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Full Length Research Paper

Evaluation of the Czech core collection of *Trifolium pratense*, including morphological, molecular and phytopathological data

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The Czech national core collection of the red clover (*Trifolium pratense* L.) was established in 2006 based on previous analyses (2004 to 2005) of morphological and some phytopathological data observed in the field conditions. Input set included 57 tetraploid accessions (varieties and newly bred varieties) and 130 diploid accessions (varieties, newly bred varieties and wild forms collected from nature) of the world collection. The core collection includes 76 accessions. Molecular and phytopathological data on the core collection were obtained after its establishment during 2007. The core collection was from the point of view of resistance to important fungal and viral pathogens evaluated. Tested plants were inoculated by spore-suspension of *Fusarium* sp. fungi and also by *Bean yellow mosaic virus* (BYMV). Microsatellites (SSRs) were used for the study of genetic diversity of all the core collections accessions. No clear difference between diploid and tetraploid accessions within the core collection was evidenced by cluster analysis of morphological data. Notable correlation was found only between the seed weight and ploidy level. The relationships among accessions were analysed with hierarchical clustering, separately for the morphological data (50 characters) and the data on SSR polymorphism (11 primers, 23 polymorphic bands). Molecular and morphological data were not significantly correlated. No differences in particular morphological characters were observed to be associated with the presence/absence of particular bands or with the overall pattern of SSR polymorphism. Dendrogram of accessions included into the Czech core collection of the red clover based on morphological data showed separation of items into seven subclusters, while dendrogram based on molecular data showed the separation of items into 13 subclusters. The core collection accessions showed some level of resistance to *Fusarium* sp. and BYMV infection. The correlation between resistance of core collection accessions to *Fusarium* sp. and BYMV pathogens was not significant.

Key words: Red clover, core collection, genetic diversity, ploidy level, microsatellites, morphological characteristics, *Fusarium* sp., BYMV.

INTRODUCTION

Red clover (*Trifolium pratense*) is one of the most important fodder crops. This species has its centre of diversity in Europe. Three basic subspecies are distinguished: subsp. *pratense*, subsp. *sativum* and subsp. *americanum* (Kubat, 1995). It is important to collect old varieties and landraces, because red clover has been cultivated in Europe for several hundred years.

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On the other hand, plants coming from natural populations growing under different ecological conditions are important sources of variability. These sources are an important resource of genes of resistance to diseases, pests, abiotic stresses and a prerequisite for the development of new varieties (Jahufer et al., 1994).

The study of genetic resources in fodder crops has a long tradition in the Czech Republic (Vacek, 1963; Pelikan et al., 2005; Vymyslicky et al., 2007). During the last 50 years, about 2000 fodder crops accessions have been gathered, evaluated, described and stored in gene banks, but many characters have not yet been evaluated. About half of the accessions are wild accessions collected from nature. The importance of wild species/forms is increasing as input materials for breeding programmes, for use in organic farming and in nature conservation.

Genetic resources of clover species and the possibilities of their use were studied by some researchers – Boller et al. (2003), Drobna (2004) and Onal Asci (2011) studied the red clover; Drobna and Zakova (2001) studied the white clover. The study of variation in the collections has been mostly based on morphological, phenological and agronomical characteristics of individual accessions. The relationships between studied characteristics are complicated and it is necessary to use multidimensional statistics. By the application of the multidimensional statistics the varieties could be differentiated or aggregated according to evaluated characteristics (Uzik and Zofajova, 1997). Cluster analysis is a useful tool for classification of the samples. We can ask if the samples have some common traits. The correlations of agronomically important traits could be studied also. Complicated relations among many variables gathered from the analyses and the selection of samples for core collections are in most cases based upon multidimensional statistics (Weihai et al., 2008). Kouame and Quesenberry (1993) used the cluster analysis for the classification of the red clover collection as the base for creating a core collection. Molecular markers recently became one of the most frequently used methods for studying genetic diversity (Varshney et al., 2008; Al-Ruqaishi et al., 2008). Simple sequence repeats (SSRs) are short tandem repeat units of between 1 and 6 bp in length (Tautz, 1989). The variation in the number of repeats present in these loci determines differences in length of the amplified fragments (Manifesto et al., 2001). Microsatellite loci proved to be highly polymorphic and useful as genetic markers in many plant species (Smith et al., 1997).

Red clover is damaged seriously by fungi from the genus *Fusarium* sp. (species most often mentioned are *F. oxysporum*, *F. solani*, *F. avenaceum*) and *Bean yellow mosaic virus* (BYMV). These pathogens cause decrease of persistence of clover plants after infection. With the respect to the present status of fodder crops and their complex biology, the mass application of direct control methods of these pathogens cannot be expected in the near future. Effective control measures will include a combination of suitable crop management and the use of resistant varieties. For this reason the continuous study and development of breeding materials with desirable characteristics is important. The aim of the study was to describe and analyse 76 Czech red clover core collection accessions by morphological, molecular and phytopathological data in detail. Gathered data were statistically analysed with the main aim to find: (1) correlations among characteristics and the ploidy level, (2) correlation of morphological and molecular data, and (3) to find relation of SSR polymorphism with some agronomically important characteristics relating to pathogen resistance.

MATERIALS AND METHODS

The Czech core collection was established in 2006. It was based on various evaluations and tests. In 2004, original seed samples gathered from the Czech national gene bank were used for detailed evaluation. Seed characteristics were evaluated on original seeds. After this evaluation the seeds were used for pre-growing of small plants that were later planted in the field. In the next year, 2005, morphological and yield characteristics were evaluated. Visual phytopathological evaluation was also included. Based on this data the core collection was established and in 2007 molecular evaluation was performed. Core collection origins were also phytopathologically evaluated on plants grown from original seeds in the greenhouse from the point of view of level of resistance to fungal pathogens.

Morphological and yield evaluation

The 76 accessions of the red clover (*T. pratense*) core collection (50 diploid and 26 tetraploid) - varieties, newly bred varieties and wild forms collected from nature, were included into detailed evaluation of morphological and yield characteristics. 30 plants of each accession were cultivated in field spacing of 50 × 50 cm. 50 characteristics were evaluated at 10 randomly selected plants of each accession (Table 1). Average values from 10 individuals were used for further analyses. Morphological, yield and resistance data were recorded as binary or in ordinal scale according to Czech national descriptor list for the genus *Trifolium* (Uzik et al., 1985); multistate character seed shape was rearranged to pseudobinary variables. All the evaluated characteristics were transformed into the nine-point scale according the Czech national descriptors list (Uzik et al., 1985). For the evaluated quantitative characteristics, the point estimations of the mean value were calculated.

Phytopathological evaluation

The level of resistance of all 76 accessions was tested according to the methods of Nedelnik (1986) and Pokorny (1989) on plants grown in controlled greenhouse conditions. Each accession of the core collection was evaluated by the average grade of disease

Table 1. Differences between diploids and tetraploids within morphological characteristics.

Characteristic	Significance Mann-Whitney
Cotyledons pubescence	0.000*
Cotyledons size	0.000*
Root collar thickness	0.236
Leaf rosette shape (from wide branched to erect)	0.230
Leaf rosette size	0.296
Spaced plant morphobiotype (from without rosette to with rosette)	0.166
Spaced plant shape (from wide branched to erect)	0.491
Spaced plant number of stems	0.511
Stem shape in diameter rounded (1) or oval (0)	0.471
Stem colour	0.838
Stem thickness	0.000*
Stem length	0.282
Stem pubescence	0.112
Stem number of internodes	0.219
Stem length of middle internode	0.206
Leaflet terminal shape (from rounded to lanceolate)	0.640
Leaflet terminal colour	0.837
Leaflet terminal margin	0.166
Leaflet terminal pubescence	0.017
Leaflet terminal mark intensity	0.470
Leaflet terminal length	0.200
Leaflet terminal width	0.000*
Leaflet terminal area	0.788
Inflorescence shape (rounded – narrow cylindrical)	0.526
Inflorescence length	0.119
Inflorescence width	0.067
Inflorescence number of flowers	0.006
Inflorescence number per stem	0.631
Seed shape ovate	0.491
Seed shape elliptical	0.002
Seed shape cordate	0.166
Seed shape rounded	0.009
Seed colour (darkness)	0.169
Weight of 1000 seeds	0.000*
Vegetation period until the beginning of flowering (earliness)	0.026
Frost hardiness	0.000*
Survival of plants during vegetation	0.796
Virus disease resistance	0.343
Rust resistance	0.022
Powdery mildew resistance	0.185
Northern anthracnose of clover resistance	0.962
Southern anthracnose of clover resistance	0.797
Ascochyta concentris leaf spot resistance	0.760
Stand height at the beginning of flowering	0.517
Stand number of cuts during the year	0.049
Green matter yield	0.114
Hay yield in the stand	0.343
Plant yield of green biomass	0.861
Plant seed yield	0.893
Dry matter content of nitrogen elements	0.041
Dry matter content of fibre	0.536

Differences marked by * are significant at $P<0.05$ by applying Bonferroni adjustment.

Table 2. Some yield characteristics obtained during the evaluation of individual plants.

Accession name	Country of origin	Ploidy level	Dry biomass weight in bunch (kg)	Seed weight in bunch (g)
143/1995	CZE	2	0.46	0.58
309/1999	SVN	2	0.14	0.91
84/1999	CZE	2	0.47	0.14
Amos	CZE	4	0.52	0.2
Arlington	USA	2	0.45	0.57
Barfiola	NLD	4	0.19	0.14
Brita	SWE	2	0.7	5.34
Bryza	POL	2	0.32	1.95
Concorde	USA	2	0.55	0.45
Divin	FRA	2	0.2	2
Dolina	CZE	4	0.41	0.16
Dolly	CZE	4	0.46	0.12
Essex Broad Red	GBR	2	0.19	0
G 27	NZL	2	0.38	0.88
Gibridnij Pozdnespelij	SUN	2	0.51	0.97
GKT Junior	HUN	2	0.36	3
Granta	GBR	2	0.28	0.72
Grasslands Hamua	NZL	2	0.3	1.01
Grasslands Turoa	NZL	2	0.48	0
Hayakita	JPN	4	0.27	0.67
HK	USA	2	0.32	0.81
Hokuseki	JPN	2	0.5	0.34
Horal	CSK	2	0.43	1.52
Hungarotetra	HUN	4	0.43	0.06
HZ-F	CZE	4	0.4	0.16
Chlumecký	CSK	2	0.19	2.12
Jokioinen	FIN	2	0.33	0.56
Karim	FRA	2	0.41	0.82
Kenland	USA	2	0.42	0.63
Kirsinai	LTU	4	0.68	0.12
Kolpo	NOR	4	0.59	0.09
Krusevacka 27	YUG	4	0.3	0.17
Kvarta	CZE	4	0.28	0.59
Lossam	FRA	4	0.16	0
Magura	SVK	4	0.34	0.12
Makimidori	JPN	2	0.53	0.53
Manuela	SVK	4	0.38	1.13
Marathon	USA	2	0.46	0.39
Markus	DEU	4	0.55	0.07
Mistral	FRA	2	0.52	1.33
Napoca-Tetra	ROM	2	0.46	0.91
Nemaro	DEU	2	0.38	2.45
Nesson	GRC	2	0.09	0
Norlac	CAN	2	0.59	1.03
Ottawa	CAN	4	0.34	0.22
Pacific	CAN	2	0.32	0
Parka	POL	2	0.45	0.37
Piemontese	ITA	2	0.27	0
Quin	GBR	2	0.32	1.2
Radegast	CZE	4	0.63	0.16

Table 2. Contd.

Accession name	Country of origin	Ploidy level	Dry biomass weight in bunch (kg)	Seed weight in bunch (g)
Ranespelij VIK 7	SUN	2	0.48	0.86
Reddy	USA	2	0.45	1.45
Reichesberger	AUT	2	0.32	4.09
Reichesberger Neu	AUT	2	0.34	1.31
Renova	CHE	2	0.18	1.23
RF*2 South	USA	2	0.52	0.23
RH	CZE	4	0.49	0
Sara	SWE	4	0.56	0.2
Select-Tetra	ROM	2	0.39	0.32
Sigord	SVK	4	0.37	0.08
Start	CZE	2	0.26	3.46
Suez	CZE	2	0.46	0.59
SW Volm	SWE	2	0.38	0.87
Tabor	CZE	2	0.38	1.5
Tatra	CSK	4	0.39	0.53
TB-12	CZE	2	0.68	0.98
Tempus	CZE	4	0.47	0.42
Triel	FRA	2	0.23	0
Trifomo	NLD	4	0.51	0.52
Triton	SWE	4	0.5	0.2
Vanessa	CHE	4	0.37	0.07
Vendelín	CZE	2	0.51	0.57
Verdi	FRA	2	0.34	1.77
Vesna	CZE	4	0.49	0.14
Viglana	SVK	2	0.38	0.09
Walter	CAN	2	0.61	0.45

(AGD) for *Fusarium* sp. and infected plants percentage (IPP) for BYMV, respectively (Table 2). From the isolates of *F. avenaceum* gathered during past years from infected plants in the fields with verified virulence, a 5×10^6 spore/ml inoculum concentration was prepared. Seedlings with decapitated root tip were immersed into the inoculum for 5 min, after which the plants were put into test tubes with agar cultivation medium. Cultivation was performed in cultivation room with controlled light conditions (12 h of light). After the cultivation period of six weeks, the evaluation according to the four point scale (R – resistant, S – susceptible, MS - moderately susceptible and HS - highly susceptible) was performed and AGD was calculated. However, three accessions were not tested because of very poor germination. In case of the BYMV, the high virulence strain J 27 was used for inoculation. 20 plants of each accession of the red clover were mechanically inoculated on the 2nd and 3rd leave stages. The evaluation was made according to a four-degree scale (1 – mosaics, clearing the veins, 2 – mild twisting, necrosis of the veins, 3 – large necrosis, dwarfishness and 4 – plant dead) on the 14th, 24th and 30th day after inoculation (DAI). In the 53rd DAI, the IPP and the indices of infection were determined, and then the plants were harvested. On the 73rd DAI, the number of dead plants was recorded. Four accessions were not tested because of very poor germination. The level of resistance was determined for both pathogens (Table 2; HS – highly susceptible, MS – moderate susceptible and S – susceptible).

Molecular analyses

The DNA of the clover plants was extracted from leaves harvested from 13 to 15 plants per accession by commercial GenElute™ Plant Genomic DNA Miniprep Kit (Sigma). DNA concentrations were determined using Lightwave II (WPA). All samples were diluted to a concentration of 20 ng/µL and stored at -80°C.

The polymerase chain reaction (PCR) was performed in a 20 µL volume containing 1x DyNAzyme buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.2 µM of each primer, 2 U of DyNAzyme™

II DNA polymerase (Finnzymes, Espoo, Finland) and 50 ng DNA template. PCR was performed in TC-512 (Techne). Thermocycler was programmed according Herrmann et al., (2006) for 95°C (4 min) for initial denaturation of DNA strands, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C and 30 s at 72°C, then followed further by 10 same cycles with annealing temperature 53°C and 10 min at 72°C as final extension. PCR products were visualised by ethidium bromide staining after electrophoresis on 3% agarose gels running in Tris-borate-EDTA (TBE). For the amplification of the products, 11 novel SSR markers from *Trifolium repens* were used (Barrett et al., 2004). In previous studies (Jungmannova and Repkova, 2005; Soldanova, 2007), it was confirmed that SSR markers from *T. repens* are applicable for analyses of *T. pratense*. These SSR markers are considered as new microsatellite loci for red clover. The SSR polymorphism was recorded as presence or absence of particular bands. The number of alleles per locus varied from three to eight with an average of 4.4. The polymorphism information content (PIC) values ranged from 0.40 to 0.86.

Statistical analyses

The relationships among accessions were tested with hierarchical clustering, separately for the morphological data (50 characteristics) and the data on SSR polymorphism (11 primers, 23 polymorphic bands). The morphological variables were initially standardized on range. The dendrogram of accessions based on morphological data was subsequently constructed in SYN-TAX 2000 program (Podani, 1994) with the unweighted pair-group method with arithmetic averages (UPGMA) method, using Euclidian distance as the dissimilarity measure. The clustering of accessions based on molecular data was done with the UPGMA method using Jaccard's similarity coefficient. The branch support in the UPGMA dendrogram of molecular data was calculated based on 2000 bootstrap replications using the WINBOOT program (Yap and Nelson, 1996). The matrices of dissimilarity coefficients from the cluster analysis based on morphological and that of molecular data were compared using the Mantel test. The test was performed in the software POPTOOLS (Hood, 2005) using 999 random iterations for the evaluation of statistical significance. The pair-wise relationships among morphological characteristics were tested with nonparametric Spearman correlations. The complex relationships among characteristics were inspected in SYN-TAX 2000 by UPGMA clustering of variables, with correlation as the dissimilarity measure. The differences between diploid and tetraploid accessions (Table 1) were tested separately for each morphological characteristic by the Mann-Whitney test in SPSS 8.0 (SPSS Inc., 1998).

The possible relationship of the presence of particular polymorphic bands with recorded morphological, yield and resistance characteristics was tested by the Mann-Whitney test, separately for each band and each morphological characteristic. The significance of tests concerning presence/absence of a particular band over all morphological variables was adjusted by Bonferroni correction for multiple comparison experiments. The analysis was done for all accessions together as well as for diploids and tetraploids separately. Further, a matrix of inter-accession dissimilarity was calculated separately for each morphological variable. Each of the matrices was consequently compared with the existing dissimilarity matrix of accessions based on molecular data (Jaccard's similarity coefficient) by the Mantel test. The correlation of overall pattern among accessions based on morphological and SSR polymorphism data was finally evaluated by the Mantel test, comparing the existing inter-accessions dissimilarity matrices used for the accession clustering. Mantel tests (Mantel, 1967) were performed in the PopTools 2.6.9 program (Hood, 2005). Significances of all tests were calculated as one tailed, based on 999 permutations; in the case of multiple comparisons the significances were Bonferroni adjusted.

RESULTS

The first step was to analyse if there are any differences between diploid and tetraploid accessions using evaluated morphological traits. From the results, no clear difference between diploid and tetraploid accessions within the core collection was evidenced by cluster analysis of morphological data. Compared to diploids, tetraploids accessions have more hairy cotyledons, larger cotyledons, thicker stems, wider terminal leaflets, higher seed weight and lower frost hardiness (Bonferroni adjusted $P<0.05$, Table 1). Some basic yield characteristics are presented in Table 2. Dry biomass weight in bunch ranged from 0.09 to 0.7 kg. Seed weight in bunch ranged from 0 to 5.34 g. An interesting fact is that the best results in both parameters were reached by the variety Brita (SWE). The worst was the variety Nesson (GRC) having no seeds and the lowest dry biomass yield.

Dendrogram of morphological characteristics used for the evaluation of the Czech core collection of the red clover was constructed. The cophenetic correlation of the resulting dendrogram (Figure 1) was 0.65. The mutual relationships of morphological characters within the core collection are also shown in Figure 1. Morphological characters were mostly not correlated. Notable correlation was found only between the seed weight and ploidy level (Spearman correlation/rs = 0.8, $P<0.001$). Molecular and morphological data were not significantly correlated (Mantel test, one tailed, $P = 0.188$). Cophenetic correlations of the dendrogram based on morphological and molecular data were 0.75 and 0.87, respectively. No differences in a particular morphological characters were observed to be associated with the presence/absence of particular bands or with the overall pattern of SSR polymorphism (Bonferroni adjusted $P>0.1$).

The dendrogram of materials included into the Czech core collection of the red clover based on morphological data (Figure 2) shows separation of items into seven subclusters (only one consist of two members), with no outliers present within the core collection. In contrast, the dendrogram based on molecular data (Figure 3) shows the separation of items into 13 subclusters (in comparison to morphological data) – 10 of these have more than two members, one has only two members and two consist of only one member. One outlier was found within the first division of the core collection (variety Kirsinai), with the presence of only three polymorphic bands (out of 23 possible). The reason why only three

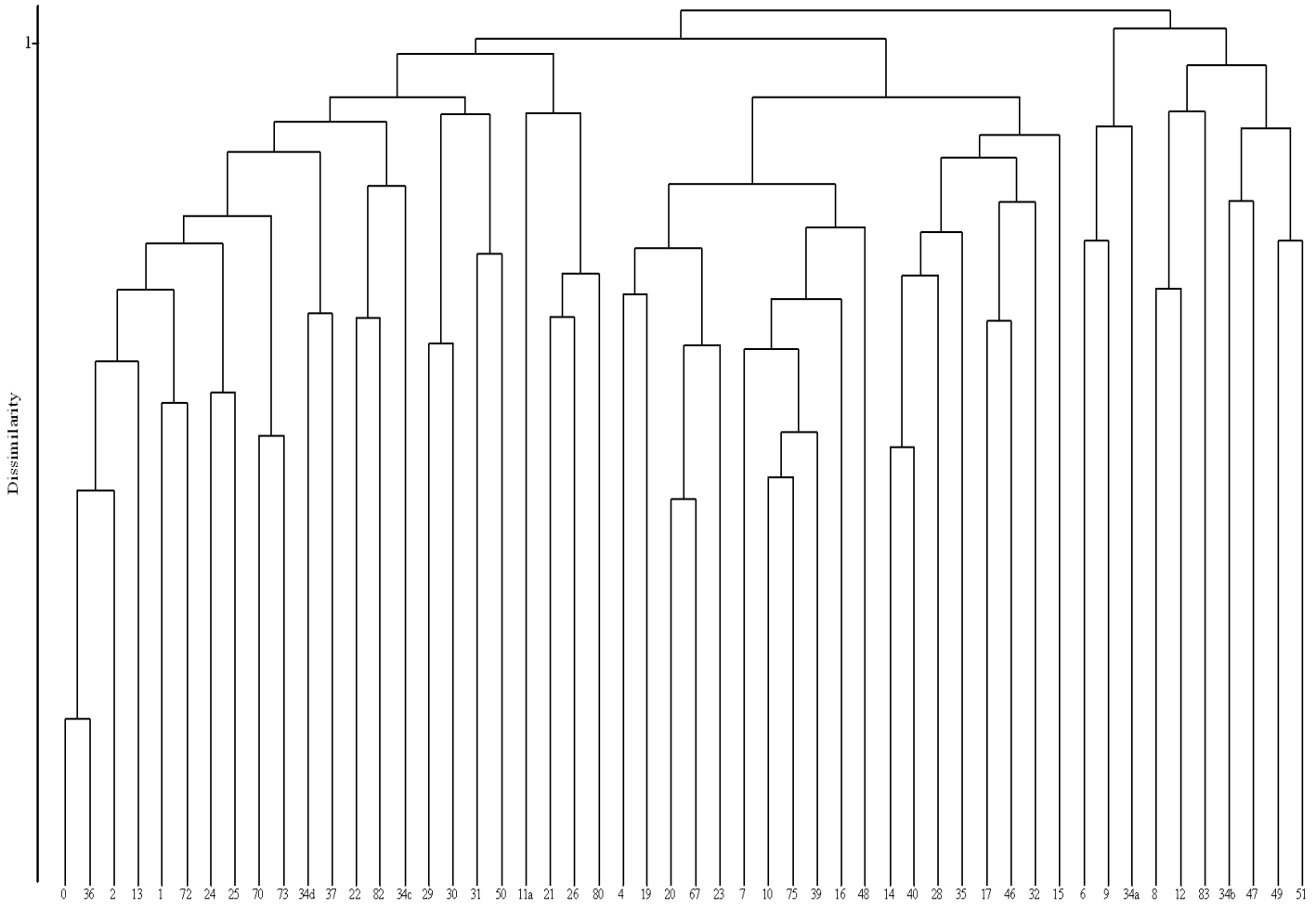

Figure 1. Dendrogram of morphological characteristics used for the evaluation of the Czech core collection of red clover. Characters: Ploidy level, 0; Weight of 1000 seeds, 36; Cotyledons size, 2; stem thickness, 13; cotyledons pubescence, 1; green matter yield, 72; leaflet terminal length, 24; leaflet terminal width, 25; stand number of cuts during the year, 70; hay yield in the stand, 73; seed shape rounded, 34d; vegetation period until the beginning of flowering (earliness), 37; leaflet terminal pubescence, 22; dry matter content of nitrogen elements, 82; seed shape cordate, 34c; inflorescence length, 29; inflorescence width, 30; inflorescence number of flowers, 31; Southern anthracnose of clover resistance, 50; stem shape in diameter rounded (1) or oval (0), 11a; leaflet terminal margin, 21; leaflet terminal area, 26; plant seed yield, 80; root collar thickness, 4; leaflet terminal shape (from rounded to lanceolate), 19; leaflet terminal colour, 20; stand height at the beginning of flowering, 67; leaflet terminal mark intensity, 23; leaf rosette size, 7; spaced plant number of stems, 10; plant yield of green biomass, 75; frost hardiness, 39; stem number of internodes, 16; powdery mildew resistance, 48; stem length, 14; survival of plants during vegetation, 40; inflorescence shape (rounded – narrow cylindrical), 28; seed colour (darkness), 35; stem length of middle internode, 17; virus disease resistance, 46; inflorescence number per stem, 32; stem pubescence, 15; leaf rosette shape (from wide branched to erect), 6; spaced plant shape (from wide branched to erect), 9; seed shape ovate, 34a; spaced plant morphobiotype (from without rosette to with rosette), 8; stem colour, 12; dry matter content of fibre, 83; seed shape elliptical, 34b; rust resistance, 47; Northern anthracnose of clover resistance, 49; *Ascochyta concentris* leaf spot resistance, 51.

bands appeared by this variety is not known. The core collection accessions showed some level of resistance to *Fusarium* sp. and BYMV infection. In case of *Fusarium* sp. eight accessions were mean susceptible, 65 were highly susceptible. No accession was classified as susceptible and resistant (Table 3). The highest level of AGD was found in the varieties Kolpo (NOR) and RH (CZE), while the lowest AGD was found by Ruin (GBR) and Ranespelij VIK 7 (SUN).

Four accessions were classified as susceptible to BYMV, 49 were moderately susceptible, and 19 highly susceptible. No accession was classified as resistant. The highest disease incidence (DI) was found in the varieties Lossam (FRA) and Essex Broad Red (GBR). The lowest DI was found in the wild population TROU 143/1995 (CZE) and the variety Reddy (USA).

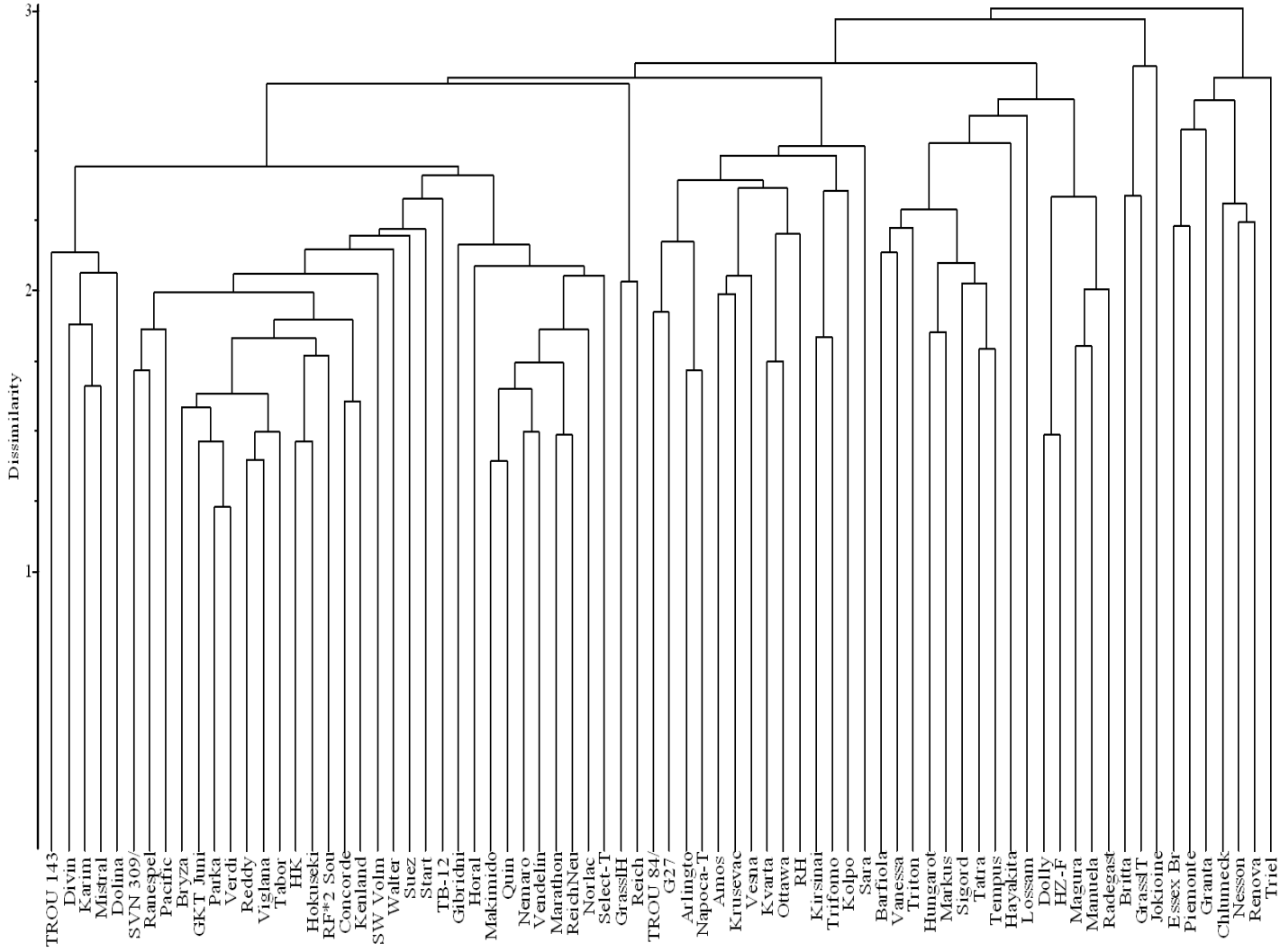

Figure 2. Dendrogram of accessions included into the Czech core collection of red clover based on morphological data.

The correlation between resistance of core collection accessions to *Fusarium* sp. and BYMV pathogens was not significant at P>0.5 (0.197). In our core collection, there is no one accession with a good level of combined resistance to both *Fusarium* sp. and BYMV. On the other hand, there is no accession susceptible to both pathogens.

DISCUSSION

The aim of the study was to describe in detail 76 diploid and tetraploid red clover core collection accessions by morphological, molecular and phytopathological data including an evaluation of resistance to important fungal and viral pathogens. Tested plants were inoculated by spore-suspension of *Fusarium* sp. fungi and also by BYMV inoculum. Microsatellites (SSRs) were used for the study of genetic diversity of all the core collections accessions. Gathered data were statistically analysed with the main aim to find; (1) correlations among characters and the ploidy level, (2) correlation of morphological and molecular data and (3) to find correlation among individual agronomically important characters and SSR polymorphism.

Based on our results, no clear difference between diploid and tetraploid accessions within the core collection was evidenced by cluster analysis of morphological data. Compared to diploids, tetraploids accessions have more hairy cotyledons, larger cotyledons, thicker stems, wider terminal leaflets, higher seed weight, and lower frost hardiness. Particularly, the correlation of ploidy level and the seed weight is well known in many species. Bretagnolle et al. (1995)

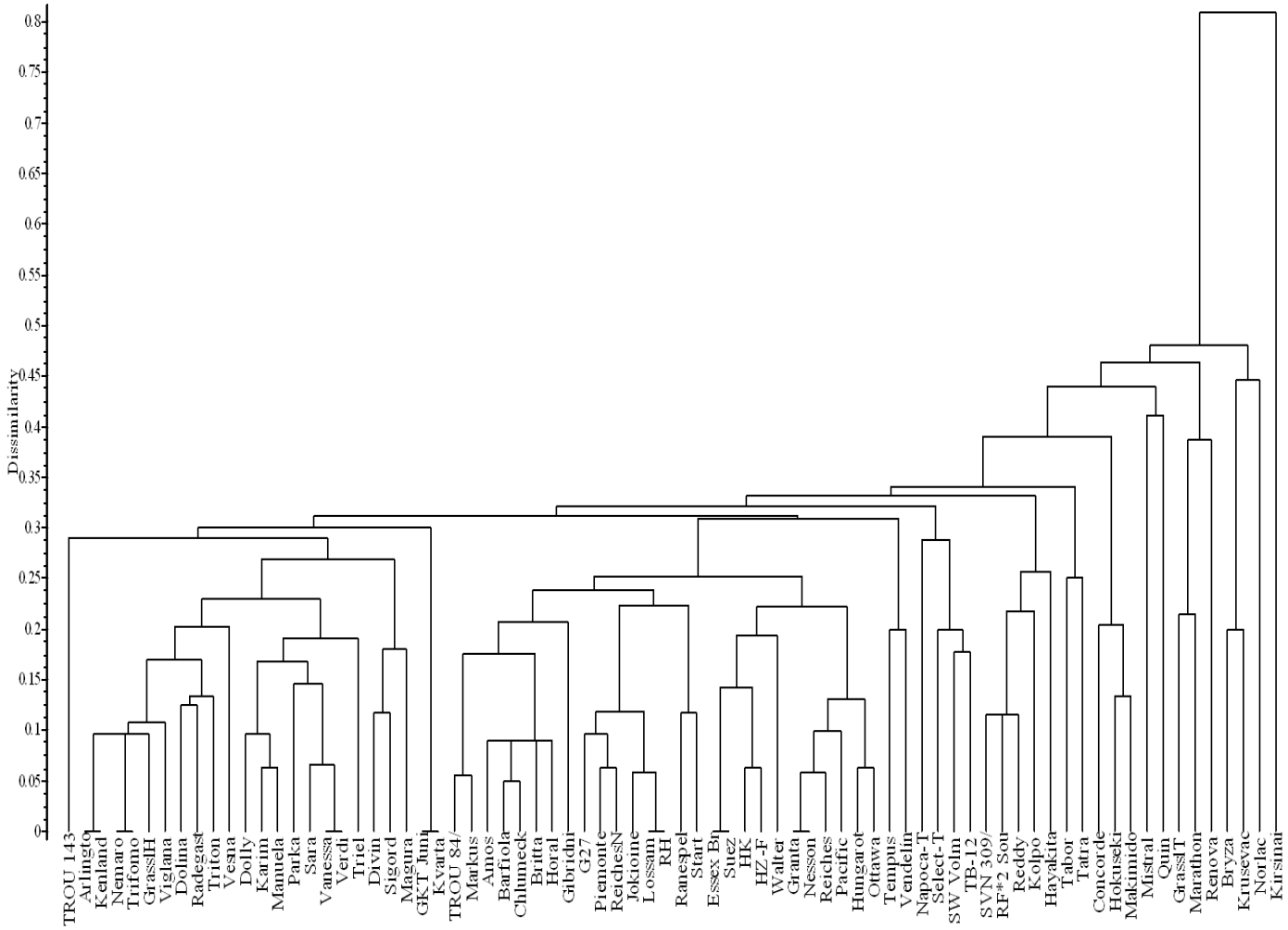

Figure 3. Dendrogram of accessions included into the Czech core collection of red clover based on molecular data.

reported that there is a dependence of seed size and ploidy level on seed germination and seedling growth, but there is no simple consequence of differences in seed size between diploids and their related tetraploids of *Dactylis glomerata*.

In our study, no significant correlation was detected between the observed patterns of morphological and molecular variation. The same results were documented in many other crops (e.g., Bruschi et al., 2003 - *Quercus petraea*; Martinez et al., 2003 - *Vitis vinifera*; Greene et al., 2004 - *T. pratense*; Zhang et al., 2010 – *T. repens*). Royo and Itoiz (2004) assumed that there are several reasons for low congruence between classifications based on morphological and molecular data but the most important reason is that the genetic regulation of one or another marker is different and that the phenotypical expression is conditioned by the state of development of the plant, by agricultural practices and by environmental conditions. The lack of separation between diploid and tetraploid accessions can be due to the fact that tetraploid accessions were often derived from diploid natural accessions using colchicine. That is why the core collection was established by using all the accessions together, regardless of the ploidy level. The final ratio of 2n and 4n accessions, when comparing the core collection to the initial data set, is similar (0.52 and 0.44 respectively).

The accessions of core collection can be used as the starting material for breeding. The combinations of resistance to the several pathogens would be advantageous for breeders, but the correlation between resistance of core collection accessions to *Fusarium* sp. and BYMV pathogens was not found in our data. Unfortunately, we did not find accessions with good level of combined resistance to both *Fusarium* sp. and BYMV. Nedelnik and Pokorny (1992) also did not find any

Table 3. Resistance level of core collection accessions.

Variety	Accession	n	*Fusarium* sp.		BYMV	
			AGD	Category	IPP	Category
143/1995	CZE	2	2.25	HS	40	S
309/1999	SVN	2	1.9	MS	65	MS
84/1999	CZE	2	1.95	MS	55	MS
Amos	CZE	4	2.75	HS	60	MS
Arlington	USA	2	2.25	HS	75	MS
Barfiola	NLD	4	2.65	HS	95	HS
Brita	SWE	2	2.75	HS	95	HS
Bryza	POL	2	2.4	HS	53	MS
Concorde	USA	2	2.3	HS	60	MS
Divin	FRA	2	2.9	HS	75	MS
Dolina	CSK	4	2.7	HS	55	MS
Dolly	CZE	4	2.65	HS	70	MS
Essex Broad Red	GBR	2	2.7	HS	100	HS
G 27	NZL	2	Not tested		90	HS
Gibridnij Pozdnespelij	SUN	2	2.75	HS	90	HS
GKT Junior	HUN	2	2.8	HS	80	MS
Granta	GBR	2	2.95	HS	Not tested	
Grasslands Hamua	NZL	2	2.6	HS	65	MS
Grasslands Turoa	NZL	2	Not tested		67	MS
Hayakita	JPN	4	2.2	HS	80	MS
HK	USA	2	2.65	HS	55	MS
Hokuseki	JPN	2	Not tested		Not tested	
Horal	CSK	2	2.5	HS	95	HS
Hungarotetra	HUN	4	2.25	HS	65	MS
HZ-F	CZE	4	2.45	HS	55	MS
Chlumecky	CSK	2	2.55	HS	80	MS
Jokioinen	FIN	2	2.85	HS	88	HS
Karim	FRA	2	2.8	HS	60	MS
Kenland	USA	2	1.95	MS	77	MS
Kirsinai	LTU	4	2.5	HS	70	MS
Kolpo	NOR	4	3.0	HS	60	MS
Krusevacka 27 (K27)	YUG	4	2.55	HS	70	MS
Kvarta	CSK	4	2.25	HS	95	HS
Lossam	FRA	4	2.4	HS	100	HS
Magura	SVK	4	2.35	HS	60	MS
Makimidori	JPN	2	2.9	HS	Not tested	
Manuela	SVK	4	2.7	HS	60	MS
Marathon	USA	2	2.25	HS	80	MS
Markus	DEU	4	2.3	HS	60	MS
Mistral	FRA	2	2.55	HS	78	MS
Napoca-Tetra	ROM	2	2.55	HS	80	MS
Nemaro	DEU	2	2.2	HS	50	S
Nesson	GRC	2	2.25	HS	65	MS
Norlac	CAN	2	2.95	HS	83	MS
Ottawa	CAN	4	2.2	HS	90	HS
Pacific	CAN	2	2.85	HS	Not tested	
Parka	POL	2	2.45	HS	60	MS
Piemontese	ITA	2	2.5	HS	81	HS
Radegast	CSK	4	2.8	HS	75	MS
Ranespelij VIK 7	SUN	2	1.85	MS	65	MS

Table 3. Contd.

Variety	Accession	n	*Fusarium* sp.		BYMV	
			ADI	Category	IPP	Category
Reddy	USA	2	2.1	HS	45	S
Reichesberger	AUT	2	1.9	MS	90	HS
Reichesberger Neu	AUT	2	2.4	HS	65	MS
Renova	CHE	2	2.9	HS	85	HS
RF*2 South	USA	2	2.5	HS	55	MS
RH	CZE	4	3.0	HS	60	MS
Ruin	GBR	2	1.8	MS	66	MS
Sara	SWE	4	2.85	HS	85	HS
Select-Tetra	ROM	2	2.25	HS	80	MS
Sigord	SVK	4	2.55	HS	50	S
Start	CZE	2	2.15	HS	95	HS
Suez	CZE	2	1.75	MS	70	MS
SW Volm	SWE	2	2.95	HS	71	MS
Tabor	CSK	2	1.9	MS	55	MS
Tatra	CSK	4	2.5	HS	90	HS
TB-12	CZE	2	2.45	HS	62	MS
Tempus	CSK	4	2.3	HS	88	HS
Triel	FRA	2	2.9	HS	80	MS
Trifomo	NLD	4	2.7	HS	83	HS
Triton	SWE	4	2.2	HS	70	MS
Vanessa	CHE	4	2.25	HS	75	MS
Vendelin	CZE	2	2.1	HS	55	MS
Verdi	FRA	2	2.6	HS	85	HS
Vesna	CSK	4	2.0	HS	80	MS
Viglana	SVK	2	2.85	HS	70	MS
Walter	CAN	2	2.85	HS	60	MS

AGD, Average grade of disease; IPP, infected plants percentage; R, resistant; S, susceptible; MS, moderately susceptible; HS, highly susceptible.

correlation between BYMV and *Fusarium* sp. pathogens. In the last 20 years, many authors have discussed root rot disease of red clover (Rufelt, 1985; Skipp and Christensen, 1986; Nedelnik, 1993; Venuto et al., 1995). The control of root rot is difficult, and that is why the resistant material is necessary for successful red clover growing. In case of BYMV the situation is similar. For this reason, a breeding programme for resistance to these pathogens was started at the Research Institute for Fodder plants in Troubsko in the 1990s (Nedelnik and Pokorny 1992; Pokorny et al., 2003). Red clover forage yield remains a prime breeding target for improved variety development. Phenotyping is still the most used method in breeding of red clover, especially in the first stage. Many red clover selection programs are based on space planted nurseries (Riday, 2009). Tetraploids are used in contemporary intensive agriculture, while diploids were used in the first half of the 20th century. Tetraploids give higher yields of green biomass, but the dry matter yields are similar. Diploids have smaller cells and water content. The highest yields in stand were obtained by the varieties Sara - SWE (4n) and Vendelin - CZE (2n). Generally, the seed yields are not dependable only on ploidy level but also on the field conditions.

Since the 1990s, diversification of varieties has started. Accessions of domestic origins usually dominate in each country. Tetraploid variety Kvarta and diploid variety Start are the most often grown in the Czech Republic. Diploid accessions are better for extreme conditions (having better frost hardiness), for organic agriculture and for species-rich mixtures (Figure 1). In Central Europe and in the Czech Republic, there is optimum for the red clover growing in medial positions. Dendrogram of accessions included into the Czech core collection of the red clover based on morphological data shows separation of items into seven subclusters, while dendrogram based on molecular data shows the separation of items into 13 subclusters. Varieties from the same country were not

clustered together, so we can conclude that our core collection well corresponds with existing diversity within red clover. In morphological data, no visible pattern can be observed. Concerning molecular data, we found two outliers – varieties Kirsinai and TROU 143/1995 (wild form collected in the Czech Republic). The accession TROU 143/1995 also has the lowest disease incidence (Table 3), which can be a very promising material for breeding programmes.

Our core collection is practically used in selections of materials in breeding processes by both the Czech and foreign breeders. All the evaluated characters are available via information system of the Czech national gene bank, so the users of our core collection can effectively select parents for starting the breeding process. For this reason especially, our core collection is preferentially maintained in the Czech national gene bank. Seed samples of the core collection have been primarily subjected to safety duplication in foreign gene banks.

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