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Hybridization and Introgression in *Carduus nutans* and *C. acanthoides* Reexamined

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ABSTRACT. The present paper provides a reexamination of an often cited example of hybridization and introgression in plants, that involving two introduced thistle species, *Carduus nutans* ($2n = 16$) and *C. acanthoides* ($2n = 22$), which occur sympatrically in Grey Co., Ontario, Canada. Evidence gathered from a range of characters (morphology, allozymes, flavonoids, molecular data, chromosome numbers, and artificial hybridization and backcrossing experiments) revealed that both hybridization and backcrossing occur in this region. The two species were well separated morphologically, while the hybrid swarms exhibited the complete range of morphological variation evident for both species as well as intermediate types. There was a very close correlation with reduced fruit set and intermediacy in morphology. In contrast, a putative introgressed population, I1, exhibited a distinct shift towards *C. acanthoides,* with almost all individuals corresponding to an *acanthoides* or intermediate phenotype. Chromosome data from hybrid swarms and the introgressed population indicated a range of numbers from $n = 8$ to 11. Genetic identities based on allozyme frequencies was 0.68 between the two species and was 0.81 and 0.87 among the *C. acanthoides* and *C. nutans* populations, respectively. The three hybrid swarms showed equal similarity to both parental types, i.e., identity values of 0.82–0.83, while I1 was very similar to *C. acanthoides.* Allozyme data also confirmed the hybrid nature of the swarms with either additive or intermediate patterns. Flavonoid profiles were very similar in the two species. All individuals of *C. acanthoides* have the same four compounds, while populations of *C. nutans* were more variable and distinguished from *C. acanthoides* by the presence of up to three additional flavonoids. The hybrid swarms exhibited the complete range of flavonoid profiles, including characteristic *acanthoides* profiles, *nutans* profiles, and mixtures of the two. In artificial hybridization studies, *C. nutans* was the most frequent maternal parent, although both species served equally well as backcross parents. The F_1 hybrids have $2n = 19$ chromosomes and were almost completely sterile. Analyses based on 17 restriction enzymes, showed that the chloroplast genome and the transcribed region of the nuclear ribosomal genome of the two *Carduus* species are highly conserved; although some variation was detected in the ribosomal DNA spacer region. Although there was evidence of introgression in populations within the hybrid zone, patterns revealed by different characters were not always congruent. The results suggested that introgression may well be bidirectional. The Grey Co. region appears to represent a stable zone of hybridization in which the nature of the *Carduus* populations has changed little in the past 30 years.

Introgression, the infiltration of part of the genome of one species into the gene pool of another, occurs through repeated backcrossing of hybrids and parental taxa (Anderson 1949). Both introgression and its prerequisite hybridization have long been accepted as important factors in plant evolution (Heiser 1949, 1973; Stebbins 1959, 1969). Whereas the occurrence of hybridization and backcrossing in hybrid swarms is well documented in plant species, there are very few strongly documented cases of introgression. Although introgression can be expected to result only after numerous backcrossing events and is therefore best studied over time, few follow-up studies of hybrid swarms have been made. Such studies are important in assessing the evolutionary and ecological impact of both hybridization and introgression, as can be seen by the results of a recent reexamination of introgression of *Helianthus annuus* L. into *H. bolanderi* A. Gray (Reiseberg et al. 1988). The authors tested the hypothesis, first advanced by Heiser (1949), that introgression had resulted in the formation of a distinct, weedy race of *H. bolanderi* and found that evidence from isozyme and DNA studies did not support it.

The present paper provides a reexamination of an often cited example of hybridization and introgression in plants, that involving two introduced thistle species, *Carduus nutans* L. ($2n = 16$) and *C. acanthoides* L. ($2n = 22$). Hybridization between the two species was studied in Grey County, Ontario, Canada over a 10 year period (Moore and Mulligan 1956, 1964; Mulligan and Moore 1961). Their evidence for hybridization was based primarily on cytological comparisons and hybrid indices constructed using a number of discrete morphological characters. A clear correlation between chromosome number and hybrid index values was reported, whereas greenhouse studies provided evidence that among the F_1 hybrid progeny there was a preponderance of seedlings with the chromosome number of *C. acanthoides*. This led the authors to suggest that ecological and gametic selection in this area was favoring the production and survival of plants with the chromosome number and morphology of *C. acanthoides*, and that introgression of *C. nutans* genes into the *C. acanthoides* genome was occurring.

In the present study, the same region was visited to determine to what extent the predictions of Moore and Mulligan were realized after 30 years, specifically to determine if changes had occurred in the hybrid zone such that *C. acanthoides*/hybrid-like populations had become prevalent and to determine whether introgression from *C. nutans* was correlated with this expansion. To accomplish this purpose, a wide variety of characters was compared. These included: morphological and cytological characters; results from artificial hybridization, backcrossing, and fertility studies; and flavonoid, allozyme, and molecular characters. Measurements included the more traditional morphological and cytological characters used by Moore and Mulligan to facilitate direct comparisons with their studies. Canonical analysis, useful in discriminating hybrids and parental taxa (Adams 1982), was also employed using quantitative morphological characters. To understand the long-term ecological implications of hybridization, studies of plant fertility, fecundity, and artificial hybridization were included. Allozyme, flavonoid, and molecular data were obtained because of their potential use as genetic markers in the detection of hybridization (see reviews by Bohm 1987; Crawford 1983, 1985; Doyle 1987; Palmer 1987) and introgression. Genetic divergence between the two species was investigated using both allozyme data and restriction fragment length polymorphism of both chloroplast DNA and nuclear ribosomal DNA.

Materials and Methods

The study area chosen (Grey County, Ontario) was the same as that of Moore and Mulligan (1956, 1964; Mulligan and Moore 1961) (fig. 1). In this region, the two species and hybrid swarms occupy rolling pastureland and gravel pit sites. Three populations of each of the two species, *C. nutans* (N1, N2, N3) and *C. acanthoides* (A1, A2, A3), plus three hybrid swarms (H1, H2, H3) were sampled. Hybrid swarms were defined as those populations that contained plants of both species as well as intermediate individuals. Samples were also collected from one population (I1) described by Mulligan and Moore (1961) as introgressed. The latter was included so that a comparison could be made between hybrid swarms and putative introgressed populations. Specific site and habitat details are listed in table 1 and shown in figure 1 in relation

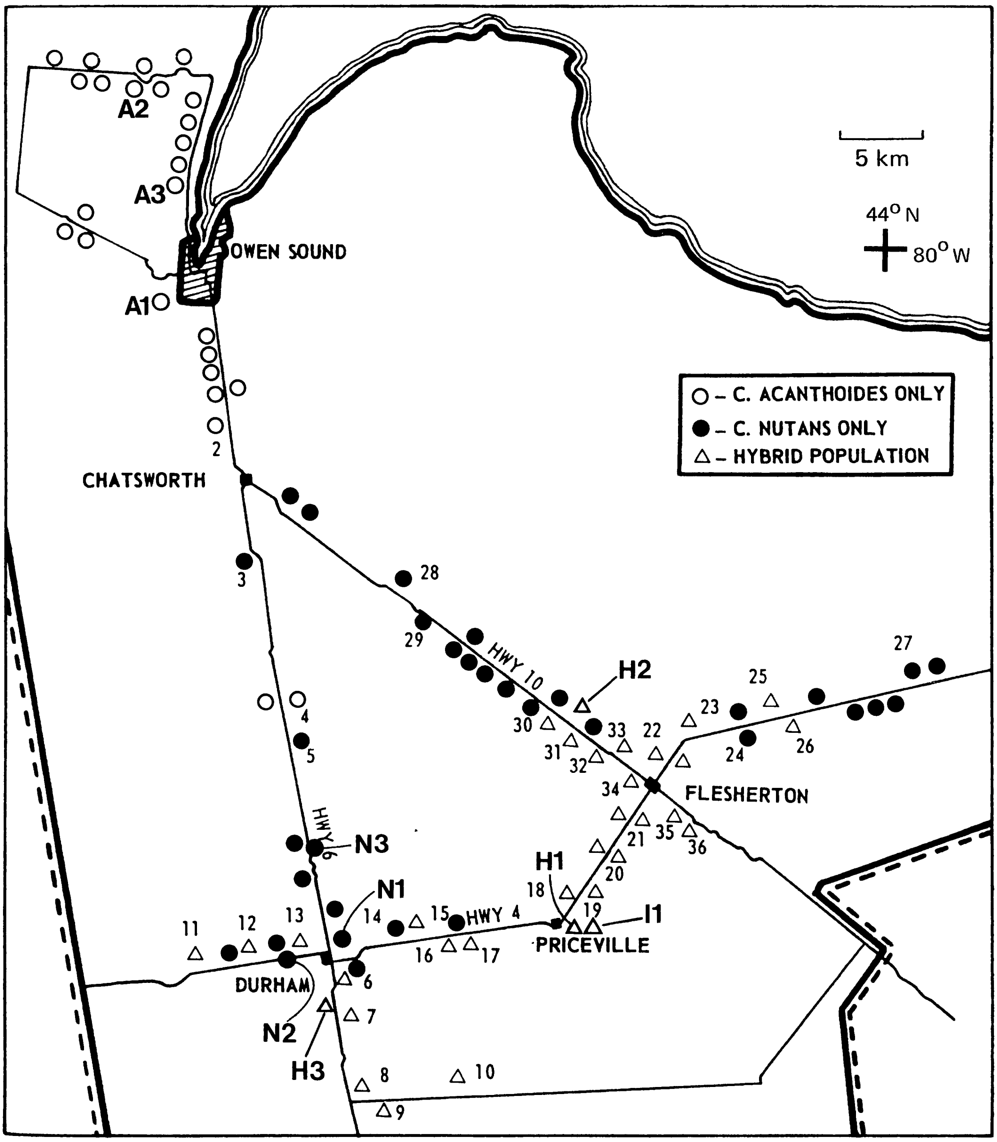


FIG. 1. Study site locations for three populations of *Carduus acanthoides* (open circle and A1, A2, A3) and *C. nutans* (solid circle and N1, N2, N3), three hybrid swarms (triangle and H1, H2, H3) and introgressed population (triangle and I1). Map from Mulligan and Moore (1961).

to the original study sites of Moore and Mulligan. Voucher specimens from each of the populations are at MTMG and DAO.

The samples for all analyses were collected from 40 well-spaced individuals in each of the populations. From each of these plants, several capitula at different stages of development were collected. These were used in morphological measurements, pollen fertility analyses, and fruit production estimates. Leaf samples were collected for flavonoid analyses and fruit samples collected to provide a source of seedlings for allozyme and molecular analyses. In addition to the 40 plants sampled as above, immature flower buds were collected for meiotic chromosome counts from each of 20 morphologically intermediate individuals in each of the three hybrid swarms and from 20 individuals in population I1.

Morphology. The two species traditionally have been separated using qualitative characters, such as nodding vs. erect capitula, solitary vs. clustered capitula, naked vs. spiny peduncles, reflexed vs. erect phyllaries, degree of constriction at base of phyllaries, and white vs. black-colored phyllary tips (Moore and Frankton 1974). These characters were used by Moore and Mulligan (1956) to construct hybrid indices. Morphological measurements were made on 20 of the 40 individuals of each of populations A1 to A3 and N1 to N3; on all 40 individuals for populations H1, H2, H3, respectively; and 30 of the 40 individuals from population I1. Two capitula per individual were scored for the 10 quantitative characters listed in table 2 and four qualitative characters described below. These include for *C. nutans* vs. *C. acanthoides*, respectively: white vs. black-colored phyllary tips, reflexed vs. erect phyllaries, presence vs. absence of constriction at base of phyllary, and absence vs. presence of spines on the peduncle. Character states are shown as T-arms (*C. nutans* phenotype), no arms (*C. acanthoides* phenotype) and a straight arm (intermediate phenotype) in figure 2. Population and species means were calculated and one-way analyses of variance were performed on each of the 10 quantitative characters. Species differences were assessed using canonical analyses (Rao 1962, Program S015, Engineering and Statistical Research Centre) of the three populations of each species. Each of the hybrid swarms was plotted separately in the same canonical space defined by the above six populations.

Plant Fertility. Estimates of both pollen fertility and fruit set were obtained. Pollen fertility was estimated for each of the 40 individuals per population using standard cotton blue lactophenol (Radford et al. 1974). Percent fertility was based on scoring 100 grains per individual. Although some differential staining was observed, percent overall staining was high and no malformed grains were detected. The results were therefore considered inconclusive and have not been included.

The number of mature fruits per capitulum was counted for at least two capitula of each of the 40 individual field-collections. Fruit number was divided into 21 classes (0–25, 26–50, . . . 475–500, and >500 per capitulum), and the higher value for the two capitula selected as the value for a given individual. On the bases of the 21 classes, three separate frequency histo-

TABLE 1. Location, habitat, and population density of the nine populations studied. [a] Collection numbers are in parentheses.

Species & population	Location Habitat, population density
C. acanthoides	
A1 (212)[a]	Lot 10, Conc. 2, Derby Twp., Grey Co. Pasture, plant widely spaced
A2 (214)	Lot 12, Conc. 20, Keppel Twp., Grey Co. Disturbed wasteground, plants dense to widely spaced
A3 (251)	Lot 26, Conc. 2, Sarawak Twp, Grey Co. Abandoned gravel pit, wasteground, dense stand
C. nutans	
N1 (216)	Lot 24, Conc. 2E, Glenelg Twp., Grey Co. Gravel field, wasteground, plants dense to widely spaced
N2 (217)	Lot 55, Conc. 2W, Bentinck Twp., Grey Co. Pasture, cattle, plants dense to widely spaced
N3 (221)	Lot 15, Conc. 1E, Glenelg Twp., Grey Co. In and along edge of wheatfield, plants widely spaced
Hybrid Swarms	
H1 (222)	Lot 15, Conc. 1N, Artemesia Twp., Grey Co. Pasture, sheep, plants dense to widely spaced
H2 (252)	Lot 117, Conc. 1E, Artemesia Twp., Grey Co. Gravel pit, disturbed wasteground, dense stands
H3 (218)	Lot 28, Conc. 1W, Bentinck Twp., Grey Co. Pasture, cattle, plants widely spaced
Introgressed	
I1 (270)	Lot 21, Conc. 1S, Artemesia Twp., Grey Co. Pasture, cattle, plants widely spaced

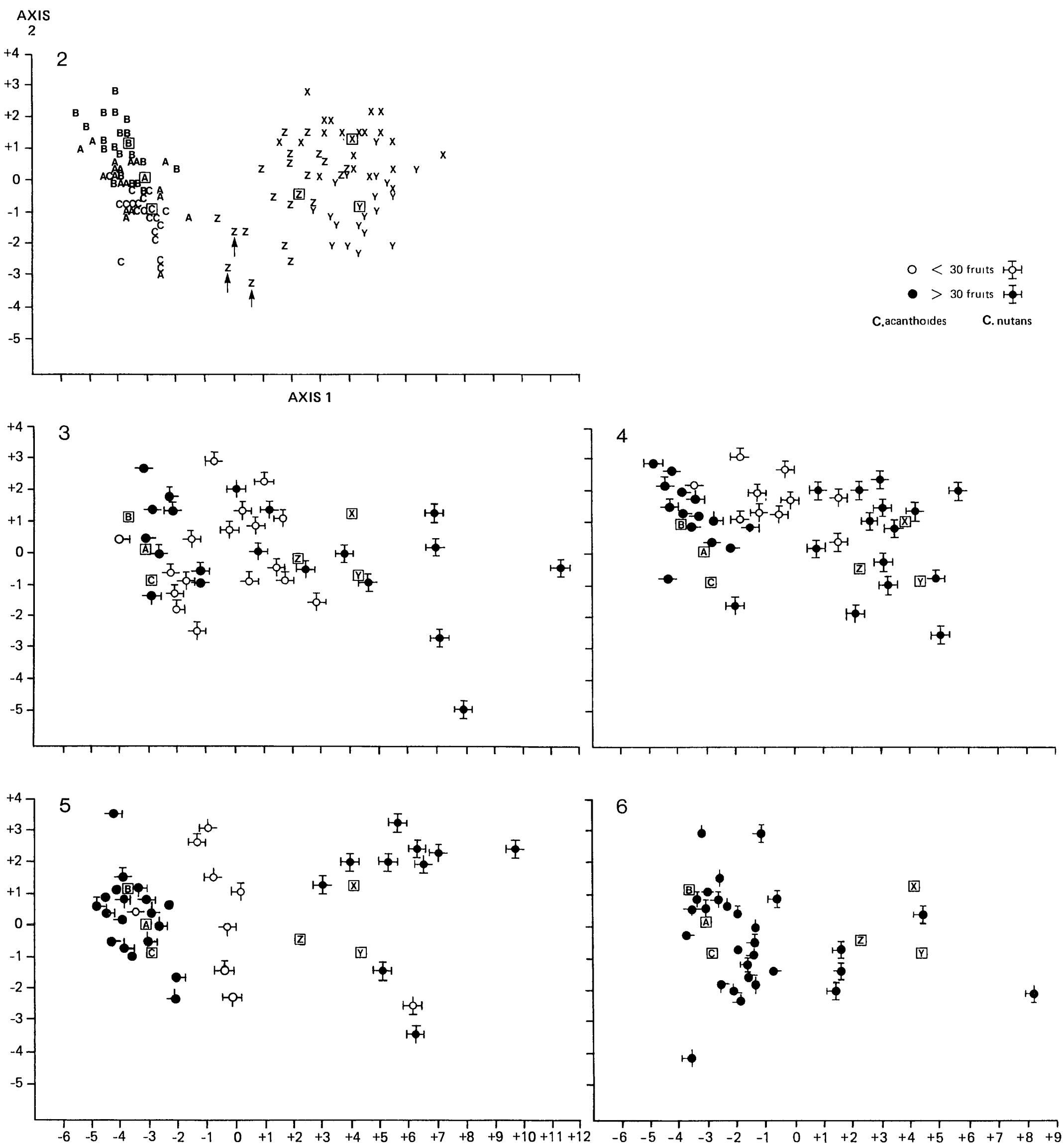
TABLE 2. Population and species means for ten quantitative characters used in the canonical analyses. [a] Sample size given in parentheses.

Species & population	Capitulum diameter (mm)	No. flowers per head	No. phyllaries per head	Corolla length (mm)	Anther length (mm)	Pappus length (mm)	Phyllary length (mm)	Phyllary width (mm)	Peduncle length to first spine (cm)	Peduncle length to first leaf (cm)
C. acanthoides										
A1 (20)[a]	9.81	129	136	18.0	6.13	11.9	6.15	1.16	0.13	0.70
A2 (20)	9.91	121	143	18.9	6.33	12.8	5.39	1.09	0.18	0.68
A3 (20)	8.67	112	116	17.8	5.91	11.5	5.71	1.24	0.11	1.25
Species mean	9.50	121	132	18.2	6.14	12.0	5.76	1.16	0.14	0.86
C. nutans										
N1 (20)	17.4	294	149	24.3	7.58	18.6	10.1	2.47	1.68	1.68
N2 (20)	15.9	284	166	23.8	7.63	16.9	11.3	2.65	1.76	2.17
N3 (20)	14.1	198	154	22.5	6.99	15.4	8.43	2.14	1.14	1.24
Species mean	15.8	258	156	23.5	7.40	17.0	9.92	2.42	1.52	1.68
Hybrid Swarms										
H1 (40)	15.3	237	166	21.7	7.07	15.0	10.8	2.03	0.61	0.87
H2 (40)	13.7	199	149	20.9	6.83	14.9	7.87	1.85	0.65	0.83
H3 (40)	13.5	201	154	21.5	7.06	15.1	8.34	1.91	0.71	1.49
Hybrid mean	14.3	212	156	21.3	6.99	15.0	8.99	1.95	0.66	1.03
Introgressed										
I1 (30)	12.3	163	133	19.4	6.64	13.6	8.09	1.31	0.40	0.73
Standard deviation[2]	3.0	76	21	2.23	0.73	2.18	2.36	0.53	0.82	0.88

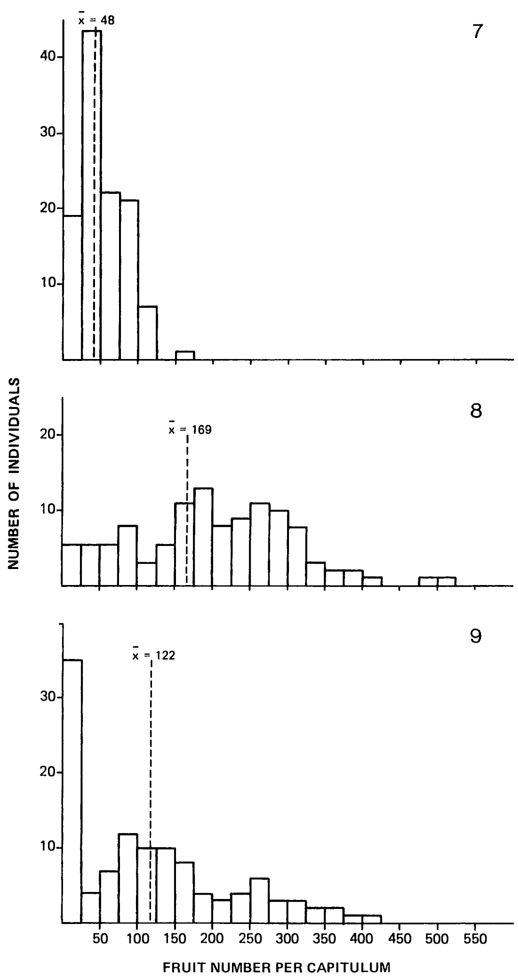
grams were constructed for all individuals of *C. acanthoides* A1 to A3 (fig. 7), *C. nutans* N1 to N3 (fig. 8), and the three hybrid swarms H1 to H3 (fig. 9), respectively, and the three group means calculated using the median of each class (i.e., 12.5, 37.5, etc.). Note that the fruit-eating weevil, *Rhinocyllus conicus* Froelich, an introduced biological control agent for *C. nutans* (see review by Desrochers et al. 1988a), was present on most individuals (some but not all capitula) of *Carduus nutans* in parental and hybrid swarm populations and that fruit number in infested individuals was obviously reduced. Infructescences from *C. acanthoides* were not infested by the insect.

Chromosome Number. Chromosome counts were determined from HCl-alcohol-carmine stain preparations (Snow 1963) of inflorescence buds. Counts were determined for each of 20 morphologically "intermediate" (as determined by eye) individuals in each of the three hybrid swarms and from 20 individuals in population I1. In addition, counts were obtained from each of five plants corresponding to the *C. acanthoides* and *C. nutans* morphological phenotypes in hybrid swarm population H3.

Allozymes. Seedlings were grown from field-collected fruits of each of 40 mother plants in each population and maintained as families. Forty seedlings, one seedling per maternal parent, were surveyed by starch gel electrophoresis in each of the 10 populations. An additional 200 seedlings, representing five progeny per maternal parent, were sampled for Pgi-2 in population I1. Crude extracts for electrophoresis were obtained from fresh leaf samples of 2 to 3 week-old seedlings. The enzyme extraction procedures and methods of horizontal starch gel electrophoresis are given in Desrochers et al. (1988b). A tris-citric system at pH 8.3 was used for aspartate aminotransferase (AAT), leucine aminopeptidase (LAP), phosphoglucose isomerase (PGI), and triose phosphate isomerase (TPI). A tris-citric system at pH 7.8 was used for glyceraldehyde-3 phosphate dehydrogenase (G3PD) and phosphoglucose mutase (PGM). A histidine-HCl system at pH 7.0 was used for isocitrate dehydrogenase (IDH). A histidine



FIGS. 2–6. Canonical analyses. 2. Graph on the first and second canonical axes of the 20 individuals of each of the three populations of *Carduus acanthoides* (A1, A2, A3 shown as A, B, and C, respectively) and *C. nutans* (N1, N2, N3 shown as X, Y, and Z, respectively) based on 10 quantitative characters listed in table 2. Population means are indicated by boxed letters A, B, C, X, Y, and Z. 3–6. Individual canonical variates for populations H1, H2, H3, and I1, respectively, plotted in the same canonical space defined in figure 2. Fruit set is shown as an open circle for less than 30 fruits per capitulum and closed circles for greater than 30 fruits per capitulum. The four qualitative characters are shown for each individual of the hybrid swarms as side arms, and include for *C. nutans* vs. *C. acanthoides*, respectively: white vs. black-colored phyllary tips (upper position), reflexed vs. erect phyllaries (right), presence vs. absence of constriction at base of phyllary (bottom), and absence vs. presence of spines on the peduncle (left) for *C. nutans* and *C. acanthoides*, respectively. Character states are shown as T-arms (*C. nutans* phenotype), no arms (*C. acanthoides* phenotype), and a straight arm (intermediate phenotype).



FIGS. 7–9. Fruit production histograms. 7. Three populations of *C. acanthoides* combined, $N = 113$. 8. Three populations of *C. nutans* combined, $N = 115$. 9. Three hybrid swarms combined, $N = 115$. Means indicated by dashed lines.

system at pH 5.7 was used for malic enzyme (ME), malate dehydrogenase (MDH), and 6-phosphogluconate dehydrogenase (6PGD). Isozyme and allozyme status of the bands was confirmed by an examination of segregation patterns of the progeny of selfed heterozygotes for several of the polymorphic enzyme loci, including Aat-1, Aat-3, Lap, Pgi-2, Tpi-1, and Tpi-2 (Appendix). The segregation ratios of selfed heterozygotes did not differ significantly from 1:2:1, indicating Mendelian segregation of codominant alleles. For the other polymorphic loci (Idh-1, Idh-2, Me, and 6Pgd-3), the genetic bases could be readily interpreted from the segregation ratios in half-sib families, which were consistent with the patterns described for these enzymes in other diploid species (Gottlieb 1982). The locus specifying the isozyme with the most anodal migration was designated 1, the next 2. At each locus, the alleles specifying the allozymes were designated by their mobilities relative to the bromophenol blue front for all systems. Frequencies for each allozyme are given in table 3. Standard genetic identities (table 4) were computed for all 10 population pairs utilizing the methods of Nei (1972); estimates included data for monomorphic loci.

Flavonoids. Individual flavonoid profiles were generated on polyamide TLC plates developed in an aqueous solvent (water-butanol-acetone-dioxane; 70:15:10:5) using leaf material from 40 individuals from each of the three hybrid swarm populations, 20 individuals from each of the six parental populations and 20 individuals from the introgressed population I1. Individual spots in each profile were identified by co-chromatography with compounds previously identified from bulk samples of the two species (Bain and Desrochers 1988).

Artificial Hybridization. Allozyme markers at the polymorphic loci Pgi-2, Tpi-1, and Tpi-2 (fig. 10), were used to set up 18 paired crosses of *C. nutans* × *C. acanthoides* in the greenhouse. Emasculation of the hundreds of flowers per capitulum would not have been practical. By crossing plants homozygous for different alleles, it was possible to separate F_1 seedlings into either progeny arising from selfing (homozygous genotype identical to the maternal genotype) or F_1 hybrids (heterozygous genotype with alleles representing both parents). The homozygous genotype of the 36 parental plants, which included representatives from all six parental populations, had been previously determined in the allozyme survey described above. Each paired cross (table 6) was kept separate in the greenhouse. Several dozen crosses within each pair were made by gently rubbing together capitula with visible pollen. All resulting fruits were kept separate for each mother plant and germinated. The seedlings were screened for allozyme genotype, which allowed separation into F_1 hybrids and F_1 half-sibs arising from selfing. The seedlings were grown to maturity in the greenhouse. The morphology, chromo-

TABLE 3. Allelic frequencies at 11 loci in three populations of each of *Carduus acanthoides* (A1, A2, A3) and *C. nutans* (N1, N2, N3); three hybrid swarms (H1, H2, H3), and one introgressed population (I1). All individuals were monomorphic for the same alleles for G3pd-1, G3pd-2, Pgi-1, Pgm-1 and Pgm-2. The number of individuals sampled = 30–40 per population. Alleles are designated by the mobility of their enzyme products relative to the bromphenol blue front.

		C. acanthoides			Introgressed	Hybrid Swarms			*C. nutans*		
Locus	Allele	A1	A2	A3	I1	H1	H2	H3	N1	N2	N3
Aat-1	.69	0.03	0.11	—	0.09	0.53	0.33	0.38	0.85	0.63	0.89
	.67	0.15	—	—	0.06	0.05	—	0.02	0.13	0.08	0.11
	.62	0.82	0.89	1.00	0.85	0.42	0.67	0.60	0.02	0.29	—
Aat-2	.59	0.87	0.82	0.81	0.79	0.95	0.76	0.80	0.75	0.68	0.83
	.53	0.13	0.18	0.19	0.21	0.05	0.24	0.20	0.25	0.32	0.17
Aat-3	.59	0.66	0.09	0.26	0.47	0.42	0.29	0.26	0.20	0.15	0.04
	.53	0.29	0.89	0.74	0.49	0.58	0.71	0.14	0.80	0.85	0.96
	.44	0.05	0.02	—	0.04	—	—	0.60	—	—	—
Idh-1	.49	1.00	1.00	1.00	1.00	1.00	0.93	0.86	0.93	0.98	0.99
	.47	—	—	—	—	—	0.07	0.14	0.07	0.02	0.01
Idh-2	.40	0.02	0.15	0.07	0.14	0.26	0.29	0.14	0.39	0.38	0.40
	.26	0.98	0.85	0.93	0.86	0.74	0.78	0.86	0.61	0.62	0.60
Lap	.63	0.04	0.02	0.03	0.27	0.26	0.07	0.04	—	—	—
	.60	0.54	0.38	0.40	0.40	0.68	0.59	0.70	1.00	0.97	0.96
	.56	0.52	0.42	0.31	0.14	0.06	0.30	0.20	—	0.03	0.03
	.55	—	—	0.26	0.19	—	—	0.06	—	—	0.01
Me	.24	1.00	1.00	0.88	0.63	0.53	0.53	0.66	—	0.09	—
	.21	—	—	0.12	0.37	0.47	0.47	0.34	1.00	0.91	1.00
6Pgd-3	.36	0.73	0.51	0.78	0.88	0.82	0.85	0.84	0.68	0.59	0.72
	.29	0.12	0.49	0.22	0.10	0.18	0.15	0.16	0.32	0.41	0.28
	.22	0.15	—	—	0.02	—	—	—	—	—	—
Pgi-2	.49	0.21	0.54	0.41	0.20	0.12	0.12	0.18	—	—	—
	.46	—	—	—	—	—	0.03	0.02	—	—	—
	.43	0.79	0.46	0.59	0.80	0.88	0.81	0.77	0.88	0.86	0.88
	.36	—	—	—	—	—	0.04	0.03	0.12	0.14	0.12
Tpi-1	.70	0.94	0.83	0.91	0.85	0.98	0.99	0.97	0.97	0.89	1.00
	.61	0.06	0.17	0.09	0.15	0.02	0.01	0.03	0.03	0.11	—
Tpi-2	.58	—	0.09	0.19	0.05	0.01	0.15	0.01	0.16	0.10	0.15
	.49	1.00	0.91	0.81	0.95	0.99	0.84	0.99	0.84	0.90	0.85
	.41	—	—	—	—	—	0.01	—	—	—	—

some number, flavonoid profiles, and plant fertility (including pollen fertility and fruit set as described above) of the F_1's was determined. Morphological measurements of the F_1's and backcross progeny (described below), chromosome counts, and meiotic pairing will be reported elsewhere (Warwick, in prep.). Results of the flavonoid analyses have been reported in Bain and Desrochers (1988).

Fruit production of 97 F_1 hybrids was determined after selfing (i.e., from five bagged capitula per plant), and after outcrossing (i.e., an additional five capitula were rubbed with those of other F_1 hybrids). The latter was done as a check to ensure that the observed infertility in the hybrids was not due to self-incompatibility within an individual. Using the allozyme marker system described above, 23 F_1 hybrids were backcrossed to the parental species, including 18 paired crosses of *C. nutans* × F_1 and five paired crosses of *C. acanthoides* × F_1 (table 7). Fewer plants of *C. acanthoides*, with the appropriate

TABLE 4. Genetic identities for each pair-wise population comparison and among the three groups: *C. acanthoides* (A1, A2, A3); hybrid swarms (H1, H2, H3); *C. nutans* (N1, N2, N3); and the introgressed population (I1).

	C. acanthoides			Hybrid Swarms			*C. nutans*		
Species & population	A1	A2	A3	H1	H2	H3	N1	N2	N3
A2	0.819								
A3	0.761	0.835							
H1	0.811	0.849	0.896						
H2	0.790	0.835	0.851	0.914					
H3	0.770	0.777	0.828	0.891	0.942				
N1	0.645	0.736	0.741	0.815	0.873	0.806			
N2	0.662	0.748	0.737	0.867	0.928	0.860	0.869		
N3	0.558	0.668	0.659	0.761	0.812	0.753	0.857	0.875	
I1	0.853	0.882	0.903	0.951	0.906	0.889	0.734	0.782	0.651
C. acanthoides		0.805							
Hybrid swarms		0.823			0.916				
C. nutans		0.684			0.831			0.867	
Introgressed		0.879			0.915			0.722	

allozyme genotype, were available to set up backcross pairs, as many of these plants did not bolt after the same six week vernalization treatment applied to *C. nutans* and the F_1 hybrids. The allozyme genotype of F_2 seedlings resulting from these crosses were determined and progeny separated into F_2 backcrossed hybrids and F_2 half-sibs arising from selfing.

Molecular Data. Genetic divergence between the two species was investigated using restriction fragment length polymorphism of both chloroplast DNA and nuclear ribosomal DNA. Plant material for this study included five "family" sets each consisting of one plant representing the *C. nutans* maternal parent, one plant representing the paternal *C. acanthoides* parent, and their F_1 hybrid. These were selected from reciprocal crosses #1 to #5 listed in table 6.

Total cellular DNA from each of the individual plants was isolated from 5 g fresh leaves harvested at the rosette stage. The tissue was homogenized and frozen in 30 mM Tris/HCl (pH 8.0), 30 mM EDTA, 6% SDS. It was then extracted at room temperature with phenol/chloroform/isoamyl alcohol (25:24:1, V/V/V) and chloroform/isoamyl alcohol (24:1, V/V). Sodium acetate (pH 5.5) was added to the aqueous supernatant to give a 0.3 M solution, and the DNA was precipitated with 2 volumes of ethanol at −80°C. After centrifugation, the pellet was suspended in 2 mM Tris/HCl (pH 7.7), 5 mM NaCl, and 0.1 mM EDTA or further purified by CsCl/ethidium bromide density gradient centrifugation.

Restriction enzymes used in this study are shown in table 8. Routinely 1–2 μg DNA were digested for 4 hr using 10 units of enzyme in incubation conditions specified by the supplier (Boehringer Mannheim Canada). Restriction fragments were separated by electrophoresis in 0.8% agarose gels in 89 mM Tris, 2.5 mM EDTA, and 89 mM boric acid. After denaturation in 0.5 N NaOH, 1.5 M NaCl for 30 min, and neutralization in 1.5 M NaCl, 1 M Tris/HCl (pH 8.0), the DNA was transferred by blotting (Southern 1975) to Biodyne membranes and dried at 80°C for 2 hr under vacuum.

The probe for nuclear ribosomal DNA was derived from pTA71 (Gerlach and Bedbrook 1979), being an rDNA repeat unit of *Triticum aestivum* L. subcloned in pUC19 (Amp^r). rDNA is the set of nuclear genes that encode three of the ribosomal RNA components of cytoplasmic ribosomes (18S, 5.8S, and 25S). The genes are transcribed as a single unit and are found in arrays of tandemly repeated units, consisting of structural genes separated by intergenic spacers (Rogers and Bendich 1987). For chloroplast DNA, two sets of pUC9-based plasmids were used, which together represented 75% of the *Petunia* chloroplast genome (Sytsma and Gottlieb 1986). Set A was comprised equally of clones p19 (1.5 kb), p12 (7.6 kb), p14 (4.6 kb), and p1

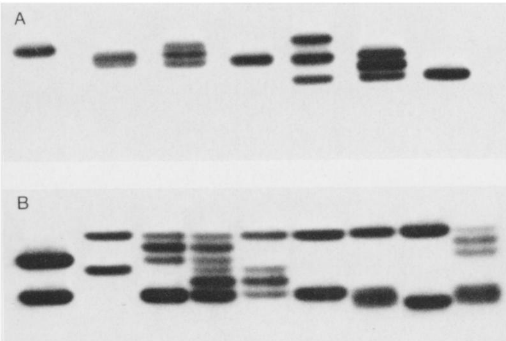


FIG. 10. A. Phosphoglucose isomerase variation patterns at locus 2. Homozygotes for alleles 0.49, 0.43, and 0.36 are shown in lanes 1, 4, and 7, respectively; heterozygotes for alleles 0.46/0.43, 0.49/0.43, 0.49/0.36, and 0.43/0.36 are shown in lanes 2, 3, 5, and 6, respectively. B. Triose phosphate isomerase variation patterns at locus 1 with alleles 0.70 and 0.61 and locus 2 with alleles 0.58, 0.49, and 0.41. At locus 1, homozygotes for allele 0.70 are shown in lanes 2, 5–8, and for allele 0.61 in lane 1 and the heterozygote 0.70/0.61 in lanes 3, 4, and 7. At locus 2, homozygotes for allele 0.58 is shown in lane 2, for allele 0.49 in lanes 1, 3, and 6, and for allele 0.41 in lane 8; heterozygotes for 0.58/0.49 in lanes 4 and 5 and for 0.49/0.41 in lanes 7 and 9.

TABLE 6. Artificial hybridization: Results for 18 paired reciprocal crosses between *C. nutans* and *C. acanthoides*.

	C. nutans ♀ parent			*C. acanthoides* ♀ parent		
Paired reciprocal cross	No. progeny screened	No. F_1s	(%)	No. progeny screened	No. F_1s	(%)
1	157	88	(56)	54	0	(0)
2	48	37	(77)	100	0	(0)
3	24	1	(4)	100	0	(0)
4	148	44	(30)	100	0	(0)
5	202	29	(14)	100	0	(0)
6	136	27	(20)	100	0	(0)
7	95	52	(55)	62	0	(0)
8	235	53	(23)	55	0	(0)
9	205	63	(31)	56	1	(2)
10	378	25	(7)	55	2	(4)
11	275	11	(4)	55	0	(0)
12	120	7	(6)	15	1	(7)
13	191	62	(32)	110	4	(4)
14	348	53	(15)	55	0	(0)
15	202	64	(32)	55	0	(0)
16	201	30	(15)	55	0	(0)
17	336	54	(16)	55	0	(0)
18	138	44	(32)	36	0	(0)
Total	3439	744	(22)	1218	8	(0.7)

(23 kb); set B consisted of clones p16 (4.1 kb), p3 (21 kb), p6 (15.3 kb), and p10 (9.0 kb). Probe DNA was labelled by nick translation with deoxycytidine 5′-(^{32}P)triphosphate (3000 Ci mmol^{-1}) from Dupont Canada. DNaseI and DNA polymerase I were supplied by Boehringer Mannheim Canada. Hybridization conditions were as described by Maniatis et al. (1982), except 50% formamide was included in the hybridization mixture and the temperature used was 42°C. Autoradiography was carried out with XAR-2 film. Membranes were stripped of probe in 50% formamide at 65°C for 1 hr and reprobed successively.

RESULTS

Morphology and Plant Fertility. Results of the canonical analyses based on the 10 quantitative morphological characters listed in table 2 are shown in figures 2–6. Figure 2 shows the three populations of each species. The two canonical axes account for ca. 90% of the total variation of the canonical space. The two species are well separated morphologically. The se-

TABLE 5. Flavonoid variation in three populations of each of *C. acanthoides* (A1, A2, A3), *C. nutans* (N1, N2, N3), in three hybrid swarms (H1, H2, H3) and one introgressed population (I1). Sample size for each population shown in parentheses.

	No. of individuals									
	C. acanthoides			*C. nutans*			Hybrid Swarms			Introgressed
Flavonoid	A1 (20)	A2 (20)	A3 (20)	N1 (19)	N2 (19)	N3 (20)	H1 (40)	H2 (40)	H3 (40)	I1 (22)
Luteolin 7-O-digalactoside	20	20	20	4	0	6	33	24	32	19
Luteolin 7-O-rutinosidel/7-O-diglucoside	0	0	0	15	17	16	1	17	16	6
Apigenin 7-O-glucoside	0	0	0	4	13	8	1	6	7	0

TABLE 7. Artificial hybridization: Results for reciprocal backcrosses between the F_1 hybrids and *C. nutans* (18 paired crosses) and *C. acanthoides* (5 paired crosses). [a] In crosses where the F_1 served as ♀ parent, all seeds which germinated were backcrosses. [b] In crosses where the F_1 served as the ♂ parent, many seeds were produced, numbers given are the numbers tested.

Cross	Backcross pair																		Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
F_1 × *C. nutans*																			
♀ F_1 × ♂ *C. nutans*																			
No. seed produced	6	5	28	11	3	7	4	6	5	0	0	0	0	0	0	0	0	0	75
No. backcross progeny[a]	2	1	11	9	2	0	0	3	4	—	—	—	—	—	—	—	—	—	32
♀ *C. nutans* × ♂ F_1																			
No. seedlings tested[b]	60	90	90	120	90	90	90	90	90	120	120	90	72	60	90	90	90	90	1632
No. backcross progeny	1	3	0	3	0	5	0	12	0	4	9	1	7	6	2	0	0	0	53
F_1 × *C. acanthoides*																			
♀ F_1 × ♂ *C. acanthoides*																			
No. seed produced	52	85	97	0	21														255
No. backcross progeny[a]	13	25	31	0	6														75
♀ *C. acanthoides* × ♂ F_1																			
No. seedlings tested[b]	44	98	4	13	45														204
No. backcross progeny	16	4	0	8	0														28

quential *F*-tests revealed that phyllary width, capitulum diameter, and number of phyllaries were the three most important characters in distinguishing the populations. Figures 3–5 show the three hybrid swarms and figure 6 the introgressed population, all plotted in the canonical space defined by the parental populations. The four qualitative characters used to separate the species are shown as side arms on the dots representing the canonical variates for individuals. See the figure captions for further explanation. The three hybrid swarms are similar in that they all exhibit the range of variation for the quantitative data evident for both species, as well as intermediate morphological types. In the hybrid swarms, there is a complete segregation of the four qualitative characters, with individuals exhibiting all possible combinations. There is a very close correlation with reduced fruit set and intermediacy in morphology. In contrast, population I1 (fig. 6) exhibits a distinct shift towards *C. acanthoides*, with almost all individuals corresponding to an *acanthoides* or intermediate phenotype. No "true" *nutans* phenotypes are apparent; the two plants corresponding to *C. nutans* in quantitative characters, show an intermediate phenotype in at least one of the four qualitative characters.

Fruit production data for the three *C. acanthoides* populations, the three *C. nutans* populations, and the three hybrid swarms are shown in figures 7–9. The average number of fruits per capitulum for individuals of *C. acanthoides* is 48, whereas fruit production in both populations of *C. nutans* and the hybrid swarms is higher and more variable with means of 169 and 122 fruits per capitulum, respectively. As described earlier, fruit production estimates for *C. nutans* may have been biased downwards by the presence of *Rhinocyllus conicus*.

Chromosome Number. In hybrid swarm H3, individuals with the *nutans* phenotype are all $n = 8$, while intermediates and *acanthoides* phenotypes are all $n = 11$. However, in the other two hybrid swarms, H1 and H2, although most plants with intermediate morphologies have chromosome numbers corresponding to one or the other of the parental counts, a few individuals produce unbalanced meiotic products; i.e., tetrads of pollen with $n = 8, 9, 10$, and 11 chromosomes. The results for the latter two populations are similar to those of Moore and Mulligan (1956), who found a range of chromosome numbers from $n = 8$ to 11 in their hybrid populations. For population I1, 10 of the plants surveyed have chromosome numbers corresponding to *C. acanthoides*, two have $n = 8$ (*C. nutans*) and one produced irregular meiotic products with $n = 10, 11$.

TABLE 8. Chloroplast DNA restriction patterns. The number of fragments detected by hybridization with each of the two probes was the same for all plants tested.

Enzyme	No. of fragments per individual: Probe A	Probe B
AvaI	10	10
BamHI	12	10
BclI	12	10
BglII	12	15
BstEII	4	0
ClaI	15	14
DraI	4	10
EcoRI	13	13
HindIII	8	5
HpaI	4	5
NcoI	10	5
NdeI	8	9
SacI	4	6
SphI	3	2
SspI	15	20
StuI	4	4
XbaI	2	9
Total	140	147

Allozymes. Allele frequencies for each population are given in table 3. The genetic identities for each pair-wise population comparison are presented in table 4. The value 0.68 between the two species is comparable to that reported for many congeneric species (Crawford 1983). Values for the three conspecific or parental *C. acanthoides* populations are 0.81 and those for the three parental *C. nutans* populations 0.87. These mean values are certainly on the low end of the scale reported for conspecific populations (Crawford 1983). Similar results were obtained by Desrochers et al. (1988b) for conspecific populations of *Carduus nutans* from across Canada and the northern United States (I = 0.80–1.00), suggesting that our somewhat lower values are not restricted to the Grey Co. populations, but are characteristic of these species. Greater differentiation among populations is expected in species with mixed mating systems, which has been demonstrated for both of the species in Grey Co. (Warwick 1987; Warwick and Thompson 1989), as compared with a completely outcrossing species. The three hybrid swarms show high and equal similarity to both parental types, i.e., identity values of 0.82–0.83.

Allozyme data also confirm the hybrid nature of the swarms with either additive or intermediate patterns. Alleles diagnostic for one or the other species are found in all of the hybrid swarms. For example, at Pgi-2, the two species share a common allele 0.43, but each has a diagnostic allele 0.49 and 0.36 for *C. acanthoides* and *C. nutans*, respectively. Two of the hybrid swarms contain all three alleles and even a new allele at low frequency. For Me, the nearly diagnostic alleles for each species occur at intermediate frequencies in the swarms. For Idh, two of the swarms contain the allele 0.47, which is found only in *C. nutans*. Two rare alleles are observed at Pgi-2 and Tpi-2 and are unique to the hybrid swarms.

Allozyme data for I1 show that the population is very like *C. acanthoides*, with the presence of Pgi-$2^{0.49}$ and $^{0.43}$ and 6Pgd-$3^{0.22}$ and the absence of Pgi-$2^{0.36}$ (*C. nutans* allele), and similar allele frequencies at Aat-1, Aat-2, and Idh-1. Allele frequencies at Me are similar to those of the hybrid swarms with a high frequency of $^{0.21}$, the nearly diagnostic allele for *C. nutans*.

Flavonoids. Seven flavonoids were identified from *C. nutans* and *C. acanthoides*, six glycosides and one aglycone (Bain and Desrochers 1988). The flavonoid profiles are very similar in the two species, differing only in the presence or absence of one or two related glycosides (table 5). All 60 individuals in the three parental populations of *C. acanthoides* surveyed have the same flavonoid profile of four compounds. Parental populations of *C. nutans* are more variable and distinguished from *C. acanthoides* by the presence of up to three additional flavonoids. Luteolin 7-O-digalactoside appears diagnostic for *C. acanthoides*. Although it occurs at a very low frequency in two of the three parental populations of *C. nutans* examined in this study, it was absent in populations of *C. nutans* collected outside the study area (Desrochers et al. 1988b) and absent from several individuals in the hybrid swarms. Although the six individuals from N3 that possess luteolin 7-O-digalactoside have a distinct *C. nutans* phenotype for discrete morphological characters, they show a tendency towards *C. acanthoides* for quantitative morphological features (fig. 2). The combination of chemical and morphological characters suggests the influence of *C. acanthoides* on this population. The hybrid swarms exhibit the complete range of flavonoid profiles, including characteristic *acanthoides* profiles, *nutans* profiles, and mixtures of the two. Frequency values of the compounds in the hybrid swarms are intermediate between the two species (table 5). Hybrid swarm H1 is chemically very similar to *C. acanthoides*, but as can be seen from figure 3, it shows a wide range of morphological variation. In the hybrid swarms, flavonoid compounds characteristic of *C. nutans* are found in some individuals with *acanthoides*-like morphology. Population I1, like H1, is chemically very similar to *C. acanthoides*, with the presence of diagnostic *C. nutans* flavonoids in only a few individuals.

Artificial Hybridization. Crossing results from the two species are summarized in table 6. When *C. nutans* served as the maternal parent, 744 (22%) of the 3439 seedlings surveyed were F_1 hybrids. In contrast, when *C. acanthoides* was the maternal parent only 8 (0.6%) of the 1218 seedlings surveyed were F_1 hybrids. Successful F_1 hybrid production varies among pairs, from 4 to 77% with *C. nutans* as maternal parent and from 0 to 7% with *C. acanthoides* as the maternal parent. Chromosome counts confirmed that progeny with parental allozyme patterns had the same number as *C. nutans* ($2n = 16$) or *C. acanthoides*) ($2n = 22$), an association that is consistent with self-fertilization. Counts for heterozygous allozyme genotypes gave an intermediate chromosome number ($2n = 19$) consistent with a normal haploid contribution from each parent, with no apparent loss or rearrangement of chromosomes. The F_1 hybrids are almost completely sterile, as only nine fruits were obtained from ca. 500 selfed capitula and several hundred $F_1 \times F_1$ crosses. This sterility would not appear to be due to pollen infertility, as staining of F_1 hybrid pollen is 100 percent.

The results of backcrossing the F_1 hybrids with the parental genotypes are summarized in table 7. Once again, the nature of the maternal parent determined the success of the cross. Only 75 fruits were obtained from the 18 pairs of female F_1 × male *C. nutans*, 32 (43%) of these germinated, all of which were backcrosses. A total of 255 fruits were obtained from the five pairs of female F_1 × *C. acanthoides*; 75 (29%) of these germinated, all of which were backcrosses. These results are in agreement with the very low selfing rates seen in the F_1 hybrids. A total of 1632 seedlings were surveyed from female *C. nutans* in the backcrosses; most seedlings were the products of selfing with only 53 (3.2%) of the progeny arising from backcrossing. A total of 204 seedlings were surveyed from female *C. acanthoides* in the backcrosses, of these 28 (13.7%) were backcrosses. Both parental species are, therefore, capable of backcrossing with the F_1 hybrids. As a female parent, *C. acanthoides* is more successful when backcrossed with F_1's, than it is when hybridized with *C. nutans*.

Molecular Data. Our results, based on 17 restriction enzymes, show that the chloroplast genome of the two *Carduus* species is highly conserved. The number of restriction fragments detected with each of the two probes was the same for all plants tested (table 8). Examples of the highly conserved restriction banding patterns are shown in figure 11 for five enzymes (*Ava*I, *Bcl*I, *Bgl*I, *Nco*I, and *Nde*I). However, variation was detected in *C. acanthoides* in one region of the chloroplast genome (less than 2 kb in size) that hybridized to probe B; this was most clearly evident in *Ava*I fragments (fig. 11, lanes 1–3). Among the five individuals of *C. acanthoides*, there were two *Ava*I fragment length variants, three had the restriction pattern shown in lane 2 and the other two individuals had patterns similar to that of *C. nutans* as shown in lane 1. Differences in *Ava*I fragment lengths may be explained by an insertion/deletion rather than a loss of a restriction site. As expected, given the maternal inheritance of chloroplast DNA (Palmer 1987), the F_1 hybrids had the same *Ava*I restriction patterns as the maternal parent, *C. nutans*, as shown for example in figure 11 (lane 3).

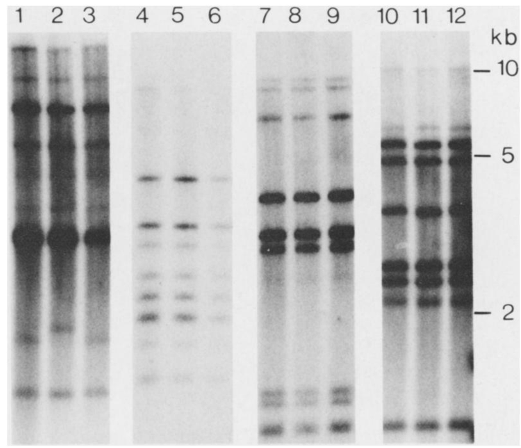


FIG. 11. Restriction fragment patterns for *Ava*I, *Bcl*I, *Bgl*II, and *Nde*I digests (lanes 1–3, 4–6, 7–9, and 10–12, respectively) detected by hybridization to the *Petunia* chloroplast DNA probe A (lanes 4–9) and probe B (lanes 1–3 and 10–12). The three samples in each set correspond to *C. nutans* (lanes 1, 4, 7, 10), *C. acanthoides* (lanes 2, 5, 8, 11), and their F_1 hybrids (3, 6, 9, 12).

Of the 17 restriction enzymes screened (table 8), only seven cleaved within the nuclear rDNA repeat unit of both species. Four restriction enzymes (*Nde*I, *Eco*RI, *Sac*I, and *Bam*HI) produced both a cluster of usually faint bands corresponding to a variable number of high molecular weight fragments as well as a number of smaller fragments (1, 1, 2, and 3, respectively). The latter fragments were evident in all the plants surveyed in both species (fig. 12, lanes 1–4, respectively). These would appear to be the result of cleavage of restriction sites within the transcribed regions of the repeat unit of the ribosomal DNA and are consistent with the restriction map proposed by Tucci and Maggini (1986) for *Carduus nutans*. Autoradiograms of *Sac*I and *Bam*HI were complicated by the apparent partial cleavage of some restriction sites (most likely due to methylation of the nuclear DNA), resulting in the frequent occurrence of extra bands, which were the size of the conserved smaller fragments combined (fig. 12, lane 5). Two enzymes (*Bst*EII and *Nco*I) cut the repeat unit once, producing only a closely related group of high molecular weight fragments (fig. 12, lanes 6–8 and 9–12, respectively). For all enzymes that cleaved within the rDNA repeat, a comparable group of fragments appeared in the autoradiograms as a cluster of bands which varied in number, relative position, and intensity (presumably due to variable homology to the probe). These fragments would appear to represent intergenic spacer length variation both among repeat units within an individual and between different individuals for at least *C. nutans*. The exact number of variants is unclear as the resolution of these high molecular weight fragments (ca. 10 kb) was poor. Nevertheless, two obvious variants were evident among the individuals of *C. nutans* for *Nco*I; four of the five individuals had a double band (lane 9) and one

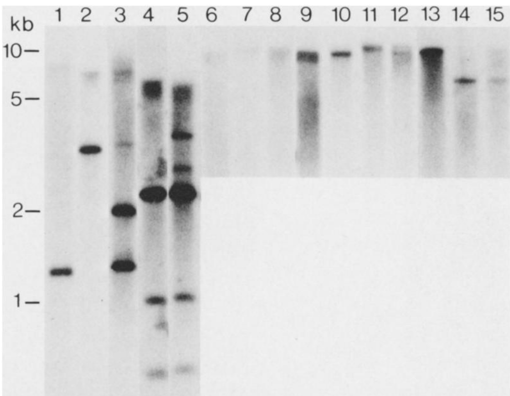


FIG. 12. Ribosomal DNA restriction fragment patterns for *Nde*I (lane 1); *Eco*RI (lane 2); *Sac*I (lane 3); *Bam*HI (lanes 4, 5); *Bst*EII (lanes 6-8); *Nco*I (lanes 9-12); and *Bcl*I (lanes 13-15). *Carduus nutans* (lanes 1-6, 9, 10, 13), *C. acanthoides* (lanes 7, 11, 14), and F_1 hybrids (8, 12, 15). Lanes 6-8, 10-12, and 13-15 represent a family set.

had a single band (lane 10), which was different from the single band for *C. acanthoides* (lane 11). Variation was also detected with *Bcl*I. Four of the five individuals of *C. nutans* had one dominant band (lane 13) consistent with only one restriction site in the repeat unit. There appeared to be an additional site in the intergenic spacer region for all five individuals of *C. acanthoides* and in one of the five plants of *C. nutans* (as shown in lane 14 for *C. acanthoides*). Presumably smaller fragments produced by restriction at this additional site in the intergenic spacer region were not detected due to lack of homology of this segment to the probe. In contrast to the variation seen among individuals of *C. nutans*, all five individuals of *C. acanthoides* had similar restriction patterns for *Nco*I and *Bcl*I. The restriction banding patterns for the corresponding F_1 hybrids appear to combine the parental phenotypes (seen most clearly in lane 12 for *Nco*I digests).

DISCUSSION

Our studies have provided strong evidence that hybridization is an important evolutionary force in the sympatric range of the two *Carduus* species. Crossing data provided insights into the most likely pattern and path of hybridization and introgression. It is not clear why *C. nutans* is the most frequent maternal parent in interspecific hybrids, as both species show varying degrees of self-incompatibility (Warwick 1987), which would presumably affect the success of the crosses in a similar manner. Perpetuation of the resulting F_1 hybrids seems unlikely and their significance as an evolutionary unit seems low, given the sterility of these forms. However, the apparent inferiority of *C. acanthoides* as a maternal parent is lost in subsequent backcross events (with the F_1's) when both species serve equally well as backcross parents. The propensity of F_1 hybrids to hybridize with both parental taxa indicates that introgression, leading to the inclusion of new alleles and gene arrangements, is reciprocal. This phenomenon has not been specifically documented in the field, but our data suggest that introgression is occurring not only in the direction of *C. acanthoides* as suggested by Moore and Mulligan, but also perhaps into *C. nutans*.

As a hybrid swarm evolves from slightly introgressed to strongly introgressed, the loss of one parental type and a shifting of the population mode towards the range between the F_1s and the other parental type would be expected (Grant 1981). While it is easy to document hybridization, documenting introgression in natural populations from morphological and historical inferences remains difficult largely because other factors may explain such variational patterns, including intraspecific variation, convergent evolution, retention of ancestral characters, and phenotypic plasticity (Bloom 1976; Heiser 1973; Reiseberg et al. 1988). To detect introgression, species specific diagnostic or nearly diagnostic genetic markers must be present in the donor species. Such genetic markers have been demonstrated in this study, most clearly with respect to the presence of the flavonoid luteolin 7-O-digalactoside. Extensive surveys of *C. nutans* populations outside the hybrid zone in Canada and the northern United States indicated that, except for a single population of uncertain taxonomic affinities from British Columbia, none possess luteolin 7-O-digalactoside (Desrochers et al. 1988b). Within the hybrid zone, characters suggesting introgression were most strongly correlated in N3, a *C. nutans* population, where a few individuals show both the presence of the digalactoside and a somewhat intermediate morphology. The presence of the allozyme $Me^{0.21}$ (nearly diagnostic for *C. nutans*) in one of the populations of *C. acanthoides* (A3) is perhaps evidence of introgression from *C. nutans*, but is not persuasive for neither *C. nutans* nor hybrid swarms have been recorded as occurring in this region of Grey Co. in recent times.

According to Mulligan and Moore (1961), population I1 had changed from a typical hybrid swarm (containing both parental species and hybrid types) in 1956 to a "wholly hybrid population, with the majority of plants approaching *C. acanthoides* in morphology" by 1961. This population appears stabilized and to have changed very little over the past 30 years. It can be regarded as showing evidence of introgression in having a predominant *C. acanthoides* mode morphologically and no true *C. nutans* types, the presence of diagnostic *nutans* flavonoids in a few individuals, and a high frequency of individuals with the *acanthoides* chromosome number. Allelic frequencies of population I1 were most similar to *C. acanthoides*, but with a higher frequency of the nearly diagnostic *nutans* $Me^{0.21}$ allele. On the other hand, there was no evidence for the *nutans* Pgi-$2^{0.36}$ allele in this population. The lack of congruence for all characters is not surprising for, as Harrison (1986) points out, the mosaic-like structure of hybrid zones (i.e., areas of "clinal" variation interspersed in pockets of "parental" forms) may well result in a differential effect on patterns of variation in morphology, allozymes, and other characters. Indeed, his studies on hybrid zones of field crickets provided strong evidence for differential and asymmetric introgression, with morphological integrity maintained despite considerable introgression of alleles at enzyme loci.

The molecular data suggest that speciation in these two *Carduus* taxa has occurred with little or no apparent divergence of either the chloroplast genome or the transcribed region of the nuclear ribosomal genomes. This was in contrast to the genetic divergence of the nuclear genome estimated by allozyme data, where species divergence was estimated at 0.68, a value within the range of that typically encountered between congeneric species (Crawford 1983; Gottlieb 1981). A similar lack of concordance between chloroplast DNA and isozymic divergence has been described for a number of species comparisons; e.g., in the genus *Hordeum* (Clegg et al. 1984) and *Lisianthius* (Sytsma and Schaal 1985).

However, variation was detected in one region of the chloroplast genome in one of the *Carduus* species, resulting in two *Ava*I fragment variants. This was of interest for, in general, very low levels of intraspecific variation in cpDNA have been reported in plants (Palmer 1987). Nevertheless, two surveys of a larger number of conspecific individuals, i.e., *Lupinus texensis* Hook. (Banks and Birky 1985) and *Pinus contorta* Dougl. and *P. banksiana* Lamb. (Wagner et al. 1987), revealed intraspecific cpDNA polymorphism. Perhaps higher levels of intraspecific variation in cpDNA will be apparent when more individuals of each species have been analyzed. Our data suggests that some caution should be exercised in assuming a lack of intraspecific variation in the cpDNA of plant species, based on studies of only a few individuals.

Our results also showed variation in nuclear ribosomal repeat unit length in both *Carduus* species, with different variants coexisting in the same individual plants and among individuals of at least *C. nutans*. Resolution of these repeat unit length variants may be improved by using a probe that has more extensive homology to *Carduus* rDNA. Intergenic spacer length variants have been described within and among individuals in several plant species, including estimates of seven and nine variants in *Clematis fremontii* Wats. (Learn and Schaal 1987) and *Phlox divaricata* L. (Schaal et al. 1987), respectively, and up to 20 variants in *Vicia faba* L. (Yakura 1984, see review by Rogers and Bendich 1987 for additional examples). Heterogeneity in spacer length would appear in some cases to be due to variability in the number of subrepeating elements in the intergenic spacer region, as for example in *Vicia faba* (Yakura 1984) or in some cases may be due to unequal recombination (Rogers et al. 1986). Variation in the presence of an additional *Bcl*I site within *C. nutans* is similar to the discovery of two variants of *Carduus nutans* both having the same repeat unit length, but differing by the presence of an additional *Eco*R1 site in the intergenic spacer region (Tucci and Maggini 1986). This study sought to evaluate the utility of molecular markers in documenting hybridization and introgression events in the two *Carduus* species, such as the studies on putatively introgressed pine populations (Wagner et al. 1987) and the proposed introgression of sunflowers (Reiseberg et al. 1988). Unfortunately, the lack of diagnostic molecular markers for the two *Carduus* species may limit the use of these probes in future studies of *Carduus* hybridization and introgression.

The suggestion by Moore and Mulligan (1964) that ecological and gametic selection in areas of sympatry of the two species would result in an increase in the production and survival of plants with the chromosome number and morphology of *C. acanthoides*, was not well supported by our field observations. Their evidence for gametic selection may have been biased by small samples and the assumption that selfing rates in the species were low. Subsequent studies (Warwick and Thompson 1989) have indicated that selfing rates vary considerably among individuals of both species and may be as high as 50%. The preponderance of plants with an *acanthoides* chromosome number may have simply resulted from a greater production of progeny arising from selfing. Studies that use allozyme markers to distinguish selfed and hybrid progeny allow an independent assessment of the effects of gametic selection on chromosome numbers in the segregating progeny of both selfed F_1 hybrids and backcrosses of F_1 hybrids with the parental species (Warwick, in prep.).

The presence of two unique rare alleles at two enzyme loci (Pgi-2 and Tpi-2) in the hybrid swarms was unexpected, but not unprecedented, as the "rare allele phenomenon" has been documented in other hybrid swarms (Harrison 1986). Various explanations have been proposed to account for this pattern (Harrison 1986), such as that the rare alleles arose via intragenic recombination or that they are of selective advantage only in the hybrid swarm.

An increase in the species potential niche width through the production of new, more vigorous genotypes has been proposed as one of the evolutionary advantages of hybridization (Lewontin and Birch 1966). The idea was examined further, but not substantiated by Levin and Schmidt (1985) who compared the relative fitness of hybrids and parental taxa in *Phlox drummondii* Hook. subsp. *drummondi* and subsp. *mcallisteri* (Whitehouse) Wherry in field sites in Texas; they found no evidence of hybrid advantage in germination, survivorship, or fecundity. Mulligan and Moore (1961) suggested that the observed increase in the number of *acanthoides*-type hybrid segregates in Grey Co. was attributable to an adaptive advantage under local conditions. However, the stability of the hybrid zone in the past 30 years and the lack of evidence for spread of introgressed *acanthoides*-type populations in the hybrid zone does not support their predictions for the increased spread and takeover of these introgressed forms.

Anderson (1949) suggested that interspecific gene flow among ecologically differentiated species meeting at an ecotone would be mediated primarily by introgressants ecophysiologically similar to the parental species rather than by intermediate hybrid segregants. The recent studies by Ellstrand et al. (1987) on *Arctostaphylos* provide support for this viewpoint. Our results would also support this suggestion, as the F_1 hybrids are almost self-sterile and there is a preponderance of parental chromosome numbers in the hybrid swarms.

In conclusion, Grey County, Ontario would appear to represent a fairly stable zone of hybridization between the two *Carduus* species. At present, both are well established in the region and continued production of new hybrid swarms is predicted where the two are in close proximity. In general, direct evidence for the long-term stability of hybrid zones is not available, but indirect evidence suggests that some zones have persisted for thousands of years (Harrison 1986). Hybrid zones may remain stable due to reduced hybrid fitness or to differentially adapted parental system (Barton 1979; Levin and Schmidt 1985). Our studies provide evidence for stabilized long-term maintenance of introgressed populations of *C. acanthoides*, similar to that provided by Meyn and Emboden (1987) for *Salvia mellifera* E. Greene and *S. apiana* Jepson. In addition, evidence for introgression into *C. nutans* is also presented. Introgression, however, has not resulted in major changes to the *Carduus* hybrid zone over the last 30 years; it remains localized and seemingly insignificant in its effect. No evidence of any widespread introgression was found and interspecific gene flow appears to be possible only through the extension of the hybrid zone (i.e., not via dispersed introgression). The movement of gravel, as suggested by Moore and Mulligan, to be responsible for the establishment of the existing hybrid zone, could also result in its extension, for *Carduus* populations are still common in gravel pits in this region. Whether other effective means of long-distance dispersal exist is unclear.

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APPENDIX. Genetic bases for enzyme patterns. Segregation ratios for selfed heterozygotes and results of Chi-Square analyses to test for Mendelian segregation of alleles a and b in a 1:2:1 ratio. All values were not significant at $P < 0.05$, df $= 2$.

Genotype$^{a/b}$	No. of progeny a/a	a/b	b/b	Total no.	Chi-square value
Aat-1$^{.69/.62}$	29	49	22	100	2.04
Aat-1$^{.67/.62}$	19	56	20	95	3.42
Aat-3$^{.59/.53}$	25	45	30	100	1.50
Aat-3$^{.53/.44}$	32	50	18	100	3.92
Lap$^{.63/.56}$	39	90	51	180	1.60
Lap$^{.60/.56}$	19	55	26	100	1.98
Lap$^{.63/.55}$	23	48	29	100	0.88
Lap$^{.56/.55}$	26	45	19	90	1.10
Pgi-2$^{.49/.43}$	64	139	67	270	0.44
Pgi-2$^{.46/.43}$	29	45	26	100	1.18
Pgi-2$^{.43/.36}$	14	36	14	64	1.00
Tpi-1$^{.70/.61}$	25	48	27	100	0.24
Tpi-2$^{.58/.49}$	20	52	18	90	3.04
Tpi-2$^{.49/.41}$	32	51	17	100	4.54