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ON THE POLYMORPHISM FOR CYANOGENESIS IN NATURAL POPULATIONS OF TRIFOLIUM REPENS L. IN THE NETHERLANDS I. DISTRIBUTION OF THE GENES Ac AND Li

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SUMMARY

The cyanogenic phenotypes of 19 populations of adult *Trifolium repens* plants were determined. Substantial differences were found, both in the frequency of Ac- as in Li-phenotypes. The differences found are not attributable to large climatic differences, but to factors acting on a local scale. Ac and Li are not independently assorted: six out of sixteen populations studied showed a positive linkage disequilibrium. As Ac and Li are not genetically linked, the association must be the result of epistatic selection favouring Ac-Li-phenotypes. The relative importance of deterministic forces, i.e. natural selection, and stochastic ones (founder effects, drift) in determining the phenotypic frequencies found in The Netherlands are discussed.

1. INTRODUCTION

The study of the manner in which plants defend themselves against herbivores is being met with increasing interest (ROSENTHAL & JANZEN 1979). These plant-animal interactions are not only important from a purely scientific point of view; the incorporation of natural defense mechanisms in economically important crops is essential in integrated pest control.

Most authors nowadays are confident that cyanogenesis is in fact a defense mechanism (see LEVIN 1976 and JONES 1972) for discussion and references). A peculiarity of the cyanogenic system lies in the fact that the attacked plants produce hydrogen cyanide, a substance poisonous for the plant itself. However, HCN does not freely occur in the plant but is produced in damaged cells. Two components are necessary for HCN production, a cyanogenic glycoside and a hydrolyzing enzyme, generally a β-glucosidase. In *T. repens* the cyanogenic glycosides are linamarin and lotaustralin, which occur in varying proportions in the vacuole. The hydrolyzing enzyme is linamarase, localized in the cell wall (KAKES 1985). Enzyme and substrate are thus separated by plasmalemma and tonoplast, and HCN is only produced after rupture of the cell:

$$\text{linamarin} + H_2O \xrightarrow{\text{linamarase}} \text{glucose} + \text{aceton} + \text{HCN}.$$

More than 2000 species of angiosperms in approximately 110 families are now recorded to be cyanogenic (SAUPE 1985). However, only a few of them

are known to be polymorphic sensu FORD (1964) having cyanogenic and acyanogenic plants in the same population.

The genetics of the cyanogenic polymorphism have been long known (ATWOOD & SULLIVAN 1943) and the biochemistry of cyanogenesis has been extensively studied (CONN 1980, HUGHES & MAHER 1973, KAKES & EELTINK 1985).

In most populations of *T. repens* four phenotypes with respect to linamarase and linamarin/lotaustralin occur together. The polymorphism is caused by two genes: Ac is responsible for the presence/absence of linamarin/lotaustralin, and Li for the presence/absence of linamarase. Such a situation is a challenge for the ecological geneticist, since it opens the possibility for a comparison of plants with and without the presumed defensive system. Indeed the presence of populations with an incomplete system, i.e. containing linamarase, but lacking the appropriate substrate and vice versa, is an enigma. Unlike one would expect, Ac and Li are not genetically linked (ATWOOD & SULLIVAN 1943).

The distribution of the cyanogenic phenotypes in W. Europe has been studied by DADAY (1954a). He found a high frequency of cyanogenic plants in Southern Europe, with a steady decline towards the North and East. As the frequency of the dominant alleles of Ac and Li was correlated with the January isotherm, he presumed that low winter temperatures favoured the acyanogenic phenotypes (DADAY 1958).

The conclusion was strengthened by the additional presence of an altitudinal cline (DADAY 1954b). Direct experiments, by transplantation and by growth under controlled conditions, show differences between the four phenotypes but do not explain their geographical distribution. DADAY concluded that the differences in the frequency of the cyanogenic phenotypes were caused by genes linked to Ac and Li. He supposed that Ac and Li formed a part of the genotypes coadapted to warm and dry, versus cool and moist climatic conditions (DADAY 1965). There could however, be a more direct relationship between climate and cyanogenesis: snails and slugs, important predators of white clover, and the only ones that show a definitively proven preference for acyanogenic *T. repens* (CRAWFORD-SIDEBOTHAM 1972, DIRZO & HARPER 1982, ENNOS 1981b) are not only more abundant in southern Europe, but are also active in winter and early spring, the main growing season for *T. repens* in the Mediterranean region. ENNOS (1981b) suggested that selective grazing could be the most important selective factor in very young plants and this could be another reason for the advantage of cyanogenic plants in Southern Europe, as the seeds germinate in early spring.

The above mentioned facts and suppositions do not explain the cyanogenic polymorphism, as they would lead to the expectation that in all populations, or at least the Southern ones, Ac and Li would be fixed, which is definitely not the case. In order to explain the polymorphism, one has to show that the advantage conferred by cyanogenesis is balanced by a force or forces acting in the opposite direction. A number of models have been proposed to explain balancing selection, i.e. heterozygote superiority, frequency dependent selection,

heterogeneity of the environment. ENNOS (1981a) found evidence for still another type of balancing selection in *T. repens*, higher yield of Li/li mixtures, compared to Li and li in pure stands.

It is important to realize that chemical defense, as any other evolutionary strategy, is subject to a cost and benefit comparison. The advantage in terms of increased biomass, must be at least balanced by the metabolic costs of producing and storing the defensive compounds. The actual values of cost and assets will be dependent on (micro) climatic factors, both directly, by determining the photosynthetic rate, as indirectly by determining competition and predation.

In the course of a broader investigation of the ecological genetics of the cyanogenic system in *T. repens*, we report here on the fine scale distribution of the cyanogenic phenotypes in natural populations of *T. repens* in The Netherlands.

2. MATERIAL AND METHODS

Sampling: Adult populations were sampled by taking pieces of stolons at 2 m intervals along a regular pattern over the sampling sites. The sites were chosen using the following criteria: either the vegetation was natural, like dune and riverbank sites, or, if man made (dike vegetations and meadows) they should have had an extensive management with no deliberate sowing of white clover for at least 20 years. The stolons were grown in the greenhouse to plants of a suitable size.

Phenotype testing: 9–12 young, unfolded leaves were collected and divided over three culture tubes (10 × 1,8 cm). The leaves were kept at –20°C for at least one hour after which 3 drops of deïonized water was added to each tube. To the second tube of each plant 0.5 µmol of linamarin was added and to the third one 100 µl of a linamarase solution that contained 1 nKat/ml enzyme activity. A two cm strip of Whatman 3 paper soaked in a Feigl-Anger test solution (FEIGL & ANGER, 1966) was clamped in the upper part of the tubes, the tubes stoppered and read after 3 hours at 35°C against a series of CN test tubes prepared in the same way. The test series were: 1, 2, 5, 10, 50 µg HCN/tube. Only tubes with <1 µg HCN developed were considered negative (see *fig. 1* for the determination of the phenotypes).

Linamarine isolation: A linamarin solution was prepared from *Linum usitatissimum* L. as follows: 100 g of one week old seedlings were cut just under the cotyledons and put into a 2 l flask filled with a reflux cooler containing 1 l boiling ethanol 96%. After boiling for 10 min, the mixture was cooled to

	+ H_2O	+ substrate	+ enzyme
Ac-Li-	+	+	+
Ac-lili	–	–	+
acacLi-	–	+	–
acaclili	–	–	–

Fig. 1. The four different phenotypes for Ac and Li of *Trifolium repens* and the method to distinguish them.

Table 1. Frequency of the cyanogenic phenotypes in 19 Dutch populations of *Trifolium repens*.

Nr	Station name	Number of plants (between brackets: %)					% of plants with the dominant phenotype	
		AC-Li	Ac-lili	acacLi-	acaclili	n	Ac-	Li-
92	Dijkwater	0 (0)	1 (4)	5 (20)	19 (76)	25	4	20
94	Lindendijk	18 (26)	0 (0)	18 (26)	32 (48)	68	26	53
95	Vlaanderendijk	6 (32)	0 (0)	2 (11)	11 (57)	19	32	50
96	Poederoyen	0 (0)	2 (7)	5 (19)	20 (74)	27	7	19
98	Empel	0 (0)	1 (2)	0 (0)	47 (98)	48	2	0
100	Lek 1	0 (0)	0 (0)	0 (0)	46 (100)	46	0	0
101	Lek 2	5 (8)	3 (5)	1 (2)	53 (85)	62	13	11
102	Lek 3	0 (0)	0 (0)	0 (0)	47 (100)	47	0	0
103	Vroongronden	1 (2)	4 (8)	2 (4)	41 (86)	48	10	7
104	Wageningen 1	0 (0)	2 (3)	1 (1)	66 (96)	69	3	1
105	Wageningen 2	0 (0)	1 (3)	1 (3)	38 (94)	40	3	3
107	Boksum, plot 7	29 (25)	38 (33)	15 (13)	34 (29)	116	58	38
108	Boksum, plot 17	7 (32)	8 (36)	0 (0)	7 (32)	22	68	32
109	Mirns	8 (23)	8 (23)	5 (14)	14 (40)	35	46	37
–	Havelte	2 (10)	0 (0)	2 (10)	16 (80)	20	10	20
113	Egmond 1	6 (8)	55 (76)	1 (1)	10 (15)	72	85	10
114	Egmond 2	8 (2)	19 (50)	1 (3)	10 (26)	38	71	24
116	Budel 2	13 (19)	16 (23)	5 (7)	35 (51)	69	42	26
117	Budel 3	6 (9)	10 (15)	78 (10)	44 (66)	67	24	19

room temperature and filtered. The volume of the filtrate was reduced to 20 ml by vacuum distillation (Rotavapor). The crude extract was centrifuged and purified over a 50 ml Sephadex G25 fine column. It was eluted with deionized water and 2 ml fractions were collected. To 50 µl of each fraction, linamarase was added for the Feigl-Anger test as described above.

Linamarase isolation: Linamarase was prepared from linseed as follows: coarsely ground linseed was extracted with ether to remove the oil. The oilfree meal was mixed with water and quickly centrifuged (20 min at 3000 rpm 2 °C). All subsequent handling was done at ±2 °C. Aceton was added to the supernatant (10% v/v), centrifuged as above. Aceton was added to the supernatant to a total concentration of 40% v/v and centrifuged again. The supernatant was now discarded and the pellet taken up in aqua dest. Ammonium sulfate was added then (20 g/100 ml) and centrifuged again. The pellet was discarded and ammonium sulfate was added to the supernatant to a final concentration of 60 g/ml. The precipitate was taken up in aqua dest. and dialyzed against deïonized water for 12 hours. This preparation has a linamarase activity of ± 10 nKat/ml and will remain active for more than two years when stored at -20 °C.

3. RESULTS AND DISCUSSION

Table 1 and *fig. 2* summarize the results of the investigation. The first point of interest is that the frequency of Ac and Li is very heterogeneous over sites.

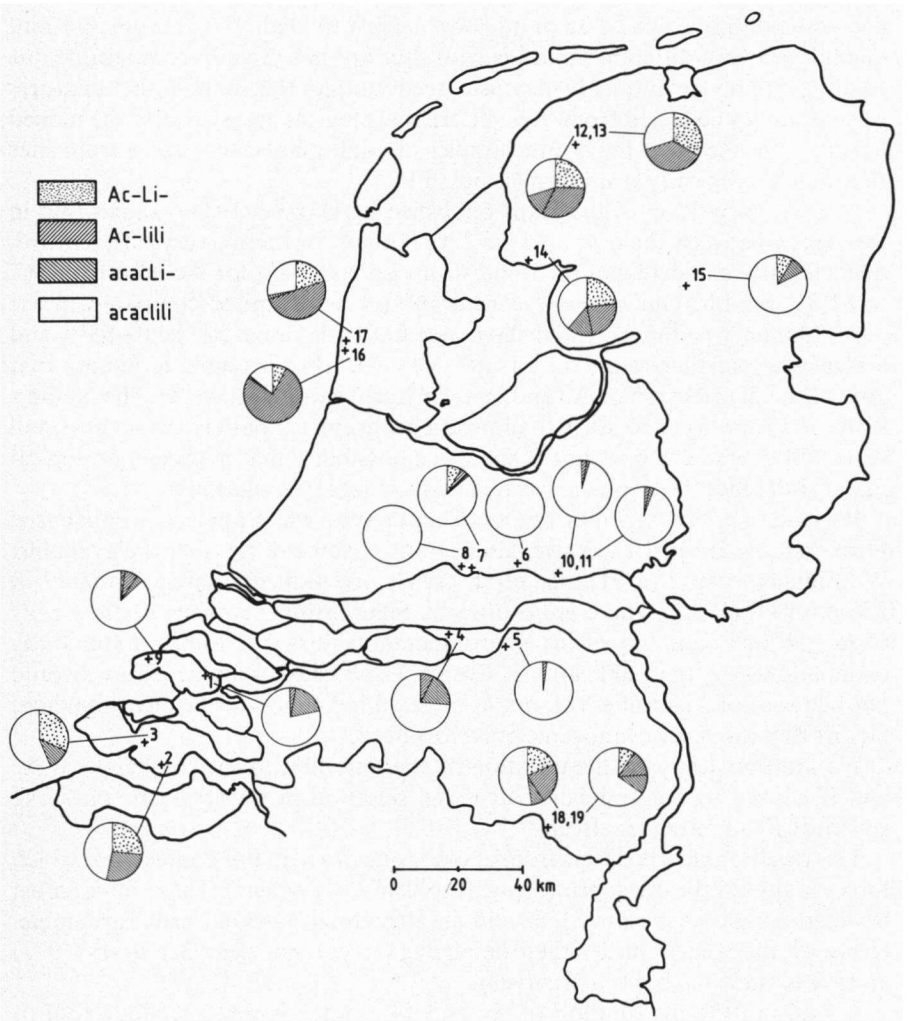

Fig. 2. Distribution map of the four possible phenotypes for Ac and Li of *Trifolium repens* L. in The Netherlands.

This is illustrated by the first two lines in *table 3*, that show significant Ac × population and Li × population interactions.

The distribution map of the four phenotypes (*fig. 2*) does not show any geographical pattern. There is no indication of clustering of populations with comparable frequencies of Ac and Li, nor of any regular trend corresponding with climatic factors. As we saw earlier, DADAY (1954a) found a relationship between the mean January temperature and the frequency of Ac and Li. The two samples in Daday's study originating in the Netherlands, viz. Groningen and Breda seem to fit very well in the broad pattern of increasing frequencies of Ac and Li with

decreasing latitude. The data of our sites nearest to Daday's, Boksum and Lindendijk are very different from his sites, but are not directly comparable due to his sampling technique. Daday used seed samples that were in fact mixtures of subsamples collected from 'five different ecological areas around the named locality'. On the other hand, our samples of adult plants were taken from sites that were ecologically as uniform as possible.

A closer inspection of the maps published by DADAY (1954a) shows that in the region between the 0 °C and the 2 °C January isotherm, very different frequencies of Ac and Li can be found: from 8.3 to 85.5% for Ac- and from 9.7 to 81,5% for Li-. Our estimates, from an area very limited compared to the 0–2 °C January isotherm region show a comparable range for Ac (0–85%) and a somewhat smaller range for Li- (0–53%). Clearly, the climatic factors that govern the distribution of Ac and Li over Europe do not have, in The Netherlands, the variation necessary to impose a geographical pattern on such a small scale. (Of course this does not rule out the possibility that microclimatological factors influence the frequency of Ac and Li of local populations).

We made a brief description of the collection sites, which appears in condensed form, in *table 2*. More extensive descriptions of some of the sites are available; Wageningen 1 en 2 in ELBERSE et al. (1983) and Boksum parcel 7 and 17 in ENNIK et al. (1982). There is no obvious relationship between the frequency of Ac and/or Li and any of the environmental factors determined in our study or mentioned in the publications. Even if established, such a relation would not be proof of a causal effect, but at most an indication of the environmental factors that influence cyanogenic polymorphism.

We are thus left with the question: is the variation in the frequency of Ac and Li caused by deterministic forces e.g. selection or by stochastic ones like genetic drift and founder effects?

This question clearly differs from another one raised in this context, viz. which forces maintain the cyanogenic polymorphism in *T. repens* ? These forces must be deterministic, as the latitudinal and the altitudinal clines in Europe are stable. However, the exact nature of these balancing forces is not clear. See JONES (1972) and ENNOS (1981 a, b) for a discussion.

A study of the distribution of Ac and Li as such is not a suitable tool to elucidate the forces influencing the cyanogenic polymorphism. There is one clue however. If selection through predation is one of the forces mentioned and supposing that HCN is the deterrent, then Ac and Li would show interaction in polymorphic populations, as only plants dominant for Ac and Li would be protected. In a sample of adult plants this interaction would lead to an association of Ac and Li known as linkage disequilibrium. Linkage disequlibrium was found by ENNOS (1982) in populations of *T. repens* in Engeland, and our analysis is essentially the same. The phenotypic frequency data are studied by means of a multidimensional χ^2 test. The last two lines of *table 3* show that both the Ac × Li and the Ac × Li × population interactions are highly significant. Apparently the Ac and Li plants are not independently assorted, but this feature is not consistent over populations. For that reason we estimated the linkage dis-

Table 2. Description of the sampling sites of *Trifolium repens*

Nr	Station name	Sampling date	Amersfoort coördinates	Short description
92	Dijkwater	8-1983	62.0–410.9	West facing slope of the dike, sandy clay, occasionally grazed by sheep
94	Lindendijk	8-1983	46.3–382.1	On top of the dike, clay with bricks of an abandoned road, no deliberate grazing
95	Vlaanderendijk	8-1983	42.4-390.0	Verges of a secondary road on the dike, clay, grazed by young cattle
96	Poederoyen	8-1983	133–421	Low dike at the bank of the Maas river, sand, no grazing apparent at the collection time
98	Empel	8-1983	150–416	River dunes in the Koornwaard, sand, no grazing at the collection time
100	Lek 1	6-1983	158–444	Low dike at the bank of the Lek river, sandy clay, grazed by cattle
101	Lek 2	6-1983	146–443	South facing slope of the dike at the north side of the Lek river. Clay, grazed by cattle
102	Lek 3	6-1983	142–441	River dune and low dike north of the Lek river. Sand, no apparent grazing
103	Vroongronden	7-1983	172–415.5	Land facing side of the old dunes, sand, grazed by cattle
104	Wageningen 1	7-1983	172–444	Meadow on heavy clay. Experimental field in use by the CABO. NPK fertiliser experiment*. Grazed
105	Wageningen 2	7-1983	172–144	Meadow on heavy clay. Experimental field in use by the CABO. High pH experiment*. Grazed
107	Boksum, plot 7	10-1983 + 8-1985	178–557	Extensively managed meadow, on heavy clay, grazed by cattle and sheep, used for haymaking**
108	Boksum, plot 17	10-1983	177–576	Extensively managed meadow, on heavy clay, grazed by cattle
109	Mirns	10-1983	161–540	At the foot of the 'Mirnsner Klif', sand on heavy loam with boulders. No deliberate grazing
–	Havelte	10-1984	211–536	Abandoned field on sand, no deliberate grazing
113	Egmond 1	5-1985	102–512	Dune slack, flooded in winter, sand, grazing by rabbits apparent
114	Egmond 2	5-1985	168–363	Abandoned field, now in use as recreational meadow, sand, grazing by rabbits
116	Budel 2	8-1958	168–363	Old meadow on sand, mixed with peat. Grazed by cattle
117	Budel 3	8-1958	168–362	Road verges, on sand, no apparent grazing

* see: ENNIK et al., 1982
** see: ELBERSE et al., 1983

equilibrium coëfficient D with its test statistic Q, that is χ^2 distributed with one degree of freedom (HILL 1974). The results are summarized in *table 4*. Of the 16 populations polymorphic for Ac and Li, 6 have a significantly positive value of D. There are two errors that could lead to spuriously high estimates of D: sampling errors and incorrect (biased) determination of the phenotype.

Table 3. χ^2 analysis with respect to the interactions between Ac-phenotype, Li phenotype and population. The populations from *table I* with n > 50 are used in this analysis.

Interaction	df	χ^2	p
Ac × population	6	147.86	$<10^{-4}$
Li × population	6	78.99	$<10^{-4}$
Ac × Li	1	37.66	$<10^{-3}$
Ac × Li × population	6	42.63	$<10^{-3}$

Table 4. Linkage disequilibrium in populations of *Trifolium repens* polymorphic for Ac and Li. The quantity Q has a χ^2 distribution with df = 1.

Nr	Station name	Number of plants	D	Q
92	Dijkwater	25	-0.0046	0.2618
94	Lindendijk	68	0.0978	18.5492***
95	Vlaanderendijk	19	0.1315	9.8845***
96	Poederoyen	27	-0.0079	0.4954
101	Lek 2	62	0.0276	28.1150***
103	Lek 3	48	0.0078	1.7857
104	Wageningen 1	69	-0.0002	0.0302
105	Wageningen 2	40	-0.0003	0.0263
107	Boksum, plot 7	116	0.0293	1.8241
108	Boksum, plot 17	22	0.0983	3.9201*
109	Mirns	35	0.0483	1.9244
–	Havelte	20	0.0459	8.4268**
113	Egmond 1	72	0.0013	0.0059
114	Egmond 2	38	0.0430	1.6681
116	Budel 2	69	0.0576	8.3598**
117	Budel 3	67	0.0271	4.2534*

* $p<0.05$
** $p<0.025$
*** $p<0.001$

We tried to avoid sampling errors by taking stolons at least 2 m apart along a regular pattern, usually a number of transects covering the predefined collection site. Multiple sampling of one genotype will be very rare, unless there were one or a few dominant genotypes present on the site, however improbable owing to the high genetic variability shown by populations of *T. repens* (GLIDDON & TRATHAN 1985, BURDON 1980).

Incorrect determination of phenotypes cannot be a source of error, as the semiquantitative method we used in this study is very reliable. This is proven by the consistency of replicates and by the progenies of tested plants, that in every instance correspond with the parental phenotype.

Several factors acting in the population and causing linkage disequilibrium are discussed by ENNOS (1982). His conclusion is that selection favouring the Ac-Li-phenotype is the most likely explanation for the linkage disequilibrium found by him in English populations. He recorded two populations out of ten with values of D that differed significantly from zero, of which only one below the $p = 0.01$ level. We found a much more pronounced deviation in a higher proportion of the populations studied. Part of the difference could be explained by the generally lower frequency of Ac and Li in the Dutch populations. Low frequency of the dominant alleles makes the test for D more efficient (HILL 1974). Other explanations for the difference may be stronger selective forces or populations established more recently.

The problem of decay of the linkage disequilibrium in time is also discussed by ENNOS (1982). He states that the selective forces maintaining the linkage disequilibrium would eventually lead to fixation of Ac and Li. On the other hand, in the absence of selective forces, any linkage disequilibrium initially present would decay in time, albeit slowly owing to the low turnover rate of populations of *T. repens*. ENNOS (1981b) has presented evidence that the Ac-Li-phenotype is favoured in seedling establishment. The advantage is only partly due to HCN production as I found that the presence of linamarin, irrespective of linamarase, protects young seedlings against grazing by snails (KAKES, in preparation).

In the absence of actual linkage, epistatic selection in itself is not a sufficient condition for a stable polymorphism (ROUGHGARDEN 1979). In the absence of any data on the effects of selection on homozygotes versus heterozygotes for Ac and Li, we can only speculate on the forces maintaining the polymorphism. ENNOS (1981b) suggests that competitive interaction between Li- and lili phenotypes maintains the polymorphism at this locus. However, it is difficult to discern the mechanism causing the interaction. Another possibility is that differences in the reproductive effort between phenotypes counteracting the Ac-Li-advantage, are responsible for the maintenance of the polymorphism. BURDON (1980), in a study of a *T. repens* population in North Wales, found that 20% of the clones produced 81% of the flowers. He also determined the cyanogenic phenotype, but unfortunately did not give the relationship with the flowering characteristics. Flowering characteristics of the cyanogenic phenotypes are given by DADAY (1965) but he used a commercial strain of *T. repens*. In addition, the climatic conditions in his field experiments in New South Wales are difficult to compare with those in W. Europe. Nevertheless, his study seems to indicate that at low temperatures the acaclili phenotype has a reproductive advantage. It is clear from these data that a comparative study of the reproductive effort of individual plants of *T. repens* in natural situations is indicated.

4. CONCLUSIONS

Population differentiation with respect to Ac and Li in populations of *T. repens* in The Netherlands is partly caused by selective forces acting on a local scale.

These forces may or may not be the same as those maintaining the polymorphism. However, it is not very likely that all of the variation found in the present study is caused by deterministic forces. Due to the low turnover rate of *T. repens* the history of the population becomes an important factor influencing the present constitution. Founder effects may cause some of the variation found. Genetic drift may be another cause, as we see that only some of the plants reproduce and so the effective population size could be small even in situations with a high density of *T. repens* plants.

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