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Genetic diversity in white clover and its progenitors as revealed by DNA fingerprinting

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Abstract

The genetic diversity and ancestral relationships of a number of *Trifolium* species was revealed by using the amplified fragment length polymorphism (AFLP) and the random amplified polymorphic DNA (RAPD) markers. Both markers produced few species-specific markers. Using distance and parsimony methods, in *NTSYS-pc* and *PAUP* software programs, we clearly differentiated the accessions of white clover from other closely related progenitors. The phylogenetic trees, produced by *PAUP*, also reinforced the close affinity of *T. nigrescens* and the allopolyploid white clover in support of former views that this diploid species could have been the donor of one of two genomes of the allotetraploid *T. repens*. In addition, the dendrograms, produced by *NTSYS-pc*, also indicated close affinity of *T. nigrescens* and *T. occidentale* to the accessions of *T. repens*. These data is congruent with karyological and phylogenetic affinities between the white clover and *T. occidentale.* The relationships between the examined accessions, in the *T. repens* gene pool, may be regarded to indicate the presence of shared alleles between *T. repens*, *T. occidentale* and *T. uniflorum*. Further, *T. occidentale* showed close phylogenetic relations to *T. pallescens*.

Additional key words: AFLP, ancestry, progenitors, RAPD, *Trifolium repens.*

Introduction

The genus *Trifolium* is of economic importance as demonstrated by the wide growing of at least 16 species as livestock forage and green manure crops (Gillett and Taylor 2001) and by the capacity of over 125 species to fix nitrogen through root nodulation by the bacterium *Rhizobium leguminosarum* (Sprent 2001). The major temperate pastures species include the white clover (*Trifolium repens* L.); a stoloniferous, perennial species. Despite its wide distribution, white clover is limited in terms of its adaptive range (Marshall *et al*. 1995, 1998). There has been considerable breeding progress in recent years to increase the variation of the white clover gene pool, through hybridization of white clover to the closely related species and also to the more distant species of the genus *Trifolium* (Jahufer *et al*. 2002, Abberton and Marshall 2005, Abberton 2007). More recently Hargreaves *et al*. (2009) found that islands populations of white clover in the United Kingdom are differentiated from mainland populations and genetically distinct from cultivated cultivars indicating that islands may act as refugia of *Trifolium repens* genetic diversity.

The vast majority of natural and cultivated forms of *T. repens* are tetraploid $2n=4x=32$, (Chen and Gibson 1970, 1972). Judged by the rare occurrence of multivalent formation during prophase-I of meiosis, and the regular disomic inheritance of its chromosomes, *T. repens* has been regarded as allotetraploid in origin with two ancestral genomes (Williams 1987). Based on its ability to cross with some closely related species, Gibson and Beinhart (1969) agreed with Brewbaker and Keim (1953), that diploid *T. nigrescens* ($2n=16$) is one of the ancestors of the polyploid *T. repens*, and that the other

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Abbreviations: AFLP - amplified fragment length polymorphism; RAPD - random amplified polymorphic DNA.
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ancestor may be *T. occidentale* (2*n*=16). Chen and Gibson (1972) indicated a close karyological and phylogenetic relationship between *T. repens, T. nigrescens* and *T. occidentale*, in addition to tetraploid *T. uniflorum* (2*n*=32). Kakes and Hakvoort (1994) found a general resemblance of the enzyme linamarase (Li) in *T. repens* and *T. nigrescens,* and concluded that *T. nigrescens* (or an ancestral form of it) donated the *Li* gene to *T. repens*. Meanwhile, the distribution of cyanotypes in *T. repens* and *T. occidentale* is dissimilar and regulated by different mechanisms (Kakes and Chardonnens 2000), which support the view that *T. occidentale* did not donate active *Li* alleles to *T. repens*. Kazimierski and Kazimierska (1973) had earlier reported successful crosses between *T. isthmocarpum* and *T. repens,* and proposed that *T. isthmocarpum* also may contributed to the two genomes of *T. repens* with *T. nigrescens*.

Ansari *et al*. (1999) concluded that the molecular organization of 5S and 18S-26S rDNA loci in *T. repens* and related species supports the allotetraploid origin of *T. repens*, and proposed *T. nigrescens* subsp. *petrisavii* as one of its diploid ancestors. However, a more extensive investigation on *T. nigrescens* using internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA elucidated three subspecies of *T. nigrescens* but could not identify the direct ancestor of *T. repens* (Williams *et al*. 2001). Evidence obtained from the isozymes polymorphism (Badr *et al*. 2002) indicated that the two genomes of the tetraploid *T. repens* could have been derived from hybridization between *T. nigrescens* and *T. uniflorum*. However, these data also indicated that the origin of *T. repens* is somewhat obscured by the presence of shared alleles between *T. repens* and both *T. occidentale* and *T. isthmocarpum*, suggesting introgression of genes from the latter two species into the *T. repens* genome. Phylogenetic results reported by Ellison *et al*. (2006) implicated *T. occidentale* and *T. pallescens* as likely diploid progenitors of *T. repens*. Genomic Southern hybridization and/or fluorescence *in situ* hybridization (FISH) performed on 17 *Trifolium* species/subspecies by Ansari *et al*. (2004) revealed that it is a conserved lineage-specific repeat confined to several species within the section *Lotoidea* originating in the Mediterranean region. Williams *et al*. (2008) reported evidence suggesting that *T. nigrescens* is a close relative of *T. occidentale* but not a direct ancestor of white clover. It is likely that *T. nigrescens* and *T. occidentale* are close relatives on one side of the ancestry of white clover. In the light of the recent evidence as reported by Ansari *et al*. (2004), Ellison *et al*. (2006) and Williams *et al*. (2008) it seems that the ancestry of the allopolyploid white clover remains elusive.

In this paper, we address the genetic diversity in a number of species representing the genetic pool of white clover using two molecular approaches; the random amplified polymorphic DNA (RAPD) and the amplified fragment length polymorphism (AFLP) fingerprinting that have been found efficient, reliable, and convenient for a number of applications in plant science including studies on the relationships among and within species of cultivated crops and their wild relatives. The RAPD approach is used to amplify DNA sequences with single short (9 - 10 bp) primers of arbitrary nucleotide sequence (Williams *et al*. 1990) and has been recently used to study genetic diversity and species relationships in *Trifolium subterraneum* complex (Piluzza *et al*. 2005). In *T. repens*, RAPD markers were used to characterize the USDA white clover core collection (Bortolini *et al*. 2006). The results showed a large genetic variability within the white clover core collection, probably due to its reproduction mode and ploidy level. Similarly the AFLP that was developed by Zabeau and Vos (1993) and published by Vos *et al*. (1995) has been also extensively used for the assessment of genetic diversity in plants (Mo *et al*. 2009, Sciacca *et al*. 2010). One approach utilizing AFLPs has been to elucidate the ancestors, origin and domestication history of some cultivated crops including monococum wheat (Heun *et al*. 1997), barley (Badr *et al*. 2000) and the Egyptian clover (Badr *et al*. 2008). The objective of this study is to analyze genetic diversity, as revealed by RAPD and AFLP fingerprinting, in accessions of *T. repens* from different sources and accessions of other *Trifolium* species that have been regarded as suspected progenitors or donors to its genome.

Materials and methods

The species selected for the present investigation are comprised of six accessions of *T. repens* and ten other accessions representing eight of its closely related species (Zohary and Heller 1984). These accessions cover *T. nigrescens, T. occidentale* and *T. pallescens* that have been taxonomically placed with *T. repens* in one series by Zohary and Heller (1984) and/or showed close phylogenetic affinity to it as indicated by Ellison *et al*. (2006). The other five species have shown ability to cross to *T. repens* or was proposed as progenitors for its genome (Kazimierski and Kazimierska 1973, Badr *et al*. 2002, Abberton 2007). Seed material of the 16 accessions of *Trifolium repens* L. and eight other related species used in this investigation were kindly provided by different sources (Table 1). Seeds were soaked in water for 48 h and germinated in compost in small pots. Root tips were collected from 1 to 2-week-old seedlings, pretreated with 0.05 % colchicine, fixed in 3:1 ethanol: acetic acid, and used for chromosome number determinations using the standard Feulgen squash method (Darlington and La Cour 1976). DNA was extracted from 4 - 5 genotypes per accession and pooled in for RAPD

and AFLP fingerprinting.

DNA was extracted using a modified CTAB method (Saghai-Maroof *et al*. 1984) as described in Badr *et al*. (2008). For RAPD fingerprinting, seven 10-mer oligonucleotide DNA primers of arbitrary sequences from *Operon Technologies* (Alameda, CA, USA), were independently used in PCR reactions according to Williams *et al*. (1990). Only five of the tested primers were able to reveal stable and reproducible polymorphism in the used 16 accessions; their codes are A13, B10, C20, Z13 and Z19. RAPD fingerprinting was performed in 0.025 cm^3 reaction volume containing the following reagents: 0.002 cm^3 of dNTPs (2.5 mM), 0.0015 cm^3 of $MgCl_2$ (25 mM), 0.0025 cm^3 of 10× buffer, 0.002 cm^3 of primer (2.5 mM), 0.002 cm^3 of template DNA (50 μg cm^{-3}), 0.0003 cm^3 of Taq polymerase (5 U mm^{-3}) and 0.0147 cm^3 of sterile dd H_2O. Amplification was carried out in *2400 Gene Amp* PCR thermocycler (*Perkin Elmer*, Waltham, MA, USA) as follows: one cycle at 94 °C for 4 min followed by 40 cycles at 94 °C for one min; 37 °C for one min and 72 °C for two min. The reaction was finally incubated at 72 °C for 10 min. The PCR products were separated in 1.4 % agarose gel in TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8) at 100 V for 60 min. The gels were stained in 0.2 μg cm^{-3} ethidium bromide and photographed using *BioRad 2000* (Hercules, CA, USA) gel documentation system. Each RAPD experiment was repeated twice and only stable products were scored.

The AFLP analysis was performed using the *ABI PRISM* fluorescent dye labeling and detection protocol (*Perkin Elmer*) based on the method of Vos *et al.* (1995), as described in Badr *et al.* (2008). Genomic DNA (500 ng) was double-digested with *Eco*RI and *Mse*I restriction enzymes and ligated to *Eco*RI and *Mse*I in a total volume of 0.011 cm^3 for 4 h at 37 °C. The restriction/ligation (R+L) products were diluted, pre-amplified with *Eco*RI + A and *Mse*I + C primers in a total volume of 0.020 cm^3 in a thermocycler for 25 cycles at 94 °C denaturation (20 s), 56 °C annealing (30 s), and 72 °C extension (2 min), with initial hold at 72 °C and a final hold at 60 °C for 30 min. The pre-selective amplification product was diluted 15× in 0.1 TE buffer and stored at 4 °C for amplification, or stored at -20 °C for later use. The above mentioned solution was used as a template for selective amplification using three 5'end labeled *Eco*RI + 3 primers (ACA, blue; AAG, green; and ACC, yellow) and three *Mse*I + 3 primers (CAC, CTC, and CTT). Amplification was conducted as in Badr *et al.* (2008) on an automated *ABI 310* DNA sequencer (*Applied Biosystems,* Foster City, CA, USA) with an injection time of 12 s and a run time of 30 min. The AFLP fragment profiles produced by the nine primer pair combinations were analyzed with *GeneScan* analysis software *v. 3.1* (*Applied Biosystems*), as well as printed on photographic paper for manual scoring and confirmation.

The RAPD bands, on the photographic prints, and the AFLP bands, in the size range of 50 to 500 bp were scored as two separate binary matrices where (1) is scored for presence and (0) for absence of bands across the white clover accessions and the accessions of other species. However, only polymorphic bands, scored in at least two accessions, were considered for analysis. In total 88 RAPD bands and 301 AFLP bands were scored across the 16 examined accessions. Distance trees demonstrating the genetic distance among the examined accessions, based on the RAPD and AFLP data separately and in combination, were constructed using Dice and Jaccard similarity coefficients by unweighted pair group method (UPGMA; Sokal and Michener 1958) and Neighbor-Joining (Saitou and Nei 1987) tree building methods using the software *NTSYS-pc v. 2.1* (Rohlf 2002). In addition, average distance UPGMA and Neighbor-Jjoining (NJ) trees were produced using *PAUP** software *v. 4* (Swofford 2002). In addition, the mean character difference was used to produce NJ and UPGMA trees as implemented in *PAUP* 4.0* and also to calculate bootstrap values for 1000 replicates (Felsenstein 1985) to evaluate branch support for the UPGMA and NJ trees. *PAUP* 4.0* was also used to conduct a parsimony analysis of the AFLP data, using heuristic search with *MULTREES* in effect TBR branch swapping and 100 replicate random additions.

Results

A total of 88 bands were identified in the RAPD profiles of the studied 16 accessions of white clover and its close relatives. These include 85 polymorphic bands and three unique bands in *T. montanum* produced by the three primers A13, C20 and Z19 (Fig. 1). Meanwhile, a total of 301 AFLP markers were scored. Examples of AFLP fingerprinting, as revealed, by the two primer pair combinations *MSe*I-CAC, *Eco*RI-ACA and *MSe*I-CAC, *Eco*RI-AAG are illustrated in Fig. 2.

The NJ and the UPGMA trees based on the RAPD data analysis are of similar topology (Fig. 3). In NJ tree, the four species *T. alpinum*, *T. isthmocarpum*, *T. montanum* and *T. ambiguum* were delimited together as a separate group from the accessions representing the other species. In this group, *T. ambiguum* was closer to *T. montanum* than either of these two species to *T. isthmocarpum*. The other accessions are divided into two groups; one comprised of the two accessions of *T. uniflorum* and *T. pallescens* and the other comprises *T. occidentale*, the two accessions of *T. nigrescens* and the six accessions of *T. repens*. In this group, *T. occidentale* is distinguished from the accessions representing *T. repens* and

Table 1. The source, origin, and somatic chromosome number (2*n*) of the studied accessions of *Trifolium repens* and its related species (ICARDA - International Center for Agricultural Research in Dry Areas, Aleppo, Syria; IPK TRIF - Institut für Pflanzenbau und Pflanzenzuechtung, Gatersleben, Germany; Kew Garden - Royal Botanic Garden, Kew, England; USDA - United States Department of Agriculture).

Serial	Species	Subsection	Series	Source	Origin	2n
01	*T. alpinum* L.	*Lotoidea*	*Grandiflora*	Kew 9841	Switzerland	16
02	*T. ambiguum* M.B.	*Platystylium*	*Platystylium*	USDA598978	Georgia	16
03	*T. isthmocarpum* Brot.	*Platystylium*	*Platystylium*	IPK TRIF/77/91	Portugal	16
04	*T. montanum* L.	*Platystylium*	*Platystylium*	IPK TRIF147194	unknown	16
05	*T. nigrescens* Viv.1	*Lotoidea*	*Lotoidea*	ICARDA 1482	Syria	16
06	*T. nigrescens* 2 ssp *petrisavi*	*Lotoidea*	*Lotoidea*	USDAPI 289969	Turkey	16
07	*T. occidentale* Combe	*Lotoidea*	*Lotoidea*	IPK TRIF255192	unknown	16
08	*T. pallescens* Schreb.	*Lotoidea*	*Lotoidea*	IPK TRIF80175	unknown	16
09	*T. repens* L.1	*Lotoidea*	*Lotoidea*	USDAPI239978	Portugal	32
10	*T. repens* 2	*Lotoidea*	*Lotoidea*	IPK (cult.) 4633	France	32
11	*T. repens* 3	*Lotoidea*	*Lotoidea*	USDA PI 369138	Greece	32
12	*T. repens* 4	*Lotoidea*	*Lotoidea*	IPK (cult.) 18367	Germany	32
13	*T. repens* 5	*Lotoidea*	*Lotoidea*	USDAG31224	Bulgaria	32
14	*T. repens* 6	*Lotoidea*	*Lotoidea*	USDAPI323021	Iran	32
15	*T. uniflorum* L.1	*Lotoidea*	*Grandiflora*	USDA PI 369138	Greece	32
16	*T. uniflorum*2	*Lotoidea*	*Grandiflora*	USDAPI369139	USA	32

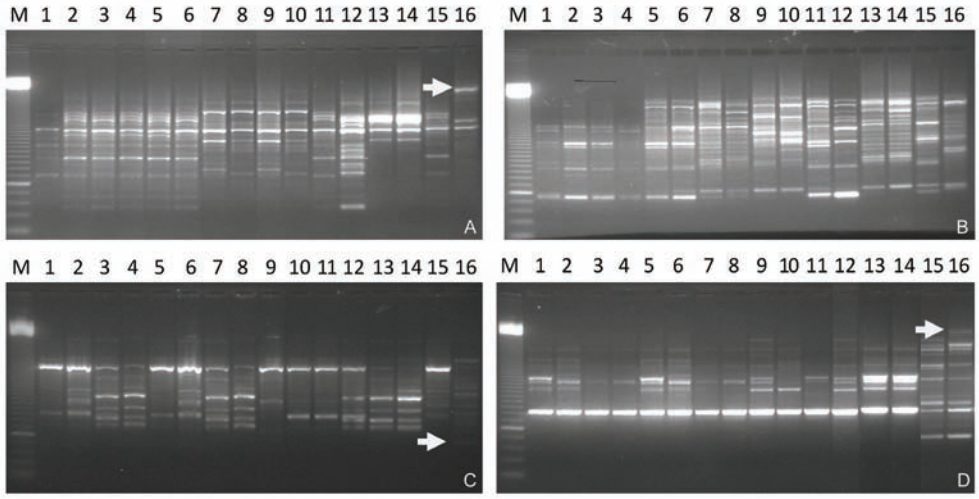

Fig. 1. Examples of the RAPD fingerprinting produced by four random primers: *A* - pr A13; *B* - pr B10; *C* - pr C20; *D* - pr Z19. The key for the numbers above the lanes is as follows: M - marker, 1 to 6 - *T. repens*; 7,8 - *T. nigrescens*; 9 - *T. pallescens*; 10 - *T. occidentale*; 11 - *T. isthmocarpum*; 12 - *T. alpinum*; 13,14 - *T. uniflorum*; 15 - *T. ambiguum*; 16 - *T. montanum* (*arrows* indicate unique bands).

T. nigrescens. In the cluster comprising the six accessions of *T. repens*, accession 5 from Bulgaria and accession 6 from Iran are delimited from the other four accessions. On the other hand, accession 3 from Greece is separated from a cluster comprised of accession 1 from Portugal, which is also differentiated from accession 2 from France and accession 4 from Germany.

The analysis of AFLP data also produced NJ and UPGMA trees of similar topology and also similar to the tree based on RAPD data (Fig. 4). In this tree, the three species *T. isthmocarpum*, *T. montanum* and *T. ambiguum* are clearly separated from the other accessions. The other accessions are divided into two groups; one comprised of *T. alpinum*, *T. pallescens* and the two samples of *T. uniflorum* with high distance between them. In this tree also the two accessions representing *T. nigrescens* and *T. occidentale* are clustered together and grouped with a second cluster that includes the six accessions of *T. repens*. In this cluster, the accessions 1, 2, and 4 are distinguished from accessions 3, 5 and 6. In the first cluster accession 1 from Portugal is differentiated from the two cultivars (accessions 2 and 4) and in the second cluster, accession 3 is differentiated from accessions 5 and 6.

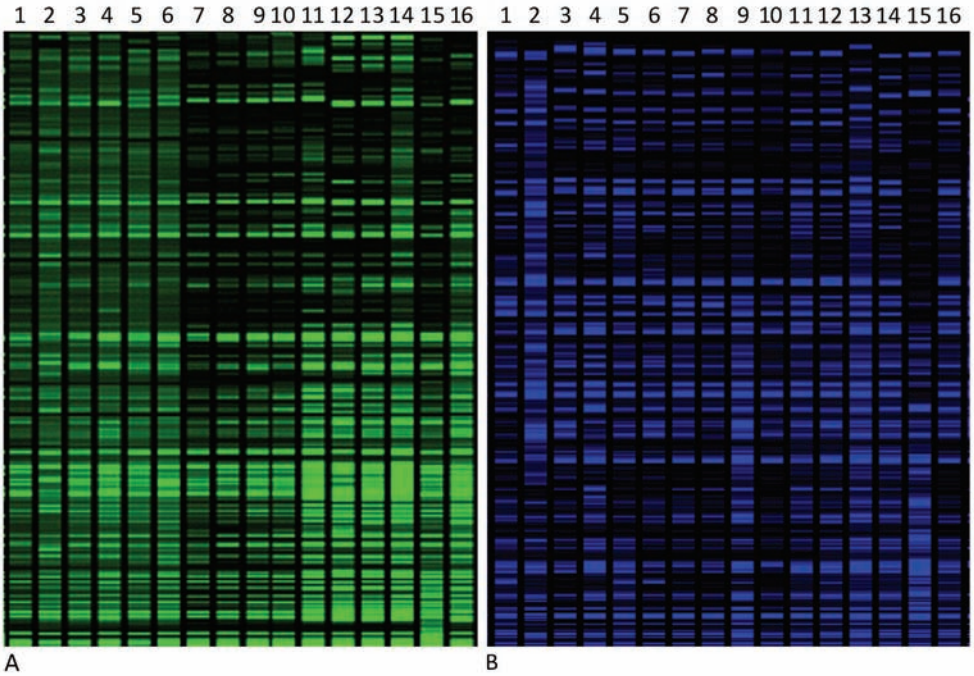

Fig. 2. Examples of the AFLP fingerprinting produced in the genome of the 16 accessions of *Trifolium repens* and its related species by the two primer pair combinations *MSe*I-CAC, *Eco*RI-ACA (*A*) and *MSe*I-CAC, *Eco*RI-AAG (*B*). The numbers above the photographs refer to the numbers of accessions as given in the legend of Fig. 1.

The analysis of AFLP and RAPD data combined using the distance method in *PAUP* (Fig. 5) produced a tree where the four species *T. alpinum*, *T. isthmocarpum*, *T. montanum* and *T. ambiguum* are delimited together as a separate group. However, *T. alpinum* is clearly distinguished from the other three species. Moreover, in this group, *T. ambiguum* is closer to *T. montanum* than either of these two species to *T. isthmocarpum*. In this tree, the two accessions of *T. uniflorum* are clearly distinct, whereas the accessions of *T. pallescens* and *T. occidentale* are associated with a group comprised of the two accessions of *T. nigrescens* and *T. repens*. Unlike the preceding tree the two accessions of *T. nigrescens* are not clustered together but are assigned separately to the group comprising the six accessions of *T. repens*. In the latter group, accession 5 from Bulgaria and accession 6 from Iran are delimited from the other four accessions; accession 3 from Greece and accession 1 from Portugal are differentiated together from the two cultivars accession 2 from France and accession 4 from Germany.

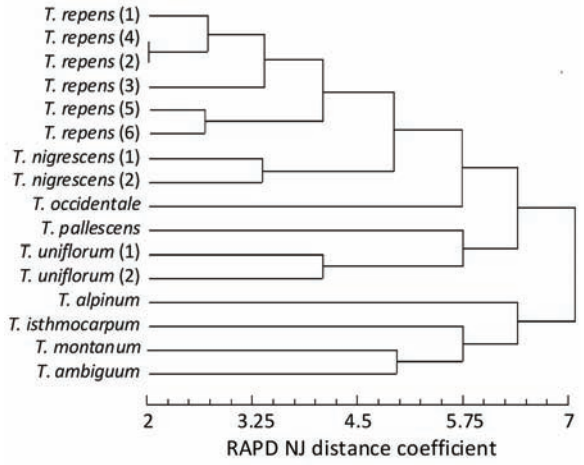

Fig. 3. NJ distance trees constructed using *NTSYSs-pc*, illustrating the relationships between the accessions of white clover and related species in its gene pool based on the analysis of RAPD data.

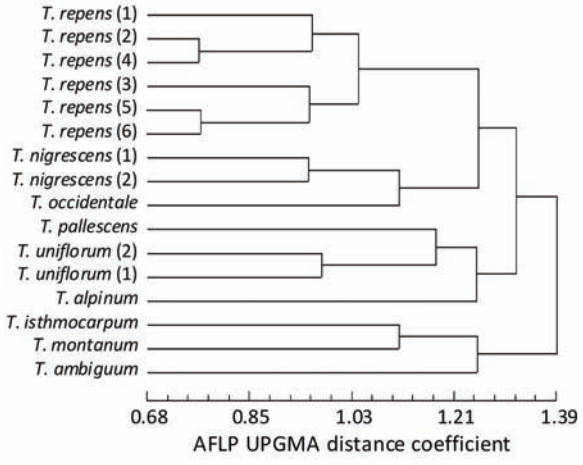

Fig. 4. UPGMA distance trees constructed using *NTSYSs-pc*, illustrating the relationships between the accessions of white clover accessions and related species in its gene pool based on AFLP data.

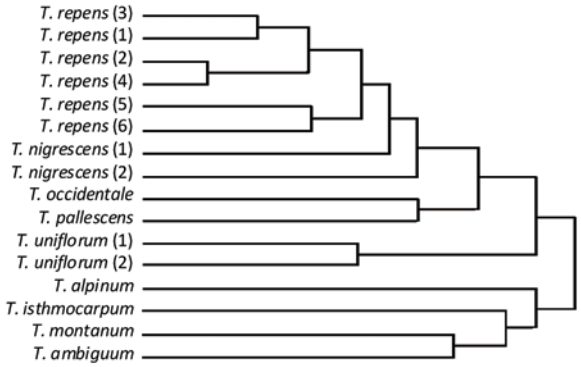

Fig. 5. A distance tree constructed using the NJ method in *PAUP*40* illustrating the relationships between accessions of white clover and other related species; based the analysis of AFLP and RAPD data combined.

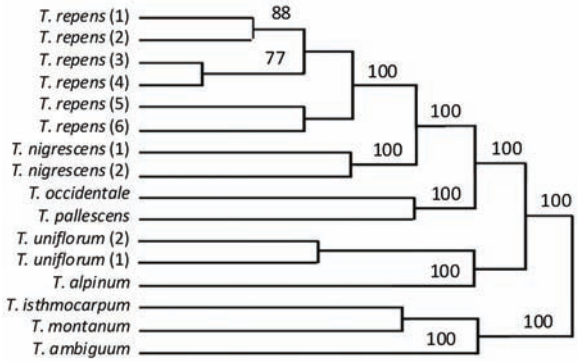

Fig. 6. A strict consensus tree illustrating the relationships among the examined accessions of white clover and other species, based on the AFLP data using *PAUP*40*; the *PAUP* bootstrap values are presented on the branches of the tree.

The parsimony analysis of AFLP data using PAUP produced strict and bootstrap trees with similar topologies to the average distance trees (Fig. 6). Like the distance tree constructed using *NTSYS*, based on AFLP data, the three species, *T. isthmocarpum*, *T. montanum* and *T. ambiguum* are clearly separated from the other accessions. Also in this tree *T. ambiguum* is distinguished from both *T. isthmocarpum* and *T. montanum*. In the accessions representing the other six species, one group is comprised of *T. alpinum* and the two accessions of *T. uniflorum* and the remaining accessions are delimited in two groups; one includes *T. pallescens* and *T. occidentale* and the other is composed of the two accessions of *T. nigrescens* and the six accessions of *T. repens*. In the PAUP analysis of AFLP data, the most equally parsimonious trees support a sister group relationship of *T. nigrescens* and the six accessions of *T. repens* but as measured by the bootstrap values, the six accessions of the white clover are clearly distinguished as a monophyletic group.

Discussion

The RAPD fingerprinting showed few species-specific bands and revealed only three monomorphic bands in the 16 accessions used in this study representing nine species in the white clover gene pool. This finding agrees with the report by Bortolini *et al*. (2006) that RAPD shows wide range of genetic diversity in the USDA core collection of white clover. The AFLPs also produced considerable genetic variation demonstrated by revealing 301 polymorphic markers across the 16 accessions used in the present investigation. The relationships among the examined accessions in the trees based on RAPD data indicated close affinity each other and distant relation to *T. repens*. This result agrees with the phylogenetic trees, based on comparisons of nuclear ribosomal DNA internal transcribed spacer and the chloroplast transfer RNA gene *trnL* intron sequences, using Parsimony and Bayesian phylogenetic analyses (Ellison *et al*. 2006). *T. isthmocarpum* was clearly separated from the other two species contrary to the taxonomic resemblance of the three species (Zohary and Heller 1984). The data obtained from the RAPD data thus do not support earlier views that *T. isthmocarpum* has contributed to the genome of the white clover (Kazimierski and Kazimierska 1973, Badr *et al*. 2002).

The relationships between the examined accessions, as expressed by the distance trees, based on the AFLP data, generally agree with the sectional grouping of the examined species as proposed by Zohary and Heller (1984). The three species, *T. isthmocarpum*, *T. montanum* and *T. ambiguum*, which have been placed in subsection *Platystylium*, are delimited together, as a separate group. The grouping of *T. alpinum* and *T. uniflorum* together, in the trees based on the analysis of AFLP data is congruent with the taxonomic delimitation of these two species together in series I of subsection *Grandiflorum* (Zohary and Heller 1984). The affinity of these two species is also congruent with the phylogenetic analysis based on ITS and cpDNA sequences (Ellison *et al*. 2006). Meanwhile, the position of *T. uniflorum*, in relation to *T. repens*, in the trees based on the RAPD data is contrary to a previously proposed hypothesis that the two genomes of the allotetraploid *T. repens* are derived from a hybridization event between *T. nigrescens* and an ancestral diploid form of *T. uniflorum* (Ansari *et al*. 1999, Badr *et al*. 2002).

Implications that *T. uniflorum*, or an ancient diploid form of it is a donor of one of the white clover genomes, as indicated by cytogenetic analysis (Chen and Gibson 1972, Ansari *et al.* 1999) and the analysis of isozyme polymorphism (Badr *et al*. 2002), is not clearly supported by the RAPD and AFLP data presented in the present investigation. Meanwhile, *T. occidentale* and

T. pallescens were identified as the contemporary taxa most likely diploid progenitors of *T. repens* in the phylogentic analyses presented by Ellison *et al*. (2006). The analysis, by these authors, based on cpDNA sequence revealed a sister relationship between *T. pallescens* and *T. repens*, implicating this taxon as the putative maternal progenitor, while ribosomal DNA-based sequence analysis demonstrated a sister relationship between *T. occidentale* and *T. repens*. In the combined analysis of RAPD and AFLP data, both species showed closer relationships to other taxa. This result is congruent with evidences provided by molecular cytogenetic survey of a satellite sequence in 16 species related to white clover gene pool that also provided no evidence to support close affinity of *T. pallescens* to *T. repens* (Ansari *et al*. 2004).

Trifolium occidentale has been suspected as a potential diploid progenitor of white clover, along with *T. nigrescens* (Chen and Gibson 1970, 1972). This view is clearly supported by the distance tress based on the RAPD data. In the Parsimony based tree, this species is grouped with *T. pallescens* supporting the close systematic relationship between the two species (Zohary and Heller 1984) and their phylogenetic affinities (Ellison *et al*. 2006). Results supporting *T. occidentale* as a potential progenitor of white clover was recently presented by Hand *et al*. (2008). These authors argued for interspecific hybridization event, which generated the modern amphidiploids of *Trifolium*. In their results, close similarity was observed between the genome of *T. occidentale* and one white clover subgenome but the affinity between *T. pallescens* and the other sub-genome was weaker suggesting that a currently uncharacterized species may be the true second progenitor. Hand *et al*. (2008) argued that this taxon may have been ancestral to an extant species, which could be identified using combination of homologous, homoeologous and paralogous classes of sequence variation. However, the hypothesis of a hybridization event involving *T. pallescens* in generating modern polyploid white clover finds no support from the AFLP and RAPD data as presented in this study.

The hypothesis that *T. occidentale* is a potential ancestor of *T. repens* is supported by the morphological similarities between them and their ability to exchange change (Gibson and Beinhart 1969). In contrast, *T. pallescens*, a montane species distributed in the Alps and Pyrenees of Europe, has rarely been included in crossing studies and molecular genetic investigations of the ancestors of *T. repens* and the progenitors of this species. Its inclusion in the molecular cytogenetic survey of 16 *Trifolium* species for the distribution of a centromeric satellite sequence (TrR350) isolated from *T. repens* by Ansari *et al*. (2004) indicated that the satellite sequence is found in several other species of sect. *Trifoliastrum* including *T. pallescens*, and thus cannot be used alone to identify the progenitors of *T. repens*. Moreover, genetic studies of cyanogenesis in white clover, which is largely attributable to presence/absence sequence variation for the *Li* linamarase gene (Olsen *et al*. 2007), showed that neither of the putative progenitors is cyanogenic. Therefore candidates for contribution of the relevant genes to white clover may only represent, as stated in the above paper, a convenient transitional nomenclature.

All trees showed *T. nigrescens* as part of the complex of species closely related to white clover and it was thought to be the source of one of its genomes (Badr *et al*. 2002, Ansari *et al*. 1999). However, more recent molecular phylogenetic evidence indicated that this may not be the case but rather *T. nigrescens* may be regarded as one of five species, which form a tight cluster in DNA based group (Ellison *et al*. 2006). This view was also implemented in the considerable variation within the species that comprises at least three distinct subspecies (Williams *et al*. 2001). The results reported here combined with evidence from isozyme polymorphism (Badr *et al*. 2002) and the general resemblance of the enzyme linamarase in *T. repens* and *T. nigrescens* (Kakes and Hakvoort 1994) support a conclusion that *T. nigrescens* (or an ancestral form of it) contributed at least one of the two genomes of white clover. This conclusion is further supported by successful crosses between *T. repens* and *T. nigrescens* (Michaelson-Yeates *et al*. 1997, Marshall *et al*. 1998, 2008). The high level of genetic identity and the small distance between these two species as revealed by the isozyme data (Badr *et al*. 2002) supported this view and confirm that *T. nigrescens* is likely one diploid ancestor of *T. repens*.

The view that *T. nigrescens* had contributed to the genome of *T. repens* has been also supported by its inclusion in the improvement programs of white clover. Hussain *et al*. (1997) demonstrated the transfer of resistance to the major pest clover cyst nematode from *T. nigrescens* to white clover. This approach has showed considerable potential for the introgression of desirable traits from the *T. nigrescens* to white clover. The development of a range of hybrids may be facilitated by the ability of white clover to produce unreduced gametes (Hussain and Williams 1997, Marshall *et al*. 2005). Marshall *et al*. (1995) described the generation of hybrids with the aim of increasing inflorescence production and seed set. Backcrosses to white clover were carried out and the progeny was intermediate between the two species in terms of reproductive characteristics. Subsequently, further backcrosses were carried out to obtain BC2 and BC3 plants which were morphologically similar to white clover and in particular were perennial and stoloniferous (Marshall *et al*. 1998). The introgression of genes from the ball clover to white clover conferring enhanced seed yield was monitored by the AFLP markers by Marshall *et al*. (2003).

In conclusion, RAPD and AFLP markers reinforce the close genetic affinity between *T. nigrescens* and

T. repens. This view is supported by the chromosomal affinities between the two species and their ability to cross freely (Brewbaker and Keim 1953, Gibson and Beinhart 1969, Ansari *et al.* 1999, Hussain and Williams 1997, Marshall *et al*. 2005) and also by evidence provided by the analysis of isozyme polymorphism (Badr *et al*. 2002). In addition, the distance trees based on the RAPD and AFLP data also indicated close affinity between *T. nigrescens* and *T. occidentale* and the accessions of *T. repens*. These data do clearly support the recent report by Williams *et al*. (2008) on the introgression between *T. nigrescens* and *T. occidentale* but do not confirm that *T. occidentale* donated the second genome of the allopolyploid white clover although congruent with karyological and phylogentic affinities between the white clover and *T. occidentale* (Chen and Gibson 1970, Ellison *et al*. 2006). As reported by Badr *et al*. (2002), the origin of *T. repens* is obscured by the presence of shared alleles between *T. repens* and *T. occidentale* and *T. uniflorum* (Chen and Gibson 1972, Ansari *et al*. 2004). In the parsimony trees, *T. occidentale* showed phylogenetic affinity to *T. pallescens*. This may be congruent with the systematic and phylogenetic affinities of these two species to *T. repens* and *T. nigrescens* (Zohary and Heller 1984, Ellison *et al*. 2006) and may support the view expressed by Badr *et al.* (2002), based on isozyme data that the genome of the white clover has been introgressed by genes from closely related species in its gene pool.

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