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POTENTIALITY OF STS FOR VARIETY DISTINCTION IN RYEGRASS

Document prepared by experts from France

POTENTIALITY OF STS FOR VARIETY DISTINCTION IN RYEGRASS

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INTRODUCTION

Distinction of the varieties in fodder crops is difficult because of the lack of discriminant morphological characters. Moreover, many of the existing characters are subject to changes according to the climatic fluctuations or are rather subjective (for example colour or growth habit). As in many other fodder crops, the varieties in ryegrass are synthetic varieties. This causes an important variation within varieties and adds to the difficulty of distinguishing them.

Therefore, there is a growing need for genetic markers, in order to characterize more accurately the varieties.

A dozen of **enzymatic markers** have already been described for the evaluation of the genetic variation in ryegrass (Charmet et al, 1994). They are useful for describing wild populations, but when commercial varieties are examined their discriminant power decreases greatly. In practice, for commercial varieties not more than 3 or 4 enzyme markers are used (Hayward. et Mc Adam, 1977 ; Greneche *et al*, 1990). In GEVES, PGI (Phospho Glucose Isomerase), ACP (Acid Phosphatase) et IDH (Isocitrate dehydrogenase) are used routinely. The varieties are described using one hundred individuals and compared using $\chi 2$ tests on the allelic frequencies. About 300 varieties have already been described and their description published periodically in the 'bulletin des variétés'. However, we would like to obtain more markers to achieve better accuracy and to diminish sample sizes.

RFLP markers have been published for ryegrass (Hayward et al ,) along with studies using **AFLP markers** (DeLoose et al; Charmet et al, comm.pers.). We still require more efficient methods than RFLP and codominant markers unlike AFLP. Thus, the present study was developped to investigate a

method which could be used on species for which few or no markers are available. Therefore we used genes known in other related species to derive STS markers for ryegrass. The present paper describes the results obtained with 10 such STS markers in the characterisation of commercial varieties.

MATERIALS AND METHODS

Plant Material

The following varieties have been used :

Magella, Pacage, Yatsyn 1 (*Lolium perenne* used for fodder); Blazer, Repell (*Lolium perenne* used for turf)

Aramo (*Lolium multiflorum,* alternative type) ; Fastyl, Lipo (tetraploïd) and Tribune (*Lolium multiflorum* , non alternative)

1 breeding population and 1 mapping population (*Lolium perenne*)

20 individuals per variety were described except for Repell which was described on 60 individuals.

DNA Amplification

The DNA was extracted using a CTAB protocol (Rogers and Bendich, 1988) The PCR conditions are given in table 1. The amplified fragments were then separated on 5% polyacrylamide gels at a voltage of 300V. The duration of the migration and the size of the expected fragments are also given in table 1.

Strategy used for finding the markers

We looked for gene sequences from gramineaes in the data bases, using « Entrez ».

Except for LP1, we kept only genes containing introns. We then looked for the closest sequences among the genes of gramineaes. When these were found, we tried to locate consensus zones flanking introns. We then defined primers in these consensus zones, using « Oligo ».

Estimation of the discrimination power of the markers by resampling methods

The power of a test is its ability to reveal differences when these exist.It can be estimated by the frequency of positive tests when it is possible to carry out tests on a great number of samples coming from the same population. Since we have not yet described big populations with our markers, we generated 2 virtual populations of 1000 individuals having the same allelic distribution as that estimated from 20 individuals from varieties 'Blazer' and 'Repell'. These 2 varieties, chosen for the tests, were the closest ones among our set of varieties. The varieties were declared distinct when the genetic distance was significantly different from zero. We chose the Rogers distance and P= 0.001. For each population, 1000 samples of N were drawn. The Rogers distances were then calculated for each pair of samples.

RESULTS

Out of 30 pairs of primers tested, 7 gave no amplification or a RAPD like amplification pattern, 7 were monomorphic on our plant samples, 16 gave polymorphic patterns with a number of alleles ranging from 2 to 8 alleles for our plants sample. Figure 1 shows the amplification products for the primers MZE and ADP .

The patterns obtained suggested a simple genetic interpretation, *i.e.* 1 gene with several alleles for most primers. All amplified products had the predicted size except CAF.

Table 2 shows the allelic distribution for 7 markers on our subset of varieties. Table 3 shows the allelic distribution for 3 isozymes on the same varieties. The polymorphism observed seems comparable for isozymes and STS : both show codominancy and a comparable number of alleles. Moreover, both concern genes of known functions. For both types of markers, some alleles seem specific to either perennial or italian ryegrass (alleles "b" and "c" from OSW, "c","d" and "e" from MZE, "a" and "d" from PGI).

Compared to the commercial varieties, the breeding population shows 2 supplementary alleles for the marker OSW.

The results on the mapping population show a good segregation and indicate that the markers are independent.

Table 4 shows the Rogers distances between 4 varieties of perennial rye-grass. They have been estimated on 3 isozyme loci (PGI-IDH-ACP : 13 alleles) or 7 STS loci (ADP, LP1, SCF, OSE, OSRB, OSW, MZE : 22 alleles)

Table 5 shows the results obtained by sampling 10 to 60 individuals in virtual populations of Blazer and Repell. The data shown are the mean distance, its standard deviation and the power of distinction, i.e. the probability to find distances significantly different from 0 (frequency on 1000 samplings).
The distances Blazer/Repell estimated using either the STS or the isozymes are similar. As expected, the standard deviation decreases as the sample size increases. It is the same for the mean distance which becomes increasingly close to the "real" distance. The over-estimation of the distance on small samples is a known phenomenon, analogous to genetic drift. The most interesting data concern the power of the test (P(D 0)). This value reaches 1 as soon as 60 individuals are used when calculating the distances with isozyme markers. In contrast, the same results is obtained with only 20 individuals when the 7 STS markers are used. This is despite the samples having the same standard deviation.

CONCLUSION

This paper describes new markers for the study of the genetic diversity of ryegrass.
The polymorphism found using these STS markers is high :16 polymorphic sequences out of 23 amplified. This is higher than the polymorphism found for wheat, barley, rice, *stylosanthes*, in similar studies (TALBERT *et al.* , 1993 ; CHEN *et al.* 1994 ; GHAREYAZIE *et al.*, 1994 ; LIU *et al.*, 1996). For these species, restriction of the amplified products was often necessary to detect polymorphism . Intravarietal polymorphism also is important. The varieties generally had 2 or more alleles per marker. Despite this fact, it was possible to distinguish the varieties from one another.
As indicated by the bootstraping on Blazer/Repell, these markers enable diminuation of the sample size to 20, which is interesting for routine uses. However, the characterisation using STS markers is time consuming and needs optimisation. Multiplexing has already been tested on a small scale and gave good results. The use of an automatic sequencer will also probably greatly improve the potential of the method.
The last point, and not the least important, is that this strategy can be used for species for which molecular markers are not available. We have also tested our markers on *Dactylis, Festuca, Poa, Phleum* and *Bromus* and obtained good results. This approach will be very useful for a lot of species, which are not economically important but for which markers are none the less needed.

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Table 1 :

Primers	Locus	Origin	Expected size	Hybridisation temperature
ADH 3/4	Alcool Déshydrogénase	Maize, barley and rice	401 pb.	53°C
MZE 1/2	Triosephosphate Isomérase	Maize, rye and rice	1202 pb.	55°C
OSW 2/3	ADP-Glucose Glycosyl Transférase	Rice, maize, barley and sorghum	414 pb.	57°C
LP1 c/d	Allergène de pollen	Ryegrass	526 pb.	58°C
PRO	Profilin (auxin binding protein)	Phleum and maïze	730 bp	62 °C
OSE 1/2	Gène LEA= =late embryogenesis abundant	Rice, barley maize and wheat	311 pb.	60°C
SCF ½	Ribulose 1-5 biphosphate carboxylase/oxygénase	Sugar cane, rice, barley and maize	402 pb.	60°C
OSRB ½	α Amylase 3	Rice, barley maize and wheat	759 pb.	60°C
ADP 1/3	ADP Glucose phosphorylase	Rice, barley and wheat	988 pb.	50°C
PHOS	Phospholipase	Rice and maize	665 bp	55°C
PGLU	Prepro glutelin	Rice and avena	380 bp	55°C
PAL	Phenylalanine ammonialyase	Rice, barley and wheat	980 bp	60°C
CAT	Catalase	Rice and barley	969 bp	55°C
SERCAR	Serin carboxypeptidase	Rice and barley	322 bp	55°C
ASP	Aspartic protease	Rice and barley	506 bp	56°C
CAF	Caffeic acid	Ryegrass	993 bp	58°C

Table 2 : allelic distribution for 7 STS

	ADP			LP1			SCF				OSE			OSRB			OSW								MZE						
	a	b	T	a	b	T	a	a'	b	T	a+a'	b	T	a	b	T	a	b	c	d	e	f	g	T	a	b	c+c'	d	e	f	T
BLAZER	0	40	40	37	3	40	33	7	0	40	14	26	40	34	4	38	32	0	0	3	1	0	0	36	10	1	27	0	0	0	40
REPELL	0	40	40	23	17	40	22	16	0	38	10	30	40	32	6	38	35	0	0	1	0	0	0	36	4	0	36	0	0	0	40
PACAGE	1	39	40	36	0	36	15	13	10	38	8	30	38	38	0	38	26	4	0	10	0	0	0	40	8	4	28	0	0	0	40
YATSIN	10	28	38	36	0	36	19	4	15	38	2	38	40	20	6	26	30	10	0	0	0	0	0	40	8	0	27	0	0	5	40
							a+a'		b	T																					
MAGELLA							19		21	40	3	37	40	37	3	40	35	0	0	5	0	0	0	40							
population				135	19	154					37	103	140	102	52	154	102	0	5	0	4	24	9	144	48	65	29	2	0	0	144
TRIBUNE							6		34	40	16	24	40	24	12	36	18	0	14	8	0	0	0	40							
FASTYL											10	28	38	27	9	36	11	0	13	9	1	0	0	34							
ARAMO							6		34	40	6	34	40	25	11	36	1	0	5	12	2	0	0	20							

Table 3 : allelic distribution for 3 isozymes

	PGI					IDH					ACP				
	a	b	c	d	T	a	b	c	d	T	a	b+c	d	e+f	T
BLAZER	57	56	95	0	208	0	74	146	0	220	116	77	21	0	214
REPELL	64	74	70	0	208	0	114	88	0	202	136	32	38	0	206
PACAGE	92	71	35	0	198	0	23	171	0	194	155	8	33	0	196
YATSIN	71	95	40	0	206	0	96	90	8	194	112	92	0	0	204
MAGELLA	30	103	66	1	200	1	73	124	0	198	114	77	7	0	198
POPULATION	79	64	9/3	1	156	0	73	83	0	156	103	38	13	2	156
TRIBUNE	30	23	4	147	204	140	43	1	0	184	72	132	0	0	204
FASTYL	4	81	0	121	206	108	79	13	0	200	55	120	3	22	200
ARAMO	4	92	7	99	202	100	102	2	0	204	30	143	0	25	198
LIPO	20	218	7	155	400						47	127	1	25	200

Table 4 : Rogers distances between 3 varieties of perennial rye-grass. Estimations from 3 isozyme loci (PGI-IDH-ACP) above diagonal, or 7 STS loci (ADP,LP1,SCF,OSE,OSRB,OSW,MZE : 22 allèles) under diagonal.

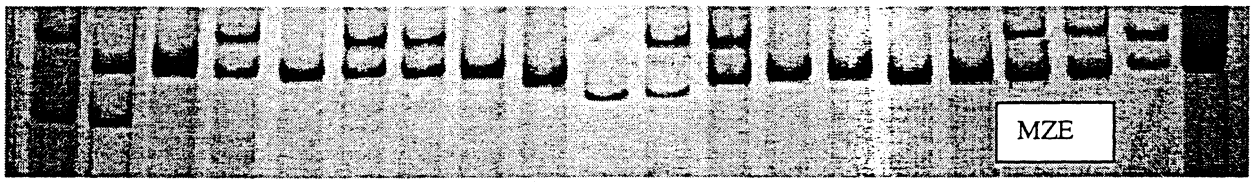
STS / ISO	BLAZER	REPELL	PACAGE	YATSIN
BLAZER	----------	0.175	0.253	0.180
REPELL	0.182	----------	0.282	0.162
PACAGE	0.186	0.224	----------	0.316
YATSIN	0.235	0.273	0.187	----------

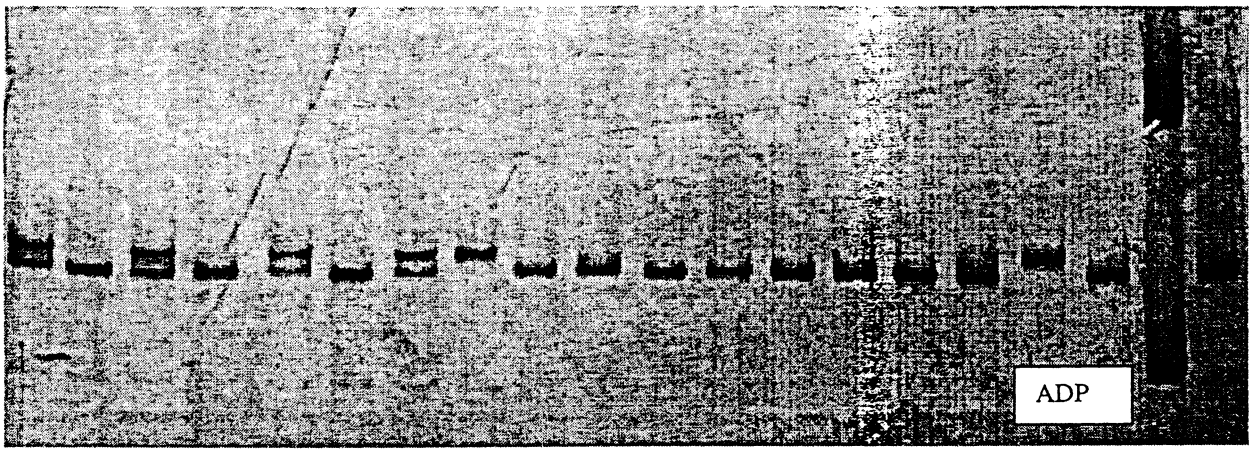
Table 5: Results of 1000 resamplings of N individuals in simulated populations of BLAZER and REPELL (1000 individuals). Dm = mean Rogers distance ; Sd = standard deviation; P(D>0) probability that the distance is significantly different from 0 with P=0.001

3 locus iso						
N	10	20	30	40	50	60
Dm	0.274	0.235	0.227	0.207	0.202	0.194
Sd	0.067	0.051	0.043	0.039	0.034	0.031
P(D>0)	0.841	0.939	0.986	0.989	0.997	1
7 locus EST						
N	10	20	30	40	50	60
Dm	0.229	0.209	0.199	0.192	0.190	0.188
Sd	0.042	0.030	0.024	0.022	0.021	0.018
P(D>0)	0.993	1	1	1	1	1

Figure 1 :

Patterns observed for the markers MZE and ADP





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