

Identification of Founding Accessions and Patterns of Relatedness and Inbreeding Derived from Historical Pedigree Data in a Red Clover Germplasm Collection in New Zealand

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ABSTRACT

Pedigree maps are useful for germplasm exploration and identification of genetic bottlenecks. The primary objective of this study was to construct pedigree maps for red clover (*Trifolium pratense* L.) accessions held in the Margot Forde Germplasm Centre (MFGC) in Palmerston North, New Zealand. Pedigree data from accessions introduced into the MFGC between 1934 and 2016 were used. Data were filtered for accessions with breeding activity. A total of 30 founding accessions was identified, of which 25 were categorized as “New Zealand Broad,” two as “English Broad,” another two as “English Giant Hybrid,” and one as “Cotswold Broad.” The first synthetic form was identified from parents derived from English Broad and English Giant Hybrid. Influencing founder accessions were also identified. A relationship between introductions, relatedness, and geographic origin was found. The inbreeding and kinship coefficients were estimated across the population. The largest number of traced generations was eight. A total of 1742 accessions was used in the estimation of relatedness coefficients, with mean relatedness of 0.005. Overall mean inbreeding was 0.56%. Among the accessions with nonzero inbreeding coefficients, inbreeding was 10.68%. A sharp increase in inbreeding was found in the 1990s. Although inbreeding remained stable in the last three decades, a relationship between inbreeding and new introductions into the collection was found ($r = 0.62$). We conclude red clover diversity has been successfully maintained at MFGC during the past 80 yr. However, risk in loss of diversity is present unless germplasm exploration techniques, such as pedigree analysis, and novel breeding patterns are undertaken.

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Abbreviations: MFGC, Margot Forde Germplasm Centre; NPGS, National Plant Germplasm System; PSC, pseudo-self-compatibility.

RED CLOVER (*Trifolium pratense* L.) is a native species in Europe, western Asia, and northwestern Africa. It is also grown widely as a fodder crop and is used as silage and hay; in a grazing system, it is often mixed with white clover (*Trifolium repens* L.) in pasture mixes (Kemp et al., 1999; Cassileth, 2010). Worldwide, red clover occupies ~4 million ha (Riday, 2010) and is an important component of pastures that sustain productivity and income for subsistence farming communities, such as the Aymara in the Andean plateau (López et al., 1998). Red clover is a key component in forage systems for its N fixation ability (Vleugels, 2013). Carlsson and Huss-Danell (2003) reported that the extent of N fixation by red clover can be up to 373 kg N ha⁻¹. Red clover can contain up to 1% estrogenic compounds. Estrogen can interfere with ewe fertility (Morley et al., 1964), and formononetin is considered the main compound responsible for this condition. Therefore, newly bred cultivars have been developed with low (0.8% formononetin) levels of estrogen compounds (Kelly et al., 1980, Cassileth, 2010). However, Sutherland et al. (1980) showed that estrogen can improve red clover's tolerance to pests, such as grass grub [*Costelytra zealandica* (White)] and black beetle [*Heteronychus arator* (Fabricius)]. There is some mixed evidence in the literature suggesting

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that isoflavonoid phytoestrogens have some human health benefits in lowering risk of osteoporosis, heart disease, and breast cancer (Patisaul and Jefferson, 2010).

Red clover is relatively drought tolerant (Vaseva et al., 2011) and provides a high-quality feed throughout summer, whereas other species are adversely affected by water deficit. When grazed infrequently during spring and summer, it can produce ~ 12 t dry matter ha^{-1} (Kemp et al., 1999). Red clover thrives in pastures via rapid establishment and tolerates poorly drained soils (Riday, 2010). Unlike white clover, red clover cannot tolerate hard, continuous grazing (Kemp et al., 1999) and is mostly used as cut feed. Red clover normally persists for 2 to 4 yr but can persist for 7 yr where favorable conditions are present (Ledgard et al., 1990; Kemp et al., 1999). The red clover cultivars ‘Tuscan’ and ‘Relish’ have been bred for persistency, productivity, and acceptable seed yields (Charlton and Stewart, 1999). Despite the extent and success of red clover cultivar releases, relatively small gains have been achieved in increasing the persistence of red clover through breeding programs. In a review of breeding for improved persistence in red clover in Chile, Ortega et al. (2014) reported a mean realized annual genetic gain of 0.4 to 0.8% annually for red clover persistence. One cultivar, Carillanca, under irrigation had a large annual gain of 2.6%.

Red clover is a diploid ($2n = 2x = 14$) with a genome size of ~ 420 Mb (Sato et al., 2005). Red clover is almost fully self-sterile but highly fertile when outcrossed (Williams and Williams, 1947). The high level of heterozygosity is attributable to the gametophytic self-incompatibility system present (Taylor, 1982). Because of the economic and agricultural importance of red clover, the number of genetic studies has increased in recent decades (Ulloa et al., 2003; De Vega et al., 2015). Abberton (2007) reviewed interspecific hybridization between red clover and its related species. These programs had been running for >50 yr, usually utilizing embryo rescue techniques. The aim of these programs was to increase persistence in the sward through crosses with wild germplasm, such as *T. medium* L. Although no new cultivars have arisen through this technique, it has expanded knowledge on the evolutionary aspects of the genus.

Red clover cultivars can be either diploid or tetraploid. Tetraploid red clovers ($2n = 28$) can outperform diploids in several aspects (Taylor, 2008), such as dry matter yield. Tetraploids are often produced by chromosome doubling of diploid lines, as described by Taylor and Quesenberry (1996). Tetraploid plants are generally larger with larger leaves and florets and have increased persistence compared with diploid plants. However, because of the large floret size, pollination can become an issue. Seed yields are therefore lower than in diploids, and this is a limiting factor to the use of tetraploid cultivars (Taylor and Quesenberry, 1996; Charlton and Stewart, 1999).

Red clover cultivars are described by ploidy level and flowering time. Early-flowering cultivars can be grown in a wider range of environmental conditions and give more frequent but lower yielding cuts than late-flowering cultivars (Abberton and Thomas, 2011).

The use of pedigrees in plant breeding is a traditional method that has proven successful across many decades (Navabi et al., 2014). A pedigree map is a visual representation of the relationships within the population, showing relatedness between individuals or groups of individuals (Acquaah, 2012). In plant breeding programs, they are useful for deciphering the population structure and visualizing existing or potential genetic bottlenecks. Phenotypic data are often projected onto the pedigree to visualize the flow of targeted traits throughout the pedigree (Shaw et al., 2016). Pedigree assembly and analysis also allow for the determination of inbreeding and kinship coefficients in a population, which are useful in assessing the status of genetic diversity in a germplasm collection. When coupled with historical information, coancestry analysis can prove a powerful tool in examining the patterns that lead to population differentiation. The inbreeding coefficient is defined as the probability of drawing two homologous alleles from an individual that are identical by descent, indicating that they result from one allele from a common ancestor. The kinship coefficient is the relatedness between individuals, indicating the proportion of alleles identical by descent shared among individuals (Hedrick, 2011).

Often, when gain for agronomic traits is limited by low genetic variation, the most appropriate way to introduce new variation is to find new germplasm from the same gene pool. Plant introductions may be of more value in the early stages of breeding programs, rather than using released red clover cultivars as parents (Taylor and Quesenberry, 1996). A successful example is the pedigree of ‘Cherokee’, a cultivar that was developed for an area of the United States where red clover had not previously been grown. The base population was made up of 75% selections from plant introductions and 25% from two older cultivars. After five selection cycles, a population was produced that had reduced dormancy and high levels of root-knot nematode (*Meloidogyne* spp.) resistance (Quesenberry et al., 1993).

The increased use of next-generation sequencing technologies has allowed plant breeding programs to accelerate their rates of genetic gain. However, in order for germplasm collections to contribute to better understanding of genetic diversity and the contained population structure, it would be highly valuable to have a detailed pedigree map (Kouamé and Quesenberry, 1993; Taylor and Quesenberry, 1996; van Berloo and Hutten, 2005; Mosjidis and Klingler, 2006; Dias et al., 2008). Plant breeding programs use pedigree data to obtain insight into

the germplasm, leading to better-informed breeding decisions (van Berloo and Hutten, 2005). Pedigree assembly can provide insights into genetic bottlenecks, inbreeding depression, and low allele complementarity.

The Margot Forde Germplasm Centre (MFGC) is located at the Grasslands campus in Palmerston North, New Zealand. It collects and maintains germplasm of ~2000 forage species, including wild and domestic germplasm, and released cultivars, including intraspecific hybrid cultivars.

By providing information resulting from stored pedigrees at the MFGC for prebreeding efforts, new avenues can be followed to address the limitations of current red clover cultivars (i.e., low persistence or high estrogen levels). To the best of our knowledge, there has been no pedigree analysis of the red clover collection in the MFGC to date.

We used historical pedigree data from the red clover collection maintained at the MFGC. The objectives of this study were (i) to create a pedigree map of the MFGC red clover collection, (ii) to identify founding accessions, and (iii) to detect patterns affecting inbreeding and kinship.

MATERIALS AND METHODS

Data Filtering

Pedigree map construction was undertaken using the MFGC database, comprising 5223 accessions from 41 countries worldwide. Accessions were curated across a timeframe of 82 years, from 1934 to 2016. Some of these accessions were developed through the application of a range of techniques, including poly- and biparental crossing. Of the total, 3291 accessions were used in the construction of the pedigree maps by Helium, a software program that visualizes large-scale plant pedigrees (Shaw et al., 2014). Subsetting was performed on the basis of missing data and whether the accessions had continued breeding (i.e., single nodes in the pedigree were removed, as they were indicative of no breeding activity, Fig. 1).

Of the total number of accessions, 1742 accessions were used for the derivation and analysis of parameters. Pedigrees associated with accessions were subset based on biparental crosses, with full or half parentage recorded. For clarity, we will refer to half- or full-sib families as “families” throughout the paper, unless specifically stated half- or full-sib. Polycrosses were excluded from the parameter analysis subset because polycrosses do not fit the allele frequency expectations of biparental crosses, and inbreeding and kinship values would have been overestimated.

An extensive search and testing of software packages did not result in an option that could handle (i) the combinations of pedigrees with both poly- and biparental crosses and (ii) the capacity to handle the number of accessions to be analyzed. The data structure was accession ID, Parent 1, Parent 2, accession date, and country of origin. Accession ID was the unique number given to the accession when it was entered into the database. Parent 1 and Parent 2 represent accession IDs that were used as parents in the cross. All parents would have been entered into the database earlier and have a unique accession ID. Accession date is the date when the accession was entered into the database. In total, only 2328 accessions had the specific

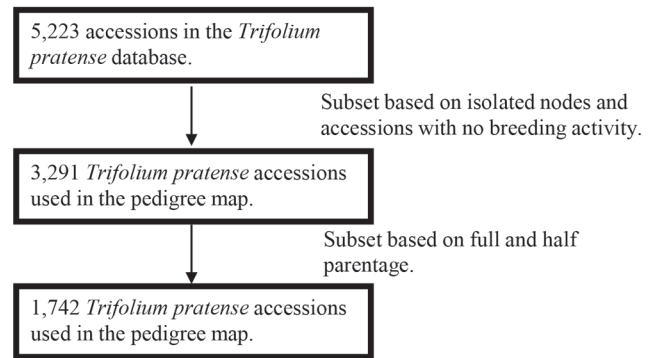


Fig. 1. Flowchart of the steps undertaken to subset and filter the *Trifolium pratense* accessions used in this study.

location of country of origin listed. Founders were defined as the accessions from 1934 to 1937 that had no parentage recorded, suggesting no prior breeding. Other accessions introduced into the database after that were considered introductions. Accessions with a total of more than 80 half- or full-sib families were regarded as influential accessions with significant footprint across the pedigree map. Accessions were clustered by the decade of entry into the MFGC database. It should be noted that the use of the term “accession” refers to any entry previously or currently stored in the MFGC. The term “Grasslands cultivar” represents accessions stored in the MFGC that were submitted as bred and released cultivars under the Grasslands trademark. The term “other cultivar” refers to cultivars bred in New Zealand or internationally by other organizations and not under the Grasslands trademark.

Data Analysis

The R package “pedigree” was used to calculate the number of offspring families, kinship, and inbreeding (Coster, 2015). The two formulas below were used in a recursive application to calculate kinship:

$$F_{yy} = 1/2(1 + F_{m_y f_y}) \quad [1]$$

$$F_{xy} = 1/2(F_{x_{m_y}} + F_{x_{f_y}}) \quad [2]$$

where F is the coefficient of inbreeding, x is not a descendant of y , and the kinship of two individuals is F_{xy} . Assuming $F_{xy} = 0$ when x and y are both from the founder population, m_y and f_y of y describe the genes from each parent that are randomly inherited, and the relationship between m_y and f_y is described by the calculated kinship coefficient between m_y and f_y . The kinship coefficient of y with y is Eq. [1]. The kinship coefficient between x and y is Eq. [2] (Fernando and Habier, 2006). A heat map was used to visualize the relatedness between 1742 pairwise comparisons of the population (Fig. 2a). The black diagonal represents perfect relationship of each accession with itself and the symmetric of diagonal elements represents kinship measures for pairs of accessions. A dendrogram was used to represent the clustering of the population based on kinship coefficients (Fig. 2b). To identify important ancestors in a particular sector of the germplasm, accessions with the highest mean kinship found at the 2000 distance coefficient were identified (solid black line in Fig. 2b), which resulted in the second or

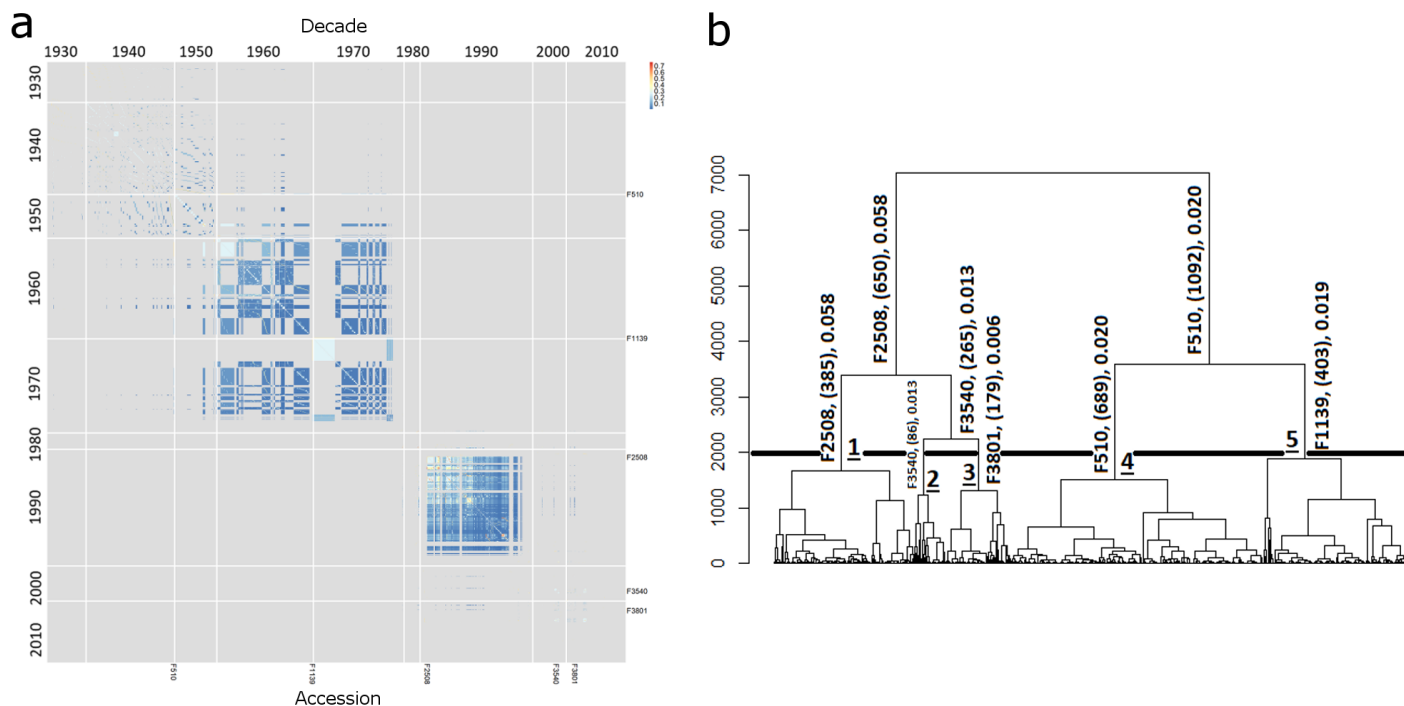


Fig. 2. (a) Kinship heat map of *Trifolium pratense* L. accessions at the Margot Forde Germplasm Centre. (b) Dendrogram of accessions included in the study. Accessions on the branches of the dendrogram at the fusion points are influencing ancestors of each cluster with the highest mean kinship in the cluster. The number of accessions in each cluster are indicated in parentheses. The solid line indicates the distance used to separate the different clusters studied. Clusters are numbered and underlined for reference.

third branch from the main branch on the dendrogram. Further insight into the history of different accessions was verified by studying individual pedigree lineages and the lineage contribution to the population in Helium.

Inbreeding (F_y) was calculated using the formula below (Crow and Kimura, 1970; Wright, 1984; Wiggans et al., 1995):

$$F_y = (H_0 - H) / H_0$$

where H_0 is the conditional probability that y is heterozygous and H is the unconditional probability that y is heterozygous at a specific locus.

RESULTS AND DISCUSSION

Pedigree Map Size, Complexity, and Offspring Family Distribution

The largest number of generations traced was eight and the minimum was 0. Of the 3291 accessions, 381 (11.58%) accessions had full parentage, 2468 (74.99%) accessions had one parent listed, and 442 (13.43%) had no parentage listed. There were 442 (13.43%) orphan lines, 2348 (71.35%) terminal lines, and 3230 relationships were identified in the pedigree. Visual inspection of the pedigree did not suggest any obvious bottlenecks.

Of the 20.44% of accessions that had recorded offspring families, the number of offspring families ranged from 1 to 356. The mean offspring family number across the whole population was 1.845. Within the accessions that had offspring families, the mean number of offspring families was 2.045 and the mode number was 1. In the

overall pedigree map, 3.73% of the accessions had only one offspring family, another 3.73% had two offspring families, 1.61% had three offspring families, and 1.21% had four offspring families. We also identified accessions with large numbers of offspring families. The largest percentage of accessions with offspring families was 3.79%, which had between 10 and 30 families.

These four accessions had >80 offspring families: F510, F709, F1139, and F2508. The first three accessions (F510, F709, and F1139) were Grasslands cultivars, whereas accession F2508 was a breeding line known as “multi-leaf selection” and was introduced into the MFGC in 1990. The accession F510 ($k = 0.019$) had 265 full-sib and half-sib offspring families, and the earliest traceable accession was introduced into the MFGC in 1952. F709 ($k = 0.014$) had 183 full-sib and half-sib offspring families and the earliest traceable accession was introduced into the MFGC in 1960. F1139 ($k = 0.019$) had 81 half-sib offspring families, and its earliest traceable accession was introduced into the MFGC in 1968. Accession F2508 ($k = 0.057$) had 356 full-sib and half-sib offspring families. Because three of the four accessions were associated with commercially available cultivars, the populations must have been phenotypically desirable.

In the 1930s, a significant number (529) of accessions was associated with germplasm collected in New Zealand (Fig. 3). The New Zealand accessions peaked in the 1940s but declined steadily thereafter until the 1990s and 2000s. Comparatively, the 1970s was a decade where the largest number of accessions (483) was imported from foreign

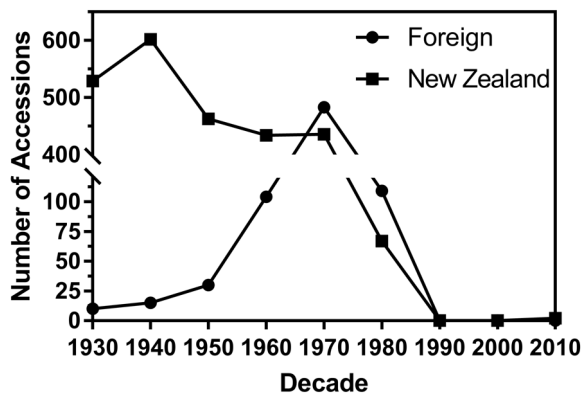


Fig. 3. The number of *Trifolium pratense* L. accessions associated with foreign countries and the number of accessions collected in New Zealand between 1930 and 2010.

countries to New Zealand. The countries of collection that contributed the most were: the United States (208), the former Soviet Union (65), Germany (34), England (28), Sweden (19), Poland (15), Belgium (13), and France (13).

Founding Ancestors and Important Introductions

Founders

A total of 30 founding accessions was identified in the red clover germplasm database. The founders are defined here as the oldest accessions with no parentage recorded, and they were introduced into the MFGC between 1934 and 1937. Of these, 25 were classified as ‘New Zealand Broad’, two were classified as ‘English Broad’, two were classified as ‘English Giant Hybrid’, and one was classified as ‘Cotswold Broad’. The first importations of red clover into New Zealand were mainly from English commercial firms.

‘Broad red clover’, an early flowering cultivar that lacks persistence, was widespread in the drier regions of the South Island and was used as the base population in breeding programs (Corkill, 1949; Wratt and Smith, 2015). By 1941, the first synthetic red clover had been formed, and some parent plants were derived from ‘English Broad’ and ‘English Giant Hybrid’. The synthetic red clover was increased, was subjected to agronomic trials and was labeled as ‘New Zealand Broad Red Clover’. This was renamed in 1964 as ‘Grasslands Hamua’.

Influencing Ancestors

There were five accessions that strongly influenced the population structure of red clover (Fig. 2b), namely F2508, F3540, F3801, F510, and F1139. Accession F2508 was listed in July 1990 and had one accession listed for parentage, F2367. Accession F2367 was collected from Turkey in August 1988. The climate of Turkey is typical of Mediterranean climate, with hot dry summers and mild to cool wet winters. Traditionally, Mediterranean phenotypes have been desirable in New Zealand, as they exhibit

drought tolerance traits. Its climatic origins could be one of the reasons why this accession influenced the population structure (Fig. 2b).

We hypothesize that adaptation to the target environments in New Zealand is a driving force behind the influence of the founding accessions. Here, we describe six relationships. Accession F3801 was listed in January 2012 and had the half parentage listed as F3540 (Fig. 1b). Accession F3540 was listed in April 2008 and arose out of a biparental cross between F2903 and F2499. Accession F2903 is a Grasslands cultivar, ‘Grasslands Sensation’ (Claydon et al., 2003), which was traced back to a poly-cross from various accessions. ‘Grasslands Sensation’ is an early-flowering diploid cultivar, which persists under grazing. Accession F2499 can be traced back to an introduction from Portugal (F2414). Two other accessions, one known as ‘Renova’, was an introduction from Belgium (F2140), and one known as ‘Changins’ was an introduction from Switzerland (F2138).

Accession F510 (Fig. 2b) was listed in April 1952 and was a progeny of accession F480. Accession F510 was defined as a highly contributing parent, with 265 offspring families. Accession F480 had no listed parentage. It was brought into MFGC in January 1950 and collected as a naturalized accession in the Manawatu region of New Zealand. Accession F1139 produced 81 offspring families and was listed in January 1968. The half parentage listed was F1002 or ‘Grasslands Hamua’, a commercial cultivar.

Diversity and Inbreeding

Diversity

The overall mean kinship from 2,201,851 pairwise combinations within the species, with kinship values ranging from 0 (no relatedness) to 1 (full relatedness), was $k = 0.005$. A total of 498 accessions (28.59%) had a mean kinship of 0, whereas 1244 accessions (71.41%) had kinship >0 , with a maximum value of $k = 0.0576$. These kinship values indicate that there are low levels of relatedness among accessions in the red clover collection (Fig. 2a).

The cumulative trend of kinship across the decades shows an increase in kinship of 0.021 from 1930 to 1970, until a plateau occurred between 1970 and 1980. A sharp increase of 0.012 occurred between 1980 and 1990. Between 1990 and 2010, there was a small increase of 0.001 (Fig. 4).

The dendrogram (Fig. 2b) has five clusters in the population. The clustering and identification of current commercial cultivars in the clusters can provide insight into the diversity present in the market and is of particular interest to the industry.

Parts of the dendrogram can be explained by groups of accessions with common countries of origin. Clusters 2 (86 accessions), 3 (179 accessions), 4 (689 accessions), and 5 (403 accessions) had some, not all, accessions associated

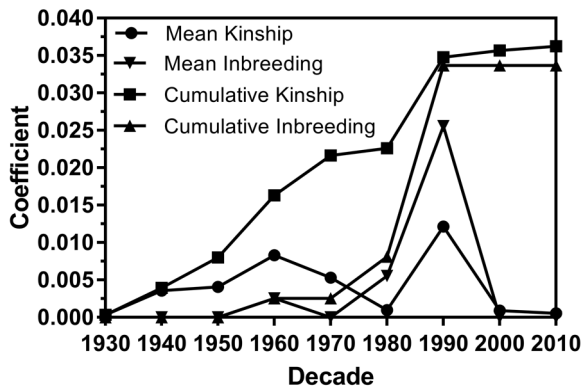


Fig. 4. The trend in cumulative and mean kinship and inbreeding in *Trifolium pratense* L. accessions at the Margot Forde Germplasm Centre between 1930 and 2010.

with country of origin. Cluster 2 had 15 accessions from Switzerland. Cluster 3 had five countries associated with accessions: Switzerland (13), Turkey (6), Spain (20), Portugal (4), and Argentina (1). Cluster 4 had 30 accessions from Turkey. Cluster 5 had seven countries associated with accessions: Portugal (4), Spain (10), Armenia (22), Georgia (30), Tajikistan (18), Greece (24), and Russia (13).

Clusters 4 and 5 were the two clusters from which commercial cultivar data could be extracted. Cluster 4 had 16 (2.32%) accessions associated with the “Grasslands cultivar” group. Accessions in Cluster 4 were introduced between October 1937 and January 1965. The geographic origin of commercial accessions within Cluster 4 was confined to New Zealand. However, 15 accessions were linked to the Manawatu region (North Island) and one to the Canterbury region (South Island). Cluster 5 had five accessions associated with “Grasslands cultivars” and three accessions were associated with “other cultivars.” The accessions in Cluster 5 were introduced between January 1968 and July 1982, notably later than those in Cluster 4. ‘Grasslands Hamua’, ‘Grasslands Turoa’, and ‘Redwest’ (Charlton and Stewart, 1999) are the three cultivars present in Cluster 5. ‘Grasslands Hamua’ and ‘Grasslands Turoa’ have similar soil and moisture requirements. However, ‘Grasslands Turoa’ is more persistent. ‘Redwest’ is a cultivar that was reselected from ‘Grasslands Hamua’ on the basis of low estrogen content. Its general agronomic traits are very similar to ‘Grasslands Hamua’.

An interesting observation is the high number of Turkish introductions in Cluster 4. In contrast, a variety of countries contributed towards the composition of Cluster 5, with fewer accessions recorded as domestic cultivars than Cluster 4.

Separation between Clusters 4 and 5 might be attributable to selection pressure. There were five records of the cultivar ‘Grasslands Hamua’ used as a parent. These accessions were split between Clusters 4 and 5, with one accession in Cluster 4 and four accessions in Cluster 5. The accession in Cluster 4 originated from the Canterbury

region, whereas all but one of the accessions in Cluster 5 originated from Manawatu. Accession F1139 (Fig. 2b), the most influencing ancestor of Cluster 5, is a progeny of ‘Grasslands Hamua’. The separation among clusters and the presence of released cultivars within different clusters suggests that breeding and selection for different target environments in New Zealand might have played a role in the genetic divergence observed.

Inbreeding, Indirect Relationships, and Unrelated Accessions

The mean inbreeding level was 0.56%; among the accessions with inbreeding coefficients >0, the mean was 10.68%. The frequency of the levels of inbreeding within the inbred accessions peaked at coefficients 0.07 and 0.13. Among the accessions with inbreeding coefficients >0, the lowest level of inbreeding was 0.015 and the highest level was 0.25. Across the whole dataset, 1651 accessions (94.78%) had an inbreeding coefficient of 0, whereas 91 accessions (5.22%) showed some inbreeding.

The combined trends of inbreeding and kinship in the red clover collection are shown in Fig. 4. Although the inbreeding levels are low, they seem to have increased across time. A correlation was found among average inbreeding per decade and the number of introductions per decade ($r = 0.62$). The trend in inbreeding from the decades of 1930 to 2010 shows no inbreeding from the 1930s to the 1970s, except for a slight increase (0.002) in the 1960s. The highest inbreeding occurred between 1980 and 1990, where it increased to 0.025. There was no further increase in inbreeding in subsequent decades (Fig. 4). The overall inbreeding value was 3.37%.

Although red clover is difficult to self, it is easily inbred (Taylor and Smith, 1980). Sib-crossing can result in severe inbreeding depression and populations, with inbreeding via selfing cannot continue beyond two to three generations. If selfing reaches past these generations, loss of vigor, fertility, and viability of pollen occurs (Taylor and Quesenberry, 1996). Because of the sensitivity of red clover to inbreeding, it is important to breed it through populations, rather than individual plants (Taylor and Quesenberry, 1996).

Although inbreeding is regarded as deleterious, it should be noted that if crosses are performed correctly, inbreeding can lead to increased genetic gain. Taylor et al. (1970) investigated the effects of one generation of selfing on pseudo-self-compatibility (PSC) and seed and forage traits to assess the degree of hybrid vigor and its correlation to persistence. It was concluded that inbred parental lines might be maintained through either vegetative reproduction or by seed through PSC. Although selfing reduced the yield and persistence rates, these were regained in single crosses. This was strengthened by Duncan et al. (1973), who found that PSC decreased with inbreeding.

They proposed a new method of inbred line maintenance, combining the favorable features of both vegetative and seed maintenance of the inbred lines. This ultimately resulted in single cross seed from different clonal sources for the production of double-cross hybrid red clover.

Germplasm Centers and the Effect of Forage Breeding Strategies

The importance of legumes is recognized by the large number of germplasm collections worldwide, with >1,000,000 accessions. Approximately 74,100 accessions of *Trifolium* are held in global collections; 53% are wild, and 14% are cultivated (Smýkal et al., 2015).

Kouamé and Quesenberry (1993) evaluated >800 accessions of red clover, originating from 41 countries and held in the Germplasm Resource and Information Network of the National Plant Germplasm System (NPGS), USDA. Their analysis found a large range of diversity across all countries, with the highest diversity contained in accessions originating from northern and eastern Europe. That study showed that clustering resulted from similar agronomic traits and it provided information for the creation of a core collection and more effective utilization of red clover germplasm. This is comparable with our study, as we also found a large range of diversity.

Mosjidis and Klingler (2006) assessed the biochemical diversity present in accessions of the USDA NPGS core subset, concluding that genetic diversity was high and that there was nearly double the variability in wild populations compared with cultivars or landraces. Dias et al. (2008) evaluated the diversity of the same core collection of 85 accessions of red clover, at both the morphological and molecular levels. An analysis of molecular variance showed that 83.6% of the variation was contained within population. This is valuable for breeding programs to use within and between population variation to breed for improved varieties.

The rate of genetic gain in red clover is low because of the long breeding cycles, complex outbreeding mating systems that suffer inbreeding depression, and interaction of genotypes with environmental factors (Annicchiarico et al., 2015). Although there has been a significant rise in the number of genetic studies in forages worldwide to suggest novel breeding strategies, the basis of forage plant breeding still largely relies on recurrent phenotypic selection. Mass selection, recurrent phenotypic selection, polycrossing, and backcrossing are the most common selection and crossing techniques in red clover worldwide (Taylor, 2008; Riday, 2010).

Breeding strategies for red clover are based mainly on mass selection, suggesting that it should be the first technique deployed in a breeding program to maintain a large population and diversity (Taylor and Quesenberry, 1996). Mass selection has been used largely for pest resistance

and persistence in red clover (Riday, 2010). Polycrossing has been sparsely used in red clover; however, progeny testing and multiple locations must be used to account for genotype \times environment interactions (Taylor and Quesenberry, 1996). In the red clover pedigree dataset, 183 accessions were identified as bred by polycross and 3108 were bred by biparental crosses. Polycrosses were identified for the decades of 1950, 1960, and 1970, with 47, 33, and 103 polycrosses, respectively.

Backcrossing is seldom used in red clover breeding, mainly because of the promotion of inbreeding depression. However, when it is used, it is in breeding programs to incorporate disease or pest resistance. Taylor et al. (1986) used backcrossing to incorporate resistance genes to a strain of *Bean yellow mosaic virus* in the cultivar 'Kenstar'. However, to date, there are no other cultivars of red clover that have been developed via the backcross method.

Unlike white clover, using related species in red clover to increase the genetic diversity has had limited success. Cleveland (1985) summarized the efforts made in hybridizing red clover with related species. However, because of most of the relatives are annuals, the perennial trait proved hard to maintain. Sterility in the progeny has been one of the biggest problems in this regard, and it is the prime reason why no commercial cultivars are available on the market for *Trifolium* interspecific hybrids. Forage species retain many of the traits of their wild progenitors because their domestication history (<3000 yr) is more recent than other crops (>10,000 yr) (Walton, 1971) and, in general, forage species are considered to be only partially domesticated (Gepts, 2004). This is shown particularly with persistence and dry matter yield under grazing, especially in New Zealand, and is one of the primary reasons why progress in breeding for complex fitness traits has been slow.

Nutman and Riley (1981) evaluated the symbiotic effectiveness of four cultivars of red clover and found that crosses between cultivars were more effective than those within cultivars, indicating heterotic effects. This shows the opportunity for breeding across gene pools, rather than within the same gene pool.

The current breeding strategies relative to the red clover accessions in the MFGC have been successful thus far, as inferred from the metrics reported here, such as number of accessions bred and generated, inbreeding and kinship levels, and the number of cultivars relevant to specific sectors of genetic diversity. The potential for breeding across newly identified breeding pools and continuing research into germplasm diversity will provide new techniques to widen the breeding pool.

CONCLUSIONS

Red clover genetic resources held at the MFGC have been valuable for germplasm diversity, and this is reflected in low kinship and inbreeding levels. International

collection expeditions and germplasm exchanges have proven successful in achieving diversification and introduction of new traits into cultivars in New Zealand and overseas. Influencing ancestors have resulted from these introductions. The newly identified population patterns and divergent clusters of germplasm will allow more informed and targeted breeding decisions. Improved populations with new additive variation can be obtained via crossing among accessions with disparate relatedness; these are considered untapped resources that will increase germplasm utilization for pre-breeding and cultivar development.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data and Germplasm Availability

Data and pedigree map queries are available upon request to the authors. Requests for MFGC germplasm may be considered depending on availability of germplasm and the purpose of the request.

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