Ninety years of Clover Breeding in Kentucky

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This is a history of red clover research in the Department of Agronomy at the University of Kentucky in the 20th Century. The program began in 1920 when Dr. E.N. Fergus was appointed as assistant in farm crops in the Department of Agronomy, where he advanced to professor and agronomist in charge of forage research, continuing until his retirement in 1960. He received his Ph.D. at the University of Chicago in 1931 (Taylor, 1985a). The goal of the red clover breeding program has always been to increase adaptability and thereby to improve yield and persistence to lower livestock production costs. To assure reliability of the crop was a part of this goal.

Beginning:

At this time, Fergus was seeking to determine why red clover was so unreliable in production. Along with W.D. Valleau, he conducted a study of clover failure in Kentucky (Fergus and Valleau, 1926). Many factors were considered, including poor fertility, low pH, and diseases. It was ultimately determined that poor adaptation of germplasm was the primary cause of clover failure. Fergus then conducted an investigation of breeding methods to improve adaptability and disease resistance in red clover. His Ph.D. thesis was concerned with self fertility as a breeding method. It was felt that one of several reasons that improvement of the crop had not been undertaken more extensively was that plants were self sterile and could not be inbred (Fergus, 1922). This was probably a comparison with inbreeding in other crops such as corn (*Zea maize*). Fergus found that red clover could be inbred to a limited extent , but he soon gave up this method of breeding in favor of mass selection.

Fergus probably was influenced in this regard by E.A. Hollowell of the United States Department of Agriculture, who was responsible for clover investigations until he retired in 1962 (Taylor, 1985a). Hollowell was instrumental in the development of Midland and Cumberland red clover varieties, and doubtless suggested the mass selection procedure to Fergus, who was also joined by Lawrence Henson in the early 1930's. Other personnel who worked on red clover at Kentucky with Fergus from time to time in the early years included E. Johnson, G. Roberts, E.J. Kinney, J.F. Freeman, E. Jewett, and P.E. Karraker.

Development of the Kenland Variety.

In the late 1920's and early 1930's, trials of seeds from farmer fields were conducted and seeds of the best of these were grown at Lexington in a soil that had been heated to kill weeds. When seedlings were four to six weeks old, they were inoculated with a spore suspension of mass cultures of the causal agent of southern anthracnose, *Colletotrichum trifolii* B&E. The plants were covered with tobacco canvas to provide a moist incubation chamber and surviving plants were allowed to produce seeds. This process was continued for several years, culminating in the release of the Kenland variety in 1947. Not only was this variety resistant to southern anthracnose, but it had greater adaptability to Kentucky conditions than other available varieties. The parent varieties of Kenland were Ky 101 and Ky 215; Virginia varieties Sanford and DeJarnette; Tennessee Anthracnose Resistant, North Carolina variety Hahn, and Missouri variety Plassmeyer (Fergus, 1963). The Kenland variety proved to be very well adapted and became the most widely used red clover variety in the United States. Part of this success was due to the inclusion of Kenland in the National Foundation

Seed Project (Garrison, 1952), a program undertaken by the USDA to provide seed of improved varieties to farmers. Seed of the Kenland variety increased from 110 metric tons (t) in 1949 to 1359 t in 1954. In 1951, 97.5% of all the Kenland seed was produced in the western states, primarily California, Oregon, Washington, and Idaho. Eighty-five percent of the 1954 production of certified seed of red clover was Kenland. No red clover variety has been so dominant in the US seed trade since that time (Wheeler and Hill, 1957).

Early Seed Investigations:

In the late 1940's and early 1950's, several master's thesis research programs were initiated to ascertain the most efficient methods for increasing seed of the new Kenland variety. One of the first of these examined the effect of certain fertilizers on seed production (Buckner, 1948). Later, Taylor (1951) studied the effect of companion grass on seed yields. Other master's theses were authored by James Shane, W.H. Stroube, and H.D. Wells. Stroube and Wells eventually received Ph.D. degrees at various institutions and obtained research positions. Much of this early research examined the number of flowers and seeds in the top, middle, and bottom of the plant as well as from top, middle, and bottom of the heads. These factors turned out to have no relationship to seed yield. The most important factor in determining seed yield was number of heads, and any treatment that adversely or beneficially influenced head number had a corresponding effect on seed yields.

Expansion:

Up to this point, research funding was at a minimum but in the early 1950's funding began to be increase, with the result that several new personnel were hired.

The first of these was N.L. Taylor (the present writer), a 1953 graduate of Cornell University who was employed July 1, 1953 to conduct red clover breeding. Shortly thereafter, W.H. Stroube, a graduate of Louisiana State University (Plant Pathology) was hired to conduct variety trials. Dr. E.A. Hollowell agreed to expand the red clover research by placing a USDA investigator, Dr. W.A. Kendall (plant physiologist), at U.K. Dean Frank J. Welsh in turn agreed to construct a greenhouse head house complex on Virginia Avenue near Scovell Hall (the main experiment station building). Dr. Kendall arrived in 1954 and a concrete block two-story head house with two attached greenhouses was completed in 1955. The upstairs of the head house was equipped for laboratories and seed storage, but part of this space was later converted to quarters where students resided at no cost in turn for caring for the greenhouses. The number of students living in these quarters ranged up to four or perhaps five at any given time, and must have numbered from 75 - 100 over the years. Some father/son pairs resided there, of course, many years apart.

Development of the Kenstar red clover variety:

Once the facilities were available, technicians to conduct the research were hired. Mr. M.G. Long became the plant breeding technician in 1954 and continued until 1984 when he retired. In his later years, he was assisted by another technician, Barbara Block. Charles Bunnell was the technician hired to assist W.A. Kendall. Field research was conducted at this time on the experiment station farms in Lexington (present site of dormitories and Seaton Center, among other buildings) and in Woodford County. The major research focus was to improve red clover by the polycross method of breeding and numerous clones were screened for persistence under field conditions (Taylor *et.*

al, 1962). Kendall examined growth of red clover at various temperatures and moisture levels as related to lack of persistence (Kendall *et. al*, 1962) and rates of respiration and photosynthesis in clones of red clover (Kendall and Taylor, 1963). A plastic greenhouse (present Seaton Center site) was erected on the experiment station farm to allow production of large numbers of cuttings (propagules). It was soon apparent that virus resistance was necessary if clones were to survive long enough for polycross progeny testing. Eventually, virus resistant clones were selected and sent to western United States to produce seed and to determine the relationship of clonal and polycross seed yields at Lexington and Prosser, Washington. Seed yields at the two locations, unfortunately, were not correlated (Taylor *et. al*, 1966). A negative correlation of earliness and persistence suggested that genetic shifts to lower persistence occurred when seed was produced in Washington. No such shift occurred at Lexington, probably because the seed crop was produced only on the second growth rather than total seasonal growth as is the practice in the western seed-producing states. A question as to the validity of the polycross method arose when certain progenies did not perform as well in mixed-progeny sown plots as they did in single-progeny plots (Taylor and Kendall, 1965).

Nevertheless, the polycross program was continued and ultimately 10 clones were selected to form a synthetic variety (Taylor *et. al*, 1968). The synthetic variety was compared to certified Kenland and was released as ‘Kenstar’ (Taylor and Anderson, 1974). Much later, it was found that seed yield of Kenstar apparently was low in the western seed producing areas, with the result that seed was not produced in the quantity needed for Kentucky and other eastern states. Consequently, Kenstar never received usage comparable to Kenland.

Interspecific hybridization:

Another approach to improving red clover is hybridization. In 1954, W.C. Harlin became the first graduate student on the project. He was assigned a thesis project of investigating means by which *Trifolium* species could be hybridized with red clover. Many hybridizations were attempted but only the crosses with *T. diffusum* were successful, even though the hybrid was sterile. Use of tetraploid parents improved fertility and resulted in the first fertile hybrid in *Trifolium* (Taylor *et. al*, 1963 and Taylor and Anderson, 1974). Unfortunately, the hybrid was less persistent than red clover. Therefore, further investigation of producing hybrids with perennials was initiated. Another graduate student examined the use of chromatographic patterns to predict successful hybridization (Collins and Taylor, 1964). This investigation showed that similar polyphenolic compounds that were producing the chromatographic patterns occurred in unrelated groups. For example, the patterns of sub-clover (*T. subterraneum)* and its close relatives were very similar to patterns for red clover and its close relatives and had little or no relationship to the possibility of producing interspecific hybrids. An interesting polyphenolic compound, pectoleragenin, isolated from *T. campestre*, apparently is associated with resistance to the alfalfa weevil in clover species. This research was conducted cooperatively with Carol Keller (1966 - 1969). All clover species possessing this compound are resistant (Keller *et. al*, 1969).

In the meantime, W.A. Kendall continued investigations on growth of red clover pollen as an aid to interspecific hybridization. The requirement for germination and growth of pollen *in vivo* were elucidated (Kendall and Taylor, 1965; Kendall *et. al*, 1971;

Kendall and Taylor, 1971).

About 1965, Dr. W. H. Stroube resigned and accepted a position at Western Kentucky University. In the early 1970's, Dr. M.K. Anderson was hired to assist N.L. Taylor in the clover research. N.L. Taylor at this time was Associate Chairman of the Department.

W.A. Kendall, assisted by graduate student D.L. Newton, attempted hybridization of some *Trifolium* species through stylar temperature treatments (Newton *et al.*, 1970). This research was repeated later by M.K. Anderson but both attempts were largely unsuccessful (Anderson and Taylor, 1973).

In 1970, W.A. Kendall resigned to accept an appointment at the USDA Pasture Laboratory at Pennsylvania State University and was not replaced at Kentucky. However, interspecific hybridization research was stimulated by the arrival of Kenneth H. Quesenberry as a graduate student. Hybrids were obtained among *T. alpestre, T. rubens, T. heldreichianum, T. noricum, T. medium* and *T. sarosiense*, species more or less closely related to red clover (Quesenberry and Taylor, 1976, 1977, 1978). A technique for doubling the chromosome number of *Trifolium* species using nitrous oxide was developed (Taylor *et. al*, 1976).

Since the start of the interspecific hybridization research, seeds of *Trifolium* species were assembled for preservation under cold storage (-5 to -15^{C}). Somatic chromosome numbers were examined (Anderson *et. al*, 1972, Giri *et. al*, 1981) as well as genetic system relationships (Taylor *et. al*, 1979). M.K. Anderson departed for North Dakota State University in 1974 but hybridization research continued (Taylor *et. al*, 1980). The cross of diploid red clover with a perennial clover species (*T. sarosiense)* was finally accomplished about 1980 (Taylor *et. al*, 1981; Collins *et. al*, 1981; Phillips *et. al*, 1982). However, the hybrid plants were sterile and doubling their chromosome numbers did not overcome the problem. Nevertheless, the groundwork for hybridizing red clover with perennial species was laid. Investigators at other locations were able to overcome the sterility problem by using tetraploid red clover in the cross and producing fertile back crosses by embryo culture.

Polyploidy:

The doubling of chromosome number to increase fertility of interspecific hybrids led to considerable research on tetraploid, triploid, and trisomic red clover. Our first research on chromosome doubling was by a graduate student, W.C. Harlin, who successfully treated red clover and *T. diffusum* with colchicine. Later, it was determined that nitrous oxide was a more efficient method of doubling because fewer chimeras and a much higher doubling rate resulted (Giri *et al.*, 1982). A few octoploid plants were produced that were invariably sterile. After tetraploids from nitrous oxide were available, it was possible to use the unreduced gamete technique to increase the population (Taylor and Giri, 1983). Our tetraploid population was not as persistent or as high yielding as comparable European diploids (Taylor, 1983). However, the tetraploid research was continued by widening the genetic base of the population (Taylor, 1985, Taylor and Berger, 1989). The Kentucky tetraploids were crossed with a Swiss tetraploid cultivar, Temara, and with other American cultivars using the unreduced gamete technique. The resultant population was at first susceptible to the disease, spring blackstem,probably caused by *Phoma trifolii* . Further selection eliminated the susceptibile .plants.In 2007,about 8000 plants were started in a greenhouse from which 1526 plants susceptible to powdery mildew were eliminated. We shipped 4000 plants to Barenbrug C. in Oregon who had expressed interest in an exclusive release. Their research personnel wished to select for seed yield in the tetraploid.. No results of this selection have been received. The tetraploid experimental which has not been named has topped the yield trials in several instances and has also been quite persistent. We have determined that the tetraploid should be sown at a slightly higher seeding rate due to larger seed size.

Further investigation showed that triploids could be produced from 4x - 2x crosses (Taylor and Wiseman, 1987). Crosses of the triploids led to the production of trisomics (Taylor and Chen, 1988). Eventually, all the seven different trisomics ($2n$ = 15) in red clover were produced and maintained vegetatively (Taylor, 1987). These were converted for seed maintenance by crossing each trisome with a self fertile stock (Taylor, 1993). These stocks are now available for studies by other investigators.

S-allele and single and doublecross hybrids:

These studies investigated the possibility of producing single and double cross hybrids using S-alleles to control crossing. Along with W.A. Kendall, it was found that red clover, normally self incompatible, could be inbred by use of elevated temperature during the selfing process (Kendall and Taylor, 1969). A graduate student, Karl Johnson, determined that homozygous S-alleles, i.e. S_1S_1 or S_2S_2, could be isolated by inbreeding (Johnston *et al*., 1968). It was further determined that when the low vigor inbreds were crossed, the progeny exhibited heterosis and vigor was restored (Taylor *et al*,. 1970). Statistical input to this research was provided by J.C. Williams, (1966 - 1969) statistician, who joined the department from the University of California at Davis (where N.L. Taylor was on sabbatical leave in 1965-1966). Pseudo self-compatibility (term used to describe self seed set in normally self-incompatible plants) declined as plants were inbred, effectively limiting inbreeding to one generation (Duncan *et al*., 1973). Some question was raised as to whether new S-alleles were generated but, due to possibility of contamination, was difficult to prove (Anderson *et al*., 1974, Taylor, 1982).

Doublecross hybrid red clover became a reality about 1970 (Anderson *et al.*, 1972) and Taylor and Anderson, 1974). The S-allele system was effective in controlling crossing but two difficulties became apparen:t: (1) inbred clones were reduced in vigor and difficult to produce and maintain in sufficient quantity and, (2) the hybrid yielded no more forage than Kenstar, a related 10-clone synthetic cultivar. To overcome the latter problem, combining ability of non-inbred clones and later combining ability of inbred clones were studied (Anderson *et al.*, 1974, and Cornelius *et al.*, 1977). The maximum number of single crosses we were able to handle was 45. This involved all possible single crosses of 10 inbred clones (nxn-1)/2. These progenies were tested at several locations, but we were not able to isolate high combiners from this limited number of crosses. Although funding did not permit more extensive testing, the utility of the program was amply demonstrated.

Phenotypic Recurrent Selection (PRS):

Two methods of breeding red clover, i.e. the polycross method and the single or double cross hybrid method yielded cultivars or germplasm. However, the labor involved was extensive necessitating consideration of using a less laborious procedure. To test the feasibility of phenotypic recurrent selection (PRS), a program to increase the intensity of flower color in red clover was conducted. In a Kenstar seed field in Fleming County, a deep red flower was observed. The plant was marked and seed was harvested at maturity. To determine the original of this unusual color, annual selection for deeper red color in normal red clover was conducted over several generations. The result was an almost purple flower color which, incidentally, was pictured on the cover of the Journal of Heredity (Cornelius and Taylor, 1981).

In view of the success with PRS for flower color, a program was initiated to improve seed and forage yield in zigzag clover. A four-generation program was successful but many more generations of selection would be necessary to change zigzag clover into a useful forage species for Kentucky conditions (Taylor *et al.*, 1984). PRS was then applied to stem length in red clover in cooperation with a Ph.D. candidate from Canada, S.R. Bowley. Although the program was successful in increasing stem length, the taller plants produced were less persistent than normal red clover both in wide and narrow spacings (Bowley *et al.*, 1984). Apparently tall plants translocate too much carbohydrate to their tops and not enough to their roots to allow for the necessary persistence. The tall genotypes should produce a very productive annual red clover. However, such a clover is not nearly as much in demand as the perennial type.

The next PRS program was for multiple-parted heads in red clover. Multiple-parted heads produce more seeds per head than normal red clover but fewer heads, so that seed yields per unit area were not increased. Concurrent selection for multiple parted heads and high seed yield might be more effective (Taylor *et al.*, 1985).

Shortly after the multiple-parted head selection was completed and published, Moss G. Long, technician, retired in 1984 after 30 years of service. He was replaced by John Nozell (1985 - 1986) who was succeeded by Ty Edmonson (1985 - 1994), Rich Mundell (1994 – 2001), Gene Olson (2002-2004), and finally by Ghabriel Roberts (2005 - present in a half-time position).

PRS was also used for improvement of flowering and seed yields in kura clover (Taylor and Cornelius, 1994). Again the program was effective in developing a first-year flowering type. However, inbreeding depression, apparently associated with using fewer than 100 plants per cycle of selection, reduced forage and seed yields. The first-year flowering types were outcrossed to unrelated Rhizo plants to restore vigor.

The next PRS program was to incorporate resistance to northern anthracnose into Kenstar cultivar genetic background. The program was effective and a germplasm (KNARS, later named Kenton) was developed (Taylor *et al.*, 1990). This program was begun in Wisconsin in cooperation with R.R. Smith when N.L. Taylor was on sabbatic leave in 1976-1977. Along with KVMRS,(later named Kenway), selection for seed yield was conducted by Kevin McVeigh (Willamette Plant Breeders). Kenton was selected for profuse blooming during two one-year cycles and was released to Production Services Intl. managed by Gary Cooper (Cornelius, OR). Kenway was released after one year of selection for profuse blooming to Smith Seed Services (Halsey, OR). Contact person is David Keister. Royalties have been received from Smith Seed Services and seed has been marketed in Eastern United States. No seed of Kenton has been marketed to date.

PRS is continuing to be used to improve kura clover for seed and forage yield, and red clover for freedom from pubescence to reduce dustiness and increase drying rate. A non-pubescent cultivar named Freedom! was released in 2001 on a exclusive basis to Barenbrug, USA (Taylor, and Collins, 2003).Subsequently, Freedom! was found to be susceptible to powdery mildew caused by *Erysiphe polygoni* .Five cycles of phenotypic recurrent selection were used to develop FreedomMR from Freedom!. (Taylor, 2008) This variety was also released on an exclusive basis to Barenbrug and royalties have been applied to the breeding program.

Genetic stability:

Soon after the National Foundation Seed Program was initiated, it became apparent that genetic shifts were important factors in loss of adaption of cultivars.

Certified seed produced in western United States was less productive and less persistent than the breeder seed that was sent west. Therefore, investigations were begun in cooperation with C.S. Garrison (USDA) to determine the causal factors involved and steps that could be undertaken to assure that such change did not occur, or at least would be minimized. Based on the results of grow-outs of seed lots increased in various locations, classes, crop years, and lineages, it was concluded that seed of Kenland red clover should be confined to the northern latitudes (north of the California border), that the registered seed class should be eliminated, that no advantages existed of third year over second crop year's production, and that any program that selected for earliness in the western states led to reduced production and persistence in the eastern states (Taylor *et al.*, 1979). One outstanding observation was the increased pubescence of clover grown in central California. Based on this evidence, pubescence is a response to stress tolerance rather than response to insect attack as has long been thought to account for pubescence in the United States- grown red clover .in contrast to that of Europe..

Investigation of genetic stability was repeated using the Kenstar cultivar. In this study, treatments were set up to generate specific seed lots rather than by using seed lots previously generated as in the Kenland study. Nevertheless, the conclusions were very similar. Negative correlations of bloom with survival was again shown, indicating that early blooming was associated with lowered persistence. The Washington location was recommended for seed increases because less shift occurred than at the California location (Taylor *et al.*, 1991).

Tissue Culture and somaclonal variation:

Creation of variability in red clover was stimulated by the possibility of increasing longevity. Although red clover is extremely variable, variation in persistence is limited. The first investigation into inducing variation occurred early with gamma and neutron irradiation of seeds (Taylor *et al.*, 1961). It was concluded that red clover, being self-incompatible, was not ideal material for irradiation studies because it could not be inbred easily and thus segregation of induced recessive genes was difficult if not possible.

Later research involved developing a suitable media for red clover explant growth (Keyes *et al.*, 1980, Collins *et al.*, 1982), and eventually for embryo rescue (Phillips *et al.*, 1982). Both approaches were successful and led to the securing of a grant to investigate somaclonal variation in red clover (Bagley and Taylor, 1987). Large numbers of somaclones were produced but unfortunately many were sterile, and those that were not sterile could not be inbred to isolate the homozygous recessive genotypes. Thus, the same problem of creating variation occurred as with irradiation. Later, an *in vitro* chromosome doubling method was devised that involved adding colchicines to tissue culture media (Anderson *et al.*, 1991).

Breeding for disease resistance:

Disease resistance has always been an important aspect in the improvement of red clover. The cultivar Kenland was bred for resistance to southern anthracnose caused by *Colletotrichum trifolii* B & E, with the result that this disease has been eliminated not only in Kentucky but in other states, wherever Kenland has been used as a parent cultivar in the development of varieties. In 1977, a program was begun to incorporate northern anthracnose resistance into Kenland and Kenstar related materials (Taylor *et al.*, 1990). (See previous section on phenotypic recurrent selection).

Virus diseases continually have been a problem in maintenance of clonal populations, which necessitated considerable investigation into causal factors and inheritance of resistance (Taylor *et al.*, 1986). The most important virus in Kentucky is bean yellow mosaic virus (BYMV) and single race resistance (hypersensitivity reaction) is controlled by a single gene. The impact of BYMV on red clover production was expanded in connection with a regional project (S-127) entitled Virus Diseases of Forage Legumes, which was discontinued in 1998. A major objective of that project was to determine the importance of virus diseases in reducing stands and yields of red and white clover. These investigations were cooperative with S.A. Ghabrial (Plant Pathology) and USDA personnel at Mississippi State University. Two experiments were established but, due to loss of red clover stands at Mississippi, these data were not published. Results of the white clover experiment were similar, and indicated that resistance to virus diseases were very important for clonal maintenance and under spaced plant conditions, but were considerably less so under broadcast field conditions (Taylor *et al.*, 1995). This research led to the release of a virus and powdery mildew resistant germplasm which is also being selected for increased seed yield in Oregon (KVMRS) (Taylor and Ghabrial, 1995).

***Trifolium* species curatorship**:

Beginning in 1954, seeds of *Trifolium* species were collected primarily for interspecific hybridization. Later, as the importance of germplasm preservation came to be realized, curatorship of the collection became the primary goal. The objectives of the program eventually became to collect, evaluate, maintain, and distribute seeds of the approximately 230 species of the genus. The importance of the collection was realized by the USDA National Plant Germplasm System (NPGS) and was supported by an annual grant of $5000. This was increased to $10,000 in 1987, but was discontinued in 1995. However, the program at Kentucky continued to the point that now about 2500 accessions representing 206 species are being maintained.

Collecting:

The USDA collection at Beltsville, Maryland, maintained by E.A. Hollowell, was transferred in part to Kentucky early in the program. This initial group of seeds of species was augmented by donations upon request from botanic gardens, of which Kew Botanic Garden, United Kingdom, is an example, and from germplasm centers of which the New Zealand collection (later to be named the Margot Forde Germplasm Centre) is an example. Other examples are the Australian *Trifolium* Genetic Resource Centre at Perth; the International Lifestock Centre for Africa, Addis Aboba, Ethiopia; and ICARDA in Leppo, Syria.

Seeds were also collected during non-federally financed collection trips and sabbatical leaves. A fairly large collection was received from Don Cornelius, Berkeley, California who had collected in Turkey. Also received at that time were seeds of California native species partly supplied by Beecher Crampton, University of California at Davis, and partly collected by N.L. Taylor during a sabbatical in 1965-66. Eastern United States species were collected during domestic travels in Kentucky (*T.*

stoloniferum and *T. reflexum*), Tennessee and Virginia (*T. calcaricum*), West Virginia (*T. virginicum*), Alabama, Texas and Mississippi (*T. reflexum*), and Florida (*T. carolinianum*). Overseas travel also led to several collections, namely New Zealand (sabbatic leave), Australia, Sweden, Scotland, Turkey, and Spain .

Formal collection trips (financed by NPGS) were begun in 1977 when R.R. Smith collected in Greece, Italy and Crete. This collection was examined for nomenclature during Taylor's sabbatic leave in Wisconsin in 1977. Other collection trips were to Romania in 1985 (Taylor and Rumbaugh), Yugoslavia (Taylor and Smith) and western United States in 1995 and 1996 (three trips; Taylor and Quesenberry). The last species collected was *T. wigginsii* from Baja California in September 2000 (funded by a $4300 grant from the National Plant Germplasm System of USDA.

Maintenance:

In the early stages of the program (approximately 1953-1960), seeds were stored in paper packets or envelopes in wooden cabinets located in the controlled temperature of the University of Kentucky seed house. Temperature was about 45^{o}F with about 40% relative humidity. Seeds did not preserve well under these conditions. Shortly thereafter, research at Prosser, Washington indicated that *Trifolium* seeds would maintain viability almost indefinitely when stored at -5 to -15^{o}C. Accordingly, all seeds were moved to non-defrosting freezers in paper envelopes inside plastic boxes. The boxes were considered necessary in case of melt down following electrical outages. Alarms were also installed. Except for occasional electrical outages, seeds have been maintained in this manner since about 1960. Poor quality seeds from the inadequate prior storage did not maintain viability well, but fresh seeds were adequately maintained.

Inasmuch as only a few seeds of an accession were received, it was necessary to increase seeds in a greenhouse for the self pollinated annuals, and under field positions in isolation for the cross pollinated, usually perennial, species

.**Evaluation**:

In connection with seed increase, opportunities existed for evaluation of genetic relationships among taxa, as well as to identify individual accessions. In the late 1970's, sufficient information was advisable to establish genetic system relationships in the genus. A total of 207 species (not all on hand) were examined and classified according to various criteria. The collection consisted of about 67% annuals and 33% perennials. 86.6% were diploid, and 6.7% were tetraploids. Species introduced from Europe varied in base chromosome numbers from five to eight and were the most diverse that Europe was the origin of the genus. Annuals were mostly self pollinated and perennials cross pollinated. Different flower colors and leafmarks were not associated with origins, climate, and other morphological and physical characteristics.

To resolve problems of nomenclature, chromosome numbers of species were determined (Giri *et al.*, 1981). The first of these groups were the hop clovers (Section *Chronosemium*) in which it was determined that chromosome number was independent of such morphological characters as yellow-green seeds, central leaflet attachment (sessile vs. elongated), and longevity (Taylor *et al.*, 1982). The next section was examined was *Mistyllus*, which contains species closely related to arrowleaf clover (*T.*

vesiculosum). It was concluded that *T. mutabile* and *T. leiocalycinum* are closely related to *T. vesiculosum* and the three should be isolated under field conditions to prevent crossing (Taylor and Giri, 1984). Crossing and morphological relationships among species within the section *Vesicaria* (the bladder clovers) indicated that the annual *T. resupinatum* (Persian clover) and *T. clusii* (reversed clover) are closely related to each other, as are the perennial *T. neglectum* and *T. fragiferum* (strawberry clover). The other two perennial species, *T. tumens* and *T. physodes* are distantly related to the other species as well as to each other (Taylor and Gillett, 1988).

Crossing and morphological relationships were also examined in the native clovers of eastern North America (Section Lotoidea). The annual species *T. carolinianum*, *T. bejariense*, *and T. reflexum* are mostly self pollinated. The perennial species *T. virginicum*, *T. calcaricum* and *T. stoloniferum* are all cross pollinated in nature but *T. stoloniferum* may set self seeds if no other parent is available. Selfing results in reduced vigor but probably does not account for the near extinction of *T. stoloniferum.* None of the species could be crossed with *T. repens* (white clover) (Taylor *et al*., 1994).

The relations of chromosome number to other characteristics were examined in zigzag clover *(T. medium*). Chromosome numbers ranged from octoploid ($2n$ = 48), to hexaploid ($2n$ = 64), to decaploid ($2n$ = 80). Numbers intermediate between the octoploid and the decaploid were found but not between the hexaploid and the decaploid. Accessions from lower latitudes (northern hemisphere) tended to be more vigorous and higher seed-yielding than those from the higher latitudes, although no accessions were found promising for forage production (Taylor, 1994).

The latest research concerns the collection, distribution, and characteristics of native American *Trifolium* species in relation to molecular classifications. Internally transcribed spacer analysis (ITS) was used to resolve taxonomic problem (Nick Ellison et al., 2008.. ITS analysis can be used to identify species that are otherwise almost impossible to identify based on conventional taxonomic methods. Seeds, live plant material or leaf material from herbarium specimens, many of which may be quite old, can be used as the source material for analysis. Preliminary results (Steiner and Taylor, 1998) indicated that several species in the curator collection (CLO in GRIN) need to be reclassified. Other results indicate that the section *Chronosemium* (hop clovers) are more distantly related to the remainder of the genus than are other genera such as *Medicago* and *Melilotus*. ITS analysis resolved important problems in the evolution of the genus, for example, the ancestry of the tetraploid *T. repens* which has been in doubt, despite much research on the subject. ITS analysis is useful to identify close relationships among species and to predict those which are more likely candidates for interspecific hybridization or those which are amenable for gene transfer.

Distribution:

Generally, if sufficient seeds are available, seeds are distributed to interested parties as requested. The seed lists are made available to other gene banks so that gaps in collections may be filled. Seeds have been exchanged with gene banks in New Zealand (Margot Forde), Australia (Kevin Foster), Icarda, Syria (Ali Shebadeh), and Miami University, Ohio (Michael Vincent) as well as others over the years. To prevent loss of valuable species seeds, a major effort was undertaken in 1997 and 1998 to back up all Kentucky seed stocks at the National Seed Storage Laboratory in Fort Collins, Colorado. The Kentucky seed collection is listed as CLO in the Germplasm Resources Information Network (GRIN). However, the list of holdings is incomplete. The latest count indicates that seed of 206 species and about 2500 accessions are stored at Kentucky. This is the largest collection of *Trifolium* species in the world. Most, but not all, are backed up at NSSL as more accessions and species are being added periodically.

Species remaining to be collected from the three centers are indicated below:

Eurasia	**Africa**	**America**
bivonae	*stolzii*	*siskiyouense*
parnassii	*chilaloense*	
congestum	*lanceolatum*	*haydenii*
pilczii		*vestitum*

wettsteinii	*ukingense*	*andinum*
prophetorum		*mucronatum*
radicosum		
saxatile		
eximium		
pachycalyx		
sintenisii		
sebastianii		

The 206 species on hand plus the 22 remaining to be collected total 228. This number is approximately the total number of species in the genus. Differences among authorities as to validity of taxons (see previous section) and occasional recognition of new species will change the total.

Summary:

The results of clover research have been summarized over the years in a series of review papers, books and book chapters. The first of these summarized all known information on red clover up to 1960 (Fergus and Hollowell, 1960). As the amount of research increased, the content of review chapters was subdivided into breeding and genetics (Taylor and Smith, 1979), and into physiology and morphology (Bowley *et al*., 1984). A major effort was *Clover Science and Technology*, a monograph sponsored by the American Society of Agronomy, consisting of 616 pages with 27 chapters and 33 authors. All aspects of clovers used in agriculture were treated, as well as taxonomy, breeding, and culture of the many clover species (Taylor, 1985). The book was dedicated to E.N. Fergus and E.A. Hollowell, the two early pioneers in clover investigations in the United States.

The next major effort was *Red Clover Science* (Taylor and Quesenberry, 1996), written while Taylor was on sabbatic leave at the University of Florida in 1992-3. Quesenberry finished his part while on sabbatic leave at Oregon State University in 1994. The manuscript was sent to as many as 20 publishers and finally Kluwer Academic Publishers of the Netherlands agreed to publish it as a contribution to their series, “Current Plant Science and Biotechnology in Agriculture”. This is the first and only book written on the science of red clover. It deals with red clover research in all countries of the world since about 1984 and was reviewed by 21 authorities assigned to the 17 chapters (226 pages).

The latest up-to-date information on kura clover, a new important forage legume, was published in 1998 (Taylor and Smith, 1998). Subject matter covered included breeding, culture, and utilization.

Finally the last book published was “*The World of Clovers”* consisting of a searchable data base on a computer disk of 228 species of the genus *Trifolium*. It provides a description and plant and seed photographs of each species. A color CD is in a sleeve attached to the black and white monograph (Gillett and Taylor, 2001).

The last major work was a review article published in Crop Science entitled ‘ A century of clover breeding developments in the United States".(Taylor,2008).This paper summarizes the current thinking concerning persistence and yield in red clover although other perennial species such as kura clover are given some consideration.

Unfinished business

A considerable number of projects are underway that should be continued. This includes cultivars that have been released, of which the Kentucky Agriculture Experiment Station has an obligation to maintain breeder seed. The first of these is Kenland, the seed of which has been maintained since 1947. Maintenance of the seed over this period has probably increased the adaptability and general hardiness of the cultivar. The second cultivar was Freedom! released in 2001. The third is Kentucky Northern Anthracnose Resistant Synthetic, KNARS (later Kenton). The .fourth red clover cultivar is Kentucky Virus and Mildew Resistant Synthetic , KVMRS (later Kenway), and the fifth is FreedomMR. One multileaf cultivar ,Lucky has been released, but has not been assigned to a seed company for marketing..One white clover cultivar, Ky Select, has been exclusively released to Saddle Butte Ag Inc.of Shedd, OR.

Breeding programs underway that need to be carried forward to a logical conclusion, i.e. release,(if merited) include the addition of mildew resistance to Kenland which is in the third cycle of selection. Possibly another cycle will be necessary before this material can be released as Kenland Plus The second red clover program involves selection for low phenolic content (reduces blackening during the curing process, with the potential of producing green rather than black hay). Thirteen cycles of field selection have been conducted and the resultant synthetic hay appears somewhat greener after curing than Freedom!, its parent cultivar. However, the synthetic is not uniform and further selection, perhaps under laboratory conditions before this material will be ready to release.

A tetraploid breeding program is underway and the first seed was increased in year 2000. Unfortunately, although tetraploid cultivars are successful in Europe, and yield more forage than diploids, this has not been the case in the United States. Whether this is due to low stress tolerance of tetraploids is unknown but is a possibility. The Kentucky tetraploid, as yet unnamed, is ready for full scale testing and further selection if necessary.

Three generations of a selection program to develop 2,4-D resistance in red clover, are complete but at least two more generations will be necessary for the required level of resistance.

The Kentucky *Trifolium* curator collection must be maintained inasmuch as it is the most complete collection of the *Trifolium* seeds in the world. Research should be continued to examine species identifications and relationships in cooperation with New Zealand and Oregon State personnel.

The kura clover breeding program has been discontinued due to inability to make much progress in breeding for early and late types. A germplasm may be released.

Conclusions:

Considerable information has been accumulated on the clovers over the years. As far as breeding is concerned, it is now apparent that the most effective method of breeding is phenotypic recurrent selection conducted annually. Heredities are high enough for most characters so that progeny tests, while effective, are too time-consuming and costly to be used for programs with limited funding. The same is true for production of double and/or single cross hybrids. These programs can be effective but are too costly.

In addition, a major change has occurred in American agriculture with the advent of the corn and soybean economy and declining emphasis on forage legumes. The low price of nitrogen has been a factor in the lessened use of legumes for livestock feeding. These changes are well documented in Chapter 15 of *Red Clover Science,* which shows the decline in seed production from about 1950 to the present. However, the recent increase in price of nitrogen has created more interest in use of forage legumes. Also, shift in seed production to the western states because of higher seed yields has placed an added burden on forage legume breeders because superior cultivars may fail because of low seed production in the west. Programs must be devised to overcome this problem.

In spite of all these difficulties, the use of red clover is expected to continue because of its unique qualities: ease of establishment, competitiveness under low light intensity, high palatability and digestibility, and general all-around adaptability to many areas of the temperature world.

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Table 1. Cultivars and other germplasm developed and released by the clover breeding program at the University of Kentucky.

Cultivars	Species	Date of release
Kenland	Red clover	1947
Kenstar	Red clover	1974
Appalow	Lespedeza	1981
Rhizo	Kura clover	1989
Kenton	Red clover	1997
Freedom!	Red clover	2001
Kenway	Red clover	2004
Lucky	Red clover	2005
Ky Select	Red clover	2006
FreedomMR	White clover	2006

Germplasms		
T. pratense x T. diffusum	Hybrid GP-17	1973
T. medium x *T. sarosiense*	Hybrid GP-18	*1978*
T sarosiense x T. alpestre	Hybrid	*1978*
Introduction bulk	Red clover	*1979*
Creeping rooted	Alfalfa GP-105-107	1980
Gene Marker Stocks	Red Clover GP-1 to 11	1982
Ky M-1	Zigzag clover GP-43	1982
Diploid and derived tetraploid	Red clover GP-12, 13	1983
BYMV Resistant	Red clover GP-52	1986

C-1	Crimson clover GP-60	1986
Trisomic source	Red clover GP-14	1987
Interspecific cross	Hybrids GP-17, 18	1989
Tetraploid 2x-4x and 4x-2x	Red clover GP-20	1989
Tetraploid *T. ambiguum* x *T. repens*	Hybrid GP-82	1990
Octoploid T. ambiguum x *T. repens*	Hybrid TP-95	1991
Ky-1	Kura clover TP-90	1991
Ky M-2	Zigzag clover TP-96	1991
Mildew and virus resistant	Red clover GP-22	1995
Hexaploid *T. ambiguum* x *T. repens*	Hybrid GP-177	1998
Self-fertile trisomics	Red clover GS-1 to 7	1993
Genetic marker	Red clover GS-8, 9, 10	1995
Tetraploids	*T. species* GS-4 to 7	1997
Genetic pool	Red clover GS-11	1997
Multiple cotyledon	Red clover GS-9	1999
Ruffled leaflet (tetraploid)	Red clover GS-12	2000
Short floret	Redv clover	2004
Accessions in GRIN with N.L. Taylor as source: 721.		

Table 2. Dates of employment of University of Kentucky personnel who conducted research on red clover.

Employee	Starting Date	Ending Date
M.K. Anderson	1969	1974
R.C. Buckner	1948	1986
Mike Collins	1985	2005
P.L. Cornelius	1972	Present
Charles Dougherty	1978	Present
Ty Edmonson	1985	1993
E.N. Fergus	1920	1960
Carol Keller	1966	1969
W.A. Kendall	1954	1970
Moss Long	1954	1984
Richard E. Mundell	1994	2001t
John Nozell	1985	1986
Gene Olson	2004	2006
Ghabriel Roberts	2006	Present
James Shane	1951	1970
Roy Sigafus	1950	1986
W.H. Stroube	1955	1965
Norman L. Taylor	1953	2001
T.H. Taylor	1955	1983
W.C. Templeton	1946	1978
Warren Thompson	1958	1973
Elizabeth Williams	1986	1989
J.C. Williams	1966	1969