Modern Methods for Genetic Improvement of *Trifolium pratense*

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Abstract

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This review focuses on trends in genetic improvement of a significant representative forage crop, *Trifolium pratense* (red clover) classified taxonomically into the agronomically outstanding family *Fabaceae*. Red clover breeding is aimed at improving traits like persistency, resistance to biotic and abiotic factors, forage yield and quality characteristics such as protein quality and stability. Isoflavone content in forage is important for cattle reproduction. Interspecific hybridization of red clover with the related wild species *T. medium* was used for the introgression of useful traits into red clover. The breeding strategy for the new variety Pramedi included hybrid plants with different DNA contents, repeated backcrosses with red clover, followed by family selection. New techniques of molecular genetics are becoming available to breeders for transferring key and complex traits into improved red clover varieties. Techniques such as marker-assisted selection and candidate gene identification can increase the speed and precision with which traits may be selected. Comparative sequence data analyses helped to identify genes for polyphenol oxidase enzymes and isoflavone synthase in red clover useful for DNA genotyping of individuals and breeding of improved high-quality red clover varieties.

Keywords: candidate gene; genetic marker; molecular genetics; red clover; wide hybridization

Throughout the history of plant breeding, breeders have endeavoured to improve crop yields and quality, always in the context of the latest knowledge and demands of users. This paper focuses on trends in genetic improvement of a significant representative forage crop, *Trifolium pratense* L. (red clover), by modern methods. The review summarizes current knowledge of the red clover genome and prospects for using molecular genetics to improve that genome.

The genus *Trifolium* L. is classified taxonomically into the tribe *Trifoliae* of the large and agronomically outstanding family *Fabaceae*. It comprises approximately 240 species (Zohary & Heller 1984) of annual and perennial herbs commonly known as clovers, both wild and cultivated. Red clover is an important forage legume that is widely cultivated in most temperate regions and is grown in pastures for grazing, sown as a companion crop, and harvested for hay (Smith *et al.* 1985; Taylor & QueenSberry 1996). It is also used as a green manure crop because of its high nutrient content resulting partly from symbiosis with nitrogen-fixing bacteria of the genus *Rhizobium*.

In Europe and worldwide, red clover breeding is focused on improving traits like persistency, resistance to biotic and abiotic factors, and forage yield and quality characteristics such as protein quality and stability, fermentable carbohydrate content, and cell wall digestibility. The benefits of protecting plant proteins from degradation in the rumen have been established. Polyphenol oxidase (PPO) enzymes catalyse the aerobic conversion of monophenols to o-phenols and finally to o-quinones (Macheix *et al.* 1991) and are present in significant quantities in red clover (Jones *et al.* 1995). The chemical processes result in the formation of a cross-linked protein-phenol complex resistant to enzymatic digestion by proteases, and this PPO mechanism may effectively reduce protein breakdown in silage (Sullivan & Hatfield 2006) and when clover is fed green directly to cattle. The inhibitory effect of PPO activity on

proteolysis, and also lipolysis (Lee *et al*. 2006), results in more than 80% of the protein being retained, thus providing improved nitrogen efficiency. These findings justify a strategy of breeding improved red clover varieties with increased PPO activity. Another trait attracting breeders' attention is the content of polyunsaturated fatty acids (PUFA), which bring well-known benefits to human nutrition. Forage abundant in PUFAs can increase the levels of PUFAs in milk, dairy products and meat. Higher concentrations of PUFAs in milk from cows fed red clover silage have been associated with the PPO activity in red clover (Lee *et al*. 2004). On the other hand, red clover forage with a high content of isoflavones, mainly formononetin (Taylor & Quesenberry 1996), can cause reproductive disorders in cows when consumed in high concentration. Isoflavones (some of which are phytoestrogens) are important plant secondary metabolites belonging mainly to a large group of substituted phenolic compounds (Yildiz 2005). They have significant antimicrobial functions in plant defences against pathogens but can also have anti-nutritional effects.

In addition to conventional plant breeding methods, useful variation in a breeding population can be generated through, for example, hybridization, wide hybridization and genome introgression, or by mutagenesis including polyploidy. Subsequent selection must be carried out on the progeny combining the best traits (with the fewest undesirable ones). The progress of molecular genetics can accelerate plant breeding particularly of outcrossing crops such as red clover. However, its high level of heterozygosity has so far hindered intensive genetic and genomic analyses.

Genome and karyotype

Red clover is an extremely polymorphic hermaphroditic allogamous diploid ($2n = 2x = 14$) with a homomorphic gametophytic self-incompatibility (GSI) system (Townsend & Taylor 1985). Zohary and Heller (1984) considered $x = 8$ to be the basic chromosome number of the genus for 80% of the species (Vižintin *et al.* 2006) while $x = 7$, 6 and 5 are derived states.

Evaluation of the nuclear DNA content of a species is an important tool in genetic diversity studies and 1C values have proved to be the most informative measure of interspecific DNA content variation. Previously published data on nuclear DNA content in red clover accessions reveal some differences. A nuclear DNA content of 1.3/2C pg was reported by Grime and Mowforth (1982). Arumuganathan and Earle (1991) performed flow cytometric measurement and revealed 2C genome size of 0.97 pg. Rizza *et al*. (2007) determined 0.92 pg and Vižintin *et al*. (2006) even 0.85 pg for a red clover genotype. Red clover has a 468 Mb genome, which is similar in size to those of *Lotus japonicus* and *Medicago truncatula*. Sato *et al*. (2005) deduced a haploid genome size of 440 Mb, as estimated by measuring the nuclear DNA content using flow cytometry (two red clover plants with 0.89 and 0.91 pg/2C), with 1 pg of DNA equivalent to 980 Mb (Bennett *et al*. 2000).

Ellison *et al*. (2006) and Vižintin *et al*. (2006) reported results of detailed and comprehensive studies of the molecular phylogenetic framework for the genus *Trifolium*. The latter authors assessed the interspecific relationships among Eurasian clovers based on sequence polymorphisms of the internal transcribed spacer region of nuclear ribosomal DNA (rDNA). *T. pratense* and *T. medium* were closely grouped in the *Trifolium* section and relationships with seven other species of this section were shown. Phylogenetic relationship is a key factor for comparative genomics within the genus or family and for exchanging knowledge in relation to the various species (Sato *et al*. 2010).

Sato *et al*. (2005) analysed a karyotype of the red clover genome by microscopic observation of prometaphase chromosomes. The lengths of the prometaphase chromosomes ranged from 5.1 to 7.4 μm, and uneven condensation patterns were observed that could be useful in chromosome identification. The genome structure of red clover using fluorescence in situ hybridization (FISH) was investigated by Sato *et al*. (2005) and Kataoka *et al*. (2012). Some chromosomes were distinguished using 26S and 5S rDNA probes. FISH image analysis showed a major 26S rDNA locus in the nucleolar organizer regions (NORs) of the short arm of chromosome 1; a less intense signal was found in the internal region on the short arm of chromosome 6 as a heterozygous locus and another signal was detected on chromosome 7. The 5S rDNA loci were detected proximal to the NOR signals on the short arm of chromosome 1 and at another two loci on the short arm of chromosome 2. These results also demonstrated polymorphisms of rRNA genes and their locations among different varieties of red clover (Kataoka *et al*. 2012). An *Arabidopsis*-type telomeric sequence (TTTAGGG)n was found to be present in red clover (Pedrosa *et al*. 2002). In addition, a centromeric satellite DNA isolated from white clover differs from that of red clover (Ansari *et al*. 2004).

Wide hybridization

Hybridization can have subtle but long-lasting consequences in plant populations. Artificial wide hybridization (mainly interspecific) of red clover with related wild species offers a greater potential for the introgression of useful traits into red clover. The main constraint of this procedure is various types of barriers to crossability (Hughes 1986). Therefore, conventional crossing procedures and the attainment of viable hybrids are entirely unsuccessful. In *Trifolium*, post-fertilization barriers are of greater importance (Kazimierska 1978; Taylor *et al.* 1980; Řepková *et al.* 2006). Various *in vitro* methods are necessary to overcome these barriers, such as embryo culture or protoplast fusion (Taylor & Quesenberry 1996). An alternative procedure currently in use is to incorporate foreign genes by genetic transformation via *Agrobacterium tumefaciens* (Khanlou *et al*. 2011).

From the 1970s to 1990s, the method of embryo culture seemed to be a promising way of overcoming the interspecific, post-fertilization barriers in *Trifolium*, even though its success was unreliable. Embryo culture provided the means to overcome the incompatibility caused by abnormal endosperm development and insufficient embryo nutrition that results in abortion (Kazimierska 1980). The efforts to exploit interspecific hybridization in *T. pratense* and interest in hybrid evaluation have continued to date. *T. pratense* has been successfully crossed with five species so far (reviewed by Abberton 2007): *T. sarosiense* Hazsl. (Collins *et al.* 1981; Phillips *et al*. 1982), *T. medium* L. (Collins *et al*. 1981; Vogt & Schweiger 1983; Merker 1984; Sawai *et al.* 1990; Isobe *et al*. 2002; Nedbalkova *et al.* 1995), *T. alpestre* L. (Collins *et al.* 1981; Merker 1988; Phillips *et al.* 1992), *T. ambiguum* M.Bieb. (Vogt & Schweiger 1983) and *T. diffusum* Ehrh. (Schwer & Cleveland 1972). These reports have mostly been limited to describing the intermediate morphological appearance of the F_1 hybrids to both parents. More recent advances have focused on evaluating morphological and agronomic traits in the F_1 progeny and on enhancing the methods for incorporating superior traits into the elite germplasm.

Hybrids between *T. pratense* ($2n = 4x = 28$) and *T. medium* (zigzag clover; $2n = 8x = 64$) have been obtained by embryo rescue (Repkova *et al.* 1991). Cytogenetic studies performed by the flow cytometric analysis of progeny in the fifth generation after open pollination of hybrids revealed plants with different DNA content consistent with 26 to 44 somatic chromosomes (Řepková *et al.* 2003). Jakešová *et al*. (2011) compared the morphological, agronomic, and reproductive traits of both parental species with plants derived from the former F_1 hybrids. The phenotypic characteristics were evaluated in 500, 745, 112, 800, and 460 hybrid plants in 5 years. There were statistically significant differences between the hybrids and *T. pratense* in nearly all of the analysed characteristics. In individual years, the stem number per plant was significantly higher in the hybrids compared to both parental species or was comparable to that of *T. medium*. This trait could have a positive impact on yield. Short rhizomes were observed in the hybrids after the harvest of the plants in the second harvest years. This trait has been studied from the viewpoint of improving persistency. The variability useful in breeding was significantly increased in resulting genotypes, and, therefore, these were used as new breeding material. The breeding strategy included hybrid plants with higher DNA content after *T. pratense* variety Tatra × *T. medium* crosses, repeated backcrosses with *T. pratense* variety Amos, followed by family selection.

The F_1 interspecific hybrids mostly display low levels of fertility and insufficient vigour. For this reason, efforts to improve seed productivity are focused on the repeated backcrossing of hybrids with cultivated species, as it has been shown that backcrossing is an effective way to increase both fertility and vigour. Indeed, in these experiments repeated open pollination helped to improve the level of fertility and vigour of the *T. pratense* × *T. medium* hybrids. Only those hybrids with higher chromosome numbers (42, 44, and more) were unsuccessful in establishing a population of fertile hybrids as a source for further selection of useful traits. In 2010, an application for Plant Breeders Rights to the variety Pramedi was filed at the Czech Plant Variety Office, which granted the rights in 2013 (variety number TPM14855 and variety code 5082339; Holders of Rights: Ing. Hana Jakešová, Breeding, Hladké Životice; Agricultural Research, Ltd.; Research Institute for Fodder Crops, Ltd. Troubsko; Masaryk University in Brno).

DNA marker systems and genetic maps

The fact that a varying portion of phenotypic variance could be caused by different environments to which the individuals are exposed is a disadvantage of selection according to phenotypes. Environmentally insensitive DNA markers enable adequate tracking

of the inheritance of important agronomic traits. For evaluating germplasm variability, identifying cultivars, and estimating genetic distance, dominant markers such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs), and inter-simple sequence repeats (ISSRs) have been widely utilized (for reviews, see Antonius-Klemola 1999; Ulloa *et al.* 2003). None of these methods requires prior knowledge of the genome sequence. In *Trifolium*, primarily RAPDs have been utilized as dominant markers for evaluating genetic diversity in *T. pratense* (Kongkiatngam *et al.* 1996; Campos & Ortega 2001; Gustine *et al.* 2002; Greene *et al.* 2004). However, the ISSR-PCR method using primers based on di-, tri-, tetra-, and pentanucleotide repeats seems particularly suitable for germplasm comparison. The advantage is the better repeatability of the ISSR methodology compared to RAPD, as reported by Rizza *et al*. (2007) for several species. The AFLP technique is a powerful and highly reliable tool capable of probing a large number of genomic loci per experiment and discriminating genetic differences. The genetic diversity of red clover has been intensively studied using AFLP markers by Kölliker *et al*. (2003) and Herrmann *et al*. (2005).

The first genetic linkage map with 256 restriction fragment length polymorphism (RFLP) markers was constructed for red clover by Isobe *et al*. (2003) using a mapping population of 188 F_1 individuals derived from a double pseudo-testcross between HR as the female parent and R130 as the male parent. HR was an elite plant selected from early progenies of the cross between Hokuseki and a Swiss variety Renova with characteristics of early flowering, erect shape, red flower colour, and middle-sized leaves. R130 originated from a Russian variety and a wild variety from the Archangelsk region which exhibits late flowering, semi-prostrate shape, white flower colour, and small leaves. Sato *et al.* (2005) developed a large collection of simple sequence repeat (SSR) markers from four types of genomic and cDNA libraries constructed from the above-mentioned genotypes HR and R130. A total of 1434 loci were detected by SSRs from this collection and another 167 RFLP loci were mapped onto seven linkage groups. Herrmann *et al*. (2006) constructed a molecular linkage map with 42 SSR and 216 AFLP loci. Isobe *et al*. (2009) constructed the first consensus high-density linkage map that is 836.6 cM long with 1414 SSRs, 181 AFLPs, and 204 RFLPs. The current genetic linkage map of the red clover genome with its sufficient number of gene-associated microsatellite markers facilitates precise mapping of quantitative trait loci (QTL) and map-based gene cloning. Such a map is necessary for characterization of new genes valuable in breeding. A total of 7,262 SSRs and 228 RFLPs are included in the Clover Garden database (http://clovergarden.jp/).

Candidate gene identification

Candidate gene identification is mostly dependent on a mapping population that is based on the cross between divergent phenotypes for a trait. Such a population of red clover useful for segregation analysis, interval mapping, and QTL investigation was constructed by Isobe *et al*. (2003). A mapping-based approach was used to identify SSRs closely linked to the GSI-locus (Riday & Krohn 2010). SSR markers developed by Sato *et al*. (2005) were used and a bi-parental cross was initiated in which the parents were known to have one self-incompatibility allele (S-allele) in common. S-allele genotypes of 100 progenies were determined through test crosses and pollen compatibility. Pseudo F_1 linkage analysis isolated the GSI-locus on red clover linkage-group 1 within 2.5 cM of markers RCS5615, RCS0810, and RCS3161. A second 256-progeny mapping testcross population of a heterozygous self-compatible mutant revealed that this specific self-compatible mutant was mapped to the same location as the GSI-locus. A linkage of 2.5 and 4.7 cM was revealed between the GSI-locus and markers RCS0810 and RCS4956, respectively. The map-based location of the GSI-locus in red clover has many immediate applications to red clover plant breeding and could be useful in helping to sequence the GSI-locus.

Because red clover is a short-lived perennial species, improving plant persistency is the most important objective in its breeding. Klimenko *et al*. (2010) developed a marker-assisted selection approach for red clover to identify candidate QTLs related to plant persistency. This is a complex trait and its investigation was associated with resistance to biotic stress caused by the most frequent pathogens *Sclerotinia trifoliorum* and *Fusarium* species (*F. oxysporum*, *F. solani*, and *F. roseum*), as well as to abiotic stress connected with low temperature (winter hardiness). Two full-sib mapping populations based on the crosses were used for QTL identification by means of SSR, RFLP, RAPD and sequence-tagged site (STS) markers. A total of 10 and 23 candidate QTL regions for plant persistency were identified in the two mapping populations.

Seed yield is another complex trait influenced by many components and difficult to improve. Herrmann *et al*. (2006) identified DNA markers linked to 38 QTLs for several key seed yield components as the first step towards marker-assisted breeding. Some QTLs were not determined independently because they were detected in the same genome region.

Screening markers for isoflavones and PPO

Particular genes participating in biosynthetic pathways have been characterized based on knowledge obtained from crop genome sequencing. Comparative analyses has helped to identify gene paralogs with the same functions in different crops. Red clover has two genes, *IFS1* and *IFS2*, for the unique enzyme isoflavone synthase that converts flavone substrates to isoflavones (daidzein and genistein) in the isoflavone pathway. Formononetin and biochanin are synthesized by the enzyme O-methyltransferase from the same intermediate (Farag *et al*. 2008). Visnevschi-Necrasov *et al*. (2013) confirmed a direct correlation between the *IFS* gene polymorphism detected by sequencing and isoflavone content in species of *Trifolium* particularly noted for formononetin. The *IFS* gene can therefore be utilized for screening *Trifolium* genotypes, including those of red clover, for formononetin. *IFS* sequences have been determined as a molecular marker for screening out genotypes with increased formononetin content.

Red clover is a high PPO legume playing a significant role in both organic and conventional farming operations. Knowledge of *PPO* genes and their structure can facilitate molecular breeding. A *PPO* gene family in red clover has been reported as a cluster of at least six genes . The three paralogous genes *PPO1*, *PPO2* and *PPO3*, having high homology and thus suggesting a recent evolutionary event, were identified by Sullivan *et al*. (2004). Multiple *PPO1* copies have been characterized. Two other single-copy *PPO* genes, *PPO4* and *PPO5*, were identified by Winters *et al.* (2009). DNA sequences show high homology within species. Red clover *PPO* genes are differentially expressed in aerial and root tissues (Sullivan *et al*. 2004). *PPO1/5* and *PPO4* have proved to be useful genetic markers for red clover breeding purposes.

Comparative genomics between model and crop species

The syntenic relationship between red clover and model genomes was first explored by Sato *et al*. (2005) by simply comparing the map locations of the red clover microsatellite markers and the corresponding best-hit genomic sequences of the model legumes *L. japonicus* and *M. truncatula*. The relationship between linkage groups seemed to be very complex on the macro-level with three exceptions: red clover linkage groups 1, 6 and 7. At the segmental level, microsyntenic relationships could be detected in all linkage groups. This showed that large synteny segments are conserved in the genomes of legumes and that anchor markers can link these segments among different species and directly to the genome sequence (Sato *et al*. 2007). Isobe *et al*. (2012) compared the genome structures of red clover, white clover, *M. truncatula*, and *L. japonicus*. Macrosynteny was confirmed again across the four legume species; however, the comparative genomic structure between white clover and *M. truncatula* had a higher degree of conservation than did that of the two clover species.

Studies based on early versions of the assembled genomic sequences later demonstrated a high degree of macrosynteny between the genomes of *M. truncatula* and *L. japonicus*, and 10 large-scale synteny blocks have been defined by direct sequence comparison (Cannon *et al.* 2006). Microsynteny provides a rapid approach for identifying gene counterparts in limited genomic regions of interest, and a high degree of microsynteny can be expected among phylogenetically diverse legume plants. Genome-wide comparison at the nucleotide level has revealed that approximately 60% of genes were conserved in the syntenic regions in the genomes of *M. truncatula* and *L. japonicus* (Cannon *et al.* 2006). Genomic information of the model legumes can be applied successfully to the identification and characterization of genes in crop legumes. Investigations adopting this strategy are currently underway.

CONCLUSION

New tools are becoming available to plant breeders and geneticists for transferring key and complex traits into improved red clover varieties. Modern breeding techniques such as marker-assisted selection can increase the speed and precision with which traits may be selected. This is based on DNA genotyping of individuals. Use of association mapping strategies eliminates the need to develop dedicated mapping populations and allows the identification of QTL within breeding germplasms. This will enable more rapid breeding of allogamous species, where

development of mapping populations and candidate gene identification are more difficult. Comparative genomics with model legumes *L japonicus* and *M. truncatula* will provide insight into the molecular genetics of red clover.

Whereas foreign varieties now prevail in the Czech Republic for most agricultural crops, domestic varieties predominate in red clover. This is a result of the high level of breeding efforts in this country. In the State Variety Book, there are 21 diploid and 22 tetraploid red clover varieties registered in the Czech Republic for certification and marketing (Bulletin of the Central Institute for Supervising and Testing in Agriculture 2013). For example, the variety Start has retained its competitiveness for decades. Stand longevity, forage yield and resistance to root rot and powdery mildew have been improved over the last seven decades. In addition, some progress has been made with resistance to *Bean Yellow Mosaic Virus* and fungal pathogens from the genus *Fusarium*. Tetraploid cultivars have increased forage yields and persistence, however, seed yields are lower than in diploid cultivars.

In the past, a given variety was evaluated primarily according to quantitative characteristics; quality was taken into account, but was not of the highest importance. Qualitative characteristics have been on the increase, however, along with the reproductive potential of cows. Ruminants are valuable for their ability to convert a fibre-rich forage diet into high-quality protein products for human consumption. Through cooperation between the plant and animal sciences, it is possible to identify how high-quality forages can be used in future to better satisfy the nutritional requirements of ruminants.

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References

Abberton M.T. (2007): Interspecific hybridization in the genus *Trifolium*. Plant Breeding, **126**: 337–342.

Ansari H.A., Ellison N.W., Griffiths A.G., Williams W.M. (2004): A lineage-specific centromeric satellite sequence in the genus *Trifolium*. Chromosome Research, **12**: 1–11.

Antonius-Klemola K. (1999): Molecular markers in *Rubus* (*Rosaceae*) research and breeding. Journal of Horticultural Science and Biotechnology, **74**: 149–160.

Arumuganathan K., Earle E.D. (1991): Nuclear DNA content of some important plant species. Plant Molecular Biology Report, **9**: 208–218.

Bennett M.D., Bhandol P., Leitch I.J. (2000): Nuclear DNA amounts in angiosperms and their modern uses – 807 new estimates. Annals of Botany, **86**: 859–909.

Bulletin of the Central Institute For Supervising and Testing in Agriculture (2013): Czech Gazette for Plant Breeders Rights and National List of Plant Varieties, No. XII/S of 30 June 2013.

Campos de Quiroz H., Ortega Klose F. (2001): Genetic variability among elite red clover (*Trifolium pratense* L.) parents used in Chile as revealed by RAPD markers. Euphytica, **122**: 61–67.

Cannon S.B., Sterck L., Rombauts S., Sato S., Cheung F., Gouzy J., Wang X., Mudge J., Vasdewani J., Schiex T. *et al*. (2006): Legume genome evolution viewed through the *Medicago truncatula* and *Lotus japonicus* genomes. Proceedings of National Academy of Science USA, **103**: 14959–14964.

Collins G.B., Taylor N.L., Phillips G.C. (1981): Successful hybridization of red clover with perennial *Trifolium* species via embryo rescue. In: Proc. 14th Int. Grassland Congress. Lexington, 168–170.

Ellison N.W., Liston A., Steiner J.J., Williams W.M. Taylor N.L. (2006): Molecular phylogenetics of the clover genus (*Trifolium* – *Leguminosae*). Molecular Phylogenetics and Evolution, **39**: 688–705.

Farag M.A., Huhman D.V., Dixon R.A., Sumner L.W. (2008): Metabolomics reveals novel pathways and differential mechanistic and elicitor-specific responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures. Plant Physiology, **146**: 387–402.

Greene S.L., Gritsenko M., Vandemark G. (2004): Relating morphologic and RAPD marker variation to collection site environment in wild populations of red clover (*Trifolium pratense* L.). Genetic Research and Crop Evolution, **51**: 643–653.

Grime J.P., Mowforth M.A. (1982): Variation in genome size – an ecological interpretation. Nature, **299**: 151–153.

Gustine D.L., Voigt P.W., Brummer E.C., Papadopoulos Y.A. (2002): Genetic variation of RAPD markers for North American white clover collections and cultivars. Crop Science, **42**: 343–347.

Herrmann D., Boller B., Widmer F., Kölliker R. (2005): Optimization of bulked AFLP analysis and its application for exploring diversity of natural and cultivated populations of red clover. Genome, **48**: 474–486.

Herrmann D., Boller B., Studer B., Widmer F., Kölliker R. (2006): QTL analysis of seed yield components in red clover (*Trifolium pratense* L.). Theoretical and Applied Genetics, **112**: 536–545.

Hughes J.W. (1986): Tissue culture derived crossing barriers. American Journal of Botany, **73**: 323–329.

Isobe S., Sawai A., Yamaguchi H., Gau M., Uchiyama K. (2002): Breeding potential of the backcross progenies of a hybrid between *Trifolium medium* × *T. pratense* to *T. pratense*. Canadian Journal of Plant Science, **82**: 395–399.

Isobe S., Klimenko I., Ivashuta S., Gau M., Kozlov N. N. (2003): First RFLP linkage map of red clover (*Trifolium pratense* L.) base on cDNA probes and its transferability to other red clover germplasm, Theoretical and Applied Genetics, **108**: 105–112.

Isobe S., Kölliker R., Hisano H., Sasamoto S., Wada T., Klimenko I., Okumura K., Tabata S. (2009): Construction of a consensus linkage map for red clover (*Trifolium pratense* L.). BMC Plant Biology, **9**: 57.

Isobe S.N., Hisano H., Sato S. *et al*. (2012). Comparative genetic mapping and discovery of linkage disequilibrium across linkage groups in white clover (*Trifolium repens* L.). Genes Genomes Genetics, **2**: 607–6017.

Jakešová H., Řepková J., Hampel D., Čechová L., Hofbauer J. (2011): Variation of morphological and agronomic traits in hybrids of *Trifolium pratense* × *T. medium* and a comparison with the parental species. Czech Journal of Genetics and Plant Breeding, **47**: 28–36.

Jones B.A., Hatfield R.D., Muck R.E. (1995): Screening legume forages for soluble phenols, polyphenol oxidace and extrakt browning. Journal of the Science of Food and Agriculture, **67**: 109–112.

Kataoka R., Hara M., Kato S., Isobe S., Sato S., Tabata S., Ohmido N. (2012): Integration of linkage and chromosome maps of red clover (*Trifolium pratense* L.). Cytogenetic and Genome Research, **137**: 60–69.

Kazimierska E.M. (1978): Embryological studies of cross compatibility in the genus *Trifolium* L. I. Hybridization of *T. pratense* L. with some species in the subgenus *Lagopus* Bernh. Genetica Polonica, **19**: 1–12.

Kazimierska E.M. (1980): Embryological studies of cross compatibility of species within the genus *Trifolium*. L. III. Development of the embryo and endosperm in crossing *T. repens* L. with *T. hybridum* L. and *T. fragiferum* L. Genetica Polonica, **21**: 37–61.

Khanlou K.M., Karimi M., Maroufi A. *et al*. (2011): Improvement of plant regeneration and Agrobacterium-mediated genetic transformation efficiency in red clover (*Trifolium pratense* L.). Research Journal of Biotechnology, **6**: 13–21.

Klimenko I., Razgulayeva N., Gau M., Okumura K., Nakaya A., Tabata S., Kozlov N.N., Isobe S. (2010): Mapping candidate QTLs related to plant persistency in red clover. Theoretical and Applied Genetics, **120**: 1253–1263.

Kölliker R., Herrmann D., Boller B., Widmer F. (2003): Swiss Mattenklee landraces, a distinct and diverse genetic resource of red clover (*Trifolium pratense* L.). Theoretical and Applied Genetics, **107**: 306–315.

Kongkiatngam P., Waterway M.J., Coulman B.E., Fortin M.G. (1996): Genetic variation among cultivars of red clover (*Trifolium pratense* L.) detected by RAPD markers amplified from bulk genomic DNA. Euphytica, **89**: 355–361.

Lee M.R.F., Winters A.L., Scollan N.D., Dewhurst R.J., Theodorou M.K., Minchin F.R. (2004): Plant-mediated lipolysis and proteolysis in red clover with different polyphenol oxidases activities. Journal of the Science of Food and Agriculture, **84**: 1639–1645.

Lee M.R.F., Olmos Colmenero J.D.J., Winters A.L., Scollan N.D., Minchin F.R. (2006): Polyphenol oxidace activity in grass and its effect on plant-mediated lipolysis and proteolysis of *Dactylis glomerata* (cocksfoot) in a simulated rumen environment. Journal of the Science of Food and Agriculture, **86**: 1503–1511.

Macheix J.J., Sapis J.C., Fleurit A. (1991): Phenolic compounds and polyphenol oxidase in relation to browning grapes and wines. CRC Reviews of Food Science, **30**: 441–486.

Merker A. (1984): Hybrids between *Trifolium medium* and *T. pratense*. Hereditas, **101**: 267–268.

Merker A. (1988): Amphidiploids between *Trifolium alpestre* and *T. pratense*. Hereditas, **108**: 267.

Nedbalkova B., Repkova J., Bartosova L. (1995): Germplasm TBZP1, TBZP2, TBZP3, TBZP4 of interspecific *Trifolium* hybrids. Scientific Studies – Research Institute for Fodder Plants, Ltd. Troubsko, **13**: 129–131.

Pedrosa A., Sansal N., Stougaard J., Schweizer D., Bachmair A. (2002): Chromosome map of the model legume *Lotus japonicus*. Genetics, **161**: 1661–1672.

Philips G.C., Collins G.B., Taylor N.T. (1982): Interspecific hybridization of red clover (*Trifolium pratense* L.) with *T. sarosiense* Hazsl. using *in vitro* embryo rescue. Theoretical and Applied Genetics, **62**: 17–24.

Phillips G.C., Grosser J.W., Buger S., Taylor N.L., Collins G.B. (1992): Interspecific hybridization between red clover and *Trifolium alpestre* using *in vitro* embryo rescue. Crop Science, **32**: 1113–1115.

Řepková J., Nedbálková B., Holub J. (1991): Regeneration of plants from zygotic embryos after interspecific hybridization within the genus *Trifolium* and electrophoretic evaluation of hybrids. Scientific Studies – Research Institute for Fodder Plants, Ltd. Troubsko, **12**: 7–14.

Řepková J., Jungmannová B., Jakešová H. (2003): Interspecific hybridisation prospects in the genus *Trifolium*. Czech Journal of Genetics and Plant Breeding, **39** (Special Issue): 306–308.

Řepková, J., Jungmannová, B., Jakešová, H. (2006): Identification of barriers to interspecific crosses in the genus *Trifolium*. Euphytica, **151**: 39–48.

Riday H., Krohn A. L. (2010): Genetic map-based location of the red clover (*Trifolium pratense* L.) gametophytic self-incompatibility locus. Theoretical and Applied Genetics, **121**: 761–767.

Rizza M.D., Real D., Reyno R., Porro V., Burgueno J., Errico E., Quesenberry K.H. (2007): Genetic diversity and DNA content of three South American and three Eurasiatic *Trifolium* species. Genetics and Molecular Biology, **30**: 1118–1124.

Sato S., Isobe S., Asamizu E. *et al.* (2005): Comprehensive structural analysis of the genome of red clover (*Trifolium pratense* L.). DNA Research, **12**: 301–364.

Sato S., Nakamura Y., Asamizu E. Isobe S., Tabata S. (2007): Genome sequencing and and genome resouecing in model legumes. Plant Physiology, **144**: 588–593.

Sato S., Isobe S., Tabata S. (2010): Structural analyses of the genomes in legumes. Current Opinion in Plant Biology, **13**: 146–152.

Sawai A., Ueda S., Gau M., Uchiyama K. (1990): Interspecific hybrids of *Trifolium medium* L. × 4*x T. pratense* L. obtained through embryo culture. Journal of Japan Society of Grassland Science, **35**: 267–272.

Schwer J.F., Cleveland R.W. (1972): Tetraploid and triploid interspecific hybrids of *Trifolium pratense*, *T. diffusum* and some telated species. Crop Science, **12**: 419–422.

Smith R.R., Taylor N.L., Bowley S.R. (1985): Red clover. In: Taylor N.L. (ed.): Clover Science and Technology. ASA-CSSA-SSSA, Madison, 457–470.

Sullivan M.L., Hatfield R.D. (2006): Polyphenol oxidaxe and o-diphenols inhibit postharvest proteolysis in red clover and alfalfa. Crop Science, **46**: 662– 670.

Sullivan M.L., Hatfield R.D., Thoma S.L., Samac D.A. (2004): Cloning and characterization of red clover polyphenol oxidase cDNA and expression of active protein in *Escherichia coli* transgenic alfalfa. Plant Physiology, **136**: 3234–3244.

Taylor N.L., Quesenberry K.H. (1996): Red Clover Science. Kluwer Academy Publishing, Dordrecht, 170–187.

Taylor N.L., Quale R.F., Anderson M.K. (1980): Methods of overcoming interspecific barriers in *Trifolium*. Euphytica, **29**: 441–450.

Townsend C.E., Taylor N.L. (1985): Incompatibility and plant breeding. In: Taylor N.L. (ed.): Clover Science and Technology. ASA-CSSA-SSSA, Madison, 365–381.

Ulloa O., Ortega F., Campos H. (2003): Analysis of genetic diversity in red clover (*Trifolium pratense* L.) breeding populations as revealed by RAPD genetic markers. Genome, **46**: 529–535.

Visnevschi-Necrasov T., Faria M.A., Cunha S.C., Harris J., Meimberg H.W.E., Curto M.A.C., Pereira M.G., Oliveira M.B.P.P., Nunes E. (2013): Isoflavone synthase (IFS) gene phylogeny in *Trifolium* species associated with plant isoflavone contents. Plant Systematics and Evolution, **299**: 357–367.

Vižintin L., Javornik B., Bohanec B. (2006): Genetic characterization of selected *Trifolium* species as revealed by nuclear DNA content and ITS rDNA region analysis. Plant Science, **170**: 859– 866.

Vogt M., Schweiger W. (1983): Study of the methodology of embryo culture in *Trifolium*. Archiv Zücht-Forschung, Berlin, **13**: 273–283.

Winters A., Heywood S., Farrar K., Donnison I., Thomas A., Webb K.J. (2009): Identification of an extensive gene cluster among a family of PPOs in *Trifolium pratense* L. (red clover) using a large insert BAC library. BMC Plant Biology, **9**: 94

Yildiz F. (ed.) (2005): Phytoestrogens in Functional Foods. Taylor and Francis Ltd., Boca Raton, 210–211.

Zohary M., Heller D. (1984): The Genus *Trifolium*. Israel Academy of Sciences and Humanities, Jerusalem, Israel.

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