GENE FREQUENCIES IN WILD POPULATIONS OF *TRIFOLIUM REPENS* L.

IV. MECHANISM OF NATURAL SELECTION

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1. INTRODUCTION

ONE of the greatest challenges to investigators of evolution is the understanding of selection mechanisms and particularly the factors which influence the gene frequencies in populations (Huxley, 1955).

Natural selection favours the establishment of close linkages between the components of well-balanced combinations. Mather (1943) proposed the linked balanced polygenic complex. Dobzhansky (1951, 1957) emphasised the co-adaptive gene complex, while Parsons and Bodmer (1961) argued that balanced polymorphisms have evolved by the accumulation of linked interacting modifiers. In spite of the numerous presentations of theoretical models, very limited experimental data is available on linked interacting polymorphic gene complexes.

Polymorphism in *Trifolium repens* may be suitable for the investigation of some aspects of the above theories. The production of lotaustralin and linamarase in *T. repens* is determined by two independent genes designated *Ac* and *Li* (Corkill, 1942; Atwood and Sullivan, 1943), while modifying genes determine the quantity of lotaustralin produced (Corkill, 1942). Crude "lotaustralin" as described throughout this paper consists of 80 per cent. lotaustralin and 20 per cent. linamarin (Melville and Doak, 1940). Lotaustralin can be hydrolysed by the cyanogenetic glucosidase, linamarase (Coop, 1940), yielding one molecule of each of methyl-ethyl-ketone, glucose, HCN and H₂O from lotaustralin; linamarin yields acetone instead of methyl-ethyl-ketone.

The geographical distribution of the *Ac* and *Li* gene frequencies in wild populations and strains of *T. repens* has been shown in previous investigations (Daday, 1954a, 1958). These studies established that natural selection formed *Ac* and *Li* gene frequency clines with a range of 0-100 per cent. in Europe, Asia and Africa where the species is indigenous; and, the formation of *Ac* and *Li* clines appeared to be in progress in U.S.A., Canada and Australia where the species has been introduced.

Two theories have been advanced to explain the mechanism of natural selection. Corkill (1952) and Jones (1962) showed that the cyanogenetic phenotypes are less favoured by animal predators;

Barber (1955), and Jones (1962) suggested that selective eating of cyanogenetic plants is reflected in the gene frequencies of populations of *T. repens* and *Lotus corniculatus* L.

Daday (1958) demonstrated a significant association between diminishing *Ac* and *Li* gene frequencies of *T. repens* and decreasing mean winter temperature and suggested that the major factor in natural selection was associated with temperature-dependent physiological processes within the plants.

The present investigation was undertaken to study the responses of the genotypes in terms of fitness components to diverse temperature and day length conditions. The observed fitness values were used to predict the gene frequency changes which would occur in each of a number of environments. These changes then were compared with the gene frequencies found in natural habitats of *T. repens*.

2. METHODS

The *T. repens* populations used in this experiment were derived from randomly selected plants of the British S100 strain of *T. repens* (controlled environment experiment) and a commercially available strain New Zealand Mother Seed (field experiment). Both populations contained all four chemical genotypes; viz. *AcLi* (lotaustralin and linamarase), *Acli* (lotaustralin only), *acLi* (Linamarase only), and *acli* (neither lotaustralin nor linamarase).

The four genotypes were distinguished by using the modified picric acid paper technique (Daday, 1954*b*). The reaction of picric acid with HCN was compared with a colour chart scale of 0 to 6.

Controlled environment experiment

The randomly selected plants of the four genotypes were grown in pots under glasshouse conditions, and exposed to the following four treatments:

- (a) Warm temperature (30° day/25° C. night) and natural short day (up to 13.35 hours for *T. repens*) in a heated glasshouse.
- (b) Warm temperature (30/25° C.) and long days (16 hours), natural daylight extended with incandescent lamps to give 20-25 ft candles at plant level, in a heated glasshouse.
- (c) Cold temperature (15/10° C.) and natural short day (up to 13.40 hours daily), in the environment-controlled glasshouse.
- (d) Cold temperature (15/10° C.) and long day (16 hours) provided by extending the natural day by lamps to give 40-50 ft candles at plant level in the controlled environment glasshouse.

Treatments (a) and (b) were applied for 75 days while those of (c) and (d) were applied for 62 days.

The number of plants per treatment varied from 28 to 81.

Field experiment

The four genotypes were transplanted when six weeks old, in July 1960, at Milton on the south coast of N.S.W., with a latitude of 34° 52', an altitude of 50 metres, a mean January (mid-summer) temperature of 22° C., and a mean July (mid-winter) temperature of 13° C. (Bureau of Meteorology, 1956). Two of the four genotypes, *AcLi* and *acli*, were transplanted at Perisher (Snowy Mountains, N.S.W. and described as alpine environment in this paper) in November 1959; with a latitude of 36° 26', and altitude of 1800 metres, a mean January temperature

of 12° C., and a mean July temperature of -2° C. One hundred and ninety-two plants were planted at 30 × 30 cm. distances and replicated four times at Milton (described as coastal environment). Three hundred and fifty plants of two genotypes were established in a spaced plant trial with seven replications at the Perisher nursery.

Observations included the number of plants in flower, number of flower heads, seed production, dry matter production, plant diameter and frost damage.

3. RESULTS

Controlled environment observations

To evaluate the relative roles of temperature, day length and their interaction as selective agents the proportion of plants of the four genotypes which flowered under various controlled conditions were observed.

The results of this investigation (table 1) show *acLi* and *acli* to be superior to the other genotypes under cold conditions and *acli* is inferior under warm, short-day conditions.

TABLE 1
*Effect of temperature and day length on the percentage of plants
flowering under controlled environments*

Temperature	Day length	Genotypes			
		<i>AcLi</i>	<i>Acli</i>	<i>acLi</i>	<i>acli</i>
Warm	Short	100	100	98 ± 2.2	85 ± 6.2
	Long	100	100	100	100
Cool	Short	6 ± 2.6	18 ± 4.7	45 ± 6.4	46 ± 6.1
	Long	15 ± 4.0	17 ± 4.6	46 ± 6.3	47 ± 6.0

These results suggest that the selective advantage of *AcLi*, *Acli*, *acLi* occurs in warm, short-day environments whereas the selective advantage of the double recessive occurs under cool conditions.

The Hardy-Weinberg law postulates stable gene frequencies in a large random mating population, in the absence of selection pressure. But if selection pressure operates, the contribution of the gametes from the unfavoured genotypes will be reduced, and gene frequencies will change. The proportionate contribution of *Ac*, *Li* gene frequencies of *T. repens* populations to the next generation will depend on the response of genotypes in a given environment. The expected changes in gene frequencies under various selection pressures were computed using the mean flowering percentage of genotypes as the sole criterion of fitness. The input gene frequencies were set at 0.50 for both *Ac* and *Li*, and the fitness values of genotypes in a short day, warm temperature environment were calculated from the proportion of plants flowering after 75 days (table 1) or 42 days in a controlled

FIG. 1.—Expected response to selection of *Ac* and *Li* gene frequencies.
— Short day/warm temperature—42 days
- - - Short day/cold—62 days
- . - Long day/warm—75 days
— Long day/cold—62 days

environment. The observations after 42 days gave indications of the early responses of genotypes. The relative fitness values at 75 days were *AcLi*, 0.2611; *Acli*, 0.2611; *acLi*, 0.2559; and *acli*, 0.2219. At 42 days the selection differential was even greater, and the relative fitness values were: *AcLi*, 0.3333; *Acli*, 0.2889; *acLi*, 0.2400, *acli*, 0.1378. The expected responses of *Ac* and *Li* gene frequencies under these two types of selection pressures were estimated and are presented in fig. 1.

Under intense selection pressure the *Ac* and *Li* gene frequencies shifted from 0.50 to 0.88 for *Ac* and to 0.78 for *Li* during 25 generations, while a further 475 generations are required to move up from these values close to 0.99, assuming non-overlapping generations. A similar pattern was found for the less intense selection pressure, but the response rate was markedly less.

In contrast to this situation the dominant alleles are at a disadvantage under cold conditions. The relative fitness values, calculated from the flowering data in table 1 are: *AcLi*, 0.0541; *Acli*, 0.1573; *acLi*, 0.3893; *acli*, 0.3993 in short days, and *AcLi*, 0.1187; *Acli*, 0.1355; *acLi*, 0.3681; *acli*, 0.3777 in long days, the *Ac* and *Li* allele frequencies fell rapidly under both conditions, *Ac* being eliminated in 13 generations and *Li* reduced to 15 per cent. in 100 generations.

Field observations

The results of the controlled environment experiment support the hypothesis that the genotypes differ in their physiological responses to temperature and day length. The investigation was extended to a more extensive field experiment.

In nature competitive ability of *T. repens* depends on asexual as well as sexual reproductive capacities, and their relative importance varies in different environments. Studies were therefore made on both flowering and morphological properties and the results are shown in table 2.

The *AcLi*, *Acli* and *acLi* genotypes have a significantly higher percentage of flowering plants than has *acli* in the coastal environment, whereas in the alpine environment the *acli* plants show a higher flowering plant percentage than *AcLi*. The magnitude of differences between genotypes varied between dates of observations, causing a significant date $\times$ genotype interaction ($P < 0.05$ and $P < 0.001$ in the coastal and alpine environments respectively). The flowering percentages of the four genotypes in spring were: *AcLi* 57, *Acli* 67, *acLi* 57 and *acli* 28, while during mid-summer all plants flowered at the coast. Most of the annual seed yield is produced in spring because the plants are damaged by the higher summer temperatures. Thus this greater differentiation of genotypes during the spring is likely to be of major importance in selection.

Differentiation between genotypes also occurred in the Snowy Mountains, but here the *acli* genotype was favoured. Early summer

observations were: *AcLi* 48 and *acLi* 85 while in mid-summer all were 100 per cent. Combined analyses of variances showed a significant ($P < 0.001$) locality $\times$ genotype interaction (V.R. = 133.06; when V.R. = 22.86 for $n_1 = 1$, $n_2 = 9$, $P = 0.001$).

When total sexual reproductive capacity is-considered, both the number of plants in flower and the number of flowers on each plant

TABLE 2
*Physiological and morphological properties of the genotypes in T. repens
in two environments*

Genotypes	Flowering plant percentages *	No. of flower heads/plant	No. of seed/head	Dry matter production	Plant diameter cm.	Frost damage scorings
Coastal environment						
<i>AcLi</i>	80.0 (63.1)†	24.5	13.8	1.23†	17.4	0
<i>AcLi</i>	86.0 (67.7)	25.5	13.9	1.09	16.0	0
<i>acLi</i>	75.0 (59.8)	22.6	4.3	1.01	14.4	0
<i>acLi</i>	62.0 (51.8)	20.6	6.1	0.95	16.0	0
L.S.D.						
P = 0.05	(1.7)	N.S.	6.0	0.04	0.78	N.S.
P = 0.01	(2.4)	...	9.1	0.06	1.13	...
Alpine environment						
<i>AcLi</i>	91.0 (72.2)	34.4	0	1.27	13.0	2.42
<i>acLi</i>	96.0 (78.6)	43.2	0	1.26	12.1	1.82
L.S.D.						
P = 0.05	(0.8)	N.S.	N.S.	N.S.	N.S.	0.53
P = 0.01	(1.3)	...	...	...	...	0.80

* Mean of flowering percentages taken in spring and summer.

† log. gm./plant.

‡ Values according to angular transformation.

are important. Although the mean number of flowers per plant do not differ significantly between the genotypes at the coast or at Snowy Mountains the trends in flower number follows the same pattern already established for percentages of plants flowering.

The seed production of the various genotypes at the coast also confirmed this pattern. No seed yield at all was recorded in the alpine environment although both experiments were provided with bees.

The conclusion from these observations is that the various genotypes when exposed to different environmental conditions, differ in sexual reproductive ability and therefore in their relative fitness under the different environments.

Since *T. repens* is a perennial and has spreading stolons, vegetative competitive ability in terms of dry matter yield and diameter of the plants was also examined.

The order of vegetative productivity in the coastal environment was *AcLi*, *Acli*, *acLi*, *acli*. The lack of any significant date of harvest $\times$ genotype interaction indicates that the order of vegetative productivity was not being determined by the different flowering behaviour of the genotype. In contrast to their performance at the coast, the *AcLi*, and *acli* genotypes did not differ significantly in alpine environment.

Since the growth habit is prostrate and uniformly spreading, the mean diameter of the plants was chosen as another estimate of competitive ability. Again in the coastal environment the *AcLi* genotype was significantly superior to the other genotypes. There was no significant difference between genotypes in the alpine environment.

TABLE 3

Computed change in frequencies of Ac and Li alleles after 100 generations of natural selection using fitness values derived from a field experiment in a coastal environment

Plant character	Expected gene frequencies	
	<i>Ac</i>	<i>Li</i>
Mean flowering plant percentages	0.9529	0.0043
Mean number of flower heads per plant	0.9273	0.0723
Mean number of seed per head	0.9847	0.3507
Mean dry matter production	0.9753	0.9629
Mean plant diameter	0.9366	0.8695

One would expect the vegetative components of competitive ability at Snowy Mountains to be influenced by the response of the genotypes to frosting, since frost occurs frequently even during the warmest summer months. To assess this effect the plants were rated for frost damage on five different occasions. Six degrees of damage were used (1 = least, 6 = most damaged). The mean and L.S.D. for frost damage rating of the two genotypes are presented in table 2.

The difference between the *AcLi* and *acli* genotypes in average damage on five dates is significant, *acli* being significantly more resistant to frost damage ($P < 0.05$).

The expected changes of *Ac* and *Li* gene frequencies under field conditions at the coast were computed using the data from table 2 to derive fitness values. Assuming the frequencies of all alleles to be 0.5 initially, the responses during 100 generations of selection were computed (table 3). The gene frequency of the *Ac* allele increased to 0.92-0.98 in all five cases, and also in two out of five cases for *Li* (0.87-0.96). In spite of the relative small number of plants on which the data is based and the consequent possibility of sampling error, the general trend is in accordance with the results from the controlled environment experiment.

The accumulation of *Ac* and *Li* alleles in an environment such as in the coastal environment can be expected because of the superior

sexual and asexual reproductive abilities of *AcLi*, *Acli* and *acLi* genotypes. In the alpine environment, despite the similarity of *AcLi* and *acli* genotypes in vegetative reproduction, *AcLi* is less fit than *acli* with respect to floral development and frost tolerance.

4. DISCUSSION

The experimental results showed the selective advantage of the *Ac* and *Li* alleles under warm-temperature (particularly short-day) conditions, and suggest their selective disadvantage under low temperatures. These results partly explain the geographical distribution of *Ac*, *Li* gene frequencies. The dominant alleles are frequent in warm climates, but rare in cold environments. An attempt has been made to determine the direct physiological basis for the selective differences. It was found that linamarase was activated by cold temperature and the HCN liberated caused an irreversible inhibition of the respiratory system of the *AcLi* genotype resulting in tissue death (Daday, unpublished). However, to date there is no known mechanism which could conclusively account for the selective advantage of the dominant alleles in warm environments.

In this study, polymorphism for the *Ac* and the *Li* loci was found to be associated with polymorphism for physiological and morphological fitness characters presumably under polygenic control. This association between the two genic systems could be due either to pleiotropic gene effects or linkage. Pleiotropy seems a less likely explanation than linkage, because there is not a complete association between investigated genotypes and reproductive capacity, whereas the alleles at the *Ac* and *Li* loci are completely penetrant and expressive with respect to their chemical products.

As discussed by Ford (1964) in his review of polymorphism, natural selection would favour a reduction in recombination within the particular chromosome segment during the development of the supergene. This reduction could arise by changes in the position or frequency of crossing-over within the bivalent. Both the frequency and position of chiasmata are under genetic control and susceptible to natural selection.

A case of close linkage between two polymorphic loci has been found in *Caprea nemoralis*. Cain and Sheppard (1950, 1954) demonstrated polymorphism in *C. nemoralis*. Cain, King and Sheppard (1960) reported that the genetic controlled main shell colours and the banding patterns are linked. The two genetic systems thus formed a supergene in *C. nemoralis*.

In *T. repens* the role of the *Ac* and *Li* loci in adaption may have been greatly enhanced by the accretion of linked loci affecting asexual and sexual efficiency. Therefore it is suggested that during the evolution of *T. repens* polygenes enhancing fitness at high or low temperatures have become linked with the dominant and recessive

alleles respectively. Selection towards high *Ac*, *Li* frequencies in warm environments could rise from the linkage to the dominant alleles of polygenes conferring high sexual and asexual reproductive capacity. The progressive reduction in the frequency of the dominant alleles in natural population with the reduction in winter temperature would result both from the direct biochemical effects of the alleles, and also from the effects of linked genes on sexual reproduction.

T. repens is an amphidiploid and one of the presumed ancestors is *Trifolium nigrescens* Viv. (Brewbaker and Keim, 1953), which contains a large amount of lotaustralin and linamarase. Biochemical polymorphism has not been found in the four populations of *T. nigrescens* examined to date (Daday, unpublished). *T. nigrescens* is restricted geographically to the Mediterranean and its environs (Hossain, 1961), while *T. repens* is spread over the majority of the temperate regions of the world (Daday, 1958). It seems possible that the chemical polymorphism of *T. repens* may have contributed to its increased adaptability.

While differential sexual and asexual reproductive ability can adequately account for selection favouring *Ac* and *Li*, the selection advantages of these genes in warm environments may not be entirely due to superior flowering capacities and vegetative growth of dominant and heterozygous genotypes. In some particular environments animal predators may also have some effect, as suggested by Barber (1955) and demonstrated by Jones (1962) under laboratory conditions. However this hypothesis offers no explanation for the formation of gene clines along temperature gradients. In the present field experiments, grasshoppers were the only predators found, and these attacked all genotypes equally.

The existence of a correlation between chemical genotypes and plant performance could have practical implications. Breeders may be able to screen populations on chemical genotypes in the laboratory prior to field testing.

Therefore it is concluded from this investigation that physiological, morphological and biochemical mechanisms appear to have been of major importance in the development of the balanced chemical polymorphism in *T. repens*.

5. SUMMARY

1. The investigations deal with the mechanism of selection for the *Ac* and *Li* genes in *T. repens*.

2. Sexual reproduction of the *AcLi*, *Accli* and *acLi* genotypes was found to be more prolific under controlled short-day and warm-temperature conditions, while *acLi*, and *accli* were at an advantage under short day and cold temperatures.

3. The fitness values exhibited in warm short-day conditions are such that the frequencies of the *Ac* and *Li* alleles are expected to increase from 0.50 to 0.99 in 500 generations of selection.

4. The *Ac* and *Li* allele frequencies are expected to fall to zero at low temperatures, regardless of day length, within 13 to 500 generations of selection.

5. Vegetative growth of the *AcLi* genotypes at a warm field site was superior to that of the other genotypes particularly *acli*.

6. The *acli* genotype was less injured by frost than *AcLi* under natural alpine conditions.

7. The consequence of the chemical interaction between linamarase and lotaustralin was discussed in relation to the biochemical disadvantage of *AcLi* under cold conditions.

8. It is suggested that alleles affecting reproductive capacity are linked with the alleles at the *Ac* and *Li* loci.

9. The interactions of these gene constellations with the environments could account for the direction and intensity of the *Ac*, *Li* gene frequency clines.

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