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Genetic structure in cultivated and wild carrots (*Daucus carota* L.) revealed by AFLP analysis

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Abstract Genetic variation within and among five Danish populations of wild carrot and five cultivated varieties was investigated using amplified fragment length polymorphism (AFLP). Ten AFLP primer combinations produced 116 polymorphic bands. Based on the marker data an UPGMA-cluster analysis and principal component analysis (PCA) separated the *Daucus* collections into three groups, consisting of the wild populations, the old varieties, and the recently bred varieties. The genetic distance between the wild populations reflected the physical distance between collection sites. Analysis of genetic diversity showed that the old varieties released between 1974 and 1976 were more heterogeneous than the newly developed F_1 hybrid varieties. The analysis of molecular variation (AMOVA) showed that the major part of the genetic variation in the plant material was found within populations/varieties. The presence of markers specific to the cultivated carrot makes it possible to detect introgression from cultivated to wild types.

Key words Genetic variation · UPGMA · AMOVA · Relationships · Crop-wild introgression

Introduction

Cultivated carrot (*Daucus carota* ssp. *sativus*) is an important vegetable grown in many areas of the world. Carrot belongs to the Apiaceae (Umbelliferae) and has a number of wild relatives such as *D. carota* ssp. *drepanensis*, *D. carota* ssp. *gummifer*, *D. carota* ssp. *maximus*, *D. carota* var. *atrorubens* and *D. muricatus* (Heywood 1983). Of the wild carrots, however, *D. carota* ssp. *carota* is the best known as it is a common taxon in temperate regions (Brandenburg 1981). The wild carrot can be weedy on cultivated land, and especially in seed-producing nurseries *D. carota* ssp. *carota* causes problems since it can hybridize spontaneously with the carrot varieties (Wijnheijmer et al. 1989). The morphology and anatomy of the wild carrots are very similar to those of the cultivated types except for characteristics of the root (Dale 1974; Wijnheijmer et al. 1989). The root of the wild carrot is whitish, and it is usually smaller and the structure more stringy than that of cultivated varietics. The taste of the wild root is perhaps the most undesirable character transferred in the hybridizations between wild and cultivated carrot, the reason why breeders and seed producers try to avoid hybridization with wild carrot. Recently, the reciprocal introgression process, namely the transfer of traits from cultivated carrot to wild carrot populations, has been the object of concern as genetically modified varieties may disperse transgenes to wild carrot populations with possible consequences to the environment (e.g. Snow and Morán Palma 1997). Many new traits have already been engineered into carrot, i.e., lines with genes encoding fungal resistance and nutritional quality have been tested in field trials (OECD 1999).

Little information is available on the genetic relationship between wild and cultivated carrot, and up till now most studies of variability in *Daucus* subspecies have described only morphological and ecological characteristics (Dale 1974; Brandenburg 1981). The difficulties in classification using morphological characteristics have accelerated the application of molecular markers for classification of taxa and studies of introgression between wild and cultivated types.

The amplified fragment length polymorphism (AFLP) technique, a polymerase chain reaction (PCR)-based DNA fingerprinting technique, was developed by Vos et al. (1995). This technique has been applied to various studies of plant genetics in many species; for instance,

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elucidation of genetic relationships between wild and domesticated taxa (Angiolillo et al. 1999; Hill et al. 1996; Maughan et al. 1996; Perera et al. 1998; Roa et al. 1997). In the study reported here the AFLP technique was used to determine the genetic relations between cultivated and wild *Daucus* (ssp. *sativus* and *carota*). Genetic distances generated from the AFLP data were estimated for five cultivated varieties, each with a different breeding history, and five wild populations collected at different sites in Denmark.

The analysis of molecular data should be different from the analysis of quantitative data because molecular data do not meet the normality assumption. Specifically developed for the application on non-normal distributed data, the statistical analysis AMOVA (Analysis of Molecular Variance, Excoffier et al. 1992) is becoming increasingly common (Huff et al 1993; Barrestt and Kidwell 1998). We applied the AMOVA to clarify the distribution of genetic variation between groups, populations/cultivars, and individuals of carrot.

Materials and methods

Plant material and genomic DNA extraction

Wild carrot (*Daucus carota* ssp. *carota*) was collected at five different places in Denmark (Hundested, Kikhavn, Karlstrup, Løgstør, and Risø, see Fig. 1). Geographical distances between the wild collections ranged from a few kilometers (between Kikhavn and Hundested) to more than 200 km (between Løgstør and Karlstrup). We obtained three old and two recent F_1 varieties of the domesticated taxon (*Daucus carota* ssp. *sativus*), were obtained from a commercial Danish breeding company, Daehnfeldt A/S (Odense, Denmark). The three old open-pollinated types, Grosa, Regol (Flakeer types) and Fancy (Nantes type) were released on the market between 1974 and 1976 by Ohlsens Enke, Denmark, and they are still commonly cultivated. The two recent F_1 inbred hybrid varieties were Napoli (from Bejo, the Netherlands) and LD945479 (bred by Dæhnfeldt A/S, Odense, Denmark). Seeds were sown in pots containing a mixture of peat and sandy loam (approximately 1:1) and grown for 30 days in a growth chamber (20ºC / 15ºC with a daylength of 16 h). At 30 days after sowing, rosette leaves from each of 13-15 individuals per population/variety were harvested, and DNA was extracted immediately or the leaves were stored at –80ºC for later use. DNA samples were prepared from the fresh or frozen leaves by the CTAB-based method described by Doyle and Doyle (1990).

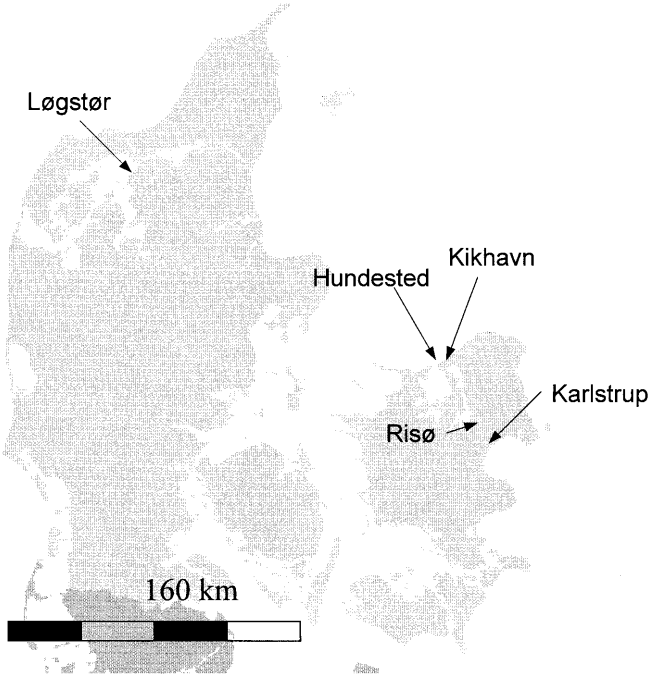

Fig. 1 Collection sites for the wild carrot populations. *Bar*: 160 km

AFLP analysis

The AFLP procedure was modified from Vos et al. (1995). Genomic DNA (160 ng) was incubated for $1^1/_2$ h at 37°C, with 1 U *Eco*RI (Amersham Pharmacia Biotech) and 2.5 U *Mse*I (New England Biolabs) in 20 µl of the buffer described by Vos et al (1995). Solutions containing equimolar amounts of both adapter strands were prepared. The strands were annealed by the method described in the Perkin Elmer manual (1995). Adapters were ligated by the method described by Vos et al. (1995); 4 µl of the adapter-ligation solution was added and the reaction was allowed to take place overnight at 37°C. The template DNA was preamplified by the method described by Vos et al. (1995), with the following modifications: 2 µl template-DNA was preamplified with 30 ng of both primers (*Eco*RI-primer and *Mse*I-primer) with one selective nucleotide. A cycle profile according to Perkin Elmer (1995) was made. The reaction products were diluted 20-fold and used as a template in the following selective amplification step. The template was amplified by the method described by Vos et al. (1995), with the following modifications: 25 ng of a non-labeled *Eco*RI-primer was used, and the 20-µl PCR sample contained 2 m*M* $MgCl_2$. Samples were amplified by the temperature cycle described by Perkin Elmer (1995). Reactions were carried out on a Techne Genius Thermocycler, with *Taq* DNA polymerase (Promega Corporation, Wis.).

DNA sequences of adapters, preselective primers with one selective nucleotide, and selective primers with two or three selective nucleotides were all designed according to Maughan et al. (1996). All oligos were synthesized at DNA Technology ApS, Science Park Aarhus, Denmark. Pre-selective amplification was carried out with four primers with one selective nucleotide. For the selective amplification, 10 primer combinations were used. In order to choose the primer combinations revealing clear, reproducible polymorphisms, we screened 29 primer combinations on a test array of plants representing all populations/varieties, and selected 10 primer combinations for further use. The nucleotide sequence of adapters, primers, and their combinations are presented in Table 1.

The amplified DNA samples were mixed with 98% formamide loading buffer, heated at 96°C for 5 min, and then quickly cooled on ice. Five of each sample was loaded and run at 80 W on 6% polyacrylamide gels according to the standard method described for DNA sequencing (Sambrook et al.1989). After electrophoresis, the amplification products were visualized by silver staining (Bassam et al. 1991). The developed gels were dried at room temperature for 24 h before scoring the bands.

Data analysis

As we used a conventional detection method for AFLP markers they appear as dominant markers. The clear and unambiguous AFLP bands were scored as present (1) or absent (0) and recorded in a data matrix. Genetic diversity was partitioned within groups of accessions (Nei 1973; Nei and Li 1979) and the genetic distance (GD) and genetic similarity (GS) were calculated using the POPGENE software (available at URL http://www.ualberta.ca/~fyeh). A dendrogram based on genetic distance (Nei 1972) was constructed by POPGENE using the unweighted pair group method using arithmetic average

Table 1 Adapters and primer sequences used in the AFLP analysis

Name	Sequence
*Eco*RI adaptor	5´-CTCGTAGACTGCGTACC-3´ 3´-CTGACGCATGGTTAA-5´
*Mse*I adaptor	5´-GACGATGAGTCCTGAG-3´ 3´-TACTCAGGACTCAT-5´
Primers of preamplification	
*Eco*RI+A or C	5´-GACTGCGTACCAATTC+A or C-3´
*Mse*I+A or C	5´-GATGAGTCCTGAGTAA+A or C-3´
Efficient primer combinations used for the selective amplification	E-AGG/M-CTG, E-ACAG/M-CTG, E-ACGG/M-CTG E-ACAG/M-CTGA E-CAC/M-ACG E-CA/M-ACG E-CAC/M-AC E-CAG/M-AG E-CAC/M-CTG E-CAG/M-CTG

Table 2 Partitioning of the genetic diversity[a] in wild and cultivated *Daucus*

Primer combination	Old cultivars			New cultivars			Wild			Overall		
	Ht	Hs	Gst	Ht	Hs	Gst	Ht	Hs	Gst	Ht	Hs	Gst
E-ACGG/M-CTG	0.31	0.29	0.04	0.26	0.12	0.39	0.41	0.34	0.15	0.44	0.28	0.35
E-AGG/M-CTG	0.31	0.30	0.05	0.16	0.11	0.22	0.32	0.28	0.12	0.43	0.25	0.37
E-ACAG/M-CTG	0.26	0.24	0.07	0.27	0.15	0.38	0.44	0.32	0.27	0.45	0.26	0.42
E-ACAG/M-CTGA	0.26	0.24	0.08	0.20	0.16	0.19	0.40	0.30	0.25	0.42	0.25	0.39
E-CA/M-ACG	0.23	0.18	0.17	0.23	0.10	0.54	0.34	0.29	0.14	0.39	0.22	0.43
E-CAC/M-ACG	0.18	0.15	0.14	0.19	0.08	0.42	0.37	0.31	0.14	0.36	0.22	0.38
E-CAG/M-AG	0.23	0.19	0.16	0.09	0.08	0.12	0.36	0.30	0.15	0.39	0.22	0.40
E-CAC/M-AC	0.20	0.15	0.15	0.12	0.11	0.09	0.36	0.32	0.12	0.39	0.23	0.39
E-CAC/M-CTG	0.28	0.24	0.14	0.15	0.11	0.19	0.40	0.33	0.19	0.43	0.26	0.39
E-CAG/M-CTG	0.27	0.23	0.12	0.18	0.13	0.20	0.33	0.26	0.21	0.41	0.23	0.42
Mean	0.25	0.22	0.13	0.19	0.12	0.35	0.37	0.30	0.18	0.41	0.24	0.41

[a] Ht, genetic diversity over all groups; Hs, genetic diversity within populations; Gst, proportion of genetic diversity between populations

(UPGMA) procedure. In order to obtain a two-dimensional grouping pattern of wild carrots and varieties we carried out principal component analysis (PCA) using the PRINCOM procedure in the SAS program package (version 6.12, SAS Institute, 1994). To estimate variance components for the AFLP phenotypes and to partition the total variance into sub-factors, i.e., among and within populations (or varieties), AMOVA analysis (Excoffier et al. 1992) was executed with the software package ARLEQUIN (available at URL http://anthropologie.unige.ch/arlequin).

Results

The 10 AFLP primer combinations used to analyze the five wild populations and five varieties produced 116 reproducible bands that were polymorphic between or within the ten accessions. The average number of polymorphic bands detected was 12 per primer combination, and the molecular weight of the selected marker-bands ranged from approximately 100 bp to 500 bp. The most efficient primer combination, which showed the largest number (18) of polymorphic band, was the E-AGG/M-CTG combination. The genetic diversity reflected the primer combination applied and ranged from 0.45 (E-ACAG/M-CTG) to 0.36 (E-CAC/M-ACG) (Table 2).

An example of the AFLP fingerprinting is presented in Fig. 2. As shown here, the patterns of markers between and/or within populations were highly polymorphic in the *Daucus* accessions. However, several bands could be found that were subspecies-specific. Figure 2 shows an apparently subspecies-specific marker monomorphic in all the cultivated carrots analyzed.

The percentage of markers present (score 1) was summarized over all loci, and this frequency differed among populations from 67% (Napoli) to 44% (Hundested) (Table 3). The F_1 inbreds had many more bands than the old varieties and wild populations but less variation in band number (small standard deviation). Measures of genetic diversity within populations (Table 2) indicated that the old varieties were more heterogeneous than the newly developed F_1 varieties.

The genetic similarity among populations (Table 4) showed that the closest relationships were between the

three old varieties Grosa, Fancy, and Regol with the Nantes type, Fancy, being more genetically distant than the two Flakeer types, Grosa and Regol. The most distant genetic relationship was found between the wild populations (except Løgstør) and the recent F_1 varieties.

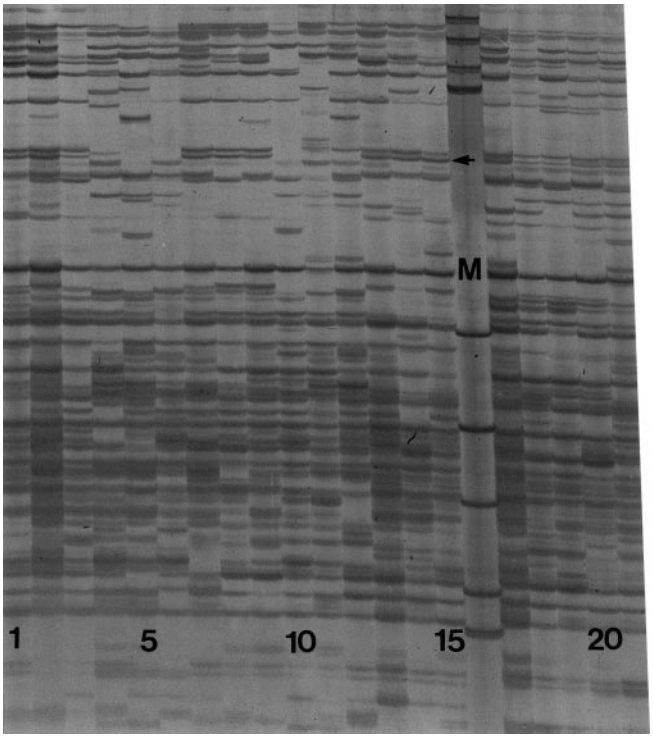

Fig. 2 AFLP profiles of *Daucus carota* ssp. *sativus*, *D. carota* ssp. *carota*, and hybrids from controlled crosses using the primer combination E-CAC/M-AC. The *arrow* indicates a specific marker for *D. carota* ssp. *sativus*. *Lanes 1–3* Regol, *4–6* Hundested, *7–9* Grosa, *10–12* Karlstrup, *13–15* Fancy, *16* size marker (540, 504, 458, 434, 267, 234, 213, 192, 184 bp), *17–21* hybrids from controlled crosses between Hundested (female) and Grosa (male)

Based on the genetic distance given in Table 4, a dendrogram of wild carrot populations and cultivated carrot varieties (Fig. 3) was generated. As shown in Fig. 3, all wild populations grouped together and are clearly separated from the cultivated carrot varieties. The F_1 hybrid varieties grouped together and are separated from the two groups of old cultivars and wild populations, respectively. Of the wild populations, the Løgstør population, a population collected in Jutland, is separate from other wild populations collected in Zealand. Wild populations that were geographically close to each other apparently also grouped together according to the AFLP data; i.e., two closely related populations collected at Hundested and Kikhavn are separated by only a short physical distance (Fig. 1). The populations from Karlstrup and Risø were also collected rather close to each other in Mid-Zealand. In order to obtain further information on the grouping of the wild populations and varieties, we carried out PCA, using AFLP band pattern as raw data. The PCA (Fig. 4) clearly reflected the relationships

Table 3 The average number of bands present at all loci, genetic distance (GD) and genetic similarity (GS) in five varieties and five wild populations of carrot. Average of 13–15 individuals

Population/cultivar	Number of bands[a]	*GD*	*GS*
Grosa	62.8(54.2)±4.9	0.1997	0.8190
Fancy	61.7(53.2)±4.8	0.2053	0.8144
Regol	61.2(52.8)±4.4	0.2445	0.7831
Napoli	78.1(67.4)±3.1	0.1157	0.8907
LD945479	70.0(60.3)±2.1	0.1278	0.8800
Hundested	51.1(44.0)±6.0	0.3169	0.7284
Kikhavn	52.5(45.2)±5.8	0.3147	0.7300
Karlstrup	54.5(47.0)±4.3	0.2789	0.7566
Løgstør	62.9(54.2)±4.3	0.2766	0.7584
Risø	59.6(51.4)±5.7	0.3160	0.7291

[a] The number of bands present at all 116 loci are given; percentage is given in parenthesis, followed by the standard deviation

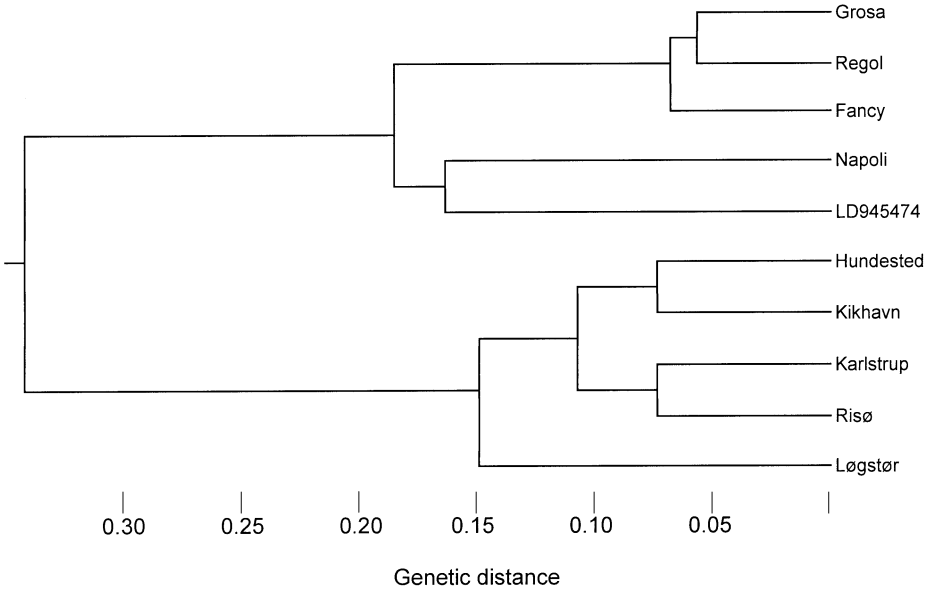

Fig. 3 Dendrogram based on AFLP polymorphisms in five wild populations and five cultivated varieties of carrot. Cluster analysis was conducted using the UPGMA method

Table 4 Genetic similarity and distance matrix. Above and below the diagonal are given Nei's (1972, 1978) genetic similarity and genetic distance

Population	Grosa	Fancy	Regol	Napoli	LD94	Hundested	Kikhavn	Karlstrup	Løgstør	Risø
Grosa		0.9455	0.9552	0.8321	0.8122	0.7119	0.7063	0.7130	0.6593	0.6939
Fancy	0.0561		0.9379	0.8515	0.8194	0.7137	0.7203	0.7111	0.6620	0.6966
Regol	0.0458	0.0641		0.8280	0.7970	0.7421	0.7364	0.7379	0.6915	0.7226
Napoli F_1	0.1838	0.1608	0.1887		0.8532	0.6243	0.6439	0.6161	0.7134	0.6678
LD F_1	0.2080	0.1992	0.2268	0.1587		0.5895	0.6235	0.5973	0.6760	0.6261
Hunsted	0.3398	0.3373	0.2982	0.4711	0.5285		0.9287	0.9253	0.8574	0.8923
Kikhavn	0.3477	0.3280	0.3059	0.4402	0.4724	0.0740		0.8826	0.8790	0.8944
Karlstrup	0.3383	0.3409	0.3040	0.4843	0.5154	0.0776	0.1249		0.8580	0.9305
Løgstør	0.4165	0.4125	0.3689	0.3376	0.3915	0.1538	0.1290	0.1532		0.8881
Risø	0.3654	0.3615	0.3250	0.4038	0.4683	0.1140	0.1117	0.0721	0.1187	

Table 5 Analysis of Molecular Variance (AMOVA) of 145 carrot individuals. The three groups were classified based on the dendrogram (Fig. 2). Mean squared deviation (MSD), variance component, and *P*-value were calculated using ARLEQUIN

Source of variation	df	MSD	Variance component	Percentage	*P*-value
Among populations/varieties	9	155.3	9.71	39.82	<0.01
Within populations/varieties	135	14.7	14.67	60.18	<0.01
Nested analysis					
Among groups	2	462.0	8.77	32.4	<0.01
Among populations/varieties	7	67.7	3.66	13.5	<0.01
Within populations/varieties	135	14.7	14.67	54.1	<0.01

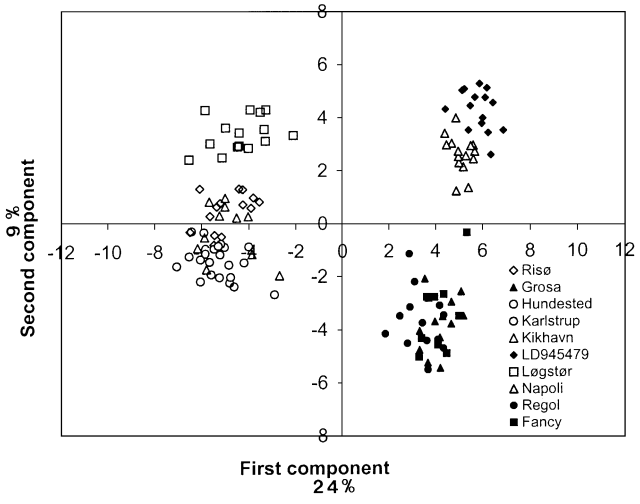

Fig. 4 Principal component analysis of 145 wild and cultivated carrot plants. The first component (*X-axis*) and second component (*Y-axis*) explain 24% and 9% of the total variation, respectively

among and between wild populations and cultivars that were also obtained from the dendrogram. The first and second component could explain 24% and 9% of the variation, respectively. The first axis obviously classified wild populations apart from the cultivated types. The second axis separated cultivated carrots into old (Grosa, Fancy, and Regol) and new (Napoli and LD945474) varieties, and the geographically distant wild population Løgstør was also separated from the other wild populations. Amova applied on our *Daucus* material indicated that a large portion of genetic variation originated from the variation found within populations/varieties (60%) compared to the variation found among populations/varieties. (Table 5). We divided the carrots into three groups based on the data from the dendrogram and used the groups in a nested AMOVA. The nested AMOVA showed that the variation present among groups (32%) was higher than that among populations/varieties (14%) and that the major part of the total genetic variation in our material originated from the variation within accessions.

Discussion

The AFLP technique can be more effective, more economic, or more precise in revealing polymorphisms than restriction fragment length polymorphisms (RFLPs) (Ajmone et al. 1998; Bohn et al. 1999), SSRs simple sequence repeats (SSRs) (Powell et al. 1996), and random amplified polymorphic DNAs (RAPDs) (Hill et al. 1996). Our AFLP analysis of carrot made a clear separation between subspecies, wild populations, and varieties. Due to our selection of only the unambiguous bands we have by far scored all of the bands arising from an AFLP primer combination; for example 1 primer combination produced more than 40 bands on average (data not shown); this value is equivalent to AFLP results reported in soybean and coconut (Maughan et al. 1996; Perera et al. 1998). Also in correspondence with results from others (i.e., Huff et al. 1993) the primer combinations we used here for revealing genetic variation had very different efficiencies (Table 2). In our study the number of primer combinations applied was large compared to other studies (Angiolillo et al. 1999; Nakajima et al. 1998; Perera et al.

1998). The appropriate number of primer combinations may depend on the plant material; an AFLP study using 3 primer combinations did not show a clear classification among wild types (ssp. *carota*) and cultivated types (ssp. *sativus*) of *Daucus carota* (Nakajima et al. 1998).

Based on genetic distance, wild populations and cultivated taxa were classified into three groups – old varieties, recent F_1 varieties, and wild carrot – reflecting that relationships were closer between the wild populations than between old and new varieties (Table 4, Fig. 3). The partitioning of the genetic variation showed that the old varieties were more heterogeneous than the newly developed F_1 inbred varieties, which was not surprising considering their different breeding history. Division of the total genetic diversity in the groups showed that the old varieties were similar to wild carrot with a relatively high genetic diversity within population/variety, higher than that found in the recent varieties (Table 2).

The genetic diversity was largest in the wild material (Table 2). Wild carrots are presumably predominantly outcrossing due to protandry (Koul et al. 1989), a reproductive habit known to enhance genetic variation. In addition, *Daucus carota* ssp. *carota* is a common wild species in Denmark, and it is likely that gene flow between populations takes place. Even though gene flow between populations tends to level out the diversity between the wild carrot populations, the genetic distance between the wild accessions was greater than between old varieties (Table 4). Apparently, in wild *Daucus* the genetic distances between populations collected in different regions reflected clinal genetic variation, suggesting that gene flow occurs as a function of distances.

The third factor that might be responsible for part of the comprehensive genetic variation found in wild carrots could be introgression between cultivated and wild carrots. Spontaneous hybridization between *Daucus carota* ssp. *carota* and *sativus* suggests that introgression may take place (Small 1984; Van Raamsdonk 1997; Wijnheijmer et al. 1989), especially if the wild carrot is a common species near seed-producing fields of cultivated carrot (Wijnheijmer et al. 1989). In our study we found that a few plants from the Risø population had a marker considered to be specific to the cultivated subspecies (data not shown). This could suggest that introgression has taken place, although more evidence is necessary to support this hypothesis.

Our AFLP results from *Daucus* show a clear classification between different taxa and varieties. The results also indicate that genetic distance between wild carrot populations is dependent on their geographic separation and that cultivated carrot varieties represent different amounts of genetic variation depending on their breeding history.

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