Molecular Analysis of Temporal Genetic Structuring in Pea (*Pisum sativum* L.) Cultivars Bred in the Czech Republic and in Former Czechoslovakia Since the Mid-20th Century

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Abstract: Changes in genetic diversity of peas bred in the Czech Republic and in former Czechoslovakia since the mid-20th century were analysed using 38 molecular marker loci, including retrotransposons and microsatellites, differentiating a total of 84 alleles. Both marker types were comparably effective in revealing the genetic diversity, with a high correlation (r = 0.81), although the pairwise genetic distances of each marker type differed. In total, 175 accessions, selected from the Czech pea gene bank collection and representing the pea cultivars collected or bred in the country, were divided into three groups according to their date of sampling or variety registration. The first group contained 70 old cultivars and landraces collected prior to 1961. The second group contained 46 cultivars released from 1961 to 1980. The third group contained 59 cultivars released between 1981 and 2004. In spite of the decline in several diversity measures, differences in allele frequencies and even allele loss in three microsatellite loci were recorded over the 70-year period, while these differences between the groups were not statistically significant. In addition, genetic heterogeneity was detected in 29 accessions (15%). This indicates that although no genetic erosion could be observed since then, it is important to monitor the genetic diversity, furthermore it highlights the vital role of germplasm collections for the crop diversity conservation.

Keywords: gene bank; genetic diversity; germplasm; microsatellites; pea; retrotransposons

The demand for productivity and homogeneity in crops has resulted in a limited number of varieties, at the cost of the loss of heterogeneous traditional local varieties (landraces), a process known as genetic erosion (Pistorius 1997). While the number of species per unit area of agricultural land is lower than in most of the natural ecosystems, agriculture has also produced amazing genetic diversity (some species with more than hundred thousand varieties) (Hammer *et al.* 2003). In the case of pea (*Pisum sativum* L.) being domesticated about 10 000 years ago (Ambrose 1995; Ellis 2011), followed by centuries of selection and breeding, this process resulted in thousands of varieties, many of which have survived and are maintained in numerous germplasm collections worldwide (Smýkal *et al.* 2011). Already in the period of rediscovery of Mendel's work, many biologists and plant breeders pointed out to the danger of losing genetic diversity (Proskowetz 1890; Schindler

1890). Later, the spread of high-yielding "Green Revolution" varieties and associated changes in crop management practices beginning in the 1960s are thought to exemplify this transition from landraces to modern varieties (Frankel & Bennett 1970). To circumvent the extinction of such genotypes, a germplasm collection strategy was first proposed by Baur (1914) and realised by Vavilov in 1920–1940. This effort led to the founding of gene banks and collecting of genetic resources of crops and their wild relatives (Vavilov 1926). However, in spite of impressive numbers of collected accessions (estimated to be around 7 million worldwide), the loss of genetic diversity of species has been reported in many commercially important crops (Harlan 1975; Esquinas-Alcázar 2005; Glaszmann *et al.* 2010). In the broadest sense, this alteration and narrowing of crop genetic diversity began with the first domestication of wild plants. Although present-day monocultures are highly productive, their reduced genetic variability leaves them with a diminished capacity to deal with novel diseases, pests, and other changes in environmental conditions. As farmers and agricultural scientists continue to focus on the world's ever-increasing food requirements, conservation of genetic diversity will play a crucial role in the development of new varieties and in meeting new environmental challenges (Esquinas-Alcázar 2005). Since the genetic diversity of a crop can change over time, the studies were devoted to comparisons of the overall genetic diversity either at temporal or spatial stratification (Russell *et al.* 2000; Le Clerc *et al.* 2005, 2006; Figliuolo *et al.* 2007; Hysing *et al.* 2008; Malysheva-Otto *et al.* 2007; van de Wouw *et al.* 2009, 2010), mostly with no clear general results.

Although morphological descriptors are still widely used in defining germplasm groups and remain the only legitimate marker type accepted by the International Union for the Protection of New Varieties of Plants (UPOV), they can be unreliable owing to the influence of the environment and also to a natural portion of genetic heterogeneity. In contrast, molecular markers accurately represent the underlying genetic variation and nowadays dominate genetic diversity research. Among the variety of markers to choose, retrotransposon-based and microsatellite markers (simple sequence repeats, SSRs) proved to be effective, thanks to their polymorphism and abundance in plant genomes (Glaszmann *et al.* 2010). Microsatellites as highly informative codominant markers are used in population genetic research ranging from the level of individuals to closely related species. On the other hand, high mutation rate and homoplasy of SSRs (Cieslarová *et al.* 2011b) make them inapplicable for studies on higher taxonomic levels. Retrotransposon-based amplified polymorphism (RBIP) developed for pea (Flavell *et al.* 2003) found its use not only in phylogenetic studies, but also for pea germplasm collections (Smýkal *et al.* 2008, 2011; Jing *et al.* 2010; Ellis 2011).

Previously we investigated changes in the genetic integrity of pea collection in the process of germplasm maintenance (Cieslarová *et al.* 2011a) and reported an evidence of genetic erosion. This study focuses on the temporal diversity changes in the Czech/Czechoslovak pea germplasm collection as assessed by microsatellite and retrotransposon markers in three time periods spanning the 70 years of pea breeding.

MATERIAL AND METHODS

Plant material

The *Pisum* germplasm collection kept in Agritec Ltd. Šumperk, Czech Republic, currently includes 1307 accessions, of which 79% are dry seed peas (*Pisum sativum* subsp. *sativum* var. *sativum* L.) and 21% fodder peas (*Pisum sativum* subsp. *sativum* var. *arvense* /L./ Poiret). The collection is guided according to general rules of the National Programme for Plant Genetic Resources of the Czech Republic and passport data are available at http://genbank.vurv.cz/genetic/resources. We have used the subset (175 accessions) of Czech/Czechoslovak accessions bred over the last 70 years, which has already been integrated into European legume diversity projects (Smýkal *et al.* 2008, 2011). Of them, 115 accessions were smooth-seeded, white flowering, dry seed peas and 60 accessions were coloured flowering, fodder peas. In addition, one hundred and sixty-four accessions were analysed within the Bioversity International funded project, aimed at the establishment of core collection methodology (Smýkal *et al.* 2010). The 175 accessions were divided according to the date of registration or germplasm entry (the latter often in the case of accessions of the first group) into three groups. The first group comprised 43 dry seed and 27 fod-

der pea accessions registered by 1961; the second group comprised 30 dry seed and 16 fodder pea accessions registered by 1980; the third group was composed of 42 dry seed and 17 fodder pea accessions registered by 2004.

DNA isolation

Young leaves from 10 randomly chosen field grown plants (but morphologically characterized as described in Smýkal *et al.* 2008) per accession were bulked together and DNA was isolated according to Smýkal *et al.* (2008) using commercial kits.

Microsatellite and retrotransposon analysis

Seven SSR markers, localized to different linkage groups (Loridon *et al.* 2005), and 31 RBIP markers (Jing *et al.* 2005) were used (Tables 1, 2). The selection of loci was done according to the previous analysis (Smýkal *et al.* 2008, 2011). The SSR and RBIP marker PCR amplifications and gel analysis were performed as described in Smýkal *et al.* (2008). RBIP alleles were scored according to Jing *et al.* (2005, 2010).

Genetic diversity analysis

Allele patterns from SSR and RBIP analysis were analysed as fragment lengths and recoded to the format required by the respective software. Both SSR and RBIP scores were converted into binary data by presence (1) or absence (0) of the selected fragment (respective allele in the case of SSR loci). The POPGENE 1.32 program (Yeh & Boyle 1997) was used to calculate the following parameters: allele frequencies at each locus for complete and each group, the number of observed alleles, the number of effective alleles, Shannon information index (*I*), gene diversity (*H*) according to Levene (1949), heterozygosity statistics (Reynolds *et al.* 1983) and Nei's unbiased genetic distance D_S (Nei 1978) for each of the three groups. Since pea is a highly self-pollinating species, heterozygous plants are rarely found. In our case, since we analysed ten plants per accession, instead of heterozygosity this value refers rather to the accession heterogeneity. Polymorphic information content (*PIC*) was calculated for each marker as described in Smýkal *et al.* (2008). Goodness of fit of genetic distances derived from different markers was assessed by the Mantel test (Mantel 1967) using NTSYS-pc version 2.2 (Rohlf 2006). F_{ST} index was computed between groups as a measure of distances between populations. The significance of F_{ST} values was tested using FSTAT 2.9.3.2 program (Goudet 1995). The factorial analysis (Principal Coordinate Analysis, PCoA) was performed with the DARwin software package (Dissimilarity Analysis and Representation for Windows v. 5.0.155, Perrier & Jacquemoud-Collet 2006), separately on SSR, RBIP and combined datasets.

Table 1. Details of microsatellite loci used in the study, modified according to Loridon *et al.* (2005)

SSR locus	Linkage group/position (cM)	No. of alleles	Fragment size (bp)	*PIC*	Shannon's information index
A-278	III/154.9	5	150–180	0.51	1.02
AD-270	III/254.3	7	230–280	0.76	1.59
A-9	IV/ 62.1	3	330–360	0.62	1.10
AD-141	VI/ 70.1	8	210–330	0.73	1.61
AB-65	VII/ 94.1	3	140–180	0.15	0.33
B-14	VII/113.9	4	430–460	0.65	1.28
AD-237	VII/152.1	7	220–340	0.75	1.60
Mean				0.60	1.22
SD				0.22	0.46

PIC – polymorphic information content; SD – standard deviation

RESULTS

Genetic diversity analysis

Seven SSR loci detected altogether 37 alleles in 175 accessions, 36 alleles in dry seed and 31 in fodder pea subset, respectively. All seven loci proved to be polymorphic, detecting 3 to 7 alleles per locus, with AD-270, AD-141 and AD-237 being the most effective with 7 to 8 alleles. The values of Shannon information index were higher than 1.5 and their *PIC* value was above 0.7 (Table 1). In the case of retrotransposon markers, 31 RBIP loci were scored, and 13 of them were monomorphic (2055-NR1, 95-R2, 2055-NR36, 1074Cyc29, 1794-1, 2385x23, 2385x64, 1006-x21, 95-x25, 64-x15, 64-x76, 45-x33, 399-9x) in this set. The frequencies of occupied and empty site, null allele (no PCR product), observed heterozygosity and gene diversity for the remaining 18 polymorphic RBIP markers are summarized in Table 2. Of these eighteen, 12 RBIP loci occasionally provided null alleles with frequencies of 0.01 to 0.35, and 16 loci detected heterozygosity/heterogeneity from 0.011 (at 2055nr23 locus) to 0.869 (at 399-80-46 locus). The analysis of individual plants revealed that it was a result of heterogeneity, often detected at several RBIP loci (data not shown), rather than individual plant heterozygosity. In total, the heterogeneity associated with the use of 10 bulked plants per accession was detected in 29 out of 175 accessions (15%). The average gene diversity was 0.346 for each of primer pairs. The 45x31, 399-80-46 and RBIP-3 were the most effective loci for the genetic diversity analysis of the set. Their Shannon information index and *PIC* had the highest values: 1.093, 0.847, 0.832 (*I*) and 0.62, 0.44, 0.44 (*PIC*), respectively (Table 2). We compared

Table 2. Shannon information index and polymorphic information content (*PIC*) of RBIP loci

RBIP Locus	Frequency of occupied site	Frequency of empty site	Null allele	Observed heterozygosity	*PIC*	Shannon's information index
RBIP-7	0.217	0.771	0.011	0.057	0.30	0.583
RBIP-3	0.686	0.194	0.120	0.023	0.44	0.832
RBIP-4	0.843	0.157	0.000	0.291	0.23	0.435
Birte-B1	0.751	0.249	0.000	0.097	0.30	0.561
B1-Agt	0.743	0.257	0.000	0.080	0.31	0.570
1006-x19	0.671	0.300	0.029	0.086	0.38	0.730
399-14-9a	0.451	0.543	0.000	0.000	0.37	0.690
399-14-9b	0.409	0.591	0.000	0.120	0.37	0.676
Birte-x5	0.511	0.483	0.006	0.040	0.38	0.724
45x31	0.363	0.283	0.354	0.063	0.62	1.093
64x45	0.546	0.431	0.023	0.006	0.40	0.780
281x40	0.080	0.920	0.000	0.160	0.14	0.279
2055nr23	0.006	0.840	0.154	0.011	0.24	0.464
281x44	0.280	0.714	0.006	0.091	0.33	0.626
Birte-x16	0.906	0.060	0.034	0.029	0.17	0.374
1794-2	0.000	0.994	0.006	0.000	0.01	0.035
1074Cyc12	0.869	0.046	0.086	0.046	0.23	0.474
399-80-46	0.469	0.486	0.046	0.869	0.44	0.847
Mean				0.115	0.32	0.598
SD				0.200	0.14	0.239

SD – standard deviation

Table 3. Gene diversity (*H*) with standard deviation values and number of accessions for different types of pea and groups (1 = x–1961, 2 = 1962–1980, 3 = 1981–2004) for SSR and RBIP markers

Type	Group	*H** (SSR)	*H** (RBIP)	Number of accessions
Dry seed	1–3	0.592 ± 0.235	0.218 ± 0.231	115
	1	0.566 ± 0.195	0.229 ± 0.236	43
	2	0.589 ± 0.259	0.220 ± 0.239	30
	3	0.538 ± 0.272	0.177 ± 0.213	42
Fodder	1–3	0.626 ± 0.196	0.214 ± 0.216	60
	1	0.612 ± 0.224	0.226 ± 0.229	27
	2	0.593 ± 0.199	0.199 ± 0.209	16
	3	0.603 ± 0.134	0.187 ± 0.216	17
Dry seed + fodder	1–3	0.625* ± 0.222	0.220 ± 0.226	175
	1	0.617 ± 0.220	0.234 ± 0.237	70
	2	0.606 ± 0.238	0.219 ± 0.228	46
	3	0.592 ± 0.222	0.187 ± 0.206	59

*differences in allele frequencies between categories resulted in higher *H* for total rather than individual categories

genetic diversity analysed separately by SSR versus RBIP markers. Although both marker types are derived from repetitive sequences, they clearly sample different proportions of the large pea genome, as the pairwise genetic distances were different (not shown). On the other hand, both marker types were comparably effective in revealing the genetic diversity, with a high correlation (r = 0.81).

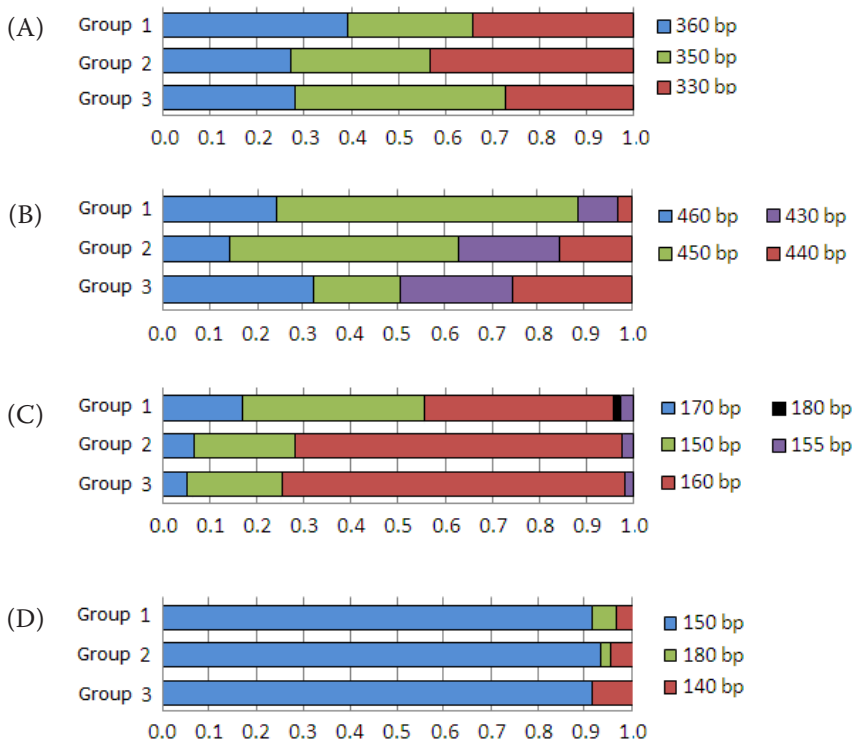

Figure 1. Changes in allele frequency at microsatellite loci; allele sizes (bp) are indicated with different bar colour and pattern; (A) locus A-9, (B) locus B-14, (C) locus AA-278, (D) locus AB-65

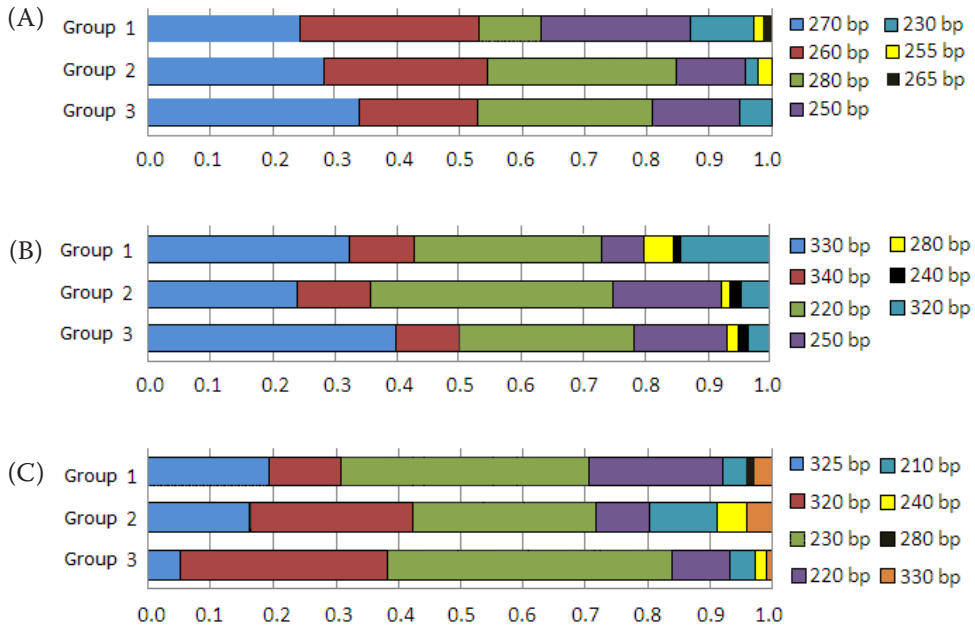

Figure 2. Changes in allele frequency at microsatellite loci; allele sizes (bp) are indicated with different bar colour and pattern; (A) locus AA-270, (B) locus AA-237, (C) locus AD-141

Temporal changes in genetic diversity

Within groups

The comparisons of gene diversity (*H*) values within groups (Table 3) revealed a decrease from the first to the third group both for microsatellite and retrotransposon markers. The changes in gene diversity in time could be detected also as changes in allele frequencies over time, which showed even the loss of some alleles in the case of AA-278, AB-65 and AD-270 loci (Figures 1–4). Average observed heterozygosity/heterogeneity was 0.06 for accessions in the first group, 0.08 in the second group and 0.04 in the third group. The analysis of individual plants revealed that it was really the result of heterogeneity and not true heterozygosity. The maximum observed heterogeneity was 0.17 (first group), 0.28 (second group) and 0.12 (third group) for the AD-141 locus.

Between groups

Based on paired comparison of the amount of genetic differentiation (F_{ST}) assessed separately by

Table 4. Pairwise comparison of genetic differentiation (F_{ST}) and Nei's unbiased measure of genetic distance (D_S) for SSR, RBIP and combined analysis

Analysis	Compared groups	F_{ST}	Unbiased D_S
SSR	1 «» 2	0.026	0.064
	1 «» 3	0.060	0.122
	2 «» 3	0.016^{NS}	0.045
RBIP	1 «» 2	0.022	0.010
	1 «» 3	0.032	0.012
	2 «» 3	0.044	0.016
Combined SSR + RBIP	1 «» 2	0.023	0.015
	1 «» 3	0.044	0.022
	2 «» 3	0.036	0.018

^{NS}not significant

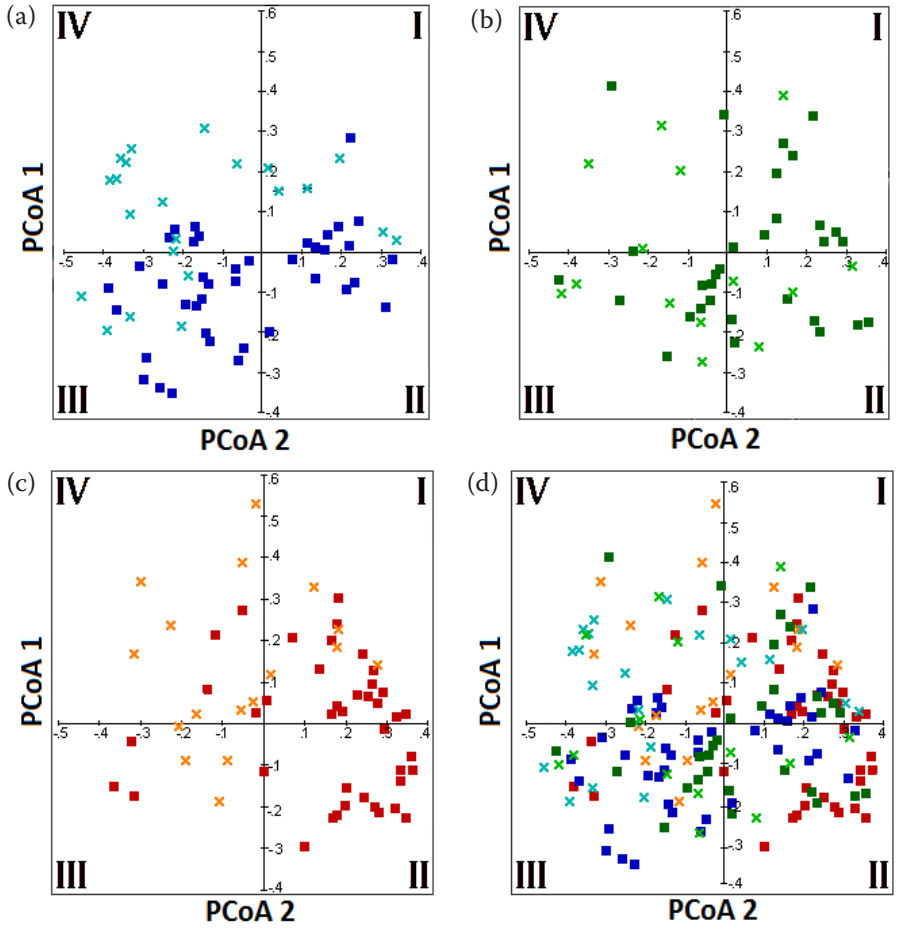

Figure 3. Principal Coordinate Analysis (PcoA) axis 1 and 2 of microsatellite data; (a) first group (blue), (b) second group (green), (c) third group (red), (d) all 175 accessions; dry seed peas are symbolized with square, fodder peas with cross symbols; ellipses represent dry seed pea and fodder pea accessions respectively

microsatellite or retrotransposon markers, accessions between the first and the third group had the highest F_{ST} index of 0.060 for SSRs (0.044 for RBIPs), between the first and the second group the value of this index was 0.026 (0.022 for RBIPs), while between the second and the third group the index had the lowest value (0.016 for SSRs, 0.032 for RBIPs). Similarly, Nei's unbiased measures of genetic distance (D_S) were highest between the first and the third group (0.122 and 0.016, respectively), followed by 0.035 (0.010 for RBIPs) between the first and the second group and they were lowest between the second and the third group (0.045) (Table 4). Similarly, the observed number of alleles (N_a) and effective number (N_e) of alleles per SSR locus and Shannon information index changed among the groups (Table 5). In the case of three SSR loci (AD-270, A-278 and AB-65) a decrease in allele number was found, while Shannon information index was a more sensitive measure and showed a decrease for all but one (B-14) loci (Table 5). Heterogeneity detected by retrotransposon markers decreased from 0.074 to 0.056 for accessions in the respective groups. The combination of both molecular markers showed similar results of group differentiation (Table 4). The first group compared to the third had the highest F_{ST} index and Nei's measures of genetic distance (D_S) (0.044 and 0.022, respectively), the second group compared to the third had the respective values 0.036 and 0.018, and accessions in the first group compared to the second had the shortest distance (0.023 and 0.015, respectively).

Among accessions

To reveal another structural level of the studied groups of accessions, the dissimilarity matrix calculated from both SSR and RBIP data was used in the factorial analysis performed by the DARwin software and also for generating the PCoA plots

Table 5. Observed number of alleles (N_a), effective number of alleles per locus (N_e) and Shannon information index (I) for all SSR loci

SSR Locus	N_a			N_e			I			
	period 1	period 2	period 3	period 1	period 2	period 3	period 1	period 2	period 3	mean
AD-270*	7	6	5	4.55	3.95	3.94	1.637	1.477	1.461	1.522
A-9	3	3	3	2.93	2.87	2.83	1.086	1.076	1.070	1.077
B-14	4	4	4	2.08	3.03	3.85	0.940	1.245	1.368	1.184
AD-237	7	7	7	4.30	3.89	3.67	1.628	1.536	1.495	1.553
A-278*	5	4	4	2.95	1.87	1.74	1.198	0.846	0.775	0.940
AD-141	7	7	7	3.87	4.90	3.01	1.543	1.732	1.340	1.538
AB-65*	3	3	2	1.19	1.14	1.18	0.347	0.283	0.290	0.307
Mean	5.1	4.9	4.6	3.12	3.09	2.89	1.196	1.170	1.114	
SD	1.9	1.8	1.9	1.22	1.29	1.07	0.464	0.492	0.443	

*decrease in the observed allele number; SD – standard deviation

(Figures 3a–c). The first five coordinates (PCoA axes) accounted for 36.63% of the observed variation in the case of microsatellites or for 40.08% in retrotransposons. The first component explained 11.5% and the second component 6.8% of the variation (11.6% and 9.78% in retrotransposons). The dry seed pea accessions formed more compact clusters (Figures 3a–c) in different quadrants of the plot in each of the three groups, in contrast to the fodder pea accessions which were more dispersed. The accession distribution as shown by both marker types suggests that similarly diverse sets of accessions are present in all three groups (Figures 5a–d). There is no clear narrowing or clustering of accessions in any time period, only small genetic shifts over the four plot quadrants (Figure 5d). When both SSR and RBIP data were combined, the first five coordinates (PCoA axes) accounted for 30.7% of the observed variation. Neither did this analysis reveal any narrowing or clustering of accessions in any time period, only small shifts over the four plot quadrants.

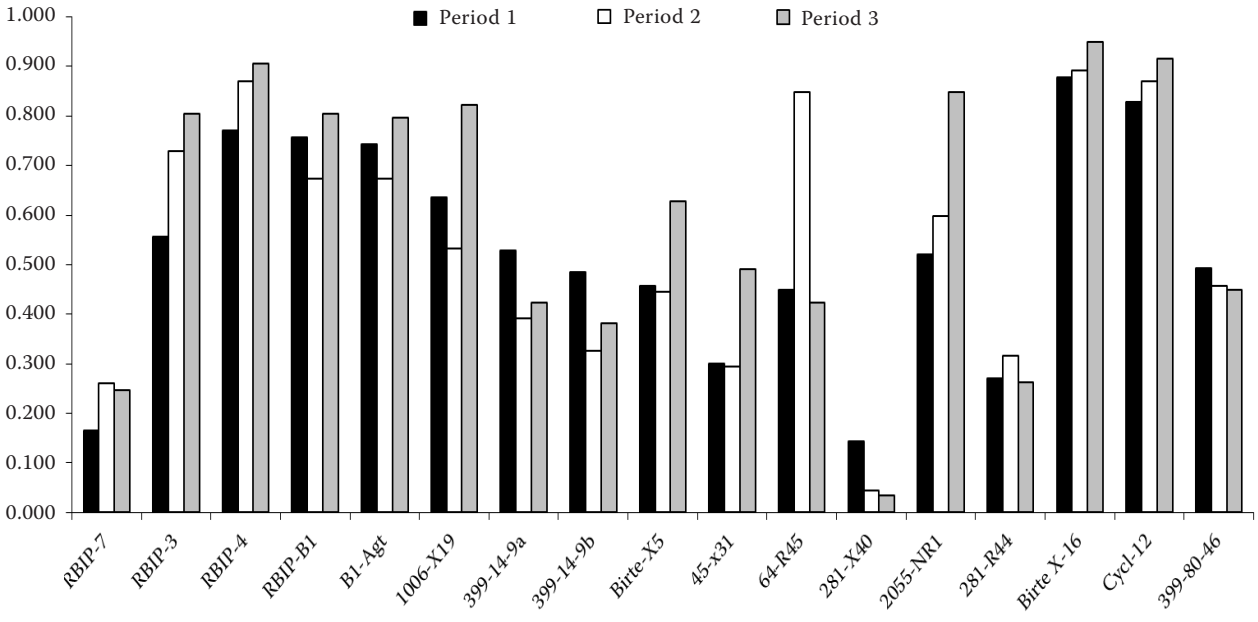

Figure 4. Frequencies of RBIP loci occupied site in accessions

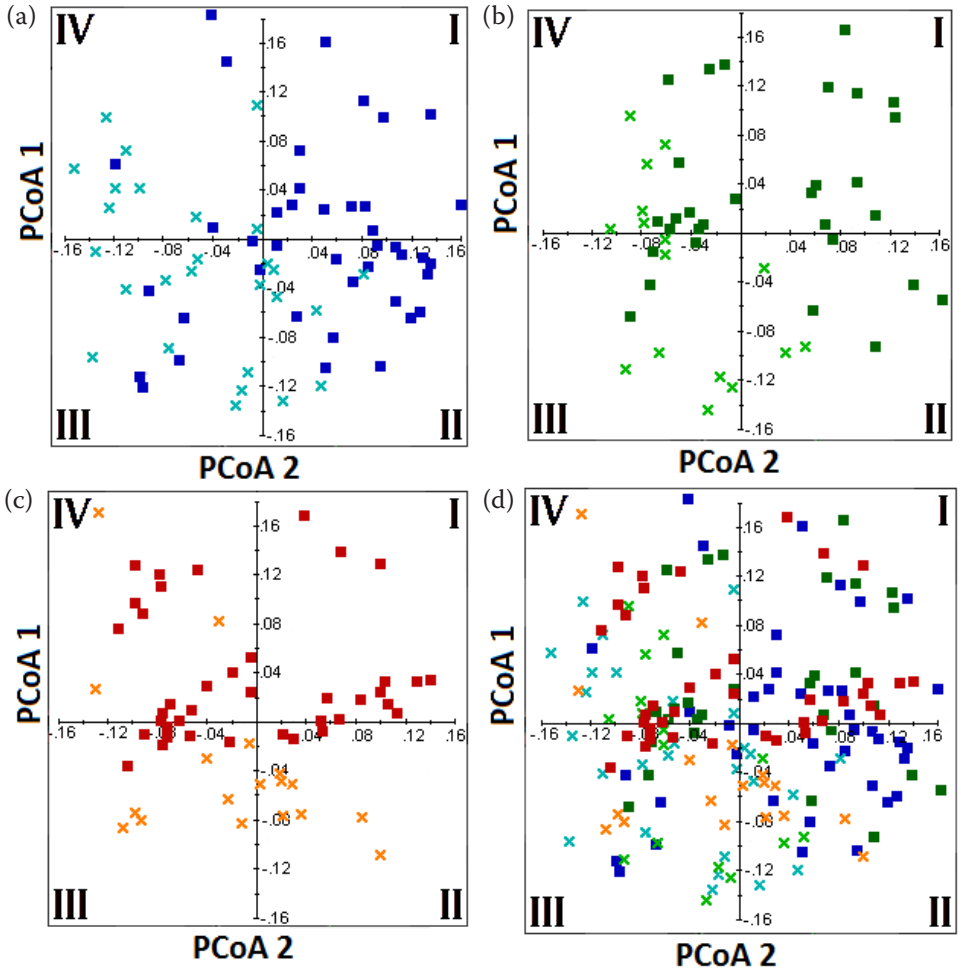

Figure 5. Principal Coordinate Analysis (PcoA) axis 1 and 2 of retrotransposon data; (a) first group (blue), (b) second group (green), (c) third group (red), (d) all 175 accessions; dry seed peas are symbolized with square, fodder peas with cross symbols

DISCUSSION

This study investigated changes in the genetic diversity of pea genetic resources over the period of 70 years by the analysis of microsatellite (SSR) and retrotransposon (RBIP) markers, while in our previous study we examined the genetic erosion in *ex situ* collections, as a result of regeneration and storage practices (Cieslarová *et al.* 2011a). We focused on pea genetic resources, where there is a limited possibility of *in situ* conservation and thus only *ex situ* germplasm collections are the essential reservoir for pea genetic diversity. We have chosen 175 accessions of Czech/Czechoslovak origin from the Czech national pea germplasm collection as this subset has the most comprehensive passport data. This set contains all 164 accessions analysed by Smýkal *et al.* (2008); however, in this study we did not focus on the population structure, but the set was divided into three groups to investigate possible changes over time. In addition, this dataset was analysed within the Bioversity International project, aimed at the establishment of core collection methodology, with available molecular and phenotypic data (Smýkal *et al.* 2010). The first group consisting of older cultivars and landraces included in the collection before 1961 represents very old varieties and landraces grown in former Czechoslovakia back in the 1940's and even earlier. The second group included accessions bred from 1962 to 1980, and the third group contained modern cultivars registered from 1981 to 2004. This division reflected changing priorities in pea breeding, which emphasized the tall plant habit in its early stages, then short pea cultivars and most recently intermediate-type pea cultivars are demanded in the market.

Breeding and genetic diversity

Three different approaches to methods to quantify genetic erosion have been recognized: (*i*) an absolute loss of a crop, variety or allele, which ignores the dynamic nature of population genetic processes, (*ii*) genetic erosion as a reduction in richness (i.e. a reduction in the total number of crops, varieties or alleles), and (*iii*) genetic erosion as a reduction in evenness. The last approach is based on diversity indices used in vegetation ecology and population genetics (van de Wouw *et al.* 2010) and uses the frequencies of alleles within a group of genotypes. In this study we have used this approach to study diversity trends in pea breeding using molecular techniques. Results from published studies vary considerably. Some studies showed a decrease in diversity over time (Russell *et al.* 2000; Fu *et al.* 2003; Reif *et al.* 2005; Figliuolo *et al.* 2007; Malysheva-Otto *et al.* 2007,), while others observed increases (Fu 2006; White *et al.* 2008) or a decrease in the diversity levels after which an increase occurred (Roussel *et al.* 2004; Qi *et al.* 2006). Our findings are consistent with the work of Le Clerc *et al.* (2006) analysing changes in the pea germplasm genetic diversity through time. After analysing 587 pea lines from the last 50 years by morphological and isozyme, selectively neutral markers, they did not observe any significant changes in genetic diversity over time, only slight genetic differentiation between the five decades. A meta-analysis aimed at 48 papers on the diversity trends (including Smýkal *et al.* 2008) showed no substantial reduction in diversity (van de Wouw *et al.* 2010). A reduction in genetic diversity was observed in the 1960s, but even there the observed reduction in diversity was only 5%. Later recovery of diversity likely reflects the greater use of exotic germplasm and crop wild relatives in the breeding process (van de Wouw *et al.* 2009, 2010). It has to be noted that most of these studies focused on the diversity released by breeders during a certain period, with the assumption that what is released by breeding programmes is a reflection of what is grown by farmers. However, breeding programmes are not always able to meet the requirements of farmers. On the other hand, a successful cultivar may be grown for many years. Consequently, in crops with a high turnover of cultivars and good acceptance by farmers, the time of cultivar release would better reflect diversity trends than in crops where cultivars are grown for many years and farmers and breeders are not very innovative, unless the time groups compared are very long (van de Wouw *et al.* 2009). Moreover, little diversity will be released during groups with little breeding activity.

Molecular data analysis

The development of molecular techniques in the last decades has made it possible to study genetic erosion at the level of alleles. Allelic richness is important from evolutionary as well as breeders' aspects as a basis for the continuous improvement and adaptation of the crop (van de Wouw *et al.* 2010). The molecular markers used in our study derive from multiple dispersed loci in the large *Pisum* genome and represent the spectrum of genetic distances between orthologous genomic regions in the germplasm (Smýkal *et al.* 2008, 2011), whereas the morphological traits are controlled by multiple genes, some of which have probably been subjected to strong direct or indirect selection during the breeding process. As a result, the molecular markers display much less of the total variance in the first axes of ordination analysis (such as PCoA) than do morphological traits, unless highly distinct accessions are analysed (Smýkal *et al.* 2008, 2011). Consequently, such analysis might not be expected to separate the germplasm into clearly separated groups, as shown in this study and in the previous one (Smýkal *et al.* 2008). Of the selected microsatellite and retrotransposon markers, 7 SSR and 18 RBIP were shown to be polymorphic and informative for the given set. It has to be noted that in order to reveal possible heterogeneity of accessions, 10 morphologically assessed plants per accession were used to form a bulk sample for DNA analysis. In comparison with single plant sampling, this assures adequate representation of the total diversity in an accession, reduces the possibility of mis-scoring and reveals heterogeneity within accessions (Cieslarová *et al.* 2011a, b). Based on our previous studies (Smýkal *et al.* 2008, 2011; Cieslarová *et al.* 2011a, b), we estimated that about 10% of the accessions of the entire collection are heterogeneous, and in the selected 175 accessions of this study this figure was approaching 15% (29 out of 175 accessions). The simplicity and unequivocal scoring of RBIP markers were previously demonstrated, while multiallelic SSR markers might be less accurate (Cieslarová *et al.* 2011a, b). We applied both markers derived from repetitive sequences and routinely used them in germplasm description (Kalendar *et al.* 2011, Smýkal *et al.* 2011). SSR markers showed a

higher polymorphism per locus (average *PIC*= 0.60, Table 1), since their polymorphism is based on various lengths (alleles) of the amplified fragment from the given locus, while for retrotransposon insertion markers the information is of only one of three possible types (average *PIC*= 0.32, Table 5). Our results showed a high correlation between the values of gene diversity obtained by SSR and RBIP analysis (r = 0.81), although the pairwise genetic distances of each marker type differed (Smýkal *et al.* 2011). As recently shown by Ellis (2011), we observed no significant correlation between the values of genetic distances derived from SSR and RBIP marker data, indicating that these two marker types sample different fractions of genetic diversity in this germplasm. We therefore suggest that combining various data types provides the better representation of diversity than using just one alone (parallel to more plants analysed per accession).

Changes in genetic diversity over time

When studying trends of allelic richness, equal sample numbers or methods to correct for unequal sample numbers have been used. Although methods to correct for different sample sizes of large populations are commonly used in ecology, such methods are less appropriate in the study reported here (van de Wouw *et al.* 2010). Le Clerc *et al.* (2006), who were able to study all cultivars that were available in a certain period, found an increased total number of alleles for both peas and maize over time, while the allelic richness showed a small (not significant) decrease. However, this decrease was more than compensated for by a larger number of cultivars in the variety lists in the more recent period. In our study, the estimates of gene diversity and changes in allele frequencies across three time groups in total indicate that plant breeding has not led to any substantial changes in the total genetic diversity of Czech/Czechoslovak pea genetic resources. We detected only not a significant decrease of gene diversity (H) over time in the investigated 175 accessions both by SSR and RBIP markers (Table 3). The first group consists of the oldest accessions, bred before 1960, and includes more landraces and heterogeneous material, which is consistent with higher gene diversity as well as with higher RBIP heterozygosity (heterogeneity) than in accessions of both more recent groups. Our results are consistent with findings of van de Wouw *et al.* (2010) and Hysing *et al.* (2008), who stated that since the 1960s and 1970s, breeders have been able to increase the diversity in released varieties by using more exotic parental accessions. We could not objectively evaluate the changes in genetic diversity in the period from the 1920s to the 1950s. Although the material from that period is included in the collection, it is not dated properly, since the collection was established in the late 1950s and all initial material had a similar year of including to the collection. Our investigated set of accessions was subdivided into three time groups, in correspondence with different breeding strategies. Although a new gene introduced into a crop should increase its overall richness, it might actually be counterproductive if it becomes very popular and all farmers switch to the cultivars with this new gene (e.g. currently the case of semi-leafless types/afila mutation of pea). Eventually, this might lead to the narrowing of genetic diversity as happened in the past in maize harbouring a specific type of male sterility. The genetic associations of the 175 accessions revealed by the principal coordinate analysis (Figures 3 and 5) were largely consistent with the genetic relationships described above, although the first two principal coordinate axes accounted only for 18.35% (SSR), 21.22% (RBIP) and 15.90% (combined) of the total variation. These accessions representing different breeding programs (tall, short and intermediate plant habit) and groups were widely spread over the plot and no clear narrowing or clustering of accessions was found in any time period, only small genetic shifts over the four plot quadrants. Although no substantial loss in genetic diversity was observed, the allelic frequencies of microsatellite loci changed and a small portion of alleles was not detected in most recent accessions. The results demonstrate the importance of monitoring the genetic diversity, and a vital role of germplasm collections for crop diversity conservation.

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References

Ambrose M.J. (1995): From Near East centre of origin the prized pea migrates throughout world. Diversity, **11**: 118–119.

Baur E. (1914): Die Bedeutung der primitiven Kulturrasen und der wilden Verwandten unserer Kulturpflanzen für die Pflanzenzüchtung. Jahrbuch der Deutschen Landwirtschaftsgeselschaft, **29**: 104–109.

Cieslarová J., Smýkal P., Dočkalová Z., Hanáček P., Procházka S., Hýbl M., Griga M. (2011a): Molecular evidence of genetic diversity changes in pea (*Pisum sativum* L.) germplasm after long-term maintenance. Genetic Resources and Crop Evolution, **58**: 439–451.

Cieslarová J., Hanáček P., Fialová E., Hýbl M., Smýkal P. (2011b): Estimation of pea (*Pisum sativum* L.) microsatellite mutation rate based on pedigree and single-seed descent analyses. Journal of Applied Genetics, **52**: 391–401.

Ellis T.H.N. (2011): *Pisum*. In: Kole C. (ed.): Wild Crop Relatives: Genomic and Breeding Resources (Legume Crops and Forages). Springer Verlag, Berlin-Heidelberg, 237–248.

Esquinas-Alcázar J. (2005): Protecting crop genetic diversity for food security: political, ethical and technical challenges. Nature Review in Genetics, **6**: 946–953.

Figliuolo G., Mazzeo M., Greco I. (2007): Temporal variation of diversity in Italian durum wheat germplasm. Genetic Resources and Crop Evolution, **54**: 615–626.

Flavell A.J., Bolshakov V.N., Booth A., Jing R., Russell J., Ellis T.H.N., Isaac P. (2003): A microarray-based high throughput molecular marker genotyping method: The tagged microarray marker (TAM) approach. Nucleic Acids Research, **31**: 1–8.

Frankel O.H., Bennett E. (1970): Genetic Resources – Introduction. In: Frankel O.H., Bennett E. (eds): Genetic Resources in Plants – Their Exploration and Conservation. IPB Handbook No. 11. International Biological Programme, London, 7–18.

Fu Y.B. (2006): Impact of plant breeding on genetic diversity of agricultural crops: Searching for molecular evidence. Plant Genetic Resources: Characterisation and Utilisation, **4**: 71–78.

Fu Y.B., Peterson G.W., Scoles G., Rossnagel B., Schoen D.J., Richards K.W. (2003): Allelic diversity changes in 96 Canadian oat cultivars released from 1886 to 2001. Crop Science, **43**: 1989–1995.

Glaszmann J.C., Kilian B., Upadhyaya H.D., Varshney R.K. (2010): Accessing genetic diversity for crop improvement. Current Opinion in Biology, **13**: 167–173.

Goudet J. (1995): FSTAT (Ver. 1.2): a computer program to calculate F-statistics. Journal of Heredity, **86**: 485–486.

Hammer K., Arrowsmith N., Gladis T.S. (2003): Agrobiodiversity with emphasis on plant genetic resources. Naturwissenschaften, **90**: 241–250.

Harlan J.R. (1975): Our vanishing genetic resources. Science, **188**: 618–621.

Hysing S.C., Sälla T., Nyboma H., Liljerotha E., Merkera A., Orforda S., Koebner R.M.D. (2008): Temporal diversity changes among 198 Nordic bread wheat landraces and cultivars detected by retrotransposon–based S–SAP analysis. Plant Genetic Resources: Characterization and Utilization, **6**: 2–10.

Jing R., Knox M.R., Lee J.M., Vershinin A.V., Ambrose M., Ellis T.H.N., Flavell A.J. (2005): Insertional polymorphism and antiquity of PDR1 retrotransposon insertions in *Pisum* species. Genetics, **171**: 741–752.

Jing R., Vershinin A., Grzebyta J., Shaw P., Smýkal P., Marshall D., Ambrose M.J., Ellis T.H.N., Flavell A.J. (2010): The genetic diversity and evolution of field pea (*Pisum*) studied by high throughput retrotransposon based insertion polymorphism (RBIP) marker analysis. BMC Evolutionary Biology, **10**: 44.

Kalendar R., Flavell A.J., Ellis T.H.N., Sjakste T., Moisy C., Schulman A.H. (2011): Analysis of plant diversity with retrotransposon-based molecular markers. Heredity, **106**: 520–530.

Le Clerc V., Banzete F., Baril C., Guiard J., Zhang D. (2005): Assessing temporal changes in genetic diversity of maize varieties using microsatellite markers. Theoretical and Applied Genetics, **110**: 294–302.

Le Clerc V., Cadot V., Canadas M., Lallemand J., Guerin D., Boulineau F. (2006): Indicators to assess temporal genetic diversity in the French catalogue: no losses for maize and peas. Theoretical and Applied Genetics, **113**: 1197–1209.

Levene H. (1949): On a matching problem in genetics. The Annals of Mathematical Statistics, **20**: 91–94.

Loridon K., Mcphee K., Morin J., Dubreuil P., Pilet–Nayel M.L., Aubert G., Rameau C., Baranger A., Coyne C., Lejeune–Henaut I., Burstin J. (2005): Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theoretical and Applied Genetics, **111**: 1022–1031.

Malysheva–Otto L., Ganal M.W., Law J.R., Reeves J.C., Röder M.S. (2007): Temporal trends of genetic diversity in European barley cultivars (*Hordeum vulgare* L.). Molecular Breeding, **20**: 309–322.

Mantel N. (1967): The detection of disease clustering and a generalized regression approach. Cancer Research, **27**: 209–220.

Nei M. (1978): Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **89**: 583–590.

Perrier X., Jacquemoud-Collet J.P. (2006): DARwin software. Available at http://darwin.cirad.fr/darwin (accessed July 2011).

Pistorius R. (1997): Scientists, Plants and Politics – A History of the Plant Genetic Resources Movement. International Plant Genetic Resources Institute, Rome.

Proskowetz E. von (1890): Welches Werthverhaltniss besteht zwishen den Landrassen landwirthschaflicher Kutlurpflanzen und den sogenanneten Zuchtunsrassen ? In: Internationaler land- und forstwirstchaflicher Congress zu Wiena 1890. Section I: Landwirthschaft. Subsection: Pflanzenbau. Frage 5. Heft 13: 3–18

Qi Y., Zhang D., Zhang H., Wang M., Sun J., Wei X., Qiu Z., Tang S., Cao Y., Wang X., Li Z. (2006): Genetic diversity of rice cultivars (*Oryza sativa* L.) in China and the temporal trends in recent fifty years. Chinese Science Bulletin, **51**: 681–688.

Reif J.C., Hamrit S., Heckenberger M., Schipprack W., Maurer H.P., Bohn M., Melchinger A.E. (2005): Trends in genetic diversity among European maize cultivars and their par-ental components during the past 50 years. Theoretical and Applied Genetics, **111**: 838–845.

Reynolds J., Weir B.S., Cockerham C. (1983): Estimation of the coancestry coefficient: basis for a short–term genetic distance. Genetics, **105**: 767–776.

Rohlf F. (2006): NTSYSpc: Numerical Taxonomy System (Ver. 2.2). Exeter Publishing, Ltd., Setauket.

Roussel V., Koenig J., Beckert M., Balfourier F. (2004): Molecular diversity in French bread wheat accessions related to temporal trends and breeding programmes. Theoretical and Applied Genetics, **108**: 920–930.

Roussel V., Leisova L., Exbrayat F., Stehno Z., Balfourier F. (2005): SSR allelic diversity changes in 480 European bread wheat varieties released from 1840 to 2000. Theoretical and Applied Genetics, **111**: 162–170.

Russell J.R., Ellis R.P., Thomas W.T.B., Waugh R., Provan J., Booth A., Fuller J., Lawrence P., Young G., Powell W. (2000): A retrospective analysis of spring barley germplasm development from 'foundation genotypes' to currently successful cultivars. Molecular Breeding, **6**: 553–568.

Schindler F. (1890): Welches Werthverhaltniss besteht zwishen den Landrassen landwirthschaflicher Kutlurpflanzen und den sogenanneten Zuchtunsrassen ? In: Internationaler land- und forstwirstchaflicher Congress zu Wiena 1890. Section I: Landwirthschaft. Subsection: Pflanzenbau. Frage 5. Heft 13: 19–24.

Smýkal P., Hýbl M., Corander J., Jarkovský J., Flavell A. J., Griga M. (2008): Genetic diversity and population structure of pea (*Pisum sativum* L.) varieties derived from combined retrotransposon, microsatellite and morphological marker analysis. Theoretical and Applied Genetics, **117**: 413–424.

Smýkal P., Hýbl M., Flavell A.J., Corander J., Ambrose M.J. (2010): Towards comprehensive pea germplasm management for future use. [Final Report of AEGIS LOA10/048 Project.] Available at http://aegis.cgiar.org/aegis_grant_scheme/first_call.html

Smýkal P., Kenicer G., Flavell A.J., Corander J., Kosterin O., Redden R.J., Ford R., Coyne C.J., Maxted N., Ambrose M.J., Ellis N.T.H. (2011): Phylogeny, phylogeography and genetic diversity of the *Pisum* genus. Plant Genetic Resources: Characterization and Utilization, **9**: 4–18.

Van De Wouw M., Kik C., Van Hintum T., Van Treuren R., Visser B. (2009): Genetic erosion in crops: concept, research, results and challenges. Plant Genetic Resources: Characterization and Utilization, **8**: 1–15.

Van De Wouw M., Van Hintum T., Kik C., Van Treuren R., Visser B. (2010): Genetic diversity trends in twentieth century crop cultivars: a meta analysis. Theoretical and Applied Genetics, **120**: 1241–1252.

Vavilov N.I. (1926): Studies on the origin of cultivated plants. Bulletin of Applied Botany 26. Leningrad.

White J., Law J.R., MacKay I., Chalmers K.J., Smith J.S.C., Kilian A., Powell W. (2008): The genetic diversity of UK, US and Australian cultivars of *Triticum aestivum* measured by DArT markers and considered by genome. Theoretical and Applied Genetics, **116**: 439–453.

Yeh F.C., Boyle T.J.B. (1997): Population genetic analysis of co-dominant markers and quantitative traits. Belgian Journal of Botany, **129**: 157.

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