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# GENETIC DIFFERENTIATION AMONG SEXUALLY COMPATIBLE RELATIVES OF *Brassica napus* L.

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Analysis of gene flow between *Brassica napus* L. and its sexually compatible relatives that could be found in the wild in Slovenia was performed by microsatellite analysis using fifteen selected primer pairs. Genotypes included in the study were obtained from the field survey of sexually compatible relatives of *B. napus* in natural habitats around Slovenia and from reference collections. Two different wild species of all the presented sexually compatible relatives of *B. napus* were found in Slovenia, *B. rapa* and *Sinapis arvensis*. The reference genotypes included varieties and wild forms from internal collections as marketable seeds or from gene banks. Reference genotypes were represented by the following species and subspecies: *B. napus* ssp. *napobrassica*, *B. napus* ssp. *napus*, *B. nigra*, *B. oleracea*, *B. rapa* ssp. *oleifera*, *Diplotaxis muralis*; *D. tenuifolia*, *Raphanus raphanistrum*, *R. sativus*, *R. sativus* var. *oleiformis*, *Rapistrum rugosum*, *S. alba* and *S. arvensis*. Estimation of gene flow described by average number of migrants was 0.72 followed by 0.20 migrants. Due to the observed gene migrations, genetic drift and selection, Hardy-Weinberg equilibrium was not met. The mean number of alleles over all loci was 16.9, the average polymorphic information content was 0.43. We found four highly divergent and polymorphic loci (Na12-C08, Na10-A08, Ni3-G04b and BRMS-050) at statistically significant level ($p<0.05$) of gene flow detected. Over all gene diversity intra-individual among populations (0.55) was lower than inter-individual among population (0.77). The results of genetic linkages based standard genetic distance and unweighted pair group method with arithmetic mean clustering method, generally divided the genotypes in three divergent groups. Similar results were obtained by principal coordinate analysis where three main groups were constructed according to three factors. A real number of genetic clusters demonstrated a

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clear separation between populations, where only one was comprised from genotypes from other origins. The 30.7 % out-crossing rate of *B. rapa* and *S. arvensis* populations from filed survey represents the potential for spontaneous inter-and intra-specific gene flow under Slovenian production area.

*Key words: Brassica napus* L./ sexually compatible relatives/ microsatellites/ gene flow/ genetic diversity/ out-crossing rate

## INTRODUCTION

*Brassica napus L.* is the most important cultivated crop species in the Brassicaceae family. The species is divided into two subspecies comprising *B. napus* ssp. *napobrassica* and *B. napus* ssp. *napus* (SNOWDON *et al.,* 2007). *B. napus* (AACC genome, 2n=38) originated through spontaneous inter-specific hybridization between *B. rapa* L. (AA genome, 2n=20) and *B. oleracea* L. (CC genome, 2n=18) (FRIEDT and SNOWDON, 2009). The origin of *B. napus* was firstly described by Triangle of U (NAGAHARU, 1935). It explains the evolution and the relationships between six oil and vegetable species from the Brassica genus and the combination of three genomes; *B. nigra* L.*, B. oleracea, B. rapa*, which are ancestral to the other three inter-specific hybrids *B. carinata* L.*, B. juncea* L. and *B. napus*. In nature, spontaneous inter-specific hybridization of *B. napus* is possible with sexually compatible species (relatives which have high pollination affinity with *B. napus*) from Brassicaceae family. The relatives of *B. napus* are cultivated as field crops, but can also appear as weeds in the farming systems or as wild plants outside cultivated areas (e.g. field edges, shelterbelts, road verges, slag heaps, embankments) (ESTHAM and SWEET, 2002; PASCHER *et al*., 2010). Species which are closely related to *B. napus* are: *B. rapa, B. juncea, B. oleracea, B. nigra, Hirchfeldia incana* L.*, Raphanus raphanistrum* L.*, Sinapis arvensis L., Diplotaxis erucoides* L.*, D. tenuifolia* L.*, D. muralis* L.*, S. alba* L.*, R. sativus* L. and *Rapistrum rugosum* L. (ESTHAM and SWEET, 2002; TREU and EMBERLIN, 2000). The possibility for inter-specific hybridization depends on physical distance between two species, synchrony of flowering, method of pollen dissemination, parental genotype characteristics and environmental conditions.

In Slovenia, *B. napus* is grown as oilseed or fodder rape and also as swedes. In 2011, it was cultivated on 4770 ha, accounting for about 1 % of the total production area (SURS, 2012). According to Jogan et al. (2001) different sexually compatible relatives of *B. napus* are spread throughout the Slovenian territory: *B. rapa, B. oleracea, B. nigra, Raphanus raphanistrum, R. sativus, D. muralis, D. tenuifolia, S. alba, S. arvensis* and *R. rugosum*. The species *S. alba, B. rapa, B. oleracea* could either be cultivated or appear as weedy or wild (PIPAN *et al*., 2012). In the case of coexistence of different production systems, including genetically modified (GM) varieties, uncontrolled gene flow between different forms of *B. napus* or sexually compatible wild relatives might have an impact on the varietal purity and crop quality (ELLING *et al*., 2009). In Slovenia, genetically modified plants are not cultivated; there has been even no field trials with GM plants yet (ŠUŠTAR-VOZLIČ *et al*., 2010).

Microsatellite data are informative and well applied for the assessment of inter- and intra-specific gene flow of *B. napus* (PIPAN *et al*., 2013). HASAN *et al.* (2006) published a comparative study using microsatellite markers isolated from *B. oleracea* and *B. rapa* genomes as progenitor species of *B. napus*. In the molecular studies of *B. napus* (oilseed rape, volunteer and feral populations) only a few highly polymorphic microsatellite markers isolated from *B. napus* were used (ELLING *et al*., 2009; PASCHER *et al.,* 2010). Those studies were performed on

intra-specific level and gave important conclusions about the origin of feral populations and distinguishing between different commercial varieties of oilseed rape.

The aim of this study was to assess the possibilities of gene flow among different subspecies of *B. napus* and across its sexually compatible relatives from Brassicaceae which are present in Slovenia using microsatellite markers. Additionally, we established informativity and transferability of selected microsatellite markers with different motif construction and formed different focal species in related Brassicaceae species. We also assessed the genetic diversity, relationships and origin of plants sampled in the field among analysed populations in comparison with the reference accessions from gene banks.

## MATERIALS AND METHODS

### Field survey

Field survey was carried out during the flowering season of biennial *B. napus* in 2008 and 2010. Prior to field survey, we identified locations where sexually compatible relatives of *B. napus* were present. According to the map of relatives persistent in Slovenia (JOGAN *et al.,* 2001; PIPAN *et al.,* 2008), internal evidences of potential locations and case studies from two national monitoring plans of oilseed rape (MEGLIČ *et al*., 2008; ŠUŠTAR-VOZLIČ *et al.,* 2008), we identified macro-locations on regional level (regions among Slovenia with higher production share of oilseed rape). We considered extension of oilseed rape production described in PIPAN *et al*., 2011. These locations were the main orientation points for actual sampling. Their spatial position was placed around transportation infrastructure including road verges, construction sites, rest areas by the roads, mounds, uncultivated areas and field margins. Among macro-locations we identified micro-locations where species of interest were actually grown, including those which were found by chance along the way.

Two different wild species of all the presented sexually compatible relatives of *B. napus* were found in Slovenia, *B. rapa* (four populations) and *S. arvensis* (twenty two populations) (Table 1). The sampled populations were included in the study.

Each sample consisted of five individual plants per one population on the micro-location (max area of $5m^2$). From each plant one young, healthy leaf was sampled. Samples were labelled and stored at $-20^oC$ for further analysis.

### Reference material

The reference genotypes included all sexually compatible species of *B. napus* which are present in Slovenia as wild or weedy plants (according to JOGAN et al., 2001) and relatives which have been widely grown in Slovenia over the past ten years including two varieties of *B. napus* ssp. *napus* (as oilseed rape grown until 1984) and *B. napus* ssp. *napobrassica* (as swede grown mainly for seed production) (Table 2). Seeds from selected genotypes of reference material were supplied from the national collections (standard seed material and certified marketable seed) and from European gene banks. Seedlings of each reference species were grown in a greenhouse until the fourth true-leaf stage. One healthy leaf from each of the five plants from one reference accession were bulked together and stored at $-20^oC$.

*Table 1. Sampled populations from field survey with spatial information (GK referees to Gauss-Krüger coordinate system) including habitat type of the location.*

| Sample name | Species | GK (Y) [m] | GK (X) [m] | Habitat type |
|---|---|---|---|---|
| D2 | *B.rapa* | 482007.91 | 113569.27 | road verge |
| D3 | *B. rapa* | 485309.31 | 114669.93 | mound |
| D4 | *B. rapa* | 484992.01 | 085683.38 | road verge |
| D5 | *B. rapa* | 512445.34 | 076370.81 | construction site |
| K2 | *S. arvensis* | 473093.34 | 112618.60 | mound |
| K3 | *S. arvensis* | 461803.84 | 119607.07 | field margin |
| K4 | *S. arvensis* | 437371.60 | 133957.45 | construction site |
| K5 | *S. arvensis* | 437126.24 | 134050.76 | construction site |
| K6 | *S. arvensis* | 451543.05 | 120956.44 | road verge |
| K7 | *S. arvensis* | 548997.40 | 084065.57 | mound |
| K8 | *S. arvensis* | 510525.07 | 089618.60 | road verge |
| K9 | *S. arvensis* | 403740.60 | 045823.22 | construction site |
| K10 | *S. arvensis* | 485309.31 | 114669.93 | road verge |
| K11 | *S. arvensis* | 512616.69 | 123611.39 | road verge |
| K12 | *S. arvensis* | 519116.18 | 121453.28 | mound |
| K13 | *S. arvensis* | 455388.24 | 098015.90 | road verge |
| K14 | *S. arvensis* | 513086.05 | 077107.46 | road verge |
| K15 | *S. arvensis* | 482007.92 | 113569.28 | road verge |
| K16 | *S. arvensis* | 467933.21 | 101205.01 | field margin |
| K17 | *S. arvensis* | 458945.66 | 100489.95 | road verge |
| K18 | *S. arvensis* | 420557.60 | 060632.74 | rest area by the road |
| K19 | *S. arvensis* | 462865.39 | 010552.51 | construction site |
| K20 | *S. arvensis* | 466086.02 | 102070.78 | rest area by the road |
| K21 | *S. arvensis* | 460128.47 | 106821.11 | uncultivated field |
| K22 | *S. arvensis* | 458945.66 | 100489.95 | road verge |
| K23 | *S. arvensis* | 447439.80 | 093705.21 | road verge |

DNA extraction

Frozen leaf material was bulked from equal weights comprised from five leaves, both for field survey material and for reference genotypes. Total DNA was extracted with BioSprint 15 DNA Plant Kit (Qiagen) on KingFisher (Thermo) isolation robot following manufacturer's instructions.

Microsatellite analysis and genotyping

Fifteen microsatellite markers were used for genotyping (Table 3). PCR reactions were performed in a final volume of 11.5 µl, containing 30 ng of genomic DNA and following reagents with starting concentrations of: 10 x PCR buffer (Biotools), 10 mM of each dNTP's, 50 mM $MgCl_2$ (Biotools), 10 µM of each primer, 10 µM 5' fluorescently labelled universal primer (6-FAM, NED, HEX) and 0. 5 U of *Taq* DNA polymerase (Biotools).

The forward primer of each microsatellite marker was appended with 18 bp tail sequence 5'-TGTAAAACGACGGCCAGT-3' (M13(-21) as described by Schuelke 2000). PCR analyses were performed on ATC 401 (Apollo Instrumentations) under the following 'touch-down' conditions: 94 °C for 4 min, fifteen cycles at 94 °C for 1 min, auto decrement temperature from 60 °C for 0.7 °C per cycle for 30 s, 72 °C for 1 min, followed by 23 cycles at 94 °C for 30 sec, 53 °C for 30 s, 72 °C for 1 min and final extension for 5 min at 72 °C. Fragment analysis was performed on 3130XL Genetic Analyzer (Applied Biosystems), the allele lengths were determined by comparison with size standard GeneScan-350 ROX (Applied Biosystems) using GeneMapper 4.0 (Applied Biosystems).

*Table 2. List of reference genotypes of B. napus and its sexually compatible relatives, including the information on variety, accession number and donor institution.*

| Sample name | Reference genotype | Variety/type | Accession number/label | Donor |
|---|---|---|---|---|
| A1 | *B. napus* ssp. | 'Hofmanova | | KIS |
| A2 | *B. napus* ssp. | 'Rumena maslena' | | KIS |
| A3 | *B. napus* ssp. *napus* | 'Bienvenue' | CR 181 | IPK |
| A4 | *B. napus* ssp. *napus* | 'Viva' | CR 1043 | IPK |
| B1 | *B. nigra* | wild | 15O0600021 | RICP |
| B2 | *B. nigra* | wild | 15O0600018 | RICP |
| C1 | *B. oleracea* var. *capitata* | 'Ditmar' | | SL |
| C2 | *B. oleracea* var. *capitata* | 'Varaždinsko' | | SL |
| D1 | *B. rapa* ssp. *oleifera* | 'Marino' | 15O0300012 | RICP |
| D6 | *B.apa* ssp. *oleifera* | 'Jugoslavsky' | 15O0300007 | RICP |
| D7 | *B. rapa* ssp. *oleifera* | 'Perko' | CR 2885 | IPK |
| E1 | *D. muralis* | wild | DIPLO 5 | IPK |
| F1 | *D. tenuifolia* | wild | | SL |
| G1 | *R. raphanistrum* ssp. | wild | RA 728 | IPK |
| H1 | *R. sativus* var. *major* | 'Acord' | 09H7300066 | RICP |
| H2 | *R. sativus* var. *radicula* | 'Zlata' | 09H7400138 | RICP |
| H3 | *R. sativus* var. *oleiformis* | 'Radical' | | KIS |
| H4 | *R. sativus* var. *oleiformis* | 'Toro' | | KIS |
| I1 | *R. rugosum* ssp. | wild | RAP 1 | IPK |
| J1 | *S. alba* | 'Achilles' | | SL |
| J2 | *S. alba* | 'Emergo' | CR 1806 | IPK |
| J3 | *S. alba* | 'Mirly' | CR 2818 | IPK |
| J4 | *S. alba* | 'Serval' | CR 1825 | IPK |
| J5 | *S. alba* | 'Siko' | CR 1817 | IPK |
| J6 | *S. alba* | 'Torpedo' | | SL |
| K1 | *S. arvensis* | wild | CR 2591 | IPK |

KIS: Agricultural Institute of Slovenia, internal seed standards; IPK: Leibniz Institute of Plant Genetics and Crop Plant Research; RICP: Research Institute of Crop Production Prague-Ruzyne; SL: Semenarna Ljubljana, certificated marketable seeds.

*Table 3. Characteristics of microsatellite markers from different sources including its origin (focal species), motif and repeat pattern and expected size of amplified fragment.*

| Locus | Focal species | Motif type | Repeat number | Expected length [bp] | Reference |
|---|---|---|---|---|---|
| BN83B1 | *Brassica* | GA/AAG | 11/4 | 186 | SZEWC- |
| BRMS- | *Brassica* | AAT/TC/TT | 4/19/3 | 186 | SUWABE et al., |
| MR187 | *Brassica* | AG/AGG | 23/5 | 146 | UZANOVA and |
| Na10- | *B. napus* | GA/CT | 21/21 | 167 | LOWE et al., |
| Na12- | *B. napus* | GT/CA | 11/11 | 158 | LOWE et al., |
| Na12-C08 | *B. napus* | GA/CT | 50/50 | 333 | LOWE et al., |
| Ni3-G04b | *B. nigra* | GA/CT | 18/18 | 123 | LOWE et al., |
| Ni4-D09 | *B. nigra* | GA/CT | 25/25 | 206 | LOWE et al., |
| Ni4-H04 | *B. nigra* | GT/CA | 14/14 | 172 | LOWE et al., |
| Ol12-D05 | *B.* | GA/CT | 32/32 | 123 | LOWE et al., |
| Ol12-E03 | *B.* | GGC/CCG | 9/9 | 117 | LOWE et al., |
| Ol13-E08 | *B.* | GA/CT | 11/11 | 171 | LOWE et al., |
| Ra2-A01 | *B. rapa* | GA/CT | 19/19 | 124 | LOWE et al., |
| Ra2-E12 | *B. rapa* | GA/CT | 32/32 | 189 | LOWE et al., |
| Ra3-H10 | *B. rapa* | GA/CT | 23/23 | 141 | LOWE et al., |

Statistical analysis

Descriptive statistics among loci for over all populations including the observed heterozygosity (Ho), the expected heterozygosity (He), the total expected heterozygosity (Ht) and the allele frequency based correlation indices (Fis, Fst and Fit) were calculated in Populations 1.2.28 (LANGELLA, 2002). Additional degree of population differentiation was calculated by multilocus estimations of over all populations with Fst and Gst statistics according to NEI and CHESSER (1983) in Genetix 4.05 (BELKHIR *et al*., 1999). The average polymorphic information content (PIC) among over all populations for the included loci was calculated by MsTollkit (PARK, 2001) add-in Microsoft Excel. Calculations of allelic patterns across populations (number of different alleles, effective alleles, locally common alleles and private alleles) including Shannon's information index was performed in GenAlEx (PEAKALL and SMOUSE, 2006). The average of corresponding probability of identity (observed frequencies of identical pairs) over all samples for specific loci was calculated by ANOVA by WEIR and COCKERHAM (1984) implemented in Genepop 4.1.0 (ROUSSET, 2008). Hierarchical island model with 20000 simulations was used for detecting loci under selection on the basis of observed heterozygosity (Ho), observed Fst and Fst P value which were calculated in Arlequin 3.5.1.2 (EXCOFFIER and LISCHER, 2010). The additional estimation of gene flow among populations was made by calculating the effective number of migrants using private allele method of SLATKIN (1985) in Genepop 4.1.0 software reporting the corrected estimated value of BARTON and SLATKIN (1986). Estimation of out-crossing rate among samples from filed survey was provided through transformation of the fixation index (t = (1-F)/ (1+F)) calculated in GenAlEx software package (PEAKALL and SMOUSE, 2006). The global Hardy-Weinberg equilibrium (HWE) for each locus within populations was estimated by Fis statistic (WEIR and COCKERHAM, 1984) using the exact test included in Genepop 4.1.0 software. The SLATKIN's (1985) distance matrix between populations of significant Fst P values with 2000 permutations was calculated in Arlequin

(EXCOFFIER and LISCHER, 2010). NEI's standard genetic distance–Ds (1972) was computed from allele frequencies with using unweighted pair group method with arithmetic mean (UPGMA) algorithm in Populations software (LANGELLA, 2002) by bootstrapping 1000 times. The tree of genetic distances was constructed using TreeView (PAGE, 1996). Principal coordinate analysis (PCoA) was performed via covariance matrix with data standardization in GenAlEx (PEAKALL and SMOUSE, 2006). Structure 2.3.3 software (PITCHARD *et al*., 2009) was included for inferring population structure using Bayesian method. By computing *ad hoc* statistic, the number of genetic clusters (K) was estimated according to EVANNO *et al.,* 2005. Twenty independent runs for each K (from 1 to 16) in the case of admixture model were performed and burning period of 10000 followed by 100000 Markov Chain Monte Carlo (MCMC) repeats were used.

## RESULTS

### Microsatellite analysis

In total, fifty-two bulked samples were analysed using fifteen microsatellite markers. High mean frequency of private alleles (0.203) and adequate number of migrants after correction for size (0.721) imply movement of individuals into new populations and introduction of new alleles into the populations (gene flow). The parameters of variability for each locus including F statistics are presented in Table 4. The mean number of alleles over all loci was 16.9, the most informative microsatellite marker was Na12-A07. For this locus the highest proportion of heterozygotes (Ho=0.697), genetic diversity between individuals (He=0.579) and PIC value (0.514) were detected. The average of total gene diversity or expected heterozygosity among all samples was very high (0.83), as estimated from the pooled allele frequencies. According to hierarchical island model (100 simulated demes and 20000 coalescent simulations) we found four highly divergent and polymorphic loci (Na12-C08, Na10-A08, Ni3-G04b and BRMS-050) with statistically significant level ($p<0.05$) of gene flow detecting. Locus by locus allele frequency based correlation indices included a set of parameters, Fis, Fst and Fit (Table 4). The average inbreeding coefficient (Fis=-0.07) was slightly negative which indicated that the individuals are more heterozygous than expected. The average degree of gene differentiation among populations over all loci (Fst) was 0.42 and the average excess of heterozygotes among populations (Fit) was 0.37. Fst and Fit values (relative to the combined population) generally reflect the probability of identical alleles by descent. Multilocus estimation (non-biased) described by NEI and CHESSER (1983) was calculated as Gst approach to account the sampling error because of large number of loci proportionally to complete the number of samples. The Gst was 0.12 representing a proportion of gene diversity measured among population. The average 1-Q values over all loci, estimating gene diversity, were calculated intra-individuals (0.55) and inter-individuals among the population (0.77) (data not shown). Shannon's information index shows the highest genetic diversity in populations of *B. rapa* and *S. arvensis* (Figure 1). On the basis of 93.3 % polymorphic loci, the out-crossing rate among *B. rapa* and *S. arvensis* from field survey was 0,693 which referees 30.7 % proportion of spontaneous gene flow between these two naturally appeared species under Slovenian production conditions.

We tested 165 locus-population combinations for Hardy-Weinberg equilibrium (HWE). Global test showed deviations from HWE ($p<0.05$) in all locus-population combinations (data not shown) due to the change of allele frequencies through generations (evolution). The mean number of alleles per locus varied from 1.308 (population of *R. rugosum*) to 9.429 (population of *S. arvensis*). The average of unbiased expected heterozygosity was 0.62 while the mean observed

heterozygosity was moderate (0.52) in all populations (Table 5). The population specific fixation index varied from -0.055 (population of *B. nigra*) to 0.463 (population of *B. oleracea*) but the populations of *S. alba* and *S. arvensis* had high Fis as well, indicating high estimation of genetic diversity in those populations (Table 5).

*Table 4. Descriptive statistics for each microsatellite locus in analysis including allele lenght, number of alleles per locus, parameters of variability (Ho, He, Ht and PIC) and correlation indices (Fis, Fst and Fit).*

| Locus | Allele | No. of | Ho | He | Ht | PIC | Fis | Fst | Fit |
|---|---|---|---|---|---|---|---|---|---|
| Na12-A07 | 162-222 | 22 | 0.69 | 0.57 | 0.910 | 0.51 | -0.204 | 0.364 | 0.234 |
| Ni4-H04 | 116-234 | 14 | 0.35 | 0.36 | 0.688 | 0.34 | 0.042 | 0.465 | 0.487 |
| Ol12-E03 | 88-145 | 14 | 0.63 | 0.51 | 0.799 | 0.46 | -0. 225 | 0.354 | 0.209 |
| Ra2-A01 | 88-144 | 14 | 0.76 | 0.54 | 0.886 | 0.48 | -0.390 | 0.384 | 0.143 |
| BN83B1 | 103-272 | 18 | 0.28 | 0.39 | 0.912 | 0.34 | 0.268 | 0.570 | 0.685 |
| Na12-C08 | 255-345 | 14 | 0.47 | 0.50 | 0.961 | 0.44 | 0.059 | 0.473 | 0.504 |
| Ni4-D09 | 124-218 | 14 | 0.30 | 0.39 | 0.768 | 0.35 | 0.243 | 0.484 | 0.609 |
| Ol13-E08 | 100-254 | 15 | 0.66 | 0.53 | 0.871 | 0.46 | -0.236 | 0.382 | 0.237 |
| Ra3-H10 | 96-202 | 18 | 0.69 | 0.53 | 0.746 | 0.47 | -0.285 | 0.277 | 0.071 |
| MR187 | 101-214 | 20 | 0.39 | 0.46 | 0.790 | 0.40 | 0. 153 | 0.412 | 0.502 |
| Na10-A08 | 107-227 | 21 | 0.64 | 0.55 | 0.859 | 0.49 | -0.144 | 0.349 | 0.255 |
| Ni3-G04b | 97-1599 | 14 | 0.39 | 0.34 | 0.844 | 0.30 | -0.130 | 0.586 | 0.532 |
| Ol12-D05 | 89-193 | 20 | 0.45 | 0.41 | 0.786 | 0.36 | -0.088 | 0.471 | 0.424 |
| Ra2-E12 | 99-257 | 16 | 0.61 | 0.53 | 0.844 | 0.48 | -0.144 | 0.363 | 0.271 |
| BRMS-050 | 105-251 | 20 | 0.48 | 0.51 | 0.837 | 0.45 | 0.070 | 0.380 | 0.423 |
| Total | | 254 | | | | | | | |
| Mean | | 16.93 | 0.52 | 0.48 | 0.83 | 0.43 | -0.07 | 0.42 | 0.37 |
| St. dev | | 3.03 | 0.16 | 0.08 | 0.07 | 0.07 | 0.20 | 0.09 | 0.18 |

Ho: observed heterozygosity, He: expected heterozygosity, Ht: total expected heterozygosity, PIC: polymorphic information content, Fis, Fst, Fit: allele frequency based correlation indices.

*Table 5. Estimates of genetic diversity of eleven populations based on population statistics including number of detected loci (NLD), average number of alleles per locus (MNA), unbiased expected heterozygosity (He), observed heterozygosity (Ho) and population specific fixation index (Fis).*

| Population | NLD | MNA | He (n.b.) | Ho | Fis |
|---|---|---|---|---|---|
| *B. napus* | 15 | 3.733 | 0.694 | 0.611 | 0.255 |
| *B. nigra* | 15 | 2.333 | 0.611 | 0.633 | -0.055 |
| *B. oleracea* | 15 | 2.200 | 0.578 | 0.367 | 0.463 |
| *B. rapa* | 15 | 6.133 | 0.819 | 0.589 | 0.377 |
| *D. muralis* | 13 | 1.692 | 0.692 | 0.692 | 0.000 |
| *D. tenuifolia* | 9 | 1.333 | 0.333 | 0.333 | 0.000 |
| *R. raphanistrum* | 15 | 1.667 | 0.667 | 0.667 | 0.000 |
| *R. sativus* | 15 | 3.000 | 0.599 | 0.356 | 0.547 |
| *R. rugosum* | 13 | 1.308 | 0.308 | 0.308 | 0.000 |
| *S. alba* | 15 | 4.067 | 0.702 | 0.576 | 0.383 |
| *S. arvensis* | 14 | 9.429 | 0.793 | 0.573 | 0.397 |
| *Mean* | 14 | 3.35 | 0.62 | 0.52 | 0.22 |

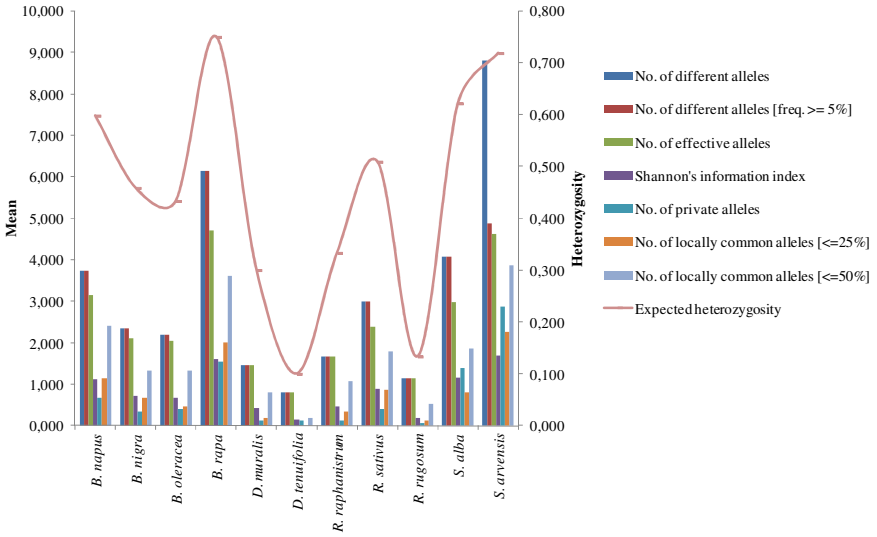


Figure 1. Allelic patterns across all populations including Shannon's information index and expected heterozygosity.

Pairwise comparisons between populations showed twenty-five statistically significant combinations. Populations of *D. muralis*, *D. tenuifolia*, *R. raphanistrum*, *R. rugosum* were not statistically significant, since only one sample per population was included (Fis value in Table 5 and Table 6).

*Table 6. Matrix of statistically significant Fst values between all pairs of populations.*

| | *B. napus* | *B. nigra* | *B. oleracea* | *B. rapa* | *D. muralis* | *D. tenuifol* | *R. raphani* | *R. sativus* | *R. rugosu* | *S. alba* | *S. arvensi* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. napus* | ** | ns | ns | + | ns | ns | ns | + | ns | + | + |
| *B. nigra* | ns | ** | ns | + | ns | ns | ns | ns | ns | + | + |
| *B. oleracea* | ns | ns | ** | + | ns | ns | ns | ns | ns | ns | + |
| *B. rapa* | + | + | + | ** | ns | ns | ns | + | ns | + | + |
| *D. muralis* | ns | ns | ns | ns | ** | ns | ns | ns | ns | ns | ns |
| *D. tenuifolia* | ns | ns | ns | ns | ns | ** | ns | ns | ns | ns | + |
| *R. raphanistrum* | ns | ns | ns | ns | ns | ns | ** | ns | ns | ns | ns |
| *R. sativus* | + | ns | ns | + | ns | ns | ns | ** | ns | + | + |
| *R. rugosum* | ns | ns | ns | ns | ns | ns | ns | ns | ** | ns | ns |
| *S. alba* | + | + | ns | + | ns | ns | ns | + | ns | ** | + |
| *S. arvensis* | + | + | + | + | ns | + | ns | + | ns | + | ** |

Significance level=0.05, ns: not significant, +: significant

A real number of genetic clusters (10) calculated using Structure, demonstrated a clear separation between populations, only one comprised of genotypes from other sites. Genetically mixed genotypes could be observed within the populations A (*B. napus*) which includes inter-specific hybrids, D (*B. rapa*) which includes genotypes from field survey, E (*D. muralis*) as wild form, I (*R. rugosum*) as wild form and K (*S. arvensis*) which also includes genotypes from field survey and wild forms (Figure 2).

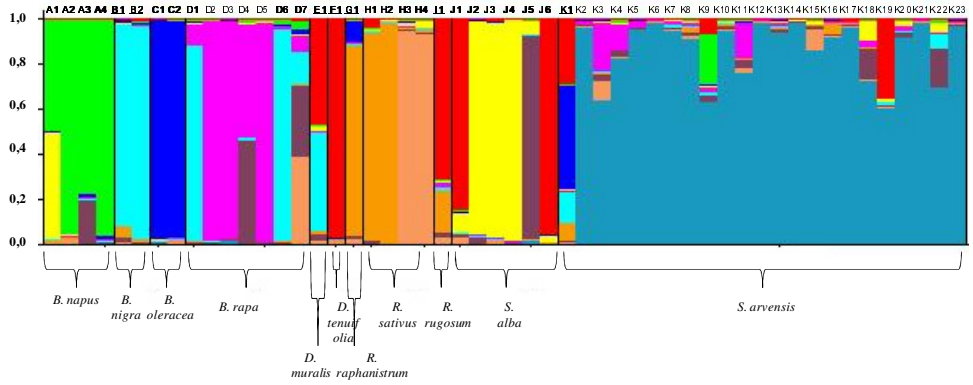


Figure 2. Bar plot (estimation of genetic diversity) of the cluster analysis (10 clusters; each column represents an individual; different colours represent different genetic groups; bold labelled individuals are reference accessions/varieties; bold and underlined labelled individuals are reference accessions of wild origin; normally labelled individuals are samples from field survey).

Phenogram based on NEI's standard genetic distance (1972) and UPGMA clustering method also demonstrated a clear separation between species referring to different descents among Brassicaceae and consequences of naturally occurred hybridizations in the past (Figure 3).

Genetic relationships between individuals and populations were analysed using PCoA. Low values of the explained variability are expected, because the origin of included reference genotypes is genetically wide and reflects naturally occurred hybridization between individuals from the field survey. The PCoA results via covariance matrix showed higher genetic linkage among species where the first three principal coordintes explained 66.2 % of the total variability between species (Figure 4).

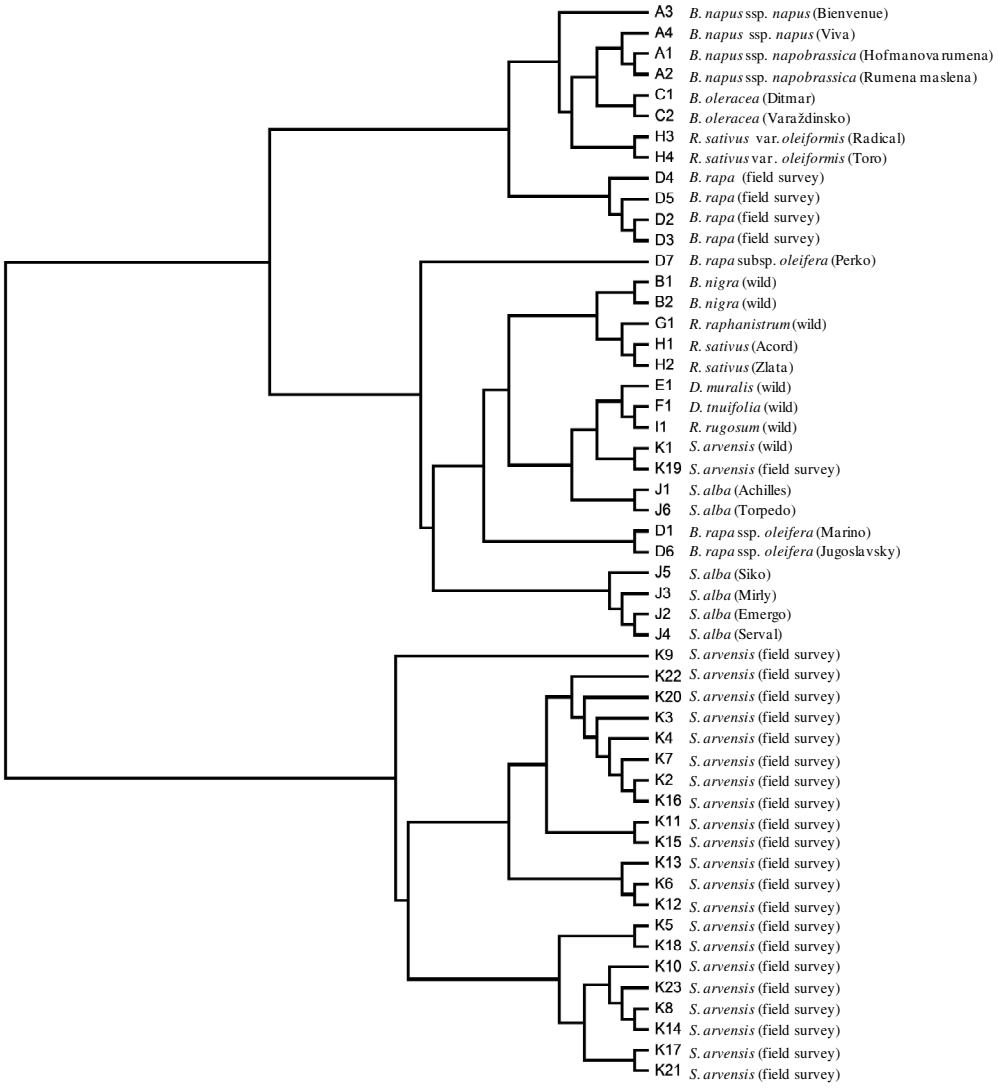


Figure 3. UPGMA phenogram of related species among Brassicaceae, based on NEI's standard genetic distance (1972).

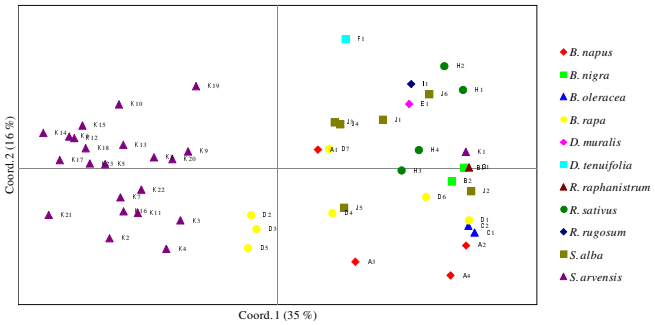


Figure 4. PCoA based on covariance matrix on fifteen loci in fifty- two analysed individuals among different species.

## DISCUSSION

Genotypes included in the study were obtained from the field survey of sexually compatible relatives of *B. napus*, naturally present on the Slovenian territory (JOGAN *et al*., 2001; PIPAN *et al.,* 2008) and from reference collections (accessions of genotypes with define origin). According to different origin of reference accessions and field samples, we analysed them as g*enus* and *species* (Table 1, 2). For this reason populations of *D. muralis*, *D. tenuifolia*, *R. raphanistrum* and *R. rugosum* included only one sample, since those species are rarely present in Slovenia (JOGAN *et al.,* 2001). Genotypes from reference accessions and from field survey were bulked since we assumed that each population originated from the same source (transport loss, soil seed bank, gene bank). The similar concept of bulking was also used by CRUZ *et. al*., 2007 and TOMMASINI *et al.,* 2003. Bulking and genotyping of exact allele lengths gave random estimation of genetic diversity among reference accessions (Figure 2). Mixed genetic structure of reference accessions of wild genotypes or varieties is related between species among Brassica, as described by NAGAHARU (1935). The observed genomes from other origins are also inter-specifically related, especially between *B. nigra* (reference wild types) and *B. rapa* (varieties 'Marino', 'Perko' and 'Jugoslavsky'). We observed linkages between *D. tenuifolia* (wild type) and *S. alba* (varieties 'Achilles' and 'Torpedo' which were/are widely grown in Slovenia), *R. rugosum* (wild type), *S. arvensis* (wild type) and *D. muralis* (wild type from the same genus). Genotypes from the field survey of *B. rapa* are genetically diverse considering the reference genotypes compared with *B. rapa* genotypes from the field survey. They are very uniform, except for one genotype (D4); it shows strong genetic relationships with *S. alba* reference accession 'Siko', which was grown in Slovenia. The consequences of spontaneous inter-specific hybridizations between Brassicaceae and *S. arvensis* were observed in field survey genotypes. The most possible hybrids were formed with *B. rapa*, *B. napus, S. alba* (especially with the varieties 'Achilles', 'Torpedo' and 'Siko'; less with 'Emergo', 'Mirly', 'Serval'), *R. sativus* (both oil varieties) and with a negligible possibility with other species. Similar relations were also found by WARWICK *et al*. (2003) where transgenic *B. napus* (herbicide resistant) and some

wild relatives like *B. rapa, S. arvensis and R. raphanistrum* were included in the gene flow study under controlled conditions (greenhouse) and/or field trials. The results based on amplified length polymorphism (AFLP) analysis (supplemented with measurements of green fluorescent protein and ploidy level) showed high frequency of hybridization between *B. napus* and *B. rapa* and low rate of gene flow between *B. napus* with *S. arvensis* and *R. raphanistrum*. In our study we observed very low gene flow and spontaneous out-crossing rate of hybrids with *B. napus*, considering the results of clustering analysis (Figure 4) and non significant Fst value between *B. napus* and *B. oleracea* populations. A special concern about spontaneous gene flow between transgenic *B. napus* and *B. oleracea* was described by FORD *et al.* (2006). They exposed that also spontaneous hybrids between transgenic *B. napus* and its "less attended" progenitor, *B. oleracea*, have important impact on the environmental flora and fauna.

Genetic analysis clearly distinguishes among *Raphanus* sp. where a strong linkage between *R. raphanistrum* (wild type) and *R. sativus* ('Accord' and 'Zlata' varieties) was found. Moreover, two groups were detected among *R. sativus*, common and oil subspecies. Among *B. napus* two groups belonging to vegetable ('Hofmanova rumena' and 'Rumena maslena') and oil ('Bienvenue' and 'Viva') subspecies were found. This repartition is described by SNOWDON *et al.*, 2007. Strong inter-specific linkage was detected between vegetable species of *B. napus* ('Hofmanova rumena') and *S. alba* ('Emergo', 'Mirly', 'Serval'), between oil species of *B. napus* ('Bienvenue') and *S. alba* ('Siko') and also with oil species of *B. rapa* ('Perko').

Good transferability of observed microsatellite markers was found among sexually compatible relatives of *B. napus,* which are present in Slovenia. The results showed that all of microsatellite markers were successfully amplified in *B. napus, B. nigra, B. oleracea, B. rapa, R. raphanistrum, R. sativus and S. arvensis;* 93% were distributed in *S. arvensis,* 87% in *R. rugosum* and *D. muralis* and only 60% in *D. tenuifolia*. These results could be used as a quick indicator of genetic distance between different species among Brassicaceae. According to the island model, we found statistically significant rate of gene exchange, detected at four loci from different focal species (two microsatellites from *B. napus*, one from *B. nigra* and one general among Brassica sp.); they were also highly informative among other observed Brassicaceae species. Considering the high allelic diversity and variation in allele sizes which are, according to HASAN *et al.* (2006), main reasons for not calculating PIC appropriately, we still have calculated PIC for individual loci. The mean PIC value from our analysis was 0.43 which is a good over all estimation of suitability of selected microsatellite markers for identification of the origin and for inter-specific hybridizations among genotypes. Similar results were obtained in a study of genotypic discrimination of two grapevine (*Vitis vinifera* L.) varieties denominations using microsatellite markers (RUSJAN *et al.,* 2012). Distribution of the calculated PIC values between different types of microsatellites (trinucleotide, dinucleotide or combined; described in Table 3) was not distinctive while the frequency of dinucleotide motifs was higher than that of the trinucleotide microsatellites in Brassicaceae, as reported by SUWABE *et al.,* 2004. Development of microsatellite markers in *B. napus* and their abundance among Brassicaceae was also assessed by PLIESKE and STRUSS (2001). Their results demonstrated high efficiency of these markers for monitoring intra-specific genetic diversity among *B. napus* (distinguishing between winter and spring varieties) and inter-specific diversity. Investigations of *B. napus* genetic diversity by RAPD method were successfully applied in distinguishing between different varieties in Lithuania (JODINSKIENE *et al.*, 2008). The occurrence and frequency of gene flow from oil varieties of *B. napus* (oilseed rape) and *R. raphanistrum* (wild radish) was assessed by

development of crop-specific transposable element (SINE) markers (PRIETO *et al.,* 2005). They mapped 19 polymorphic markers on 19 genomic regions assigned to ten linkage groups. WESTERMEIER *et al*. (2009) published methods for developing single-nucleotide polymorphism (SNP) markers in *B. napus*. They found most SNPs in non-coding regions where PIC ranged between 0.02-0.50 in a set of 86 varieties.

Over all intra-individual gene diversity among populations was lower (0.55) than inter-individual gene diversity (0.77). The described calculations explain the appearance of selection or genetic drift which consequently decreases genetic variance between populations and increases within populations (FROUKH, 2011). This can be logically explained since 50% of genotyped samples are reference genotypes from gene banks, where 73% of them originated from commercial breeding programmes. Population F statistics (Table 7) indicated a moderate inbreeding coefficient (Fis=0.39) which reflects strong genetic linkages and common descent between the observed related species among Brassicaceae. SUWABE *et al.* (2004) suggested that genomes of Brassica and other Brassicaceae should have common regions originating from the common ancestor and that such regions may be specific and essential to the genus or family. Consequently, there are possibilities for hybridization between these species in natural habitats which was confirmed by our analysis (Figure 2). The Fis value was low, since in four populations only one sample per population was included. Therefore, it is better and more illustrative to consider the Fit value which reflects the probability that two alleles in an individual are identical by descent. The value obtained was 0.48 and is considered high since different Brassicaceae species were included in our study. The Fit value obtained in the study by PASCHER *et al.* (2010) was 0.59, but only commercial varieties of *B. napus* were analysed. Low degree of gene differentiation among populations (based on allele frequencies) (Fst=0.15) indicates that rare random alleles are identical by descent. The number of private alleles and different alleles is the highest within *S. arvensis* and *B. rapa* populations (Figure 1), which also reflects spontaneous hybridizations and conservation of these alleles thought generations in natural habitats.

Over all estimation of gene migration described by average number of migrants among populations (after correction for size) was quite high (0.72) followed by 0.20 share value of mean frequency of rare alleles. Because of gene migrations, genetic drift and selection, these values do not meet Hardy-Weinberg conditions. Naturally present populations could never meet all the conditions required to achieve HWE. Their allele frequencies will change from one generation to the next and the population will evolve (NBII, 2011). According to obtained deviations of the observed from the expected heterozygosity, high population dynamics among *B. oleracea, B. rapa* and *S. arvensis* was found while the lowest one was found in *B. nigra* and *B. napus*. *S. alba, S. arvensis, R. sativus, D. erucoides* and *D. tenuifolia* are potential sources of resistance to pathogens (*Leptosphaeria maculans*, *Alternaria brassicicola*, *A. brassicae*, *A. raphani* and *Plasmodiophora brassicae*) which could be introduced to *B. napus* gene pool (SIEMENS, 2002), considering the relatively high out-crossing rate of *B. rapa* and *S. arvensis* species (30.7 %) calculated in our analysis. This proportion of out-crossing rate also represents the potential for spontaneous gene flow under Slovenian production area.

The results of genetic linkages based on NEI's standard genetic distance and UPGMA clustering method generally divided genotypes in three divergent groups (Figure 3). The first one includes samples of *B. rapa* form the field survey, reference accessions of *B. oleracea*, all reference accessions of *B. napus* and both oil varieties from *R. sativus*. SNOWDON *et al.* (2007)

explained that *B. napus* originated from spontaneous inter-specific hybridization of *B. rapa* and *B. oleracea* genomes. The oil varieties of *R. sativus* have been cultivated in Slovenia in the past and were compared with *B. rapa* samples from the field survey. All reference accessions of *B. rapa* are included in the second group along with the third *Brassica genus* which is also a member of the "Triangle of U" (NAGAHARU, 1935). The second group includes also other genotypes from Brassicaceae, except most of the *S. arvensis* samples from the field survey, which are placed in the third group. Similar results were obtained in the clustering analysis (PCoA) (Figure 4), where three main groups according to three factors, were constructed. Inter-simple sequence repeat (ISSR)-PCR technique was applied to detect microsatellite transferability between *B. rapa* and related species among *Brassica* sp. by CUI *et al*. (2008). Their results supported the hypothesis that *B. nigra* genome was divergent from *B. oleracea* and *B. rapa* genomes, which were relatively closed and consequently closer to *B. napus* than *B. nigra* genome. Similar results were also obtained in our study (see Figure 3) where samples of *B. rapa*, *B. oleracea* and *B. napus* are included in the first group, samples of *B. nigra* are also very close to the oil varieties of *B. rapa* but are included in the second group according to UPGMA clustering method.

Our study is the first one where a selected set of microsatellites was used from different focal species including *B. napus*, *B. rapa*, *B. oleracea*, *B. nigra* and specifically their primer pairs from other genetic sources among Brassicaceae. The investigated gene pool was wider since all the sexually compatible species of *B. napus* were included, the naturally present species in Slovenia and a large number of reference genotypes. The result of this study reflects the actual genetic relationships between sexually compatible relatives of *B. napus* found in the nature. The 30.7 % out-crossing rate of naturally occurred populations of *B. rapa* and *S. arevensis* reflects the potential for spontaneous intra- and inter- specific gene flow among different appeared forms of related species inside Brassicaceae family. Genetic differentiation using microsatellite markers shows on one hand exact distinction between the assessed genotypes and relationships between them. On the other hand it reflects the impact of spontaneous hybridization in the specific ecological conditions of Slovenia. The results also indicate the conservation of transferred alleles via natural spontaneous hybridization. Our findings are forming a basis for further gene flow studies among Brassicaceae as well as considering coexistence of different cropping systems.

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# GENETSKA DIFERENCIACIJA IZMEĐU SEKSUALNO KOMPATIBILNIH SRODNIKA KOD VRSTE *Brassica napus* L.

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Izvod

Analiza protoka gena između *Brassica napus* L. i njegovih seksualno kompatibilnih srodnika, koji se mogu naći u prirodi u Sloveniji, izvedena je sa mikrosatelitskom analizom korišćenjem petnaest parova početnih oligonukleotida. Genotipovi uključeni u studiju dobijeni su iz terenskog istraživanja seksualno kompatibilnih srodnika *B. napus* u prirodnim staništima širom Slovenije i iz referentnih zbirki. U Sloveniji pronađene su dve različite divlje vrste svih seksualno kompatibilnih srodnika *B. napus*, *B. rapa* i *Sinapis arvensis*. Referentni genotipovi uključuju sorte i divlje forme iz internih zbirki kao semena s tržišta ili genskih banaka. Referentni genotipovi su bili zastupljeni sledećim vrstama i podvrstama: *B. napus* ssp. *napobrassica*, *B. napus* ssp. *napus*, *B. nigra*, *B. oleracea*, *B. rapa* ssp. *oleifera*, *Diplotaxis muralis*, *D. tenuifolia*, *Raphanus raphanistrum, R. sativus, R. sativus var. oleiformis, Rapistrum rugosum, Sinapis alba i S. arvensis.* Procena protoka gena opisana prosečnim brojem migranata je bila 0,72, praćena sa 0,20 migranata. Zbog uočenih migracija gena, genetskog drifta i selekcije, Hardy-Weinberg ravnoteža nije bila postižena. Prosečan broj alela svih lokusa je bio 16,9, prosečan sadržaj informacijske vrednosti polimorfizma je bio 0,43. Od otkrivenog genskog protoka na statistički značajnom nivou ($p<0,05$) našli smo četiri vrlo različita i polimorfna lokusa (Na12-C08, Na10-A08, Ni3-G04B i BRMS-050). Rezultati genetskih veza koje temelje na standardnoj genetskoj udaljenosti i klasterskoj metodi nepondirane aritmetičke sredine, podelili su genotipove u tri glavne divergentne grupe. Slični rezultati dobijeni su analizom glavnih koordinata, gde su tri glavne grupe oblikovane na osnovu tri faktora. Stvaran broj genetskih klastera pokazao je jasnu podelu između populacija, gde samo jedna uključuje genotipove iz drugog porekla. Poljski pokus, u kojem smo kod *B. rapa* i *S. avensis* populacija primetili 30,7% tuđe oplodnje, predstavlja potencijal za spontani inter- in intra-specifični protok gena unutar slovenačkog proizvodnog područja.

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