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Mode of reproduction is detected by Parth1 and Sex1 SCAR markers in a wide range of facultative apomictic Kentucky bluegrass varieties

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Abstract

Gametophytic apomixis in Kentucky bluegrass (*Poa pratensis* L.) involves the parthenogenetic development of unreduced eggs from aposporic embryo sacs. Marker-assisted selection for the mode of reproduction in *P. pratensis* would avoid costly and time-consuming phenotypic progeny tests. We developed and tested two SCAR primer pairs that are associated with the mode of reproduction in *P. pratensis*. The SCAR primers identified the apomictic and sexual genotypes among progenies of sexual x apomictic crosses with very low bias. Furthermore, when tested on a wide range of Italian and exotic *P. pratensis* germplasm, they were able to unequivocally distinguish sexual from apomictic genotypes. This system should, therefore, allow new selection models to be set up in this species.

Abbreviations: AFLP – amplified fragment length polymorphism, BSA – bulked segregant analysis, MAS – marker-assisted selection, PCR – polymerase chain reaction, SCAR – sequence characterized amplified region

Introduction

The mating of gametes of the opposite sex is generally required for seed production. An interesting exception to this rule is apomixis, an asexual mode of reproduction through seed that avoids both meiotic reduction and egg fertilization. The essential feature of apomixis is that an embryo is formed parthenogenetically from an unreduced egg generated either from a megaspore mother cell (diplospory) or a nucellar cell (apospory) (Koltunow 1993). The genetic constitution of the apomictic embryos is, therefore, usually identical to the female parent, a trait of great interest to plant breeders. If apomixis were well understood and harnessed, it could be exploited to indefinitely propagate superior hybrids or specific genotypes bearing complex gene sets. Until the gene(s) that promote and control apomixis are molecularly understood, this trait can only be introgressed into agricultural crops through traditional breeding methods, most of which are slow and laborious and require progeny tests for the selection of apomictic genotypes after each round of backcrossing.

Among the practical choices for minimizing time and costs of breeding programs, an easy method for early selection based on molecular markers appears to be the most promising. The essential requirements are: (1) simple genetic inheritance of the trait under selection, and (2) the availability of molecular markers strongly associated with the trait.

The available data on the apomictic reproduction indicate that the first requirement is met. In Kentucky bluegrass (*Poa pratensis* L.) (Matzk 1991; Barcaccia et al. 1998) and several unrelated species such as *Panicum maximum* (Savidan 1983), *Pennisetum squamulatum* (Ozias-Akins et al. 1993, 1998; Gustine et al. 1997; Rocke et al. 1999), *Brachiaria decum-*

bens (Pessino et al. 1997), and *Tripsacum dactyloides* (Leblanc et al. 1995), apomixis seems to be under simple genetic control. Cyto-embryological and molecular analysis have revealed that only a single, or a few tightly clustered, dominant genes are required for the genetic transmission of apospory and/or parthenogenesis and that apomixis is a simply inherited system (Matzk 1991; Barcaccia et al. 1998).

The development of an appropriate set of genetic markers would then be of great, practical interest. However, the constraints are the availability of plant material amenable to genetic analysis, the resources allocated, and the laboratory facilities accessible to the breeders. As a general rule, a convenient genetic marker should be easily detectable, highly specific, reliable, and cheap.

SCARs are recently introduced molecular markers (Paran and Michelmore 1993) that qualify for all the above features. Each SCAR consists of a genomic DNA fragment at a single genetic locus that is identified by PCR using a pair of specific oligonucleotide primers. The highly stringent conditions of the PCR amplification make its detection relatively easy, very specific and requires only traces of genomic DNA (Klimyuk et al. 1993) of moderate quality, a thermal cycler and a nucleic acid detection system.

Kentucky bluegrass is a hardy, persistent, attractive forage and turf grass adapted to a wide range of soils and climates (van Wijk 1997). It has an extremely versatile mode of reproduction, which ranges naturally from nearly obligate apomixis to complete sexuality. Although this species can hybridize and absorb entire genomes, it reproduces mainly through facultative aposporous apomixis. Because different plants may show contrasting modes of reproduction, *P. pratensis* could serve as model species for investigating apomixis and its inheritance. Moreover, due to its ever-increasing importance in turf science and technology, it has captured the interest of many breeders. Varieties of *P. pratensis* are widely used in highly cared turfs (soccer, rugby, golf, etc.). The minimum requirements are uniformity, adaptation to the environment and resistance to disease. Once combined in superior genotypes, these traits could be easily propagated apomictically.

We previously detected an AFLP linkage group related to aposporous parthenogenesis in *P. pratensis* (Barcaccia et al. 1998). We now report the conversion of the two parthenogenesis-nearest flanking markers into SCARs and their potential use in MAS programs.

Materials and methods

Plant material

Controlled crosses were carried out between completely sexual clones (S1, S2, S4) and highly apomictic genotypes (RS-9, RS-18, RS-20) of *P. pratensis* (Matzk 1991; Barcaccia et al. 1997). A total of 48 plants segregating for the mode of reproduction, selected from the F_1 progeny and characterized as B_{II} hybrids on the basis of genomic DNA fingerprints and nuclear DNA contents (Barcaccia et al. 1997), were used to identify a genetic linkage group related to parthenogenesis in *P. pratensis* (Barcaccia et al. 1998). On the whole, twelve genotypes of *P. pratensis* from Italy, Germany and the Czech Republic (see Table 1) with known reproductive behavior (Matzk 1991; Mazzucato 1995) were used to develop and test SCAR.

Auxin test

Parthenogenesis was assessed on 48 F_1 plants for two years with the auxin test (Matzk 1991). The auxin treatment of panicles and classification of induced grains (i.e., grains where the endosperm is lacking) were done according to Mazzucato et al. (1996) with 20–30 specimens per plant.

Linkage analysis

Linkage analysis of markers significantly co-segregating with parthenogenesis was carried out following a pseudo-test-cross strategy with molecular data of a population of 48 F_1 plants (Barcaccia et al. 1988). A linkage group related to parthenogenesis was established with JOINMAP software Version 2.0 (Stam and van Ooijen 1995) with the 'cross-pollination' (CP) population type option. The function 'group' was applied by setting a LOD score of 3.5 and a maximum recombination frequency $r = 0.3$ to detect groups of linked markers. The map distances were calculated by the Kosambi function (Kosambi 1944).

SCAR markers

The two AFLP parthenogenesis-nearest flanking markers mapped upstream and downstream of the target locus were converted into SCAR markers by the following procedure. The restricted-amplified bands deriving from both paternal and maternal genotypes and two progeny genotypes were excised from a dried

Table 1. Information on germplasm source, ploidy level and mode of reproduction of 12 *P. pratensis* genotypes.

Plant code	Germplasm origin	Chromosome number	Mode of reproduction	References
S1	cv. Berbi (Germany)	48	sexual	1, 2
S2	cv. Berbi (Germany)	44	sexual	1, 2
S4	cv. Roznovska (CZ)	58	sexual	1, 2
BL9	ec. Balvano (SI)	64	sexual	3
CC2	ec. C. Castello (CI)	70	apomictic	2, 3, 4
MM12	ec. M. Maddalena (NI)	42	apomictic	2, 3, 4
TU8	ec. Tuoro (CI)	62	apomictic	2, 3, 4
TU13	ec. Tuoro (CI)	–	apomictic	2, 3, 4
RS7	ec. Rionero S. (SI)	50	apomictic	2, 3, 4
RS9	ec. Rionero S. (SI)	56	apomictic	2, 3, 4
RS18	ec. Rionero S. (SI)	54	apomictic	2, 3, 4
RS20	ec. Rionero S. (SI)	46	apomictic	2, 3, 4

According to and adapted from Matzk (1991), Mazzucato (1995), Mazzucato et al. (1995) and Barcaccia et al. (1998).
[a]CZ, Czech Republic; CI, central Italy; SI, southern Italy.
[b]1, Matzk 1991; 2, Barcaccia et al. 1998; 3, Mazzucato et al. 1995; 4, Mazzucato 1995.

polyacrylamide gel and eluted with 100 μl of sterile distilled water for 6 h at 4 °C. Samples were stirred frequently and finally centrifuged for 10 min at 13 000 rpm. Then, 3 μl of the supernatant containing the resuspended DNA were re-amplified using the same *Eco*RI-*Mse*-I primer combinations that yielded the markers. PCR amplification was performed under the following conditions: one cycle of 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. An aliquot of 0.7 μl of the re-amplified DNA was ligated into a pCRII-TOPO vector with the TOPO TA Cloning kit (Invitrogen). For each transformation, 20 colonies were amplified with either the *Eco*RI-*Mse*I primer combination or *Mse*I primer alone. Plasmid DNA was purified from 25 ml of an overnight culture of *Escherichia coli* using the Flexi Prep kit (Amersham Pharmacia Biotech). Plasmid sequencing was performed by the dideoxynucleotide chain termination method with Sp6 and T7 as primers according to the Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech).

Using the sequence information of each cloned AFLP band, a pair of 24-mer primers was synthesized. Each primer included the original *Eco*RI or *Mse*I site sequences with the respective three selective nucleotides plus the next 16 or 18 internal bases.

Amplification of genomic DNA with pairs of SCAR primers was done in a GeneAmp PCR system 9700 (PE Applied Biosystem). Various annealing temperatures (55–69 °C, Table 2) were tested in order to optimize the amplification profiles obtained for the parthenogenetic and sexual plants. The 25 μl reaction volume contained 1× PCR buffer (50 mM MgCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl), 5 mM dNTPs (Amersham Pharmacia Biotech), 40 pmol of each primer, 200 ng of genomic DNA and 2.5 U Platinum Taq DNA polymerase (Life Technologies). PCR was carried out in an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, primers optimal annealing temperature for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. Amplification products were separated by electrophoresis in 2% agarose (Gibco-BRL) gels.

Results

Cloning of AFLP bands and SCAR primer development

In our previous research, a bulked segregant analysis with RAPD and AFLP markers was used to identify a genetic linkage group related to the apomictic mode of reproduction (Barcaccia et al. 1998). However, during auxin test repetitions, two plants of the BSA progeny, previously identified as sexual, showed a low but reproducible level of parthenogenesis and

Table 2. Information of SCAR markers, including primer sequences, and amplification product characteristics.

SCAR marker name (5′ to 3′)	Primer sequence forward and reverse	Optimal annealing temp. (°C)	Amplified fragment size (bp)	Product content (%) C+G
*Parth*1	AATTCCCAACAACCACTGTCCCTT TAAAGAATGGTAACAGCAGCACTC	54.4	475–496	43.9
*Sex*1	AATTCCCACATCTTCAAAGGATTC TAAACATCTTACTGGCAATCGAGA	49.8	147	35.0

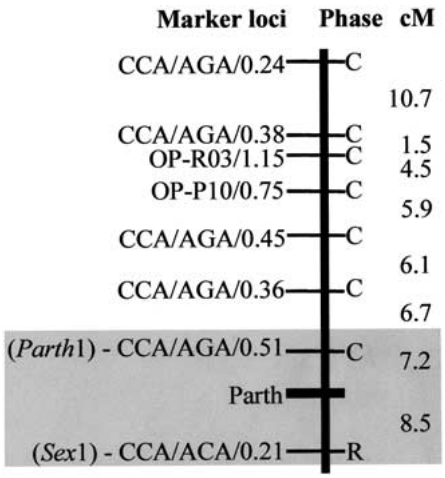

Figure 1. Genetic linkage group related to parthenogenesis in *P. pratensis*. Highlighted in light gray are the two AFLP markers converted into SCARs, whose names are given in parenthesis.

were, therefore, reclassified as parthenogenetic. This fact determined changes of recombination frequency estimates between molecular markers and the target locus and thus led to the construction of a new linkage group as shown in Figure 1.

AFLP bands corresponding to the parthenogenesis-nearest markers were cut out from the dehydrated gel, eluted in sterile water and amplified with the same primer combinations that originated the AFLP fingerprint. PCR products were then visualized in a agarose gel and cloned in a T-overhang vector.

An initial screening of cloned AFLP markers which are linked to parthenogenesis in the F_1 population was done in order to avoid the sequencing of possible *Mse*I/*Mse*I fragments co-migrating with the *Eco*RI/*Mse*I amplification products. In fact, *Mse*I/*Mse*I amplification products, unscreenable on the X-ray film, could co-migrate with the AFLP marker of interest and thus be eluted along with it. After this screening, those colonies that showed the *Eco*RI/*Mse*I amplification product only were selected and sequenced. DNA sequencing confirmed the presence of the expected primer sequences at the ends of each cloned fragment. No significant homology was found in data banks searches using the band sequences as query.

SCAR primers were designed on the basis of the sequence of each cloned fragment. The *Parth*1 primer pair was designed on the basis of the CCA/AGA0.51 AFLP marker (see Figure 1), whereas the *Sex*1 primer pair derived from CCA/ACA0.21 (see Figure 1). Each primer sequence included the restriction sites plus the next nucleotides to a final length of 24 (Table 2).

SCAR primer testing

Genomic DNAs of the progeny used for mapping were amplified by PCR under stringent conditions with each pair of primers.

The *Sex*1 pair of primers produced a single amplification product of 147 bp, whereas two different amplification products of 475 and 496 bp were generated by the *Parth*1 pair (Figure 2). Although several explanations could be invoked for the first result, a short insertion/deletion seemed the most probable cause of the second. For this reason the two bands generated by the *Parth*1 primer pair were cloned and sequenced. The alignment is shown in Figure 3. Overall sequence homology was very high (95.5%) and length variation was due to two stretches of 7 and 14 nucleotides, not found in the shorter fragment. If the two fragments are located at the same genetic locus is currently under investigation. If the two fragments would prove to be allelic, then *Parth*1 primers could be used to mark either the parthenogenetic or sexual mode of reproduction.

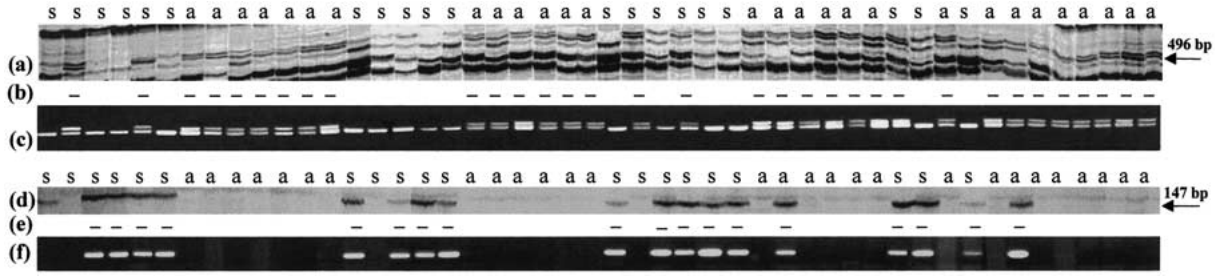

Figure 2. Patterns generated by SCAR primers *Parth*1 (c) and *Sex*1 (f) are identical to those of the relative segregating AFLPs (a and d, respectively) indicating that the cloned fragments, and so the SCARs, were derived from it. Note that the *Parth*1 SCAR primers generated two fragments instead of one (c); one monomorphic and the other polymorphic segregating as the original AFLP marker (a). Segregations of AFLP markers a and d are drawn out in b and e, respectively. Arrows indicate the AFLP fragments converted into SCARs with the relative molecular weights.

Figure 2 compares the segregation of the two AFLP and corresponding SCAR markers in the mapping progeny. As expected, their segregation coincided perfectly. Note that *Parth*1 is in coupling with the locus putatively controlling parthenogenesis while *Sex*1 is in repulsion.

Use of Parth1 *and* Sex1 *SCAR primers in MAS programs involving germplasm of wide origins*

An important quality of SCAR markers is their suitability on different genetic backgrounds. To test their efficiency in this respect, SCAR primers were tested on apomictic and sexual genotypes adapted to contrasting environments of southern and eastern Europe. The reproductive behavior, the provenance, the chromosome number, and the level of apomixis of the analyzed genotypes are reported in Table 1. The amplification pattern generated by each primer pair strongly resembled that observed in the mapping progeny and unequivocally distinguished the sexual from the parthenogenetic individuals (Figure 4).

Discussion

PCR-based molecular markers provide a highly accurate and sensitive discriminating technique for various plant breeding programs (Smith and Beavis 1996). Their use is particularly convenient when the selectable phenotype is manifested late in the plant life-cycle.

In breeding programs for the reproductive behavior, for example, where the selectable phenotype is manifested at flowering stage or even later in the following generation, PCR-based markers could be used for selecting at seedling stage or even before at seed stage to greatly reduce the time and costs involved. However, the applicability of these selection procedures requires preliminary work to develop molecular markers in association with the locus of interest.

It is still not known whether the extent of parthenogenesis reflects usable apomixis. Because haploid parthenogenesis usually occurs at low frequencies, it seems reasonable to consider the degree of parthenogenesis an estimate of aposporous parthenogenesis potentialities, and thus of the final extent of apomixis of single plants (Mazzucato et al. 1996a; Barcaccia et al. 1998). Although the question remains open, the genetic determinants for apomixis in *P. pratensis* proved to be closely linked and thus inherited together (Barcaccia et al. 2000). This means that the unreduced egg cell has a built-in tendency to autonomous development and therefore estimates of apospory, parthenogenesis and apomixis should not greatly differ (Mazzucato et al. 1996a; Barcaccia et al. 1997). In *P. pratensis*, when large populations are surveyed, some genotypes that segregate for the two main processes can be found. Such genotypes prove to be highly sexual when progeny-tested, even when impaired in only one step. However, very low fitness is the major reason for the rarity of apospory-parthenogenesis segregating genotypes (Savidan 1990). Plants that reproduce by non-recurrent apomixis, which leads to recurrent changes in ploidy level, cannot propagate themselves through generations (Nogler 1984).

We previously demonstrated that, in *P. pratensis*, parthenogenesis is regulated by a single gene or a cluster of tightly linked genes (Barcaccia et al. 1998). We now report the development and testing of a SCAR-based system suitable for breeding parthenogenesis in *P. pratensis*.

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Parth1 - AATTCCCAACAACCACTGTCCCTTGGCTCATAATAAGTTACCGTTTACAT -50
         ||||||||||||||||||||||||||||||||||||||||||||||||||
Cons   - AATTCCCAACAACCACTGTCCCTTGGCTCATAATAAGTTACCGTTTACAT -50

Parth1 - AAAGTACCACAGGATTGGCAAACAGCGGCCATACATCAACAACAACAGTG -100
         ||||||||||| ||||||||||||||||||||||||||||||||||||||||
Cons   - AAAGTACCACAGGATTGGCAAACAGCGGCCATACATCAACAACAACAGTG -100

Parth1 - TCATTCCAACAGCTTATCTACTGTCAGATTGATTCTTACAGAAAGAACAT -150
         |||||||||||||||||| ||||||| |||| ||||||||||||||||||
Cons   - TCATTCCAACAGCTTATCGACTGTCACATTGGTTCTTACAGAAAGAACAT -150

Parth1 - ACTCAGTCACCCCTTACAACAACCTCAAGAAATTTACAACATGGAACAGC -200
         ||||||||||||||||||||||||||||||||||||||||||||||||||
Cons   - ACTCAGTCACCCCTTACAACAACCTCAAGAAATTTACAACATGGAACAGC -200

Parth1 - ACATGGTCTCATTACCAAGGTTCTTACAGGGAGAATGCAAAAATAATCCA -250
         |||||||||||||||||||||| ||||||||||||||||||||||||||
Cons   - ACATGGTCTCATTACCAAGGTTCTAACAGGGAGAATGCAAAAATAATCCA -250

Parth1 - GCAACAAATACACATGCTGTTGGGAGTTGGGACACAACGCCGCGTTGGGA -300
         |||||||||||||||||||        ||||||||||||||||||||
Cons   - GCAACAAATACACATGCTGT-------TGGGACACAACGCCGCGTT---- -289

Parth1 - TGATGTGAGGTATAAATTAGAATGAAAAATGGCATCAGAACGGAACGGTG -350
                  ||||||||||||||||||||||||||||||||||||||||
Cons   - ----------TATAAATTAGAATGAAAAATGGCATCAGAACGGAACGGTG -329

Parth1 - CAAGAGTTAGCTCCGTACAGCAACATTTGCAGCACTTGGCTGCCCCACAG -400
         ||||||||||||||||||||||||||||||||||||||||||||||||||
Cons   - CAAGAGTTAGCTCCGTACAGCAACATTTGCAGCACTTGGCTGCCCCACAG -379

Parth1 - GCTTCATGGTTGATTTGGGCGCCCTGGTTTTTGTAGCTGCTACAATCTTC -450
         ||||||||||||||||||||||||||||||||||||||||||||||||||
Cons   - GCTTCATGGTTGATTTGGGCGCCCTGGTTTTTGTAGCTGCTACAATCTTC -429

Parth1 - GGAGAGCCTTTTGGAGAGCACTGAGTGCTGCTGTTACCATTCTTTA     -496
         ||||||||||||||||||||||||||||||||||||||||||||
Cons   - GGAGAGCCTTTTGGAGAGCACTGAGTGCTGCTGTTACCATTCTTTA     -475
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Figure 3. The two fragments generated by *Parth*1 primers were cloned and sequenced to ascertain whether base compositions were similar.

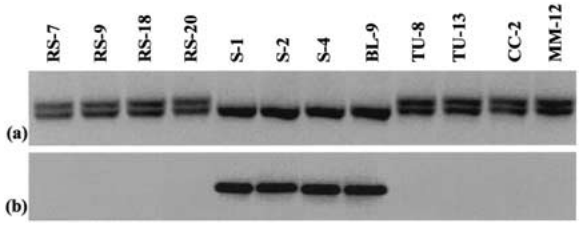

*Figure 4. Parth*1 (a) and *Sex*1 (b) SCAR primers tested on exotic germplasm sources of known reproductive behavior (Table 1).

The main features of the system are: (1) its low bias in identifying apomictic and sexual genotypes, and (2) Its applicability to a wide range of Italian and exotic germplasm.

Whenever a desirable trait has to be introgressed into an apomictic genetic background, the recurrent parent will act as pollen source and the sexual progeny needs to be selected in each backcross generation. Once the recurrent parent genotype has been recovered, selection is directed to apomictic individuals possessing the introgressed trait. Therefore in developing markers suitable for breeding *P. pratensis*, attention should be paid to minimizing the bias of apomictic and sexual genotypes discrimination in progeny of apomictic × sexual crosses.

To date, the methods most commonly used for investigating apomixis in *P. pratensis* have been cyto-embryological analyses in ovules of the mother plant (Grazi et al. 1961; Mazzucato et al. 1996a) and progeny tests based on the detection of morphological traits and/or molecular markers (Mazzucato et al. 1995; Barcaccia et al. 1997). Such procedures are often time-consuming and limited in their application. The availability of PCR-based markers linked to the mode of reproduction would help overcome these limitations by simplifying and accelerating the identification of apomictic individuals.

It is worth mentioning that MAS can be fully exploited if selectable molecular alleles are mapped on both upstream and downstream of the gene of interest. The system here presented is based on two SCAR markers, which map 7.2 and 8.5 cM to the sides of the

locus that controls parthenogenesis. If it is assumed that there is no genetic interference in this region the probability of mis-scoring a recombinant for the parthenogenesis locus is about six in one thousand. The particular configuration of the two SCARs with respect to the parthenogenesis locus, one in coupling and the other in repulsion, is functionally equivalent to a co-dominant marker locus and, thus, provides high efficiency in MAS for sexual or apomictic genotypes.

Another important quality of SCAR markers is their suitability on different genetic backgrounds. Our SCAR primers were tested on apomictic and sexual genotypes adapted to contrasting environments in Southern and Eastern Europe. *Parth*1 and *Sex*1 primer pairs produced the expected amplification profile on both apomictic and sexual genotypes. This interesting finding provides the present system with the desired validation for breeding for reproductive behavior, as well as leaving room for additional considerations.

As the genetic and, probably, also, physic, window defined by the two SCARs is too large to conclude that such a high level of conservation is due to the presence of the gene that regulates reproductive behavior, the question of the extent of the genetic diversity sampled for the analysis needs answering.

In the absence of specific studies on the topic, and allowing for the fact that the center of origin of *P. pratensis* is located in Eurasia (Wedin and Huff 1996), the Mediterranean and the eastern European areas could well be two regions of intensive diversification. The occurrence of reciprocal gene flow between these two regions is highly unlikely. An alternative hypothesis would be that the genetic basis of the European *P. pratensis* is not very large. The adaptative response to contrasting environmental pressures could be mediated by the buffering capacity of polyploidy (Grazi et al. 1961; Savidan 1990). Such a hypothesis would account for the high ploidy variability noticed in *P. pratensis* germplasm.

It is believed that apomixis, from an adaptative point of view, is a means of multiplying the presently adapted gene combinations and sexuality is a reservoir of variation to be selected for in the evolutionary process (Clausen 1961; Mazzucato et al. 1996b). However, the regulation of the ratio of the sexual versus the apomictic mode of reproduction in natural populations in an unsolved puzzle in *P. pratensis* ecology. Ecological studies have shown that sexuality is preferred when the population moves in seek of a new equilibrium point, whereas apomixis is predominant once the new equilibrium point has been reached (Wedim and Huff 1996).

The system we propose, which identified apomictic and sexual genotypes, should prove helpful in describing the dynamics of reproductive behavior against a background of changing environmental conditions.

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