

Review

Development of Genomic Resources in the Species of *Trifolium* L. and Its Application in Forage Legume Breeding

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Abstract: Clovers (genus *Trifolium*) are a large and widespread genus of legumes. A number of clovers are of agricultural importance as forage crops in grassland agriculture, particularly temperate areas. White clover (*Trifolium repens* L.) is used in grazed pasture and red clover (*T. pratense* L.) is widely cut and conserved as a winter feed. For the diploid red clover, genetic and genomic tools and resources have developed rapidly over the last five years including genetic and physical maps, BAC (bacterial artificial chromosome) end sequence and transcriptome sequence information. This has paved the way for the use of genome wide selection and high throughput phenotyping in germplasm development. For the allotetraploid white clover progress has been slower although marker assisted selection is in use and relatively robust genetic maps and QTL (quantitative trait locus) information now exist. For both species the sequencing of the model legume *Medicago truncatula* gene space is an important development to aid genomic, biological and evolutionary studies. The first genetic maps of another species, subterranean clover (*Trifolium subterraneum* L.) have also been published and its comparative genomics with red clover and *M. truncatula* conducted. Next generation sequencing brings the potential to revolutionize clover genomics, but international consortia and effective use of germplasm, novel population structures and phenomics will be required to carry out effective translation into breeding. Another avenue for clover genomic and genetic improvement is interspecific hybridization. This approach has considerable potential with regard to crop improvement but also opens windows of opportunity for studies of biological and evolutionary processes.

Keywords: breeding; clover; genetics; genomics; interspecific hybrids; QTL

1. Introduction

Grasslands cover about two-thirds of the world's agricultural area [1]. They have a crucial role in terms of food production and in the delivery of ecosystem services such as water supplies, biodiversity and carbon sequestration. Forage grasses and legumes are important components of these grasslands in many parts of the world and in temperate regions a relatively small number of grasses (e.g., *Lolium perenne*, *Festuca* spp, *Dactylis glomerata*) and legumes (*Trifolium* spp, *Medicago* spp, *Lotus* spp) dominate [1]. The application of genetics and genomics in the breeding of these species requires a focus not only on their key role underpinning meat and milk production, but also their importance in generating ecosystem services.

The genus *Trifolium* includes more than 250 species of which ten are of considerable agricultural importance [2]. Taylor [3] summarised available information on the distribution of *Trifolium* of which there are ninety perennial species including white and red clover. The centre of origin of the clovers is believed to be in the eastern regions of the Mediterranean, where the greatest variety of species is found [4]. Early cultivation of clover most likely started in southern Spain around 1000 AD. From there it spread to the Netherlands and Italy where rotational cultivation of clover was already recommended for the improvement of poor soil (Camillo Tarello in Ricordo d'agricultura, 1567). By the end of the 17th century clover had spread over most of Europe, reaching the more northern areas by the end of the 18th century [5]. Cultivated clover has generally larger leaves, blossoms earlier and more profusely than the wild counterpart, like most other forage and pasture species.

2. White Clover

White clover (*Trifolium repens* L.) is the most widely grown temperate forage legume, and the most common in pastures grazed by sheep or cattle [6]. Estimates have been made of annual global white clover sowings totalling 3–4 Mha [7]. Originally in section Lotoidea in the Zohary and Heller taxonomy [2] along with other perennials, such as *T. ambiguum* (kura clover or Caucasian clover), in the new taxonomy by Ellison *et al.* [8], it has been placed in section Trifolium. White clover is an outbreeding allotetraploid species ($2n = 4x = 32$) and its characteristic feature is its stoloniferous habit *i.e.*, it spreads by means of stolons, or horizontal stems and thus has many active growing points.

2.1. Germplasm and Breeding

Abberton and Thomas [9] recently reviewed germplasm collections of white clover and their utilisation in breeding programmes and Abberton and Marshall [10] summarised the state of breeding programmes around the world. White clover varieties are characterised by their leaf size and fall into four groups (small, medium, large and very large). Leaf size is closely related to the size of the stolons and dictates the livestock system for which the varieties are best suited. Small leaf varieties are considered suitable for continuous hard sheep grazing, medium leaf types used under rotational grazing and large or very large leaf size cultivars mainly for lax cattle grazing or conservation. Key

targets for trait improvement are persistence (closely related to stolon architecture and density), nutrient use efficiency, particularly phosphorus and water, tolerance of biotic and abiotic stresses and increased seed production. Mass phenotypic or recurrent selection approaches typical for outbreeding species, have been traditionally employed in white clover germplasm improvement breeding programmes. These have utilised field, glasshouse and controlled environment based schemes of assessment for important traits. Assessment of single plants in rows is useful for a general characterization of new germplasm resources and particularly for leaf size and evaluation of distinctiveness, uniformity and stability. For agronomic characters and performance effective evaluation is carried out in swards with the companion grasses over a period of at least three years and with an appropriate management involving either sheep or cattle depending on leaf size. Variety development in general follows the route of developing synthetics based on a small number of mother plants. Complexities arise due to the need to maintain a balanced sward with its ryegrass companion over many years. Other challenges include the need to maintain forage performance whilst enhancing seed yield for variety production and the interaction between clover, its symbionts (rhizobia and mycorrhizae) and the ruminant animals (sheep and cattle in the main) which feed on it.

White clover is an obligate outbreeding species, with a gametophytic self-incompatibility (SI) system controlled by a series of alleles at a single locus (*S*). Inbred lines described by Michaelson-Yeates *et al.* [11] were utilised in initial genome mapping work, and QTL studies were reported by Jones *et al.* [12] and Cogan *et al.* [13]. The location of the SI locus was described by Casey *et al.* [14] and utilised a characterised BAC library for white clover with partial BAC end sequencing reported by Febrer *et al.* [14]. Genetic maps in white clover have been reported by several groups [12,15–17]. They were all based on a mixture of SSR and AFLP markers [12], or exclusively SSR markers. Zhang *et al.* [16] used markers transferred from red clover and *M. truncatula*, which allowed some analysis of synteny between the three species. Wang *et al.* [17] used their map to identify QTL regions associated with salt tolerance. Subsequent mapping families have been constructed specifically to analyse genetic control of other key traits: stolon architecture and resistance to stem nematode (*Ditylenchus dipsaci*). Information about plant stolon architecture in particular has allowed studies of root architecture and water use efficiency [18,19]. A key feature of current and future studies in clovers is their close taxonomic relationship to the model legume *Medicago truncatula*. This species has been a useful source of markers for studies in clovers for some time [20], but the recent sequencing of the gene space [21] provides a strong basis for studies of microsynteny and genome sequencing in clovers as well as generating biological and evolutionary insights.

The fact that white clover is grown (and evaluated in breeding programmes) in mixed swards, typically with perennial ryegrass (*Lolium perenne* L.) highlights the unique challenges facing the white clover breeder and the importance of gaining a greater understanding of the biological and genetic bases of competition. Modern tools such as transcriptome sequencing have an important role to play in the future.

2.2. Impact of Genomics on Breeding Strategy in White Clover

Extensive and well characterised germplasm collections, understanding of key traits and the ability to develop novel population structures are all key elements underpinning the use of genomics tools.

Molecular markers such as simple sequence repeats (SSRs), Amplified Fragment Length Polymorphisms (AFLPs) and more recently single nucleotide polymorphisms (SNPs) have been used to characterise germplasm collections and genetic diversity [22,23]. In addition progress has been made in sub-genome differentiation making robust SNP identification easier [24]. A preliminary study of marker-assisted selection (MAS) in white clover has been described by Barrett *et al.* [25].

The limitations of QTL analysis based on biparental crosses or related family structures are well known [26–28], and studies on clovers as with other species are exploring association based approaches. Data on linkage disequilibrium in white clover are very limited, but it seems likely that a candidate gene approach, as is emerging from studies of other outbreeding perennials such as *Lolium perenne* will prove profitable [29]. Initial field experiments to underpin association analysis are being carried out by IBERS, Teagasc, USDA Wisconsin, Noble Foundation and based on crosses carried out at AgResearch in New Zealand. Such approaches are likely to be complementary to QTL analysis and will allow the results of previous studies to be applied more effectively in breeding programmes. The potential of heterosis in outbreeding forage legumes has been explored by Brummer [30], and some progress towards this end has been made, particularly in alfalfa [31]. Heterotic combinations of populations, derived from germplasm of different geographic origins are also underway. High throughput SNP analysis of genotypes in both these experiments would considerably improve our ability to relate genotype to phenotype. A complexity with forages is the need to not only focus on the characters that can be assessed in individual genotypes (e.g., flowering time), but the key agronomic traits that require assessment in plots and in the case of white clover, plots with perennial ryegrass.

Next generation sequencing (NGS) technologies are revolutionising the availability of genomic resources in many crops and these can also be applied to white clover. Clearly, international consortia have an important role to play, and the bioinformatic challenges are always significant. NGS technologies are becoming more accessible and increasingly the ability to understand and accurately phenotype key traits will be crucial for effective translation of genomics into commercially successful novel varieties. High throughput phenotyping (phenomics) is an important part of the solution for this challenge, and the development of phenotyping technologies both in glasshouse and field is likely to be a key endeavour for the coming years.

3. Red Clover (*Trifolium pratense* L.)

3.1. Traits of Interest

Red clover is one of the most important forage crops for feeding ruminant animals (sheep, beef and dairy cattle) in temperate agriculture, including the UK. It is a forage with a high protein content mainly used in silage production for winterfeed in livestock agriculture. Red clover production declined after the 1960s, largely due to the availability of cheap industrial production of chemical nitrogen fertilisers [3]. This drastically reduced the need for nitrogen derived from the symbiotic nitrogen fixation process occurring in the root nodules of red clover and other legume crops. This trend has seen a reversal in recent years, because of the high energy input required for N fertilizer production and its contribution to climate change and eutrophication of lakes and waterways due to N

leaching [32]. Red clover is thus gaining in importance again as there is a resurgence in more sustainable agriculture [33].

Red clover is a short-lived perennial species, but in some cases has a biennial life cycle. It exhibits significant variation in persistency, depending upon variety and adaptation [33–35]. Experience has shown that it is difficult to maintain yield beyond 4 years [34], so one of the most important targets for genetic improvement in red clover is persistency. This is an outcome of the effects of a range of biotic and abiotic factors, as well as intrinsic properties of the species. Among the biotic factors are a number of diseases of which crown rot (*Sclerotinia trifoliorum*) and a number of root rot causing fungi are the most serious [3,33,34,36]. The stem nematode (*Ditylenchus dipsaci*) is also considered to be a potentially serious disease contributing to reduced longevity of red clover. Abiotic factors include stresses such as cold, flooding and drought, while other environmental factors such as competition in mixed swards and the frequency of cutting also contribute to the overall stress on the plant. Root rot pathogens building up in the soil often seem to be the cause of the limited lifespan [34]. The fact that red clover neither form stolons, like white clover, nor tillers, like perennial ryegrass, would also appear to reduce its longevity. Changing red clover to a more stoloniferous or rhizomatous growth habit would be potentially desirable, and the former has been reported [37], but reaching these targets is not easy (see below). Improvement of persistence in red clover would thus seem most likely to come from exploiting the genetically heritable variation in some or all of the complex traits listed above, at least in the shorter term.

3.2. Genetics and Breeding

QTL mapping for persistence has not been studied extensively in red clover, but some data are available on aspects of this trait. The obligate allogamous habit of red clover has meant that genetic linkage maps are primarily based on progeny from F₁ pseudotestcrosses between two heterozygous parents. They are less optimal compared to textbook F₂ populations derived from a F₁ hybrid of two inbred grandparents, since not all the parental polymorphisms are informative in terms of linkage in the progeny. The first red clover linkage map was constructed by Isobe *et al.* [38], and contained 158 RFLP loci. Subsequently, a high density map with 1399 markers consisting of mainly SSRs and RFLPs was published [39]. Also over 26000 EST sequences were published, and grouped into 9339 non-redundant genes, providing a great basis for further genetic studies of red clover. Hermann *et al.* [40] described a map based on AFLP and SSR markers, and Zainol [41] generated a map consisting of 72 SSR markers and 67 AFLP markers, which was used to identify a total of 17 QTL related to flowering time, morphological and agronomic traits. The map of Hermann *et al.* [40] was used in a study of seed yield components, in which 38 QTL were identified. More recently, the maps of Isobe *et al.* [38], Sato *et al.* [39] and Hermann *et al.* [40] were combined with three other maps to generate an integrated map consisting of 1804 markers distributed over the 7 linkage groups and a total length of 836 cM [42].

Two recent papers have described work on QTL directly linked to persistence and its component traits. Herrmann *et al.* [43] used vigour scores after three growing seasons to identify QTL for persistence on LG3. Klimenko *et al.* [44] used two of the mapping families described above [42] to identify QTL for winter hardiness, resistance to *S. trifoliorum* and the root rot fungus *Fusarium*, and

found consistent QTL on linkage groups 1, 3 and 6. Given the complex nature of these traits, it is not surprising that the heritabilities they reported were quite low. It was also noticeable that a range of interacting QTLs were identified, highlighting the fact that pyramiding single effect QTLs may not bring sufficient improvement to breeding populations, but that it is important to ensure that all the interacting QTLs are present in the elite germplasm [44].

So far, the impact of molecular markers in red clover has been primarily on generation of genetic maps and QTL analysis. The next step would be the use of this knowledge in marker assisted selection (MAS) for breeding programmes. Red clover breeding is, like many other outbreeding forages, often based on recurrent selection or selection on maternal half-sib family progeny of polycrosses [34]. Paternity testing using a limited number of SSR markers was recently advocated as a highly efficient and cost-effective way of increasing genetic gain in outbreeding forage crops [45], and its usefulness was demonstrated with 11 SSR markers in a red clover breeding programme, by enabling the use of both maternal and paternal breeding values. Other outbreeding forage crops with breeding programmes based on half-sib family selection should theoretically be able to adopt this strategy as well.

3.3. Translational Genomics in Red Clover

While QTL analyses are important and a requisite for MAS and molecular breeding, it is still a daunting task to isolate and identify the gene(s) underlying any given QTL in a species without access to extensive genomics resources. Many forage crops including red clover have lagged behind other crops in the availability of genomics data. However, this is rapidly changing in red clover. Bacterial artificial chromosome (BAC) libraries [46] have facilitated a physical mapping project, the primary objective of which was to compare the genome of red clover to the closely related [8] model legume *Medicago truncatula* or barrel medic. Such a resource helps in understanding more about the genetics and genomics of forage crops such as red clover, to facilitate improved ways of breeding new varieties in a rapidly changing world. The physical map was based on 41 Mb of BAC end sequences and the use of fingerprint contig assembly. In order to integrate the genetic and physical maps we used the mapping family described by Zainol [41] without the AFLP markers. We developed a number of SNP markers from the available EST database and more SSR markers derived from the BAC end sequences which are available in the public database [47]. This work is near completion and will be described in more detail. An integrated physical and genetic map will assist in identifying genes underlying QTL of agronomic and biological traits and chromosomal rearrangements and translocations. Further, it will serve as an excellent scaffold for assembling the genome sequence, a project currently in progress.

The development of next generation sequencing technology has opened up further avenues for utilising these new genomic tools even in crops without a reference sequence. This is particularly true in functional genomics. NGS sequencing is emerging as a more powerful tool than existing methods such as differential display, cDNA-AFLP and even micro-array technology for quantifying gene expression in different tissues or in response to various treatments and stresses [48]. Among legume crops RNA-Seq (*i.e.*, the sequencing of the transcriptome with NGS technology), using the Illumina platform, has been employed in soybean [49,50], alfalfa [51] and chickpea [52]. It can also be used for SNP and/or SSR discovery, as for example in alfalfa [51] and chickpea [52], and many other species. While RNA-Seq does not require the availability of a reference sequence, *de novo* assembly of

transcripts without a reference to map the short reads, does have its challenges. However, programmes are now available to attend to those issues, e.g., [53,54]. Over 46,000 non-redundant transcripts have been identified in red clover. While the analysis of differentially expressed transcripts is not yet finalised, the heterozygous nature of the plant material made it possible to identify putative SNPs in over 7000 transcripts, using stringent criteria. Additionally, over 3100 SSR motifs in a little over 7000 transcripts were identified, over half of which were tri-nucleotide repeats. These genomics resources will facilitate the development of dense genetic maps, and further dissection of some of the complex traits in urgent need of genetic improvement. Further technological developments will in due course make genome-wide association mapping and genomics based breeding programmes more realistic goals for forage crops.

4. Genetics and Comparative Genomics in Subterranean Clover (*Trifolium ambiguum*)

Subterranean clover (*T. ambiguum*) is a pasture legume for more Mediterranean climes [55]. It is diploid and has a winter-annual autogamous life cycle, but crosses can be made. The basic chromosome number for subterranean clover is eight [56], which is identical to that in the reference legume *M. truncatula*. Despite these useful properties little information has been available with respect to genetics and genomics of this species. However, very recently the first genetic maps were published [57], using two F₂ families. They were based on SSR markers transferred from *M. truncatula*, red and white clover. This allowed cross-referencing and comparative genomics to be carried out with these three species. Moreover, the conservation of synteny of chromosomal segments to the other clover species was confirmed by reference to *M. truncatula*, and identification of possible locations for segmental duplication and inversion was made.

5. Interspecific Hybrids

At the beginning of the last century, Vavilov [4] recognised the importance of interspecific diversity as a potential pool of genetic resources for crop improvement. Much effort has been put into achieving introgression of desirable traits into cultivated species of *Trifolium* through interspecific hybridisation, with different levels of success. Many studies suggest that the main cause of failure has to be attributed to post-fertilisation barriers [58,59]. To overcome this, *in vitro* embryo rescue techniques have been extensively used. Despite the difficulties, this practice has gained more interest in recent years in view of global climate change and food security issues.

5.1. *T. repens* and Its Putative Progenitors

T. repens ($2n = 4x = 32$) is a tetraploid of most likely allopolyploid origin from two ancestral genomes. Its origin is considered quite recent, as suggested by the chromosome number being a multiple of the genus basic number [60,61]. An age of 4.2 million years has been estimated by Hand *et al.* [24] by comparing synonymous substitutions between homoeologous genes from the putative ancestral species. However, measurement was carried out on a limited number of genes rather than a large collection, such as an EST library. Over the years various related diploid species have been proposed as possible progenitors, namely *T. occidentale* ($2n = 16$), *T. nigrescens* ($2n = 16$),

T. pallescens ($2n = 16$). In early studies these hypotheses were based on cross-compatibility in interspecific hybrids [62–64] and karyotype similarities [65]. While the general consent is that *T. occidentale* might be the paternal genome donor, the more recent use of molecular techniques has not yet resolved the identity of the maternal donor. The work of Ellison *et al.* [8] using ITS nrDNA and cpDNA to construct a phylogenetic tree of 218 *Trifolium* species strongly indicates *T. pallescens* as the female ancestor. Following SNP comparison in eight stress-related genes from *T. repens*, *T. occidentale* and *T. pallescens*, Hand *et al.* [24] showed a close relation between *T. occidentale* and one of *T. repens* sub-genomes, while the *T. pallescens* sequences were more loosely related and somewhat less informative. This led to the argument that the real ancestor might be an extinct or yet to be discovered species related to, but different from *T. pallescens*. It would be of great interest to assess whether the same analysis carried out on the entire transcriptome would provide the same outcome. More recently, Badr *et al.* [66] sustained the former view that proposed *T. nigrescens* as the female genome donor on the basis of a phylogenetic tree constructed using AFLP and RAPD markers. Supporting this hypothesis is also the fact that, unlike *T. repens* and *T. nigrescens*, all the *T. occidentale* and *T. pallescens* accessions tested to date are acyanogenic [67,68]. Furthermore, cross-hybridisation of diploid *T. nigrescens* with artificially doubled [63,69,70] and diploid *T. occidentale* [71] can be achieved without the aid of special techniques, while hybridisation of *T. pallescens* and *T. occidentale* could only be achieved by embryo rescue (Williams, personal communication).

We have embarked on a program that exploits these hybrids to study the effects of hybridisation and/or polyploidisation on the making of the genome. The ever decreasing cost of NGS and the availability of sophisticated molecular techniques [72] mean we can now obtain and compare transcriptome and epigenome data in a relatively short time, and monitor genomic rearrangements. Changes such as gene loss and chromosome rearrangements have been shown to occur quite quickly after polyploidisation in *Brassica* [73] and wheat [74], and following interspecific hybridisation in *Brassica* [75]. These observations have implications not only in terms of evolutionary studies, but also of interspecific crop improvement. Beneficial genetic variants arisen from genome fusion and doubling have to be identified promptly and selected artificially before being eliminated during a process of genome stabilisation.

White clover wild relatives often display contrasting phenotypes for agriculturally desirable traits, such as drought-tolerance (*T. occidentale*), cold-tolerance (*T. pallescens*), high inflorescence and seed set (*T. nigrescens*), presence/absence of stolons (*T. occidentale* vs. *T. pallescens* and *T. nigrescens*), annual/perennial growth habit (*T. nigrescens* vs. *T. occidentale*). Generation of segregating progenies from these crosses would facilitate mapping these traits and, with the availability of an increasing number of whole genome sequences, the discovery of the underlying genes, with dramatic consequences on clover breeding programs.

Introgression of useful traits into white clover via interspecific crosses has already been shown in a few cases. Improved resistance to the root-knot nematode *Meloidogyne incognita* [76] and to the clover cyst nematode *Heterodera trifolii* [77], as well as increased flowering and seed set [78] were achieved by crossing white clover with *T. nigrescens*. The rhizomatous trait could also be introgressed into white clover by crossing with *T. ambiguum* ($2n = 4x = 32$) [79]. *T. ambiguum* and *T. occidentale* were also shown to improve resistance to the peanut stunt virus [80]. These examples are all very encouraging as for the potential of introgression via inter-specific crossing.

5.2. *T. pratense*

The phylogenetic relationship between *T. pratense* ($2n = 14$) and related species is not as clear as *T. repens*. As for *T. repens*, most studies are based on cross-compatibility and chromosome pairing in hybrids. The complication of red clover is that it is the only species with $n = 7$ amongst its closest relatives in the section *Trifolium* [8]. Much effort has been put into interspecific hybridisation of red clover with the main aim of improving the longevity of this short lived perennial. The first hybrid was generated by Taylor and co-workers [81] by crossing with *T. diffusum* ($2n = 16$). This hybrid was however sterile and fertile progeny could only be achieved by doubling the parents' genome prior to crossing. Although this cross is not useful in improving red clover longevity (*T. diffusum* is an annual), it could be used to improve flowering and seed yield, as shown more recently by Dabkeviciene and colleagues in backcrosses of the hybrid to *T. pratense* [82]. Successful hybrids were also generated by crossing autotetraploid *T. pratense* with the closely related diploid *T. pallidum* ($2n = 16$) [83,84]. The authors suggested that *T. pallidum* would be more valuable as an intermediary for transferring genes to red clover, rather than a source of useful germplasm *per se*. To improve red clover persistency the perennial rhizomatous species *T. medium* ($2n = 72$), *T. sarosiense* ($2n = 48$) and *T. alpestre* ($2n = 16$) have been suggested. These species are more distantly related and *in vitro* embryo rescue techniques had to be employed to overcome fertility barriers. Hybrids were obtained by crossing tetraploid *T. alpestre* and tetraploid *T. pratense* [85] and diploid *T. alpestre* with both diploid and tetraploid *T. pratense* [86]. One hybrid from the latter resembled *T. alpestre*, especially in the creeping rhizomatous growth habit, but it was both male and female sterile. Hybrids with *T. sarosiense* [87] and *T. medium* [88–91] have also been generated. In all cases some degree of rhizomatous growth was observed. Isobe *et al.* [92] characterised four generations of backcrossing and reported the disappearance of rhizomes after BC1. Nevertheless, the BC4 plants showed strong vigour and were therefore introduced in a breeding program.

5.3. *T. subterraneum* and *T. alexandrinum*

T. subterraneum and *T. alexandrinum* are annual species and common forage crops in the Mediterranean basin and Australia (*T. subterraneum*) and Middle east/India (*T. alexandrinum*). As for red and white clover, early research in interspecific hybridisation aimed at clarifying the closest relatives. For this purpose *T. subterraneum* ($2n = 16$) was shown to cross with *T. eriosphaerum* ($2n = 14$) and *T. pilulare* ($2n = 14$) [93], generating hybrids with various levels of fertility, while *T. alexandrinum* ($2n = 16$) was successfully hybridised with *T. berytheum* ($2n = 16$) and *T. salmoneum* ($2n = 16$) [94] and with *T. resupinatum* ($2n = 16$) [95]. While the interspecific hybridisation of *T. subterraneum* has not been taken any further, more efforts have recently been put into using this approach with the aim of improving *T. alexandrinum*'s resistance to biotic and abiotic stresses, tolerance to soil alkalinity and length of the vegetative period. Hybridisation with *T. apertum* ($2n = 16$) via *in vitro* embryo rescue generated 20 hybrid plants showing introgression of various desirable traits, including late flowering [96]. With the aid of embryo rescue, hybridisation was also successfully achieved with *T. constantinopolitanum* ($2n = 16$) [97] and *T. resupinatum* [98]. Some of the hybrid plants from the latter cross showed late flowering and the higher survival rate in the field compared to

T. alexandrinum. This was taken as an indication of tolerance to root and stem rot, encouraging further investigation into this route.

6. Conclusions and Future Prospects

The genus *Trifolium* contains many of the most important leguminous forage crops for grassland-based livestock agriculture. They provide a high quality, and protein-rich source of animal food and feed. Their importance is likely to increase as the need for lower input and more sustainable and resource efficient agriculture grows. Their allogamous and often polyploid nature has hampered the development of genetic and genomics resources for many of them, compared to other crops. However, the recent development of more affordable NGS technology is promising a rapid expansion in sequence and genotype data availability in clover crops. This will enable genome-wide association studies and genomics assisted breeding and genetic improvement, methods that have provided results particularly in animal breeding systems. The use of wide crosses and inter-specific hybridizations will complement these approaches, and ensure that we maximize the exploitation the large genetic variation present in these crops, not just for introgression of useful properties, but also for contributing to answer questions about the evolution of clovers. There are unique challenges for clover forage crops such as the need for phenotypic assessment of performance in plots in mixtures with grasses. Successful integration of molecular marker technology in clover genetics and breeding is however, dependent upon effective international collaborations and exchange of material.

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