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RESEARCH ARTICLE

Cytoplasmic diversity of *Brassica napus* L., *Brassica oleracea* L. and *Brassica rapa* L. as determined by chloroplast microsatellite markers

Shirin Zamani-Nour · Rosemarie Clemens · Christian Möllers

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Abstract Cytoplasmic genomes in most angiosperms are known to be maternally inherited. Oilseed rape (*Brassica napus* L.) as a natural amphidiploid species hence may carry the *B. oleracea* L. or the *B. rapa* L. cytoplasm, depending on the cross direction. The presence of either the *B. oleracea* or the *B. rapa* cytoplasm in oilseed rape has been reported to affect agronomically important traits. However, to date little is known about the cytoplasmic composition and genetic diversity of current winter oilseed rape cultivars and breeding material. The aim of this study was to assess the usefulness of 40 previously published chloroplast cpSSR markers from *Brassica* species and *Arabidopsis thaliana* (L.) Heynh. for distinguishing the cytoplasms of 49 different genotypes of *B. napus* and its diploid ancestor species. Results showed that only 14 out of the 40 tested primer combinations were suitable to distinguish the cytoplasms of a test set of 8 *Brassica* genotypes. With the 14 primer pairs 64 different cpSSR alleles were identified in the set of 49 genotypes. Cluster analysis indicated distinct groups for the cytoplasms of *B. napus, B. rapa*, and *B. oleracea*. However, an unambiguous identification and classification of the cytoplasm types was not possible in all cases with the available polymorphic set of cpSSR primer pairs.

Keywords *Brassica* · Chloroplast · Cytoplasma · Oilseed rape · Plastome · Resynthesized rapeseed · SSR marker

S. Zamani-Nour · R. Clemens · C. Möllers (✉)
Division of Plant Breeding, Department of Crop Sciences, Georg-August-Universität Göttingen, Von-Siebold-Str. 8, 37075 Göttingen, Germany
e-mail: cmoelle2@gwdg.de

Introduction

Oilseed rape (*Brassica napus* L., AACC-genome, 2n = 38) is an amphidiploid crop species, spontaneously arisen from a cross between *Brassica rapa* L. (turnip rape, AA-genome, 2n = 20) and *Brassica oleracea* L. (cabbage, CC-genome, 2n = 18). Although oilseed rape is considered to be of polyphyletic origin, it is assumed that there have been only a limited number of successful hybridization events. From an evolutionary point of view, oilseed rape is a fairly new crop. First reliable documented records are about 500 years old (Downey and Röbbelen 1989; Gómez-Campo and Prakash 1999) and results from molecular phylogenetic analyses indicate that hybridization occurred less than 10,000 years ago (Rana et al. 2004). Unlike, the divergence and separate evolution of the diploid species *Brassica rapa* and *Brassica oleracea* began between 3 and 4 million years ago (Inaba and Nishio 2002; Cheung et al. 2009).

As cytoplasmic genomes—plastome and mitochondrial genome—are maternally inherited (Bock 2007),

oilseed rape may carry either the cytoplasm of its diploid ancestors *B. oleracea* or *B. rapa*, depending on the direction of the hybridization event. Furthermore, during the past 60 years, oilseed rape has been resynthesized via interspecific crossing of various *B. oleracea* and *B. rapa* forms followed by embryo rescue and in vitro plant regeneration, so that depending on the cross direction different cytoplasms are available (Chen and Heneen 1989). However, the parental material and the direction of the crosses have not always been fully documented in the literature. The presence of either the *B. oleracea* or the *B. rapa* cytoplasm in oilseed rape has been reported to affect agronomically important traits, like oil quality (Rajcan et al. 2002), oil and protein content (Wu et al. 2005, 2006; Wang et al. 2010) and floral characteristics (Chang et al. 2011). Furthermore, genes relevant to the expression of agricultural traits are located in the plastome. For example, one unit of the heteromeric Acetyl-CoA Carboxylase (with four subunits) which is required for the de novo fatty acid synthesis in the plastids (Sasaki and Nagano 2004; Kode et al. 2005) is encoded by the chloroplast DNA (cpDNA). Furthermore, the cpDNA gene rbcL encodes for the large subunit of ribulose-1,5-bisphosphate carboxylase (Clegg et al. 1994), which is a vital enzyme for carbon fixation in the Calvin cycle. Despite of the obvious importance of chloroplast genes for many metabolic pathways (Wicke et al. 2011), to date little is known about the cytoplasmic composition of current oilseed rape cultivars and breeding material.

Preliminary results from early studies with RFLP markers (Palmer et al. 1983; Erickson et al. 1983; Kemble 1987; Song and Osborn 1992; Halldén et al. 1993) were inconsistent, indicating that oilseed rape may have a cytoplasm derived from *B. oleracea* (ole-type) or *B. rapa* (rap-type) or may have its own (nap-type). Furthermore, for hybrid breeding programmes, cytoplasmic male sterility (CMS) has been achieved through the introgression of specific cytoplasms from related species. The Polima CMS system possibly is derived from a Polish winter oilseed rape genotype (Liu et al. 1987), and the Ogura CMS system has been introduced from *Raphanus sativus* L. into oilseed rape genomic background by sexual crossing and back crossing (Pelletier et al. 1983). In a next step, protoplast fusion was performed to replace the original *Raphanus* chloroplasts by the chloroplasts of the spring oilseed rape cultivar Brutor (Pelletier et al. 1983).

Rather recently it has been discovered that chloroplast DNA does also contain microsatellite polymorphisms. Chloroplast specific SSR-markers (cpSSR) have been developed for a number of species including *Arabidopsis* (Weising and Gardner 1999; Provan 2000; Jakobsson et al. 2007; Haider 2011) and oilseed rape (Provan 2000; Flannery et al. 2006; Allender et al. 2007). Chloroplast cpSSR repeats may be found in both coding and non-coding regions but more variation were reported in the non-coding regions including introns and intergenic spacer of cpDNA (Provan et al. 2001; Jakobsson et al. 2007). Ebert and Peakall (2009) emphasized that cpSSR repeats are likely more abundant in intergenic spacer regions than in introns. Nearly all of the cpSSR markers detected are of the mononucleotide type (Flannery et al. 2006; Jakobsson et al. 2007). Using a limited set of cpSSR markers for studying cytoplasmic diversity in oilseed rape has led to differing results (Flannery et al. 2006; Jakobsson et al. 2007; Allender and King 2010). The identification of a set of most informative cpSSR primer pairs for the *Brassica* AA, CC and AACC genomes could be most useful in future work for the characterization and evaluation of the effects of different cytoplasms in oilseed rape.

The objective of the present study was to analyse a set of cpSSR primers previously developed for *Arabidopsis*, *Brassica oleracea* and for *Brassica napus* (Provan 2000; Flannery et al. 2006; Allender et al. 2007; Jakobsson et al. 2007) for their suitability to distinguish the cytoplasms of *Brassica* species. The plant material included in the study comprised wild and cultivated forms of *Brassica oleracea*, cultivated vegetable and oil forms of *B. rapa* and cultivated and resynthesized forms of oilseed rape (*B. napus*). For comparison, oilseed rape genotypes with the Polima, the Ogura cytoplasmic male sterility cytoplasm and with the *Brassica* B genome cytoplasm were included.

Materials and methods

Plant material

The 49 genotypes comprised resynthesized forms of *B. napus* L. (AACC, n = 7), current German winter oilseed rape cultivars (*B. napus* L., AACC, n = 10), spring *B. napus* cultivar Korall with its normal cytoplasm (AACC, n = 1), with the Polima male

sterility cytoplasm (n = 1), and with the *Brassica* B genome cytoplasm (n = 1), current winter oilseed rape *B. napus* hybrid cultivars with the Ogura male sterility cytoplasm (AACC, n = 2), *B. rapa* L. (AA, n = 9), wild forms of *B. oleracea* L. (CC, n = 13; *B. cretica* Lam., *B. incana* Ten., *B. villosa* Biv. 3821, *B. villosa* Biv. subsp. *bivoniana* 6581, *B. bourgeaui* Kuntze, *B. montana* Pourr., *B. macrocarpa* Guss., *B. rupestris* Raf., *B. taurica* Tzvel., *B. hilarionis* G.E.Post, *B. insularis*, *B. oleracea* subsp. *oleracea* 7695 and *B. oleracea* var. *alboglabra* (Bail.) Sun BRA 165), cultivated forms of *B. oleracea* (CC, n = 4) and one accession of *B. carinata* A. Braun (BBCC, n = 1) (Table 1, 2). The three Korall genotypes were provided by Bo Gertsson, Svalöv (Sweden). For nomenclature see Gladis and Hammer (1990, 1992).

Chloroplast SSR primers

A total of 40 cpSSR primer pairs were used for the analysis. They included 11 primer pairs previously developed for *B. napus* by Flannery et al. (2006; MF-1, MF-2, MF-3, MF-4, MF-6, MF-7, MF-8, MF-9) and by Allender et al. (2007; ChloroO, ChloroP, ChloroQ). The remaining 29 primer pairs were developed for *Arabidopsis thaliana* by Provan (2000; ATCP7905, ATCP 28673, ATCP30287, ATCP46615, ATCP66701, ATCP 70189), by Allender et al. (2007; Chla16, Chloro35, Chloro39) and by Jakobsson et al. (2007; 01, 07, 08, 11, 12, 17, 19, 21, 24, 29, 34, 37, 43, 44, 45, 47, 51, 55, 58, 60). The 20 primer pairs of Jakobsson et al. (2007) were selected from the 60 published ones so that they were representing all regions of the *Arabidopsis* chloroplast genome. The primer pair names in this publication refer to their names given in the original publications. Primers were ordered from Eurofins MWG Operon (www.eurofinsdna.com).

Purification of total DNA

Leaf samples were taken from one young plant each of the 49 *Brassica* genotypes. Total genomic DNA was isolated using the Qiagen DNeasy Plant Mini Kit (The Netherlands) and following basically the procedure described in the manual. DNA concentration was determined by using the Bio-Rad VersaFluor™ Fluorometer and the Bio-Rad Fluorescent DNA Quantitation Kit (Bio-Rad, CA, USA) containing the fluorochrome Hoechst 33258 (bisbenzimide), following the instruction manual.

PCR reaction

A total volume of 20 µl was used for each PCR reaction, containing 0.05 units/µl FIREPol Taq polymerase (Solis Biodyne; Tartu, Estonia), 1× FIREPol PCR buffer without MgCl2, 2.5 mM $MgCl_2$ (Solis Biodyne; Tartu, Estonia), 0.2 mM dNTP-Set (Bio-Budget Technologies GmbH; Krefeld, Germany), 0.05 µM M13-universal primer (23 bp)(Applied Biosystems), 0.05 µM forward primer with M13 (18 bp) tail at its 5′ end (Eurofins MWG Operon; Ebersberg, Germany), 0.05 µM unlabelled reverse primer (Eurofins MWG Operon; Ebersberg, Germany) together with 25 ng of template DNA. The PCR reaction was performed in a Biometra Thermocycler (Biometra GmbH; Göttingen, Germany) using the following two-step touchdown PCR program: 95 °C for 2 min; 5 cycles of 95 °C for 45 s, 68 °C (−2 °C/cycle) for 5 min, 72 °C for 1 min; 5 cycles of 95 °C for 45 s, 58 °C (−2 °C/cycle) for 1 min, 72 °C for 1 min; 27 cycles of 95 °C for 45 s, 47 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min and then cooled down to 4 °C after the last cycle.

Preparation of cpSSR-PCR products for capillary array analysis and identification of cpSSR alleles

Three different SSR-PCR products with different colours (FAM™, VIC™ and NED™) (Applied Biosystems) were mixed together. 2 µl of each of three PCR products were mixed and diluted 1:100 using HPLC water. Afterwards 2 µl of diluted PCR product was added to a loading mixture of 12 µl Hi-Di™ Formamide and 500 ROXTM size standard. The mixture was denatured for 2 min at 90 °C in a Thermocycler. The electrophoresis was then carried out automatically in Genetic Analyzer 3130x, a 16-capillary instrument, using POP7, 36 cm capillary and 23 s injection time. Fluorescently labelled fragments were interpreted using GeneMapper software v.3.7 (Applied Biosystems). Each locus was represented by one peak. If more than one peak occurred, unspecific binding of primers were anticipated and those primer pairs were excluded from further analysis. The maximum and minimum sizes of markers were selected in the range of 50–500 (bp). An allele height of more than 500 was

Table 1 *B. rapa*, *B. oleracea*, *B. napus,* resynthesized *B. napus* and *B. carinata* genotypes used in this study

Species	Subspecies/Genotype or accession no.	Comments/Origin
B. rapa L.	subsp. *trilocularis*/Yellow Sarson 59	Prof. Fu, Wuhan, China
	var. *nipposinica*/Mizuna	www.nelson.se; Art. No. 60233
	subsp. *oleifera*/Rex	NPZ Lembke KG, DE
	subsp. *oleifera*/Largo	Lantmännen SW Seed/DE
	subsp. *oleifera*/Tori	Brown Sarson/L. Hassan, Bangladesh
	subsp. *oleifera*/Steinacher/BAZ18101	BAZ Braunschweig, DE
	subsp. *oleifera*/Perko	Tetraploid/KWS SAAT AG, DE
	subsp. *oleifera*/Orbit	Lantmännen SW Seed, DE
	subsp. *oleifera*/Salut	Winter turnip rape
Br. cretica Lam.	subsp. *aegaea* 6344	Genbank Spain (ES)
B. incana Ten.	6564	Genbank Spain (ES)
B. villosa Biv.	3821–75	Genbank Spain (ES)
Br. villosa Biv.	subsp. *bivoniana* 6581	Genbank Spain (ES)
B. bourgeaui Kuntze	BRA 2998 (=K 9825)	Genbank Gatersleben (IPK), DE
B. montana Pourr.	BRA 1644 (=K5457)	Genbank Gatersleben (IPK), DE
B. macrocarpa Guss.	3819–75	Genbank Spain (ES)
B. rupestris Raf.	subsp. *hispida* 6580–84	Genbank Spain (ES)
B. taurica Tzvel.	BRA 2947(=K9238)	Genbank Gatersleben (IPK), DE
B. hilarionis G.E.Post	HRIGRU 12483	Genbank Great-Britain (GB)
B. insularis	BRA 3050 (=K 9321)	Genbank Gatersleben (IPK), DE
B. oleracea L.	subsp. *oleracea* 7695	Genbank Spain (ES)
B. oleracea L.	var. *alboglabra* BRA 165	Genbank Gatersleben (IPK), DE
	var. *botrytis* L./Super Regama/BRA 1381	Cauliflower/Genbank Gatersleben (IPK), DE
	var. *botrytis* L./Vasco	Cauliflower/Novartis Seeds, CH
	var. *capitata* L./Reliant	Red cabbage/Novartis Seeds, CH
	var. *gongylodes* L./Azur	Turnip, stem cabbage/Novartis Seeds, CH Switzerland
B. napus L.	Ha 699/91-4	WOSR, Breeding line/GAU Göttingen, DE
	Komando-5	WOSR, Line cultivar/KWS Saat AG, DE
	Oase-3	WOSR, Line cultivar/DSV AG, DE
	Krypton	WOSR, Line cultivar/KWS Saat AG, DE
	Charly-7	WOSR, Line cultivar/DSV AG, DE
	Favorite-6	WOSR, Breeding Line/DSV AG, DE
	DSV 2-08-1	WOSR, Breeding Line/DSV AG, DE
	ES Alienor	WOSR, Line cultivar/Euralis Semences, FR
	NK Beauty-10	WOSR, Line cultivar/Syngenta Seeds GmbH, DE
	Express 617-4	WOSR, NPZ Lembke KG, DE
	Triangle with Ogura (a) CMS cytoplasm	WOSR, Ogura CMS hybrid/KWS Saat AG, DE Germany
	Flash with Ogura CMS cytoplasm	WOSR, Ogura CMS hybrid/DSV AG, DE
	Korall	SOSR, Line cultivar/SE
	Korall with Polima CMS cytoplasm	SOSR, Line cultivar/SE
	Korall with B genome cytoplasm	SOSR, Line cultivar/SE
	H123-1	Dept. of Crop Sciences, GAU Göttingen, DE
	H10-3	Dept. of Crop Sciences, GAU Göttingen, DE
	H61	Dept. of Crop Sciences, GAU Göttingen, DE
	S3	Dept. of Crop Sciences, GAU Göttingen, DE
	S13	Dept. of Crop Sciences, GAU Göttingen, DE
	H48	Dept. of Crop Sciences, GAU Göttingen, DE
	L239	Dept. of Crop Sciences, GAU Göttingen, DE
B. carinata A. Braun	BRA 1151/90	Genbank Gatersleben (IPK), DE

WOSR winter oilseed rape, *SOSR* spring oilseed rape

Table 2 Resynthesized *B. napus* genotypes with reported cytoplasmic origin

Name	Female parent	Male parent
H123-1	*B. oleracea* L. convar. *capitata* (L.) Alef. var. sabauda L.	*B. rapa* ssp. *nipposinica* (Bail.) Hanelt
H10-1	*B. oleracea* L. convar. *capitata* f.*capitata* L.	*B. rapa* ssp. *pekinensis* (Lour.) Hanelt
H61	*B. napus* L. em. Metzg. ssp. *napus* var. *pabularia* (DC.) Rehb.	*B. rapa* ssp. *pekinensis*
S3	*B. rapa* L. em. Metzg. ssp. rapa	*B. oleracea* convar. *acephala* (DC.) Alef. var. *sabellica* L.
S13	*B. rapa* subsp. *oleifera* (DC.) Metzg. 4x	*B. oleracea* convar. *acephala* var. *medullosa* Thell. 4×
H48	*B. oleracea* convar. *capitata* var. *sabauda*	*B. rapa* ssp. *nipposinica*
L239	*B. oleracea* convar. *gemmifera* DC.	*B. rapa* ssp. *chinensis* (L.) Hanelt

Origin Department of Crop Sciences, Göttingen, Germany. For more information about H10-3 see Kräling (1986) and for the rest of the samples see Girke (2002)

preferred and of less than 100 was ignored. The presence and absence of microsatellite alleles were scored manually as 1 and 0, respectively, and data were stored as binary data in a matrix.

Cluster analysis

The binary matrix file was used to calculate genetic similarities and to perform a cluster analysis with the software NTSYSpc v2.1 (www.exetersoftware.com) using the Dice coefficient (equal to Nei-Li equation; $GSi,j = 2Ni,j/(2Ni,j + Ni + Nj)$ where GSi,j represents the similarity between the genotypes i and j, Ni,j is the total number of loci common in i and j, and Ni and Nj correspond to the number of loci found in genotypes i and j) (Nei and Li 1979). The analysis was performed as Unweighted Pair Group Method with Arithmetic mean (UPGMA). The cluster matrix was then compared to a cophenetic value matrix of the original data to produce a cophenetic correlation value as a measure of goodness of fit. Value less than 0.7 indicates very poor fit, 0.7–0.8 poor fit, 0.8–0.9 good fit and 0.9–1.0 very good fit (Rohlf 1997). Bootstrap analysis was performed using Winboot program (Yap and Nelson 1996) to verify if the number of markers was good enough to provide an accurate approximation (Halldén et al. 1994). The strength of the cluster was determined in 2,000 replicates.

Results

From the 40 chloroplast microsatellite primer pairs that were used to analyse each four *B. oleracea* and *B. rapa* genotypes and two *B. napus* genotypes, only 14 showed clear polymorphism (Table 3). The remaining ones were either monomorphic, showed no amplification (null alleles) or showed ambiguous results with two or three different alleles. In those cases, the primer pairs were not considered for the further analysis. From the eight primer pairs developed by Flannery et al. (2006) for *Brassica napus*, only MF-1, MF-2, MF-3, MF-4, MF-7 and MF-9 revealed polymorphism. The three primer pairs developed by Allender et al. (2007) for *B. napus* gave in two cases no amplification and in one case only a monomorphic peak was observed. From the six primer pairs developed by Provan (2000) for *Arabidopsis thaliana*, only one (ATCP28673) showed a useful polymorphism. Two out of the three primer pairs from Allender et al. (2007) and only five out of the 20 primer pairs from Jakobsson et al. (2007) for *Arabidopsis* revealed useful polymorphism in the test set of eight genotypes.

By applying the 14 primer pairs to the whole collection of 49 genotypes, altogether 64 polymorphic cpSSR alleles were generated (Table 4). The number of detected alleles per primer pair ranged from 2 to 10. Primer pair MF-7 produced the largest number of polymorphic alleles (10 alleles) followed by MF-3 (8 alleles) and MF-4 (7 alleles). Primer pairs ATCP28673, Chla16 and Chloro35 produced the lowest number of polymorphic allele (2 alleles for each primer combination). *B. oleracea* represented the most diverse cytoplasmic group with 13 different haplotypes and 1–6 alleles per locus (Table 5), followed by the *B. napus* group with 12 haplotypes and 1–7 alleles per cpSSR locus (Table 6). The *B. rapa* group represented the least diverse group with a total number of 6 haplotypes and 1–3 alleles per cpSSR locus (Table 7). Only one of the *B. napus*

Table 3 Allele sizes obtained with 40 cpSSR primer pairs applied to a test set of 8 genotypes for checking the specificity and degree of polymorphism

Primer name	Largo	Yellow Sarson 59	Tori	*B. montana*	Vasco	*B. hilarionis*	NK Beauty-10	Express 617-4	Considered
Flannery et al. 2006									
MF-1	187	187	187	195	195	198	195	195	Yes
MF-2	199	199	198	195	195	198	195	195	Yes
MF-3	309	309	309	310	310	313	310	309	Yes
MF-4	168	167	167	163	163	165	166	167	Yes
MF-6	183	183	183	183	183	183	183	183	No
MF-7	175	178	179	172	172	174	174	174	Yes
MF-8	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	Null	No
MF-9	333	332	332	333	333	333	331	332	Yes
Provan (2000)									
ATCP7905	161	161	161	161	161	161	161	161	No
ATCP28673	165	165	165	164	164	164	164	164	Yes
ATCP30287	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	2 peaks	No
ATCP46615	124	124	124	124	124	124	124	124	No
ATCP66701	Null	2 peaks	Null	2 peaks	2 peaks	2 peaks	Null	68	No
ATCP70189	146	146	146	146	146	146	146	146	No
Jakobsson et al. (2007)									
01	Null	Null	Null	Null	133	Null	Null	Null	No
07	173	173	173	173	173	172	173	173	Yes
08	279	279	279	280	279	279	278	278	Yes
11	238	238	238	238	238	238	239	239	Yes
12	301	301	301	301	301	301	301	301	No
17	Null	Null	Null	Null	Null	Null	Null	Null	No
19	303	303	303	303	303	304	304	303	Yes
21	326	326	326	327	327	326	326	326	Yes
24	205	205	205	205	205	205	205	205	No
29	310	310	310	310	310	310	310	310	No
34	310	310	310	310	310	310	310	310	No
37	390	Null	390	390	390	390	390	390	No
43	Null	335	335	335	335	Null	335	335	No
44	476	476	476	476	476	Null	476	476	No
45	Null	Null	477	477	477	Null	477	477	No

Table 3 continued

Primer name	Largo	Yellow Sarson 59	Tori	*B. montana*	Vasco	*B. hilarionis*	NK Beauty-10	Express 617-4	Considered
47	381	381	381	381	381	381	381	381	No
51	381	381	381	381	381	381	381	381	No
55	Null	Null	Null	Null	Null	Null	Null	Null	No
58	Null	322	322	322	322	322	322	322	No
60	322	322	322	322	322	322	322	322	No
Allender et al. (2007)									
Chla16	110	110	110	110	109	110	110	109	Yes
Chloro35	111	111	111	111	110	111	111	110	Yes
Chloro39	3 peaks	3 peaks	3 peaks	3 Peaks	3 peaks	3 peaks	3 peaks	3 peaks	No
ChloroP	Null	Null	Null	Null	Null	Null	Null	Null	No
ChloroO	Null	Null	Null	Null	Null	Null	Null	Null	No
ChloroQ	303	303	303	303	303	303	303	303	No

(a) Numbers represent allele sizes (bp). (b) 2 and 3 Peaks represent the presence of 2 and 3 alleles, respectively and lack of specificity of the primers. (c) Null represents no detectable allele with the primer pairs

haplotypes (AC7) was identical with a *B. oleracea* haplotype (C12). *B. carinata* as BBCC genome species was unique in its haplotype (Table 8), but showed high similarity with the haplotype of the *B. napus* cultivar Korall, carrying the *Brassica* B genome cytoplasm (AC12; Table 6).

The results from the UPGMA cluster analysis showed that at a genetic distance of 0.49 (Dice) the 49 genotypes form six major clusters (Fig. 1). Genetic similarity coefficients for the genotypes based on pairwise comparisons of cpSSR marker alleles ranged from 0.21 to 1.00. The cophenetic correlation value indicated with $r = 0.92$ a high goodness of fit. Bootstrap values ranged from 51.4 to 100 %. Cluster 1 comprised all *B. rapa* genotypes (A genome) and in addition the wild species *B. cretica* accession 6344 (C genome). *B. oleracea* genotypes were apparently more diverse and were found in clusters 2, 3 and 5. Cluster 2 did contain also the three *B. napus* genotypes Korall with Polima CMS cytoplasm, DSV-2-08-1 and resynthesized *B. napus* L239. Cluster 5 included mostly wild and cultivated *B. oleracea* genotypes but also the three resynthesized *B. napus* genotypes H48, H123-1 and H10-3. The majority of the *B. napus* genotypes were found in cluster 4. The two *B. napus* cultivars Flash and Triangle carrying the Ogura cytoplasm and the three resynthesized *B. napus* genotypes H61, S3 and S13 were also found in this group. Cluster 6 consisted of the two genotypes *B. carinata* Bra1151/90 and *B. napus* cultivar Korall with the B genome cytoplasm (bootstrap value = 99.8 %). Cluster 6 was most distantly related to the other clusters with the promising bootstrap value of 61.2 %.

Discussion

The ultimate aim of the present work was to identify a set of chloroplast microsatellite markers that could be used to unambiguously distinguish between the *B. rapa* and the *Brassica oleracea* chloroplast genomes and hence could be used as diagnostic markers to determine the cytoplasmic origin of amphidiploid *Brassica napus*. However, screening of 40 previously published microsatellite primer pairs for *Brassica* species and *Arabidopsis* chloroplast DNA showed that only 14 of them were useful to detect polymorphism in a test set of each four *Brassica oleracea* and *Brassica rapa* genotypes and two *Brassica napus* genotypes (Table 3).

Table 4 Total number of polymorphic alleles among 49 genotypes using 14 selected cpSSR primer pairs

Name	MF-1	MF-2	MF-3	MF-4	MF-7	MF-9	ATCP28673	07	08	11	19	21	Chla16	Chloro35
No. of alleles	4	6	8	7	10	3	2	4	4	3	4	5	2	2

Table 5 Haplotypes and allele sizes (bp) detected in *B. oleracea* (C genome)

cpSSR haplotype (n)	Name	MF-1	MF-2	MF-3	MF-4	MF-7	MF-9	ATCP 28673	07	08	11	19	21	Chla16	Chloro35
C1	*B. cretica*	187	199	310	167	173	333	165	172	280	238	303	327	110	111
C2	*B. incana*	187	195	311	163	171	333	165	172	280	238	303	327	109	110
C3	*B. villosa* 3821	187	196	307	164	178	332	164	171	280	238	307	323	110	111
C4	*B. v.* subsp. *bivoniana*	187	196	307	164	177	332	164	171	280	238	307	326	110	111
C5	*B. montana*	195	195	310	163	172	333	164	173	280	238	303	327	110	111
C6	*B. macrocarpa*	187	194	307	164	178	332	164	171	281	238	307	323	110	111
C7	*B. rupestris*	195	195	307	164	178	332	164	171	280	238	307	323	110	111
C8	*B. taurica*	187	195	310	163	172	332	164	172	279	238	303	327	109	110
C8	*B. bourgeaui*	187	195	310	163	172	332	164	172	279	238	303	327	109	110
C9	*B. oleracea*	187	195	309	163	172	332	164	172	279	238	303	327	109	110
C10	*B. insularis*	187	199	309	163	174	332	164	172	279	238	238	327	110	111
C11	*B. hilarionis*	198	198	313	165	174	333	164	172	279	238	304	326	110	111
C12	Reliant	195	195	310	163	173	332	164	173	279	238	303	327	109	110
C12	Super Regama	195	195	310	163	173	332	164	173	279	238	303	327	109	110
C12	*B. alboglabra*	195	195	310	163	173	332	164	173	279	238	303	327	109	110
C12	Azur	195	195	310	163	173	332	164	173	279	238	303	327	109	110
C13	Vasco	195	195	310	163	172	333	164	173	279	238	303	327	109	110
Total no. of alleles		3	5	5	4	6	2	2	3	3	1	4	3	2	2

The remaining 26 primer pairs were either monomorphic, did not give any amplification (null alleles) or they produced doubtful results by showing two or more peaks in the capillary electrophoresis. Although the test set comprised quite diverse genotypes, it is possible that those primer pairs showing no amplification or monomorphism, could detect polymorphism in the complete set of 49 genotypes. The number of alleles detected per cpSSR locus using the primer pairs MF-1 to MF-9 ranged from 3 to 10 in the present study (Table 4), whereas Flannery et al. (2006) reported a range from 5 to 11 alleles for those primer pairs. As in the study of Flannery et al. (2006) primer pair MF7 proved to be the most polymorphic one in this study (Table 4). Surprisingly, for most of the primer pairs there was no overlap in the fragment size range of the amplicons reported by Flannery et al. (2006) and found in the present study. For locus MF-6, e.g. Flannery et al. (2006) reported 5 different alleles with a fragment size of 155 to 164 bp, whereas in the present study only one monomorphic allele was found with an allele size of 183 bp (Table 3). Furthermore, the three primer pairs ChloroO, ChloroP, and ChloroQ (Table 3) designed by Allender et al. (2007) for *Brassica* species, did not show amplification in PCR or produced a monomorphic band in the present test set. However, two of the three primer pairs designed by Allender et al. (2007) for *A. thaliana* detected each two alleles per cpSSR locus among the 49 *Brassica* genotypes of the present study (Table 4), although allele size ranges did not overlap. Provan (2000) demonstrated cross-species amplification in *Brassica* species using *Arabidopsis* chloroplast microsatellite primers but no allele sizes were reported. Cross-species amplification of the chloroplast microsatellite primer pairs identified in *Arabidopsis* was not tested by Jakobsson et al. (2007).

Although not a single primer pair or a few primer pairs were useful to distinguish between the cytoplasms of *Brassica rapa*, *B. oleracea* and *B. napus*, the cluster analysis performed with the marker data from

Table 6 Haplotypes and allele sizes detected in *B. napus* (AC genome)

cpSSR haplotype (n)	Name	MF-1	MF-2	MF-3	MF-4	MF-7	MF-9	ATCP 28673	07	08	11	19	21	Chla16	Chloro35
AC1	Oase-3	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC1	Favorite-6	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC1	Charly-7	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC1	H61	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC1	S3	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC1	Korall	195	195	309	166	175	331	164	173	278	239	303	326	110	111
AC2	Triangle	195	195	309	166	175	331	164	172	278	239	303	326	110	111
AC2	Flash	195	195	309	166	175	331	164	172	278	239	303	326	110	111
AC2	ES Alienor	195	195	309	166	175	331	164	172	278	239	303	326	110	111
AC2	Krypton	195	195	309	166	175	331	164	172	278	239	303	326	110	111
AC3	Komando-5	195	195	309	166	174	331	164	173	278	239	303	325	110	111
AC3	Ha 699/91-4	195	195	309	166	174	331	164	173	278	239	303	325	110	111
AC4	H48	195	195	310	163	172	332	164	172	279	238	303	327	109	110
AC5	NK Beauty-10	195	195	310	166	174	331	164	173	278	238	304	326	110	111
AC6	Express 617-4	195	195	309	167	174	332	164	173	278	239	303	326	109	110
AC7 (C12)	H123-1	195	195	310	163	173	332	164	173	279	238	303	327	109	110
AC7 (C12)	H10-3	195	195	310	163	173	332	164	173	279	238	303	327	109	110
AC8	DSV 2-08-1	187	198	309	169	179	331	164	171	279	238	304	326	110	111
AC9	S13	187	195	309	166	175	331	164	173	278	239	303	326	110	111
AC10	L239	187	198	309	169	180	331	164	172	279	238	303	326	110	111
AC11	Korall[a]	187	198	308	169	179	332	164	172	279	238	303	326	110	111
AC12	Korall[b]	189	197	296	168	183	331	164	172	278	248	303	329	109	110
Total no. of alleles		3	3	4	5	7	2	1	3	2	3	2	4	2	2

[a] With Polima CMS cytoplasm

[b] With B genome cytoplasm

the 14 polymorphic chloroplast microsatellite markers revealed the existence of clearly separated groups. All individuals of *B. rapa* clustered together in one group (Cluster 1; Fig. 1). However, unexpectedly *B. cretica* as a member of the *B. oleracea* cytodeme clustered within the same group. The remaining *B. oleracea* genotypes were found in clusters 2, 3, and 5, thus confirming earlier reports describing *B. oleracea* as an 'incredible diverse' species (Mei et al. 2010; and references therein). Cluster 2 is linked to the *B. rapa* cluster 1 with a weak bootstrap value of less than 50 % (data not shown). Cluster 2 contains *Brassica insularis* and *B. hilarionis*, the spring oilseed rape cultivar Korall with the Polima male sterility cytoplasm, the resynthesized *B. napus* line L239 and the winter oilseed rape breeding line DSV2-08-1. The origin of the Polima cytoplasm is still unknown, but Erickson et al. (1986a) classified this cytoplasm as rap-type. According to Liu et al. (1987) the Polima cytoplasm is of Polish origin, but Erickson et al. (1986a, b) stated that it is probably derived from *B. juncea* (AABB, n = 18). And Palmer et al. (1983) and Erickson et al. (1983) indicated that *B. rapa* (AA) likely is the ancestral maternal parent of amphidiploid *B. juncea*. Resynthesized *B. napus* line L239 reportedly has a *B. oleracea* genotype as maternal parent (Table 2; Girke 2002). However, this report is doubtful, since L239 is low in erucic acid content of the seed oil (Girke 2002) and hence L239 may be semi-synthetic derived from a cross with oilseed rape.

Brassica villosa 3821, *B. macrocarpa*, *B. rupestris* and *B. villosa* subsp. *bivoniana* Mazzola et Raimondo 6581 were found in cluster 3 which is in support of Snogerup et al. (1990) who have mentioned that these species form a unique group together with *B. incana* and *B. insularis* with its origin in Sicily/Italy. However, in this study *B. incana* and *B. insularis* were separated from the Sicilian group. *Brassica incana* grouped together with *B. oleracea* subsp. *oleracea* 7695 and cultivated *B. oleracea* forms in cluster 5. The

Table 7 Haplotypes and allele sizes (bp) detected in *B. rapa* (A genome)

cpSSR haplotype (n)	Genotype	MF-1	MF-2	MF-3	MF-4	MF-7	MF-9	ATCP 28673	07	08	11	19	21	Chla16	Chloro35
A1	Rex	187	198	310	167	175	333	165	172	280	238	303	326	110	111
A1	Steinacher	187	198	310	167	175	333	165	172	280	238	303	326	110	111
A1	Salut	187	198	310	167	175	333	165	172	280	238	303	326	110	111
A1	Perko	187	198	310	167	175	333	165	172	280	238	303	326	110	111
A2	Mizuna	187	198	310	168	179	332	165	172	280	238	303	326	110	111
A3	Tori	187	198	309	167	179	332	165	173	279	238	303	326	110	111
A4	Largo	187	199	309	168	175	333	165	173	279	238	303	326	110	111
A5	Yellow Sars. 59	187	199	309	167	178	332	165	173	279	238	303	326	110	111
A6	Orbit	187	199	310	168	175	333	165	172	280	238	303	326	110	111
Total no. of alleles		1	2	2	2	3	2	1	2	2	1	1	1	1	1

Table 8 Allele sizes (bp) detected in *B. carinata* BRA 1151/90 (BC genome)

Allele name	MF-1	MF-2	MF-3	MF-4	MF-7	MF-9	ATCP28673	07	08	11	19	21	Chla16	Chloro35
Allele size	189	197	295	168	183	331	164	160	278	248	303	329	109	110

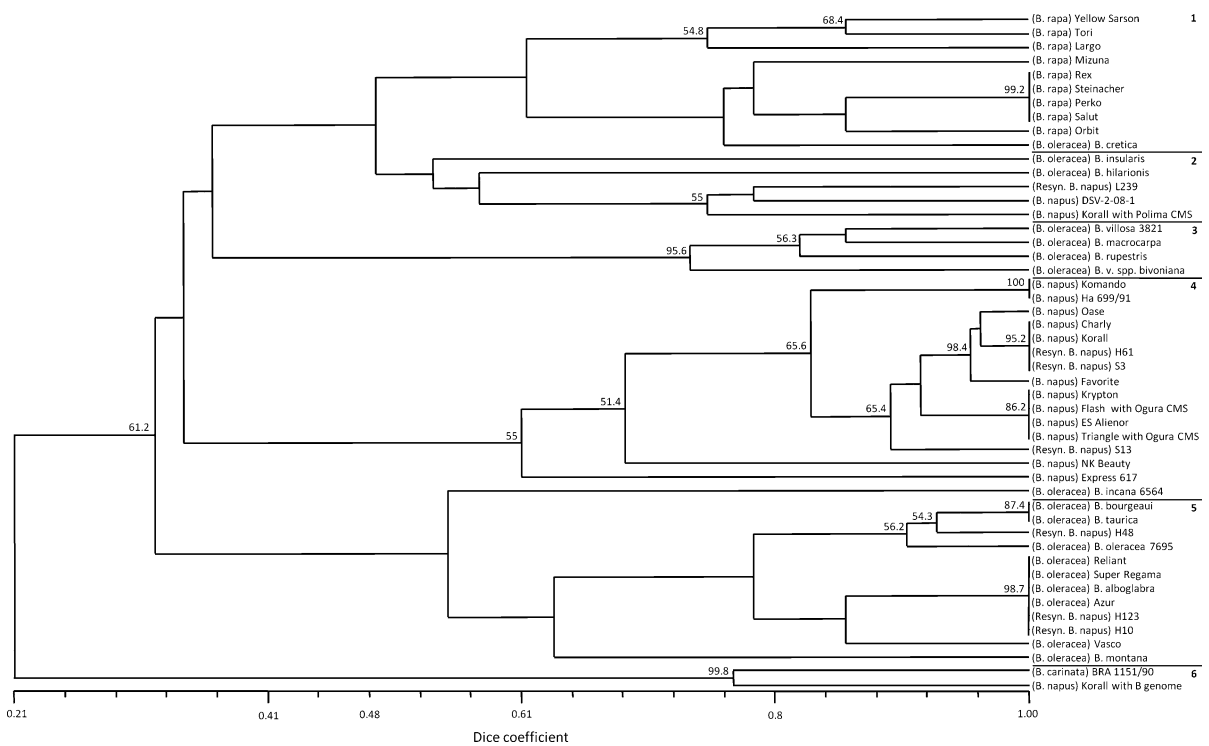

Fig. 1 UPGMA dendrogram of 49 different *Brassica* species based on cpSSR markers. Each cluster is separated with the *line* within the *gray box* at the right hand side and numbered from 1 to 6. Bootstrap values >50 % are indicated above the corresponding branch. B. r. *B. rapa*, B. n. *B. napus*, Resyn. B. n. Resynthesized *B. napus,* B. o. *B. oleracea*, CMS cytopl. Cytoplasmic Male Sterility cytoplasma

separation of *B. incana* from the Sicilian group and its close relationship to *B. oleracea* subsp. *oleracea* has also been documented by other authors (Lázaro and Aguinagalde 1998a,b; Allender et al. 2007; Mei et al. 2010). In the present study, *Brassica insularis* grouped together with *B. hilarionis* and *B. napus* genotypes in cluster 2. Reports in the literature about the relationship of *B. insularis* to the Sicilian group are ambiguous (Lannér 1998; Lázaro and Aguinagalde 1998a,b).

Most of the *B. napus* winter oilseed rape cultivars grouped together in cluster 4. Furthermore, two of the three resynthesized *B. napus* lines (S3 and S13, Table 2) with *B. rapa* as maternal parent clustered in this group. And as the two hybrid cultivars Flash and Triangle with the Ogura male sterility cytoplasm contain the chloroplasts of spring oilseed rape cultivar Brutor (Pelletier et al. 1983), which has previously been found to carry a rap-type cytoplasm (Song and Osborn 1992), it is not surprising that the two Ogura hybrids also cluster in group 4.

Cluster 5 contains many of the wild and cultivated forms of *B. oleracea*. The close relationship between *B. taurica*, *B. bourgeaui* and B. *oleracea* subsp. *oleracea* 7695 is in agreement with results of Lannér et al. (1997) and Lannér (1998). *Brassica montana* which is also found in cluster 5 has previously been considered to be an intermediate taxon between the Sicilian group and *B. oleracea* subsp. *oleracea* (Lázaro and Aguinagalde 1998b), although RFLP-cpDNA analysis has shown that *B. montana* is related to both, the *B. rapa* and the *B. oleracea* cytoplasm (Song and Osborn 1992). Interestingly, cultivated forms including *B. oleracea* Reliant (var. *capitata*, Red cabbage), Super Regama (var. *botrytis*, Cauliflower) and Azur (var. *gongylodes* L., Turnip/stem cabbage) are sharing the same haplotype (C12) together with the wild species *B. alboglabra*. The cultivated forms are also closely related with the wild *B. oleracea* subsp. *oleracea.* Song et al. (1990) pointed out that *B. alboglabra* along with the wild *B. oleracea* subsp. *oleracea* can be the ancestors of cultivated forms of *B. oleracea.* The two resynthesized *B. napus lines* H123-1 and H10-3 are also found in cluster 5. This fits well to their reported origin with *B. oleracea* being their maternal parent (Table 2).

Finally, cluster 6 is quite distantly related to all other clusters. It contains *Brassica carinata* BRA1151/90 and the spring oilseed rape cultivar Korall with the *Brassica* B genome cytoplasm. This finding is in line with early reports by Uchimiya and Wildman (1978), Erickson et al. (1983) and Palmer et al. (1983) and the recently published work of Allender and King (2010) indicating that *B. carinata* with the nuclear genome BBCC harbours the *B. nigra* (BB genome) cytoplasm.

In conclusion, the results of the present study show for a new set of *Brassica rapa*, *Brassica oleracea* and *Brassica napus* winter oilseed rape genotypes that even with a comparatively large number of chloroplast microsatellite markers, an unambiguous differentiation of the cytoplasm types is not possible. As in previous work, oilseed rape was found to form its own cluster separated from *B. oleracea* and *B. rapa* (e.g. Erickson et al. 1983; Palmer et al. 1983; Halldén et al. 1993; Flannery et al. 2006; Allender and King 2010). Allender and King (2010) concluded that multiple hybridization events including different maternal genotypes may be the reason for this. Results from the present study also show that transferability of primer pairs from different material groups is limited, because of lack of amplification (null alleles), lack of polymorphism and the occurrence of doubtful results. Furthermore, ranges of allele sizes found in the present study deviated partly from allele sizes reported in other work using the same primer pairs. This could also be indicative for multiple hybridization events and/or for an increased mutation rate of chloroplast microsatellite markers (for discussion, see Jakobsson et al. 2007; Allender and King 2010). An increased mutation rate of chloroplast microsatellite markers could perhaps explain to some extend the development of a separate *B. napus* cytoplasm within a relative short time of evolution (c.f. Introduction). Bootstrap values of lower than 50 % obtained in this study indicate that relationships may change if results from more polymorphic chloroplast markers would be included in the study. However, considering the results published so far, it is questionable if chloroplast microsatellite markers are the right choice for quickly determining the cytoplasmic origin of oilseed rape genotypes. The recently established 'DNA barcode of land plants' initiative (CBOL Plant Working Group 2009; Hollingsworth et al. 2011) might point towards an easier method to distinguish among the *Brassica* cytoplasms by sequencing conserved regions of plastidic genes, like e.g. the rbcL- and the matK gene.

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