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Ancestors of white clover (*Trifolium repens* L.), as revealed by isozyme polymorphisms

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Abstract Isozymes were used to study the putative ancestors of white clover (*Trifolium repens* L.). Ten enzymes were examined, and 18 loci were resolved via starch-gel electrophoresis for accessions representing *T. repens*, *Trifolium isthmocarpum*, *Trifolium nigrescens*, *Trifolium occidentale* and *Trifolium uniflorum*, in addition to two more distantly related species, *Trifolium alpinum* and *Trifolium purseglovei*. Nei's genetic identities indicate that *T. uniflorum* and *T. nigrescens* are the closest relatives of *T. repens*. The isozyme data thus support a hypothesis that the two genomes of the tetraploid *T. repens* could have been derived from hybridization between *T. nigrescens* and *T. uniflorum*. This conclusion is further supported by shared alleles between *T. repens*, *T. nigrescens* and *T. uniflorum*. However, 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 that introgression of genes from the latter two species into *T. repens* may also have taken place. High values of genetic identity are shared between *T. occidentale* with *T. nigrescens* and *T. uniflorum*, also indicative of introgression. Alternatively the presence of shared alleles among the five species may reflect their recent common ancestry.

Keywords White clover · Isozymes · Introgression

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Introduction

Genetic variation within crop species and between their wild relatives is of major interest to population geneticists and plant breeders. The study of genetic relationships between crop species and their wild relatives, and the determination of their wild progenitors, have been considered important to understanding crop evolution. In recent years, a number of PCR-based DNA markers have been developed to evaluate genetic variation at the infraspecific and interspecific levels (outlined by Wolfe and Liston 1998). However, isozyme polymorphisms are also used effectively to assess genetic relationships among individuals, populations and closely related species (Soltis and Soltis 1989; Murphy et al. 1996). In fact, isozymes may be more useful when infraspecific taxa are compared, since the assumption of homology can be more accurate (Klass 1998).

Within *Trifolium*, isozymes have been widely used. For example, Collins et al. (1984) determined genetic relationships for subterranean clover (*Trifolium subterraneum*) cultivars and found them to be highly polymorphic at different loci. Hickey et al. (1991) studied genetic variation in *Trifolium stoloniferum*, compared to *Trifolium reflexum, Trifolium hybridum* and *Trifolium pratense*, and found limited gene flow among *T. stoloniferum* populations compared to the other three species. Molina-Freaner and Jain (1992) used isozymes to study population variation of the colonizing species *Trifolium hirtum* (rose clover) using Californian and Turkish cultivars. Isozyme polymorphism was also used for cultivar identification of white clover (*Trifolium repens*) and red clover (*T. pratense*) by Sawada and Yamouchi (1994) and Kongkiatongam (1995), respectively. In these two species, in addition to *Trifolium riograndense*, considerable infraspecific variation was reported (Lange and Schifino-Wittmann 2000).

T. repens is one of the most important forage legumes in temperate regions, significant to sheep, beef cattle and dairy industries. Therefore, there are continuous requirements for breeding cultivars of higher quality, better

adapted to harsh environments. However, seed production is variable and often low due to a number of reproductive and climatic factors (Marshall et al. 1995). Improvement of characters influencing seed production may be achieved by interspecific hybridization with closely related species, as a means of extending the range of heritable variation useful in a breeding program in *T. repens* (Marshall et al. 1995; Hussain et al. 1997). Understanding its life history and determining its ancestry are closely related to these objectives.

The vast majority of natural and cultivated forms of *T. repens* are tetrapoid 2n = 4*x* = 32, whereas diploid cytotypes have been encountered in a few chromosome counts in this species (Federov 1969; Chen and Gibson 1971; Zohary and Heller 1984; Badr 1995). 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). A number of previous studies have addressed the ancestors of *T. repens*. Based on its ability to cross with some closely related species, Gibson and Beinhart (1969) agreed with Brewbaker and Keim (1953), that diploid *Trifolium nigrescens* (2n = 16) is one of the ancestors of polyploid *T. repens*, and that the other ancestor may be *Trifolium occidentale* (2n = 16). Chen and Gibson (1971) indicated a close karyological and phylogenetic relationship between *T. repens, T. nigrescens* and *T. occidentale*, in addition to tetraploid *Trifolium uniflorum* (2n = 32). Chen and Gibson (1972) suggested that these species might share a common genome indicated by their ability to make successful crosses and the occurrence of chromosome pairing in their hybrids. Kazimierski and Kazimierska (1973) also reported successful crosses between *Trifolium isthmocarpum* and *T. repens,* and proposed that it and *T. nigrescens* may have contributed the two genomes of *T. repens*.

Kakes and Hakvoort (1994) accepted the view that *T. repens* resulted from hybridization between *T. occidentale* and *T. nigrescens,* and utilized the pattern of cyanogenic polymorphism, in addition to morphology and meiosis, to study its origin. They 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*. This gene was shown to be linked to genes controlling vegetative and reproductive characters. More recently Kakes and Chardonnens (2000) found that the distribution of cyanotypes in *T. repens* and *T. occidentale* are dissimilar, regulated by different mechanisms. These findings lend support to the view of Kakes and Hakvoort (1994) that *T. occidentale* did not donate active Li alleles to *T. repens*. Recent studies (Ansari et al. 1999) on the molecular organization of 5S and 18S-26S rDNA loci in *T. repens* and related species support the allotetraploid origin of *T. repens*, and *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 could not pinpoint which of three subspecies of *T. nigrescens* was the direct ancestor of *T. repens* (Williams et al. 2001).

The view that *T. nigrescens* is a diploid ancestor of *T. repens* has generally been accepted (Marshall et al. 1995; Hussain et al. 1997). However, evidence for the ancestor that could have donated the other genome is still conflicting. The objective of this study is to assess the ancestry of *T. repens* by analyzing isozyme polymorphisms in representative accessions of *T. repens* and the species that have been proposed as possible donors of its genome, i.e., *T. isthmocarpum, T. nigrescens, T. occidentale* and *T. uniflorum*. To confirm the close relationship of these species, material of two more distantly related species is also included.

Materials and methods

Seed material representing 14 accessions of *T. repens*, six of *T. nigrescens*, five of *T. occidentale*, four of *T. isthmocarpum*, three of *T. uniflorum* and one each of *Trifolium alpinum* and *Trifolium purseglovei* were used. We included material of two samples of *T. nigrescens* ssp. *petrisavii* because Ansari et al (1999) concluded that it is the most probable genome donor to *T. repens*. However, refer to Williams et al. (2001) for additional information on the three *T. nigrescens* subspecies. The source and origin of the accessions are listed in Table 1. Seeds were grown under greenhouse conditions, and leaves of a total of 325 individual plants were used. Root tips were collected from 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). Plants of each accession were grown to maturity, and their identification was confirmed. Specimens were deposited at MU, vouchered by H. Sayed-Ahmed or M.A. Vincent (Miami University).

One or two leaflets of each plant were extracted in a spot plate, in 4–5 drops of cold buffer (0.029 M borax, 0.017 M sodium metabisulfite, 0.2 M sodium ascorbate, 0.016 M sodium diethyldithiocarbamate, 0.02 M Tris-HCl, pH = 7.5, with 5% PVP-40 and 0.1% 2-mercaptoethanol) and ground with a few grains of fine sterile sand (Werth 1985). The leaf extract was absorbed onto 3M Whatman 3 × 11-mm filter paper wicks and used immediately or stored in a –70 °C freezer until use. Ten enzymes [adenylate kinase (ADK; EC2.7.4.3), fructose 1,6 diphosphate dehydrogenase (FDP; EC3.1.3.11), glucose-6-phosphate isomerase (GPI; EC5.3.1.9), isocitrate dehydrogenase (IDH; EC1.1.142), leucine amino peptidase (LAP; EC3.4.11.1), malate dehydrogenase (MDH; EC1.1.1.37), 6-phosphogluconate dehydrogenase (6-PGD; EC1.1.1.44), phospho-glucomutase (PGM; EC5.4.2.2), shikimate dehydrogenase (SKDH; EC1.1.1.25) and triose-phosphate isomerase (TPI; EC5.3.1.1)] were resolved on 13% starch gels employing five buffer systems as described by Soltis et al. (1983), with some modifications in the staining procedures. Buffer system I was used to resolve GTP, IDH, 6-PGD, PGM and SKDH; Buffer II was used to resolve LAP; Buffer III was used to resolve GPI and TPI; Buffer IV was used to resolve ADK, MDH and SKDH; and Buffer V was used to resolve FDP, IDH, MDH and PGM. The number of plants loaded onto a single gel varied between 20 and 35. Representative plants were routinely included to ensure accurate interpretation of allele homology. Interpretation of banding patterns followed standard principles (Murphy et al. 1996). Loci were numbered consecutively from the anodal end, and alleles at each locus were labeled alphabetically in the same direction.

Alleles and inferred genotypes were directly scored for each isozyme locus, and allele frequencies were calculated. A few loci were unresolved in some accessions, and were treated as missing data in subsequent analyses. Based on allele frequencies, the following estimates of genetic variation were calculated for each accession and species: the proportion of polymorphic loci (*P*), the

Table 1 Source, origin, and chromosome number of *Trifolium* accessions

Species	Source[a]	Origin	2n
T. alpinum	Kew 9841	Switzerland	16
T. isthmocarpum 1	Vincent MU-116	Copenhagen 1726	16
T. isthmocarpum 2	IPK TRIF/77/91	Portugal	16[b]
T. isthmocarpum 3	USDA PI 517110	Morocco	16[b]
T. isthmocarpum 4	USDA PI 244679	India	16[b]
T. nigrescens 1	Vincent MU-133	Copenhagen 1732	16
T. nigrescens 2	Kew 32753	Greece	16
T. nigrescens 3	Kew 31561	Greece	16
T. nigrescens 4	ICARDA 1482	Syria	16
T. nigrescens 5 ssp. *petrisavii*	USDA PI 289969?	Turkey	16[b]
T. nigrescens 6 ssp. *petrisavii*	USDA PI 289969?	Cyprus	16[b]
T. occidentale 1	Kew 55332	England	16
T. occidentale 2	AZ 3297	IPK	16[b]
T. occidentale 3	Vincent MU 199	France	16[b]
T. occidentale 4	IPK TRIF 254/92	France	16[b]
T. occidentale 5	IPK TRIF 255/192	unknown	16[b]
T. purseglovei	Kew 65791	Zaire	16
T. repens 1	IPK (cult.) 141	Sweden	32
T. repens 2	IPK (cult.) 175	Italy	32
T. repens 3	IPK (cult.) 171	England	28
T. repens 4	IPK (cult.) 7332	Sweden	32
T. repens 5	IPK (cult.) 7331	Netherlands	32
T. repens 6	IPK (cult.) 7330	Italy	28
T. repens 7	IPK (cult.) 4633	France	32
T. repens 8	IPK (cult.) 17442	Germany	32
T. repens 9	IPK (cult.) 3793	Netherlands	28
T. repens 10	IPK (cult.) 18367	Germany	32
T. repens 11	Helsinki 2	Finland	32
T. repens 12	Tri-star seed co.	USA	32
T. repens 13	Hamburg 623	Germany	32
T. repens 14	Latvia (cult.)	Latvia	32
T. uniflorum 1	USDA PI 369138	Greece	32[b]
T. uniflorum 2	AZ 4382	Australia?	32[b]
T. uniflorum 3	USDA PI 369139	unknown	32[b]

[a] AZ – AgResearch, New Zealand
Hamburg – Botanischer Garten der Universitate Hamborg, Germany
Helsinki – University of Helsinki Botanical Garden
ICARDA – International Center for Agricultural Research in Dry Areas, Aleppo, Syria
IPK – Institut fuer Pflanzenbau und Pflanzenzuechtung, Gatersleben, Germany
Kew – Royal Botanic Garden Kew, England
Tri Star – Tri Star seed Co., Georgia, USA
Vincent MU – Dr. Michael Vincent, Miami University, Oxford, Ohio, USA
USDA – United States Department of Agriculture
[b] Chromosome counts are cited from the literature. All others are first reports from this study

mean number of alleles per locus (K), the mean number of alleles per polymorphic locus (K_p), the mean observed and expected heterozygous loci (H_0, H_e) and the number of unique alleles. Nei's genetic identities and distances (Nei 1972, 1973) were calculated using the software program GENESTAT (Whitkus 2000) and the following four genetic diversity measures were calculated: total genetic diversity of polymorphic loci (H_t), mean genetic diversity within populations (H_s), genetic diversity among populations (D_{st}), and differentiation among populations (G_{st}). A phenogram based on Nei's genetic distance, using the unweighted pair group method with arithmetic average (UPGMA) was generated (Sokal and Michener 1958) using the sequential, agglomerative, hierarchical and nested clustering method (SAHN) as defined by Sneath and Sokal (1973), using the NTSYS-pc software program (Rohlf 1993).

Results

Eighteen loci were resolved for the ten enzymes in the 34 accessions examined. Two of the examined enzymes (IDH and SKDH) have one locus each, while the remaining eight enzymes have two loci. Three of the 18 loci are monomorphic for all samples (Fdp-1, Lap-2 and Tpi-1), while the remaining 15 loci are polymorphic. Four loci are polymorphic in *T. alpinum*, eight in *T. isthmocarpum*, ten in *T. occidentale*, 13 in *T. nigrescens*, 11 in *T. repens*, six in *T. uniflorum* and two in *T. purseglovei*. However, Mdh-2 was resolved only in *T. isthmocarpum* and *T. occidentale*; 6Pgd-2 was not resolved for *T. repens*, Adk-2 was not resolved for *T. nigrescens*, Gpi-2 was not resolved for *T. isthmocarpum*, and Lap-1 and Tpi-1 were not resolved for *T. uniflorum*. Some alleles are only shared by a few species: Pgm-2c is shared by *T. repens* and *T. nigrescens*, Gpi-1e is shared by *T. nigrescens* and *T. occidentale*, and Gpi-1c is shared by all three species. In addition, Gpi-2c is shared by *T. repens*, *T. nigrescens* and *T. uniflorum*. *T. repens* has four unique alleles (Gpi-2d, Gpi-2e, Idh-c and Lap-1c), *T. nigrescens* has two (Mdh-1d and Fdp-2b) and *T. occidentale* has two (Gpi-1d and Mdh-2c). However, *T. uniflorum* has no unique alleles (Table 2).

Estimates of allelic diversity are also presented in Table 2. The proportion of polymorphic loci (P) is most variable among accessions of *T. nigrescens* and *T. occidentale*, and least variable for *T. isthmocarpum*. The mean number of alleles per locus (K) is highest for *T. repens*, and slightly lower in *T. nigrescens* and *T. occidentale*. The mean number of alleles per polymorphic locus (K_p) is most variable among accessions of *T. repens* and *T. nigrescens*. The mean proportion of observed heterozygous loci (H_0) is highest in *T. nigrescens*, followed by *T. occidentale*. This value is lower for *T. alpinum* and *T. purseglovei*, which were each represented by single accessions. Genetic diversity statistics

Table 2 Number of individuals (N) examined, proportion of polymorphic loci (*P*), mean number of alleles per locus (*K*), mean number of alleles per polymorphic locus (K_p), mean observed heterozygous loci (H_0), mean expected heterozygous loci (H_e), and number of unique alleles

Accessions	N	*P*	*K*	K_p	H_0	H_e	Unique alleles
T. alpinum	7	0.24	1.24	2.00	0.09	0.10	0
T. isthmocarpum 1	6	0.24	1.24	2.00	0.09	0.10	0
T. isthmocarpum 2	12	0.25	1.25	2.00	0.12	0.12	0
T. isthmocarpum 3	3	0.25	1.25	2.00	0.10	0.11	0
T. isthmocarpum 4	5	0.25	1.25	2.00	0.10	0.11	0
T. nigrescens 1	6	0.35	1.35	2.00	0.16	0.17	1
T. nigrescens 2	8	0.53	1.67	2.25	0.25	0.27	0
T. nigrescens 3	8	0.69	1.88	2.27	0.30	0.32	0
T. nigrescens 4	6	0.50	1.75	2.50	0.24	0.26	0
T. nigrescens 5 ssp. *petrisavii*	3	0.64	1.79	2.22	0.13	0.37	0
T. nigrescens 6 ssp. *petrisavii*	7	0.47	1.80	2.71	0.25	0.27	0
T. occidentale 1	8	0.56	1.69	2.22	0.25	0.26	0
T. occidentale 2	9	0.25	1.33	2.33	0.13	0.14	1
T. occidentale 3	7	0.31	1.21	2.00	0.10	0.10	0
T. occidentale 4	8	0.31	1.31	2.00	0.14	0.14	0
T. occidentale 5	7	0.21	1.21	2.00	0.10	0.11	0
T. purseglovei	7	0.07	1.07	2.00	0.03	0.04	0
T. repens 1	4	0.38	1.50	2.33	0.18	0.20	1
T. repens 2	8	0.36	1.36	2.00	0.13	0.14	0
T. repens 3	10	0.44	1.44	2.00	0.17	0.17	0
T. repens 4	12	0.36	1.36	2.00	0.15	0.16	0
T. repens 5	8	0.38	1.44	2.17	0.17	0.18	0
T. repens 6	28	0.57	1.86	2.50	0.25	0.26	0
T. repens 7	15	0.50	1.60	2.20	0.24	0.25	0
T. repens 8	20	0.44	1.63	2.43	0.18	0.19	0
T. repens 9	7	0.38	1.44	2.17	0.14	0.15	0
T. repens 10	8	0.31	1.44	2.40	0.16	0.17	0
T. repens 11	15	0.50	1.75	2.50	0.19	0.20	0
T. repens 12	26	0.50	1.56	2.13	0.19	0.20	0
T. repens 13	9	0.50	1.67	2.33	0.22	0.24	0
T. repens 14	20	0.40	1.50	2.75	0.37	0.38	0
T. uniflorum 1	6	0.20	1.20	2.00	0.07	0.07	0
T. uniflorum 2	7	0.27	1.27	2.00	0.14	0.15	0
T. uniflorum 3	4	0.33	1.33	2.00	0.14	0.16	0
Species							
T. alpinum	7	0.24	1.24	2.00	0.10	0.10	0
T. isthmocarpum	26	0.53	1.59	2.11	0.23	0.23	0
T. nigrescens	38	0.82	2.24	2.50	0.38	0.39	2
T. occidentale	39	0.64	2.00	2.54	0.31	0.31	2
T. purseglovei	7	0.07	1.07	2.00	0.04	0.04	0
T. repens	190	0.69	2.31	2.91	0.25	0.26	4
T. uniflorum	17	0.47	1.60	2.29	0.22	0.23	0

are presented in Table 3, and include total gene diversity (H_t), within accession diversity (H_s), among accession diversity (D_{st}) and differentiation among accessions (G_{st}). *T. nigrescens* and *T. occidentale* have the highest total diversity (H_t). *T. nigrescens* exhibits the highest within accession diversity (H_s), while *T. uniflorum* has the highest among accession diversity (D_{st}) and differentiation among accessions (G_{st}). The resulting diversity statistics may be biased by the sampling strategy of a greater number of accessions examined for *T. repens*.

Nei's genetic identity (I) values (Table 4) indicate that pairwise comparisons are highest for *T. repens* and *T. uniflorum*, and *T. nigrescens* and *T. uniflorum*. *T. repens* is genetically closest to *T. uniflorum* (I = 0.957) and *T. nigrescens* (I = 0.934), followed by *T. occidentale* (I = 0.843) and *T. isthmocarpum* (I = 0.851). *T. alpinum* and *T. purseglovei* are clearly distant from the other five species. Nei's genetic pairwise distances between accessions are reflected in the UPGMA tree (Fig. 1). Most of the *T. repens* accessions cluster together, closest to *T. nigrescens*. A couple of *T. repens* accessions fall outside this major cluster, and are closer to *T. uniflorum* and *T. nigrescens*.

Table 3 Total gene diversity (H_t), within accession diversity (H_s), among accession diversity (D_{st}) and differentiation among accessions (G_{st})

Loci	H_t	H_s	D_{st}	G_{st}
T. alpinum	0.10 ± 0.05	0.10 ± 0.05	0.00	0.00
T. isthmocarpoum	0.27 ± 0.04	0.13 ± 0.04	0.11	0.44
T. nigrescens	0.41 ± 0.06	0.28 ± 0.05	0.12	0.31
T. occidentale	0.35 ± 0.05	0.17 ± 0.05	0.14	0.44
T. purseglovei	0.07 ± 0.04	0.04 ± 0.04	0.00	0.00
T. repens	0.26 ± 0.05	0.20 ± 0.05	0.06	0.24
T. uniflorum	0.27 ± 0.05	0.13 ± 0.05	0.14	0.54

Table 4 Nei's genetic identities (above) and distances (below)

Species	1	2	3	4	5	6	7
1. *T. alpinum*		0.6470	0.713	0.709	0.752	0.616	0.617
2. *T. isthmocarpum*	0.435		0.888	0.787	0.686	0.851	0.816
3. *T. nigrescens*	0.339	0.119		0.933	0.748	0.934	0.907
4. *T. occidentale*	0.344	0.239	0.069		0.683	0.843	0.897
5. *T. purseglovei*	0.260	0.377	0.291	0.381		0.698	0.687
6. *T. repens*	0.485	0.162	0.067	0.170	0.359		0.957
7. *T. uniflorum*	0.483	0.203	0.097	0.109	0.375	0.044	

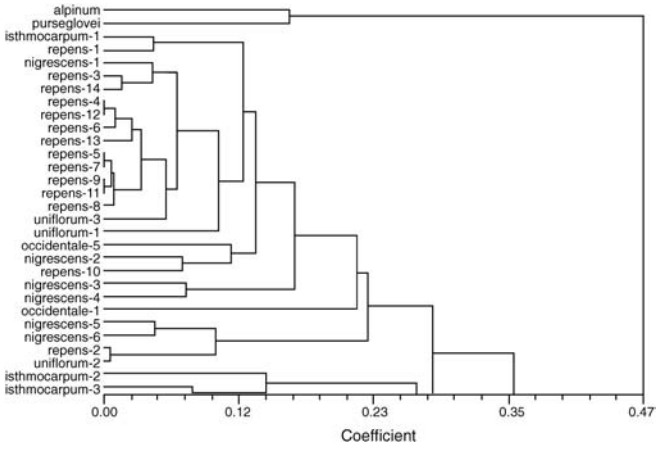

Fig. 1 Phenogram (UPGMA) of accessions of *T. repens* and allied species, based on Nei's genetic distance for isozyme loci

Discussion

Most evidence supports the hypothesis that *T. repens* is as an allotetraploid that resulted from hybridization between unreduced gametes of two different species (Gibson and Beinhart 1969; Chen and Gibson 1972), although it has been suggested (Cleveland 1985) that *T. repens* originated as an autotetraploid, perhaps of *T. occidentale*. However, this hypothesis is contrary to the results reported by Kakes and Hakvoort (1994) and Kakes and Chardonnens (2000) that *T. occidentale* did not donate the Li allele to *T. repens*. Furthermore, the work of Williams (1987) showed that chromosomes in the pollen mother cells of *T. repens* pair in bivalents at prophase-I of meiosis, which is indicative of an allopolyploid origin. Additionally the work of Ansari et al. (1999) confirmed this conclusion by demonstrating the presence of two genomes in the karyotype of *T. repens*, with one genome having a pair of NOR chromosomes while the other does not.

A number of authors agree that *T. nigrescens* donated one of the two genomes of *T. repens* (Michaelson-Yeates 1986; Kakes and Hakvoort 1994; Marshall et al. 1995; Hussain et al. 1997). The high level of genetic identity and the small distance between these two species as revealed by the isozyme data, also support this view and confirm that *T. nigrescens* is likely to be one ancestor of *T. repens.* Judged by the affinity between *T. uniflorum* and *T. repens*, it may also be concluded that *T. uniflorum* may have donated the other genome to *T. repens*. *T. uniflorum* is regarded as an autotetraploid with 2n = 32, and with autoploid-like chromosome behavior at meiosis (Cleveland 1985). The autoploid nature of this species is also confirmed by the organization of rDNA fragments that revealed two pairs of NOR chromosomes in its karyotype (Ansari et al. 1999). The postulated genome donated by *T. uniflorum* to *T. repens* was most likely donated by a diploid ancestral form of *T. uniflorum* or through the hybridization of unreduced gametes of *T. nigrescens* with diploid gametes of tetraploid *T. uniflorum*. In addition, the isozyme data, which supports *T. nigrescens* and *T. uniflorum* as the progenitors to *T. repens*, are not consistent with the clonal perennial nature of *T. repens*. However, these traits in *T. repens* could have been acquired subsequent to speciation to increase survival and colonization ability.

In addition to the close relationship of *T. repens* to both *T. nigrescens* and *T. uniflorum*, the isozyme data show close affinity with *T. occidentale* and *T. isthmocarpum*. These four species are considered close relatives of *T. repens* (Cleveland 1985), and may hybridize with each other. Studies on chromosome pairing in their hybrids showed that *T. repens*, *T. nigrescens* and *T. occidentale* have chromosomes with high pairing affinities and might share one basic genome (Chen and Gibson 1970a, b, 1971). The findings by Kakes and Hakvoort (1994) and Kakes and Chardonnens (2000) that *T. occidentale* did not donate the Li allele to *T. repens* lend support to our hypothesis that *T. nigrescens* and an ancestral form of *T. uniflorum* are the immediate progenitors of *T. repens*. In addition, the affinity between *T. nigrescens* and *T. occidentale*, as expressed by the isozyme data, is congruent with their ability to form stable hybrids with each other. These data confirm the results of earlier cytogenetic studies by Gibson and Beinhart (1969), Chen and Gibson (1970a, b, 1971, 1972) and more recent molecular cytogenetic studies by Ansari et al. (1999) in indicating that all these species are close relatives. These species are to be considered the primary gene pool of *T. repens*. The affinities among one accession of *T. isthmocarpum* and two accessions of *T. occidentale*, and the major group comprised of the accessions of *T. repens*, *T. uniflorum* and *T. nigrescens*, that includes the core group of 11 accessions of *T. repens*, may be indicative of possible introgressions of some genes as a result of occassional natural hybrids between these species.

In summary, genetic identity values support our conclusion that an ancestral form of *T. uniflorum* and *T. nigrescens* are the likely donors of the two genomes of *T. repens*. This conclusion is further supported by shared alleles between *T. repens*, *T. nigrescens* and *T. uniflorum*. However, it is not unlikely that the genomes of these species could have been introgressed by genes from *T. occidentale* and to a lesser extent by *T. isthmocarpum*. The high values of genetic identity and the low values of genetic distance between *T. occidentale* and each of *T. nigrescens* and *T. uniflorum* may be indicative of introgression of genes from the former species into the genomes of the latter two species. Some of these genes may have been subsequently introduced into the genome of *T. repens*.

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References

Ansari HA, Ellison NW, Reader SM, Badeva ED, Friebe B, Miller TE, Williams WM (1999) Molecular cytogenetic organization of 5S and 18S-26S rDNA loci in white clover (*Trifolium repens* L) and related species. Ann Bot 83:199–206

Badr A (1995) Electrophoretic studies of seed proteins in relation to chromosomal criteria and species relationships of some taxa of *Trifolium*. Taxon 44:183–191

Brewbaker JL, Keim WF (1953) A fertile interspecific hybrid in *Trifolium*. Am Nat 87:323–326

Chen CC, Gibson PB (1970a) Meiosis in two species of *Trifolium* and their hybrids. Crop Sci 10:188–189

Chen CC, Gibson PB (1970b) Chromosome pairing in two interspecific hybrids of *Trifolium*. Can J Genet Cytol 12:190–794

Chen CC, Gibson PB (1971) Karyotype of fifteen *Trifolium* species in section *Amoria*. Crop Sci 11:441–445

Chen CC, Gibson PB (1972) Barriers to hybridization of *Trifolium repens* with related species. Canad J Genet Cytol 14:591–595

Cleveland RW (1985) Reproduction cycle and cytogenetics. In: Taylor NL (ed) Clover science and technology. Soil Science Society of America, Madison, Wisconsin, USA, pp 71–104

Collins WJ, Rossiter RC, Haynes Y, Brown ADH (1984) Identification of subterranean clover cultivars and their genetic relationships by isozyme analysis. Aust J Agric Res 35:399–411

Darlington CD, La Cour F (1976) Handling of Chromosomes, 6th edn. George Allen and Unwin Ltd, London

Federov A (1969) Chromosome numbers of flowering plants. Akademija Nauk SSSR, Leningrad

Gibson PG, Beinhart G (1969) Hybridization of *Trifolium occidentale* with two other species of clover. J Hered 60:93–96

Hickey RJ, Vincent MA, Guttman SI (1991) Genetic variation in running buffalo clover (*Trifolium stoloniferum*, Fabaceae). Conserv Biol 5:309–316

Hossain M (1961) A revision of *Trifolium* in the Nearer East. Notes Roy Bot Gard Edinburgh 23:387–481

Hussain SW, Williams WM, Woodfield DR, Hampton JG (1997) Development of a ploidy series from a single interspecific *Trifolium repens* L × *T. nigrescens* Viv. F1 hybrid. Theor Appl Genet 94:821–831

Kakes P, Hakvoort HWJ (1994) On the origin of the cyanogenic polymorphism in *Trifolium repens* L. J Evol Biol 7:201-215

Kakes P, Chardonnens AN (2000) Cyanotypic frequencies in adjacent and mixed populations of *Trifolium occidentale* Coombe and *Trifolium repens* L are regulated by different mechanisms. Biochem Syst Ecol 28:633–649

Kazimierski T, Kazimierska EM (1973) Investigation of hybrids in the genus *Trifolium* L. V. Fertility and cytogenetics of the hybrid *Trifolium nigrescens* and *T. isthmocarpum* Brot. Acta Soc Bot Pol 42:567–589

Klass M (1998) Application and impact of molecular markers on evolutionary and diversity studies in *Allium*. Plant Breed 117:297–308

Kongkiatongam P, Waterway MJ, Fortin M, Coulman BE (1995) Genetic variation within and between two cultivars of red clover (*Trifolium pratense* L): comparison of morphological, isozyme and RAPD markers. Euphytica 84:237–246

Lange O, Schifino-Wittmann MT (2000) Isozyme variation in wild and cultivated species of the genus *Trifolium* L (Leguminosae). Ann Bot 86:339–345

Marshall AH, Michaelson-Yeates TPT, Aluka P, Meredith M (1995) Reproductive characters of interspecific hybrids between *Trifolium repens* L and *T. nigrescens* Viv. Heredity 74: 136–145

Michaelson-Yates TPT (1986) Phosphoglucoisomerase variation in *Trifolium repens*. Genetica 70:53–58

Molina-Freaner F, Jain SK (1992) Isozyme variation in Californian and Turkish populations of the colonizing species *Trifolium hirtum*. J Hered 83:423–430

Murphy RW, Sites JWJ, Buth DJ, Haufler CH (1996) Proteins, isozyme electrophoresis. In: Hillis DM, Moritz C, Mable BK (eds) Molecular systematics. Sinauer Associates, Sunderland, Massachusetts

Nei M (1972) Genetic distance between populations. Am Nat 106:283–292

Nei M (1973) Analysis of gene diversity in subdivided populations. Proc Natl Acad Sci USA 70:3321–3323

Rohlf FJ (1993) NTSYS-pc. Numerical taxonomy and multivariate analysis system. Applied Biostatistics Inc, New York

Sawada H, Yamouchi K (1994) Identification of white clover (*Trifolium repens* L) clones using isozymes. Japan J Grass Sci 39:488–496

Sneath PHA, Sokal RR (1973) Numerical taxonomy. Freeman, San Francisco

Sokal RR, Michener CD (1958) A statistical method for evaluating systematic relationships. Univ Kansas Sci Bull 28:1409–1438

Soltis DE, Haufler CH, Darrow DC, Gastony GJ (1983) Starch-gel electrophoresis: a compilation of grinding buffers, gel and electrode buffers and staining schedules. Am Fern Jour 73: 9–27

Soltis DE, Soltis PE (eds) (1989) Isozymes in plant biology. Advances in Plant Science Vol 4. Dioscorides Press, Portland

Werth C (1985) Implementing an izozyme laboratory at a field station. Virginia J Sci 36:53–76

Whitkus R (2000) GENESTAT, a pc program for computing genetic statistics from allele frequency. Distributed by the author (Sonoma State University)

Williams WM (1987) White clover taxonomy and biosystematics. In: Baker MJ, Williams WM (eds) White clover. CAB International, Wallingford, pp 323–342

Williams WM, Ansari HA, Ellison NW, Hussain SW (2001) Evidence of three subspecies in *Trifolium nigrescens* Viv. Ann Bot 87:683–691

Wolfe A, Liston A (1998) Contribution of PCR-based methods to plant systematics and evolutionary biology. In: Soltis DE, Soltis PS, Doyle JJ (eds) Molecular Biology of Plants II DNA Sequencing. Kluwer Academic Publishers, Boston Dordrecht London, pp 43–86

Zohary M, Heller D (1984) The genus *Trifolium*. The Israel Academy of Science, Jerusalem