Pak. J. Bot., 46(3): 779-787, 2014.

GENETIC DIVERSITY ANALYSIS OF *BRASSICA NAPUS/BRASSICA CAMPESTRIS* PROGENIES USING MICROSATELLITE MARKERS

LAILA FAYYAZ[1], FARHATULLAH[1*], M. ASHIQ RABBANI[2], SIDRA IQBAL[1] MEHWISH KANWAL[1] AND IFFAT NAWAZ[1]

[1]*The University of Agriculture, Peshawar, Pakistan*
[2]*Plant Genetic Resources Institute, National Agricultural Research Centre, Islamabad, Pakistan*
[*]*Corresponding author's e-mail address: drfarhat@aup.edu.pk*

Abstract

Genetic diversity and relationship of F_2 segregating progenies of interspecific crosses between *B. napus* N-501/*B. campestris* C-118 were studied. A set of 90 genotypes (2 parental lines and their 88 F_2 progenies) was characterized separately using 24 microsatellite or SSR markers to cover the diversity as broadly as possibly present in them. In initial screening only 12 out of 24 SSR primers combination amplified DNA fragments, while the remaining 12 SSR primers did not amplify DNA fragment therefore those 12 SSR molecular markers were not used for further analysis. The 12 SSR primer combinations generated a total of 33 alleles, of that 32 were polymorphic loci, whereas only one was monomorphic locus. Primers BRMS-19 and BRMS-40 were highly polymorphic producing 4 bands each. Primer Ra2-D04 was less polymorphic and it produced only one band. The proportion of polymorphic loci was 95.83% which indicates high genetic diversity among the progenies. The average number of polymorphic alleles per locus was 2.66. The PIC values ranged from 0.395 for primer Ra2-E03 to 0.726 for primer BRMS-019 with an average genetic diversity (PIC value) of 0.584 per locus. Seven primers showed PIC values above 0.5 (50%) indicating high genetic diversity in the studied plant materials. Pair-wise similarity indices among 90 genotypes ranged from 0.3 to 0.95. Dendrogram obtained through UPGMA clustering of F_2 progenies depicted eight main groups using similarity coefficient of 0.70. The progenies could be similar to their parents if they have the same banding patterns as that of the parents and could be distinguished from each other by the combination of fragments which are repeatedly present in one progeny and absent in the other. Considerable genetic diversity has been found among the F_2 segregating progenies and their parents using SSR markers thus, SSR analysis proved to be a useful tool.

Introduction

Brassica napus L. (genome AACC; 2n = 38) is an amphidiploid species that originated through spontaneous interspecific hybridizations between turnip rape (*Brassica rapa* L. syn. *campestris*; AA, 2n = 20) and cabbage (*Brassica oleracea* L; CC, 2n = 18) genotypes (Kimber & McGregor, 1995). Oilseed brassica species (*Brassica napus; B. campestris* and *B. juncea*) are now the 3rd most important source of edible vegetable oil in the world after palm and soybean (Zhang & Zhou, 2006; Turi *et al*., 2012; Ali *et al*., 2013; Zada *et al*., 2013, 2013a). There is subdivision of oilseed rape into winter and spring types (Bus *et al*., 2011). Canola-type cultivars having low level of erucic acid (<2%) and glucosinolate (<30µM) of *B. napus* and *B. rapa* (Downey & Rimmer, 1933) are widely grown commercially. Genetic diversity in germplasm is the base of exploiting and utilizing desirable genes for genetic improvement of open-pollinated varieties and hybrids (having high level of heterosis). For commercial hybrid development, the knowledge of genetic diversity is important (Ahmad *et al*., 2014; Shinwari *et al*., 2013 & 2013a; Sultan *et al*., 2013). Interspecific hybridization can easily be forced in the family brassicaceae where gene flow is very limited under natural conditions (Sandhu & Gupta, 2000). In addition to biochemical and morphological characterization (Azam *et al*., 2013) molecular characterization is essential for identifying genetic relationship as molecular markers can detect high degree of polymorphism and are not sensitive to environmental and developmental variation (Ahmad *et al*., 2014; Rabbani *et al*., 2010; Zeb *et al*., 2011).

Molecular markers closely linked to genes of interest are very useful for marker-assisted selection and pyramiding of two or more genes into a single genotype in Brassica breeding programs (Ahmad *et al*., 2013). Among different types of molecular markers, microsatellite also known as simple sequence repeats (SSRs) have been utilized for different purposes in plant breeding and genetics including genetic mapping, cultivar discrimination and detection of genetic diversity (Gupta & Varshney, 2000). SSRs are tandemly repeated nucleotide motifs 1–6 bp (Dib et al., 1996; Dietrich et al., 1996). They are co-dominant markers, reproducible, highly efficient, detect high level of polymorphism and are evenly distributed (Morgante & Olivieri, 1993). Microsatellites surpass other DNA based markers such as RFLPs are co-dominant but they have hybridization based problems and RAPDs have low reproducibility. It had been documented that SSRs can analyze better genetic diversity than other molecular markers (Eujayl et al., 2001). SSRs provided an abundant class of polymorphisms in crops including soybean (Maughan et al., 1995), wheat (Prasad et al., 2000), barley (Pillen et al., 2000), rice (Cho et al., 2000) and maize (Senior et al., 1998). SSRs were used to assess the relationships among brassica species and numerous SSR primer sequences were already publicly documented (Snowdon & Friedt, 2004). Simple sequence repeats also have certain demerits like they are expensive and time consuming.

For the present study we selected microsatellites or SSR markers. The aim of our investigation was to study the extent of variation between B. napus and B. campestris parental lines and their respective F2 population through SSR markers. We also characterized

the pattern of genetic diversity in parental lines to identify potential parents for commercial hybridization. This will help geneticists to predict which parent is more dominating in this cross.

Materials and Methods

Plant materials: A set of 90 genotypes including eighty eight progenies from a cross between B. napus x B. campestris (N-501×C-118) along with their respective parental lines. Segregating progenies of interspecific cross (N-501×C-118) were maintained till F2 generation to cover as much genetic diversity as possible. The research work was conducted at The University of Agriculture Peshawar Khyber Pakhtunkhwa and Plant Genetic Resources Institute (PGRI), National Agricultural Research Centre (NARC), Islamabad Pakistan.

SSR primers: A PCR-based technique, SSR, which successfully amplifies open reading frame, was tested on this cross. Initially twenty four SSR primer combinations selected from the collection available at genomic library of PGRI, NARC, Islamabad were tested on this cross. After pre-screening 12 primer pairs were chosen out of 24, because of their polymorphism, repeatability and the number of markers per gel in 90 samples. List of the microsatellites analyzed, motif type, forward/reverse primer sequences, expected allele size and annealing temperature are given in Table 1.

Table 1. List of the microsatellites analyzed, motif type, forward (F)/reverse(R) primer sequences, expected allele size and annealing temperature.

SSR Primer	Motif type	Primer sequence (5′–3′)	Expected size (bp)	Annealing temperature
BRMS-008	di TC/AG	F:AGGACACCAGGCACCATATA R:CATTGTTGTCTTGGGAGAGC	145	55
BRMS-019	di GT/CA	F:CCCAAACGCTTTTGACACAT R:GGCACAATCCACTCAGCTTT	220	55
BRMS-027	di GA/CT	F:GCAGGCGTTGCCTTTATGTA R:TCGTTGGTCGGTCACTCCTT	205	56/57
BRMS-029	di GA/CT	F:AACAAATGACACACACCACACT R:ATTGAAAATCTTAACCGTGAAG	232	56
BRMS-040	di GA/CT di GT/CA	F:TCGGATTTGCATGTTCCTGACT R:CCGATACACAACCAGCCAACTC	283	55
Na10-E02	di GA/CT	F:TCGCGCATGTAATCAAAATC R:TGTGACGCATCCGATCATAC	136	55
Na10-G10	di GA/CT	F:TGGAAACATTGGTGTTAAGGC R:CATAGATTCCATCTCAAATCCG	237	58/59
Na12-B09	tri GGC/CCG	F:ACGGAAGATCAAACAGCTCC R:TGAGCGACCCATTCTTTAGG	208	59/60
Na12-C07	di GA/CT	F:ACTCAACCCCACAAACCTG R:AGTTCCCCGGATCCGATTAG	184	58
Na12-D04	di GT/CA	F:ACGGAGTGATGATGGGTCTC R:CCTCAATGAAACTGAAATATGTGTG	281	59/60
Na12-E02	tri TTG/AAC	F:TTGAAGTAGTTGGAGTAATTGGAGG R:CAGCAGCCACAACCTTACG	127	55/56
Na14-D07	tri GGC/CCG	F:GCATAACGTCAGCGTCAAAC R:CTGCGGGACACATAACTTTG	134	55
Ni2-All	di GA/CT	F:AACAAACAAGAGTCGAATACGG R:AATGCCCTCTAACTGAGCCC	226	58/60
Ol10-A05	di GA/CT	F:TGTAATAACCCGACCCATCC R:CTCTCTCGCTCTCTCGATCC	220	55-58
Ol10-F11	tri GGC/CCG	F:TTTGGAACGTCCGTAGAAGG R:CAGCTGACTTCGAAAGGTCC	149	55
Ol10-H02	tri GGC/CCG	F:AACAGGAAGAAACGACGAGG R:AGAGAGCCATGAGAAGCACC	220	55
Ol11-H02	tri AAT/AAG	F:TCTTCAGGGTTTCCAACGAC R:AGGCTCCTTCATTTGATCCC	189	55
Ra2-A11	di GA/CT	F:GACCTATTTTAATATGCTGTTTTACG R:ACCTCACCGGAGAGAAATCC	332	55
Ra2-D04	di GT/CA	F:TGGATTCTCTTTACACACGCC R:CAAACCAAAATGTGTGAAGCC	176	55
Ra2-E03	di GA/CT	F:AGGTAGGCCCATCTCTCTCC R:CCAAAACTTGCTCAAAACCC	279	55-56
Ra2-E11	di GA/CT	F:GGAGCCAGGAGAGAAGAAGG R:CCCAAAACTTCCAAGAAAAGC	198	55
Ra2-E12	di GA/CT	F:TGTCAGTGTGTCCACTTCGC R:AAGAGAAACCCAATAAAGTAGAACC	189	55
Ra2-F11	di GA/CT	F:TGAAACTAGGGTTTCCAGCC R:CTTCACCATGGTTTTGTCCC	240	54/55
Ra2-H06	di GT/CA	F:GAATTCAGAGGTATCTACACGGC R:TAACAAAGACCCTGCGTTCC	212	55/57

DNA extraction: The plant material was grown in mini-pots and placed in incubator having a temperature of 25°C. After three to four weeks, young leaves of the seedlings were harvested from each progeny of this cross separately for DNA extraction. Total cellular DNA was extracted using a standard cetyl trimethyl ammonium bromide (CTAB) extraction protocol of Doyle & Doyle (1990) with some modifications.

Young leaves of the selected seedlings were clipped off and were ground in sterilized pistil and mortar but we used an alternate procedure. A 300-400mg fresh leave samples were crushed into powered in 1.5ml microcentrifuge tube. The samples were homogenized with pre-warmed extraction buffer (500µl of 2% CTAB, 100mM Tris-HCl, pH 8.0, 1.4M NaCl, 20mM EDTA, 0.5% SDS) with added 0.2% marceptoethanol. The samples were then incubated in water bath for 30 minutes to 1 hour at a temperature of 65°C. The tubes were cooled to room temperature and 300-500µl of chloroform-isoamyl alcohol (24:1) was added to each sample and mixed manually for 15 to 20 minutes by inverting the tubes up and down to precipitate the DNA. The mixture was centrifuged for 10 minutes at 15000 rpm. After centrifugation, we got two phases in centrifuge tubes; the upper aqueous phase was supernatant containing DNA, while the lower chloroform phase contained some degraded proteins, lipids, and many secondary compounds and cell debris. Supernatant was shifted into another tubes with great care to prevent the merging of two phase and the remaining debris were discarded. A 0.6 volume (300µl) of cold isoproponal was added to the supernatant and mixed gently to precipitate nucleic acids. Tubes with supernatant were kept at -20˚C for several hours to overnight for more precipitation.

The samples were centrifuged next day for ten minutes at 12000 rpm to get the pellet of DNA. Poured off supernatant carefully as some pellets were still loose even after this longer spin and pellet would drain off with supernatant. The pellet was washed twice with ice cold 70% ethanol (v/v) to remove the impurities or chemical residues and then air-dried by inverting the tubes on tissue paper. After drying, pellet was dissolved in 50µl of TE buffer (10mM Tris-HCl, 1mM EDTA pH 7.4 for further use. RNA was removed by adding 1µl of RNase (10mg/ml). Spun tubes at 10,000 rpm for 10 minutes. The DNA was stored at 4°C for shorter period (up to one week) or -20°C for longer period. After isolation, concentration of total genomic DNA of each genotype was monitored by visual assessment of band intensity in comparison with lambda DNA molecular standards of known concentrations, i.e., 100, 300 and 500ng using 0.8% agarose gel. DNA of each genotype was further diluted to working concentration for PCR analysis.

PCR amplification: The protocols of Hasan *et al*., (2006) were used for DNA amplification. The final volume of reaction mixture was 20µl containing 14.4µl of deionized ddH2O, 2µl of 10×Dream Taq buffer (with added 20mM of $Mgcl_2$), 0.4µl dNTPs (10mM each), 1.0µl of SSR forward/reverse primers, 20µM (20 pmols/µl) and 0.2µl of Taq DNA polymerase (5 Units/µl) (Fermentas Life Sciences). The amplification reaction was carried out in a PCR thermo cycler under the following profile: initial strand separation had 1 cycle at 94°C for 4 minutes, 35x cycles of (Denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute) and one cycle of final extension at 72°C for 7 minutes followed by 4°C temperature to hold samples until removed from PCR machine. Electrophoresis of PCR products was carried out using 2% gene choice high resolution agarose gels (w/v) at 100V for 1.30 h. the samples were loaded in the gels in such a way that parent N-501 was loaded in extreme left well proceeding ladder and parent C-118 was loaded in the extreme right well preceding ladder. The wells in-between the parent were loaded with amplified DNA of F_2 segregating populations. The gels were stained internally with 8µl per 100 ml ethidium bromide. Gels were photographed under UV Doc Gel Documentation System (JICA, Japan).

Data analysis: Only fragments that were polymorphic among the progenies and could be clearly scored, used in data analysis. SSR gel images were scored visually. Each DNA fragment was treated as an independent unit. Clearly distinguishable bands were scored as 1 representing the presence of fragment and 0 to denote the absence of fragment. Data were entered into a spreadsheet to form 0-1 matrix. The molecular size of PCR product was calculated through matching with a size-calling standard of 20bp DNA ladder. The ladder contains the following 15 discrete fragments: 300, 280, 260, 240, 220, 200, 180, 160, 140, 120, 100, 80, 60, 40, and 20 bp. The bright bands (100, 200 and 300bp) served as reference bands. Pairwise comparisons of the populations and parents were used to generate genetic similarity coefficient. Genetic similarity was obtained by Dice algorithm which was described as follows by Nei & Li (1979):

$$\text{Similarity (F)} = 2Nab/ (Na + Nb)$$

where Na and Nb is the number of scored fragments of respective individual, Nab is the number of common fragments between individuals ‘a’ and ‘b’. The SAHN module of NTSYSpc (Version 2.01, Exeter Software, Setauket, NY, USA) was used for constructing a dendrogram based on the UPGMA algorithm (unweighted pair group method with arithmetic average). Polymorphism information content (PIC) was used as follows:

$$PIC = 1 - \Sigma (pi)^2$$

where *pi* is the frequency of *i*th allele in a population.

Results

The representative gel shows bands amplified using primer BRMS-040 (Fig. 1). The given primer produced 4 amplified bands at 260, 200, 180 and 100bp observed through matching with 20bp DNA ladder. The fragment size of 260bp was observed in all F2 plants and parent C-118 except plant number 1, 2, 3, 4, 6, 7, 9, 13, 16, 19, 20, 21, 23, 24, 25, 26, 28, 31, 32, 34, 36, 39, 40, 42, 43, 44, 47, 48, 52, 53, 54,

56, 57, 58, 59, 60, 63, 65, 66, 69, 70, 71, 75, 76, 77, 79, 80, 82, 84, 86, 87, 88 and parent N-501. The next fragment size of 200bp were amplified by all the F_2 plants and both parents N-501 and C-118, while plants number 1, 2, 3, 4, 5, 8, 12, 14, 17, 22, 24, 31, 43, 47, 51, 55, 56, 62 and 86 have not amplified this fragment size. The fragment size of 180bp was observed in all F_2 plants and parent N-501 except plants number 2, 4, 5, 7, 8, 10, 11, 14, 25, 26, 27, 28, 29, 30, 31, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 47, 49, 61, 62, 63, 64, 66, 67, 68, 70, 79, 84 and parent C-118. The fragment size of 100bp was observed in all F_2 plants except plants number 1, 3, 6, 7, 8, 17, 18, 19, 20, 21, 23, 25, 27, 29, 30, 31, 32, 41, 42, 43, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88 and both parents N-501 and C-118. Plants number 6, 19, 20, 21, 23, 32, 52, 53, 54, 57, 58, 59, 60, 71, 75, 76, 77, 80, 82, 87 and 88 showed banding pattern that were similar to parent N-501 amplified at two fragment size (220 and 200bp). Plants number 27, 29, 30, 41, 49, 61 and 64 gave banding pattern that were similar to C-118 amplified at all the two fragment size (260 and 200bp). Rest of the plants showed banding pattern that were different from both of the parents this observation may support the segregating nature of F2 progenies.

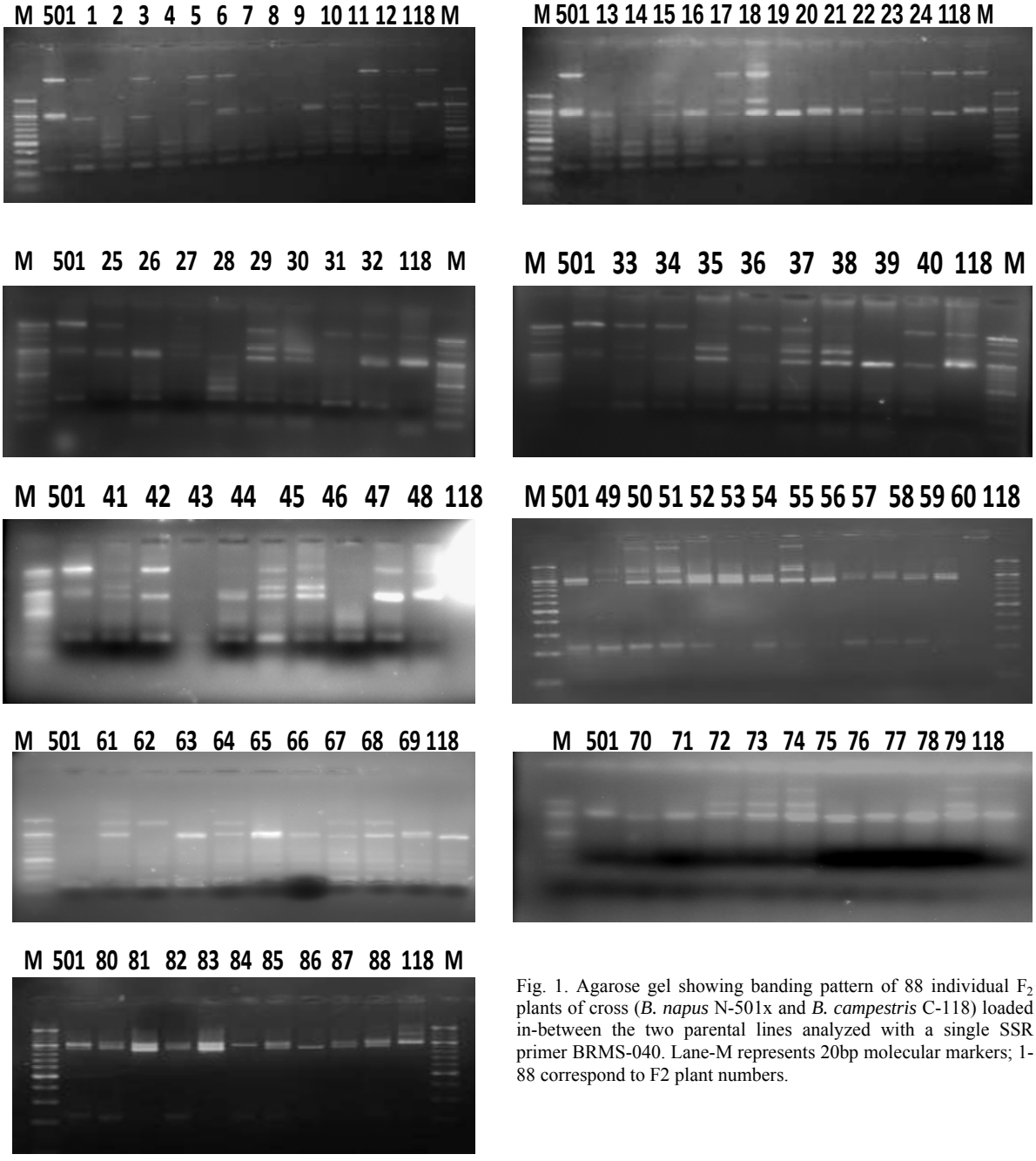

Fig. 1. Agarose gel showing banding pattern of 88 individual F_2 plants of cross (*B. napus* N-501x and *B. campestris* C-118) loaded in-between the two parental lines analyzed with a single SSR primer BRMS-040. Lane-M represents 20bp molecular markers; 1-88 correspond to F2 plant numbers.

The progenies could be distinguished from each other by means of a combination of fragments which is repeatedly present in one progeny and absent in the other or it may be identical to one parent and different from the other parent. A total of 33 distinct bands were generated using the 12 SSR primers. From these, 32 bands were polymorphic. An average of 2 polymorphic bands per primer was observed. It can be observed that these primers produced diverse numbers of amplified and polymorphic bands (Table 2). Ra2-D04 produced the least number of polymorphic bands (only one), while BRMS-19 and BRMS-40 produced the greatest number of polymorphic bands (4 bands). The same Table 2 also indicates the percent polymorphic loci of the 12 primers. The percentage ranged from 50-100% with a mean of 95.83%. This information is important especially for future studies to choose appropriate primer for assessing genetic diversity and relationships of F2 progenies to their parents and among themselves.

Polymorphism information content (PIC): Polymorphism information content (PIC) was calculated for each of the 12 SSR primers to measure allele diversity at each locus. The PIC values ranged from 0.395 for primer Ra2-E03 to 0.726 for primer BRMS-019 with an average genetic diversity/PIC value of 0.584 per locus (Table 2). Seven primers showed PIC values above 0.5 (50%) indicating high genetic diversity in the studied plant material. The maximum genetic diversity of 73% was observed for these genotypes. Pair-wise similarity indices among 90 samples ranged from 0.3 to 0.95.

Cluster analysis: To further determine the genetic relationships of F2 progenies with parents and among themselves based on microsatellite, cluster analysis was performed. Dendrogram obtained through UPGMA clustering depicted different grouping of F_2 populations. Progenies clustered into eight main groups using similarity coefficient of 0.70 (Fig. 2). Group I was further sub-divided into five sub-clusters, among them parent N-501 grouped with hybrid plant 5. Cluster two had three plants coded as 1, 3, and 6. In third cluster two plants 2 and 4 were grouped together. Cluster four had three genotypes 13, 14, 15 and cluster 5 contained plant number 41. Group II was sub-divided into five sub-clusters: cluster one had two plants 7 and 8. Cluster two had three plants 10, 11 and 12, cluster three had plant 16, 17 and 18 and fourth cluster had three plants 19, 20 and 23, while cluster five comprised of 22 and 24 (Table 3).

Group III was further sub-divided into nine sub-clusters forming the largest group of the dendrogram. Eight genotypes were present in cluster one i.e., 37, 39, 40, 47, 38, 45, 46 and 49 while, cluster two was comprised of six hybrids 42, 57, 53, 54, 56 and 58. Cluster three had four plants coded as 50, 51, 52 and 55. Cluster four was grouped separately and had only one plant numbered as 44 that had not grouped with the rest of the population progenies in this major group. Cluster five had plant number 61, 62, 63 and 64 grouped together. Plant number 65 and 69 formed sixth cluster. Cluster seven was generated by grouping of plants number 66, 67, 68 and 70. Cluster eight was grouped separately and had only one plant 43 that had not showed similarity with the rest of the genotypes in this major group III. Cluster nine had three plants coded as 48, 59, and 60. Group IV had three distinct sub-clusters; first one consisted of one genotype numbered 71 that had not grouped with genotypes of other two clusters. Cluster two was formed by the grouping of three plants number 72, 73 and 74. Cluster three had two genotypes, i.e., 75 and 76. In group V there was one cluster generated by two plants numbered as 21 and 32. Group VI had three sub-clusters: first one consisted of five F_2 progenies number 25, 27, 29, 26 and 30. Cluster two had four hybrids coded as 28, 36, 34 and 31. Cluster three had plant number 33 and 35. Group VII was further sub-divided into five sub-clusters: cluster one had plant number 77, while cluster two was comprised by three hybrids numbered 78, 80, and 81. Cluster three was constructed by plant number 79 and parent C-118. Cluster four contained plant number 82, 83, 85 and 84, whereas fifth cluster had plant number 86, 88, and 87. The plant number 9 was found in group VIII in dendrogram (Table 3). The higher numbers of bands for each primer reflected the existence of larger genetic diversity among the progenies under investigation.

Discussion

The 12 SSR markers used in this research revealed considerable genetic diversity within F_2 populations. The 12 SSR primer combinations amplified a total 33 alleles with an average number of 2.66 polymorphic alleles per locus. Other researchers had also reported the average number of alleles per locus as 2 (Uzunova & Ecke, 1999), 3.9 (Rudolph *et al*., 2000) and 4.44 (Tonguc & Griffiths, 2004). Similarly, lower number of amplified fragments per primer for brassica germplasm was reported by Yu *et al*., (2005) who detected 4.0 loci per primer. However, Teklewold & Becker (2006) observed 8.7 to 9.8 marker loci per primer in *Brassica carinata*. These differences might be due to the protocols used or material used by those workers.

Allele size ranged from 60-330 base pairs in SSR markers used. The observed polymorphic bands in present research were in accordance to previous findings on brassica (Cartea *et al*., 2002; Teklewold & Becker, 2006). The primer BRMS-019 and BRMS-040 gave maximum polymorphic bands (4) which were supported by the study of Pradhan *et al*., (2004) and Roman *et al*., (2004) who stated that higher numbers of bands for each primer reflected the existence of larger genetic diversity among the genotypes under investigation. Primers with higher polymorphic bands are more desirable in studying genetic diversity and discrimination of the genotypes. In our study for a number of primer combinations more polymorphic loci were detected than were mapped by Lowe *et al*., (2002) for a few SSRs not all mapped loci could be identified. This was supported by Hasan *et al*., (2006) who stated that this is not unexpected when comparing a large set of genotypes with selected cross parents.

Table 2. List of the microsatellites analyzed, observed size, and amplified alleles, polymorphic alleles, rate of polymorphic loci and PIC values.

Marker	Observed size (bp)	Amplified alleles	Polymorphic alleles	Rate of polymorphic Loci (%)*	PIC value
BRMS-008	80-180	3	3	100	0.646
BRMS-019	100-250	4	4	100	0.726
BRMS-040	100-260	4	4	100	0.725
Na10-E02	60-200	3	3	100	0.655
Na14-D07	130-150	2	2	100	0.490
Ol10-A05	130-180	2	2	100	0.490
Ol11-H02	200-220	2	2	100	0.497
Ra2-A11	220-330	3	3	100	0.628
Ra2-D04	170-180	2	1	50	0.481
Ra2-E03	280-300	2	2	100	0.395
Ra2-E11	60-200	3	3	100	0.651
Ra2-E12	150-200	3	3	100	0.632
Total	**----**	**33**	**32**	**----**	**7.016**
Average	**----**	**2.75**	**2.66**	**95.83**	**0.584**

$$\text{*Rate of polymorphic loci} = \frac{\text{Number of polymorphic bands}}{\text{Number of amplified bands}} \times 100$$

Table 3. Groups formed by 88 individual plants of a cross (N-501×C-118) and their parents.

Group	Sub-cluster	No. of plants	Numbering
I	Cluster 1	2	501, 5
	Cluster 2	3	1, 3, 6
	Cluster 3	2	2, 4
	Cluster 4	3	13, 14, 15
	Cluster 5	1	41
II	Cluster 1	2	7, 8
	Cluster 2	3	10, 11, 12
	Cluster 3	3	16, 17, 18
	Cluster 4	3	19, 20, 23
	Cluster 5	2	22, 24
III	Cluster 1	8	37, 39, 40, 47, 38, 45, 46, 49
	Cluster 2	6	42, 57, 53, 54, 56, 58
	Cluster 3	4	50, 51, 52, 55
	Cluster 4	1	44
	Cluster 5	4	61, 62, 63, 64
	Cluster 6	2	65, 69
	Cluster 7	4	66, 67, 68, 70
	Cluster 8	1	43
	Cluster 9	3	48, 59, 60
IV	Cluster 1	1	71
	Cluster 2	3	72, 73, 74
	Cluster 3	2	75, 76
V	Cluster 1	2	21, 32
VI	Cluster 1	5	25, 27, 29, 26, 30
	Cluster 2	4	28, 36, 34, 31
	Cluster 3	2	33, 35
VII	Cluster 1	1	77
	Cluster 2	3	78, 80, 81
	Cluster 3	2	79, 118
	Cluster 4	4	82, 83, 85, 84
	Cluster 5	3	86, 88, 87
VIII	Cluster 1	1	9

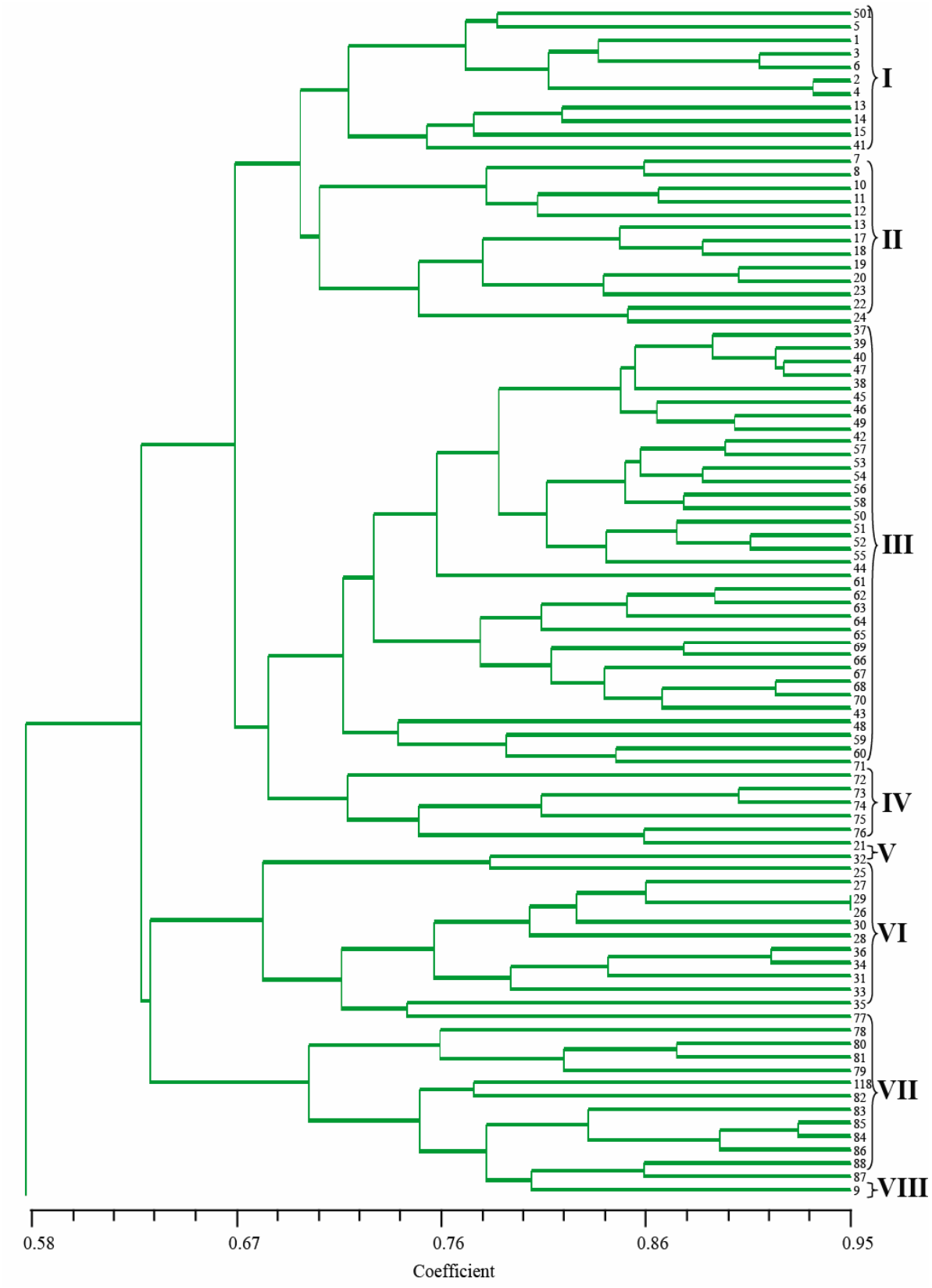

Fig. 2. The dendrogram of parents i.e. *B. napus* (N-501) and *B.campestris* (C-118) along with 88 individual F2 PROGE of cross (N-501xC-118) constructed to study genetic diversity using 12 SSR markers. Numbering correspond to individual plant number.

Polymorphic information content (PIC) is the important feature of the molecular study. Seven out of 12 primers had PIC value greater than 0.5 resulting in unique SSR fingerprints. These results were in agreement with that obtained by Tonguc & Griffiths (2004), who used 13 SSR primer pairs with similar PIC values to differentiate 49 out of 54 cultivars of broccoli, cabbage and cauliflower. PIC values ranged from 0.04 to 0.80 with an average of 0.50 in *B. napus* (Plieske & Struss, 2001). The PIC value is a function of allele number and frequency. Thus, markers with more alleles had larger PIC values. For example, BRMS-019 and BRMS-040 represented the largest number of alleles (4) had the highest PIC (0.72). Averages PIC of all SSR markers were 0.58 indicating that the hybrids were genetically diverse. Sinior *et al*., (1998) stated that if there is no heterozygosity, PIC and diversity index will be identical. Therefore, in homozygote genotypes, there is no need to estimate gene diversity and information from PIC will be sufficient. The present study detected 96% polymorphism which was in agreement with other researcher's studies: 56% polymorphism in 11 parental lines of oilseed rape using 34 reproducible primers was detected by Yu *et al*., (2005) while Teklewold & Becker (2006) noticed 20-83% polymorphism in 43 Ethiopian mustard cultivars using 50 RAPD primers**.** Two of the hybrid progenies 27 and 29 were not differentiated in our study and perhaps were very closely related.

Conclusions

From the data obtained, several conclusions can be drawn: (i) Genetic differences were observed among F_2 progenies and their parents. (ii) F_2 progenies produced bands that were either similar to or different from parents N-501 and C-118. (iii) The F_2 progenies were clustered into different groups giving strong indications that F_2 was a segregating population with high genetic diversity. (iv) SSR markers proved to be useful in the germplasm characterization of *B. napus* (N-501) and *B. campestris* (C-118) species.

Acknowledgements

The authors gratefully acknowledge the financial support from Higher Education Commission Islamabad Pakistan through project ILPUFU and Pakistan Agricultural Research Council, Islamabad under the Research for Agricultural Development Program (RADP) for this study. We would also like to thank the Plant Genetic Resources Institute, National Agricultural Research Center, Islamabad for extending laboratory facilities for the accomplishment of present investigation.

References

Ahmad R., Farhatullah, C. F. Quiros, H. Rehman and Z. A. Swati. 2014. Genetic diversity analyses of Brassica napus accessions using SRAP molecular markers. *Plant Genet. Resour*. 12(1): 14–21 (doi:10.1017/S147926211300021X).

Ahmad R., Farhatullah, R.S. Khan and C. F. Quiros. Inheritance of fertility restorer gene for cytoplasmic male-sterility in B. napus and identification of closely linked molecular markers to it. Euphtica. 194(3):351-360 (doi: 10.1007/s10681-013-0942.y).

Ali Y., Farhatullah., H. Rahman., A. Nasim., S. M. Azam and A. Khan. 2013. Heritability and correlation analysis for morphological and biochemical traits in *brassica carinata*. Sarhad J. Agric. 29(3): 359-369.

Azam, S.M., Farhatullah, A. Nasim, S. Shah and S. Iqbal. 2013. Correlation studies for some agronomic and quality traits in Brassica napus L. Sarhad J. Agric. 29(4): 547-550.

Bus, A., N. Korber, R.J. Snowdon and B. Stich. 2011. Patterns of molecular variation in a species-wide germplasm set of *Brassica napus. Theor. Appl. Genet*., 123: 1413-1423.

Cartea, M.E., A. Picoaga, P. Soengas and A. Ordas. 2002. Morphological characterization of kale populations from northwestern Spain. *Euphytica*, 129: 25-32.

Cho, Y.G., T. Ishii, S. Temnykh, X. Chen, L. Lipovich, S.R. McCouch, W.D. Park, N. Ayres and S. Cartinhour. 2000. Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). *Theor. Appl. Genet*., 100: 713-722.

Dib, C., S. Faure, C. Fizames, D. Samson, N. Drouot and A. Vignal. 1996. A comprehensive genetic map of human genome based on 5,264 microsatellites. *Nature*, 380: 152-154.

Dietrich, W.F., J. Miller, R. Steen, M.A. Merchant, D. Damron-Boles and Z. Husain. 1996. A comprehensive genetic map of the mouse genome. *Nature*, 380: 149-152.

Downey, R.K. and S.R. Rimmer. 1993. Agronomic improvement in oilseed Brassica. *Adv. Agron*., 50: 1-66.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12: 13-15.

Eujayl, I., M. Sorrels, M. Baum, P. Walters and W. Powell. 2001. Assessment of genotypic variation among cultivated durum wheat based on EST-SSRs and genomic SSRs. *Euphytica*, 119: 39-43.

Gupta, P.K. and R.K. Varshney. 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica*, 113: 163-185.

Hasan, M., F. Seyis, A.G. Badani, J. Pons-Ku¨hnemann, W. Friedt, W. Lu¨hs and R.J. Snowdon. 2006. Analysis of genetic diversity in the *Brassica napus* L. gene pool using SSR markers. *Genet. Resour. Crop Evol*., 53: 793-802.

Kimber, D.S. and D.I. McGregor. 1995. The species and their origin, cultivation and world production. In: *Brassica Oilseeds: Production and Utilization*. (Eds.): D. Kimber and D.I. McGregor. CABI Publishing, Wallingford, UK, pp.1-9.

Lowe, A.J., A.E. Jones, A.F. Raybould, M. Trick, C. Moule and K.J. Edwards. 2002. Transferability and genome specificity of a new set of microsatellite primers among *Brassica* species of the U triangle. *Mol. Ecol. Notes*, 2: 7-11.

Maughan, P.J., M.A. Saghai-Maroof and G.R. Buss. 1995. Microsatellite and amplified length polymorphisms in cultivated and wild soybean. *Genome*, 38: 715-728.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. *Plant J*., 3: 175-182.

Nei, M. and W.H. Li. 1979. Mathematical models for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA*, 76: 5269-5273.

Pillen, K., A. Binder, B. Kreuzkam, L. Ramsay, R. Waugh, J. Forster and J. Leon. 2000. Mapping new EMBL-derived barley microsatellites and their use in differentiating German barley cultivars. *Theor. Appl. Genet*., 101: 652-660.

Plieske, J. and D. Struss. 2001. Microsatellite markers for genome analysis in *Brassica*. I. Development in *Brassica napus* and abundance in *Brassicaceae* species. *Theor. Appl. Genet*., 102: 689-694.

Pradhan, A., G. Yan and J.A. Plummer. 2004. Development of DNA fingerprinting keys for the identification of radish cultivars. Australian *J. Exp. Agric*., 44: 95-102.

Prasad, M., R.K. Varshney, J.K. Roy, H.S. Balyan and P.K. Gupta. 2000. The use of microsatellites for detecting DNA polymorphism, genotype identification and genetic diversity in wheat. *Theor. Appl. Genet*., 100: 584-592.

Rabbani M.A., M. S. Masood, Z. K. Shinwari and K. Y. Shinozaki.2010.Genetic analysis of basmati and non-basmati Pakistani rice (*Oryza Sativa* L.) cultivars using microsatellite markers. *Pak. J. Bot*., 42(4): 2551-2564.

Roman, S., D. Lenka, C. Vladislav and O. Jaroslava. 2004. Fluorescence based AFLPs occur as the most suitable marker system for oilseed rape cultivar identification. *J. Appl. Genet*., 45: 161-173.

Rudolph, B., M.I. Uzunova and W. Ecke. 2000. Development of microsatellite markers for the analysis of genetic diversity in rapeseed (*Brassica napus* L.). 3rd ISHS International Symposium on Brassica/12th Crucifer Genetics Workshop. 5-9 Sep., Horticulture Research International Wellesbourne, CV35 9EF, UK.

Sandhu, S.K. and V.P. Gupta. 2000. Interspecific hybridization among digenomic species of *Brassica. Crop Improv*., 27: 195-197.

Senior, M.L., J.P. Murphy, M.M. Goodman and C.W. Stuber. 1998. Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Sci*., 38: 1088-1098.

Shinwari, S., A.S. Mumtaz, M.A. Rabbani, F. Akbar and Z.K. Shinwari. 2013a. Genetic divergence in Taramira (*Eruca sativa* L.) germplasm based on quantitative and qualitative characters. *Pak. J. Bot*., 45(SI): 375-381

Shinwari, S., F. Akbar , M.A. Rabbani, A.S. Mumtaz and Z.K. Shinwari. 2013. Evaluation of Genetic diversity in different genotypes of *Eruca sativa* from Pakistan by SDS-Page analysis. *Pak. J. Bot*., 45(4): 1235-1240

Snowdon, R.J. and W. Friedt. 2004. Molecular markers in Brassica oilseed breeding: current status and future possibilities. *Plant Breed*., 123: 1-8.

Sultan, M., N. Zakir, M.A. Rabbani, Z.K. Shinwari1 and M.S. Masood. 2013. Genetic diversity of guar (*Cyamopsis tetragonoloba* L.) landraces from Pakistan Based on RAPD Markers. *Pak. J. Bot*., 45(3): 865-870.

Teklewold, A. and H.C. Becker. 2006. Geographic pattern of genetic diversity among 43 Ethiopian mustard (*Brassica carinata* A. Braun) accessions as revealed by RAPD analysis. *Genet. Resour. Crop Evol*., 53: 1173-1185.

Tonguc, M. and P.D. Griffiths. 2004. Genetic relationships of Brassica vegetables determined using database derived sequence repeats. *Euphytica*, 137:193-201.

Turi, N. A., Farhatullah, M.A. Rabbani and Z.K. Shinwari. 2012. Genetic diversity in the locally collected *Brassica* species of Pakistan based on microsatellite markers. *Pak. J. Bot*., 44(3): 1029-1035.

Uzunova, M.I. and W. Ecke. 1999. Abundance, polymorphism, and genetic mapping of microsatellites in oilseed rape (*Brassica napus*). *Plant Breed*., 118: 323-326.

Yu, C.Y., S.W. Hu, H.X. Zhao, A.G. Guo and G.L. Sun. 2005. Genetic distances revealed by morphological characters, isozymes, proteins and RAPD markers and their relationships with hybrid performance in oilseed rape (*Brassica napus* L.). *Theor. Appl. Genet*., 110: 511-518.

Zada, M., N. Zakir, M.A. Rabbani and Z.K. Shinwari. 2013. Assessment of genetic variation in Ethiopian mustard (*Brassica Carinata* A. Braun) germplasm using multivariate techniques. *Pak. J. Bot*., 45(S1): 583-593

Zada, M., Z.K. Shinwari, Nahida Zakir and M.A. Rabbani. 2013a. Study of total seed storage proteins in Ethiopian mustard (*Brassica carinata* A. Braun) germplasm. *Pak. J. Bot*., 45(2): 443-448.

Zeb, A., Z.K. Shinwari and T. Mahmood. 2011. Molecular markers assisted genetic characterization of some selected Wild *Poaceae* species. *Pak. J. Bot*., 43(5): 2285-2288.

Zhang, G. and W. Zhou. 2006. Genetic analyses of agronomic and seed quality traits of synthetic oilseed *Brassica napus* produced from inter specific hybridization of *Brassica campestris* and *Brassica olearacea. J. Genet*., 85: 45-51.

(Received for publication 17 September 2012)