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Diversity Array Technology Markers: Genetic Diversity Analyses and Linkage Map Construction in Rapeseed (*Brassica napus* L.)

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Abstract

We developed Diversity Array Technology (DArT) markers for application in genetic studies of *Brassica napus* and other *Brassica* species with A or C genomes. Genomic representation from 107 diverse genotypes of *B. napus* L. var. *oleifera* (rapeseed, AACC genomes) and *B. rapa* (AA genome) was used to develop a DArT array comprising 11 520 clones generated using *Pst*I/*Ban*II and *Pst*I/*Bst*N1 complexity reduction methods. In total, 1547 polymorphic DArT markers of high technical quality were identified and used to assess molecular diversity among 89 accessions of *B. napus*, *B. rapa*, *B. juncea*, and *B. carinata* collected from different parts of the world. Hierarchical cluster and principal component analyses based on genetic distance matrices identified distinct populations clustering mainly according to their origin/pedigrees. DArT markers were also mapped in a new doubled haploid population comprising 131 lines from a cross between spring rapeseed lines 'Lynx-037DH' and 'Monty-028DH'. Linkage groups were assigned on the basis of previously mapped simple sequence repeat (SSRs), intron polymorphism (IP), and gene-based markers. The map consisted of 437 DArT, 135 SSR, 6 IP, and 6 gene-based markers and spanned 2288 cM. Our results demonstrate that DArT markers are suitable for genetic diversity analysis and linkage map construction in rapeseed.

Key words: Diversity Array Technology; genetic diversity; genetic linkage mapping; *Brassica* species; rapeseed

1. Introduction

The Brassiceae tribe consists some of the world's most important oilseed and vegetable crops, such as *Brassica napus* L., (rapeseed, genomes = AACC, $2n = 4x = 38$), *Brassica rapa* L. (Indian mustard, genome = AA, $2n = 2x = 20$), *Brassica juncea* L. (genomes = AABB, $2n = 4x = 36$), and *Brassica oleracea* L. (cauliflower, broccoli, Brussels sprout, cabbage, and kale, genome = CC, $2n = 2x = 18$). *Brassica napus* originated as a result from spontaneous hybridization between *B. rapa* and *B. oleracea*[1] and is believed to be originated in the Mediterranean region of south-western Europe where native *B. rapa* and *B. oleracea* overlap,[2] although no wild populations exist. Today, rapeseed is one of the leading sources of

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vegetable oil, oil-meal, fodder, and serves as the raw material for a broad range of industrial products including bio-fuel, especially in European countries.

Various molecular marker system based upon restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA, amplified fragment length polymorphism (AFLP®), simple sequence repeats (SSRs), sequence related amplified polymorphisms, and single nucleotide polymorphism (SNP) have been developed[3–7] and further applied in rapeseed genetics and breeding research.[2,8–15] Most of these markers are assayed on low-throughput agarose or polyacrylamide gel system, although markers based upon SSR and AFLP can be assayed on highly parallel genotyping platforms such as capillary electrophoresis systems, yet they are expensive to assay per data point.[16]

Rapeseed breeding programmes require an efficient, cost-efficient and reproducible marker platform that is amenable for whole genomic analysis especially for pedigree and association analysis,[17,18] mapping-as-you-go,[19] large-scale molecular evaluation of germplasm collections and for genome-wide selection of desirable alleles.[20] Current polymerase chain reaction (PCR)-based marker technologies are not practicable in terms of consumable and labour costs for such applications.

DNA hybridization-based technologies such as some SNP technologies and Diversity Arrays Technology (DArT) are suitable for such applications. SNP markers are recognized as 'markers of choice' due to their abundance and distribution in the genomes and the ability to screen populations at relatively low cost.[21] In the recent years, a large number of SNP markers has been identified in *B. napus* and related species.[22,23] However, the identification of SNPs and validation in the relevant germplasm especially those identified by sequencing candidate genes remains costly to implement for the routine marker-assisted selection, as it requires high-quality sequence information. This limits the broad application of SNP markers for rapeseed improvement using these strategies. Many genes of agronomic importance such as flowering time genes; *Flowering Locus C* (*FLC*) and *Flowering Locus T* (*FT*) occur in multiple copies in *Brassica* genomes.[24,25] The presence of multiple copies of genes in amphidiploid rapeseed poses a great challenge to develop allele-specific SNP markers. To date, only a few genome-specific SNP markers are currently available for marker-assisted selection although the ability to resolve allelic variation in members of gene families has been demonstrated.[26,27] In addition, a large number of SSR and SNP markers have been developed in private consortia and are not readily accessible to the rapeseed breeding programmes. The development of 'genotyping by sequencing' and multiplex sequencing strategies using next generation DNA-sequencing technologies offer to generate massive amount of genetic data for various applications.[28,29] However, these technologies are currently in the development stage and bioinformatics tools to handle such massive data in polyploid *Brassica* species are still being developed.[30] These limitations currently restrict the capacity of breeding programmes to routinely conduct genome-wide marker surveys.

DArT performs well in many polyploid species and does not require any DNA-sequence information. DArT marker analysis is a sequence-independent microarray-based genotyping platform, and enables high multiplexing; simultaneous typing of several hundred to several thousands of polymorphic loci spread over the genome.[31,32] DArT polymorphisms results from nucleotide polymorphisms within restriction enzyme (RE) recognition sites and indels, and the high fidelity of the RE can provide better reproducibility compared with PCR-based assays that are based on lower fidelity selective primer annealing. DArT markers have been developed in more than 40 plant species including in wheat, durum, barley, oats, lupin (www.diversityarray.com) and have been employed extensively for construction of molecular maps,[33–36] identifying trait-marker associations,[37–40] assessment of genetic diversity,[34,41,42] association mapping,[43] and routine genotyping in various crops for varietal identification.

In this study, we report on (i) the development of a robust, cost-effective, and high-throughput DArT marker platform for rapeseed, and (ii) demonstrate the usefulness of DArT markers in the assessment of genetic diversity and linkage mapping.

2. Materials and methods

2.1. Plant material

Eighty-nine accessions of *B. napus*, *B. juncea*, *B. rapa* and *B. carinata* comprising contemporary rapeseed cultivars and elite lines from Australian breeding programmes were chosen for molecular diversity analyses along with accessions from China, Germany, Japan, India, France, and Ethiopia (Table 1). Seed samples were procured from the Australian National Brassica Germplasm Improvement Programs (Wagga Wagga and Horsham) and Australian Temperate Field Crops Collection, Horsham.

DArT markers were tested for their performance in linkage mapping using a new *B. napus* doubled haploid (DH) mapping population named 'BnaLMDH'.[44] The founding parents of this population were 'Lynx-037DH' and 'Monty-028DH'; these DH lines were derived from the open-pollinated spring

Table 1. List of genotypes, their country of origin, and species used for DArT analysis

Genotype ID	Country of origin	Species	Breeding programme[a]	Genetic status
03-p74-6	China	*B. napus*	Unknown	Breeding line
04-p34	China	*B. napus*	Unknown	Breeding line
44C73	Australia	*B. napus*	Pioneer	Cultivar
44Y06[a]	Australia	*B. napus*	Pioneer	Cultivar
45C05	Australia	*B. napus*	Pioneer	Cultivar
45C75[a]	Australia	*B. napus*	Pioneer	Cultivar
45Y77[a]	Australia	*B. napus*	Pioneer	Cultivar
46C04	Australia	*B. napus*	Pioneer	Cultivar
46C76[a]	Australia	*B. napus*	Pioneer	Cultivar
46Y78	Australia	*B. napus*	Pioneer	Cultivar
A-19890[a]	Unknown	*B. napus*	USDA	Breeding line
Ag-Comet	Australia	*B. napus*	Ag-Seed	Cultivar
Ag-Emblem	Australia	*B.napus*	Ag-Seed	Cultivar
Ag-Muster[a]	Australia	*B. napus*	Ag-Seed	Cultivar
Ag-Outback[a]	Australia	*B. napus*	Ag-Seed	Cultivar
Ag-Spectrum[a]	Australia	*B.napus*	Ag-Seed	Cultivar
ATC93184-1[a]	Unknown	*B. carinata*	USDA	Breeding line
ATC94044-1[a]	Ethiopia	*B. carinata*	USDA	Breeding line
ATR409	Australia	*B. napus*	Ag-Seed	Cultivar
ATR-Barra	Australia	*B. napus*	Ag-Seed	Cultivar
ATR-Beacon	Australia	*B.napus*	AgVic	Cultivar
ATR-Cobbler	Australia	*B.napus*	Nugrain	Cultivar
ATR-Hyden	Australia	*B. napus*	Ag-Seed	Cultivar
ATR-Marlin	Australia	*B. napus*	Ag-Seed	Cultivar
ATR-Signal	Australia	*B. napus*	Nugrain	Cultivar
ATR-Stubby	Australia	*B. napus*	Ag-Seed	Cultivar
ATR-Summitt	Australia	*B. napus*	Ag-Seed	Cultivar
AV-Jade	Australia	*B. napus*	Ag-Seed	Cultivar
AV-Opal	Australia	*B. napus*	Ag-Seed	Cultivar
AV-Ruby	Australia	*B. napus*	Ag-Seed	Cultivar
AV-Sapphire	Australia	*B. napus*	Ag-Seed	Cultivar
Barossa[a]	Australia	*B. napus*	NSWA	Cultivar
BravoTT	Australia	*B. napus*	Nuseed	Cultivar
Carousel-10[a,b]	Europe	*B. napus*	Unknown	Selection
Charlton	Australia	*B. napus*	AgVic	Cultivar
Colt[a]	Unknown	*B. rapa*	Unknown	Cultivar
Drakkar	France	*B. napus*	INRA	Cultivar
Dunkeld	Australia	*B. napus*	AgVic	Cultivar
Eureka[a]	Australia	*B.napus*	NSWA	Cultivar
Expander[b]	Germany	*B. napus*	BGRC	Cultivar
Fan023	China	*B. napus*	Unknown	Breeding line
Fan028	China	*B. napus*	Unknown	Breeding line
Fan168	China	*B. napus*	Unknown	Breeding line
FlindersTTC	Australia	*B. napus*	Ag-Seed	Cultivar
Georgie	Australia	*B. napus*	NSWDPI	Cultivar
Grouse	Australia	*B. napus*	NSWA	Cultivar

Continued

Table 1. Continued

Genotype ID	Country of origin	Species	Breeding programme[a]	Genetic status
HurricaneTT	Australia	*B. napus*	Pacific Seeds	Cultivar
Hyola50	Australia	*B. napus*	Pacific Seeds	Cultivar
Hyola60	Australia	*B. napus*	Pacific Seeds	Cultivar
Hyola61	Australia	*B. napus*	Pacific Seeds	Cultivar
Iwao-natane[a,b]	Japan	*B. napus*	Unknown	Cultivar
Karoo	Australia	*B. napus*	AgVic	Cultivar
Lantern	Australia	*B. napus*	NSWDPI	Cultivar
Lisora[b]	Germany	*B. napus*	DSV	Cultivar
Major[a,b]	France	*B. napus*	Unknown	Cultivar
Maluka[a]	Australia	*B. napus*	NSWA	Cultivar
Marnoo	Australia	*B. napus*	AgVic	Cultivar
Monty	Australia	*B. napus*	NSWA	Cultivar
Mutu98-1[a,b]	Japan	*B. napus*	NSWA	Breeding line
Mystic	Australia	*B. napus*	AgVic	Cultivar
Nindoo	Australia	*B. napus*	AgVic	Cultivar
Norin22[a,b]	Japan	*B. napus*	Unknown	Cultivar
OasisCL[a]	Australia	*B. juncea*	AgVic	Cultivar
Oscar	Australia	*B. napus*	NSWDPI	Cultivar
P624	China	*B. napus*	Unknown	Breeding line
Purler	Australia	*B. napus*	NSWDPI	Cultivar
Qu1104	China	*B. napus*	Unknown	Breeding line
Rainbow[a]	Australia	*B. napus*	AgVic	Cultivar
Range	Australia	*B. napus*	Ag Seed	Cultivar
Ripper	Australia	*B. napus*	NSWDPI	Cultivar
Rivette	Australia	*B. napus*	NSWDPI	Cultivar
RocketCL	Australia	*B. napus*	Pacific Seeds	Cultivar
RottnestTTC	Australia	*B. napus*	Nuseed	Cultivar
RSO94-67 (98-18)	Unknown	*B. napus*	Unknown	Breeding line
Scoop	Australia	*B. napus*	NSWA	Cultivar
Seetha[a]	India	*B. juncea*	Unknown	Cultivar
Shiralee[a]	Australia	*B. napus*	NSWA	Cultivar
Skipton	Australia	*B. napus*	NSWDPI	Cultivar
StormTT	Australia	*B. napus*	Pacific seeds	Cultivar
Surpass400[a]	Australia	*B. napus*	Pacific Seeds	Cultivar
Surpass402CL[a]	Australia	*B. napus*	Pacific Seeds	Cultivar
Surpass404CL[a]	Australia	*B. napus*	Pacific Seeds	Cultivar
Surpass501TT[a]	Australia	*B. napus*	Pacific Seeds	Cultivar
Surpass603CL[a]	Australia	*B. napus*	Pacific Seeds	Cultivar
Tarcoola	Australia	*B. napus*	NSWDPI	Cultivar
Tatyoon	Australia	*B. napus*	AgVic	Cultivar
TawrifficTT	Australia	*B. napus*	Nugrain	Cultivar
TERI(OO)R9903[a]	India	*B. napus*	TERI	Breeding line
ThunderTT	Australia	*B. napus*	Pacific Seeds	Cultivar
TornadoTT	Australia	*B. napus*	Pacific Seeds	Cultivar
Tranby	Australia	*B. napus*	AgWA	Cultivar
WarriorCL	Australia	*B. napus*	NSWDPI	Cultivar
Wesbarker[a]	Australia	*B. napus*	AgWA	Cultivar

Continued

Table 1. Continued

Genotype ID	Country of origin	Species	Breeding programme[a]	Genetic status
Wesreo	Australia	*B. napus*	AgWA	Cultivar
Wesroona[a]	Australia	*B. napus*	AgWA	Cultivar
Yickadee[a]	Australia	*B. napus*	NSWA	Cultivar
Yu 178	China	*B. napus*	Unknown	Cultivar
Zhongyou 821	China	*B. napus*	Unknown	Cultivar

BGRC, Institut fur Pflanzenbau und Pflanzenzuchtung, Braunschweig (Germany); DSV, Deutsche Saatveredelung (Germany); AgWA, Department of Agriculture, Western Australia; NSWA, NSW Department of Agriculture (now NSW Department of Primary Industries - NSWDPI); USDA, United State Department of Agriculture; AgVic, Victorian Department of Agriculture.
[a]Genotypes were also used for testing suitability of DArT in differentiation of different species.
[b]Genotypes are winter/semi-spring types and unmarked are spring types.

B. napus varieties 'Lynx' and 'Monty', respectively, using the microspore culture methodology described by Cousin and Nelson.[45] Lynx is a high oleic, low linolenic, and European spring variety; seed was provided by O. Sass (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth, Germany). Monty is an Australian spring variety with typical canola quality seed oil characteristics;[46] Monty-028DH seed was provided by Canola Breeders Western Australia Pty Ltd (Perth, Australia). The DH parental lines Lynx-037DH and Monty-028DH were crossed reciprocally to produce F_1 seeds. A single F_1 plant from each reciprocal cross was used as microspore donor for microspore culture using the same methods as used for the parental DH lines production. Self-seed of the primary DH progeny were multiplied either during the summer of 2004–05 at Manjimup Horticultural Research Centre (Manjimup, Australia) or during the winter of 2005 at The University of Western Australia's Shenton Park field station (Perth, Australia).

2.2. DNA isolation

DNA was obtained from 131 'BnaLMDH' lines along with parental and F_1 controls using a standard CTAB method[44] or using Illustra Nucleon Phytopure Genomic DNA Extraction Kits (GE Healthcare).

2.3. Development of B. napus DArT array

Initially, the most frequently used seven methods of complexity reduction (all based on a combination of *Pst*I RE and a single 'frequently cutting' RE) were tested in several *Brassica* accessions by resolving products of representation amplification on 1.2% agarose gel. After this initial test, two methods with the most heterodispersed (no observable banding) smears of PCR products were selected: *Pst*I/*Bst*NI and *Pst*I/*Ban*II (data not reported). The DArT markers were designated with the prefix '*BrPb*' where '*Br*' indicates for Brassica, '*P*' for *Pst*I (primary enzyme used) and '*b*' for *Ban*II (secondary RE used) followed by a number corresponding to their unique clone ID.

2.4. Preparation of genomic representations

The *Pst*I/*Bst*NI genomic representations were prepared as described before for barley,[32] while *Pst*I/*Ban*II representations were prepared similarly as for sorghum according to a previous report.[36] Briefly, ~50 ng of genomic DNA was digested with either *Pst*I and *Bst*NI or *Pst*I and *Ban*II RE combinations and resulting fragments ligated to a *Pst*I overhang compatible oligonucleotide adapter. A primer annealing to this adapter was used in PCR reaction to amplify complexity reduced representation of a sample. Amplification products were either used for cloning in marker development process or labelled with fluorescent dyes and hybridized to DArT array in genotyping process.

2.5. Library construction and array printing

For library construction, we used 107 *Brassica* genotypes (Supplementary Table S1). Amplified *Pst*I restriction fragments from all accessions were cloned into pCR2.1-TOPO vector (Invitrogen, Australia) and 10 libraries were generated (Supplementary Table S2) as described by Jaccoud *et al.*[31] The white colonies containing genomic fragments inserted into pCR2.1-TOPO vector were picked into individual wells of 384-well microtitre plates filled with ampicillin/kanamycin-supplemented freezing medium. There were 7680 clones in *Pst*I/*Bst*NI and 3840 clones in *Pst*I/*Ban*II libraries (a total of 11 520 clones). Inserts from these clones were amplified using M13F and M13R primers in 384 plate format, a subset of PCR products were assessed for quality (10% of 25 μl PCR reaction) through gel electrophoresis and all remaining PCR products dried, washed and dissolved in a spotting buffer as described previously.[47] Microarrays were printed with spot duplication on SuperChip poly-L-lysine slides (Thermo Scientific,

Australia) using a MicroGrid arrayer (Genomics Solutions, UK).

2.6. DArT genotyping

Each sample was assayed using methods described above for library construction. Genomic representations were assessed for quality through gel electrophoresis in 1.3% agarose and labelled with fluorescent dyes (Cy3 and Cy5) as described previously.[32] Labelled targets were than hybridized to printed DArT arrays for 16 h at 62°C in a water bath. Slides were washed as described by Kilian *et al.*,[47] dried initially by centrifugation at $500 \times g$ for 7 min and later in a desiccator under vacuum for 30 min. The slides were scanned using Tecan LS300 scanner generating three images per array: one image scanned at 488 nm for reference signal measures the amount of DNA within the spot based on hybridization signal of FAM-labelled fragment of a TOPO vector multiple cloning site fragment and two images for 'target' signal measurement: one scanned at 543 nM (for Cy3-labelled targets) and one at 633 nm (for Cy-5-labelled targets).

2.7. Array image processing and polymorphism scoring

All the images were analysed with the DArTsoft v. 7.4.7 (DArT P/L, Canberra, Australia) software. The same software was used to score polymorphic markers in a binary manner (for the presence of marker in the representation as '1' and for the absence as '0') as described previously.[32] For quality control, 30% of genotypes were genotyped in full technical replication. Clones with $P > 77\%$, a call rate >97%, and 100% allele-calling consistency across the replicates were selected as markers. *P*-value represents the allelic-states variance of the relative target hybridization intensity as a percentage of the total variance.[34] The informativeness of the DArT markers was determined by calculating the polymorphism information content (PIC), within the panel of diverse accessions (Table 1) according to Anderson *et al.*[48]

2.8. Genetic diversity analysis

Pair-wise genetic similarity matrix was calculated from binary DArT matrices using the Jaccard's coefficient (J) where $J = a/(n - d)$ and 'a' is the number of fragments in common, 'd' is the number of fragments absent, and 'n' is the total number of DArT loci scored.[49] Similarities matrices were converted to genetic distances according to Swofford and Olson.[50] The dendrogram was generated based on Jaccard's coefficient with Unweighted Pair-Group Method using Arithmetic average (UPGMA) method in Sequential, Agglomerative, Hierarchical and Nested Clustering module[51] of Numerical Taxonomy and Multivariate Analysis System for personal computers software (NTSYS-pc), version 2.21.[52] The marker data were processed using the SIMINT module in NTSYS to compute a correlation among the columns for the 1000 bootstrap samples. The dendrogram was visualized using the program TREEVIEW implemented in NTSYSpc. The MXCOMP subroutine was used to calculate a cophenetic correlation matrix between the genetic similarity matrix and the tree matrix to measure goodness-of-fit implemented in the NTSYSpc package. Multivariate principle component analysis (PCA) based on genetic similarity matrices were used to resolve the genetic relationship and overall diversity among different accessions.

2.9. Linkage map construction

The 'BnaLMDH' mapping population of 131 DH lines along with parental and F_1 controls were genotyped with the initial marker discovery DArT array (Version 1.0). Highly polymorphic DArT clones in the set of diverse rapeseed germplasm (Supplementary Table S1) were selected and arrayed for routine DArT analysis (Version 2.0). A subset of 91 DH lines was also genotyped with Version 2.0 DArT array as described previously.

SSR primer sequences were obtained from Lowe *et al.*[53] and from the Agriculture and AgriFood Canada Brassica Microsatellite Consortium (for more information, see http://brassica.agr.gc.ca). Genetic map locations for the majority of these markers were reported previously.[54] PCR and fragment analysis methods were as described by Nelson *et al.*[55] In addition, intron polymorphism (IP) primer sequences and methods were reported by Panjabi *et al.*[56] New gene-based markers were developed based on publicly available cDNA sequences for fatty acid desaturase and FLC genes.[44] All polymorphic markers were scored using genotype codes 'A' (Lynx-037DH allele), 'B' (Monty-028DH allele), and '–' (missing value).

Linkage mapping was conducted with the aid of MultiPoint 2.1 (MultiQTL Ltd, Haifa, Israel), which uses the 'evolutionary optimization strategy'.[57] We followed the general approach described by Nelson *et al.*[58] with minor modifications. Markers showing significant allele segregation distortion (i.e. diverging from the Mendelian expectation of 1A:1B) were excluded from the analysis with DArT markers (which were scored in a dominant manner) and treated more strictly than the other markers types (which were codominant). DArT markers with very severe segregation distortion (χ^2, $P < 0.0001$) were discarded before commencing linkage mapping on

the basis that these may represent >1 locus, while DArT markers with less extreme segregation distortion (χ^2, $P < 0.001$) were included in the analysis but only as 'attached' markers (see explanation of this term below). Some codominant SSR, IP, and gene-based markers with very significant segregation distortion (χ^2, $P < 0.0001$) were included in the linkage analysis but were only permitted to act as 'attached' markers.

Iterative clustering analysis began at recombination frequency: rf = 0.14 and was increased at 0.02 increments to a maximum of 0.30. Linkage groups were assigned chromosome names and orientated using microsatellite markers mapped in reference populations.[54] Recombination frequencies between high-quality 'framework' markers were transformed to Kosambi distances in centiMorgans. 'Redundant' markers (those with identical map positions to their respective framework markers) and lower quality 'attached' markers (those with less well-supported map positions) were integrated into the most appropriate positions in framework marker map. As a quality control measure, marker genotyping scores that introduced singletons into the genotyping matrix of framework markers were re-checked to distinguish apparently true double crossover events from scoring errors. Care was taken not to over-correct the data such that apparently true double crossover events were retained in the scoring matrix. After correcting clear marker scoring errors, genetic interval sizes were recalculated.

3. Results

3.1. Assessment of genetic diversity

A total of 1547 high-quality DArT markers were polymorphic in a set of 89 contemporary cultivars and elite rapeseed lines from breeding programmes. The call rate ranged from 78.9 to 100% with an average of 96.7% and scoring reproducibility was 100% for all selected markers. The PIC values of individual DArT markers ranged from 0.02 to 0.5 (original data not shown), with an average of 0.3. Similarity coefficients (J) based on the binary matrix between individual lines ranged from 0.50 to 0.93.

The hierarchical cluster analysis discriminated all 89 genotypes of rapeseed into three major clusters (I, II, and III; Fig. 1), but clear differences were observed within these clusters, which were generally consistent with their phenology and genetic lineage. The cluster I consisted of a large number of accessions requiring vernalization for flowering such as 'Fan023', 'Fan 028', 'Fan168', 'Qu1104', 'Yu178', 'Zhongyou821', 'P624', '03-p74-6', '04-p34', 'Carousel-10', 'Major', 'Expander', 'Drakker', and 'Mutu 98-1' (Fig. 1). These varieties originated from China, France, and Japan (Table 1). Clusters II and III consisted of the Australian cultivars or their derivatives. In Cluster III, 'RocketCL', 'Surpass400', 'Surpass402CL', 'Surpass404CL', 'Surpass501TT', 'Surpass603CL', 'Hyola 50', and 'Hyola60' (bred by Pacific Seeds); 'Marnoo' and 'Tatyoon' (bred by Department of Primary Industries, Victoria); and 'TawrifficTT' (bred by Nuseed) showed strong grouping. Several subclades were evident in cluster II, and comprised a range of rapeseed cultivars bred by different Australian breeding programmes (Table 1), as many of them share the same parents in their pedigrees.[59] The cultivars released by Pioneer: '46C40', '45C05', '46C76', '44C73', '46Y78', '45Y77', and '44Y06' showed tight genetic relationship with 'Ag-Spectrum' and 'Rainbow' and grouped together in subclade in cluster II. Likewise, a discrete grouping of triazine tolerant cultivars ('ATR'- and 'TT') was also evident in cluster II. These groupings were strongly related to geographic origin, parentage and selection history (http:/www.ipaustralia.gov.au/pbr/). For example, '45C05' and '46C76' had Rainbow and Dunkeld in their pedigrees, respectively. Despite being of the same parentage 'Major' and 'Wesreo' (Major/Oro) varieties did not show tighter grouping, and may be heterogeneous or heterozygous.

Ultrametric cophenetic and genetic distance matrices that were used to generate the phylogenetic tree using the hierarchical clustering method, showed a 'good' fit (Z: cophenetic correlation coefficient = 0.8), indicating that the cophenetic distance is in congruence with the distance matrices obtained from the DArT marker data (Supplementary Fig. S1). Three principal components (first three axes), explaining 25% of variation, were sufficient to represent most of the structured information generated with multivariate analysis and reconfirmed the groupings of different accessions resultant from the UPGMA-based phenogram (Supplementary Fig. S2).

In order to test if the current DArT array is suitable for genetic analysis of other *Brassica* species, we analysed a subset of 32 genotypes representing *B. rapa*, *B. juncea*, *B. carinata* and investigated their genetic relationships in relation to selected *B. napus* cultivars representing 'spring' and 'winter' types from different breeding programmes (Table 1). Both cluster and PCA analyses differentiated different species of *Brassica*, as expected (Fig. 2, Supplementary Fig. S3). For example, cultivars/breeding lines of *B. napus*, *B. rapa*, *B. carinata*, and *B. juncea* clustered into distinct I–III groups. Within the *B. napus* cluster, two distinct subclades representing 'winter and semi-spring cultivars' ('Carousel-10', 'Major', 'Norin 22', 'Iwao natame', and 'Mutu') and 'spring cultivars' were clearly evident. Clustering of genotypes was consistent with their pedigrees and/or their origin (breeding programmes).

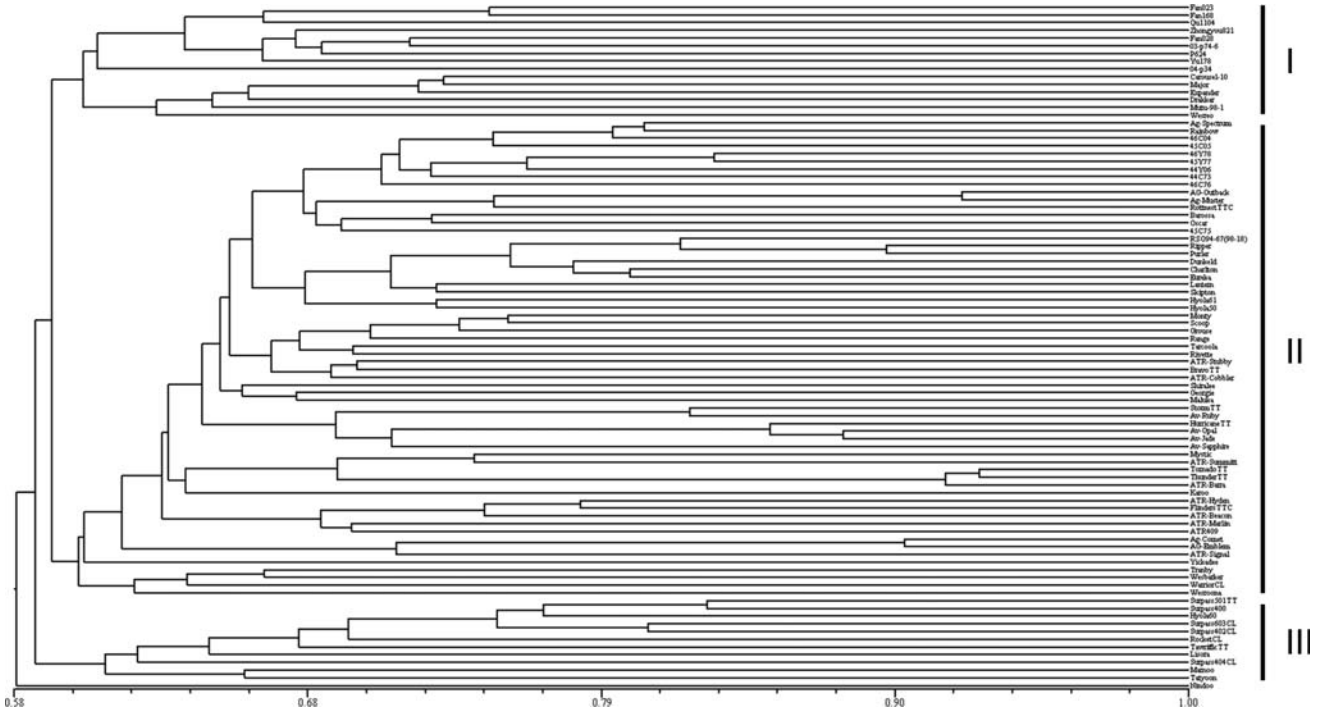

Figure 1. Dendrogram showing genetic similarity among 89 accessions of oilseed rape based upon the binary matrix obtained from 1547 DArT polymorphic loci, generated using the unweighted pair-group method with arithmetic mean analysis and Jaccard's coefficient in SAHN module of the NTSYSpc version 2.21h.

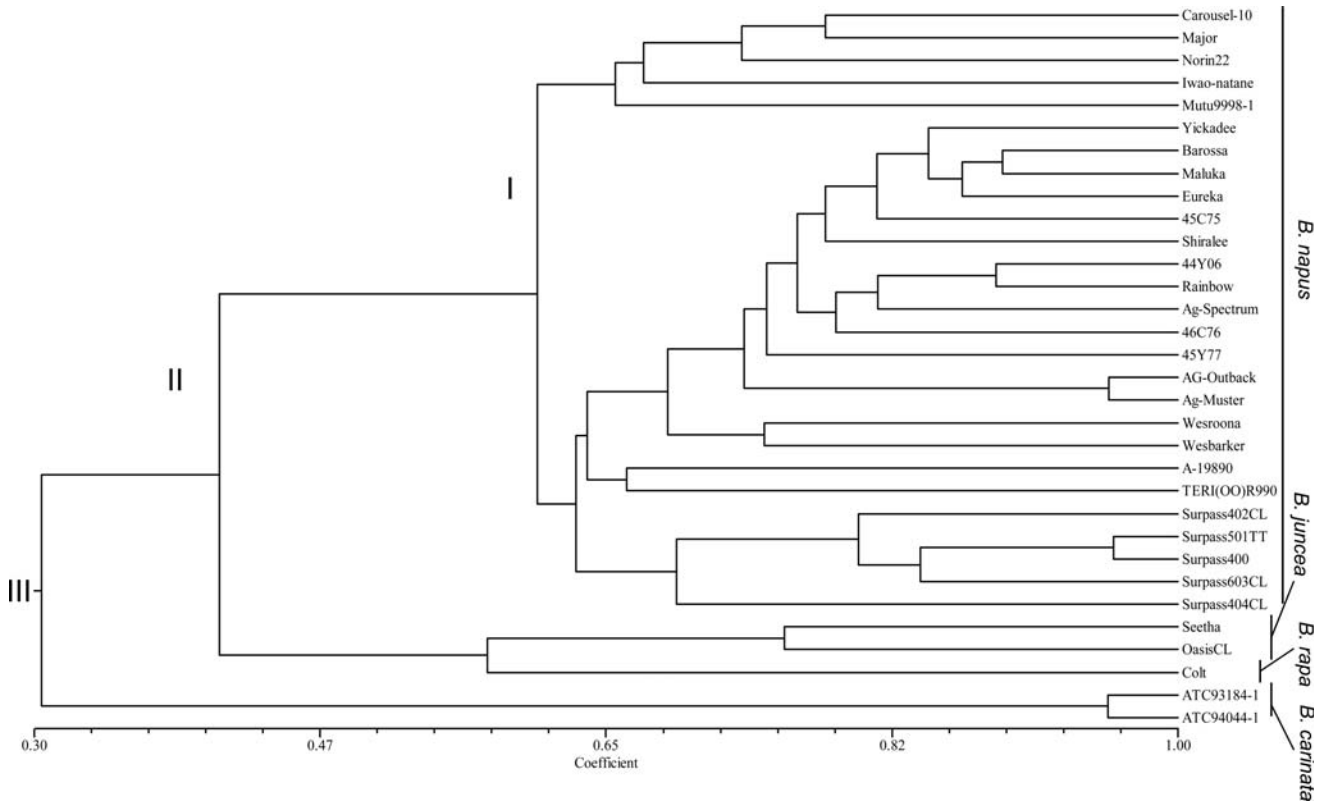

Figure 2. Dendrogram showing the genetic relationship among 32 accessions of *B. napus*, *B. rapa*, *B. juncea*, and *B. carinata*. Binary matrix obtained from DArT polymorphisms were used to generate dendrogram using the unweighted pair-group method with arithmetic mean analysis and Jaccard's coefficient.

Likewise, 'Ag-Outback' and 'Ag-Muster', and cultivars with suffix 'Surpass' share ancestry among themselves. For a general overview of Australian canola variety pedigrees, see Cowling.[59] PCA of DArT data of 32 accessions also supported the grouping identified by cluster analysis. The top three principal components (dimensions) explained 58.6% of the genetic variation (Supplementary Fig. S3).

3.2. *Identifying single-copy DArT markers for linkage mapping*

In the 131 DH lines screened with the DArT array Version 1, 1171 DArT markers passed the standard DArT quality criteria. These criteria include minimal call rate for markers (>80%) and high reproducibility of technical replicates (average scoring consensus >99.7%). Given that *B. napus* is an allotetraploid species with strong residual homoeologous relationships among chromosomes, it was considered likely that some DArT markers would detect pairs of homoeologous loci rather than single loci. Therefore, χ^2 tests were used to test each DArT marker for goodness-of-fit for a one-locus model (1:1 segregation ratio of parental alleles) and for two-locus models (1:3 or 3:1 ratio of parental alleles). Only those fitting the single-locus model were retained for linkage mapping purposes. Of 1171 DArT markers scored, 644 were monomorphic, 415 had allelic ratios consistent with the one-locus model (1:1; $P < 0.001$), and 112 had allelic ratios consistent with the two-locus model (3:1 or 1:3; $P < 0.001$). In the 91 DH lines screening the Version 2 array, 1020 DArT markers passed the standard DArT quality criteria (as above). Of these, 552 were monomorphic, 351 had allelic ratios consistent with the one-locus model (1:1; $P < 0.001$) and 117 had allelic ratios consistent with the two-locus model (3:1 or 1:3; $P < 0.001$). Markers that were putatively single-locus were combined from both DArT analyses to give 444 DArT loci that were used for subsequent linkage mapping. There were few inconsistencies between independent DArT analyses, which were entered as missing values in the combined DArT marker set.

3.3. *Linkage mapping*

In total, 584 marker loci (comprising 437 DArT, 135 SSR, 6 IP, and 6 gene-based markers) were used to generate a *B. napus* linkage map based on the BnaLMDH mapping population (Fig. 3, Supplementary Fig. S4; all scoring data provided in Supplementary Table S3, and summarized in Supplementary Table S4). Of these 584 loci, 329 were high-quality, non-redundant 'framework' markers, 209 were 'redundant' markers (i.e. co-segregated with respective framework markers), and 46 were lower quality 'attached' markers with less well-defined map positions. In consistent with the haploid chromosome number for *B. napus* ($n = 19$), there were 19 linkage groups with the shortest linkage group (A08) being 61.3 cM, the longest (C4) being 186.2 cM, and a total map length of 2288 cM (Supplementary Table S4). There were also two small clusters (Cluster-1 and Cluster-2) comprising four and three loci, respectively. There were also four markers (brPb-661033, brPb-662131, brPb-809033, and sN2675) that remained unlinked.

Using SSR markers with known map locations, all linkage groups were unambiguously assigned chromosome names and orientated relative to the reference map.[54] Alignment to the reference map revealed that this map encompasses ~90% of the known *B. napus* genome with notably incomplete coverage of chromosomes A07 (bottom half) and A08 (top half). DArT marker were unevenly distributed, with the proportion of DArT loci ranging from 31% (chromosome C5) to 90% (chromosome A03), with an average of 70% across all chromosomes (Supplementary Table S4).

Several regions of the linkage map comprised markers with allelic segregation ratios diverging from the Mendelian expectation (1:1 for a DH population; Supplementary Table S3). The most significantly skewed deviations (χ^2, $P < 0.001$) were on linkage groups A01, A03, and A06 and encompassed both DArT and SSR markers. We found evidence of high frequency of homoeologous recombination between chromosomes A07 and C6, as shown by the tendency of A07 and C6 to cluster together during the linkage mapping process, which had to be manually separated.

4. Discussion

We developed and applied a high-throughput DArT marker array for the first time in rapeseed. We demonstrated that this whole-genome profiling technology is useful for establishing phylogenetic relationships among varieties and elite breeding *Brassica* lines and for construction of linkage map that has extensive genome coverage of the rapeseed genome.

4.1. *DArT markers for genetic diversity assessment*

In this study, we utilized up to 1547 DArT markers for the assessment of genetic diversity among accessions of rapeseed and related species. A number of these markers were distributed across the genome in the 'BnaLMDH' population (Fig. 3). Previously, only a limited number of markers were used to analyse genetic diversity in rapeseed. For example, Wang *et al.*[12] used 18 SSR primer pairs to generate 112 polymorphic features for genetic diversity

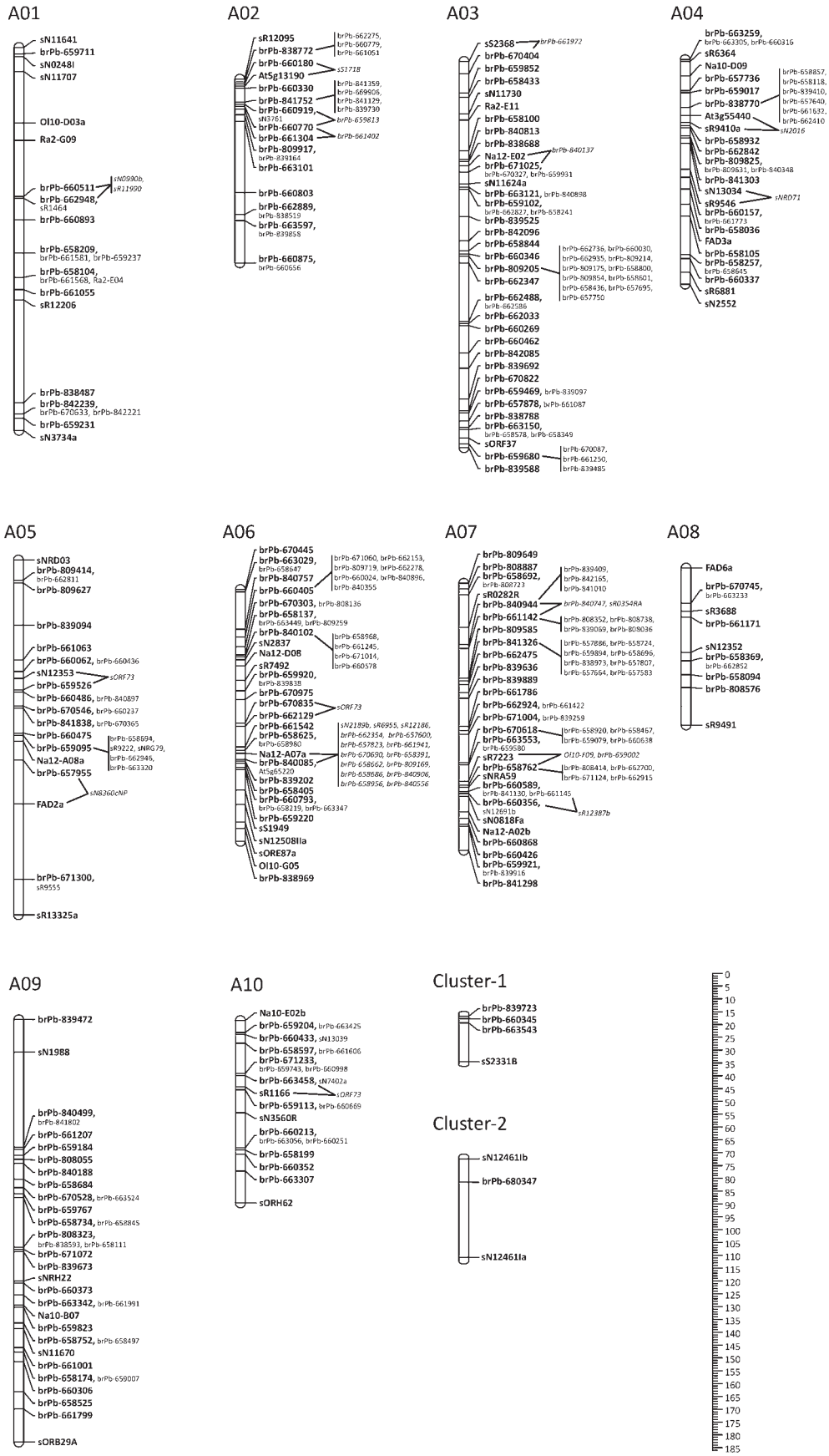

Figure 3. Graphical representation of the genetic linkage map constructed in the DH population from Lynx/Monty. Map distances are given in centiMorgan (cM) on the left of sample linkage groups.

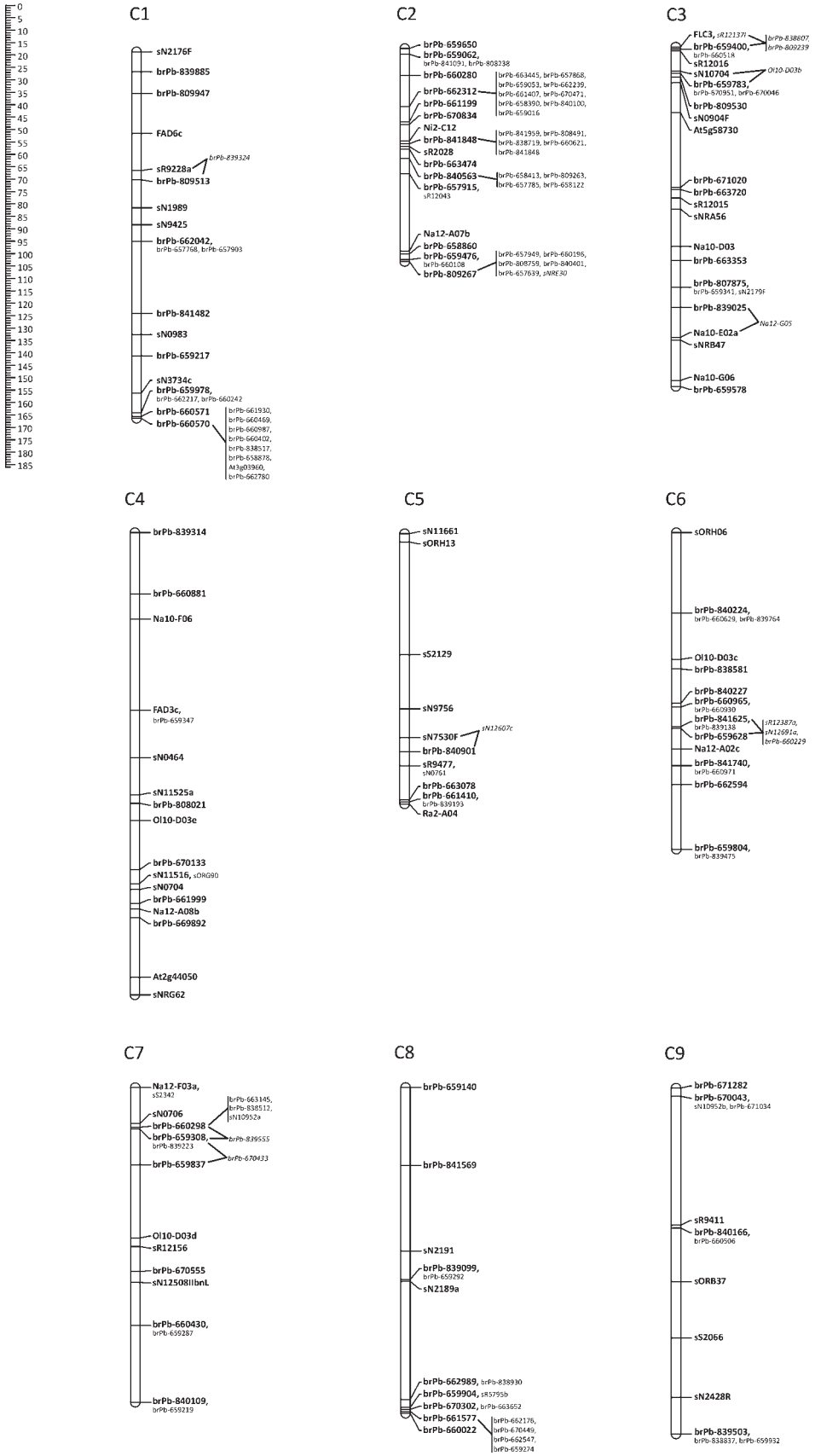

Figure 3. *Continued*

analysis of 48 Australian rapeseed cultivars while Chen *et al.*[60] used 55 SSR primer pairs to generate 287 polymorphic features for genetic diversity analysis of 72 cultivars. DArT markers with extensive genome coverage, developed in this study, will provide better estimate of the extent of genetic diversity. DArT markers have the additional advantage of being based largely on SNP variation, which more closely reflects normal genome evolutionary behaviour compared with SSRs that have high rates of mutation.[61] Therefore, this DArT resource constitutes a significant improvement in marker density, and possibly quality, compared with that possible with marker technology previously available for rapeseed.

We employed both cluster and PCA analyses for studying genetic diversity and population structure. Results suggested that genetic diversity exists within the rapeseed germplasm. Most of the Australian varieties were grouped into distinct clusters according their common ancestry.[59,62] For example, 'Yickadee', 'Maluka', 'Eureka', 'Shiralee', 'Rainbow', '45C75', '44Y06', 'Ag-Spectrum', '46C76', and '45Y77' share pedigrees with each other[59] (Patel personal communication. http://www.ipaustralia.gov.au/pbr/index.shtml). This confirms that gene flow into Australian breeding programmes was restricted before the time of release of these varieties.[59] Furthermore, all Australian varieties up to the year 2000 were developed from the same set of donor sources collected from Japan, France, Poland, Germany, Sweden, and Canada.[59] However, some accessions overlapped or distributed across well-defined clusters, for example 'Karoo' (Fig 1). This could be due to the fact that rapeseed (syn canola) is a predominantly self-pollinated crop; however, outcrossing may occur, ranging from 5 to 36% under field conditions.[63,64] Therefore, heterogeneity and heterozygosity in some varieties, such as 'Karoo', is very likely. This phenomena has been reported in previous studies,[11] especially in the Australian cultivars which were developed using open-pollinated pedigree selection without 'intentional selfing'.[62] Furthermore, many breeding programmes have intentionally released 'genetically mixed' varieties to cope with environmental variation and biotic stresses.[63,64] Phenograms also clearly differentiated 'winter' and 'spring' types, which represent to different gene pools as described previously using RFLP markers.[8,65] Our results are consistent with previous studies which showed that rapeseed accessions can be grouped on the basis of flowering habit (winter and spring type), and on the basis of origin by geographical region and breeding organizations.[8,9,12,65–67]

DArT markers also enabled us to assess genetic diversity among four agricultural *Brassica* species that are extensively being used in rapeseed improvement programmes. Although we have not sampled extensively these species, DArT markers allowed us to differentiate *B. rapa* (AA), *B. napus* (AACC), *B. juncea* (AABB), and *B. carinata* (BBCC) on their polymorphism patterns. Therefore, the current DArT platform is suitable for genotyping of different *Brassica* species especially those having 'A' and 'C' genomes and may facilitate alien gene introgression from related species.

4.2. *Implication of diversity*

This DArT marker analysis supports previous studies that show that rapeseed accessions from Australia, Europe, and China are genetically differentiated.[60] Crossing between these differentiated sources can be used to enlarge genetic diversity in rapeseed breeding programmes.[59] Genetic distance estimates would assist breeding programmes to maximize the level of variation in their germplasm and to exploit high heterosis in hybrid varieties. DArT allelic profiles produced in this study will also be useful for fast and accurate DNA fingerprinting of elite Australian rapeseed genotypes and identifying loci associated with traits of agronomic importance using association mapping strategy.

4.3. *Genome-wide coverage of DArT markers*

A genetic map of 'BnaLMDH' was constructed using 437 DArT, 135 SSR, 6 IP, and 6 gene-based markers. These markers provided good coverage across all 19 linkage groups, corresponding to all 10 chromosomes of A and 9 chromosomes of C genome of *B. napus*. Good genome coverage could be partly attributed to the enrichment of the genic regions with the use of *Pst*I. The map of 'BnaLMDH' population covering 2288 cM is comparable with the previous genetic linkage maps of *B. napus*, spanning distances of 1173–2619 cM.[68–70] Approximately 10% of the DArT loci were found to be duplicated in this study. Therefore, a limited effect of homoplasmy is anticipated on the usage of DArT in diversity analysis in allotetraploid *B. napus*.

5. Conclusion

We developed the DArT platform for genetic analysis of *Brassica* genomes and demonstrated its usefulness in genetic diversity assessment and genetic map construction. Our results have proved that DArT technology is amenable for various downstream applications such as cluster analysis and map construction, including for trait-marker association analyses.[66] It can supplement marker systems that cannot be analysed in a highly parallel genotyping format, such as SSR markers. SSRs are suitable to distinguish heterozygotes due to their, in general, co-dominance. Besides,

excessive stutter bands and overlapping bands due to coamplification of loci also limit the usefulness of SSR markers in rapeseed. In light of the current paucity of high-density SNP assay formats for rapeseed and the embryonic nature of genotyping by sequencing technologies, the development of the DArT platform for rapeseed is a significant development for identifying trait-marker association, genetic diversity, and population genetics studies and whole-genome selection in this important crop species.

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Supplementary Data: Supplementary Data are available at www.dnaresearch.oxfordjournals.org.

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