Diversity and Evolution of *B. napus* Chloroplast Genome

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Abstract

Chloroplast genomes (cpDNAs) are a vital resource for studying plant genome diversity, origin and evolution. *B. napus*, an important oilseed crop, is a recently formed allotetraploid between *B. rapa* and *B. oleracea*. In this chapter, we explored the genetic diversity and evolutionary origin of the three types of *B. napus* cpDNA. We exclusively assembled and characterized the complete cpDNAs of nine *B. napus* accessions using Illumina whole-genome sequence data for this study. Based on the genetic diversity and phylogenetic analysis of three cytotypes with its progenitor species, we provide a possible explanation for the origin of the most common nap-type cpDNA in *B. napus* genome. Overall, this study discusses the diversity, evolution and origin of the *B. napus* chloroplast genome and also provides new resources for *Brassica* breeding and evolutionary studies.

10.1 Introduction

The cpDNAs are cytoplasmic genomes, which are conservatively inherited uniparentally mostly via maternal inheritance and play various roles other than photosynthesis. For example, biochemical processes such as fatty acid synthesis, nitrogen metabolism and immune response in plants are associated with cpDNA function (Mullet 1988; Birky 1995; Jansen and Ruhlman 2012). The cpDNA is a circular genome with a size about 59–218 kb and contains a typical quadripartite structure, with a pair of inverted repeats (IRs) flanked by large and small single-copy regions (Chumley et al. 2006; Jansen and Ruhlman 2012; Delannoy et al. 2011). The IRs play important role in intermolecular homologous recombination in order to produce isomeric structure of cpDNA (Palmer et al. 1983). Highly conserved nature of the cpDNA makes them vital tool in studying genetic and

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genome diversity and phylogenetic and systematic evolutionary analyses (Shu et al. 2015). Development of cpDNA-based markers for species authentication and barcoding has been comparatively easier than the nuclear genome (Nock et al. 2011; Kim et al. 2013). Due to the mostly maternal inheritance, the cpDNA has high advantage in tracking down the parental origin or parentage in interspecific hybrid (Allender and King 2010).

B. napus (AACC, $2n = 4x = 38$) belongs to genus *Brassica* and is an economically important oilseed crop yielding food, biofuels, and lubricants (Bonnema 2011). It is an allotetraploid plant with recent evolutionary ($\sim$7500 years) and domestication history (<500 years) (Röbbelen et al. 1989). As a natural allopolyploid, *B. napus* originated from hybridization between two diploid species, *B. rapa* (AA, $2n = 2x = 20$) and *B. oleracea* (CC, $2n = 2x = 18$) (Parkin et al. 1995). Depending on the artificial or natural cross direction, *B. napus* cytoplasm may be derived from either of its progenitors. A recent study about exploration of *B. napus* and its progenitor genome exposes the high-level genome rearrangement caused by non-homeologous exchanges between the parental sub-genome in *B. napus* (Cheung et al. 2009; Chalhoub et al. 2014). However, due to extensive homeologous recombination, high-level rearrangements were observed which hinder control of chromosome pairing that leads to unstable hybrid formation (Chang et al. 2011; Leflon et al. 2006; Chalhoub et al. 2014; Sharpe et al. 1995). Unlike *B. napus*, *B. juncea* (AABB, $2n = 4x = 36$), which is also an important allotetraploid from the genus *Brassica*, has remained considerably unchanged since its polyploidization from progenitor species, *B. rapa* (AA, $2n = 2x = 20$) and *B. nigra* (BB, $2n = 2x = 16$). The genetic map developed from the natural and synthetic parents of *B. juncea* exhibited disomic inheritance and comparison of its A and B subgenomes revealed collinearity with their respective progenitor diploid genomes (Axelsson et al. 2000).

Primary results based on the *B. napus* cpDNAs suggest that *B. napus* has three kinds of cytotypes which may have derived from *B. oleracea* (ole-type), *B. rapa* (rap-type) and its own (nap-type) (Hu et al. 2011; Allender and King 2010; Qiao et al. 2015). Various studies based on partial or complete cpDNA of *B. napus* could not bring a clear conclusion about the maternal origin of the *B. napus* cpDNA (Mei et al. 2011; Song and Osborn 1992; Qiao et al. 2015). To date, numerous controversies have arisen from attempts to decipher the molecular mechanisms underlying the origin and evolution of the *B. napus* genome (Zamani-Nour et al. 2013). In this investigation, we explore the diversity and evolutionary origin of the *B. napus* cpDNA genome based on complete chloroplast genome sequence of 11 *B. napus* accessions (Table 10.1).

10.2 Chloroplast Genome Assembly and Characterization of Nine *B. napus* Accessions

Advancement of next-generation sequencing technology (NGS) has offered remarkable advantage in understanding the genomes. Low-coverage (1× haploid equivalent) whole-genome shotgun (WGS) sequences from the nine *B. napus* accessions were used to assemble complete and error-free chloroplast genome by dnaLCW method (Kim et al. 2015b) (Table 10.1; Fig. 10.1). All nine accessions used in this study are inbred lines. It is important to note that the accessions M083 (Bn-2) and H165 (Bn-7) are derived from multi-parental and synthetic origin, respectively. Accession Bn-2 is a Asian semi-winter type oilseed rape, which was derived from double haploid (DH) line of various multiple crossing with inbred lines (Liu et al. 2005). Likewise, accession Bn-7 was obtained by embryo rescue and chromosome doubling of an interspecific haploid from the cross between *B. oleracea* ssp. *capitata* var. *sabauda* and *B. rapa* ssp. *chinensis* (Jesske et al. 2013).

Each cpDNA of the nine assembled *B. napus* consists of a typical quadripartite structure with size range from 152,833 to 153,502 bp. Unlike cpDNA, mitochondrial genome (mtDNA), another organelle genome, exhibited high

Table 10.1 Summary statistics of *B. napus* accessions and chloroplast genome assembly

ID	Chloroplast genome						NGS read summary		Accession name	Genotype description	Genbank No
	Size (bp)	LSC (bp)	SSC (bp)	IR (bp)	Mean coverage (*x*)	GC%	Amounts (Mbp)	Genome coverage (*x*)			
Bn-1	153,453	83,227	17,794	26,216	456	36.32	2,126.6	1.9	Zhongsuang11	Asian winter type	KU324625
Bn-2	153,502	83,301	17,775	26,213	1,287	36.35	1,273.8	1.1	M083	Asian semi-winter type	KU324626
Bn-3	153,453	83,227	17,794	26,216	260	36.36	13,900.7	12.3	Aburamasari	South asian origin	KU324627
Bn-4	153,452	83,226	17,794	26,216	264	36.35	8,679.2	7.7	Avisol	European winter type	KU324628
Bn-5	153,472	83,247	17,793	26,216	196	36.35	6,029.7	5.3	Darmor-Bzh	European winter type	KU324629
Bn-6	153,471	83,225	17,814	26,216	1,949	36.35	13,323.1	11.8	Grüner Schnittkohl	Siberian Kale type	KU324630
Bn-7	153,368	83,137	17,837	26,197	2,205	36.35	15,928.4	14.1	H165	Resynthesized oilseed	KU324631
Bn-8	153,452	83,227	17,793	26,216	2,255	36.36	14,923.5	13.2	Swede Sensation NZ	Rutabaga type	KU324632
Bn-9	153,450	83,228	17,790	26,216	633	36.35	14,105.3	12.5	Yudal	South asian origin	KU324633
Bn-NCBI	152,833	83,003	17,760	26,035		36.35			ZY036	Asian semi-winter type	GQ861354
BnCp-1 (pan)	152,831	83,002	17,759	26,035		36.32			Pangenome		Qiao et al. (2015)

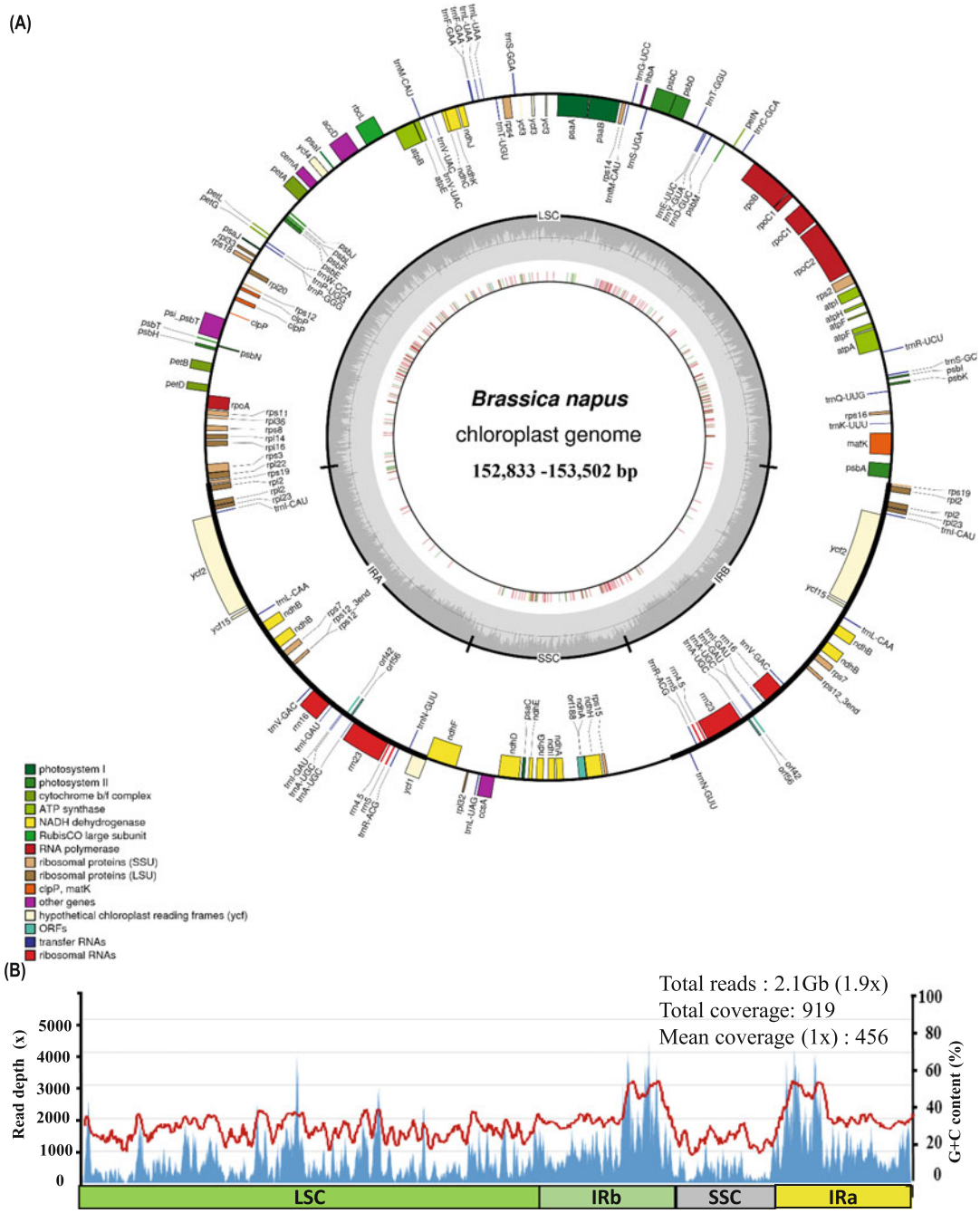

Fig. 10.1 Chloroplast genome structure of *Brassica napus*. **a** Gene map of *B. napus* chloroplast genome sequence was created using OGDRAW (Lohse et al. 2013). Genes transcribed clockwise and counterclockwise are indicated on the outside and inside of the large circle, respectively. Genes associated with different functional groups are color coded. Four parts of chloroplast genome and GC content are indicated on the middle circle. The innermost circle represents the sequence variation as SNP (red bar), INDEL (green bar) and copy number variation (blue bar). **b** Estimation of coverage of chloroplast genome by mapping of raw reads on Bn-1 cpDNA with GC distribution (red graph)

diversity and evolution in *Brassica.* Around 140 kb (219,747–360,271 bp) size variation was observed among the mtDNAs of *B. rapa* and *B. oleracea* and allopolyploids (Yang et al. 2015; Chang et al. 2011), suggesting that cpDNAs are vastly more conserved structure than mitochondrial genomes. The genome annotation based on DOGMA tool and manual curation has revealed 113 individual genes including 74 protein-coding genes, 30 tRNA, 4 rRNA and five open reading frames, which is similar to the reported *B. napus* (Bn-NCBI) cpDNA (Hu et al. 2011; Wyman et al. 2004). The overall GC content is 36.3%, which is parallel to its close relatives such as *B. rapa* (36.3), *B. oleracea* (36.3), *B. nigra* (36.3), *Raphanus sativus* (36.3) and *A. thaliana* (36.2). We also observed the differences in terms of copy numbers; the mean cpDNA coverage for a haploid genome was found to have 11-fold variation (196–2255 copies) based on clc_reference mapping approach. The newly developed cpDNAs of the nine accessions with complete annotation can be accessed from the Genbank with accession numbers listed in Table 10.1.

10.3 Diversity and Phylogenetic Relationship of *B. napus* cpDNA

Though the cpDNAs are considered to be evolving slowly and are generally highly conserved, considerable variations were observed in the coding and non-coding regions especially in rapidly evolving regions such as intergenic spacers and intronic regions (Zeng et al. 2012). cpDNA markers derived from disparity sites were widely accepted for numerous applications including genetic differentiation, cytoplasmic diversity, molecular barcoding, monitoring transgene introgression and population and phylogenetic studies (Flannery et al. 2006; Woo et al. 2013; Shu et al. 2015; Kundu et al. 2013; Wang et al. 2012). Owing to artificial breeding programs and intentional introgression, increases in genetic diversity in *B. napus* have been achieved. In addition, exploring the allelic variation responsible for the genetic changes will provide a way for crop enhancement and dissecting complex agronomic traits (Qian et al. 2006; Song et al. 1994; Wang et al. 2014; Szadkowski et al. 2010). However, using a limited set of cpDNA markers may cause inaccurate results which raise concerns on drawing the right conclusion (Allender et al. 2007; Allender and King 2010; Flannery et al. 2006).

We have obtained complete chloroplast genome sequences of nine *B. napus* accessions which includes the three types of cpDNAs such as, rap-type, ole-type and nap-type based on homology with cp genomes from *B. rapa*, *B. oleracea* and *B. napus*–unique, respectively (Qiao et al. 2015; Allender and King 2010) (Table 10.1). Genome-wide cpDNA nucleotide similarity search for 13 *Brassica* accessions including 11 *B. napus* including two previously reported *B. napus* cpDNA (Bn-NCBI and BnCp-1) and its progenitors (*B. rapa* and *B. oleracea*) has revealed high homology within *B. napus* (98.9–100%) (Table 10.2). Despite the conserved gene content and gene order in those of 11 accessions, more than 450 genetic variations were observed including 332 single nucleotide polymorphisms (SNPs), 118 insertions and deletions (INDELs) and 4 copy number variation (CNVs) (Fig. 10.1a). The differential nucleotide count analysis showed 0–1554 and 7–1549 variations sites as intra- and interspecies diversity, respectively (Table 10.2).

B. napus cpDNAs were highly diverged with *B. oleracea* and *B. rapa*. Alignment by mVISTA tool showed high genome conservation in the genic regions than intronic and intergenic spacer regions. Similar to other angiosperms, the non-coding regions of the cpDNA show high sequence divergence than the coding regions (Li et al. 2015). Out of 113 genes, *rpoC1*, *rpob*, *rbs12*, *psbB*, *rpL16* and *ycf1* are potential hotspot regions for development of barcoding markers in the 11 *B. napus* accessions (Hollingsworth et al. 2011; Kim et al. 2015a). Among the 11 *B. napus* accessions, Bn-2, (multi inter-crossed synthetic *B. napus*) and Bn-7 (resynthesized origin: synthetic *B. napus*) are highly diverged with other *B. napus* cpDNA (Fig. 10.2). High amount of genetic differentiation compared with the

Table 10.2 Inter- and intra-species similarity and diversity of *B. napus* chloroplast genome with its progenitors

Similarity (%)/variation sites (n)[a]	Bn-NCBI	Bn-1	Bn-2	Bn-3	Bn-4	Bn-5	Bn-6	Bn-7	Bn-8	Bn-9	BnCp-1 (pan)	Bo-NCBI	Br-NCBI
Bn-NCBI	ID	99.5	99	99.5	99.5	99.5	99.5	98.9	99.5	99.5	99.9	98.9	99.1
Bn-1	680	ID	99.4	100	99.9	99.9	99.9	99.3	99.9	99.9	99.5	99.3	99.4
Bn-2	1,402	811	ID	99.4	99.4	99.4	99.4	99.3	99.4	99.4	99	99.3	99.9
Bn-3	680	0	811	ID	99.9	99.9	99.9	99.3	99.9	99.9	99.5	99.3	99.4
Bn-4	675	7	816	7	ID	99.9	99.9	99.3	99.9	99.9	99.5	99.3	99.4
Bn-5	696	28	835	28	23	ID	99.9	99.3	99.9	99.9	99.5	99.3	99.4
Bn-6	698	30	839	30	27	48	ID	99.3	99.9	99.9	99.5	99.3	99.4
Bn-7	1,554	958	955	958	952	973	978	ID	99.3	99.3	99	99.9	99.4
Bn-8	681	1	812	1	8	29	31	959	ID	99.9	99.5	99.3	99.4
Bn-9	686	6	817	6	13	34	36	964	5	ID	99.5	99.3	99.4
BnCp-1 (pan)	37	644	1,392	644	651	672	672	1,533	645	650	ID	99	99.1
Bo-NCBI	1,549	953	936	953	947	968	971	7	954	959	1,528	ID	99.4
Br-NCBI	1,381	790	39	790	795	814	818	913	791	796	1,371	910	ID

[a]Upper and lower diagonal shows the percentage of similarities and number of sequence variations among the chloroplast genomes analyzed

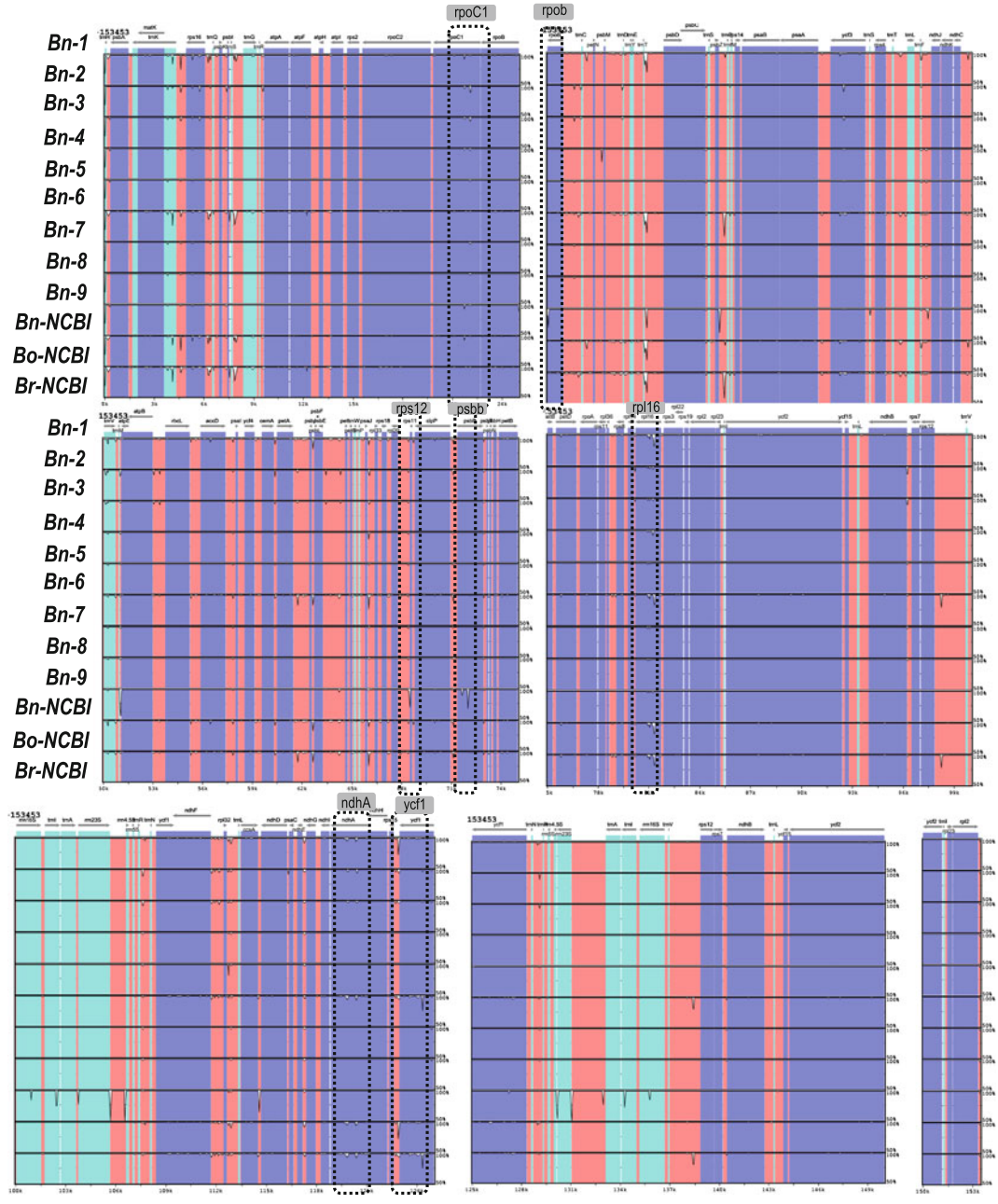

Fig. 10.2 Sequence comparison and visualization of 10 *B. napus* cpDNA with its progenitor genomes. Complete cpDNA sequence-based identity plot was developed by mVISTA. Genome regions are color coded; blue block, conserved gene; sky-blue block, tRNA and rRNA; red block, intergenic region. Prominent genic regions for molecular validation were marked as dotted box

recently developed accession (Bn-2 and Bn-7) suggest that the established accessions have undergone rapid evolutionary changes (Fig. 10.2). Furthermore, understanding the diversity in the gene pool will help for breeding improvement and hybrid formation.

The cpDNA provides high-resolution data, thus widely accepted for population and

phylogenetic analyses (Bailey et al. 2006; Panda et al. 2003; Wang et al. 2005). The cpDNA-based phylogenetic analysis has revealed better understanding of evolution and domestication in wild and cultivated rice species (Kim et al. 2015b). Moreover, using this approach, species with less or moderate differentiation can be distinctively classified. To date, partial cpDNA sequences of *B. napus* provided an ambiguous conclusion for the diversity of *B. napus* (Allender and King 2010; Qiao et al. 2015). Here, we have generated a phylogenetic relationship based on the complete cpDNA sequences of 23 *Brassica* accessions including several morphotypes from *B. rapa* (Br1-5) and *B. oleracea* (Bo1-5) (Fig. 10.3). Comparative phylogenetic analysis of complete cpDNA of 11 *B. napus* accessions with five *B. rapa* (Br1-5) and five *B. oleracea* (Bo1-5) categorized the taxa into three clades and clearly distinguishes each species and subspecies. Among the 11 *B. napus* accessions, nine accessions were grouped into a unique clade which follows the nap-type and the remaining two Bn-2 and Bn-7 were associated with *B. oleracea* (ole-type) and *B. rapa* (rap-type), respectively. This suggests that the nap-type is the major type of cpDNA in the *B. napus* genome which also corresponds with previous findings (Qiao et al. 2015; Allender and King 2010) cytotypes. Hence, our analysis also supports that the nap-type cytoplasm is a major type in *B. napus*.

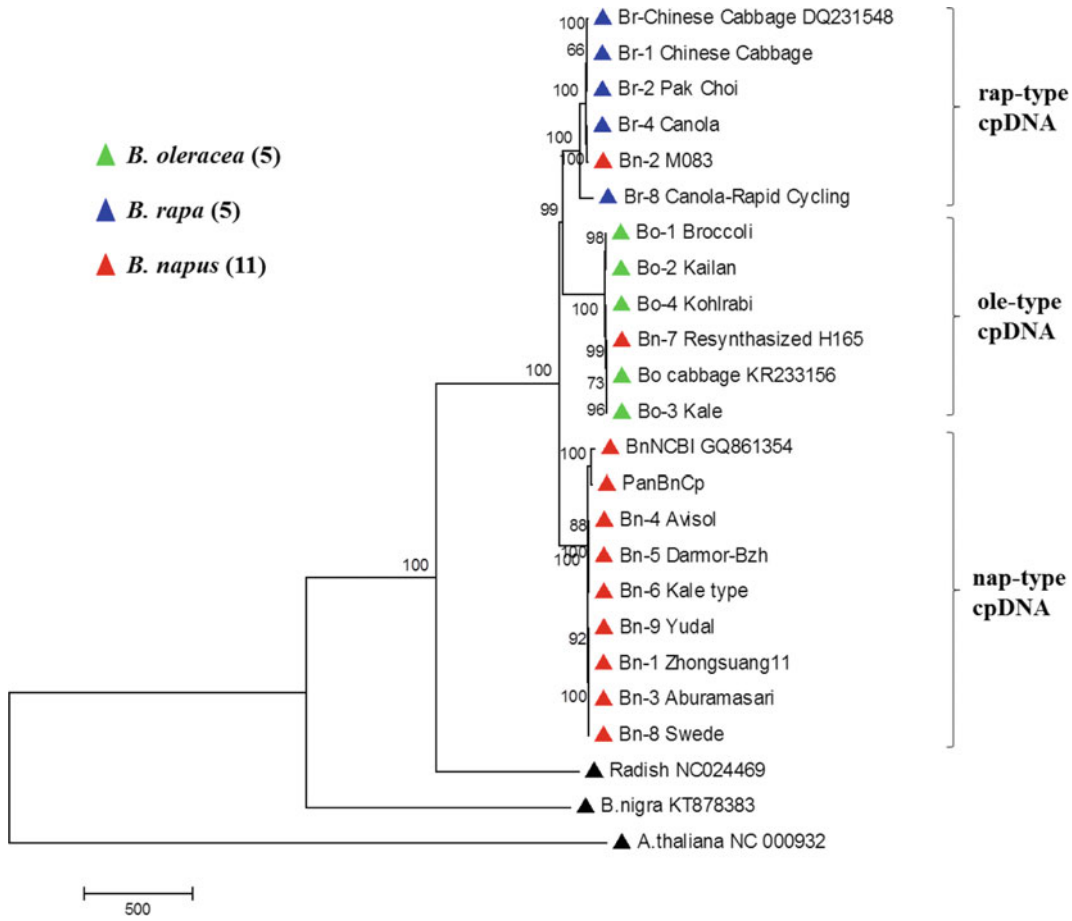

Fig. 10.3 Phylogenetic analysis based on complete chloroplast genome sequences of *B. napus* accessions and its relative species. Complete cpDNA of 11 *B. napus*, five *B. rapa* (Br1-5), five *B. oleracea* (Bo1-5), *R. sativus*, *B. nigra* and *A. thaliana* used to develop neighbor-joining tree with 1000 bootstrap replications by MEGA6

10.4 Origin and Evolution of the *B. napus* Chloroplast Genome

Polyploidization is a major evolutionary force in the evolution of *Brassica* species. *Brassica* diploids, *B. rapa* (AA), *B. nigra* (BB), *B. oleracea* (CC) evolved from a common hexaploid ancestor (Sharma et al. 2014) around 13 million years ago (mya) (Gupta 2013; Yang et al. 2006). Two distinct lineage of *Brassica* diploids (rapa/oleracea and nigra lineage) that have formed around 9–13 mya with hexaploid ancestor were clearly explained by plastid genome analysis (Sharma et al. 2014; Kaur et al. 2014). The allotetraploid *B. napus* (AC) was formed quite recently around 7500 years ago by hybridization and polyploidization of the diploid progenitor *B. rapa* and *B. oleracea* (Chalhoub et al. 2014). The cpDNA is one of the important tools in identifying parental origins and in clearly elucidating the origin for many species including rice, wheat and apple (Zou et al. 2015; Haider 2012; Nikiforova et al. 2013). Lack of wild relatives and various cytogenetic and genomic studies on *B. napus* support its polyphyletic origin (Warwick et al. 2003).

Unlike other two tetraploids, *B. juncea* (AB) and *B. carinata* (BC), *B. napus* chloroplast did not follow with either of the parental genomes (A or C genome) (Li et al. 2017). Studies have been performed to clarify the genetic relationships of the major diploid and tetraploid *Brassica* species, but the origin of the chloroplast in the AC genome is still unclear (Qiao et al. 2015; Allender and King 2010). Furthermore, the cpDNA of the *B. napus* has unique origin (nap-type) and its genetic relationship with its diploid ancestors remains controversial (Qiao et al. 2015). cpDNA analysis based on *rpo* regions of 488 *B. napus* accessions revealed that more than 92% were associated with nap-type and differentiated with their ancestor (Qiao et al. 2015). Identification of exact origin of the *B. napus* will help to understand genome for stable hybrid formation, overcome self-incompatibility and creation of fertile plants required for crop improvement.

B. napus has three cytotypes including two diploid progenitors type and nap-type, which is also well supported by previous analysis (Qiao et al. 2015; Allender and King 2010). Since the origin of the Bn-2 (multi inter-crossed origin) and Bn-7 (synthetic origin with *B. rapa* as a maternal parent) were obvious, it is possible that the cytoplasm of the Bn-2 and Bn-7 could be grouped into *B. oleracea* and *B. rapa* genotypes, respectively. In addition, because the *B. napus* genome has high sexual compatibility with close relatives such as *B. rapa*, *R. sativus* and *Sinapis alba*, it is possible that *B. napus* cytoplasm may have derived from close relatives by natural or artificial crossing (Wang et al. 2005; Warwick et al. 2003). For example, Polima and Ogura cytoplasmic male sterility (CMS) lines achieved through introgression of cytotypes derived from polish winter oilseed rape and radish (Witt et al. 1991; Pellan-Delourme and Renard 1988). However, we could not identify any off types or CMS types among the 11 accessions since all *B. napus* accessions have clearly grouped into three cytotypes. Recently, cpDNA analysis of more diverse *B. rapa* genotypes revealed two types of chloroplast genomes rapa-type1 (= rap-type) and rapa-type2 (= nap-type). Though the rap-type chloroplast genome is found to be common to *B. rapa*, rapa-type2 is unique for some Italian Broccoletto genotypes of *B. rapa* (Li et al. 2017). Further analysis indicated that the Italian Broccoletto genotype is expected to be the donor for the nap-type chloroplast genome of *B. napus*. Moreover, nap-type chloroplast genome was maintained in the Italian Broccoletto genotype by geographical isolation or maternal dominance since its divergence (4.7 mya), and the Italian Broccoletto genome was utilized as the maternal parent to generate the AC genome 7500 years ago (Li et al. 2017).

10.5 Conclusion and Perspectives

Chloroplast genome has been applied to decode the plant evolution and systematics (Jansen and Ruhlman 2012). Studies on *B. napus* chloroplast genome has revealed three cytotypes in which

the nap-type (>92%) was of unknown origin and discrete to both parental progenitors, *B. rapa* and *B. oleracea* (Qiao et al. 2015). Artificial *B. napus* lines which were developed by interspecies hybridization has widened its genetic diversity which helps increase its environmental adaptability, improved production and quality (Qian et al. 2006). However, genetic factors such as self-incompatibility, unbalanced gametes and environmental causes such as biotic and abiotic stress hinder further improvement of *B. napus* (Leflon et al. 2006; Cifuentes et al. 2010). Identification of the original parents does not only clarify the evolutionary history but also enables the closer investigation of chromosome pairing mechanisms to produce stable artificial *B. napus* hybrids. Furthermore, agronomically important elite alleles that are present in the progenitors will help to improve the crop management and production.

Using the reconstructed chloroplast genome sequences of various *B. napus* accessions, we investigated the genetic diversity and evolution. The comparative genomics studies revealed that cpDNAs were well diversified among the *B. napus* and with its progenitors. Inter- and intra-cytotype variations including the recently developed synthetic *B. napus* will serve as important resources for *Brassica* breeding and evolutionary analysis. Phylogenetic analysis revealed that *B. napus* carry three kinds of cytotypes, rap-type, ole-type and nap-type, and comparative analysis with its progenitors revealed that the Italian Broccoletto genotype is the possible source for the origin of nap-type cp genome. Our study provides further evidence to clarify the phylogenetic origin and evolution of the three cytotypes of *B. napus* chloroplast genome. However, it is still not clear how the rapa-type2/nap-type chloroplast genome became the common maternal parent for most (92%) of AC genomes, although the Italian Broccoletto genotype is not prevalent in the A genome.

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