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Development of SCARs useful for species identification in the genus *Lolium*

Valérie Delozier, Didier Peltier, Raymond Jalouzot*

Groupe de biochimie et de biologie moléculaire végétales, laboratoire de génétique, U.F.R. sciences, 2, bd Lavoisier,
49045 Angers cedex, France

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Abstract – In order to obtain some species-specific sequences, random amplified polymorphic DNAs were analysed in two varieties belonging to *Lolium multiflorum* Lam. and four belonging to *Lolium perenne* L. First, analysis was performed on bulks of individuals in order to screen oligonucleotides allowing amplification of species-specific bands. Then, we tried to amplify eight selected markers from 34 individuals belonging to *L. perenne* L. var. 'baltic' and 34 belonging to *L. multiflorum* Lam. var. 'wewo westerworld'. Southern hybridizations were performed in order to enhance detection of amplification products and to verify fragments homologies. We assessed the level of representation of putative markers in those varieties. Two markers were retained as species-specific ones. The sequences of both markers showed a strong homology to each other, allowing us to produce codominant sequence characterized amplified regions (SCARs). They allowed us to distinguish between the two studied varieties and were efficient enough to discriminate 13 other varieties in bulk analyses. (© Inra/Elsevier, Paris.)

L. multiflorum Lam. / *Lolium perenne* L. / RAPD marker / SCAR

Résumé – Développement de SCAR pour l'identification d'espèces dans le genre *Lolium*. Nous avons utilisé la technique RAPD (ADN polymorphe amplifié au hasard) sur deux variétés de *Lolium multiflorum* Lam. et quatre de *Lolium perenne* L., dans le but de développer des régions amplifiées de séquence connue (SCAR). Dans un premier temps, nous avons trié les amorces générant des profils polymorphes entre ces espèces, sur des mélanges d'individus. Puis nous avons amplifié les huit marqueurs RAPD sélectionnés, à partir de l'ADN de 34 individus pour la variété « baltic » de *L. perenne* L. et pour la variété « wewo westerworld » de *L. multiflorum* Lam.. Nous avons réalisé des hybridations afin d'accroître la sensibilité de la détection des produits d'amplification et de vérifier l'homologie de séquence entre fragments de même taille. Nous avons ainsi pu estimer le degré de représentation de ces marqueurs potentiels dans ces deux variétés. Finalement, seuls deux marqueurs spécifiques des deux variétés étudiées ont été retenus. Ces marqueurs ont montré une forte homologie de séquence et nous ont permis de dériver des SCARs codominants. Ils ont également permis de différencier 13 autres variétés lors d'analyses en mélanges de plantes. (© Inra/Elsevier, Paris.)

L. multiflorum Lam. / *Lolium perenne* L. / marqueurs RAPD / SCAR

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* Correspondence and reprints
jalouzot@univ-angers.fr

1. INTRODUCTION

The genus *Lolium* contains most of agronomically important temperate forage grass species. *Lolium perenne* L. and *L. multiflorum* Lam. are two species from this genus widely cultivated. *L. perenne* L. is used in mixes for greens or pastures while *L. multiflorum* Lam. is harvested to make hay or silage.

L. perenne L. and *L. multiflorum* Lam. can naturally cross-hybridize. Studying interspecific crossing, Naylor [17] states that, despite the fact that the two species overlap in their geographical distribution, they can be distinguished morphologically but not consistently in genetical terms, since no difference in chromosome organization was found and they can interbreed without difficulty; so *L. multiflorum* Lam. was first considered as a *L. perenne* L. subspecies [3]. Terrell [29], quoted by Charmet and Balfourier [1], distinguished eight species in the genus: two of them are *L. perenne* L. and *L. multiflorum* Lam. More recently, Charmet and Balfourier [1], analysing isozyme polymorphism, have not revealed any specific allele allowing differentiation of these two species. However, the mean allelic frequency of some alleles, such as Pgi2-d, Prx-b or Dia-b alleles, clearly allowed us to distinguish between the two species. Stammers et al. [27] using RAPD markers (random amplified polymorphic DNA) estimated genetic distance between six *Lolium* and nine *Festuca* species. They found that *Lolium* species are very close, Nei's genetic distance [18] ranging from 0.032 to 0.068. Charmet et al. [2] performed RAPD and RFLP analyses, PCR-amplifications of six non-coding regions of chloroplast DNA and sequencing internal transcribed spacers (ITSs) on eight species of ryegrass (*Lolium*) and 11 species of fescue (*Festuca*). In all studies, *L. perenne* L. and *L. multiflorum* Lam. appeared to be very close. The ITS-derived phylogenetic tree appeared to be the one with the best resolution and revealed that, among outbreeding *Lolium* species, perennial ryegrass seems to have differentiated last.

Although *L. perenne* L. and *L. multiflorum* Lam. are easily morphologically recognizable at the plant stage, they are very similar at the seed one. This fact makes purity analysis of seed batches very difficult.

Controls are performed after germination on 10-day-old plantlets, a procedure that is long and tedious. We, therefore, plan to use molecular assay in order to detect specific contaminants. The statutory policy relative to quality of seed batches rejects those containing more than one seed of *L. multiflorum* Lam. out of 25 000 of *L. perenne* L. in a batch and vice versa.

Previous works reported the use of molecular hybridization in order to detect contaminant in cytoplasmic male sterile seed stocks [16, 24] with a sensitivity of one out of 1 000. This involves having at disposal a sequence characteristic of the contaminant. Screening for such a sequence can be performed using various techniques such as RAPD analysis [30, 31], RFLP [15, 20] or AFLP [7]. Out of these, we chose random amplified polymorphic DNA (RAPD), a technique derived from polymerase chain reaction (PCR) technology [22]. Although its reproducibility has been discussed [13, 21], it represents a convenient tool for marker screening. It has been proved to limit time cost, the amount of DNA required and to reveal polymorphic loci.

The aim of this work was to identify RAPD markers present in all individuals belonging to the *L. perenne* L. species but which do not exist in any individual belonging to the *L. multiflorum* Lam. species and vice versa. These markers could then be used to generate sequence characterized amplified regions (SCARs) [19]. The higher sensitivity and reproducibility of the PCR technique using SCARs rather than RAPD makes it usable to determine whether a batch of seeds for one of these two species of *Lolium* contains seeds belonging to the other *Lolium* species described or not.

Nevertheless, commercial varieties of *Lolium perenne* L. and *L. multiflorum* Lam. are synthetic varieties and therefore the level of heterozygosity is significative. It means that genetic differences exist between individuals belonging to the same variety. One can envisage the case in which a marker is absent only in 5 % of individuals. If such individuals are contaminating a batch of seeds, they could not be detected. Thus, we planned to work with DNA

extractions from individual plants in order to estimate the level of representation of selected markers.

We realized RAPD amplifications of DNA from bulks and then from 34 individual plants for each variety. Homologies between the amplified products were checked by hybridization. This paper describes the distribution of eight RAPD markers in two varieties of *Lolium* and the potential use of the two species-specific markers retained to detect contamination.

2. MATERIALS AND METHODS

2.1. Biological materials

Plant batches of about 30 individuals were supplied and the absence of contamination was checked by the Station nationale d'essais des semences (Beaucouzé, France). They are batches 35467, 35318, 35319, 35363 and 35074 for *L. perenne* L. and 25, 373 and 35437 for *L. multiflorum* Lam. A second subset of varieties was further studied, namely Herbie, Bastion and Barcredo for *L. perenne* L., and Adret, Tonyl, Vitesse and Barspectra for *L. multiflorum* Lam.

Studies at the individual level were performed on 34 plants from *L. perenne* L. var 'baltic' and 34 from *L. multiflorum* Lam. var. 'wewo westerworld' supplied by J. Lallemand (GEVES, Magneraud, France).

2.2. DNA isolation and analysis

DNA was isolated from individual plant leaf tissues with the DNeasy mini extraction kit (Qiagen) according to the suppliers, or from leaf mixes of all the plants, according to Dellaporta et al. [4].

RAPD reactions were performed with 25-ng template DNA, 0.2 mM random primer (Operon), 100 mM each 4 dNTP (Eurogentec), 1.5 unit Taq DNA polymerase (Promega), 2 mM MgCl₂, 75 mM Tris-HCl pH9, 20 mM (NH₄)₂SO₄, 0.01 % (w/v) Tween 20 in a final volume of 25 µL. The reaction mix was overlaid with two drops of mineral oil.

Since variations have been reported with Taq DNA polymerase brands [13], the same Taq DNA polymerase

brand was used for amplifications carried out for a given primer.

DNA amplifications were performed on a Crocodile III (Appligene) thermal cycler programmed for one cycle of 4 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and finally one cycle for 1 min at 94 °C, 1 min at 36 °C and 20 min at 72 °C.

Amplification products were analysed by electrophoresis in 1.4 % agarose gels and visualized by ethidium bromide staining. The sizes of the amplified fragments were estimated by comparison with the 1-kb DNA ladder from BRL.

RAPD fragments, used for cloning or labelling, were recovered from agarose gels according to the technique described by Tautz and Rentz [28].

2.3. Cloning of the selected fragments

RAPD fragments, recovered from agarose gels, were cloned in the pGEM-Teasy Vector (Promega) following the manufacturer's instructions.

2.4. Hybridization

DNA was transferred from gels to Hybond™-N Nylon membranes (Amersham) by capillary Southern blotting [23, 26]. Transferred DNA was bound to the membranes by 3 min of UV-ray exposure.

The bands of interest were excised from another RAPD gel electrophoresis and eluted from agarose on a rock wool filter or excised from plasmid DNA by restriction enzyme. The eluate was radiolabelled by random priming with the kit Ready To Go (Pharmacia Biotech) according to the manufacturer's instructions.

Hybridizations were performed overnight at 65 °C in hybridization buffer 5 × SSPE (20 × SSPE: 3.6 M NaCl, 0.2 M NaH₂PO₄, 0.02 M EDTA), 0.5 % (w/v) SDS). Blots were washed twice at low stringency (2 × SSPE, 0.1 % (w/v) SDS) and room temperature, then at medium-high stringency (1 × SSPE, 0.1 % (w/v) SDS) and 65 °C, and finally at high stringency (0.1 × SSPE, 0.1 % (w/v) SDS) and 65 °C.

After washes, blots were autoradiographed using X-ray films (Kodak).

2.5. Sequencing

Sequencing data of the inserts were obtained from Cybergene (Saint-Malo, France). The nucleotide sequences determined for both strands were analysed with PCGene programs.

3. RESULTS

First, we checked the reliability of RAPD amplifications with our DNA extracts. Control experiments were carried out on five amounts of *Lolium* DNA ranging from 10 to 100 ng. The results (not shown) confirmed that patterns obtained were identical. Likewise, amplifications, using a given amount of 25 ng DNA per reaction, were performed, each time with new reaction mixes, in order to test the reproducibility of this technique. Patterns obtained after electrophoresis and ethidium bromide staining were roughly identical. Only minor bands showed variable intensities. Though, minor bands were not selected as potential polymorphic markers.

A total of 66 10-mer primers were screened. First, the primers were used for RAPD reactions performed on DNA extracted from genotypes mixes from various species. Out of the primers tested, four gave no amplification products and 17 lead to unclear profiles. This allowed us to identify primers generating profiles containing one or more bright bands present in only one species. Such a profile is shown in figure 1. Two very bright polymorphic bands are amplified with primer OPH-18. OPH-18 550-bp marker is present in all samples belonging to *L. perenne* L. but absent in all samples belonging to *L. multiflorum* Lam. On the contrary, OPH-18 512-bp marker is present in all samples belonging to *L. multiflorum* Lam. but absent in all samples belonging to *L. perenne* L. Seven primers leading to eight polymorphic bands were thus selected (table I).

We further checked the presence of a polymorphic band at the individual level. Amplifications were carried out on DNA from 34 different individuals from one variety of *L. perenne* and from one

Figure 1: Ethidium bromide-stained (Bet) agarose gel of amplification products obtained with DNA isolated from mixes of plants with oligonucleotide OPH-18. M: molecular weight marker (1-kb ladder from BRL). Lanes 1–3: *L. multiflorum* Lam. mixes; lanes 4–8: *L. perenne* L. mixes. Arrows are pointed out on two potential markers. OPH-18 550 bp is present in all samples belonging to *L. perenne* L. and absent in all samples belonging to *L. multiflorum* Lam. OPH-18 512 bp is present in all samples belonging to *L. multiflorum* Lam. and absent in all samples belonging to *L. perenne* L.

variety of *L. multiflorum*. In order to confirm homology between DNA fragments of the same size in different genotypes and to increase detection sensitivity, bands were eluted from gels after electrophoresis of amplification products realized on DNA extractions from mixes of several individuals and they were either directly ($\alpha^{32}\text{P}$)-dCTP-labelled and used as probes on capillary Southern blotting, or cloned beforehand to be used as probes. In the case of hybridizations realized with fragments eluted from RAPD gels as probes, one major signal is detected at the same size as the probe but many other minor signals are revealed (results not shown), indicating that the gel-isolated fragment was contaminated with other sequences, in sufficient quantity so as to generate a non-specific background in hybridization as demonstrated previously by Lanner et al. [11].

Table I. Selected markers generating an interspecific polymorphism between *L. perenne* L. and *L. multiflorum* Lam.

Primer	Primer sequence	Marker specificity	Approximate size of the marker (in base pairs)
70-23	5'-GAGACGTCCC-3'	<i>L. multiflorum</i> Lam.	500 bp
OPH-01	5'-GGTCGGAGAA-3'	<i>L. multiflorum</i> Lam.	400 bp
OPH-18	5'-GAATCGGCCA -3'	<i>L. multiflorum</i> Lam.	512 bp
OPH-18	5'-GAATCGGCCA -3'	<i>L. perenne</i> L.	550 bp
OPG-06	5'-GTGCCTAACC-3'	<i>L. perenne</i> L.	700 bp
OPH-04	5'-GGAAGTCGCC-3'	<i>L. perenne</i> L.	700 bp
OPH-06	5'-ACGCATCGCA-3'	<i>L. perenne</i> L.	750 bp
70-27	5'-AGCCTGACGC-3'	<i>L. perenne</i> L.	370 bp

In some experiments, the results obtained after ethidium bromide staining were improved by hybridization data. Some bands, at the same size as the marker, that were not detectable after electrophoresis and ethidium bromide staining were revealed, indicating that the sequence was present but faintly amplified with the RAPD technique. Thus, screening for a species-specific sequence is improved by the additional work implied by hybridization. Actually, if a marker, whose presence was only detected in one species after coloration, could be revealed after hybridization in one or more individuals of the other species, it could still not be considered as a species-specific one.

A species-specific marker must possess two properties: presence in all individuals from one species; and absence in all individuals from the other species. Out of eight markers, six were proved to be not species specific. We could distinguish three cases: markers which are present in all individuals of one species but also in some individuals of the other species, such as the 70-23 (500 bp) marker; markers which were present at a high level in one species but not in all individuals and which were also revealed in few individuals of the other species, such as the OPH-01 (400 bp), OPH-04 (700 bp) and OPH-06 (750 bp) markers; markers which were absent in all individuals belonging to one species but not present in all individual of the other species, such as the OPG-06 (700 bp) marker.

In a second step, we studied the distribution of these six markers at the individual level. It means that we determined how many individuals from *L. perenne* possess one, two or more markers first

retained as specific to *L. multiflorum*, and vice versa. Seventeen individuals from *L. perenne* and 13 from *L. multiflorum* exhibit one of these six markers. Likewise, five individuals from *L. perenne* and two from *L. multiflorum* possess two of these six markers. No individual has more than two markers first retained as specific to the other variety.

The other markers retained, OP H-18 550 bp and OPH-18 512 bp, appeared species specific in the studied sample as they were amplified in all genotypes belonging to *L. perenne* L. var. 'baltic' and *L. multiflorum* Lam. var. 'wewo westerworld', respectively, and absent in all genotypes belonging to *L. multiflorum* Lam. var. 'wewo westerworld' and *L. perenne* L. var. 'baltic', respectively. Hybridization results showed a strong homology between OPH-18 550 bp and OPH-18 512 bp as these two markers cross-hybridize. Both markers were cloned and the sequences obtained after sequencing are shown in figure 2: OP H-18 512 bp shows a 37-bp deletion with regards OP H-18 550 bp and only five mismatches. Computer-based sequence-similarity searches performed with the BLAST network service revealed that the sequence showed strong homology (over 95 % identity) with monocot chloroplastic gene coding for cytochrome b 559 (psbE and psbF genes). Two primers (RG1 and RG2) were derived from the sequences (figure 2) leading to amplification of 488-bp and 451-bp fragments for *L. perenne* L. and *L. multiflorum* Lam., respectively. Their presence were further checked at the individual level (figure 3). In order to confirm the species specificity of both markers, seven other varieties were further studied. The analyses were

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OPH-18 550 bp - GAATCGGCCAAACGAGTATTTCACGGAAAGACGACAAAGGGAATTCGGTTA -50
OPH-18 512 bp - GAATCGGCCAAACGAGTATTTCACGGAAAGACGACAAAGG-AAATTCGGTTA -49
OPH-18 550 bp - ATAACCGACCGTTTGGATTCTTTAGAACAACTCGATGAATTTAGTAGATC -100
OPH-18 512 bp - ATAACCGACCGTTTGGATTCTTTAGAACAACTCGATGAATTTAGTAGATC -99
OPH-18 550 bp - CCTTTAGGAGGCCCTCAATGACCATAGATCGAACCTTATCCTATTTTACA -150
OPH-18 512 bp - CCTTTAGGAGGCCCTCAATGACCATAGATCGAACCTTATCCTATTTTACA -149
OPH-18 550 bp - GTGCGATGGCTGGCTATTACGGGACTAGCTATACCTACTGTTTTTTTCTT -200
OPH-18 512 bp - GTGCGATGGCTGGCTATTACGGGACTAGCTATACCTACTGTTTTTTTCTT -199
OPH-18 550 bp - GGGATCAATATCAGCAATGCGATTTCATCCAAAGATAACAAAATTCGAAC -250
OPH-18 512 bp - GGGATCAATATCAGCAATGCGATTTCATCCAAAGATAACAAAATTCGAAC -249
OPH-18 550 bp - TATAGAACTATGACACAATCAAAACCGAATGAACAAAATGTTGAATTGAA -300
OPH-18 512 bp - TATAGAACTATGACACAATCAAAACCGAATGAACAAAATGTTGAATTGAA -299
OPH-18 550 bp - TCGTACCAGTCTATACTGGGGTTTATTACTCATTTTGTACTGTGCTGTTT -350
OPH-18 512 bp - TCGTACCAGTCTATACTGGGGTTTATTACTCATTTTGTACTGTGCTGTTT -349
OPH-18 550 bp - TATTTTCCAATTACTTCTTCAATTGATAGAAAGAAAGATAGTTCCTCAATTA -400
OPH-18 512 bp - TATTTTCCAATTACTTCTTCAATTGATAGAAAGAAAGATAGT----- -391
OPH-18 550 bp - CTTCTTCAATTGATAGAGAGAAAGATAGTAATAAGAAATCTCTTATCCCA -450
OPH-18 512 bp - -----AATAAGAAATCTCTTATCCCA -412
OPH-18 550 bp - TTTGGAAGGATACCCGTCCTTATACTATCCATGACTGTTTTGCTCTATA -500
OPH-18 512 bp - TTTGGAAGGATACCCGTCCTTATACTATCCATGACTGTTTTGCTCTATA -462
OPH-18 550 bp - GCATGACCACCTTGATAAAATGTGAGGAAAGTAGGGGAAATGCGCGATTTC -550
OPH-18 512 bp - GCATGACCACCTTGATAAAATGTGAGGAAAGTAGGGGAAATGCGCGATTTC -512

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Identity : 509 (99.4%)

Number of gaps inserted in: OPH-18 550 bp : 0

Number of gaps inserted in: OPH-18 512 bp : 2

Figure 2. Alignment of the two markers sequences: OPH-18 550 bp and OPH-18 512 bp. The underlined sequences correspond to the primers synthesised for PCR analysis.

performed on 35 plant (approx.) bulks. After amplification using primers RG1 and RG2, the three *L. perenne* L. varieties exhibit the 488-bp fragment and the four *L. multiflorum* Lam. varieties exhibit the 451-bp fragment (figure 4), although other minor bands were detected.

As the final aim of our work was the detection of contaminant in seed batches, we determined the sensitivity of detection of the species-specific sequence. The PCR detection limit of markers was estimated at 10 pg of template DNA using various amount of *Lolium* DNA and the PCR parameters described in Material and methods (data not shown).

4. DISCUSSION

We have chosen to use RAPD as a tool to screen species-specific sequences, the final aim of this

Figure 3. Ethidium bromide-stained agarose gels of PCR amplification products obtained with oligonucleotides RG1 and RG2, synthesized after OPH-18 550-bp sequencing, on DNA isolated from individual plants. M: molecular weight marker. A) Lanes a-p: individuals from *L. perenne* L. var 'baltic'; I: Italian ryegrass DNA. B) Lanes a-p: individuals from *L. multiflorum* Lam. var 'wewo westerworld'; P: perennial ryegrass DNA. Arrows indicate the potential interspecific markers (OPH-18 488 bp and OPH-18 451 bp) obtained after bulk analysis.

work being to develop sequence characterized amplified regions (SCARs) [9]. A good SCAR for detection of specific contamination should be present in all individuals belonging to one of the two species and absent in all individuals belonging to the other species. Although Kesseli et al. [9] reported some trouble concerning reproducibility of the RAPD technique, Penner et al. [21] showed that patterns can be reproducible among laboratories if reaction conditions are strictly followed. Furthermore, Koller et al. [10] showed for apple cultivars that PCR amplifications patterns were robust over a wide range of DNA concentrations. In preliminary analyses, we confirmed that RAPD

Figure 4. Ethidium bromide-stained agarose gels of PCR amplification products obtained with oligonucleotides RG1 and RG2 (hybridization temperature for amplification: 55 °C), on three varieties from *L. perenne* L. (lanes a–c) and four from *L. multiflorum* Lam. (lanes d–g). M: molecular weight marker. *L. perenne* L. varieties: a, Herbie; b, Bastion; c, Barcredo. *L. multiflorum* Lam. varieties: d, Adret; e, Tonym; f, Vitesse; g, Barspectra. Arrows indicate the species-specific markers, OPH-18 488 bp and OPH-18 451 bp.

technique is reproducible and supports some variations in DNA concentration.

In bulk analysis, the sensitivity of RAPD to detect a poorly represented marker is about six individuals in a 30-individual bulk [14]. Thus, markers first retained as absent in one species could be present in five out 30 individuals but not detected. Then, we checked 34 individuals, for each variety chosen in each species, in order to refine the previous screening and to eliminate markers which were revealed to be not species specific after plant to plant analysis.

4.1. RAPD markers and varietal identification

In bulk studies, we observed RAPD bands characteristic of each species, that is to say, present in one species and absent in the other one. Nevertheless, for six of the eight selected markers, plant to plant analysis revealed the presence of these bands in some individuals from the other species or the absence in some individuals of the

species. The proportion of such individuals has been determined for each of these markers. Our results are in agreement with Charmet and Balfourier [1], who distinguished the two species only by the mean allelic frequency of some isoenzyme. One can similarly use the markers revealed in bulk analysis for varietal identification. The estimation of the frequency of each marker in a variety could be performed as follows: moving PCR parameters (hybridization temperature, cycles number, etc.) would allow us to define experimental conditions for the detection of a band, after ethidium bromide staining, only if the level of presence exceeds a definite threshold. Accumulating such data for a sufficient number of markers will then allow varietal identification. This involves cloning and sequencing of the selected markers in order to synthesize the longer primers (20-mers) needed for PCR. Nevertheless, this approach cannot be used to detect *L. multiflorum* Lam. contamination in a batch of *L. perenne* L. seeds and vice versa, as the studied marker is present at a variable level in both populations. Actually, if markers, spread at a low level over population, are detected, we will not be able to determine if the results are due to a contamination or if the population behaves slightly differently from the control.

4.2. RAPD as a tool to screen species-specific SCARs

We revealed two species-specific markers in the sample studied as they were present in all tested genotypes belonging to one species and absent in all tested genotypes belonging to the other. Hybridization experiments revealed that both markers showed a very strong homology. Their sequences allowed us to determine that they are chloroplastic ones, OPH-18 512 bp showing a deletion with regards the OPH-18 550 bp marker. The spreading out of both markers was confirmed on seven more varieties of *Lolium*. Other studies should be performed to analyse the distribution of both types in natural populations. This will demonstrate whether cytotypes are spread in some popula-

tions of both species, especially in the sympatric zone.

We can wonder if the variations between both markers could be related to an evolution event for both species. The natural dispersion of both species is different, *L. perenne* L. having a preference for wet and cool soils [25], whereas *L. multiflorum* Lam. is a natural species of irrigated and warmer areas, but all authors admit genetic proximity between both species whatever the evolutionary theory considered. According to Malik and Thomas [12], all allogamous species in the genus *Lolium* are derived from a common ancestor, from which *L. rigidum* is the closest form. Both chloroplast genomes could have evolved leading to two different chlorotypes. For Essad [6], quoted by Malik and Thomas [12], this ancestor could be *L. perenne* L. while, for Jenkin [8] quoted by Essad [5], *L. perenne* L. would be the result of South to North evolution of *L. multiflorum* Lam., with successive mutations. Both hypotheses could support an insertion or a deletion in the chloroplast genome during the radiation of the ancestor species.

Although both species studied are genetically very closely related, and genetically heterogeneous, the RAPD technique was shown to be convenient for detecting species-specific sequences. Thus, two homologous chloroplast sequences were revealed. Screening of other markers would improve detection of contaminants. If some individuals from a species did not exhibit one species-specific marker, it seem unlikely that they would lack three or more species-specific markers.

The detection limit was estimated at 10 pg of DNA template for each marker. This means that at least 250 ng of DNA (25 000 seeds $\times$ 10 pg) would be required to detect one single contaminant in a batch. In order to avoid the use of such a DNA amount for PCR, it would be better to fractionate, prior to DNA extraction, the batch into ten or more sub-samples of seeds. Even in this case, the crucial condition would be the homogeneity of the grinding. Actually, if a single seed contaminates a batch the homogeneity of the powder must be perfect without loss of any material in order to guarantee that each DNA extract is representative of the seed

batch composition. Such a precision could only be reached with automated grinders which have to be developed or adapted for seed DNA extraction.

Screening for species-specific sequences for grasses, which are mostly cultivated as synthetic varieties and so highly heterozygous, represents a delicate problem. An appropriate combination of RAPD, bulk analysis and plant to plant analysis, allowed us to isolate two species-specific markers convenient for the detection of contamination.

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