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Spatial and Temporal Assessment of *Brassica napus* L. Maintaining Genetic Diversity and Gene Flow Potential: An Empirical Evaluation

Vladimir Meglič and Barbara Pipan

Additional information is available at the end of the chapter

Abstract

Unpredicted persistence of all forms of *B. napus* present in the agro-ecosystem is the most common consequence of preservation and self-recruitment of seeds originating from soil seed bank. In nature, spontaneous intra- and inter-specific hybridization of *B. napus* is possible with sexually compatible species from the Brassicaceae family. The aim of this chapter is (a) to identify the distribution pattern and population dynamics of volunteers and feral populations along statistical regions in Slovenia; (b) to assess the global diversity of naturally appearing *B. napus* plants; (c) to evaluate the genetic differentiation between volunteers and feral populations; (d) to obtain the spatial and temporal distribution of spontaneous pollination potential and estimation of gene flow conservation; (e) to find the empirically assigned out-crossing rate of *B. napus* under a fragmented landscape structure, during 4-year monitoring; and (f) to observe that ecologically, evolutionary, and agronomically oriented studies could be conducted at the DNA level using short sequence repeat (SSR) markers. In total, we collected 261 samples of volunteer and feral populations. Our results showed that alleles from both volunteer and feral populations were distributed in three genetic clusters with relatively similar levels of diversity. Naturally occurring out-crossing rate is 13.71%. The global Mantel correlation coefficient of genetic and spatial relatedness between genotypes is 0.044.

Keywords: *Brassica napus* L., feral populations, volunteers, spontaneous pollination, out-crossing rate, temporal and spatial distribution, SSR markers, genetic diversity, population structure

1. Introduction

Pollination relations occur among all existing forms of *Brassica napus* L. from different habitats; crops (mainly oilseed rape varieties), volunteers (grown from seed losses in previous years inside cultivated areas), and feral populations (appearing outside cultivation areas, mainly along the transportation infrastructure) [1, 2]. In the case of coexistence of different cropping systems which includes genetically modified (GM) oilseed rape production, introduction of transgenes in *B. napus* or related species is possible [3–7]. In nature, spontaneous inter-specific hybridization of *B. napus* is possible with sexually compatible species (relatives that have high pollination affinity with *B. napus*) from the Brassicaceae family. Villaseñor and Spinosa-Garcia [8] reported 7.3% of alien flowering plants in Mexico including 45 species and 25 genera from Brassicaceae family compared with 5.1% of its alien floras of the world determined by Pysek [9]. The relatives of *B. napus* are cultivated as field crops, but can also appear as weeds or wild outside cultivated areas (e.g., field edges, shelterbelts, road verges, slag heaps, embankments) [4, 6, 10]. Unpredicted persistence of all existing forms of *B. napus* in the agro-ecosystem is the most common consequence of preservation and self-recruitment of seeds originating from soil seed bank [11–15]. Because of its physical characteristics, the seed is very mobile and therefore disposed to spillage. Uncontrolled seed loss represents the potential for the appearance of volunteer and feral populations of *B. napus* inside and outside production areas; *B. napus* seed remains viable in the soil for several years [16, 17]. The population dynamics of these plants is dependent on the soil seed bank potential and on the complex interactive characteristics of the genotype, soil, and agro-climatic factors [18–23]. Pollen transfer is a primary source of gene flow and has direct influence on the level of genetic exchange within and among plants, depending on the landscape context within which it occurs [24, 25]. Non-native *B. napus* invasions and migrations are possible by vehicles, which act as vectors of long-distance dispersal [26, 27]. The spread of biological propagules, both pollen and seeds, plays a pivotal role in a number of fundamental ecological and evolutionary processes [28]. Dispersal is a process of central importance for the ecological and evolutionary dynamics of populations and communities, because of its diverse consequences for gene flow and demography [29]. The presence of undefined pollination in both natural and agricultural systems presents the potential for spontaneous intra- and inter-specific hybridization, reflected in the genetic structure and biodiversity of *B. napus*.

B. napus originated through spontaneous inter-specific hybridization (followed by polyploidization) between turnip rape (*B. rapa* L.; genome AA, 2n = 20) and cabbage (*B. oleracea* L.; genome CC, 2n = 18), resulting in an allotetraploid genome comprising the full chromosome complements of its two progenitors. Spontaneous hybridization between *B. rapa* and *B. oleracea* (from Europe and Asia) occurred due to contemporary cultivation of both species in a small geographic area in the Mediterranean region [30].

B. napus is a self-pollinated plant species with a variable out-crossing rate, influenced by genotype and environmental conditions. Due to the variable out-crossing rate, intra- and inter-specific gene flow may occur in nature [30–32]. Inside cultivation areas, the common rate of out-crossing is from 20 to 30% [23]. The out-crossing rate between different varieties with full fertility is up to 0.1% on the field-to-field scale, while in varieties with incorporated male sterility (bait plants; they produce no pollen on their own and represent the worst case scenario on

the out-crossing rate), it is higher than 1% [23, 33]. Out-crossing potential is most prominent on field margins and starts diminishing after 10 m; however, pollination at greater distances is not excluded. This is more frequent in cases where there are no other flowering plants in the surroundings of the donor plant/cultivated crop. The out-crossing rate is significantly influenced by proportions between donor and recipient plants [23].

Different marker systems including short sequence repeat (SSR) markers are used for genetic characterization of agro-economically important plant species [10, 34–37]. To assess the molecular variation, genetic structure and gene flow potential among *B. napus* genome on a spatial and temporal scale, proved to be best suitable applying several molecular marker systems (RAPD, AFLP, SINE, ISSR, and SSR) [1, 6, 38–40]. There are also newly developed DNA marker types (e.g., SNP, KASP-SNP) and NGS (Next Generation Sequencing) based applications (e.g., GWS, GBS, RAD) [41–44] for genotyping and breeding purposes of *B. napus*.

Fragmented landscape and small-sized field structure reflect the heterogeneous growth conditions in several parts of Europe and world. The presence of ecological barriers like landscape structural elements (small woods, hedges, overgrown paths, and hills) and the influence of different agro-climatic conditions manage pollen and seed distribution [45]. Consequently, the persistence of *B. napus* plants originating from seed in soil seed banks enables gene flow potential on a spatial and temporal scale, reflecting in the crop quality, seed purity, and long-term biodiversity. Therefore, the aim of this study is to empirically estimate the out-crossing potential of *B. napus* gene transfer, under a fragmented landscape (10 statistical regions) in Slovenia and study the conservation of spontaneous gene flow into *B. napus* genome on a temporal level (4-year period). Through analysis of genetic diversity and calculation of population genetics parameters, implemented by advanced bioinformatics procedures, this study represents the important agronomical, biological, and ecological baselines. The presented results are provided on a DNA level, which is the most reliable way to determine changes in the genetic composition of *B. napus* genome on a spatial and temporal scale. Our goals were (a) to identify the distribution pattern and population dynamics of volunteers and feral populations along statistical regions in Slovenia; (b) to assess the global diversity of naturally appearing gene pool structure of *B. napus*; (c) to evaluate the genetic differentiation between volunteers and feral populations; (d) to obtain the spatial and temporal distribution of spontaneous pollination potential and estimation of gene flow conservation; (e) to find the empirically assigned out-crossing rate of *B. napus* under a fragmented landscape structure during a 4-year period of monitoring; (f) to observe that due to genetic diversity and population genetics parameters, ecologically, evolutionary, and agronomically oriented studies could be conducted at the DNA level using highly informative SSR markers.

2. Materials and methods

2.1. Study area

For the purpose of the study, we have selected macro-locations on a regional level—regions along Slovenia with high crop production share of *B. napus* (as oilseed rape) [2]. Therefore, from all statistical regions (12) of Slovenia, 10 were included in our research (Osrednjeslovenska-OSR,

Gorenjska-GOR, Jugovzhodna Slovenia-JVS, Notranjsko-kraška-NTK, Obalno-kraška-OBK, Podravska-POD, Pomurska-POM, Savinjska-SAV, Spodnjeposavska-SPS, and Zasavska-ZAS) (**Figure 1**). Inside those regions, we identified agrotopes (field edges, meadows, loess slopes, shelterbelts, field margins, field paths, etc.) and ruderal habitats (road verges, railway embankments, slag heaps, construction sites, rest areas by the roads, uncultivated areas, mounds, roundabouts, etc.) as main orientation points for field survey. Meanwhile, volunteer populations were sampled inside field margins as weedy plants in other cultivated crops.

2.2. Field survey

Field survey was conducted in a 4-year period from 2007 to 2010 every year during the flowering time of the biennial *B. napus* (third week of April and first week of May). We sampled five young leaves from each individual plant per population from each micro-location on an area of approx. 5m² including a minimum of five plants of *B. napus*. Sampled leaves were frozen (−20°C) and stored for DNA analysis.

2.3. DNA extraction

The leaf apex of each sample from the five young plants was bulked for DNA extraction with BioSprint 15 DNA Plant Kit (Qiagen) on a KingFisher (Thermo) isolation robot following the optimized method according to manufacturer's instructions.
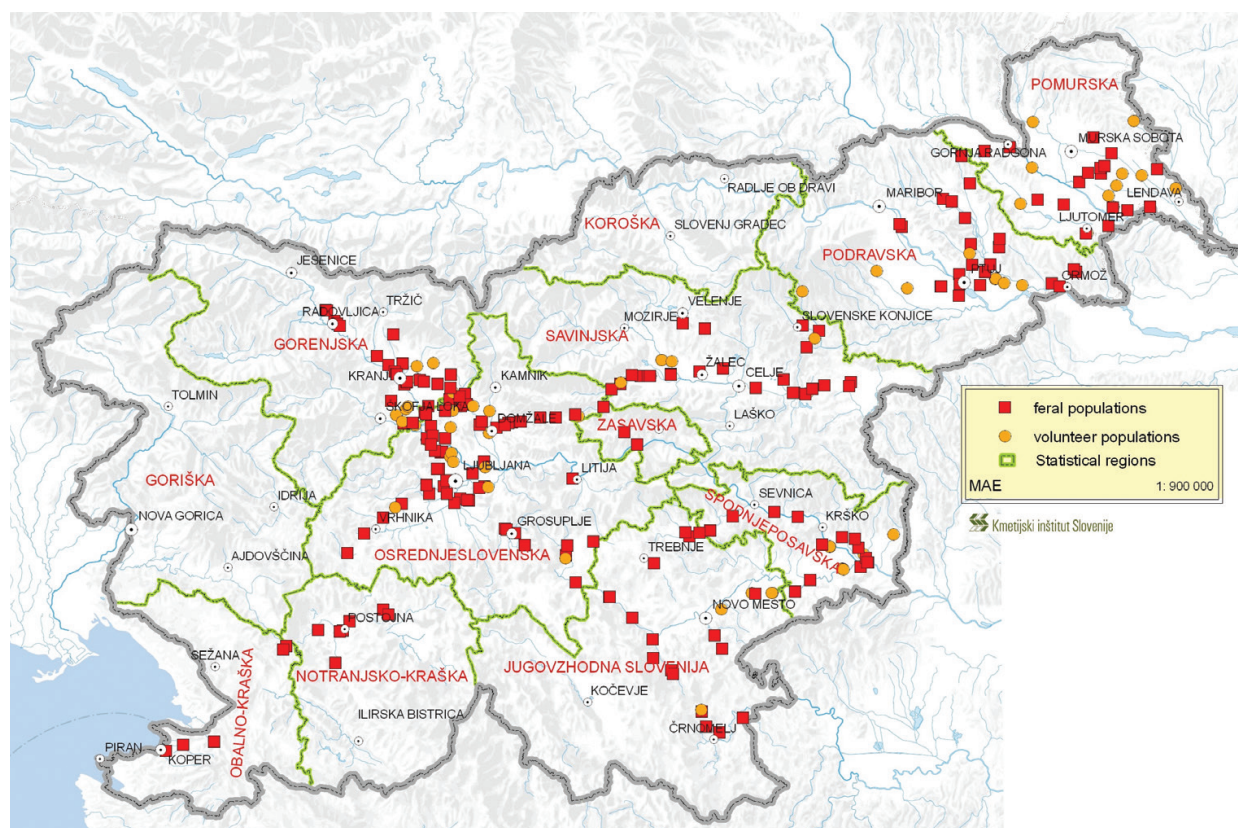
Figure 1. Sampling locations of feral and volunteer populations of *B. napus* in 2007–2010 along Slovenian statistical regions.

2.4. Genotyping procedure

A total of 45 nuclear SSR markers originating from different Brassicaceae family species, with various nucleotide repeat motives (listed in **Table 1**) were used. Thirty-seven SSR markers (with Na, Ol, Ni, Ra) were developed by Lowe et al. [46]; two SSR markers (with BRMS) were published by Suwabe et al. [47]; two SSR markers (with MR) were by Uzanova and Ecke [48]; one SSR marker (named BN83B1) was developed by Szewc-McFadden et al. [49]; and two SSR markers (with RES) were published by Wang et al. [50]. PCR reactions were performed on a final volume of 11.5 μl, containing 30 ng of genomic DNA and the following reagents with initial concentrations of: 10 x PCR buffer (Biotools), 10 mM of each dNTPs, 50 mM MgCl$_2$ (Biotools), 10 μM of each primer, 10 μM 5' fluorescently labeled universal primer (6-FAM, NED, HEX), and 0. 5 U of Taq DNA polymerase (Biotools). The forward primer of each SSR was appended with 18 bp tail sequence 5'-TGTAAAACGACGGCCAGT-3' (M13(−21) as described by Schuelke [51]. PCR analyses were performed on ATC 401 (Apollo Instrumentations) under the following "touch-down" conditions, dependent on each primer pair: 94°C for 4 min; 15 cycles at 94°C for 1 min; auto decrement temperature from 60 (62)°C at 0.7°C per cycle for 30 s; 72°C for 1 min, followed by 23 cycles at 94°C for 30 s; 53°C for 30 s; 72°C for 1 min; and final extension for 5 min at 72°C. Fragment analysis was performed on a 3130XL genetic analyzer (ABI); the allele lengths were determined by comparison to a size standard GeneScan-350 ROX (ABI) using GeneMapper 4.0 (ABI).

2.5. Data analysis

Parameters of genetic diversity among loci including ranges of allele lengths (Ra), numbers of alleles (n), frequencies of null alleles (No), and probability of identity (PI) were calculated using Identity v.1.0 [52]. MsToolkit [53] was used to evaluate expected heterozygosities (He), observed heterozygosities (Ho), and polymorphic information content (PIC). Locus-specific fixation indices and deviations of volunteer and feral populations from the Hardy–Weinberg equilibrium (HWE) were calculated using the GenAlEx v.6.4. [54]. Detecting the loci under selection was performed using Arlequin v.3.5.1.2 software [55] with 20,000 simulations. FSTAT v.2.9.3.2 [56] was used to determine allelic richness (R) as a measure of the number of alleles independent of sample size after 2000 permutations. The calculations of population statistics parameters at the spatial and temporal level including numbers of different alleles (Na), numbers of private alleles (Np), numbers of effective alleles (Ne), number of locally common alleles, fixation indices (F), population-specific expected heterozygosities (He), Shannon's information index (I), and pairwise Nei's genetic correlations were obtained using GenAlEx v.6.4 [54]. The out-crossing rate (t) was calculated from the fixation index using the equation $t = (1 − F) / (1 + F)$ described by He et al. [57]. Gene flow among volunteer and feral populations was estimated by calculating the effective number of migrants (m) using the private allele method of Slatkin [58], implemented by Genepop v.4.1 [59]; the corrected estimated value of Barton and Slatkin were reported [60]. Two common estimators of volunteer and feral population differentiation (Fst and Rst as standard parameters of genetic distance) are Fst, based on allele identity, and Rst, which incorporates the SSR-specific stepwise mutation model. Calculations of both estimations were performed using GenAlEx v.6.4 [54], where the estimation of RST was evaluated by AMOVA with 999 permutations. Pairwise genetic and geographic (log10 [lat, long]) uniformity between genotypes in the 4-year period, was established by 999 permutations with the Mantel test [61]. The mean within region pairwise values (r), according

Locus	Repeat motif	Ra[bp]	n	He	Ho	N_0	PI	PIC	F
Na12-A07	$(GT/CA)_{11}$	160–190	13	0.601	0.473	0.077	0.197	0.573	0.003
Na12-B05	$(GA/CT)_{18}$	135–221	27	0.862	0.767	0.034	0.030	0.845	0.005
Na12-C08	$(GA/CT)_{50}$	259–349	21	0.711	0.388	0.169	0.107	0.691	0.008
Na12-E05	$(GT/CA)_{10}$	102–176	21	0.730	0.866	−0.078	0.099	0.704	0.003
Na12-G05	$(GA/CT)_{50}$	144–254	22	0.876	0.749	0.069	0.027	0.858	0.005
Na14-E11	$(GA/CT)_{29}$	108–184	16	0.722	0.871	−0.077	0.117	0.678	0.001
Na14-G02	$(GA/CT)_{17}$	139–215	16	0.817	0.729	0.045	0.059	0.791	0.008
Ni3-G04b	$(GA/CT)_{18}$	99–171	20	0.690	0.747	−0.033	0.138	0.641	0.002
Ni4-D09	$(GA/CT)_{25}$	162–246	22	0.911	0.932	−0.008	0.016	0.899	0.002
Ni4-E08	$(GA/CT)_{47}$	105–195	11	0.422	0.436	−0.017	0.394	0.388	0.005
Na12-A08	$(GA/CT)_{28}$	137–205	17	0.778	0.415	0.203	0.066	0.753	0.003
Na12-E06a	$(GA/CT)_{23'}$	162–252	12	0.816	0.730	0.052	0.055	0.790	0.006
Na12-C06	$(GA/CT)_{37}$	153–285	19	0.895	0.702	0.095	0.213	0.880	0.002
Na10-A08	$(GA/CT)_{21}$	107–217	21	0.700	0.723	−0.012	0.118	0.677	0.002
Na14-H11	$(GT/CA)_{10}$	102–182	15	0.758	0.969	−0.120	0.091	0.725	0.005
BN83B1	$(GA)_{11} (AAG)_4$	135–232	13	0.414	0.201	0.139	0.393	0.396	0.011
MR183	$(TG)_{11}$	80–116	12	0.743	0.938	−0.106	0.108	0.699	0.001
Ni4-G04	$(GA/CT)_{60}$	260–348	10	0.619	0.350	0.147	0.205	0.562	0.007
Ni4-H04	$(GT/CA)_{14}$	132–134	16	0.872	0.253	0.334	0.026	0.848	0.029
Ol10-D03	$(GA/CT)_{20}$	106–190	21	0.793	0.938	−0.089	0.067	0.767	0.003
Ol11-D12	$(GA/CT)_{52}$	111–209	32	0.931	0.685	0.145	0.009	0.920	0.007
Ol11-G11	$(GGC/CCG)_5$	99–197	15	0.831	0.959	−0.065	0.051	0.806	0.004
Ol11-H02	$(AAT/AAG)_{18}$	128–218	12	0.802	0.713	0.047	0.070	0.772	0.003
Ol12-A04	$(GA/CT)_{17}$	120–202	15	0.449	0.410	0.023	0.328	0.428	0.002
Ol12-B05	$(GA/CT)_{36}$	122–244	20	0.516	0.405	0.069	0.256	0.493	0.004
Ol12-D05	$(GA/CT)_{32}$	101–193	17	0.770	0.665	0.048	0.068	0.745	0.003
Ol12-D09	$(GGC/CCG)_4$	103–193	11	0.703	0.875	−0.095	0.118	0.653	0.007
Ol12-E03	$(GGC/CCG)_9$	94–257	13	0.854	0.921	−0.045	0.039	0.832	0.001
Ol12-F11	$(GT/CA)_{14}$	124–254	17	0.707	0.660	0.023	0.130	0.678	0.016
Ol13-E08	$(GA/CT)_{11}$	126–232	17	0.729	0.770	−0.043	0.118	0.684	0.002
Ra2-A01	$(GA/CT)_{19}$	98–144	12	0.671	0.910	−0.149	0.171	0.611	0.001
Ra2-A10	$(GT/CA)_{107}$	170–296	13	0.648	0.313	0.195	0.177	0.608	0.007
Ra2-E03	$(GA/CT)_{18}$	187–319	19	0.589	0.334	0.165	0.226	0.555	0.010
Ra2-E04	$(GA/CT)_{19}$	96–218	18	0.657	0.925	−0.155	0.174	0.593	0.002
Ra2-E12	$(GA/CT)_{32}$	115–281	24	0.782	0.769	0.018	0.069	0.757	0.002

Locus	Repeat motif	Ra[bp]	n	He	Ho	N_0	PI	PIC	F
Ra2-F11	$(GA/CT)_{34}$	151–307	29	0.797	0.929	−0.068	0.065	0.768	0.003
Ra2-G09	$(GA/CT)_{19}$	168–266	21	0.740	0.461	0.159	0.096	0.717	0.007
Ra3-E05	$(GT/CA)_{65}$	183–285	11	0.656	0.735	−0.038	0.165	0.607	0.003
Ra3-H10	$(GA/CT)_{23}$	122–202	13	0.779	0.823	−0.025	0.072	0.760	0.006
BRMS-036	$(CA)_{10}(GA)_4$	100–178	15	0.813	0.976	−0.082	0.055	0.786	0.001
BRMS-050	$(AAT)_4(TC)_{19}(TTC)_3$	143–215	14	0.361	0.292	0.047	0.473	0.345	0.013
MR187	$(AG)_{23}(AGG)_5$	101–189	18	0.600	0.450	0.103	0.175	0.579	0.001
RES1	$(CCT)_5$	104–199	16	0.812	0.912	−0.058	0.064	0.782	0.002
RES6	$(ATG)_7$	148–223	10	0.373	0.244	0.088	0.426	0.352	0.003
BN6A2	$(GATT)_4$	93–133	9	0.596	0.415	0.116	0.191	0.556	0.003
Total			756				2.480×10^{-46}		
Mean			16.8	0.709	0.661	0.028		0.679	0.005

[*]Range of allele lengths (Ra), number of alleles (n), expected heterozygosity (He), observed heterozygosity (Ho), estimated frequency of null alleles (No), probability of identity (PI), polymorphic information content (PIC), and fixation index (F).

Table 1. Parameters of genetic diversity within volunteer and feral populations among loci[*].

to geographic and genetic distance, was calculated by 999 permutations and 1000 bootstraps using GenAlEx v.6.4 [54]. To assess the genetic structure of volunteer and feral populations, a Bayesian method was used. This analysis was performed using the model-based software Structure v.2.3.3 [62] that infers the number of genetic groups K present in a sample by comparing the posterior probability for different numbers of putative populations specified by the user and assigning individuals, giving a percentage of membership (Q value), for these clusters. The admixture model with 100,000 MCMC (Markov chain Monte Carlo) repetitions and 10,000 burn-in periods were used. Eleven independent runs were performed without prior information on groups assuming correlated allele frequencies. Temporal changes of genetic structure among volunteer and feral populations were estimated in PCoA (principal coordinate analysis) via covariance matrix with data standardization using GenAlEx v.6.4. [54].

3. Results

3.1. The dataset

In the 4-year period, 261 samples were collected in total—66 samples of volunteer populations and 195 samples of feral populations within 10 statistical regions in Slovenia (**Figure 1**).

3.2. Evaluation of genetic diversity

Genotypic results for 45 analyzed loci are summarized in **Table 1**. All loci were 100% polymorphic in both volunteer and feral populations. The selected set of SSR markers is highly applicable for genetic differentiation analysis within *B. napus* genome, suggesting high mean PIC

value (0.679) and low total PI value (2.480×10^{-46}) (**Table 1**). The most informative locus with the highest PIC value was Ni4-D09, which originated from *B. nigra* genome (**Table 1**). Global genetic diversity (mean He value, **Table 1**) between all naturally present volunteer and feral populations in Slovenia is 0.709. Positive and low mean N_0 value (**Table 1**) suggests that there was negligible mutation activity within the included SSR regions in *B. napus* genome, during the 4-year period.

According to the exact HWE test, both volunteer and feral populations do not meet HWE conditions ($P < 0.05$) for any of the 45 loci, which is confirmed by the mean positive value of F (0.005) (**Table 1**), indicating spontaneous random mating and inbreeding potential. These findings reflect the characteristics of natural populations during the 4-year monitoring of non-cultivated *B. napus* populations. Significant changes ($P < 0.05$) in genetic structure of all included genotypes at each locus were detected for loci Ra3-H10 and NA10-A08; it is assumed that the level of gene flow for those loci was influenced by microevolution and natural selection. The calculated values of different alleles (Na = 12.40), private alleles (Np = 1.13), and fixation index (F = 0.072) within volunteer populations were lower compared to feral populations, where Na was 15.67, Np reached 4.40, and F was 0.074. Naturally occurring out-crossing rate among feral populations during the 4-year period on the national level is 13.71%; the global out-crossing rate among volunteer populations is lower (13.47%). These comparisons indicate the favorable introduction and conservation of new alleles via spontaneous gene flow in nature in self-recruited generations of feral populations.

The MCMC structure of 45 SSRs showed moderate genetic structure. When Evanno's [63] ad hoc estimator of the real number of clusters was used, it indicated modes at K = 3 (**Figure 2**). The average genetic distances between genotypes in the first cluster is 0.794 (Fst = 0.062), following 0.627 (Fst = 0.169) in the second cluster and 0.646 (Fst = 0.092) in the third genetic cluster.

3.3. Regional-spatial assessment of gene flow in fragmented field landscapes

Genetic diversity and allelic structure of volunteer and feral populations along statistical regions are presented in **Figure 3** and **Table 2**. According to the highest values of expected heterozygosity (He) and Shannon's information index (I), the most genetically diverse genotypes are from JVS (He = 0.731; I = 1.779), SAV (He = 0.726; I = 1.729), OSR (He = 0.688; I = 1.627), and POM (He = 0.662; I = 1.482) regions (**Figure 3**). The highest number of private alleles, Np = 0.867, was detected among genotypes from OSR (**Figure 3**); the out-crossing rate inside this region reached 10.45%. The highest out-crossing rate was calculated within SAV (*t* = 18.75%) and
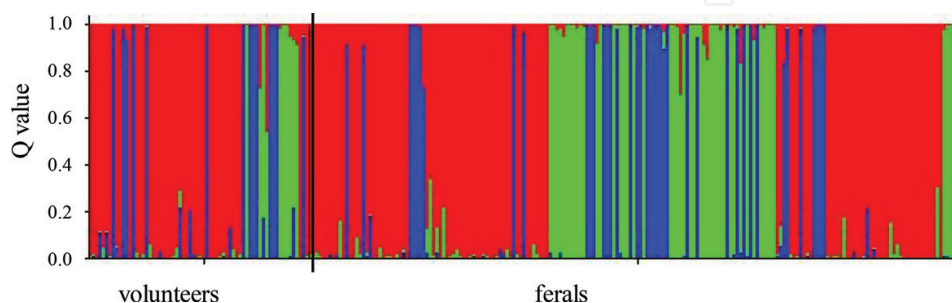
Figure 2. Genetic structure of volunteer and feral populations, according to three genetic clusters.
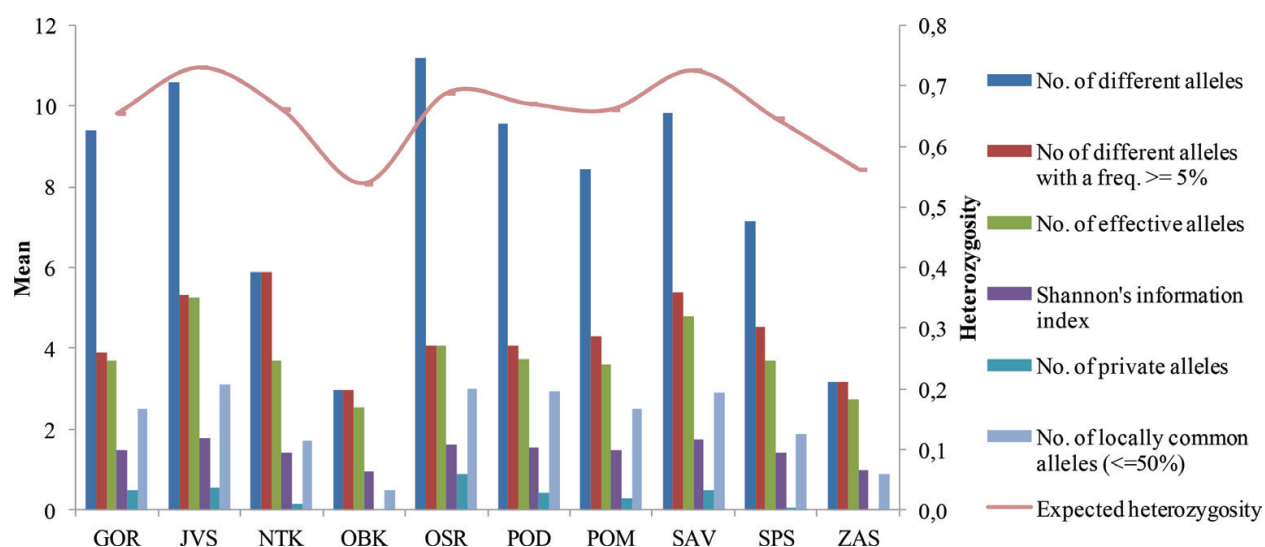
Figure 3. Genetic patterns according to spatial distribution of volunteer and feral populations.

JVS (t = 18.31%) regions. The differences between the highest Np and low t values in the OSR region indicate the favorable potential of gene flow conservation in feral and volunteer populations; this is in contrast with the JVS and SAV regions, where the level of spontaneous gene flow was high, but conservation into naturally occurred populations, was low.

The estimation of R_{ST} (using stepwise mutation model) using AMOVA showed 4% molecular variability among statistical regions. High genetic relatedness between genotypes from different

	GOR	JVS	NTK	OBK	OSR	POD	POM	SAV	SPS	ZAS
GOR	*	0.032	0.020	0.076	0.006	0.010	0.010	0.017	0.012	0.070
JVS	0.857	*	0.034	0.096	0.026	0.027	0.030	0.015	0.038	0.066
NTK	0.919	0.850	*	0.093	0.019	0.019	0.022	0.020	0.022	0.072
OBK	0.820	0.724	0.761	*	0.073	0.079	0.077	0.085	0.088	0.122
OSR	0.977	0.874	0.921	0.826	*	0.009	0.009	0.012	0.013	0.067
POD	0.958	0.874	0.921	0.801	0.963	*	0.010	0.015	0.016	0.063
POM	0.963	0.865	0.911	0.813	0.963	0.955	*	0.014	0.017	0.067
SAV	0.929	0.923	0.915	0.772	0.941	0.934	0.940	*	0.021	0.061
SPS	0.957	0.842	0.913	0.788	0.951	0.937	0.936	0.919	*	0.068
ZAS	0.770	0.763	0.764	0.711	0.774	0.789	0.774	0.785	0.778	*

*Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of "No difference" across the regions as determined by permutation. The lowest mean r value was calculated across POD region (63.3%), where r was outside U and L limits reflecting the highest genetic and geographic difference of included genotypes along this region.

Table 2. Values of pairwise comparisons of feral and volunteer populations according to statistical regions, Nei's genetic identity (under diagonal) and F_{ST} values (above diagonal).
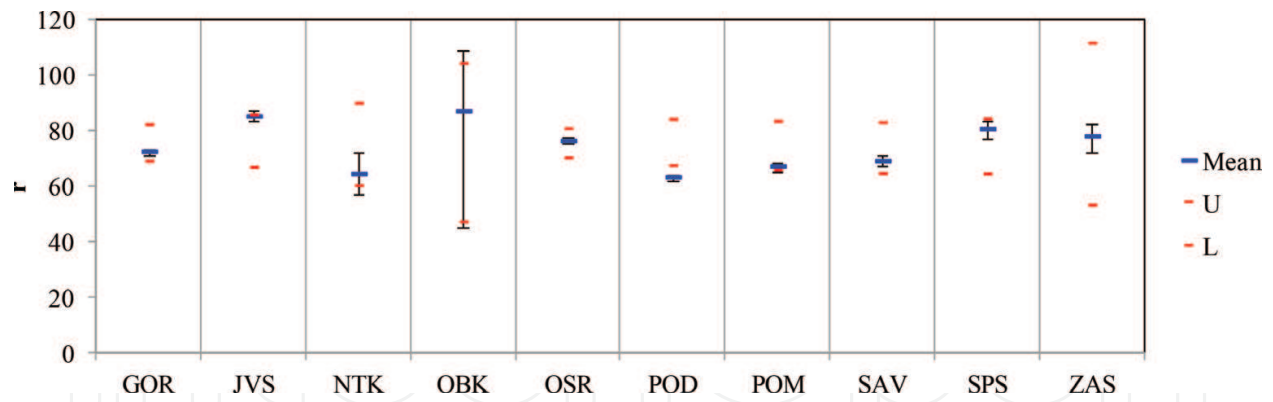
Figure 4. Mean within region pairwise values (r), according to geographic and genetic distance.

regions was also confirmed with pairwise comparisons between genotypes from different geographical areas, based on Nei's genetic identity and F_{ST} values (**Table 2**). The highest pairwise genetic correlation was calculated between genotypes from the OSR and GOR regions (0.977), which corresponds to the lowest F_{ST} values, based on allele frequencies between these two geographic areas (F_{ST} = 0.006) (**Table 2**). These two regions are geographically neighboring areas (**Figure 1**).

According to the results from **Table 2**, the included genotypes are relatively homogenously dispersed along all geographic areas and no grouping of genetically similar genotypes within statistical regions was observed. This finding was confirmed by a global Mantel test, which compares the genetic and geographic distance matrix of all 261 genotypes. The Mantel correlation coefficient of genetic and spatial relatedness between genotypes was low, but positive (rxy = 0.044, P = 0.01), due to minor spatial linkage on the basis of genetic structure. The summary of the mean within region pairwise values, based on genetic and geographic distance, is presented in **Figure 4**.

3.4. Temporal distribution of landscape gene flow and conservation of genetic variation

Temporal distribution of genetic variation, according to 100% polymorphic loci during the 4-year monitoring is presented in **Table 3**. Increasing values of Np, m, and molecular variance for every successive year, signify the gene flow potential, distribution, and conservation of new alleles into *B. napus* genome in a relatively short period. However, for allelic richness, the highest contribution was determined in 2010 (see **Table 3**).

According to PCoA results, there is a decreasing pattern of genetic linkages between all genotypes from 2007 to 2010 (**Figure 5**). This genetic differentiation reflects the spontaneous gene flow through the 4-year period in the surveyed agro-ecosystem.

4. Discussion

According to the 4-year field monitoring, volunteer/feral populations appeared within statistical regions, where *B. napus* have been widely cultivated as oilseed rape (OSR, 56; GOR,

45; POD, 36; JVS, 32; POM and SAV, 29). The actual regional cultivation of *B. napus* in 2009 was reported by Pipan et al. [2], where the highest proportion of oilseed rape production was inscribed along POM and POD regions. There was no volunteer or feral population found inside Goriška and Koroška region. Distribution of volunteer and feral populations (**Figure 1**) represents the highly-developed *B. napus* persistence under the Slovenian fragmented landscape structure, according to soil seed bank potential as a consequence of seed movements. The regional pattern of *B. napus* presence indicates that volunteer or feral populations most commonly originate from seed losses. Zhu et al. [17] report that seed losses during harvest could be limited to 0.7–1.1% of total seed production under Chinese farming systems. Consequently, uncultivated forms of *B. napus* colonize mostly pioneer habitats, such as waste sites, cultivated grounds, rubble tips, arable fields, riverbanks, road sides, and tracks [6, 64].

In this study, spatial and temporal determination of genetic changes on 45 loci inside the *B. napus* genome was proven to be useful and informative—there was low probability of identity value (PI = 2.480 × 10^{-46}) and high polymorphic content value (PIC = 0.679) (see **Table 1**) among single species. These values also reflect the equal distribution of alleles among volunteer and feral genotypes. SSR markers are suitable to identify varieties of *B. napus* (e.g., [6, 39, 65]). A high level of genetic differentiation within the same species was obtained in our study. The composed structure of some SSR repeat motives, which originated from *Brassica sp.* (BN83B1, PIC = 0.396; BRMS-050, PIC = 0.345), could have a negative effect on the information content (**Table 1**). We would like to emphasize the highly distinctive loci RES1 (PI = 0.782, **Table 1**) developed from the sexually compatible relative of *B. napus*, *Raphanussativus* [50]. This study confirmed the finding reported by Elling et al. [38], Hasan et al. [39], Suwabe et al. [47], and Bond et al. [66] that SSR markers originating from related *Brassica* species are highly applicable in investigations of *B. napus* gene pool.

Variable out-crossing rate, being a biological characteristic of *B. napus*, is 5–47% [30]. Likewise, empirically determined out-crossing rate in Slovenia was 13.6% and represents the spontaneous gene flow potential of *B. napus* under a fragmented landscape structure during a 4-year period. Moreover, the ability for introgression and conservation of spontaneous gene flow into *B. napus* genome through (self-recruited) generations in nature is possible. According to the increasing pattern of Np and m values in each following year during the 4-year period (**Table 3**), proves that genetic changes within volunteer/feral populations are reflected temporally. This finding is confirmed by PCoA distribution, where genetic relatedness between genotypes decreased (**Figure 5**) and the proportion of molecular variance during the 4-year period increased (**Table 3**). Additionally, genetic diversity within feral populations was higher, compared to volunteers due to uncontrolled pollination and introduction of new genes into feral populations. Pascher et al. [6] reported that feral populations shared less than 50% of the SSR alleles among 8 loci, compared to commercial varieties, which were cultivated in the previous year along the same region. Our results showed that alleles from both volunteer and feral populations were distributed in three genetic clusters (**Figure 2**) with relatively similar level of diversity. Considering this, we assume that high proportion of spatially and temporary distributed agro-biodiversity of *B. napus* gene pool was observed (global He = 0.709, F = 0.005; **Table 1**). Temporal determination among volunteers and feral populations was described by R, a measure of independent quantitative comparison of genetic diversity between all years. Overall, the most genetically diverse genotypes were

Parameter of population diversity and genetics	Ecological interpretation	2007	2008	2009	2010
Ne	Allelic diversity	4.01	3.80	3.64	4.17
Np	Estimation of spontaneous gene flow conservation into naturally appearing populations	0.58	0.93	0.98	1.64
F	Estimated level of spontaneous gene flow	0.03	0.01	0.07	0.05
t (%)	Actual gene flow potential	5.74	2.81	13.27	12.52
Molecular variance (%)	Conservation of naturally occurring spontaneous gene flow	1.64	1.78	2.77	6.1
m	Level of gene flow	2.16	3.36	4.41	5.47
R	Basic genetic diversity parameter; allelic richness	3.41	1.67	3.23	5.64

Table 3. Ecologically important parameters of population genetics for genetic diversity distribution in 4-year sampling period.

determined in 2010, additionally confirmed with the highest Ne value (**Table 3**), indicating the ability and introduction of new alleles through spontaneous pollination of *B. napus* in nature.

Our study suggests that there is no specific distribution of genetically similar genotypes present within the same statistical region. Conversely, the proportion of shared molecular variability of volunteers/feral populations between regions is high (96%). These large-scale genetic similarities could be caused by common ancestry from commercial varieties of *B. napus*
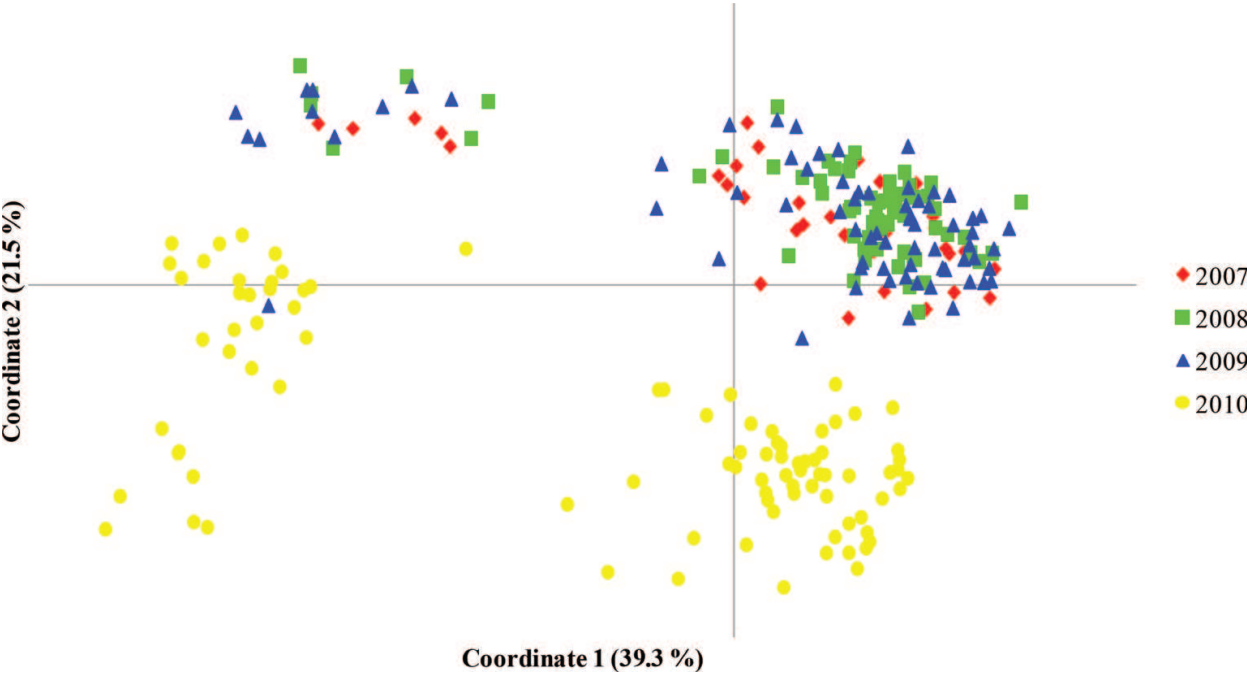
Figure 5. PCoA temporal distribution of genotypes.

(oilseed rape), which were cultivated in the observed statistical regions. Pasher et al. [6] observed that genetic similarities among feral populations could be caused by selection favoring or eliminating certain alleles of loci linked to the markers, or by pollination and hybridization with sexually compatible relatives. However, Mantel correlation coefficient between genetic and geographic distance matrix assigned a low level of spatially and genetically related distribution among genotypes. The highest spatially distributed genetic diversity was observed in the JVS and SAV regions (He >0.700; **Figure 3**); the highest numbers of locally common alleles (< 50%) with a frequency > 5% (**Figure 3**) were detected along the JVS and OSR regions. Most likely, the highest potential for gene flow conservation into natural *B. napus* populations (highest Np values) was determined within the OSR region (**Figure 3**) due to favorable agro-climatic and geographic conditions. The most genetically heterogeneous genotypes, according to their spatial position, were formed along the POD region (**Figure 3**).

5. Conclusions

Distribution of volunteer and feral populations represents the highly developed *B. napus* persistence under the Slovenian fragmented landscape structure, according to soil seed bank potential as a consequence of seed movements. The regional pattern of *B. napus* presence indicates that volunteer/feral populations most commonly originate from seed losses. In this study, spatial and temporal determination of genetic changes on 45 loci within *B. napus* genome was proven to be useful and informative. Empirically determined out-crossing rate in Slovenia was 13.6% and represents the spontaneous gene flow potential of *B. napus*, under a fragmented landscape structure during a 4-year period. This calculation reflects that the actual large-scale situation is an important basis for ecological, agronomical, and ecological evaluation of spontaneous pollination potential of *B. napus* in this agro-ecosystem. Moreover, the ability of introgression and conservation of spontaneous gene flow into the *B. napus* genome through (self-recruited) generations in nature is possible. Our study suggests that there is no specific distribution of genetically similar genotypes present within the same statistical region.

Our empirically obtained results show the existing potential of large-scale spontaneous pollination and gene flow conservation into the *B. napus* gene pool in a short time period under a fragmented landscape structure. Genetic diversity of naturally present *B. napus* plants and spatially and temporally determined conservation of genetic variation, is proven to be successfully assessed using SSR markers, due to biologically, agronomically, evolutionary, and ecologically important parameters.

Acknowledgements

Author details

Vladimir Meglič* and Barbara Pipan

*Address all correspondence to: vladimir.meglic@kis.si

Crop Science Department, Agricultural Institute of Slovenia, Ljubljana, Slovenia

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