

Genetic resources in *Trifolium* and their utilization in plant breeding

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Abstract

Clovers (*Trifolium* species) are a large and widespread genus of legumes. Recent work supports the Mediterranean origin of the genus in the Early Miocene period, and centres of diversity for clovers occur in the Eastern Mediterranean, East Africa and South America. A number of clovers are of agricultural importance as forage species in grassland agriculture around the world, particularly in temperate areas. White clover (*Trifolium repens* L.) is the most important legume of grazed pastures, and red clover (*T. pratense*) is widely cut and conserved as a winter feed. We consider the current state of collected resources in clovers, which have been collated in the most important databases. These are European Internet Search Catalogue (EURISCO), germplasm resources information network and system-wide information network for genetic resources together with the collection held at AgResearch, New Zealand. In total, collections have been made of 204 species with 48 species having more than 100 accessions in these databases. As expected, the majority of accessions are of the agriculturally important species. The geographical origin of collected material is outlined and, for the major species, accessions are broken down according to their status, e.g. wild population, breeders' lines. We then describe some of the ways in which genetic resources of white and red clovers and their relatives have been used in the breeding of these species. These include introgression of stress tolerance traits, targetting improvements in resource use efficiency and increasing seed yield.

Keywords: clovers; germplasm collections; improvement

Introduction

The genus *Trifolium* includes more than 250 species of which ten are of agricultural significance with the most important being white clover (*Trifolium repens* L.) and red clover (*T. pratense* L.) (Zohary and Heller, 1984). Taylor (2003) summarized information on the distribution of perennial *Trifolium* of which there are 90 including white and red clovers. Clovers are of global agricultural significance as forage species, i.e. they are either grazed or fed to livestock. They are particularly important in temperate areas. White clover is the major legume of grazed pastures in most parts of the world.

Estimates of global white clover sowings totalling 3–4 Mha annually have been made (Mather *et al.*, 1996). In Europe, the greatest use of this species is in the more northern and western parts of the continent, but it is difficult to obtain reliable estimates of clover content on a wide scale of either recently reseeded or more established pastures. This review aims to describe some features of the state of collected and curated germplasm in *Trifolium* and to give examples of how genetic resources have been characterized and used in the breeding of white and red clovers.

Clovers in genebanks

Genetic resources of clovers have been assembled in a number of important collections around the world.

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Among these is the EURISCO database (<http://eurisco.ecpgr.org>), which brings together data from collections held by 51 organizations across Europe. EURISCO is based on a European network of *ex situ* National Inventories and makes these data accessible around the world. For clovers, the major contributors are collections held in the UK, Spain, Russia and Italy. The clover collection in the germplasm resources information network (GRIN) database of the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS) (<http://www.ars-grin.gov/npgs>) is derived from germplasm from 74 countries around the world. GRIN is a web server providing information on plants, animals and microbes maintained by the USDA–ARS. The great majority of the accessions are held by the USDA Plant Introduction Stations at the University of Georgia and Washington State University. The system-wide information network for genetic resources (SINGER) database is the germplasm information exchange network of the Consultative Group on International Agricultural Research (CGIAR) coordinated by International Centre for Research in the Semi-Arid Tropics (ICRISAT) (<http://www.singer.cgiar.org>) and consists of the collections of the CGIAR institutes which, for clovers, is predominantly held at ICARDA with a smaller collection at International Livestock Research Institute (ILRI). All three databases use the Food and agriculture organization/International Plant Genetic Resources Institute multi-crop passport descriptor list, an international standard to facilitate germplasm passport information exchange (<http://eurisco.ecpgr.org/documents>) and the GRIN taxonomic nomenclature checker to standardize the spelling and use of synonyms. A significant clover genebank is held by AgResearch, New Zealand (Margot Forde Forage Germplasm Centre). Clearly, the agriculturally important clovers are heavily represented in these genebanks, although lower numbers of accessions are present for a large number of species (Table 1).

Only those species with total accessions of more than 100 in the four databases are shown. There are a further 156 species with fewer than this number of accessions. It should be noted that a number of these species may also be important with respect to future breeding efforts. Among these are the species most closely related to the putative diploid ancestors of white clover, namely *T. pallescens* and *T. occidentale*. Other species such as *T. elegans* may be locally of agricultural importance, and consideration with respect to conservation and use needs to be given to all of them. *T. diffusum* has been used in programmes of interspecific hybridization with red clover (Strzyewska, 1995).

Important questions are the extent to which current collections represent an appropriate level of geographical coverage around the globe and to what

degree the major collections are sampling independent geographical regions.

Collected populations in the EURISCO database are predominantly European (Fig. 1). The GRIN database shows a large number of accessions from southern Europe and parts of Asia as well as the Pacific coast of North America (Fig. 2). Parts of Africa, particularly Northern Africa and Ethiopia, are well represented in the SINGER collections (Fig. 3).

The majority of the accessions in all the major *Trifolium* collections are from wild Populations, but significant numbers are landraces, traditional cultivars or improved material (Table 2).

Use of genetic resources in breeding new varieties of white clover and red clover

Breeding of perennial legumes has been reviewed by Abberton and Marshall (2005).

White clover (*T. repens* L.) is a perennial legume, which can survive for many years and which is typically utilized by livestock under grazing, or less commonly, cutting for silage. It is utilized in mixed swards with a grass; in the UK (and many other countries), this is generally perennial ryegrass (*Lolium perenne* L.). Clearly, an important attribute is nitrogen fixation, and a white clover contribution to a mixed sward of around 30% (dry matter averaged across a season) might be expected to fix the equivalent of approximately 250 kg N/ha per year. An important trait is the possession of creeping stems or stolons, and a well-developed network of stolons is important for grazing tolerance, winter hardiness and persistence. This is, therefore, an important breeding criterion for white clover. Other species with stolons such as *T. stoloniferum* may also have potential under grazing systems. Pest and disease resistance, resource use efficiency and compatibility with the grass companion are also important targets of white clover breeding. In recent years, greater focus has been given to increasing resource use efficiency, particularly water (including enhancing drought tolerance) and also phosphorus.

A number of significant white clover breeding programmes are being carried out around the world including those in New Zealand (Williams *et al.*, 2007), Australia (Lane *et al.*, 1997; Jahufer *et al.*, 2002), the USA (Taylor, 2008) and UK (Abberton and Marshall, 2010). These are largely focused on particular environments and management systems but share the objective of more fully realizing the potential of white clover to contribute to livestock nutrition and soil fertility. This is not only based on the value of nitrogen fixation but also the high protein, high quality feed supplied by

Table 1. Numbers of accessions of *Trifolium* species in the EURISCO, GRIN and SINGER databases and the Margot Forde Forage Germplasm Centre, AgResearch, New Zealand. Only those species with greater than 100 total accessions are shown. Species names have been standardized for spelling and synonyms using the GRIN Taxonomic Nomenclature Checker^a

Species	Accessions	AgResearch	EURISCO	GRIN	SINGER
<i>pratense</i>	10,113	1569	7114	1354	76
<i>repens</i>	8488	4040	3546	804	98
<i>subterraneum</i>	6623	639	5387	323	274
<i>fragiferum</i>	1101	567	211	250	73
<i>glomeratum</i>	1061	67	854	28	112
Unidentified <i>Trifolium</i> sp.	1053	53	650	50	300
<i>hybridum</i>	1016	147	617	233	19
<i>resupinatum</i>	851	45	163	217	426
<i>campestre</i>	794	63	184	113	434
<i>cherleri</i>	588	18	289	58	223
<i>alexandrinum</i>	504	28	212	127	137
<i>arvense</i>	391	77	176	54	84
<i>tomentosum</i>	372	20	44	18	290
<i>tembense</i>	371	12	5	135	219
<i>stellatum</i>	360	7	108	18	227
<i>scabrum</i>	355	12	54	33	256
<i>semipilosum</i>	353	43	7	82	221
<i>ambiguum</i>	348	115	65	155	13
<i>angustifolium</i>	329	18	122	44	145
<i>striatum</i>	304	30	231	21	22
<i>medium</i>	303	68	131	103	1
<i>hirtum</i>	298	18	86	77	117
<i>purpureum</i>	296	4	49	21	222
<i>rueppellianum</i>	270	33	1	67	169
<i>incarnatum</i>	268	64	132	54	18
<i>montanum</i>	216	41	114	60	1
<i>lappaceum</i>	216	16	64	38	98
<i>spumosum</i>	215	8	27	19	161
<i>pilulare</i>	185	1	9	8	167
<i>argutum</i>	180	37	11	8	124
<i>burchellianum</i>	176	24	5	45	102
<i>alpestre</i>	176	34	82	60	
<i>nigrescens</i>	172	31	37	45	59
<i>steudneri</i>	165	14		64	87
<i>physodes</i>	138	68	19	22	29
<i>echinatum</i>	128	19	21	23	65
<i>cryptopodium</i>	128	1		30	97
<i>aureum</i>	127	26	50	37	14
<i>dubium</i>	126	48	53	17	8
<i>isthmocarpum</i>	125	12	12	21	80
<i>clusii</i>	121	5	3	6	107
<i>decorum</i>	119	15		30	74
<i>simense</i>	113	2	1	25	85
<i>baccarinii</i>	111	8		33	70
<i>pauciflorum</i>	107	10	7	22	68
<i>pannonicum</i>	106	21	52	33	
<i>scutatum</i>	105		5	4	96
<i>rubens</i>	104	18	58	28	
<i>bullatum</i>	103			3	100

^a EURISCO Catalogue (<http://eurisco.ecpgr.org>); date of data consultation (05 July 2010); USDA ARS Catalogue (<http://www.ars-grin.gov/npgs>); date of consultation (15 July 2010); CGIAR SINGER Catalogue (<http://www.singer.cgiar.org>); date of data consultation (15 July 2010); AgResearch Catalogue (<http://www.agresearch.co.nz/seeds/>) date of data consultation (15 July 2010).

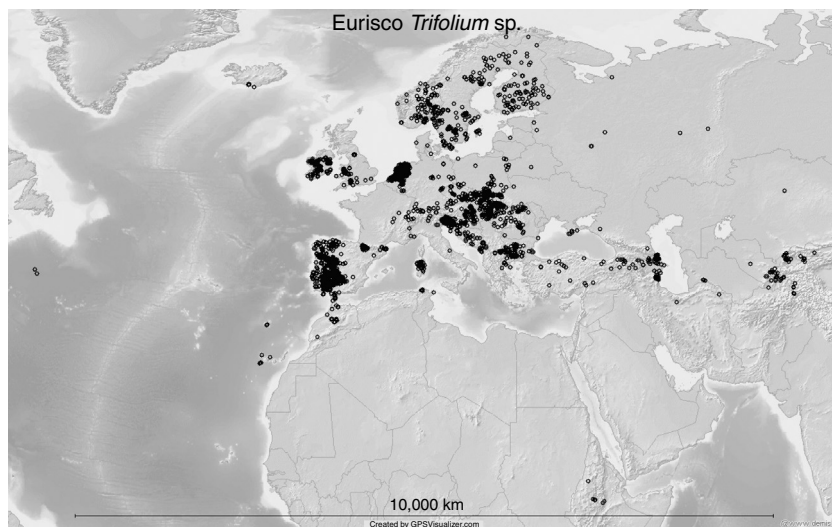


Fig. 1. Distribution of *Trifolium* collection sites for accessions in the EURISCO database.

white clover. There is also some evidence that white clover can have a beneficial effect on aspects of soil structure (Mytton *et al.*, 1993).

White clover is an outbreeding allopolyploid species ($2n = 4x = 32$) with considerable levels of heterozygosity found within individuals and populations. It also shows considerable genotype \times environment interactions and phenotypic plasticity. Lack of genetic variation *per se* is therefore not a major issue in the breeding of white clover. However, in many cases, the extent of variation within a particular breeding pool is not great enough to allow rapid gains under selection for desired improvements, and the use of *ex situ* genetic resources has been critical in the development of new varieties. For a small number of important traits, significant programmes of interspecific hybridization have been undertaken (see below).

White clover is generally classified for agricultural use according to leaf size. Small leaf size varieties are suitable for continuous or intensive grazing by sheep, medium leaf size types for rotational grazing, large leaf types and very large leaf size varieties are used for cattle grazing or cutting for silage. Ladino white clover is a distinctive form of germplasm with very large leaves, lacking cyanogenic glucosides. It is typically used for hay and occurs naturally in the permanent meadows of southern Lombardy with landraces commercialized under the name of 'Gigante lodigiano'. Annichiarico (1993) reported on a study of variation in Ladino landraces and populations as a step towards the germplasm conservation and use in breeding.

In some cases, collection of genetic resources has been targeted at particular traits based on the environmental conditions at the collection sites. Thus, in the

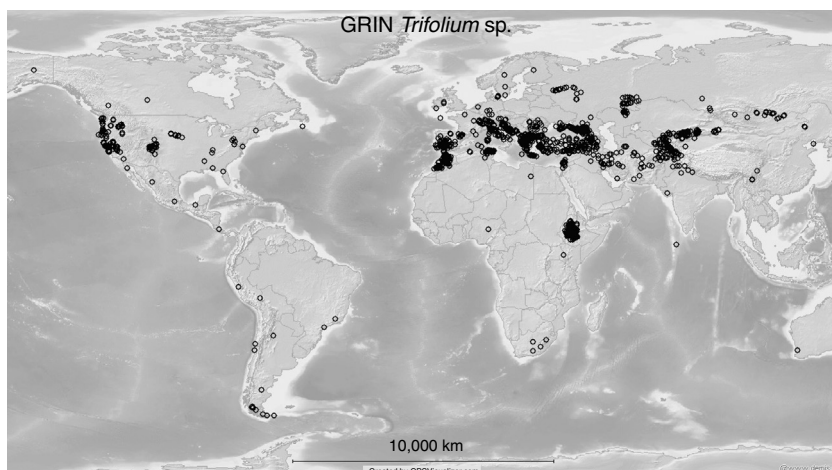


Fig. 2. Distribution of *Trifolium* collection sites for accessions in the GRIN database.

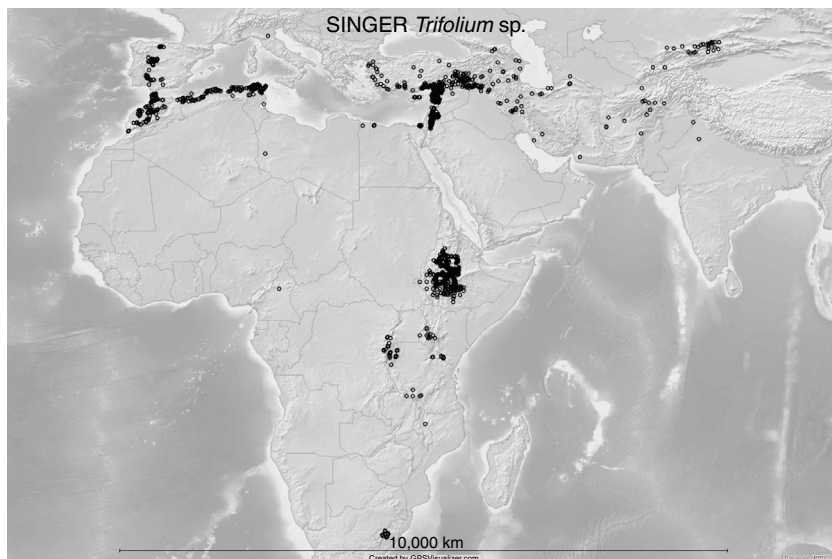


Fig. 3. Distribution of *Trifolium* collection sites for accessions in the SINGER database.

UK, cold tolerance and winter hardiness are important attributes with respect to the persistence of white clover. Selection in more extreme environments (Switzerland) successfully identified novel germplasm that was introduced into the breeding programme at Aberystwyth (Rhodes and Ortega, 1996). This germplasm was analyzed with respect to its cold tolerance under controlled conditions in freezing chambers and in parallel assessed for its agronomic performance. The two varieties AberHerald and AberCrest in the medium and small leaf size categories developed in this way showed both improved winter hardiness and early spring growth leading to higher spring and total yields (Rhodes *et al.*, 1994).

Clearly, collection strategies need, among other criteria, to consider the current and likely future needs of breeding programmes. This includes resource use efficiency, e.g. effective use of slurry and manure with grass/clover swards and reduced requirement for phosphorus. Abberton and Warren (2006) described a

case study of collecting white clover germplasm from sites subject to current and potential change in land use practices. They describe how material collected from fields under traditional agricultural management in Poland was used in the development of novel white clover pre-varietal lines with enhanced phosphorus use efficiency.

Crop wild relatives, their distribution and potential use are an important aspect to consider with respect to the conservation and improvement of any crop. Ellison *et al.*, 2006 provided evidence for *T. pallescens* and *T. occidentale* as the likely diploid progenitors of the allotetraploid white clover. Genetic resources of these species are likely to be important in the future improvement of white clover.

The role of interspecific hybridization in the improvement of clovers was reviewed by Abberton (2007). With respect to white clover, major programmes have been undertaken in the UK and in New Zealand. In the UK, crosses have been made between white clover and

Table 2. Biological status of *Trifolium* accessions in the EURISCO, GRIN and SINGER databases

Biological status	Accessions	EURISCO	GRIN	SINGER
Advanced/improved cultivar	2970	2270	700	
Breeding/research material	3133	2854	205	74
Not recorded	5505	5505		
Others	2023	365	1658	
Traditional cultivar/landrace	2446	1655	644	147
Unknown sample status	830			830
Weedy	60	57		3
Wild	17,845	9190	2917	5738

T. nigrescens (ball clover). The latter is a diploid annual species, which is profusely flowering and has high seed yield relative to white clover. A backcrossing programme has produced lines with the agronomic characteristics of white clover but increased seed yield. A second crossing programme introgressed the rhizomatous trait from *T. ambiguum* M. Bieb (Kura clover or Caucasian clover) and has produced lines with greatly enhanced drought tolerance in comparison with its white clover parent and control varieties.

The *in situ* conservation of *T. occidentale*, *T. pallescens*, *T. ambiguum* and *T. nigrescens* are priorities. Within Europe, Lamont *et al.* (2001) also highlighted a number of other clovers as important for *in situ* conservation: *T. fragiferum* (strawberry clover), *T. cberleri*, *T. hirtum* and *T. subterraneum*.

A unique conservation resource in white clover has been identified recently. Hargreaves *et al.* (2010) show that populations on the island of Hirta in the St Kildan archipelago (the remotest part of the British Isles, in the Outer Hebrides) are highly differentiated from UK mainland populations and are genetically distinct from cultivated varieties.

Use of genetic resources is not confined to the introgression of genes from germplasm adapted to widely different climates. In some cases, the origin may be in close proximity to regions where the variety developed will be commercially successful. An example is the breeding of the small leaf variety of white clover, AberAce. The original gene pool from which this variety was developed drew heavily on populations collected in the Welsh uplands that had been subjected to decades of grazing by sheep. This variety has an exceptionally dense network of stolons and very good ground cover and persistence under such grazing regimes around the UK.

Red clover is an important perennial legume in many parts of the world, e.g. Western and Northern Europe, parts of Russia, Japan and the USA (Taylor and Smith, 1995). It often fulfils a similar function as alfalfa or lucerne where environmental conditions or soil type are not suitable for the latter. It has a very different growth habit than white clover with upright stems emerging from a meristematic 'crown'. Red clover is high yielding but with much less tolerance of grazing than white clover and is typically cut (two, three or more times a year) for silage.

Red clover varieties are classified by ploidy level and by flowering date or maturity. Tetraploid varieties are artificially produced by chromosome doubling of diploid lines. Early flowering or 'double cut' varieties are widely grown and give two more or less equal conservation cuts and subsequent lower yielding cut(s). Late flowering or 'single cut' types give a greater proportion of their yield

at the first cut. Red clover's traditional importance lies predominantly with organic farming systems where it is used as a source of nitrogen in rotations. However, in recent years, it has been utilized more in conventional systems, and its benefits in terms of high protein content have been better realized through improved silage technology. It is often grown in single species stands but also commonly in mixture with a grass; in the UK, this is often Italian or hybrid ryegrass. It is also utilized alongside a cereal such as wheat in an intercropping combination and is considered an important target for pollinating insects, in particular bees, in a number of environments where these insects are a conservation priority. The breeding of red clover has recently been reviewed (Boller *et al.*, 2010). Important breeding objectives are yield, persistence and pest and disease resistance. Red clover in the silo shows lower breakdown of protein (and hence propensity to cause nitrogenous pollution) than white clover or alfalfa (*Medicago sativa* L.). This is believed to be due to the action of the enzyme polyphenol oxidase, which aids the establishment of protein–quinone complexes (Owens *et al.*, 1999).

Programmes of interspecific hybridization between red clover and related species have been carried out and were reviewed by Abberton (2007). The main emphasis has been increasing longevity through crosses with more persistent species, particularly *T. medium*, but to date these have had little commercial impact. An important example of genetic resources in red clover is the Swiss Mattenkee landraces described by Kolliker *et al.* (2003). Amplified fragment length polymorphism analysis by these authors showed that these landraces form a genetically distinct group with important implications for conservation and breeding. In subsequent work, Herrmann *et al.* (2005) showed that the 'ancestry of red clover landraces is primarily found in introduced cultivars in natural wild clover populations'.

Molecular techniques are increasingly being employed to characterize genetic resources in forage legumes (Kolliker *et al.*, 2009). Their use in forage legumes has hitherto been limited, but George *et al.* (2006) used simple sequence repeats to assess diversity in white clover cultivars and Mosjidis and Klinger (2006) carried out a genetic diversity on the core subset of US red clover germplasm using isozymes. However, recent developments building on the genome mapping of both white and red clovers (Isobe *et al.*, 2003; Jones *et al.*, 2003) and the physical mapping of the latter (Abberton, unpublished) have set the stage for the use of large numbers of single nucleotide polymorphisms in new approaches (e.g. those based on association genetics) to linking phenotype to genotype across a wide range of genetically diverse material.

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