be addressed: Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

age relationships of these RFLP markers in *B. campestris* is presented and compared with the *B. oleracea* synteny map.

Materials and methods

An F_2 population of 61 *B. campestris* plants was derived from a single F_1 inter-subspecies hybrid plant from the cross between turnip (*B. campestris* ssp. *rapifera* cv 'Yorii Spring', accession number B493, obtained from Jonny's Selected Seeds, Albion, Me., as female) and mock pak-choi (*B. c.* spp. *parachinensis*, cv 'Kwan Hoo Choi', accession B233, Redwood City Seeds, Redwood City, Calif., as male). These parent accessions are morphologically and phylogenetically divergent. Each parent used in this cross and the resulting F_1 and F_2 populations were tested for isozyme markers. Isozymes analyzed included PGI (phosphoglucose isomerase), PGM (phosphoglucose mutase), MDH (malate dehydrogenase) and GOT (glutamine oxaloacetate transaminase) as described (Quiros et al. 1987).

DNA was isolated as described (Saghai-Maroof et al. 1984), digested with the restriction endonuclease EcoRI, electrophoresed in a 0.8% agarose gel and transferred to nylon membranes (Hybond-N, Amersham) under alkaline conditions as per manufacturer's instructions. The clones used (primarily cDNA) were previously located to *B. oleracea* monosomic alien addition line chromosomes (McGrath et al. 1990). Briefly, cDNA clones were derived from *B. napus* seedlings (Harada et al. 1988), genomic clones were isolated from either *B. oleracea* of *B. napus* (Hosaka et al. 1990), and the napin seed storage protein gene (pN_2) was provided by Dr. M. Crouch (Crouch et al. 1983). Clone nomenclature is the same as previously reported (McGrath et al. 1990). Cloned DNA sequences were isolated from the vector, radiolabelled with ^{32}P using random hexanucleotides as primers (Feinberg and Vogelstein 1983) and hybridized for 16–20 h in 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml fish sperm DNA (Maniatis et al. 1982). Filters were washed at 65 °C with 0.2 × SSPE and 0.1% SDS prior to autoradiography.

Both parents were from open-pollinated accessions and were expected to be heterozygous at many loci. As both parental individuals and the F_1 plant were unavailable at the time of DNA extraction, polymorphism for RFLP markers in this F_2 population was determined through the analysis of seven randomly selected F_2 plants. Selfed progeny of both parents and the F_1 plants (two to five individuals each) served as controls for F_2 RFLP segregation analysis. In all cases, alleles in the segregating F_2 population were represented in these pooled control populations and could be ascribed to their respective parent. Segregation and linkage analyses were performed with computer algorithms LINKAGE-1 (Suiter et al. 1983) and MAPMAKER (Lander et al. 1987), respectively. Linkage group assignment was arbitrary.

Results

Genetic mapping in B. campestris

Of 45 probes tested, 69% revealed at least one polymorphism in the turnip × mock pak-choi F_2 population using the single restriction enzyme EcoRI. A total of 49 loci were identified using 3 genomic and 28 cDNA clones as probes, and four isozyme markers (Table 1). Only 6

Table 1. Segregation of marker genes in the turnip X 'Kwan Hoo Choi' F_2 population. Duplicate loci are indicated by a letter suffix

Marker name	Homozygous turnip	Heterozygous	Homozygous mock pak choi	χ^2	*P*
Isozyme					
Got-1 [a]	–50–		9	2.99	0.08
Mdh-1	14	–43–		0.01	0.94
Pgi-2	14	28	17	0.46	0.80
Pgm-3	12	25	22	4.76	0.09
Genomic clones					
B370	15	30	14	0.05	0.98
YLC19	15	30	14	0.05	0.98
YLC25	17	27	14	0.59	0.75
cDNA clones					
INF8 [b]	23	23	10	7.82	0.02 [e]
INF9a	4	13	11	3.64	0.16
INF9b	5	10	4	0.16	0.92
INF10 [b]	12	18	10	0.60	0.74
ING9	14	29	15	0.03	0.98
BN5	16	19	16	3.31	0.19
BN6a	26	21	12	11.54	0.01
BN6b	12	30	15	0.47	0.79
BN6c	12	32	15	0.73	0.70
BN7 [c]	–45–		14	0.05	0.82
BN8	23	24	12	6.15	0.05
BN12	14	29	11	0.63	0.73
BN31	10	28	9	1.77	0.41
BN32 [b]	11	25	16	1.04	0.60
BN33 [b]	25	23	11	9.51	0.01 [e]
BN55 [b]	12	27	20	2.59	0.27
BN98a	6	23	12	2.37	0.31
BN98b	16	30	12	0.62	0.73
BN116a	17	30	11	1.31	0.52
BN116b	15	23	14	0.73	0.69
BN120	14	31	13	0.31	0.86
BN121a	15	–38–		0.31	0.58
BN121b	13	29	16	0.31	0.86
BN121c	12	–42–		0.22	0.64
BN122a	12	33	13	1.14	0.57
BN122b	13	–45–		0.21	0.65
BN127a	19	36	4	10.49	0.01 [e]
BN127b	15	28	16	0.19	0.91
BN128 [c]	–47–		12	0.68	0.41
BN129a	12	34	14	1.20	0.55
BN129b	–42–		14	0.00	1.00
BN129c	13	35	8	4.39	0.11
IL9a	15	29	15	0.02	0.99
IL9b	13	37	9	4.36	0.11
MS1	17	32	11	1.47	0.48
CA3 [d]	25	–34–		9.50	0.01 [e]
CA12 [b]	14	34	11	1.68	0.43
COT44a	17	27	15	0.56	0.76
COT44b [c]	–47–		12	0.68	0.41
LEA76 [c]	–45–		15	0.00	1.00
N_2a (napin)	–41–		19	1.42	0.23
N_2b (napin)	16	–44–		0.09	0.77

[a] Novel phenotype appearing in this F_2 population
[b] Single copy sequences
[c] Apparent duplication in turnip
[d] Apparent duplication in mock pak-choi
[e] Distorted segregation ratios

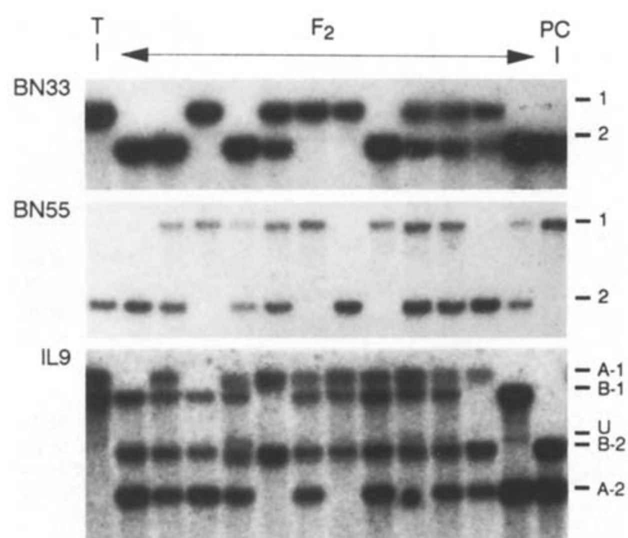

Fig. 1. Co-dominant segregation of RFLP loci in the turnip × mock pak-choi F_2 population. Both probes BN33 and BN55 disclose single copy loci with two alleles (*indicated by numbers*). Probe IL9 showed two co-dominant loci (*indicated by letters*), with an additional poorly resolved band of reduced intensity (*U*). *T* = Turnip parental type, *PC* mock pak-choi parental type; all others are F_2 individuals

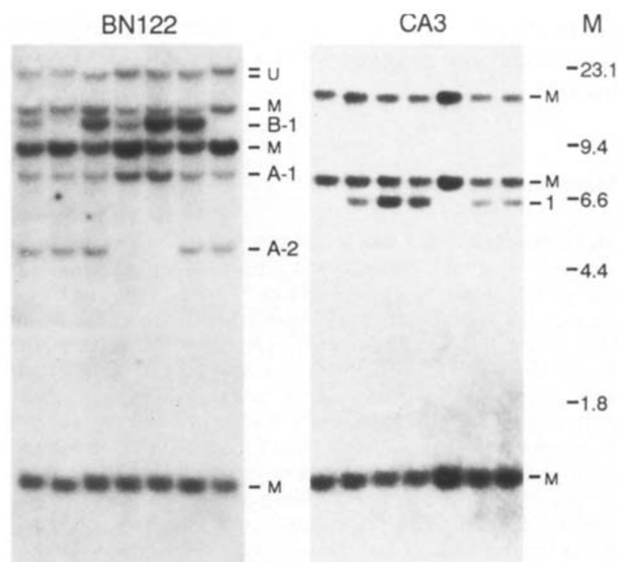

Fig. 2. Dominant segregation patterns observed in the turnip × mock pak-choi F_2 population. Monomorphic fixed (*M*) fragments were seen with most probes used. Unresolved (*U*) fragments showed insufficient resolution to be reliably scored (*BN122*). *Letters* designate the locus and *numbers* identify the allelic fragments scored. Molecular weight markers are indicated in kilobase pairs (*lane M*)

probes (19%) detected a single unique locus with two alleles (e.g., BN33 and BN55, Fig. 1). Duplicate loci were segregating for 11 probes (35%) (e.g., IL9, Fig. 1). The remaining probes detected monomorphic fragments in addition to a segregating locus. While these monomorphic fragments are as yet unproven to be individual loci, their presence on large-sized DNA fragments and their near ubiquity suggest at least some represent duplicated loci (e.g., see Fig. 2). Thus, the proportion of duplicated genes reported here likely represents a minimum estimate for *B. campestris*. Pooled χ^2 values for all co-dominant segregating loci (as well as apparent nulls) suggested no significant deviation from expected Mendelian segregation ratios ($\chi^2 = 3.43$, $P = 0.18$). However, the 6 loci showing distorted segregation ratios were each skewed towards the maternal (turnip) allele ($P < 0.05$ in Table 1). Four of these (*BN6-1, INF8, CA3* and *BN33*) were clustered on *B. campestris* linkage group A (Fig. 3).

Thirty-one loci listed in Table 1 were genetically linked into eight groups, covering 262 centiMorgans (cM) of the *B. campestris* genome. These groups were arbitrarily designated A through H (Fig. 3). Three single copy loci (*INF8, INF10* and *BN33*) were linked on group A (Fig. 3). Of the 11 probes where duplicate loci were segregating, 3 showed linkage between duplicate copies (Fig. 3). The remaining duplications were unlinked.

In general, co-dominance was exhibited by RFLP and isozyme markers. However, 13 loci in this *B. campestris* population were scored as dominant/null characters (Table 1). No bias in the overall distribution of apparent null phenotypes was observed (i.e. six were scored null in turnip, seven in mock pak-choi) (Table 1). Probes disclosing these loci always showed additional monomorphic bands in addition to the scored locus. For the majority of these (i.e., 6 RFLP loci and 2 isozyme loci) complex patterns were generated that may have hindered identification of an alternate allele. For example, locus *BN122a* displayed two alleles (five heterozygotes and two homozygotes are shown, Fig. 2), but an alternate allele for locus *BN122b* was not identified. Additional bands were evident which appeared polymorphic but were poorly resolved (such as in the high molecular weight region of BN122). Often, additional bands of low intensity showed polymorphisms that were difficult to score unambiguously (e.g., the unresolved band of IL9, Fig. 1). Zymogram patterns for both MDH and GOT were complex, and presumably obscured alternate alleles. For MDH, a fast migrating band derived from turnip was scored. For GOT, a novel and apparently labile form was scored in the F_2 but was not observed in either parent or the F_1. This novel GOT phenotype was precisely correlated with a sublethal virescent leaf phenotype (McGrath and Quiros 1991).

Five other loci showed dominant/null phenotypes, and their inheritance was not complicated by poorly resolved fragments or weak hybridization (e.g., *CA3*, Fig. 2). However, each hybridized to additional monomorphic fragments that may harbor an alternate

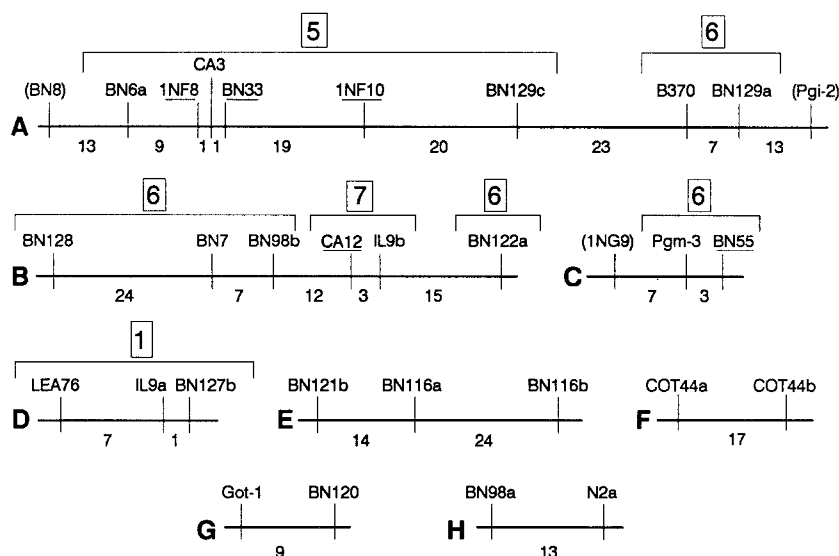

Fig. 3. Linkage groups of *B. campestris*, indicated by *letters A through H* at *left* of group. *Boxed numbers* above a *B. campestris* linkage group indicate the putative corresponding *B. oleracea* synteny group (data from McGrath et al. 1990). Single copy loci are *underlined.* Markers mapped in *B. campestris* but not *B. oleracea* are in *parentheses*

allele and may be disclosed by utilizing additional enzymes. Four of these apparent duplications were specific to turnip (*BN7, BN128, COT44b* and *LEA76,* Table 1), and one (*CA3*) was peculiar to mock pak-choi. Two duplicated loci (*BN7* and *BN128*) were linked on group B, suggesting this region has rearranged during the evolution of *B. campestris.* Locus *COT44b* is linked to *COT44a,* perhaps due to a short range duplication in turnip (Fig. 3).

Comparison of B. campestris and B. oleracea maps

Each of more than 100 cDNA clones tested recognized discrete DNA sequences in both *B. campestris* and *B. oleracea.* With more than 95% of the probes at least one polymorphism was detected between these two species. At a gross level, the complexity of the hybridization pattern was similar for a particular probe in both species. For example, probes hybridizing to multiple fragments in one species hybridized with multiple fragments in the other (due to either extensive heterozygosity or duplicate genes or both), and no change in copy number was observed for single copy loci.

The majority of probes utilized for interspecific comparison were located to *B. oleracea* synteny groups 1, 5, 6 and 7, and defined 5, 9, 11 and 7 *B. oleracea* loci, respectively (McGrath et al. 1990). Due to the duplicated nature of the *B. oleracea* genome some probes disclosed multiple loci. Thus, multiple markers for four *B. oleracea* chromosomes were tested, and the remaining *B. oleracea* synteny groups were each represented by two markers. Along with two isozyme markers (PGM and GOT), a total of 27 marker probes were comparable between species. Of these 27, 16 retained an association between the *B. oleracea* and *B. campestris* maps (Fig. 3).

Portions of four *B. oleracea* synteny groups could be superimposed on four of the eight *B. campestris* linkage groups (Fig. 3). *Brassica campestris* group A combined markers from two *B. oleracea* synteny groups, one larger region containing 6 loci from synteny group 5 (which also included the region of skewed segregation ratios in *B. campestris)* and a shorter region apparently derived from synteny group 6. Three of these loci were single copy in both *B. campestris* and *B. oleracea.* Another region was syntenic between *B. oleracea* group 1 and *B. campestris* linkage group D, however the synteny was interrupted by a locus (*IL9a*) not identified in *B. oleracea.* It is possible that this locus is syntenic, but was unable to be mapped in *B. oleracea* due to a lack of polymorphism. Six additional loci from *B. oleracea* synteny group 6 were distributed among *B. campestris* linkage groups B and C. Linkage group B was interrupted by insertion of two markers from *B. oleracea* group 7. Since one of these markers (*CA12*) was single copy in both species, it appears that this association has changed during or after speciation.

Discussion

A major result encountered in RFLP mapping of *Brassica* chromosomes is the demonstration of extensive gene duplication. A high proportion of the *B. campestris* genome is duplicated at the cDNA level: 28 cDNA probes disclosed 42 loci (1.5 loci/probe), and few duplicate genes were linked. Similar results using the same set of probes have been obtained for *B. oleracea* (McGrath et al. 1990). The genic complement of *B. campestris* and *B. oleracea* also appears similar both at the level of nucleotide sequence and gene copy number, as all tested probes hybridized with both species and no change in copy number

for single copy loci was detected. These results are consistent with the cytological evidence that these diploid *Brassica* species are derived secondary polyploids (reviewed in Prakash and Hinata 1980) and share a common ancestor.

Phylogenetic analysis indicates that turnip forms a distinct subspecies group from mock pak-choi (Song et al. 1990; J. M. McGrath and C. F. Quiros, unpublished). Map distortion was observed along a relatively large chromosome segment containing the only single copy gene cluster, suggesting genome divergence within *B. campestris*. Subspecies-specific duplications or deletions were inferred for at least 5 RFLP loci segregating in this *B. campestris* population, some showing linkage. Similar results of linked dominant/null RFLP alleles were also obtained through RFLP mapping of the *B. oleracea* inter-subspecies cross broccoli × cabbage (Slocum et al. 1990). The effects of sub-species specific gene duplications or deletions may contribute to morphological diversity within species. The extent of genome differentiation, manifested by map distortion and intraspecific gene duplication/deletions, can be tested by surveying progeny from additional *Brassica* inter-subspecies hybrids.

Comparison of gene synteny between related species can be a powerful tool to investigate the evolution of plant genomes, particularly when orthologous RFLP loci can be readily inferred: for example, among diploid solanaceous plants where the majority of cDNA RFLP loci appear to be single copy (e.g., in tomato, Bernatzky and Tanksley 1986). Using a common set of clones, Bonierbale et al. (1988) showed that the potato and tomato genomes were nearly homosequential, but extensive chromosome rearrangements have occurred between the tomato and pepper genomes (Tanksley et al. 1988). However, a difficulty in comparing synteny between species with a high proportion of duplicated genes arises in the assignment of orthologous loci, since it is rarely clear which fragments represent the same gene in different species and which fragments were duplicated prior to speciation. Both maize and sorghum (Graminae) show a higher level of gene duplication than tomato (Heletjaris et al. 1988; Hulbert et al. 1990). Using a common set of RFLP markers derived from maize and applied to sorghum, linkage group relationships between species were often conserved, and considered orthologous (Hulbert et al. 1990).

In comparing synteny between *Brassica* species, two parsimonious assumptions were made: (1) unique single copy loci were orthologous between species and (2) when duplicated loci were considered, conserved syntenic associations (i.e., shared linkage between species) were orthologous linkage blocks. Results indicate linkage blocks have been conserved between *B. oleracea* and *B. campestris*, but also that chromosome repatterning has accompanied chromosome number changes, as some loci have altered syntenic associations during or after speciation. Similar conclusions of chromosome repatterning between these species were indicated by Slocum (1989) through a comparative analysis of *B. oleracea* and *B. campestris* RFLP maps based on random genomic clones. Given the level of gene duplication in these species (estimated to be between 35% and 81%), it is possible that single copy loci actually represent reciprocally silenced paralogs. Circumstantial evidence would argue against this conclusion. First, both species shared the same set of single copy loci. If reciprocal silencing had occurred, presumably at random, then some single copy loci in one species would be expected to be duplicated in the other. This was not observed. Second, 3 single copy loci were linked in *B. campestris* and the same loci were located on one *B. oleracea* chromosome. Both linkage groups showed measured effects. In *B. campestris*, segregation distortion in this region was observed. In *B. oleracea*, this chromosome provided the only visual diagnostic for an alien addition chromosome (McGrath and Quiros 1990). Such effects are indicative of genetic differentiation, and their chromosome specificity may be related to the single copy region of this chromosome. Finally, the existence of orthologous linkage blocks would allow sexual recombination between genomes. Inter-genomic recombination in *Brassica* remains to be unequivocally demonstrated, but it has been inferred. For example, transfer of clubroot resistance from *B. campestris* to *B. oleracea* (Chiang and Crête 1983) and putative inter-genomic recombination and chromosome substitution events observed in the construction of *B. campestris-oleracea* alien chromosome addition lines (Quiros et al. 1987; McGrath et al. 1990) may have been facilitated through homeologous chromosome pairing.

The origin of morphological variation between cultivars of *B. oleracea* and *B. campestris* has not been adequately explained (although distinguishing morphological characters are generally reported to be under oligogenic control, Yarnell 1956), and may in part be the result of extensive nucleotide substitution or chromosome rearrangements, either is capable of altering patterns of gene expression. Chromosome rearrangement, either localized duplication/deletions within species or chromosome repatterning between species, may be an important mechanism for generating diversity in *Brassica*. It is possible that extensive gene duplication buffers an otherwise adverse effect of deletions and that varieties within species may be characterized by a set of differential gene duplications or deletions.

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