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Isozyme polymorphism in a collection of Spanish and French perennial ryegrass populations

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Summary — Twenty-eight natural populations of perennial ryegrass (*Lolium perenne L*) collected from a latitudinal and a longitudinal gradient in Spain, Portugal and France were screened for allozyme diversity at ten loci. Population genetic statistics were found to be of the same magnitude as those previously reported for other outbreeding species (average number of alleles per locus = 2.82, observed heterozygosity = 0.289 and expected heterozygosity = 0.312). Genotype frequencies at most collection sites did not deviate significantly from Hardy-Weinberg expectations. Gene diversity was mainly explained by the within population component. The between population differentiation (F_{ST}) averaged over seven loci was 0.073, which only accounted for 7.9% of the whole diversity. Non-metric multidimentional scaling carried out on the matrix of Cavalli chord distances, based on allelic frequencies, showed that the global differentiation between the populations was partly explained by the latitude and the altitude of the collection sites. Thus, a south–north cline was observed for ACP2-20 and PGI2-20 alleles. In the same way, more SDK1-30 and PGI2-20 alleles were found in populations from higher altitudes. Hypotheses on the origin of such clinal trends are briefly discussed.

allozymes / genetic diversity / *Lolium perenne L* / natural populations

Résumé — Polymorphisme isoenzymatique au sein d'une collection franco-espagnole de populations naturelles de ray-grass anglais. Vingt-huit populations naturelles de ray-grass anglais collectées suivant un gradient longitudinal et latitudinal en Espagne, au Portugal et en France ont été évaluées pour la diversité isoenzymatique de dix loci. Les valeurs des statistiques de diversité génétique apparaissent du même ordre de grandeur que celles rapportées pour d'autres espèces allogames (nombre moyen d'allèles = 2,82, hétérozygotie moyenne observée = 0,289, hétérozygotie moyenne espérée = 0,312). Les fréquences génotypiques dans la plupart des populations sont conformes à celles attendues sous équilibre panmictique. La diversité génétique est expliquée essentiellement par la composante intrapopulation, tandis que la diversité interpopulation ne représente que 7,9 % de la diversité totale. Une analyse factorielle appliquée sur la matrice des distances de Cavalli obtenues à partir des fréquences alléliques a montré que la différenciation globale entre les populations était associée à la latitude et l'altitude des lieux de collecte. Ainsi, pour les allèles ACP2-20 et PGI2-20 une variation clinale sud/nord a été observée. De même, dans les populations provenant des lieux les plus élevées, les allèles SDK1-30 et PGI2-20 ont été trouvés en fréquences plus élevées. Des hypothèses sur l'origine possible de tels clines sont brièvement discutées.

allozymes / diversité génétique / *Lolium perenne* L / populations naturelles

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INTRODUCTION

Perennial ryegrass (*Lolium perenne L*) is the second most frequently sown fodder grass in Spain, after annual ryegrass (*L multiflorum*) with 2000 and 3000 tonnes of seed consumed per year, respectively (Piñeiro and Perez, 1992). The UE agricultural policy towards limitation of cereal production and encouragement of a non-agricultural use of land may further increase the interest for grass species such as *Lolium,* especially for use in unfavorable areas such as the north of Spain. Because perennial ryegrass is mainly cultivated in the oceanic regions of Europe, a sample of populations has been collected in 'natural' meadows (very old meadows with no record of recent sowing with important cultivars), following a latitudinal and a longitudinal gradient from the northwest oceanic coast of Spain to the north of France in order to obtain a diverse germplasm for plant breeding in southwestern Europe.

As genetic resources are primarily of use for plant breeders, who look for adaptive traits, an assessment of these genetic resources for their ability to have good seasonal growth was carried out and will be presented in another paper (Balfourier et al, 1997).

Survey of isozyme variation in natural populations of forage grasses have already been reported for ryegrass (Hayward, 1985; Arcioni et al, 1988; Oliveira and Charmet, 1988; Charmet et al, 1993; Balfourier and Charmet, 1994; Charmet and Balfourier, 1994; Loos, 1994). Variation at isozyme loci is generally considered as selectively neutral (Kimura, 1983). However, sometimes, allelic frequencies of isozyme are correlated with environmental factors, thus possibly reflecting selection pressures (Nevo et al, 1986).

The aims of this isozyme study were to investigate 1) the importance of genetic differentiation in relation to the geographic distance of populations (genetic markers supposed neutral) and 2) whether there were alleles showing latitudinal, altitudinal or longitudinal gradients that could be markers of adaptative traits.

MATERIALS AND METHODS

Isozyme procedures

Twenty-eight wild populations of perennial ryegrass were collected as seed from Spain, Portugal and France in 1990. Out of these 28 populations, 16 were sampled from Spain (Nos 1–16), one from Portugal (No 17), and 11 from France (Nos 18–28).

Populations were sampled to represent latitudinal and longitudinal gradients, sites ranging from latitude 41.45 to 50.71°N and from longitude 9.10°W to 2.74°E, and altitudes ranging from 10 to 1050 m. Each population was collected as seed from at least 50 plants taken from an ecologically homogeneous area of 100–1000 m^2. These conditions are thought to yield a sample of seeds representative of the original population (Tyler et al, 1984). Unlike in more northern countries, most plant are able to head in southern countries under moderate grazing. Therefore, collecting seeds does not bias the population of living plants too much.

From each of the 28 populations, approximately 100 plants were studied using isozyme electrophoresis techniques following Hayward and McAdam (1977), Ostergaard et al (1985), Pollans and Allard (1985) and Greneche et al (1991). Slices of two starch gels and two different buffer systems were used, which permitted the study of nine enzyme systems, giving ten readable loci. Histidine–citrate buffer was used for acid phosphatase (ACP, EC 3 1 3 2), isocitrate dehydrogenase (IDH, EC 1 1 1 42), malate dehydrogenase (MDH, EC 1 1 1 3 7), phosphogluco-isomerase (PGI, EC 5 3 1 9), phosphogluco-mutase (PGM, EC 2 7 5 1), peroxidase (PRX, EC 1 11 1 7) and shikimate dehydrogenase (SKD, EC 1 1 1 25). Tris-citrate–lithium-borate buffer was used for glutamate oxaloacetate-transaminase (GOT, EC 2 6 1 1) giving two readable loci, and superoxide-dismutase (SOD, EC 1 15 1 1). Allele nomenclature and isozymes procedures were those of Hayward et al (1995).

Statistical analysis

Allelic frequencies were determined by direct allele counting. Standard stastistics for characterizing genetic variability were computed for all accessions using the BIOSYS 1 program (Swofford and Sealander, 1981). The following population genetic statistics were computed: mean number of alleles per locus (A), average observed heterozygosity (H_O) and expected heterozygosity under panmixia (H). Wright's (1965) fixation indices were calculated: F_{IT} represents the fixation of the individuals relative to whole population; F_{IS} gives the fixation of individuals within populations. Both parameters measure the surplus or deficit of heterozygotes, and can become negative; F_{ST} is the fixation index that represents the differentiation level of the populations, and is equivalent to Nei's (1977) G_{ST} for biallelic loci. The distances between all populations, based on allelic frequencies, were calculated using the chord distance of Cavalli-Sforza and Edwards (1967). The populations were scaled in a two-dimensional space using non-metric scaling (Kruskal and Wish, 1978). This is a class of methods for estimating the coordinates of a set of objects in a space of specified dimensionality from data measuring the distances between a pair of objects. Regression analysis was used to investigate the relationship between the first

two axes obtained by non-metric scaling and some explanatory variables (altitude, latitude and longitude of the collection site).

Logistic regression analysis is often used to investigate the relationship between binary and ordinal response variables and explanatory variables (Cox and Snell, 1989; Collett, 1991). In this study, logistic regression analysis was carried out between the allelic frequencies of the populations and the altitude, latitude and longitude. All these analyses were carried out using the 'S' programing environment (Becker et al, 1988).

RESULTS

Population diversity indices are summarized in table I. Mean number of alleles per locus and average heterozygosities found in this sample of ryegrass populations are, respectively: A = 2.82, H_O = 0.289 and H = 0.312. Table II shows the summary statistics of allelic frequencies in the populations studied. All the enzymatic systems are polymorphic. A total of 41 alleles has been found. The PGI2 locus contained the highest number of alleles, with up to eight alleles, while SOD1 and MDH1 loci contained the fewest, with only three alleles. Of 41 alleles, 21 (§) can be considered as common widespread according to the classification of Brown (1978), eight (*) as rare widespread (mean frequency less than 5%, but present in more than half of the populations), and 12 (**) as rare and sporadic. Eight alleles of

Table I. Parameters of genetic variability at ten loci in 28 populations, including average number of alleles per locus (A), observed heterozygosity (H_O) and expected heterozygosity (H).

Pop No	*Origin*	*Location*	*A*	H_O	*H*
1	Spain	Galicia	2.7	0.218	0.250
2	Spain	Galicia	2.6	0.212	0.226
3	Spain	Galicia	2.3	0.222	0.211
4	Spain	Galicia	2.8	0.282	0.284
5	Spain	Galicia	3.0	0.305	0.371
6	Spain	Galicia	2.9	0.285	0.307
7	Spain	Galicia	2.8	0.250	0.280
8	Spain	Asturias	3.2	0.358	0.398
9	Spain	Asturias	2.9	0.315	0.334
10	Spain	Asturias	3.0	0.314	0.344
11	Spain	Asturias	2.8	0.200	0.216
12	Spain	Asturias	2.8	0.284	0.315
13	Spain	Asturias	3.4	0.369	0.429
14	Spain	Cantabria	2.4	0.258	0.286
15	Spain	Pais Vasco	2.7	0.261	0.297
16	Spain	Pais Vasco	3.4	0.328	0.378
17	Portugal	Vila Real	2.9	0.327	0.317
18	France	Pyrénées Atlantiques	2.9	0.275	0.302
19	France	Charente Maritime	3.1	0.314	0.346
20	France	Charente	2.3	0.253	0.266
21	France	Morbihan	3.1	0,336	0.348
22	France	Manche	2.9	0.327	0.319
23	France	Calvados	3.1	0.314	0.322
24	France	Orne	2.8	0.254	0.314
25	France	Seine Maritime	2.6	0.334	0.320
26	France	Pas de Calais	2.4	0.284	0.305
27	France	Somme	2.7	0.323	0.324
28	France	Somme	2.7	0.296	0.323
mean			2.82	0.289	0.312
se			0.38	0.069	0.071

Table II. Average frequencies of alleles detected in the Spanish and French ryegrass populations.

Allele	*Mean freq*	*Min*	*Max*	*Allele*	*Mean freq*	*Min*	*Max*
PGI2-10 **	0.007	0	0.107	SOD1-10 **	0.004	0	0.069
PGI2-20 §	0.300	0.083	0.540	SOD1-20 §	0.052	0	0.235
PGI2-28 **	0.001	0	0.013	SOD1-30 §	0.943	0.760	1
PGI2-30 §	0.521	0.310	0.712	PRX1-30 §	0.053	0	0.255
PGI2-40 §	0.063	0.006	0.146	PRX1-40 §	0.947	0.745	1
PGI2-45 *	0.046	0	0.118	PRX1-50 **	< 0.001	0	0.007
PRI2-50 *	0.048	0	0.320	IDH1-20 *	0.019	0	0.113
PGI2-60 **	0.015	0	0.115	IDH1-30 §	0.492	0.085	0.887
ACP2-20 §	0.369	0.007	0.770	IDH1-40 §	0.475	0.107	0.915
ACP2-30 §	0.534	0.162	0.966	IDH1-50 *	0.014	0	0.171
ACP2-40 §	0.066	0	0.202	SKD1-10 **	< 0.001	0	0.007
ACP2-50 *	0.031	0	0.164	SKD1-20 *	0.034	0	0.154
GOT2-10 **	0.011	0	0.160	SKD1-30 §	0.914	0.825	0.995
GOT2-20 §	0.313	0.015	0.701	SKD1-40 §	0.051	0	0.140
GOT2-30 §	0.668	0.269	0.985	PGM1-10 **	< 0.001	0	0.015
GOT2-40 **	0.008	0	0.152	PGM1-20 §	0.608	0.395	0.792
GOT3-20*	0.024	0	0.192	PGM1-30 §	0.373	0.197	0.545
GOT3-30 §	0.865	0.722	1	PGM1-40 **	0.018	0	0.081
GOT3-40 §	0.099	0	0.221	MDH1-10 **	0.001	0	0.110
GOT3-50 **	0.012	0	0.153	MDH1-20 §	0.961	0.775	1
				MDH1-30 *	0.032	0	0.210

§ = common allele; * = rare widespread allele; ** = rare and sporadic allele.

Table III. Wright's fixation indices.

Loci	F_{IS}	F_{IT}	F_{ST}
PGI2	–0.018	0.033	0.050
ACP2	0.105	0.198	0.104
GOT2	0.187	0.268	0.100
GOT3	0.099	0.164	0.071
SOD1	0.118	0.187	0.078
PRX1	0.280	0.328	0.066
IDH1	0.020	0.141	0.124
SKD1	0.041	0.076	0.036
PGM1	0.023	0.062	0.040
MDH1	0.005	0.092	0.087
mean of seven loci	0.039	0.109	0.073

this last category reach a frequency of 5% or more in at least one population, while the others have a very low frequency of less than 2%.

The values of Wrights' fixation indices for each locus averaged across all loci are given in table III. The fixation indices are lower for PGI2, ACP2, GOT3, IDH1, SKD1, PGM1 and MDH1 compared with the other loci, showing that populations are in panmictic equilibrium for these loci. PRX1, GOT2 and SOD1 loci show higher fixation indices F_{is} and F_{it}, which illustrate their deficits in heterozygotes. The discrepancy between these three loci and the other seven loci may be accounted for by some misinterpretation of gel patterns. Therefore we used only the seven loci PGI2, ACP2, GOT3, IDH1, SKD1, PGM1 and MDH1 in averaging F statistics. The mean level of between population differentiation (F_{ST}) is 0.073. The among population differentiation can also be expressed as a proportion of the diversity index H (mean heterozygosity expected under panmixia) by $D_{ST} / H = F_{ST} / (1–F_{ST})$ (Nei, 1977). In this study ($D_{ST} / H = 0.073 / 0.927 = 0.079$) only 7.9% of the genetic diversity is accounted for by among population differentiation.

The results from non-metric multidimentional scaling of the distances between the populations are presented in figure 1. The first two axes account for 23.1 and 19.4% of the total variance, respectively. This figure shows that the northern French populations are grouped at the bottom right corner of the figure, indicating that these populations have relatively short distances between them. With regards Spanish populations, most of them are scattered all over the plot except some northwest populations that are grouped together, such as Sp1, Sp2 and Sp3 and Sp4, Sp5 and Sp6.

Linear regression carried out on the first two axes of non-metric scaling showed that the second axis is significantly explained by latitude ($r = 0.571$, $P < 0.01$).

The logistic regression between allelic frequencies and altitude, latitude and longitude reveals several significant ($P < 0.001$) relationships. Results of deviances (Collett, 1991) are given in table IV for the most significant relationships. Two alleles show a trend toward a latitudinal clinal variation: ACP2-20 and PGI2-20 are more frequent in the north than in the south as presented in figure 2 for the PGI2-20 allele. On the other hand, in populations from higher altitudes, more SDK1-30 and PGI2-20 alleles were found; figure 3 illustrates the logistic regression between SDK1-30 allele frequency and altitude of collection site.

DISCUSSION

As reported in other publications, native populations of perennial ryegrass collected in Spain and France showed a wide range of isozyme variation (Oliveira and Charmet, 1988).

The average genetic diversity indices A (2.82) and H (0.312) of this study are higher than those reported by Hamrick and Godt (1990) for outcrossing, wind pollinating species. Our diversity indices are very similar to those reported by Hayward (1985) in 40 natural populations from Britain (A = 3.09, H = 0.372) on five loci, by Oliveira and Charmet (1988) in populations from northwestern Spain (A = 3.16, H = 0.395) on seven loci, by Charmet et al (1993) for 60 perennial ryegrass populations from France and seven polymorphic isozyme loci (A = 2.75, H = 0.270) and Loos (1994) for 60 European populations and cultivars of *L perenne* and five loci (A = 2.70, H = 0.308). The means of both parameters were higher in these studies than in the work of Hamrick and Godt (1990) because none of the loci studied here were monomorphic.

The mean value F_{IS} of 0.039 is comparable with the values reported by Hayward and McAdam (1977) for *L perenne* cultivars, Oliveira and Charmet (1988) for *L perenne* Galician populations, Charmet et al (1993) for French perennial ryegrass populations and Loos (1994) for European populations and cultivars of *L perenne L.* This low value leads to the conclusion that collected populations are nearly panmictic.

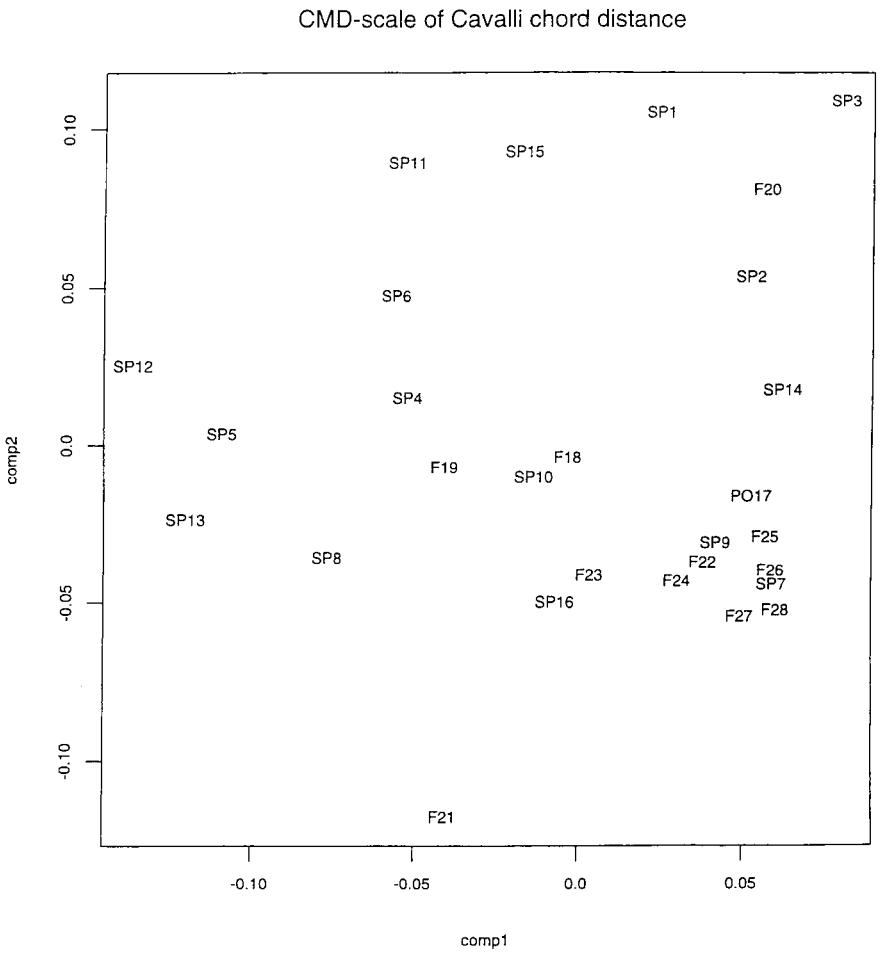

Fig 1. Scatterplot of 28 *L perenne* populations, based on the Cavalli chord distances calculated from the allelic frequencies for ten allozymes loci. For the populations, the following abbreviations were used: SP, Spain; PO, Portugal; F, France.

The fact that most of the total gene diversity is accounted for by the within population variation component agrees with Hamrick and Godt (1990). This suggests that sampling few populations for conservation of genetic resources could be sufficient to preserve most of the species isozymic variation. Large effective population size must be used to avoid genetic drift.

Most alleles showed no geographic structuration. As reported by Sokal (1986), such an absence of geographic structure could be explained by near panmixia over the whole geographic ranges. The panmixia hypothesis is supported by the high estimate of average gene flow among populations. Such a high gene flow among populations might be accounted for by long-distance seed transport by animals, or even pollen transport by wind if we assume very large neighborhood sizes in a continuous population model.

Table IV. Table of deviance explained by logistic regression.

Alleles	*Altitude*	*Latitude*	*Longitude*
PGI2-20	24.06 **	52.80 **	1.02
ACP2-20	13.85 **	77.08 **	0.03
SDK1-30	28.15 **	2.46	1.33

** = significant at 0.001 level.

However, some alleles showed clinal patterns of geographic variation, the most clear being that of the ACP2-20 (former ACP2-a) and PGI2-20 (former PGI2-a), which show a clinal trend from southwest to northwest. There are two evolutionary hypotheses to account for these existing clinal variation in allele frequencies: it could be explained either by selection of different alleles along a geographical or ecological gradient (Endler, 1977), or as a consequence of migration and long-range dispersal along a geographical axis (Wijsmann and Cavalli-Sforza, 1984). Other isozyme surveys reported a south–north cline for different alleles in a collection of French perennial ryegrass populations (Charmet et al, 1993) and in a European collection of ryegrass populations from England to Italy (Loos,

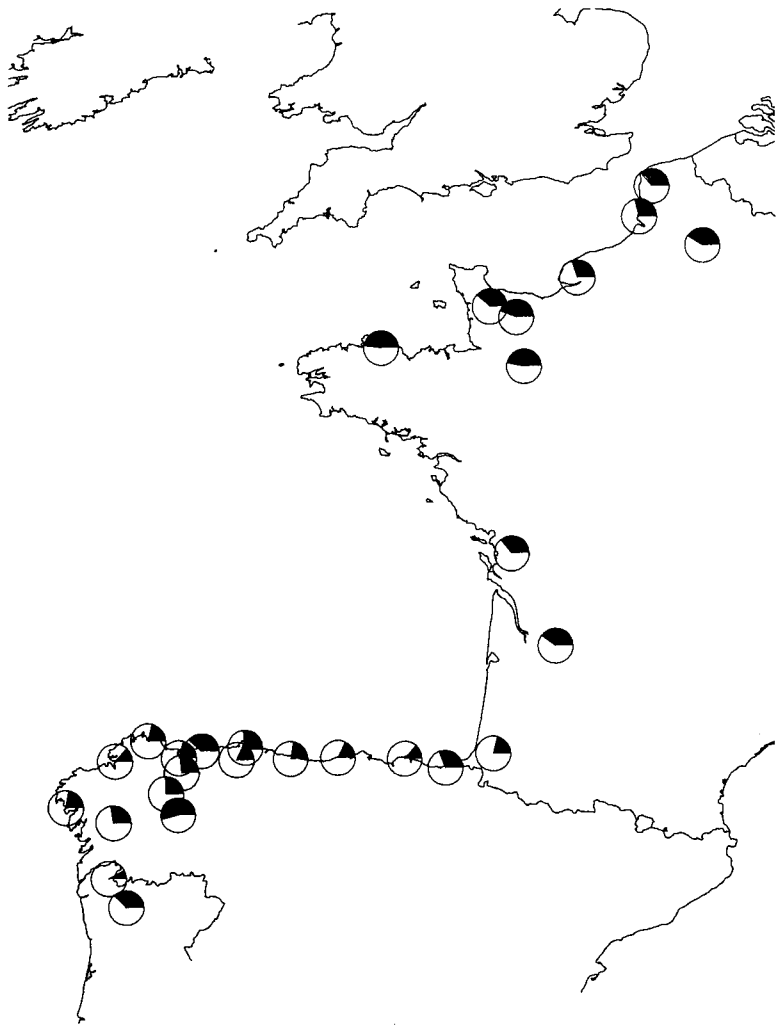

Fig 2. Geographic patterns of variation for PGI2-20 allele in 28 populations of *L perenne*. Filled sectors are proportional to allele frequency.

1994). These authors seems to prefer the migration hypothesis.

We also observe that the PGI2-20 allele seems to be linked with altitude. This could probably be explained by the selection hypothesis; in that way, Humphreys (1992) observed a consistent association between water soluble carbohydrate content and genotype at the PGI2 locus. This locus is involved in the increase in water soluble carbohydrate content, producing an osmotic effect responsible for a better frost tolerance. In these conditions, it is possible to imagine that altitude of evolution site (and consequently low temperature) interacts with the enzymatic reaction and acts as a selection pressure (Balfourier et al, 1997). As for the relationships between SKD1-30 allele and altitude, if we consider the neutral theory, we can also suppose that this locus is closely linked to a quantitative trait locus, which is selected by altitude.

Thus, some relationships seem to exist between few alleles and geographical data of evolution site, illustrating a geographical structure of the diversity. However, it is difficult to make conclusions about the origin of the structure: for ACP2-20 and PGI2-20 alleles linked with latitude, the migration or colonization hypothesis appears attractive but, in that case, a similar cline of variation should have been observed for all loci (Barbujani, 1988). In fact, it would be necessary to realize such a study on a larger geographical scale and with more numerous populations, as realized on forest tree species where similar south–north clines have been suggested (Michaud et al, 1994; Leonardi and Menozzi, 1995; Zanetto and Kremer, 1995). All these authors explain such clines as a consequence of colonization since postglacial refugia zones. In this way, to support the selection hypothesis, it would be necessary to sample populations on several distant climatic

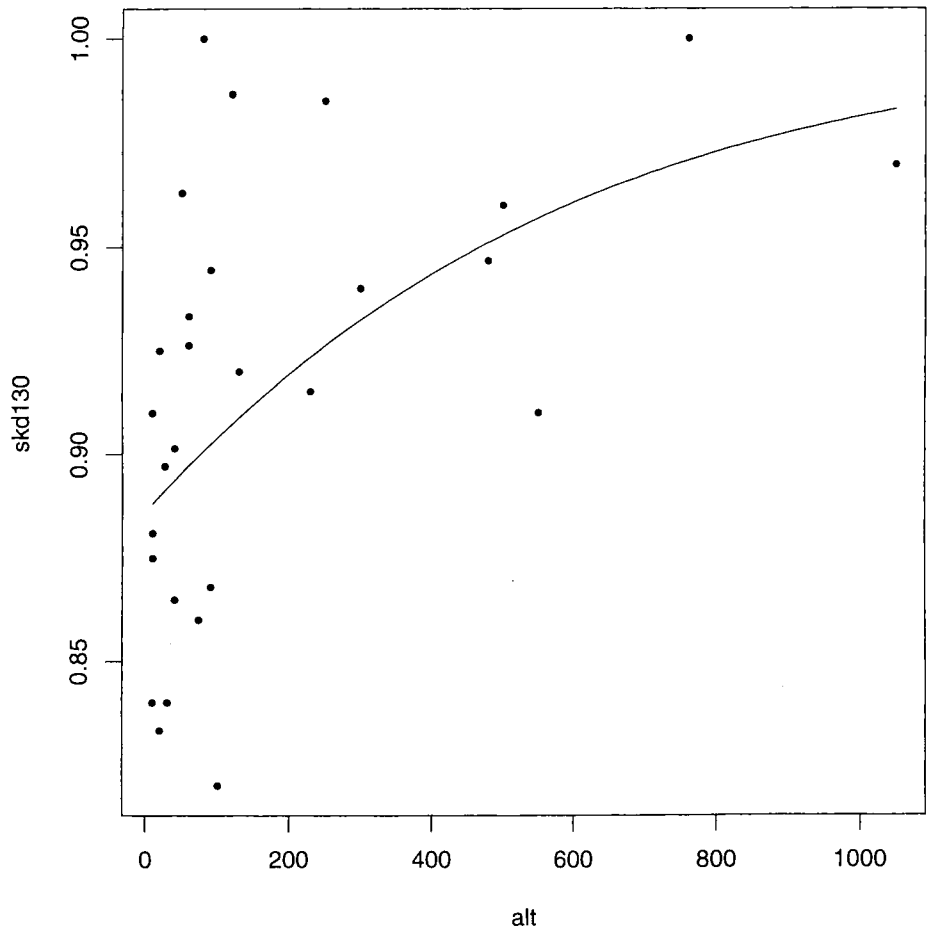

Fig 3. Logistic regression between altitude and SKD1-30 allele frequency.

and geographical gradients, as did Lumaret (1984) in *Dactylis glomerata L.* These studies are in process at the present time and will be presented in a further paper.

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