Flow cytometry as a tool to quickly check for major taxonomic inaccuracies in collections

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Introduction

The taxonomy and identification of *Daucus* species and subtaxa has been problematic due to conflicting taxonomic schemes and the morphological plasticity of the groups concerned. This is a major issue for genebank collections wishing to verify the taxonomic identity of donated material; many of these groups are unidentifiable when seed are ripe as the plant is dead or in an advanced state of senescence. There are often uncertainties concerning the classification of donated material, and without easy access to taxonomic expertise in the *Daucus* group, it is a challenge to resolve them.

Warwick Genetic Resources Unit holds a collection of 246 accessions of crop wild relatives in the genus *Daucus*. While the taxonomic identities of some have been confirmed in collaboration with external experts, others have not yet been checked. Developing further tools to assist in the process would assist Warwick GRU to provide better classified germplasm to users. New tools would allow the investigation of an accession without necessarily growing plants to a mature flowering stage which takes space and resources better utilized for regeneration. Flow cytometry is a rapid method of investigating genome size in leaf and other plant tissue samples. It is very effective in confirming the identity of *Brassica* species with the different A, B or C genomes (and amphiploid species which combine two genomes in each individual).

Daucus species are known to have karyotypes (chromosome numbers) ranging from n=9 to n=11 (Pimenov et al. 2003). Additionally the size of the genome (DNA quantity) may vary as well as chromosome number, and higher numbers of chromosomes don't always correspond to a larger genome size. It is possible that flow cytometry may offer a quick and relatively cheap assessment of the genome size of accessions, which may permit more accurate classification. It would probably not enable clear distinction among subspecific taxa but it would potentially be able to highlight cases of gross misidentification.

Methods

Five individuals of 55 accessions (see Appendix 1) were grown up in pots at Warwick GRU. Leaf samples were taken from mature plants where available and sent to Plant Cytometry Services Ltd, The Netherlands for analysis by flow cytometry. Cell nuclei were stained with DAPI dye, and the fluorescence was quantified to provide an indication of the amount of DNA in each cell. An internal control (*Buxus sempervirens*) was run with each sample to enable an estimation of relative genome size. This method does not provide an absolute quantification of the amount of DNA in each sample, as the dye only binds to adenine (A) and thymine (T) in the DNA molecule, and the AT:GC ratios will be different in each *Daucus* species and in *Buxus*. The results however indicate relative genome sizes.

In parallel, many accessions were evaluated at Agrocampus Ouest, Angers, France by Emmanuel Geoffriau and J.P. Reduron to verify the taxonomic classification by morphological methods. This was extremely helpful in confirming the correct taxonomic

classification of the accessions and allowing the flow cytometry results to be properly assessed. The data presented are a combination of the two approaches.

Digital images of material sampled for cytometry testing were also taken, including leaf scans.

Results

Flow cytometry results were obtained from 48 accessions. Other accessions completed their life cycle too rapidly to be included in the sampling programme. Using *B. sempervirens* as an internal standard, the ratio of sample DNA to standard ranged from 0.72 to 2.88. All results are given in Appendix 1. A summary of the results for each species is given in Table 1, excluding five accessions thought to be heterogeneous or hybrid. Figure 1 gives a graphical plot of the mean DNA ratio +/- one standard deviation for three *D. carota* subspecies. It appears that accessions classified as subspecies *gummifer* have slightly larger DNA ratios than subspecies *carota* or *sativus*. There is a significant difference in the DNA ratio of *D. c. gummifer* and the other two subspecies (Kruskal-Wallis Hc = 11.6, p=0.003).

Table 1. Summary of flow cytometry results showing the number of accessions and total number of individuals tested per taxon. The supplied taxonomic identities of some accessions were found to be synonyms when checked using GRIN taxonomy; these are noted in the comments column.

Taxon	N (accessions)	N (individuals)	Mean DNA ratio	s.d.	Comments
D. carota carota	15	61	0.77	0.02	
D. carota gummifer	5	25	0.80	0.06	
D. carota commutatus	2	10	0.83	0.05	
D. carota major	1	2	0.76	0.02	
D. carota sativus	4	12	0.76	0.03	
D littoralis	2	4	2.42	0.33	Including *D. glaber*
D. glochidiatus	1	5	1.86	0.01	
D. muricatus	2	2	1.53	0.01	
D. pusillus	2	7	0.83	0.11	Including *D. hispidifolius*, *D. montevidensis*
D. duriena	1	2	1.0	n/a	

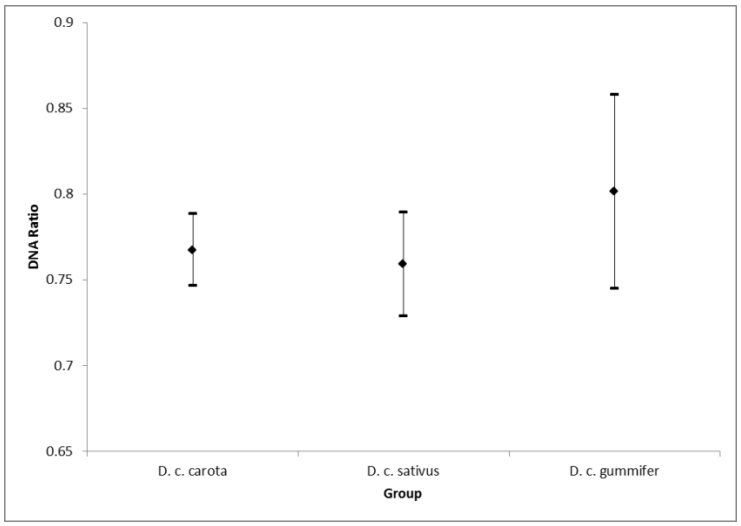

Figure 1. Mean and standard deviations of the ratio of DNA quantity with *B. sempervirens* for all individuals from three *D. carota* subspecies.

Discussion

The results indicate that differences in the DNA ratio will allow certain taxonomic identities to be excluded (various *Daucus* species such as *D. glochidatus* and D. *littoralis* showed a very different ratio of DNA to the internal standard used) however the technique is not suitable for definitively confirming the identity of an accession, particularly at the subspecies level. The differences seen may be worth investigating further; *D. c. gummifer* appears to have a larger genome in terms of DNA quantity than *D. c. sativus* or *D. c. carota*. Using DAPI as a dye has some limitations, namely that the AT:GC ratio varies among species so it cannot give a truly quantitative measure of genome size. Additionally, to have an accurate measure of DNA quantity (C-value or pg DNA per cell), one would need to treat the samples to remove other nucleic acids such as RNA first, and this method of sample preparation was outside of the scope of the project.

The digital images taken including photographs of growth habit and leaf scans will be added to the images available for Warwick GRU material, and the database has already been updated where necessary to reflect the taxonomic assessment suggested by the Agrocampus Ouest analysis.

References

Iovene M, Grzebelus E, Carputo D, Jiang J, Simon P.W. 2008. Major cytogenetic landmarks and karyotype analysis in *Daucus carota* and other Apiaceae. American Journal of Botany 95(7):793-804 (doi: 10.3732/ajb.0700007).

Pimenov MG et al. 2003. Karyotaxonomical analysis in the Umbelliferae. Science Publishers, Enfield, NH.

Appendix 1. Full results for all individuals tested using flow cytometry. The taxonomic comments indicate further information provided by E. Geoffriau or indications of synonymy. N indicates haploid number of chromosomes per cell (reported by Pimenov et al. 2003 or Iovene et al. 2008)

				DNA ratio to internal standard								
ACCENUM	**Genus**	**Species**	**Subtaxa**	**Ind. 1**	**Ind. 2**	**Ind. 3**	**Ind. 4**	**Ind.5**	**DNA ratio mean**	**s.d.**	**N**	**Taxonomy comments**
13807	*Daucus*	*guttatus*		2.16	2.18	n/a	n/a	n/a	2.17	0.01	10	(syn. *bicolor*)
8250	*Daucus*	*broteri*		2.55	n/a	n/a	n/a	n/a	2.55	n/a	10	
13967	*Daucus*	*carota*		0.75	0.76	n/a	n/a	0.74	0.75	0.01	9	mixed – including *carota, mauritanicus* and *sativus* x wild hybrids
13808	*Daucus*	*carota*	*azoricus*	0.78	0.86	0.84	0.79	0.81	0.82	0.035	9	
6666	*Daucus*	*carota*	*carota*	0.80	0.80	0.80	0.79	0.79	0.80	0.01	9	
6668	*Daucus*	*carota*	*carota*	0.76	0.78	0.81	n/a	n/a	0.78	0.02	9	*carota* var. *carota*
6673	*Daucus*	*carota*	*carota*	0.77	0.79	0.78	n/a	0.77	0.78	0.01	9	
6679	*Daucus*	*carota*	*carota*	0.84	0.83	0.87	0.86	0.87	0.85	0.02	9	similarities with *D. carota* var. *maritimus*
6680	*Daucus*	*carota*	*carota*	0.79	0.79	n/a	n/a	n/a	0.79	n/a	9	var. *carota*
7385	*Daucus*	*carota*	*carota*	0.74	0.76	0.76	0.75	n/a	0.75	0.01	9	var. *carota*
8001	*Daucus*	*carota*	*carota*	0.74	0.77	0.75	0.77	0.73	0.75	0.02	9	var. *carota*
8002	*Daucus*	*carota*	*carota*	0.75	0.78	n/a	0.78	0.79	0.78	0.02	9	possibly var. mauritanicus
9246	*Daucus*	*carota*	*carota*	0.75	0.76	n/a	0.75	0.79	0.76	0.02	9	var. *carota*
9257	*Daucus*	*carota*	*carota*	0.79	0.75	0.78	0.80	0.75	0.77	0.02	9	var. *carota*
12423	*Daucus*	*carota*	*carota*	0.78	0.77	0.75	0.74	0.74	0.76	0.02	9	var. *carota*
13962	*Daucus*	*carota*	*carota*	0.76	0.72	0.77	0.76	0.77	0.76	0.02	9	possibly var. *mauritanicus*
13963	*Daucus*	*carota*	*carota*	0.73	n/a	n/a	n/a	n/a	0.73	n/a	9	var. *carota*
13964	*Daucus*	*carota*	*carota*	0.76	0.77	n/a	n/a	n/a	0.77	0.01	9	var. *carota*
13966	*Daucus*	*carota*	*carota*	0.77	0.78	0.78	0.77	0.77	0.77	0.01	9	var. *carota*
13968	*Daucus*	*carota*	*carota*	0.78	0.77	0.76	0.72	0.74	0.75	0.02	9	var. unknown
7192	*Daucus*	*carota*	*carota* x *gummifer*	0.77	0.78	0.81	0.81	0.80	0.79	0.02	9	intermediate type
8710	*Daucus*	*carota*	*carota* x *gummifer*	0.74	0.80	0.81	0.80	0.80	0.79	0.03	9	intermediate type
7386	*Daucus*	*carota*	*commutatus*	0.77	0.80	0.77	0.80	0.78	0.78	0.02	9	var. *tenuisectus*
8728	*Daucus*	*carota*	*commutatus*	0.87	0.86	0.90	0.87	0.89	0.88	0.02	9	

				DNA ratio to internal standard								
ACCENUM	**Genus**	**Species**	**Subtaxa**	**Ind. 1**	**Ind. 2**	**Ind. 3**	**Ind. 4**	**Ind.5**	**DNA ratio mean**	**s.d.**	**N**	**Taxonomy comments**
6667	*Daucus*	*carota*	*gummifer*	0.87	0.89	0.92	0.93	0.89	0.90	0.02	9	*gummifer* var. *fontanesii*
7996	*Daucus*	*carota*	*gummifer*	0.75	0.76	0.74	0.73	0.72	0.74	0.01	9	*gummifer* var. *gummifer*
8706	*Daucus*	*carota*	*gummifer*	0.81	0.80	0.78	0.76	0.78	0.79	0.02	9	*gummifer* var. *fontanesii*
8713	*Daucus*	*carota*	*gummifer*	0.80	0.80	0.79	0.80	0.81	0.80	0.01	9	
9289	*Daucus*	*carota*	*gummifer*	0.76	0.79	0.77	0.79	0.80	0.78	0.02	9	
9200	*Daucus*	*carota*	*major*	0.74	0.78	0.75	0.75	n/a	0.76	0.02	9	
7388	*Daucus*	*carota*	*sativus*	0.75	0.76	0.78	0.75	0.72	0.75	0.02	9	
10311	*Daucus*	*carota*	*sativus*	0.75	0.74	n/a	n/a	n/a	0.75	0.02	9	cv. Kintoki
12400	*Daucus*	*carota*	*sativus*	0.81	0.82	n/a	n/a	n/a	0.82	0.01	9	cv. Altringham
13886	*Daucus*	*carota*	*sativus*	0.73	0.76	0.74	n/a	n/a	0.74	0.01	9	donated as *D. littoralis*
13965	*Daucus*	*carota*	*sativus x*	0.77	0.79	0.73	0.77	0.78	0.77	0.02	n/a	cultivated x wild?
7191	*Daucus*	*carota*		0.75	0.77	0.77	0.79	0.78	0.77	0.01	9	
9233	*Daucus*	*carota*		0.72	0.72	0.72	0.70	0.67	0.71	0.02	9	
13969	*Daucus*	*carota*		0.78	0.77	n/a	0.76	0.77	0.77	0.01	n/a	hybrid wild x cultivated
8252	*Daucus*	*carota*	*sativus*	0.75	0.75	0.73	n/a	n/a	0.74	0.01	9	donated as *carota*? *hispanicus*?
7188	*Daucus*	*duriena*		≈1.00	≈1.00	n/a	n/a	n/a	n/a	n/a	10 or 11	confirmed
7997	*Daucus*	*littoralis*		2.88	2.43	n/a	n/a	n/a	2.66	0.31	10	confirmed
12577	*Daucus*	*littoralis*		2.12	2.25	n/a	n/a	n/a	2.19	0.09	10	(syn. *glaber*)
8251	*Daucus*	*glochidiatus*		1.86	1.86	1.86	1.85	n/a	1.86	0.01	11	
7189	*Daucus*	*pusillus*		0.78	0.79	0.77	0.74	0.75	0.77	0.02	10	(syn. *hispidifolius*)
8254	*Daucus*	*pusillus*		≈1.00	≈1.00	n/a	n/a	n/a	n/a	n/a	10	(syn. *montevidensis*)
7998	*Daucus*	*muricatus*		1.52	n/a	n/a	n/a	n/a	1.52	n/a	11	
13880	*Daucus*	*muricatus*		1.54	n/a	n/a	n/a	n/a	1.54	n/a	11	
12600	*Daucus*	*sp*		0.72	0.74	0.72	n/a	n/a	0.73	0.01	n/a	