Theor Appl Genet (1992) 83:940–946

Outcrossing and hybridization in wild and cultivated foxtail millets: consequences for the release of transgenic crops

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Received July 20, 1991; Accepted October 1, 1991
Communicated by J. Mac Key

Summary. Outcrossing rates within the wild green foxtail, *Setaria viridis*, and the cultivated foxtail millet, *S. italica*, are very low. However, spontaneous interspecific hybridizations in the experimental garden occurred in both directions at rates ranging from 0.002% to 0.6% according to plant density and distance between parents. Offtypes found in farmers' fields where foxtail millet is cultivated were shown to have originated from such interspecific crosses. Differences in the *Eco*R1 patterns of chloroplast DNA between cultivated and wild plants indicated that reciprocal crosses do occur in the field. These findings indicate that even a largely selfing cultivated species may exchange genetic information with wild relatives at rates that may cause problems if transgenic cultivars are released.

Key words: Species complex – Outcrossing – Hybridization – Chloroplast DNA

Introduction

One of the problems that might arise from the release of transgenic crops on a wide scale is the risk of transferring the transformed genes to wild relatives of the crops. Economically and ecologically important weeds might benefit or originate from such gene transfers, then shift ecological balances. The most important parameter by which to assess this risk is the gene flow between the "artificial" and wild populations. Hybridization alone provides a sufficient potential drive for the wild and weed relatives of crops to obtain genes never before present in their lineages. The natural transfers of genes through interspecific pollen exchanges and their consequences on wild populations have been poorly documented. Only very limited data are available from studies on cereal domestication, e.g. in maize (Doebley et al. 1987), rice (Oka and Chang 1959), barley (Zohary 1960) and sorghum (Doggett and Majisu 1968). As suggested by Harlan (1975), wild/weed/crop systems are basic aspects of plant evolution in man-disturbed areas. One such system is the *Setaria* species complex.

Setaria italica (L) Beauv., the foxtail or Italian millet, is used as bird seed or as a food staple in China and India. It was probably domesticated 6,000 years ago (Naciri and Belliard 1987). Its closest wild relative is *S. viridis* (L) Beauv., the green foxtail, a weedy grass of maize fields, vineyards, orchards, roadsides and wastelands. Both species are diploid ($2n=2x=18$) and may intercross giving more or less sterile hybrids; thus they probably form the same genetic species (de Wet et al. 1979; Till-Bottraud and Brabant 1990). In the temperate zone three other wild species are common: *S. verticillata*, an autotetraploid, morphologically similar to *S. viridis* but with retrorse bristles; *S. faberii*, an allotetraploid common in North America and China but not found in Europe; and *S. glauca*, morphologically quite different from *S. viridis* and in fact itself a ploidy complex.

Some of us have been working on foxtail millet as a model of herbicide-resistant crops, and consequently as a model for risk assessment. Cytoplasm-inherited resistance to triazine herbicides has been transferred from a wild *S. viridis* to the crop through classical breeding (Darmency and Pernès 1985, 1989). Although this resistant material has no transgenic origin, one must ask to what extent its use may lead to the spread of the resistance within wild and weedy populations of *Setaria*. Species of the *Setaria* complex are thought to be highly self pollinating, but within the cultivated type, "off types"

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Table 1. Experimental design of the outcrossing and hybridization between species studies. The description of the plant is given in Table 2

♀	Cross	♂	♀/♂	Marker[b]	Distance[c]	Design	Conditions	Number of replicates
Outcrossing								
cv1	×	cv3, cv4, cv5	1/60	Collar color	40 cm	Square	Field 1981	3
cv2								
vir1		vir1[a]						
vir2	×	vir2	1/20	Esterase	25 cm	Square	Field 1988	2
vir3		vir3						
Same plants			1/1	Esterase	0 cm	Same bag	Greenhouse	2
Hybridization between species								
Experiment 1								
cv3	×	vir1	1/60	Esterase	17 cm	Square	Field 1985	1
vir1	×	cv3	1/60	Esterase	17 cm	Square	Field 1985	1
Experiment 2[a]								
cv6, cv7, cv8	×	vir1, maj1	1/8	Collar color	60 cm	Circle	Field 1988	6
Experiment 3[a]								
cv8	×	maj1, vir1	2/5	Collar color	38 cm	Alternate lines	Field 1989	2
vir4, maj2	×	cv5	2/5	Collar color	38 cm	Alternate lines	Field 1989	2
Experiment 4								
cv6	×	vta1						
vta2	×	cv5	1/15	Collar color and isozymes	40 cm	Square	Field 1987	3
vir3	×	gl1						
gl2	×	vir5						

[a] All of the crosses possible between the three genotypes were made individually
[b] Two types of genetic markers were used to identify the hybrids: collar color pigmentation (the ♀ is homozygous recessive unpigmented and the ♂ homozygous dominant pigmented) and isozymic markers (esterase)
[c] The distance given is that between the ♀ parent and the closest ♂ parent

often occur. Sterile hybrids between the crop and *S. verticillata* can be observed in millet fields in France (Poirier-Hamon and Pernès 1986). Moreover, botanists recognize a type intermediate between *S. viridis* and *S. italica*: *S. viridis* var '*major*' (Gaud.) Posp., the giant green foxtail. This vigorous weedy type probably originated from hybridization between wild and cultivated plants followed by stabilization (Darmency et al. 1987).

The aim of the research presented in this paper was to measure the outcrossing rates of the different species and to estimate the gene flow between these species in natural and artificial conditions.

Material and methods

Outcrossing and hybridization between species

One experiment was designed to estimate "normal" outcrossing within *S. italica*, and two for *S. viridis* (one under "normal" and one under forced conditions where two spikes were bagged together). The hybridization between species was studied in four different types of experiments (denoted 1–4); all are described in Table 1. The origin of the plant material is given in Table 2.

Spontaneous interspecific hybrids

Origin of plants. Plants that looked intermediate between *S. italica* and *S. verticillata* were transplanted from a millet field in France (Maine-et-Loire). Chromosome counts were carried out on root tips. The growth and flowering of these plants were observed in the greenhouse.

In the same way, 11 plants intermediate in phenotype between *S. italica* and *S. viridis* were transplanted from two fields in Maine-et-Loire where *S. italica* is currently cultivated (hvi1–hvi10 originated from the same field and hvi11 from another). They fit the description of the var '*major*,' but are suspected to be offspring of interspecific hybrids. The progeny was observed in the experimental garden at Orsay in 1989 for seed and spike characters.

Analysis of restriction fragments of chloroplast DNA was performed on offsprings of 6 intermediate plants (hvi1, hvi2, hvi6, hvi9, hvi10 and hvi11) and on reference lines of *S. italica* ('cv6,' 'cv7' and 'cv10') and *S. viridis* (vir6, collected in the same field as hvi11) to determine the female parent of the putative hybrids.

Table 2. Description of the cultivars of *S. italica* and types of *S. viridis* (var '*minor*' or '*major*'), *S. verticillata* and *S. glauca* used in the experiments

Code	Stock reference	Variety or cultivar	Origin
S. viridis			
vir1	99–79	minor	China (Heilungkiang)
vir2	29–80	minor	France (Tours)
vir3	202–82	minor	France (St Rémy de Provence)
vir4	175–82	minor	France (Puy de Dôme)
vir5	201–82	minor	France (St Rémy de Provence)
vir6	109–88	minor	France (Maine et Loire)
maj1	98–79	major	China (Heilungkiang)
maj2	176–82	major	France (Charente maritime)
S. italica			
cv1	62–79	Anda	China (Heilungkiang)
cv2	12–80	Bourgogne	France (Maine et Loire)
cv3	58–79	Tax of poor soils	China (Heilungkiang)
cv4	65–79	Red Glutinous	China (Heilungkiang)
cv5	67–79	Change with aging	China (Heilungkiang)
cv6	14–80	Burganjou	France (Maine et Loire)
cv7	15–80	Trois Mois	France (Maine et Loire)
cv8	61–79	Amende 4	China (Heilungkiang)
cv9	73–83	Sarbazan	France (Landes)
cv10	13–80	Anjou	France (Maine et Loire)
S. verticillata			
vta1	367–83		France (Pyrénées Orientales)
vta2	366–83		France (Maine et Loire)
S. glauca			
gl1	197–82		France (St Rémy de Provence)
gl2	198–82		France (St Rémy de Provence)

Isolation of chloroplast DNA. Chloroplasts were isolated in a medium of high ionic strength (Bookjans et al. 1984). Young leaves were homogenized at 4 °C in a Waring blender for 3 × 5 s at low speed in 10 ml/g fresh weight NaCl buffer (50 m*M* TRIS-HCl, pH 8.0, 1.25 *M* NaCl, 25 m*M* EDTA, 0.1% BSA, 7 m*M* β-mercaptoethanol). The homogenate was filtered through a 35 µm nylon net, and the filtrate centrifuged at 1,200 *g* for 5 min (IEC, rotor 210). The pellet was resuspended once using a paintbrush in the homogenization buffer and then centrifuged again at 1,200 *g* for 5 min. The pellet (corresponding to 5–10 g fresh material) was lysed with 3 ml buffer C [50 m*M* TRIS-HCl pH 8, 20 m*M* EDTA containing 2% laurylsarcosinate and 100 µg/ml proteinase K (Boehringer Mannheim)] for 1 h at 20 °C. The lysate was treated twice with a phenol-chloroform mixture, and the nucleic acids were precipitated from the aqueous phase overnight at −20 °C after the addition of 50 mg NaCl and 2.5 volumes of cold ethanol. The nucleic acid pellet was recovered by centrifugation and dissolved in 1.3 ml of buffer C. Then, 1.57 g CsCl and 20 µl of an ethidium bromide solution (10 mg/ml) were added. After careful mixing the solution was poured into a quickseal tube (Beckman, TLV rotor, TL100 ultracentrifuge). Each tube was filled with buffer C, sealed and centrifuged at 90,000 rpm for 3 h at 18 °C (San et al. 1990). The ctDNA band was collected at 360 nm. Ethidium bromide was eliminated by a butanol treatment. The solution was diluted with 4 vol buffer D (10 m*M* TRIS-HCl pH 8.0, 1 m*M* EDTA, 50 m*M* NaCl), and the DNA precipitated overnight at −20 °C after the addition of 2.5 vol of cold ethanol. The DNA pellet was recovered by centrifugation and dissolved in buffer TE (10 m*M* TRIS-HCl, 1 m*M* EDTA, pH 8.0).

Table 3. Outcrossing rates of *S. italica* and *S. viridis*. For more details on the parents see Table 1

Parents		Offspring		
♀	♂	Total	Number of hybrids	H (%)
S. italica				
cv1	cv3+4+5	632	0	0
cv1	cv3+4+5	540	0	0
cv1	cv3+4+5	873	3	0.34
cv2	cv3+4+5	630	4	0.64
cv2	cv3+4+5	614	9	1.47
cv2	cv3+4+5	433	4	0.92
S. viridis				
vir1	vir2	93	1	1.08
vir2	vir1	108	0	0
vir2	vir3	114	0	0
vir3	vir1	92	2	2.17

ctDNA restriction analysis. One to three micrograms of ctDNA was digested in 30 µl reaction buffer with sufficient enzyme to give complete digestion. The restriction fragments were separated by electrophoresis on 0.7% agarose gels (Vedel et al. 1976; Quétier and Vedel 1977). The 1-kb ladder (Bethesda Research Laboratories) was used as a molecular weight standard.

Results

Outcrossing rates within each species

The outcrossing rates for *S. italica* and *S. viridis* ranged between 0% and 2.2% under natural conditions (Table 3). When outcrossing was highly favored by putting spikes from different plants in close contact inside the same bag, it could reach up to 4% with the wild plant (Table 4). In some instances, none or only very few hybrids were found (crosses using 'cv1' as female parent), which could be due to some difference in the flowering time of the two parents (although different cultivars were used to provide pollen over a long period), to some incompatibility system (although this seems unlikely in a selfing species) or to the fact that the rates were very low and the sample sizes not big enough to get good estimates.

Interspecific hybridization rates

In experiment 1 the hybridization rates between *S. italica* and *S. viridis* were 0.3–0.6% (Table 5). No clearcut dif-

Table 4. "Maximum" outcrossing rates in *S. viridis* (spikes in close contact within the same bag). As seed shedding occurs right after maturity, which is not synchronous for all of the seeds of a spike, the progeny from both parents was collected together in the bag and analyzed as Parent 1, Parent 2 or hybrid. For more details on the parents see Table 1

Parents		Offspring				
P1	P2	P1 type	P2 type	Hybrid	Total	H (%)
vir1	vir2	93	111	5	209	2.45
vir1	vir3	98	98	7	203	3.75
vir2	vir3	168	175	14	375	4.08

Table 5. Hybridization between species

Parents[a]		Offspring		
♀	♂	Total	Number of hybrids	H (%)
Experiment 1: distance = 17 cm				
cv5	vir1	345	2	0.58
vir1	cv5	366	1	0.27
Experiment 2: distance = 60 cm				
cv8	vir1	40,300 ± 1,000	7	0.017
cv8	maj1	40,000 ± 2,000	6	0.015
cv6	maj1	32,000 ± 1,200	69	0.22
cv7	maj1	24,300 ± 1,600	124	0.51
Experiment 3: inter-row distance = 75 cm				
cv8	vir1 + maj1	700,000 ± 30,000	13	0.002
vir4	cv5	1,350	2	0.15
maj2	cv5	4,000	8	0.20
Experiment 4: distance = 40 cm				
cv6	vta1	1,000	0	0
vta2	cv9	1,000	5	0.50
vir3	gl1	1,000	0	0
gl2	vir5	1,000	0	0
	distance > 4 m			
cv6	vir5	1,000	1	0.10

[a] cv, *S. italica*; vir or maj, *S. viridis*; vta, *S. verticillata*; gl, *S. glauca*
For more details on the parents see Table 1

ference appeared in reciprocal crosses: two hybrids were obtained with the cultivated plant as female, and one with the wild plant as female. These hybrids were partially sterile, but thousands of seeds could be collected from each plant grown in the greenhouse. In contrast to seeds from wild plants, there was a high germination rate in the F_2 generation.

Experiment 2 was designed to test gene transfers from wild *S. viridis* to cultivated plants under non-crop conditions. The flowering of wild plants vir1 and maj1 started at the same time as that of cv 'cv8,' at the end of June. In both cases, around 40,000 seedlings were obtained from seeds collected from the 6 cultivated plants, of which 1.6×10^{-4} were hybrids (Table 5). Cultivars 'cv6' and 'cv7' flowered at the end of July when the wild maj1 showed 17 flowering spikes per plant and was as tall as the cultivated plants. High hybridization rates were observed: 22×10^{-4} with 'cv6' and 51×10^{-4} with 'cv7' (Table 5).

Experiment 3 was designed to estimate the gene flow in the border of a field; a total of 700,000 seedlings of the cv 'cv8' were analyzed, of which 13 were hybrids (Table 5). Here, the rate of hybridization was 0.19×10^{-4}. The low number of hybrids did not allow to find any differences between rows or between pollen sources (7 hybrids with vir1 and 6 with maj1). The number of seeds tested from the wild vir2 and maj2 plants was much lower because of the low number of seeds collected in the bags and bad germination. Hybridization rates were 15×10^{-4} with vir4 and 20×10^{-4} with maj2 (Table 5). However, the real hybridization rate must be lower because the wild plants produced seeds long after the cultivar had finished flowering: the total seed yield of the wild plants could not be estimated completely, but has been previously shown to be 2 or 3 times more than the amount collected in an equivalent experiment (Darmency et al. 1987), so that the hybridization rate could be rather close to 5×10^{-4}.

Experiment 4 was designed to detect gene flow between *S. italica* and *S. verticillata* on the one hand and between *S. viridis* and *S. glauca* on the other. One hybrid was detected between plants of *S. italica* ('cv6') and *S. viridis* (vir3) even though, they were separated by other plants (plots with *S. verticillata* and *S. glauca*) and a distance of more than 4 m. Natural hybridization between *S. italica* and *S. verticillata* was obtained only with the wild parent as female at the rate of 0.5% (Table 5). These hybrids were all triploid and almost totally sterile: 1 plant produced 3 seeds (out of approximately 3,000 to 4,000 flowers) of which only 2 germinated. The pollen of this F_1 hybrid was 97.7% unstained by the Alexander (1969) coloration technique. The two F_2 plants resembled the female parent (*S. verticillata*), were less sterile than the F_1 (27% of the pollen was unstained by the Alexander coloration, and they produced some seeds) and have recovered a stable chromosome number (4×). Natural and artificial crosses between *S. viridis* and *S. glauca* did not succeed (Table 5).

Spontaneous hybrids in the field

The chromosome number of plants intermediate between *S. italica* and *S. verticillata* transplanted from the field was found to be 27, i.e. the plants were triploids (Fig. 1). They were very similar to the artificial hybrids obtained in the laboratory. This confirmed their interspecific origin. No seeds could be collected on these plants, but neither were seeds usually found on these plants in the field.

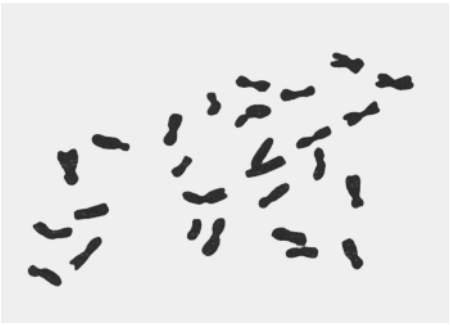

Fig. 1. Metaphase plate of a wild plant of intermediate morphology between *S. italica* (2n=2x=18) and *S. verticillata* (2n=4x=36). The number of chromosomes is 3x=27

Table 6. Segregation of the natural hybrid progenies hvi1 to hvi8 for seed shedding, seed-coat color, pericarp color and polyphenol oxydase activity (PPO)

Family	Total	Seed shedding		Seed-coat color		Pericarp color		PPO	
		+	−	Pale	Dark	Pale	Dark	+	−
hvi1	10	2	8	4	6	9	1	6	4
hvi2	10	2	8	4	6	7	3	7	3
hvi3	113	63	50	26	87	113	0	113	0
hvi4	111	60	51	111	0	111	0	0	111
hvi5	12	3	9	5	7	8	4	7	5
hvi6	8	2	6	2	6	7	1	6	2
hvi7	17	4	13	6	11	14	3	11	6
hvi8	107	32	75	47	60	94	13	63	44

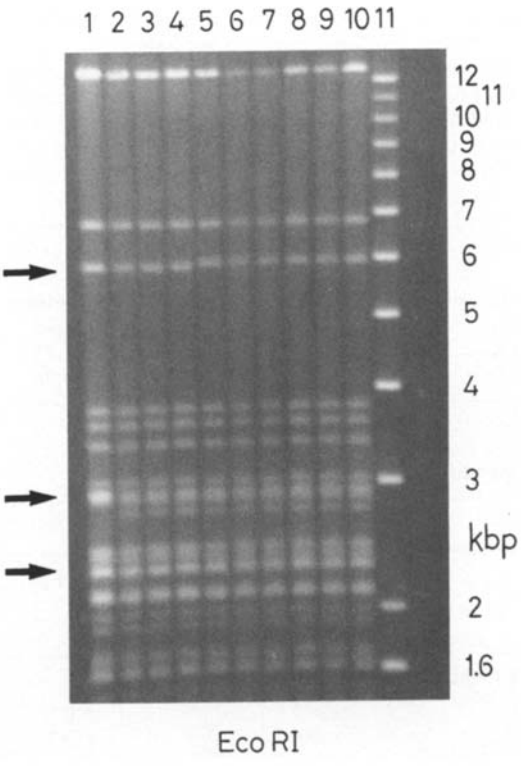

Fig. 2. *Eco*RI restriction patterns of chloroplast DNA from *Setaria* plants. *Lanes 1–3* cultivated millet (cv10, cv6 and cv7), *lanes 4–9* hybrids (hvi1, hvi2, hvi9, hvi10, hvi6 and hvi11), *lane 10* wild green foxtail millet (vir6), *lane 11* the 1-kb ladder. *Arrows* indicate differential bands at the 2, 3 and 6 kb levels. Only lane 4 (hvi1) out of the six hybrids tested has a cultivated chloroplast type

The progenies of plants that had been collected as *S. viridis* var. '*major*' in the field (hvi1 to hvi11) were analyzed for characters that usually differentiate wild millets from the cultivated ones. These progenies segregated for one, two, three or four of the criteria used (Table 6). The segregations observed in some progenies having enough plants were in agreement with those described in the literature. For seed shedding, the hvi3 and hvi4 progenies showed 9:7 (shedding:non-shedding) segregations ($\chi^2=0.01$ and 0.21, respectively) which would be expected from a two major loci hypothesis (Darmency and Pernès 1987). The hvi11 progeny showed a 1:3 segregation ($\chi^2=1.37$), which is expected in a F_3 with only one dominant allele. For seed-coat color, the hvi8 progeny showed a 9:7 (dark:pale) segregation ($\chi^2=0.00$), which was expected according to Till-Bottraud and Brabant (1990), whereas the hvi3 progeny could be an F_3 with a 1:3 segregation ($\chi^2=0.24$) and hvi4 could be homozygous. For pericarp color, the hvi8 progeny showed the 13:3 segregation ($\chi^2=3.02$) found in previous F_2 studies (Darmency and Pernès 1987; Till-Bottraud and Brabant 1990) while the two hvi3 and hvi4 progenies were homozygous. These two progenies were also homozygous for polyphenol oxydase (PPO) activity, but segregation for PPO activity in the hvi8 progeny probably involved two loci although only one has always been reported in the literature (Li et al. 1945; Kawase and Sakamoto 1982; Darmency and Pernès 1987; Till-Bottraud and Brabant 1990). We therefore suggest that these plants result from natural hybridizations between *S. italica* and *S. viridis*. However, as some progenies were fixed for some characters, the plants collected from the fields cannot be F_1 hybrids. These hybridization events are probably not uncommon: the plants studied here were easily recovered from two different fields.

The restriction fragment analysis of chloroplast DNA of *Setaria* showed four differential bands at the 2, 3 and 6 Rb levels. The presence or absence of these bands allowed us to distinguish three different patterns (Fig. 2). One pattern was obtained for only 'cv10', another for 'cv6,' 'cv7' and hiv1 and a third one for vir6 and the other putative hybrids. This result means that hvi1 is the descendant of an interspecific hybridization with a cultivated plant as the female parent. In contrast, the other

putative hybrids show the same cytoplasm as wild plants collected from the same field – that is they are certainly descendants of an interspecific cross with a wild plant as the female parent.

Discussion

Outcrossing rates were very low (0.3–4% in closely mixed plants) within both *S. italica* and *S. viridis*. This is in agreement data on *S. italica* obtained from the literature (0.09–7.5%, Takashi and Hoshino 1934; Li et al. 1935) and confirms the general idea that they are largely selfing species. Spontaneous interspecific hybridizations between *S. italica* and *S. viridis* under field conditions also occurred in both directions. In some cases their rates were of the same magnitude as the outcrossing rates we found within each species, which favors the hypothesis that there is in fact only one genetic species.

When experiments simulated one cultivated plant escaped from a cultivated field and growing amongst wild plants, the interspecific gene flow appeared to be lower (0.016%). However, this depends mainly on the earliness of flowering of the cultivar. Ten to thirty times as many hybrids (0.2–0.5%) were found with late flowering cultivars as the density of wild flowers increased with time: wild *Setaria* produce a high number of tillers and their reproductive effort increase with time (Till-Bottraud 1988). When experiments simulated the border of a millet field, hybridizations were less frequent: 0.002% of the flowers of the cultivar were fertilized by pollen from wild plants, and approximately 0.05% of the wild flowers were fertilized by pollen of the cultivar. In terms of risks, we therefore have to separate clearly what may arise after the migration of cultivated seeds in a natural habitat from what may arise with gene flows at the border of the field.

In the case of a millet cultivar resistant to triazine herbicides, the gene encoding for this new agronomic trait is located on the chloroplast DNA. As biparental plastid inheritance has not been reported to date, pollen cannot be a potential vehicle for spreading the resistance in wild populations. However, resistant hybrids can be obtained at a low rate on spikes of cultivated plants fertilized by pollen from wild plants. As a first approximation, we may expect 40 resistant hybrids in a 1-ha field.

In the case of a new agronomic trait inherited as a Mendelian gene, the pollen of the cultivated species may transfer this trait to wild plants in the border of the field at a rate 20 times higher than for the reciprocal cross. Hybrid yield will depend on the density of the wild plants, but contrary to what has occurred above with cultivated plants, all of the seeds will fall onto the soil and approximately 3,200 hybrids in 1-ha field can be expected.

There is no data to support the idea that hybridization events have different probabilities according to the type of the mother. The fact that we found only one hybrid with the cultivated cytoplasm is in agreement with the bias expected with differences in the amount of pollen produced by the two types of plants: the cultivated millet produces much more pollen than the wild type so that a cross of a wild ovule with a cultivated pollen is far more probable than the reverse.

The germination and survival of these hybrids is another point in our concern about gene flow between wild and crop plants, but the fact that offspring from interspecific hybrids have been found in fields where *S. italica* is currently grown is proof of a continuous gene flow between the crop and the wild species. Moreover, stabilization of the progeny of the hybrid after the fixation of numerous traits and selection on various habitats are likely to lead to the vigorous giant green foxtail, *S. viridis* var '*major*,' which is a more troublesome weed than its wild parent. Coming back to the strategy for transgenic crops, the fact that we found a hybrid with the cultivated type of cytoplasm is of great importance for risk assessment, as this hybrid would have received any of the introduced cytoplasmic gene. The present results, obtained on a selfing species, lead us to point out the urgent need for risk assessment studies previous to the release of any genetically modified organism.

Acknowledgements. The authors wish to thank Dr. J. Mac Key for comments on the manuscript. Part of this work was supported by a grant from INRA (X.R.).

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