Investigation of pollen competition between wild and cultivated sorghums (*Sorghum bicolor* (L.) Moench) using simple sequence repeats markers

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Abstract In self-compatible plant species stigmata receive a mixture of self and outcrossed pollen and competition between them is expected to play a major role in determining the pollen-mediated gene flow. The use of male sterile bait plants in field trials to demonstrate the rate of gene flow is questionable due to lack of pollination competition. However, little direct evidence has been published. A field experiment of male sterile and male fertile sorghum pollen recipient bait plants was conducted to evaluate pollen competition between wild and cultivated sorghums and the effects of pollen competition on gene flow assessment. Pollen competition between wild and cultivated sorghums was estimated from two-component pollen mixtures of wild and cultivated sorghum (1:1 ratio) applied to wild, cultivated and male-sterile maternal bait plants. Paternity was determined in the progeny using two diagnostic Simple Sequence Repeat markers. The study found that self pollen has higher seed-siring success. Maternal genotype influences the siring ability of the pollen donor components which significantly deviated from the 1:1 pollen loads. The study showed that published estimates of gene flow derived from studies using male-sterile bait plants seriously overestimate gene flow and that pollen competition may be a significant factor influencing outcrossing rates. The results suggest that the predominant direction of gene flow is from cultivated to wild sorghum, potentially leading to introgression of crop genes into wild sorghum. Pollen competition should be taken into account in gene flow estimation, since presence of self-pollen can account for over half of seed produced irrespective of maternal genotype.

Keywords *Sorghum bicolor* · Pollen competition · Outcross pollen · Self pollen · Pollination · Pollen-mediated gene flow

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Introduction

In many interfertile plant species, stigmata capture a mixture of self and outcross pollen. However, when

such plant species occur sympatrically, they may be reproductively isolated by the action of one or more mechanisms (Grant 1992; Ramsey et al. 2003). Pollination by another species may be prevented by species-specific pollinators (Grant 1992; Wolf et al. 2001) or by differences in peak flowering periods. However, for species that rely generally on wind pollination, the likelihood of receiving pollen loads comprising a mixture of conspecific (i.e., same species) and heterospecific (i.e., another species) pollen is expected to be high. In such cases, pollen competition may affect gene flow.

Though pollen competition is often proposed to be an important factor of reproductive isolation, there are few studies of wild-crop pollen competition. To our knowledge pollen competition between cultivated sorghum and its wild relatives has not been documented. Generally, the extent to which pollen competition occurs between cultivated crops and their wild relatives has been unclear, because tests of the predictions of the pollen competition have used mainly cultivated crops (Winsor et al. 2000).

Cultivated sorghum and its closely related wild relatives are classified as species *Sorghum bicolor* (L.) Moench, which comprises of subspecies *bicolor* (cultivated sorghum) and *verticilliflorum* (wild sorghum). *S. bicolor* is a herbaceous species with an annual lifecycle. It is an important crop species providing food, fodder and fuel particularly in semi-arid regions of Africa. Here, cultivated sorghum and its wild relatives exist in sympatric ranges, they are interfertile and have overlapping flowering windows (Doggett 1988; Harlan 1995; Muraya et al. 2009 unpublished data).

In many areas where wild sorghum is found, cultivated sorghum forms an important element of traditional agroecosystems, thus allowing gene flow and introgression between cultivated and wild sorghum gene pools. The wild relatives of sorghum serve as a potential genetic resource for sorghum breeding programmes (Rooney and Smith 2000; Rosenow and Dahlberg 2000). Resistance to major pests and diseases of cultivated sorghum are found in wild sorghum species such shoot fly, sorghum midge, downy mildew and sorghum ergot (Bapat and Mote 1982; Karunakar et al. 1994; Franzmann and Hardy 1996; Sharma and Franzmann 2001; Kamala et al. 2002; Komolong et al. 2002). *Striga*-resistance mechanisms such as low germination stimulant production, germination inhibition, and low haustorial initiation activity have also been found in wild sorghum (Ejeta et al. 2000; Ejeta 2007; Rich et al. 2004). In addition, the grain starch properties of wild sorghum are unique and could be used to improve digestability of feed sorghum for intensive livestock industries (Dillon et al. 2007).

A prerequisite for using wild species as germplasm is sexual compatibility for successful hybridisation and backcrossing. Hence, utilisation of wild sorghum requires an understanding of the biological nature of incompatibility systems that regulate gene flow between wild and cultivated sorghum. Furthermore, with the development of genetically modified sorghum cultivars under way, there is a need to understand the reproductive isolation mechanisms between the two subspecies for proper management of their coexistence. Introduction of novel, transgenic traits into crops and subsequent gene flow could lead to introgression of such traits into populations of wild relatives occurring in sympatric ranges (Gealy et al. 2003; Snow et al. 2003; Fuchs et al. 2004). Transgenic traits such as resistance to diseases, herbicides and insects could enhance fitness in some cases and might allow weedy relatives to become more abundant (Snow et al. 2003). Genetically engineered (transgenic) sorghum cultivars may have a potential to negatively affect the natural wild sorghum populations through the evolution of more aggressive weeds and affect biodiversity by possible extinction through genetic swamping of wild sorghum species (Ellstrand et al. 1999).

Previously, gene flow studies have often been conducted using male sterile plants as pollen bait plants (Timmons et al. 1995, 1996; Squire et al. 1999; Thompson et al. 1999; Schmidt and Bothma 2006). In such simplified experiments quantifying gene flow is straightforward since all pollen comes from the source plants and consequently, all offspring results from the source pollen. However, competition between self pollen and outcross pollen is not considered. On the other hand, using male fertile bait plants requires the ability to distinguish whether offspring were fertilised by pollen from source plants or bait plants. Consequently, gene flow may often be overestimated due to experimental designs which eliminate pollen competition between source and bait plants. The study presented here used SSR markers to determine pollen competition between cultivated

sorghum and two of its wild relatives as well as the effects of pollination competition on gene flow.

Materials and methods

A controlled pollination experiment was conducted in the field in the short-rain season of 2007/2008 at the research station of the Kenya Agricultural Research Institute (KARI) at Kiambere, Kenya, to test the competitiveness of pollen from wild and cultivated sorghum using male-sterile and male-fertile bait plants. Two male sterile lines (ATX623 and ICSA88), two wild sorghum populations (Acc26 and Acc74), and two cultivated populations (Acc126 and Acc154) were used for this purpose. Acc26 and Acc74 are wild sorghum accessions belonging to *S. bicolor* spp *verticilliflorum* race verticilliflorum and arundinaceum, respectively. Acc126 and Acc154 are cultivated sorghum accessions belonging to *S. bicolor* spp *bicolor* race caudatum and durra,

respectively. Two-component pollen mixtures were produced by blending pollen from a wild and a cultivated population (Fig. 1). The mixture was prepared by first combining pollen from ten individual plants of each of the pollen donor populations and then mixing the two bulks on a 1:1 volume basis. The resulting two-component pollen bulk was divided into four equal portions. The first portion was used to pollinate three cultivated sorghum plants, the second to pollinate three wild sorghum plants and the third and fourth to pollinate the male-sterile lines ATX623 and ICSA88, respectively. The pollen bulk was applied thrice per treatment on three consecutive days. Pollination was carried out between 09:00 and 12:30 a.m. During the entire flowering period, all plants chosen as pollen recipients were bagged to preclude pollination from unknown pollen sources. In the evening before pollen collection, panicles of pollen donors were bagged to avoid pollen from unintended sources. Pollen was collected and mixed shortly before pollination. During the growth period

Fig. 1 Pollen bulking and pollination scheme: *1* and *2* refers to the bulks formed by mixing pollen from two pollen donor components. Each of the male-sterile line consisted of two sets of treatment plants, with each set receiving pollen from either *1* or *2*. Each treatment had three replicates. Mixing of pollen was done in a way to exclude application of pollen bulk (mixture) to a recipient parent that has been used as a pollen donor: including pollen of pollen-recipient plant into the pollen bulk will make it impossible to distinguish self-pollination from cross-pollination resulting from crosses with its own pollen in the pollen mixture, leading to underestimation of cross-pollination
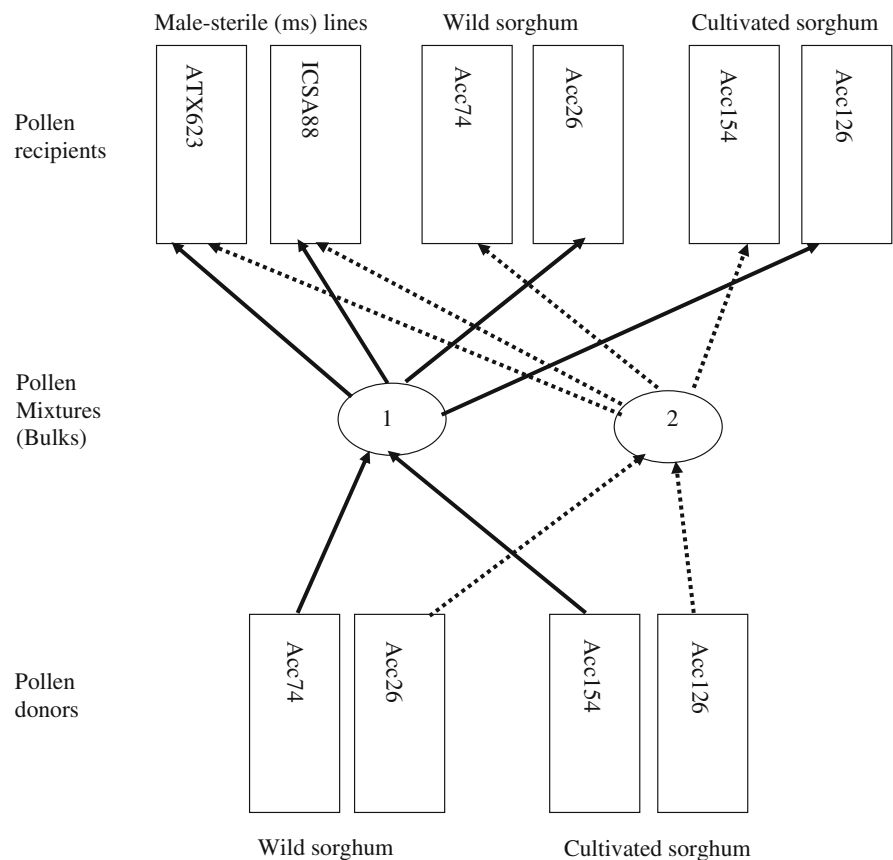
prior to flowering and pollination, leaf samples were taken from all pollen recipient and donor plants for genotyping. At maturity all seeds from pollen recipient plants were harvested.

DNA extraction and genotyping

A random sample of 100 seeds of each of the recipient plants, amounting to 300 seeds per treatment (a treatment refers to a set of pollen-recipient plants of a given population which received the same pollen mixture), were grown in a greenhouse at the University of Hohenheim. A special germination method was used to overcome seed dormancy (Muraya and Parzies unpublished): Seeds were placed in Eppendorf tubes, arranged in a rack and warm water (37°C) was added. The rack containing the Eppendorf tubes was placed in a hot water bath at 37°C overnight (12 h). The Seeds were then planted in pots in compost in the greenhouse and grown for 2 weeks. Two-week old leaves were harvested and lyophilized.

Total genomic DNA was extracted from 5 cm long pieces of young dry frozen leaves using a modified CTAB protocol (Mace et al. 2003). Concentration and quality of the DNA was assessed using electrophoresis of 1 μl of extracted DNA on a 0.7% (w/v) agarose gel followed by normalization of the concentration at 50 ng/μl. DNA from the parents was screened for polymorphism using a panel of 20 SSR markers obtained from generation challenge project (Brown et al. 1996; Taramino et al. 1997; Kong et al. 2000; Schloss et al. 2002; Agropolis-CIRAD-Genoplante unpublished) in order to identify markers that can discriminate among the parents used in each of the treatments. Two genomic SSR markers were identified that met this requirement for each parent combination (Tables 1, 2).

Polymerase chain reaction and fragment analysis

A set of four sorghum SSR markers (Tables 3) was used for genotyping the extracted genomic DNA. The PCR reactions were performed in a volume of 10 μl each containing 1× PCR buffer [20 mM Tris–HCl (pH 8.4), 50 mM KCl], 1.5 mM $MgCl_2$, 0.25 μM of each fluorescent labelled forward and unlabelled reverse primers, 0.2 mM dNTPs, 0.5 unit per reaction of *Taq* polymerase and 2 μl (100 ng) template DNA.

The amplification reaction consisted of a denaturing step of 3 min at 94°C, followed by 40 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min followed by one terminal extension step at 72°C for 10 min and consecutive storage of amplification products at 4°C. All PCR reactions were performed on an MJResearch icl PTC-100 thermocycler. Fragment analysis was carried out on automated laser fluorescence sequencer (MegaBACE). DNA of the parents and the seven progenies were included in all reactions as controls. Progeny were subsequently classified as either self, outcross with cultivated sorghum or outcross with wild sorghum.

A paired *t* test was used to check for significant differences between means of the recipient plants between pollen mixture treatments. The means of each recipient plant was computed from its 100 progeny, to calculate how many progeny were self, outcrossed with cultivated sorghum or outcrossed with wild sorghum paternal genotype.

Table 1 Simple sequence repeat (SSR) marker pairs used to discriminate progeny paternity in different pollen-recipient-donor treatments

Pollen recipient	Pollen donors		Pair of discriminative SSR marker
Parent 1	Parent 2	Parent 3	
26	74	154	Xtxp021 and Xtxp141
74	26	126	Xtxp021 and Xtxp57
154	26	126	Xtxp021 and Xtxp141
126	74	154	Xtxp021 and Xtxp57
ATX623	26	126	Xtxp021 and sbAGB02
ATX623	74	154	Xtxp021 and Xtxp141
ICSA88006	26	126	Xtxp021 and sbAGB02
ICSA88006	74	154	Xtxp021 and Xtxp57

Table 2 Allelic sizes of the SSR markers that were used to discriminate between the parental lines in different pollen-recipient-donor treatments (PRDT)

Treatments	PRDT	Markers		Treatments	PRDT	Markers	
		Xtxp21	Xtxp141			sbAGB02	Xtxp21
Pollen recipient	26	167/167	133/151	Pollen recipient	ATX623	118/118	169/169
Pollen donor	74	163/163	147/147	Pollen donor	26	98/98	167/167
Pollen donor	154	175/175	163/163	Pollen donor	126	96/96	181/181
		Xtxp21	Xtxp57			Xtxp21	Xtxp141
Pollen recipient	74	163/163	224/224	Pollen recipient	ATX623	169/169	157/157
Pollen donor	26	167/167	234/234	Pollen donor	74	163/163	147/147
Pollen donor	126	181/181	250/250	Pollen donor	154	175/175	163/163
		Xtxp21	Xtxp141			sbAGB02	Xtxp21
Pollen recipient	154	175/175	163/163	Pollen recipient	ICSA88006	102/102	179/179
Pollen donor	26	167/167	133/151	Pollen donor	26	98/98	167/167
Pollen donor	126	181/181	147/147	Pollen donor	126	96/96	181/181
		Xtxp21	Xtxp57			Xtxp21	Xtxp57
Pollen recipient	126	181/181	250/250	Pollen recipient	ICSA88006	179/179	252/252
Pollen donor	74	163/163	224/224	Pollen donor	74	163/163	224/224
Pollen donor	154	175/175	246/246	Pollen donor	154	175/175	246/246

Table 3 Designation, repeat motifs and forward and reverse primer sequences of the simple sequence repeat (SSR) markers used for genotyping

Marker	Motif	Forward primer (5′-3′)	Reverse primer (5′-3′)
Xtxp057[a]	$(GT)_{21}$	GGAACTTTTGACGGGTAGTGC	CGATCGTGATGTCCCAATC
Xtxp021[a]	$(AG)_{18}$	GAGCTGCCATAGATTTGGTCG	ACCTCGTCCCACCTTTGTTG
Xtxp141[a]	$(GA)_{23}$	TGTATGGCCTAGCTTATCT	CAACAAGCCAACCTAAA
SbAGB02[b]	$(AG)_{35}$	CTCTGATATGTCGTTGTGCT	ATAGAGAGGATAGCTTATAGCTCA

[a] Kong et al. (2000)

[b] Taramino et al. (1997)

Results

The percentage of sired seeds following self- and cross-pollination differed significantly in all treatments involving male-fertile bait plants ($P = 0.05$; Table 4). For all maternal plants, except Acc154, the cross-pollination percentage contributed by the cultivated and wild pollen deviated significantly from the 1:1 ratio in which the pollen mixture was applied. In male-fertile plants, the selfing percentage exceeded that of cross-pollination. Cultivated plant outcross pollen showed a higher seed-siring success than wild plant outcross pollen on a cultivated maternal plant. On the other hand, the wild plant outcross pollen was more competitive than cultivated plant outcross pollen on a wild plant. In cultivated sorghum male-sterile bait plants, the cultivated plant pollen was more competitive than the wild plant pollen on all maternal plants.

The selfing percentage ranged from 55 to 86% among the receptor plants (Table 4). Greater selfing proportions were observed in cultivated than in wild sorghum. The percentage of seeds from those analyzed that were fertilized by the cultivated sorghum donor ranged from 9 to 62%, while the offspring fertilized by the wild sorghum donor ranged from 2 to 49%. The success of wild sorghum pollen was higher on wild receptor plants and that of cultivated sorghum pollen higher on cultivated receptor plants.

Table 4 The occurrence of selfing and cross-pollination in different pollen-recipient plant combinations with pollen from different donor plants conducted at KARI-Kiambere during the short rains, 2008

Pollen recipient	PD pair[a]		Pollination (%)		P value	CPP (%)[b]		P value
	W[c]	C[d]	Self[e]	Cross[f]		Cross (C′)[g]	Cross (W′)[h]	
Acc26 [W]	Acc74 [W]	Acc154 [C]	55.00	45.00	0.002	15.33	29.67	0.007
Acc74 [W]	Acc26 [W]	Acc126 [C]	55.67	44.33	0.003	13.67	30.67	0.007
Acc154 [C]	Acc26 [W]	Acc126 [C]	86.00	14.00	0.004	9.00	5.00	0.290
Acc126 [C]	Acc74 [W]	Acc154 [C]	86.33	13.67	0.005	11.33	2.33	0.020
ATX623 [MS[i]]	Acc26 [W]	Acc126 [C]				51.67	48.33	0.004
ATX623 [MS[i]]	Acc74 [W]	Acc154 [C]				51.00	49.00	0.040
ICSA88 [MS[i]]	Acc26 [W]	Acc126 [C]				61.67	38.33	0.001
ICSA88 [MS[i]]	Acc74 [W]	Acc154 [C]				59.67	40.33	0.002

[a] *PD pair* pollen donor

[b] *CPP* contribution of pollen donors to cross-pollination

[c] *W* wild sorghum pollen donor

[d] *C* cultivated sorghum pollen donor

[e] *Self* selfing percentage

[f] *Cross* cross-pollination percentage

[g] *Cross (C′)* cross-pollination with cultivated pollen donor

[h] *Cross [W′]* cross-pollination with wild pollen donor

[i] *MS* the cultivated male sterile lines

The sample size for each pollen recipient was 300 progeny. *P* values indicate the test conducted on treatment means

Discussion

Pollination mixes containing equal amounts of outcross pollen from cultivated and wild sorghum showed different reproductive success depending on whether the maternal plant was a cultivated or wild sorghum. This implies that pollen donors had different reproductive success. In many self-compatible plants, the stigma captures a mixture of self and outcross pollen. However, the proportion of self or outcross pollen will not necessarily reflect the proportion of the resulting self- to cross-pollinated seed, respectively, because a number of mechanisms may lead to differential fertilization success (Lyons et al. 1989; Rieseberg and Willis 2007; Widmer et al. 2009). These include maternal control of pollen germination, pollen tube growth rate and seed abortion (Marshall and Ellstrand 1988; Swanson et al. 2004; Ruane and Donohue 2007). Hodnett et al. (2005) reported that outcross pollen tubes showed major inhibition of growth in sorghum pistils and seldom grew beyond the stigma. Mechanisms by which female plants choose mates are important since by influencing the paternity, females may be able to improve offspring fitness.

There are a number of consequences that non-random fertilisation, due to pollen competition, may have within a plant population. Pollen competition favouring outcross pollen may result in a decrease in the production of selfed progeny and hence reduce the extent of inbreeding depression (Charlesworth and Charlesworth 1992). In species where self-pollination is the predominant mating system, we expect that selfing would have a competitive advantage over cross-pollination. Generally, a flexible mating system should be more favourable for natural populations, such as wild sorghum, which are subdivided into small isolated units. Selfing is advantageous for solitary plants if no other pollen is around; in contrast, outcrossing allows for heterosis and the production of genetically variable offspring. A flexible mating system may enable self-compatible plants to take advantage of self-fertilisation or cross-pollination depending on the circumstances. Pollen competition can also affect the number of seeds that a

plant sires (Marshall 1991; Bernasconi 2003). Differential pollen competition is an important fitness component.

In Kenya wild and cultivated sorghums occur sympatrically, thus potentially allowing gene flow between the two taxa. Therefore, pollen competition as an isolation mechanism is expected to play a role in the co-existence of wild and cultivated sorghum. Our study showed that pollen competition depends on the pollen source-recipient relationship. In wild sorghum populations, cross-pollination with other wild sorghum plants had a competitive advantage in comparison to that from cultivated plants. On the other hand, in cultivated populations cross-pollination with cultivated plants had a competitive advantage in comparison with wild plants. However, generally wild sorghum showed a relatively higher percentage of cross-pollination than cultivated sorghum. Thus, wild sorghum seems to be at a higher risk of being fertilized via pollen mediated gene flow than cultivated sorghum, which may become relevant in the case of undesired pollination, e.g., by transgenic pollen donors.

Another factor that may favour competitiveness of cultivated sorghum, though not studied here, is a greater pollen production of cultivated sorghum relative to that of wild sorghum (own field observation) which may lead to a higher proportion of cultivated pollen landing on wild sorghum than observed in our controlled experiments. This may alter the competitive advantages of cross-pollination in favour of cultivated sorghum under field conditions. Together with the low cross-pollination of cultivated sorghum (on average ratio of 1:6; Table 4) this may further limit the introgression of wild sorghum genes into the domesticated sorghum gene pool. Therefore, gene flow is likely to occur mainly from cultivated to wild sorghum. Payro' de la cruz et al. (2005) also suggested that gene flow occurs mainly from cultivated Phaseolus vulgaris to its wild relatives. The present study is in agreement with their findings. Thus, both studies corroborate a general trend of the direction of gene flow from cultivated to wild plant species.

Gene flow estimates using male-sterile bait plants are usually overestimated. This is because the donor pollen is not diluted by pollen from the receptor plants. Our results suggest that in the case of cultivated sorghum gene flow will be reduced approximately six fold if male-fertile instead of male-sterile bait plants are used, while in wild sorghum gene flow will be reduced approximately 1.2 fold. Our result is consistent with observations of previous studies in maize (Wang et al. 2006) and question the rationale of using male-sterile bait plants in field trials to assess rates of pollen mediated gene flow. The results obtained in this study further suggest that the actual gene flow from cultivated to cultivated sorghum is likely to be much lower than estimates reported in previous studies using male-sterile bait plants (Schmidt and Bothma 2006). Thompson et al. (1999) furnished proof of gene flow of 49% at 100 m from the source using male-sterile oilseed rape (Brassica napus L.). However, Paul et al. (1995) reported gene flow of 0.1% at 10 m using male-fertile oilseed rape bait plants. Pollen competition between bait and source plants is the most likely explanation for the dramatic differences observed for gene flow when using male-sterile and male-fertile bait plants. The development of molecular markers has made it possible to use male fertile bait plants as the parentage of progeny can now be determined unambiguously.

Conclusion

The present study revealed that self pollen is more competitive than outcross pollen in male-fertile sorghum bait plants and outcross pollen competitiveness is dependent on the maternal genotype. The results of this study suggest that published estimates of gene flow derived from studies using male-sterile plants overestimate gene flow and that pollen competition may be a significant factor influencing outcrossing rates. Therefore, in addition to the outcrossing rate and the amount of pollen dispersal relative to distance, pollen competition should be taken into account in future attempts of estimating pollen-mediated gene flow.

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