***Rhizobium* Population Genetics: Genetic Variation Within and Between Populations from Diverse Locations**

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Polyacrylamide gel electrophoresis (PAGE) of isoenzymes of chromosomal origin, and plasmid profile analyses, were used to investigate genetic variation within and between natural populations of *Rhizobium leguminosarum* biovar *trifolii* from diverse sources. PAGE showed that, within the biovar in general, relatively few alleles existed for the enzyme loci studied. There was a strong genetic disequilibrium between loci, suggesting that chromosomal recombination is rare. Both PAGE and plasmid profile analysis revealed differences in the degree of strain polymorphism at the sites examined. At sites which were acidic, improved (by liming) and neutral, strain variation was found to be low, intermediate and high, respectively.

INTRODUCTION

The baiting of soil samples with a single white clover (*Trifolium repens*) cultivar under growth-room conditions to obtain indigenous *Rhizobium leguminosarum* biovar *trifolii* has been shown to provide samples of rhizobia unbiased in respect of the strains identified by enzyme polymorphism and plasmid profile (Harrison *et al.*, 1987; and our unpublished results). Not only can an examination of enzyme polymorphism using polyacrylamide gel electrophoresis (PAGE), be used to give an indication of the degree of chromosomal uniformity within a collection of *Rhizobium* (Young, 1985) but it can also be used to describe degrees of strain heterogeneity in natural populations. Plasmid profile analysis, through agarose gel electrophoresis, is also particularly useful in the investigation of the latter (Glynn *et al.*, 1985; Bromfield *et al.*, 1987).

These techniques have proved to be very useful in an examination of these aspects in *Escherichia coli* populations. Restricted chromosomal variation has been demonstrated using enzyme electrophoresis by a number of workers (Milkman, 1975; Selander & Levin, 1980; Causant *et al.*, 1981). Causant *et al.* (1981) also examined cryptic plasmids which, by contrast, were very variable. The presence of chromosomal linkage disequilibrium was again shown by Whittam *et al.* (1983) and by Ochman & Selander (1984), who suggested that populations are clonal and that certain clones recur throughout unrelated populations.

Work by Young (1985) and Young *et al.* (1987) on single populations suggests that chromosomal variation is also limited in the species *R. leguminosarum*. However, other studies (Noel & Brill, 1980; Dughri & Bottomley, 1983*a, b*, 1984; Bromfield *et al.*, 1986; Thurman & Bromfield, 1988) have indicated the existence of heterogeneous groups of strains within natural populations. The degree of heterogeneity demonstrated clearly relies heavily on the identification method employed, and a group of isolates homogeneous for one characteristic may vary in respect of another (Noel & Brill, 1980; Dughri & Bottomley, 1983*a*).

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Abbreviations: BGAL, β -galactosidase; ET, electrophoretic type; G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase.

As the results of such work have depended on the method used, the host and other environmental factors, they are not strictly comparable. A widespread comparison of populations of a single biovar from sites of varying geography and ecology, using the same evaluation methods, has not been carried out. Such a study of the genetic variation in indigenous populations of *R. leguminosarum* biovar *trifolii* would be of both ecological and agronomic interest. Here, an investigation is reported which used PAGE of chromosomal isoenzymes and agarose gel electrophoresis of plasmid DNA to screen isolates from ten geographically and ecologically diverse sites.

METHODS

Soil sampling and strain isolation. Soil samples were collected from ten geographically and ecologically varying sites (Table 1). Ten samples, taken on a transect at 2 m intervals, were removed from each location. This was achieved by loosening the top 10 cm of soil with a sterile trowel followed by the removal of a 'core' in a sterile 30 ml universal bottle (Sterilin). These soils were then used to inoculate seedlings of white clover (*Trifolium repens*) cv. Nesta grown on Jensen's (1942) nitrogen-free agar slopes at 16 °C under a 16 h light/8 h dark cycle. The seedlings were derived from seeds that had been surface sterilized with 20% (v/v) sodium hypochlorite, rinsed thoroughly in sterile distilled water and germinated on water agar.

Inoculum was prepared by mixing 10 g soil (1 g from each sample point on the transect) with 90 ml quarter-strength Jensen's (1942) N-free liquid medium at 4 °C by agitation on a wrist-action shaker for 1 h at 250 cycles min⁻¹. In addition, at locations where within-site variation was considered (Table 2), four of these samples from alternate sampling points (4 m intervals) were chosen for sub-site analysis. In this case 10 g soil from each sample was treated as above.

From both parent and sub-site soil suspensions the resulting 10⁻¹ dilutions were diluted by a further factor of ten and 1 ml aliquots of these suspensions were used to inoculate single plants (one hundred per soil sample). The pH of each soil was also determined by measuring the pH of 10 g soil shaken in 90 ml sterile deionized water as above (mean of three readings). The populations of *Rhizobium* were estimated by Brockwell's most probable number plant infection method (Brockwell, 1963; Vincent, 1970).

After 6 weeks, one nodule was removed at random from each plant, crushed in a microtitre plate (Sterilin) and streaked out on TY agar plates (Beringer, 1974). Each strain was subcultured to ensure *Rhizobium* purity. Isolates were tested for nodulation of clover cv. Nesta to authenticate them as *Rhizobium*; all were positive.

Enzyme electrophoresis. All isolates were examined by PAGE of three enzymes (Young, 1985; Harrison *et al.*, 1987): glucose-6-phosphate dehydrogenase (G6PD), superoxide dismutase (SOD) and β -galactosidase (BGAL). The alleles of each locus were labelled alphabetically with a single letter in order of descending PAGE mobility. Each combination of variants for a particular strain for the three single enzyme bands was treated as a distinct genetic class or electrophoretic type (ET) (Caugant *et al.*, 1981).

Analysis of plasmid profiles. Random samples of 40 and 30 isolates from each major site and sub-site, respectively, were examined by agarose gel electrophoresis for variation in plasmid size and distribution. Strains were grown in PA medium (Hirsch *et al.*, 1980) overnight (to a concentration of approximately 10⁷ cells ml⁻¹) following inoculation with actively growing culture. Rhizobial cell lysis and the electrophoretic method were by slight modification of the method of Eckhardt (1978) described by P. R. Hirsch (personal communication). One ml of cell culture was centrifuged and to the pellet was added 50 μ l of lysis mix (1 mg lysozyme ml⁻¹ and 1 unit RNAase ml⁻¹ in 25% (w/v) sucrose, 0.025 M-Tris/HCl). This mixture was immediately transferred to the sample wells of a 0.65% (w/v) agarose gel. Behind the wells was incorporated a single wide well containing 0.4% (w/v) agarose/1% (w/v) SDS. A BRL horizontal gel kit was used to run gels at 150 V for 4 h in Tris/borate buffer (pH 8.3). Gels were stained in a solution of 1 μ g ethidium bromide ml⁻¹ for 40 min. Plasmid sizes were estimated by calibration against the relative mobilities of a number of plasmids of known size (Harrison *et al.*, 1988).

Analysis of genetic and genotypic diversity. Analysis of genetic diversity has been employed by Young (1985) and Young *et al.* (1987) and is based on the gene diversity measure of Nei (1978). The genetic diversity of each locus, $h = 1 - \sum X_i^2$, where X_i = frequency of the i^{th} allele: frequency = (no. of times an allele occurs)/(total no. of isolates).

Genotypic diversity, hx , is also represented by the formula $hx = 1 - \sum X_i^2$; but here X_i is the frequency of the i^{th} ET; its use as a means of indicating variation in bacterial populations has been described by Selander *et al.* (1986).

RESULTS AND DISCUSSION

Implications with regard to general genetic variation within the biovar trifolii

Limitation of allelic variation is demonstrated through an examination of the data in Table 3 in terms of the total mean genetic diversity of the enzymes G6PD, SOD and BGAL (Table 4a) for isolates from all the sites combined. Mean genetic diversity, $\bar{h}$, is the sum of the genetic

Table 1. *Sites from which isolates were sampled*

Site	Grid ref.	pH	Approx. no. of cells g ⁻¹ *	Site description
Tal-y-Bont (T) Dyfed	SN 677914	4.3	60	Acidic peaty podsol
Bronant 1 (B1) Dyfed	SN 626673	4.4	60	
Bronant 2 (B2)	SN 635685	5.9	2 × 10 ⁴	
Pwllpeiran (Pw)	SN 773748	6.1	2 × 10 ⁵	Previously acidic peaty podsol, improved through liming (3 yrs previously)
Bowstreet (BS) Dyfed	SN 617846	6.2	6 × 10 ⁴	Previously acidic mineral soil, improved through liming (5 yrs previously)
Frongoch (F) Dyfed	SN 605827	6.3	6 × 10 ⁴	
Penglais (Pen) Dyfed	SN 595823	6.5	6 × 10 ⁵	
Costessey (C) Norfolk	TG 184214	6.7	2 × 10 ⁷	
Garveston (G) Norfolk	TG 018078	6.9	2 × 10 ⁶	
Blackawton (Bn) Devon	SX 805513	7.5	6 × 10 ³	

* Fiducial limits are determined by dividing or multiplying numbers by 6.6.

Table 2. *Summary of sub-sites examined*

Sub-site	pH	Approx. no. of cells g ⁻¹ *
Bronant 1/1 (B1/1)	4.4	60
Bronant 1/2 (B1/2)	4.2	60
Bronant 1/3 (B1/3)	4.2	60
Bronant 1/4 (B1/4)	4.5	60
Bronant 2/1 (B2/1)	5.7	6 × 10 ²
Bronant 2/2 (B2/2)	5.8	2 × 10 ³
Bronant 2/3 (B2/3)	6.1	6 × 10 ⁴
Bronant 2/4 (B2/4)	6.0	2 × 10 ⁴
Pwllpeiran/1 (Pw/1)	6.1	2 × 10 ⁶
Pwllpeiran/2 (Pw/2)	6.0	2 × 10 ⁴
Pwllpeiran/3 (Pw/3)	6.0	6 × 10 ⁴
Pwllpeiran/4 (Pw/4)	5.9	6 × 10 ³
Garveston/1 (G/1)	6.2	6 × 10 ⁵
Garveston/2 (G/2)	6.4	2 × 10 ⁵
Garveston/3 (G/3)	5.7	6 × 10 ⁶
Garveston/4 (G/4)	7.1	6 × 10 ³

* Fiducial limits are determined by dividing or multiplying numbers by 6.6.

Table 3. *Number of isolates of each ET obtained from particular sites*

Site	No. of isolates of ET indicated*:											Totals
	MFK	MFM	MFP	MSK	MSM	MSQ	MSS	SSM	SSQ	SSS	Others	
T	0	44	0	0	6	1	0	0	0	0	0	51
B1	0	17	1	0	0	1	0	0	0	0	0	19
B2	0	5	0	0	10	27	8	6	30	0	0	86
Pw	3	6	0	0	8	8	0	17	40	0	8	90
BS	10	15	7	5	8	7	2	0	1	0	0	55
F	21	33	0	15	14	0	0	0	0	0	5	88
Pen	1	48	24	3	6	0	1	0	0	0	0	83
C	0	9	0	4	36	0	15	5	7	0	0	76
G	0	10	10	5	34	0	19	0	0	0	1	79
Bn	5	7	5	3	7	0	8	34	3	18	4	94
Totals	40	194	47	35	129	44	53	62	81	18	18	721

* The three letters indicate the electrophoretic mobility class of the enzymes G6PD, SOD and BGAL, respectively.

Table 4. *Genetic and genotypic diversity at each site (a) and sub-site (b)*

Mean genetic diversity $\bar{h} = h_{G6PD} + h_{SOD} + h_{BGAL}/3$, where h_{G6PD} , h_{SOD} and h_{BGAL} are the genetic diversity values for the G6PD, SOD and BGAL loci respectively; 3 is the number of loci examined. Genetic diversity, $h = 1 - \sum X_i^2$, where X_i is the frequency of the i^{th} allele (frequency = no. of times an allele occurs/total number of isolates). Genotypic diversity, $h_x = 1 - \sum X_i^2$, where X_i is the frequency of the i^{th} ET (frequency = no. of times each ET occurs/no. of isolates). Numbers of alleles and ETs are given in parentheses where appropriate.

	h_{G6PD}	h_{SOD}	h_{BGAL}	$\bar{h}$	h_x
<i>(a) Site</i>					
T	0.00 (1)	0.24 (2)	0.04 (2)	0.09	0.24 (3)
B1	0.00 (1)	0.10 (2)	0.20 (3)	0.10	0.19 (3)
B2	0.48 (2)	0.11 (2)	0.49 (3)	0.36	0.75 (6)
Pw	0.42 (3)	0.28 (2)	0.57 (3)	0.42	0.73 (9)
BS	0.04 (2)	0.30 (2)	0.72 (5)	0.35	0.83 (8)
F	0.00 (1)	0.47 (2)	0.52 (3)	0.34	0.48 (5)
Pen	0.00 (1)	0.21 (2)	0.50 (4)	0.24	0.58 (6)
C	0.28 (2)	0.23 (2)	0.52 (4)	0.34	0.71 (7)
G	0.03 (2)	0.39 (2)	0.60 (4)	0.34	0.70 (6)
Bn	0.49 (2)	0.35 (2)	0.64 (7)	0.49	0.80 (11)
Total site diversity*	0.37 (3)	0.49 (2)	0.65 (7)	0.50	0.86 (17)
<i>(b) Sub-site</i>					
B1/1	0.00 (1)	0.1 (2)	0.16 (3)	0.09	0.16 (3)
B1/2	0.00 (1)	0.00 (1)	0.00 (1)	0.00	0.00 (1)
B1/3	0.00 (1)	0.00 (1)	0.00 (1)	0.00	0.00 (1)
B1/4	0.00 (1)	0.00 (1)	0.00 (1)	0.00	0.00 (1)
All B1 sub-sites combined	0.00 (1)	0.05 (2)	0.08 (3)	0.04	0.08 (3)
B2/1	0.45 (2)	0.45 (2)	0.45 (2)	0.45	0.46 (2)
B2/2	0.49 (2)	0.36 (2)	0.36 (2)	0.40	0.64 (3)
B2/3	0.46 (2)	0.31 (2)	0.57 (3)	0.45	0.80 (6)
B2/4	0.46 (2)	0.34 (2)	0.43 (2)	0.40	0.70 (4)
All B2 sub-sites combined	0.50 (2)	0.37 (2)	0.47 (3)	0.45	0.70 (6)
Pw/1	0.51 (2)	0.30 (2)	0.61 (3)	0.47	0.80 (8)
Pw/2	0.47 (2)	0.26 (2)	0.56 (3)	0.43	0.74 (7)
Pw/3	0.43 (2)	0.28 (2)	0.50 (2)	0.40	0.69 (4)
Pw/4	0.36 (2)	0.36 (2)	0.49 (2)	0.40	0.58 (3)
All Pw sub-sites combined	0.44 (2)	0.30 (2)	0.55 (3)	0.43	0.72 (8)
G/1	0.00 (1)	0.48 (2)	0.66 (5)	0.38	0.77 (6)
G/2	0.00 (1)	0.43 (2)	0.57 (4)	0.33	0.72 (5)
G/3	0.00 (1)	0.34 (2)	0.54 (4)	0.29	0.67 (5)
G/4	0.00 (1)	0.32 (2)	0.58 (4)	0.30	0.71 (5)
All G sub-sites combined	0.00 (1)	0.41 (2)	0.66 (5)	0.36	0.72 (6)
Total sub-site diversity*	0.45 (2)	0.41 (2)	0.63 (6)	0.50	0.82 (11)

* The total site and sub-site diversity describe the genetic diversity and genotypic diversity values for all isolates from all sites and sub-sites respectively, considered as single populations.

diversity for each locus over the number of loci examined and represents the proportion of loci at which two individuals, chosen randomly from the population, can be expected to differ. Milkman (1975), working with *E. coli*, assumed that the effective population size, N_e , had exceeded 10^{10} for at least $4N_e$ generations, in which case a genetic diversity of 0.97 would be expected. If we assume that we are dealing with populations of a genetically effective size at least as large as that predicted for *E. coli*, then the mean genetic diversity, $\bar{h}$, for all sites combined ($\bar{h} = 0.50$), which is also the value determined by Young (1985), appears relatively low by comparison. The numbers of alleles per locus are comparable with those found by Young (1985) (three, two and seven for G6PD, SOD and BGAL respectively in this study, compared with four, three and seven), which further indicates lower allelic variation in *Rhizobium* compared

with *E. coli* (alleles per locus in *E. coli*, five, six and 19 for G6PD, SOD and BGAL respectively; Selander & Levin, 1980).

As in the study of Young (1985), strong genetic disequilibrium occurred between loci, in that the number of ETs found was not as might be envisaged from the random coalescence of polymorphism at each locus. This was well demonstrated at the Blackawton site, where the possible number of ETs, based on the number of different alleles at each locus, was 28, although the observed number was only 11.

Genetic disequilibrium, combined with the absence of strong allelic variation, means that isolates from diverse sources may be allotted to comparatively few ET classes, a situation which also occurs with *E. coli* (Caugant *et al.*, 1981; Ochman *et al.*, 1983; Ochman & Selander, 1984). This indicates that chromosomal variation in the biovar *trifolii* is limited, either because there is repeated selection for particular genetic lineages or because chromosomal recombination is rare.

Differences in ET genetic variability between sites

Although genetic variation appeared low in the biovar in general and many ETs recurred at a number of sites (Table 3), a chi-square analysis showed that the frequency of the four most widespread ETs (MFM, MSM, MSS and MSK) varied significantly between sites ($\chi^2 = 213.22$, 27 degrees of freedom), indicating that populations did have individual genetic identities.

A chi-square analysis of the occurrence of the five most abundant (not widespread) ETs (MFM, MSM, SSQ, SSM and MSS) also indicated a significant difference in the distribution of ETs ($\chi^2 = 594.94$, 36 degrees of freedom).

Populations at different sites differ principally in the presence or absence of a few ETs and their relative proportions, rather than through the occurrence of many radically different ETs.

The amount of genetic variation appeared to be strongly dependent upon the pH of the soil of origin, which ranged from 4.32 at Tal-y-Bont to 7.52 at Blackawton. The number of ETs per site was positively correlated with pH ($r = 0.799$, $P < 0.001$), as was the mean genetic diversity, $\bar{h}$ ($r = 0.870$, $P < 0.001$). The number of ETs found at Pwllpeiran and Bronant 2 was, however, greater than would be expected from this correlation. This is probably due to an increase in population size and variability following liming.

Genotypic diversity, hx (Table 4a) is a measure of the probability of isolating two different ETs from a population at any single time and provides a good representation of genetic variation at the various locations by allowing consideration of ETs rather than loci. Because hx is based on the proportions of ETs, in addition to the number of ETs present, high hx values in a population may be attributed to an even distribution, as well as to the presence of a great variety of ETs. This means that genotypic diversity values may be used profitably to demonstrate differences in qualitative and quantitative variation between sites and in particular to show the dominance of a few ETs at certain sites. Variation expressed in this manner re-inforced the correlation of variation with pH ($r = 0.831$; $P < 0.001$).

Low genotypic diversity and mean genetic diversity values at the two Welsh acidic sites were the result of the occurrence of the particularly dominant ET, MFM (Table 3). This is perhaps explained by the plasmid profile analysis of representative strains. A single plasmid profile (three plasmids of sizes > 550 , 500 and 197 kb) was indicated for all the MFM isolates at these sites and it might be reasonable to assume that they are the same strain. This strain was not only recurrent at these sites but also appeared in much fewer numbers at Bowstreet and Bronant 2. Although this strain was widespread at relatively adjacent sites, further studies may reveal that its high occurrence in acid soils is due to possession of particular traits providing an ecological advantage in these conditions.

Genetic variation was not dependent upon the number of cells per g of soil at each site, which varied from less than 60 at Tal-y-Bont and Bronant 1 to 2×10^7 at Costessey (Table 1); correlation values for $\bar{h}$, hx and no. of plasmid profiles against cells g^{-1} were 0.10, 0.19 and 0.33, respectively. The number of cells g^{-1} was also independent of pH, a result contrary to other published reports (Jones *et al.*, 1964; Jones, 1966).

Table 5. *Number of isolates of a particular ET at each sub-site*

Sub-site	No. of isolates of ET indicated:										Totals
	MFK	MFM	MFP	MSK	MSM	MSQ	MSS	SSM	SSQ	Others	
B1/1	0	33	1	0	0	2	0	0	0	0	36
B1/2	0	9	0	0	0	0	0	0	0	0	9
B1/3	0	21	0	0	0	0	0	0	0	0	21
B1/4	0	7	0	0	0	0	0	0	0	0	0
Totals	0	70	1	0	0	2	0	0	0	0	73
B2/1	0	28	0	0	0	0	0	0	51	0	79
B2/2	0	19	0	0	0	25	0	0	35	0	79
B2/3	0	16	0	0	9	21	8	7	23	0	84
B2/4	0	17	0	0	8	26	0	0	30	0	81
Totals	0	80	0	0	17	72	8	7	139	0	323
Pw/1	4	7	0	4	8	8	0	15	27	3	76
Pw/2	2	9	0	3	7	6	0	14	31	0	72
Pw/3	0	14	0	0	12	0	0	20	38	0	84
Pw/4	0	16	0	0	0	0	0	14	39	0	69
Totals	6	46	0	7	27	14	0	63	135	3	301
G/1	0	12	15	3	27	0	18	0	0	4	79
G/2	0	14	12	4	37	0	17	0	0	0	84
G/3	0	12	7	2	40	0	25	0	0	0	86
G/4	0	11	4	7	32	0	20	0	0	0	74
Totals	0	49	38	16	136	0	80	0	0	4	323
TOTALS	6	245	39	23	180	88	88	70	274	7	1020

Differences in ET genetic variability between sub-sites

The ET data from the sub-sites (Table 5) largely reflected the patterns from the 'parent' site, although there were several deviations in proportions. Greater variation in terms of the number of different ETs, mean genetic diversity, $\bar{h}$, and genotypic diversity, h_x (Table 4b), was again exhibited at sites of higher pH. The frequency counts of any single ET at all sub-sites were transformed by taking their square roots and the data analysed considering the values in groups according to their parent site, using a one-way analysis of variance. *F* tests, using data for the most frequently occurring ETs over all sub-sites, were significant in all cases except MFM (MSQ, $P < 0.05$; SSQ, $P < 0.001$; MSM, $P < 0.001$; MSP, $P < 0.001$; MFM, NS), indicating greater variation between, rather than within sites.

Differences in ETs between sub-sites were based, to a greater extent than at the parent sites, on quantitative and proportional, as opposed to qualitative differences and were significant in the cases of Pwllpeiran and Bronant 2. Chi-square analyses of the most frequently occurring ETs (SSQ, SSM, MFM, MSM and SSQ, MFM, MSQ, MSM respectively) gave significant values ($\chi^2 = 52.283$ and 102.72 for 12 degrees of freedom).

Although the sub-site data indicated that distinct populations could exist within sites, restriction of allelic variation was again emphasized ($\bar{h} = 0.50$ for all combined sub-sites; Table 4b).

On the basis that the data from sub-sites of the same major location indicate a relatedness of adjacent populations in terms of their chromosomal status and that similarities, albeit to a lesser extent, also occurred between adjacent major sites (Frongoch and Penglais, Garveston and Costessey; see grid references in Table 1) it is suggested that between-site variation might be influenced by geographical separation.

Plasmid profile variation at different locations

Plasmid analyses of representative isolates further demonstrated the differential variation existing within the various sites of contrasting ecology. Where possible, 40 isolates per site were classified (19 at Bronant 1). The number of different plasmid profiles varied from three at

Fig. 1. Number of different plasmid profiles identified at each site (a) and sub-site (b). In (a), the pH value for each site is shown; there was a positive correlation between number of plasmid profiles and pH ($r = 0.90$, $P = > 0.001$).

Bronant 1 to 40 at Penglais, Garveston and Blackawton (Fig. 1a), showing that isolates having the same plasmid profile varied from being in the majority, through many, to few at the acidic, improved and neutral sites, respectively. A strong correlation was again present between genetic diversity (this time in the form of plasmid profile variation) and pH ($r = 0.90$, $P > 0.001$).

Plasmid profile variability at any particular sub-site appeared to be representative of the parent site (Fig. 1b). In many cases the profiles found were identical to representatives of the parent site. This was especially obvious at the acidic, Bronant 1 sub-sites, where the MFM strain with a characteristic plasmid profile represented the majority of isolates. At the other extreme, however, the profiles from the Garveston sub-sites were, as in the case of the parent site, greatly variable.

Complementary methods of identification are particularly useful in examining strain polymorphism in populations subject to variation. In this investigation, as in those of other workers (Glynn *et al.*, 1985; Dughri & Bottomley, 1983a, b, 1984), the use of an additional method has been able to indicate a greater degree of strain variation than suggested by the primary method used alone (for instance at Blackawton, 11 variants were identified by PAGE, whereas 40 were revealed by plasmid profile analysis). Plasmid profile analysis can, therefore, be used to re-evaluate the degree of strain variation at particular sites. An examination of genotypic diversity, *h_x*, over the ten sites might indicate that the Bronant 2, Pwllpeiran and Bowstreet sites possess greater isolate variation than Frongoch, Penglais, Costessey and

Garveston (0.75, 0.73, 0.83 versus 0.48, 0.58, 0.71, 0.70, respectively). However, examination of their plasmid profiles indicates that the converse is true (15, 16 and 33 versus 36, 40, 39 and 40 different profiles, respectively) and that chromosomal variation is more limited at the latter group of sites.

In conclusion, it can be seen that many alleles and ETs are of universal occurrence, implying a certain degree of chromosomal conservation in the biovar *trifolii* as a whole. Sites had their own identities with respect to the variation present and qualitative ET variation within sites was dependent upon the pH of the site. There was a high degree of quantitative, rather than qualitative, difference between sites. This was especially so in the case of adjacent sites where the similarity in ET range of populations in close proximity to each other was demonstrated. The potential for variation to occur appeared to be limited in that it was based on the distribution of a few distinct lineages which seem to be subject to strong genetic disequilibrium. The plasmid profile data were more variable, particularly at unstressed sites, and were useful when used in conjunction with enzyme typing in demonstrating a correlation between genetic variation and environmental stress.

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