High Cross Pollination Frequency in Rice Landraces in Field Condition

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Abstract

Based on a plethora of experimental observations, cross pollination frequency (CPF) in rice (*Oryza sativa* L.) is described in all rice biology text books and official publications to be very low, rarely exceeding 2%. Our previous study reported in 2021 the first contrary evidence of CPF >80%. Here we present the results of our recent experiments involving 25 pairs of rice landraces, and establish that CPF tends to increase with the flower exposure duration (FED) overlap between the pollen parent and ovary parent, and that CPF may reach 100% when the flower exposure overlap exceeds 40 min. This study corroborates our previous finding that the success of cross pollination in rice crucially depends on FED, to which no attention was given in previous rice outcrossing experiments over a century.

Introduction

The flower opening time (FOT) and the flower exposure duration (FED, measured as the period between flower opening time and closing time) are crucially important for the success of pollination and fertilization in rice plants. Although several studies have reported the range of FOT and flower closing time (FCT) of different landraces (**Table 1**), none of them documented the FED of the pollen parent (PP) and ovary parent (OP) landraces in the crossing experiments.

Table 1. Published Records of Earliest and Latest FOT for Rice Cultivars from Different Countries.

	Indica		***Japonica***		
Country of Origin	**Earliest FOT**	**Last FOT**	**Earliest FOT**	**Last FOT**	**Author Reference**
India	07:00	12:00			18
	09:53	11:16			19
Long day *Short day*	8:55 9:18	11:36 12:19	 9:37	 12:10	20; 21 20; 21
Indonesia	09:00 10:44	13:00 12:00	11:01	-	22 19
Nepal			10:51	11:30	19
Myanmar	07:00 10:38	09:00 11:31			23 19
China	09:01	10:47	11:44	12:02	19
Japan	10:08	11:42	10:19	12:35	19
The Philippines	09:00 09:55	11:30 11:15			24 19
USA	08:00	17:00	10:10	11:42	25; 19
			10:14	13:00	26

The most crucial period for successful cross pollination is the pollen recipient flower's exposure duration, calculated as the difference between the PP's FCT and OP's FOT, or alternatively, the OP's FCT and PP's FOT, which is denoted here as the 'effective exposure duration' (EED). The CPF is likely to be proportionately high only when EED > 0. In other words, if the flowers of the ovary parent (OP) close even a second before the FOT of the pollen parent (PP), the FED overlap between the two parents is zero, and consequently, there would be no cross pollination. Oblivious to this overlap of the crossing pair's exposure duration (or EED as defined here), all previous experimental studies [1-9] as well as authentic reference

books[10,11] inform that cross pollination frequency (CPF) in cultivated rice (*Oryza sativa*) rarely exceeds 2%. This received wisdom of very low CPF is also reported from experiments involving crossing between cultivated and transgenic rice cultivars [12-15]. The only report of over 9% CPF, in one instance, came from an experiment by Bae et al.[16], yet the CPF did not exceed 1% "in the field condition" (ref. 16, p. 168). We surmise that in all these experiments [1-9, 12-15], near-zero overlap of the FED between the PP and OP might be the only factor resulting in extremely low CPF reported in those experiments, and therefore the received wisdom of extremely low CPF in cultivated rice is a severe underestimation of the actual value in real life conditions [17]. Our 2019 study (published in 2021, ref. 17) reported for the first time that CPF exceeded 81% in a pair of rice landraces, whose respective FEDs had significantly overlapped.

In continuation of our previous experiment in cross pollination conducted in 2019, we conducted similar experiments in subsequent years using 17 pairs of landraces during winter of 2020 and 8 pairs during summer of 2021. The range of flowering period of each pair was almost identical, albeit with varying FOT. We report here the results of the new sets of experiment to test our conjecture that the CPF in cultivated rice is usually very high unless the overlap between the FEDs of the crossing pairs is zero or near-zero.

Materials and Methods

Study site

The experiment was conducted at Basudha conservation farm (19°42′32.0″N, 83°28′8.4″E), located in Rayagada district, Odisha, India, during *aman* season (June–December) of the year 2020 and *boro* (Januiary – March) of 2021. The uniformly sandy loam soil of the farm was enriched with decomposed farm yard manure, farm-grown phosphate solubilizing microbial (PSM) inocula, and leaf mulching, and received no synthetic agrochemicals for the past 25 years.

Selection of landraces

The experiment was conducted with rice landraces in two seasons, first with the short-day (*aman*) season landraces, flowering in winter (October-November 2020); and subsequently, with the photoperiod-insensitive landraces flowering in long day (*boro*) season (April-May 2021).

Based on the previously recorded booting dates and unmistakable contrasting morphological characters of the landraces[27], we selected a total of 25 landraces from Basudha's repertoire of 1480 landraces, based on matching anthesis dates and FOT, and 5 visually distinctive morphological characters (**Table 2**).

Selection of morphological characters

Each of the 25 landraces had already been assessed for 56 morphological characters to maintain genetic purity over the past 26 years (http://cintdis.org/basudha). The landraces were paired such that the morphological characters were unmistakably contrasting between the PP and OP in each pair. Most of these characters were previously employed in crossing experiments by many authors[2]- [9].

Table 2: Countries of Origin and Heritable Morphological Characters[27,[28] of the Landraces Used in Pair-wise Cross-Pollination Experiments.

Landrace Code	Origin	Pollen- or Ovary Parent	Flag Leaf Attitude	Awn: Max Length (mm)	Hull Colour	Pericarp Colour	Kernel: Notched Belly %
AA19	India	PP	Droopy	0	Straw	Brown	0
B103	Sri Lanka	OP	Semi Erect	0	Straw	Light Brown	0
V17	India	PP	Erect	62	Black furrows	White	>80%
C34	India	PP	Erect	14	Black	Black	0
C44	India	OP	Semi Erect	0	Gold furrows	White	0
DD16	India	OP	Erect	25	Gold furrows	White	0
G25	India	OP	Horizontal	0	Brown furrows	White	0
H01	India	OP	Erect	0	Brown (tawny)	White	0
H19	India	OP	Erect	0	Gold furrows	White	0
H40	Sri Lanka	OP	Erect	0	Brown furrows	Light Brown	0
J33	India	PP & OP	Horizontal	0	Gold furrows	White	0
Q11	India	PP & OP	Semi Erect	11	Straw	White	0
K131	India	PP	Droopy	0	Gold furrows	White	>80%
L59	India	PP	Horizontal	0	Brown furrows	White	0
M120	India	PP	Erect	37	Gold furrows	White	0
M121	India	PP	Semi Erect	0	Brown (tawny)	White	0
M84	Philippines	OP	Erect	0	Straw	Light Brown	0
M63	India	OP	Erect	0	Straw	White	0
M91	India	PP & OP	Erect	0	Brown (tawny)	White	0
M94	India	PP	Semi Erect	3	Brown (tawny)	Brown	0
N01	India	PP & OP	Semi Erect	82	Straw	Light Brown	0
S72	India	OP	Erect	0	Straw	White	0
S87	India	OP	Horizontal	6	Straw	White	0
S20	India	OP	Erect	0	Straw	White	0
T05	India	PP	Semi Erect	0	Purple furrows	Black	0

Experimental design

The experimental design of the crossing pairs was identical to our previous experiment [17]. Experiments were set up on Basudha farm in two cultivation seasons – *Aman* (sown in June 2020, harvested in December 2020), and *Boro* (sown in February 2021, harvested in May 2021). During *Aman* 2020, 13 pairs of landraces were Selected, and 5 of the 13 pairs were replicated in two plots each. Thus the total number of plots was 18. In each plot, a single seedling of a landrace, demarcated as the pollen recipient ovary parent (OP), was planted in the centre, around which 24 seedlings of a different landrace as the pollen parent (PP) were planted in a 5 x 5 matrix with 25 cm-spacing (**Fig. 1**, Plot A1 and Plot A2). The central position of the OP plant ensured the possible reception of pollen from all the surrounding PP plants, regardless of wind direction and speed. All seedlings were uniformly 14-day old when transplanted.

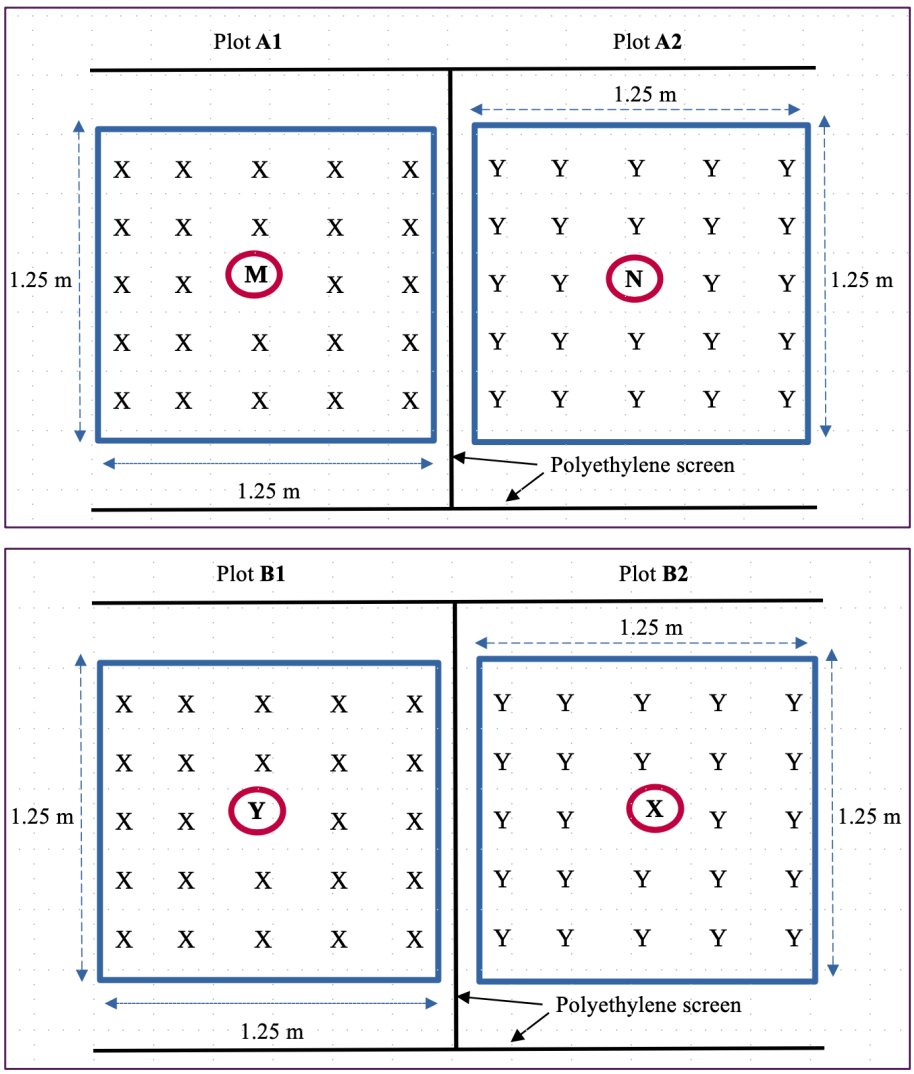

Fig. 1. Planting Design of Two Experimental Plots with a Single Ovary Parent (OP, *in red circle*), Surrounded by 24 Pollen Parents (PP), Planted in a Density of 16 m^{-2}. Plot A1 and Plot A2 Represent Different Pairs of PP and OP, planted during *Aman* season. Plot B1 and Plot B2 Represent Two Reciprocal Pairs (the OP in Plot B1 is the PP in Plot B2) Planted During *Boro* Season.

During *boro* season, 8 landraces in 8 discrete plots were planted as the PP, around a different landrace planted in the middle as the OP in each plot. The arrangement of the each pair was reciprocally reversed in a corresponding neighbouring plot, such that each PP in a plot was planted as OP, and vice cersa, in another plot (**Fig. 1**, Plot B1 and Plot B2). The total number of experimental plots with different pairs of landraces flowering during summer 2021 was 8, in 8 reciprocal pairs.
At the booting stage, a thin polyethylene sheet was vertically placed as a barrier to pollen flux between the neighbouring plots. The height of the barrier was 1.5 m, always above the top panicle level in each plot.

Recording of FOT, FCT, FED and EED of the parental pairs
After the anthesis of each landrace, we recorded the first flower opening time (FOT) and the last floret closing time (FCT) in each varietal plot. The methods are given in detail in ref. [19]. The longest duration of flowers remaining open (= flower opening duration, FED) was the interval between the FOT and the FCT of the last floret on a given varietal plot. As the transfer of pollen is possible only during the overlap of FED of OP and PP, we calculated the effective exposure duration (EED) of the OP florets, or the time widest window available for the OP's florets receiving pollen, as described in the checker box (**Table 2**).

Table 3: Calculation of Effective Exposure Duration of OP for Pollination Success. ['>' = later than]

Differenec in FOT and FCT between OP and PP	**If $FOT\|_{OP} > FOT\|_{PP}$**	**If $FOT\|_{PP} > FOT\|_{OP}$**
$FCT\|_{OP} > FCT\|_{PP}$	EED = $FCT\|_{PP} - FOT\|_{OP}$	EED = $FCT\|_{PP} - FOT\|_{PP}$
$FCT\|_{PP} > FCT\|_{OP}$	EED = $FCT\|_{OP} - FOT\|_{OP}$	EED = $FCT\|_{OP} - FOT\|_{PP}$

Collection of F1 progeny seeds
After maturity of seeds, panicles of the F1 progeny were harvested from the single OP plant from each plot. The OP panicles of *Aman* season were harvested in January 2021, and the OP panicles from *Boro* season were harvested in April 2021.

Cultivation of F1 plants for assessment of gene flow frquency
All the seeds from each panicle with labels bearing the parental pairs and corresponding plot numbers were germinated in discrete nursery beds in June 2021, and then transplanted in July 2021 in two rows in separate plots.

Characterisation of the F1 offspring and CPF estimation

Every plant of the F1 progeny was visually examined for the presence of morphological characters at maturity, namely, Flag leaf angle at maturity, and the length of the longest awn (if present). The panicles of each plant were were harvested after maturity in January 2021, and their seeds were assessed for hull (= lemma and palea) colour, pericarp colour and the frequency of 'notched belly' trait (**Table 2**), to discern if any trait was inherited from the respective PP of the progeny.

Assessment of gene flow frequency

The detection of at least one of the 5 contrasting characters of the PP in the F1 progeny would indicate the success of cross pollination. Since all these characters are a result of interactions of multiple genes, crosses between two landraces with contrasting traits is likely to produce offspring with variable quantifiable morphotypes. In particular, at least one of the offspring of a long-awned PP crossed with an awnless OP may possess smaller awns on most grains. In this case, the presence of awn would be a clear evidence of inheritance of the PP trait, even if the exact length of the awn in the offspring does not match the awn length of the PP.

The cross pollination frequency (CPF) is a measure of the inheritance of a morphological character c_i characteristic of the PP (but absent in OP), and was calculated as

$$\text{CPF} = \max\,[n(c_i)/N], \text{ with } i = 1 \text{ to } 5 \qquad \text{(eq. 1)}$$

where $n(c_i)$ is the number of individuals in F1 generation bearing c_i and N is the total number of F1 offspring. The 5 characters of each parental landrace are described in Table S1. When none of the offspring inherits any PP trait, CPF = 0.

Results

Table 4 and **Fig. 3** show the phenological details of flowering in the PP and OP landraces in all experimental plots in three cultivation seasons, as well as the critical segment of FED (CFED) calculated from the FOT and FCT of respective OP and PP. Using a total of 16 landraces as PP and 18 landraces PP, we planted selected pairs in a total of 26 experimental plots (including 5 replicates), of which 18 plots were established during *Aman* and 8 plots during *Boro* season (**Table 4** and **Suppl. Table S1**). However, the F1 progeny of one of these crosses (in Plot A11, in **Table 4**) was lost due to insect pest attack before harvest. Therefore, we discarded the pair Q11 x S87 from our final data for CPF estimation. Furthermore, The F1 progeny of two crosses (Plots B2 and B4 in **Table 4**) produced >98% sterile grains. Thus, the final dataset is comprised by 17 plots including 3 replicated plots, during *Aman* and 8 plots during long day (*Boro*) season. The range of the EED (in min) for each pair is shown in the right margin of **Figure. 3**.

Table 4: Phenology of Flowering of the 16 Pollen Parents (PP) and 18 Ovary Parents (OP) Selected in Cross Pollination Experiments over Two Seasons.

Season	Plot No.*	Lan-drace as PP	FOD	FOT	FED (min)	Lan-drace as OP	FOT	FED (min)	EED (min)	No. of Offs-pring #
Aman 2020	A1	C34	22/09	10:12	122	H19	10:19	64	64	22
	A2		22/09		122	M63	09:26	104	58	68
	A3a	K131	26/09	10:08	67	M84 a	09:48	80	60	122
	A3b		26/09			M84 b	09:48	80	60	107
	A4		26/09			S20	09:58	82	67	196
	A5	V17	01/10	10:06	73	M84	09:48	80	62	130
	A6a		01/10		73	S20 a	09:58	82	72	82
	A6b		01/10		72	S20 b	09:58	82	73	56
	A7a	M120	02/10	10:10	77	C44 a	09:54	55	39	16
	A7b		02/10		78	C44 b	09:54	55	39	22
	A8		02/10		77	H40	09:32	114	76	34
	A9	M121	15/10	10:22	57	Q11	10:11	66	55	74
	A10a	Q11	11/10	10:11	66	DD16 a	10:19	41	41	93
	A10b		11/10			DD16 b	10:19	41	41	85
	A11$	Q11	11/10	10:12	65	S87	10:06	54	48	110
	A12a	T05	15/09	10:35	23	S87 a	10:06	54	23	22
	A12b		15/09		23	S87 b	10:06	54	23	24
	A13		15/09		23	K169	09:42	98	23	89
	A14	L59	28/10	10:59	79	G25	10:32	58	31	49
Boro 2021	B1	AA19	04/05	10:05	60	B103	09:43	77	55	54
	B2$	B103	04/05	09:47	73	AA19	10:03	56	56	0
	B3	M94	07/05	10:26	65	H01	10:13	65	52	66
	B4$	H01	07/05	10:13	60	M94	10:28	63	45	0
	B5	N01	14/04	09:47	71	S72	09:54	78	64	78
	B6	S72	14/04	09:58	78	N01	09:47	67	56	28
	B7	M91	14/04	10:03	70	J33	10:10	69	63	41
	B8	J33	14/04	10:12	71	M91	10:09	67	64	80

* Pairs marked *a* and *b* are replicated experiments with the same OP.
$ Data of progeny lost/ discarded (see text).
# No. of fertile seeds from a single panicle of the OP.

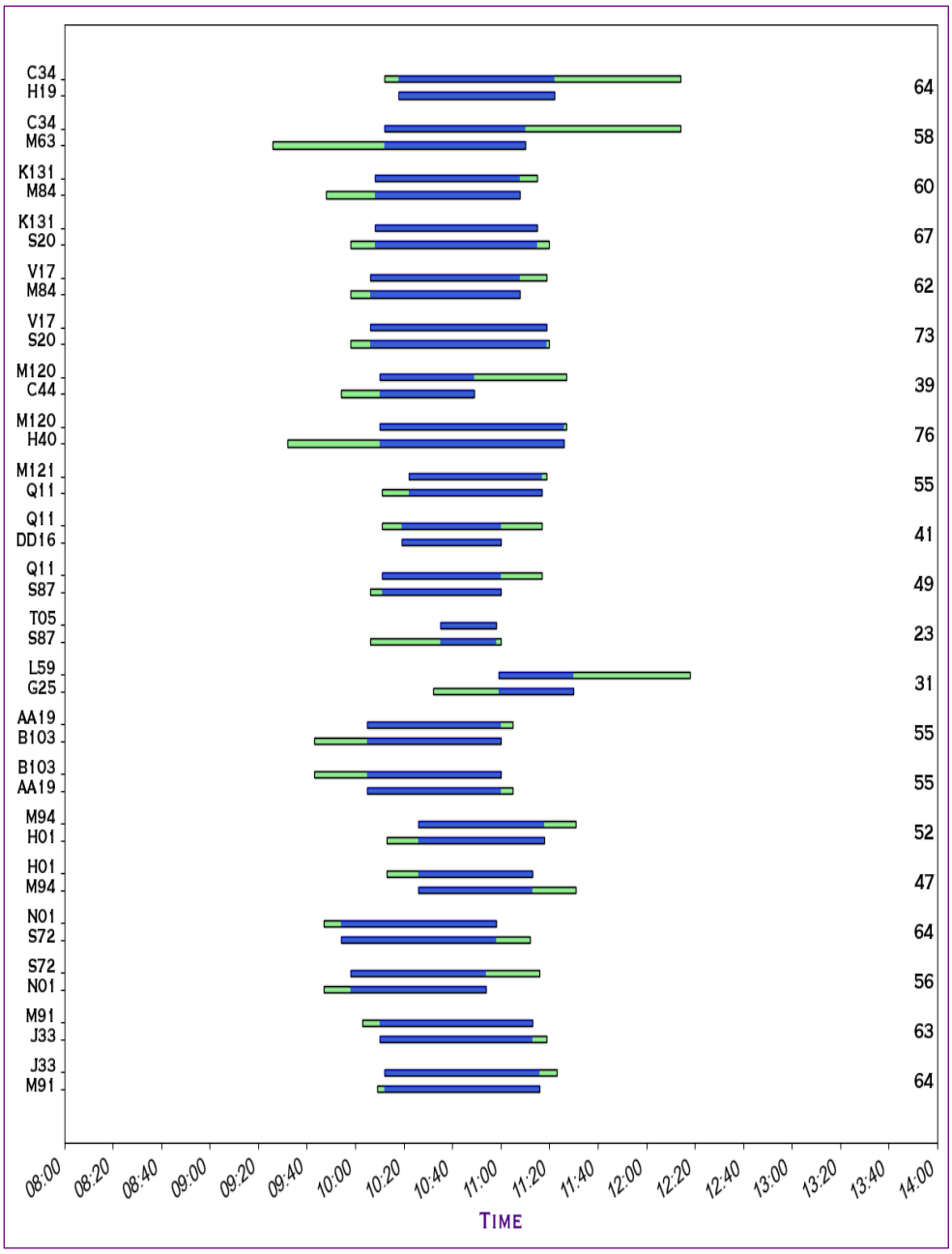

Fig. 2. The Range of FED (green bar), Beginning from FOT and Ending at FCT, with the Overlapping Period (blue) Indicating Effective Exposure Duration (EED) for Each Pair of Landraces. The EED (min) for each pair is shown on the right margin.

F1 sterility in some crosses

The data presented in **Table 4** and **Fig. 2** reveal that the EED of all pairs of landraces in this experiment ranged from 23 min (Plot A12A and Plot A12B) to 76 min (Plot A8). The mean of EED was 50.3 min during *Aman* season, and 56.8 min during *Boro* season. In the observed field condition of unhindered pollen flux from PP on all sides to OP florets, each OP was expected to yield a progeny with a non-zero probability of inheritance of at least 1 trait from the corresponding PP.

The crosses between the landraces in Plot B2 and in Plot B4 produced only sterile grains (**Table 4**). Some of the other crosses also yielded a high proportion of sterile grains, thereby reducing the panicle density by a considerable extent. The crosses between the landraces grown in Plot A1, Plot A7a, Plot A&b, Plot A12 a, Plot A12 b, Plot B6 and Plot B7 produced about 40% - 50% of sterile grains borne on the respective OP's panicle. When the proportions of sterile grains were plotted against EED for all crosses, the slope of regression was significant at 95% confidence level (**Fig. 3**).

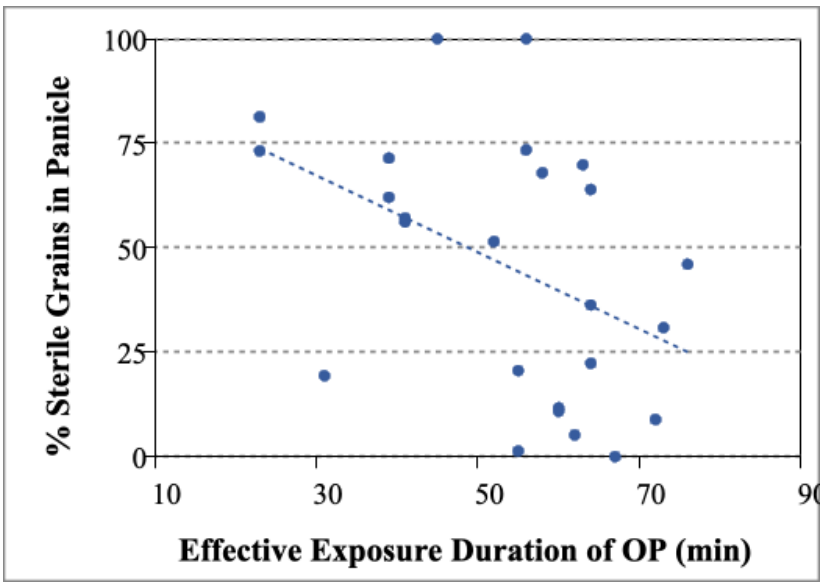

Fig. 3: Regression of % Sterility of Grains in the Progeny of Crossed Parents against EED (min). Slope of regression = —0.92, $df = 24$, $R^2 = 0.19$, $t = 2.32$, $p < 0.05$.

High frequency of gene flow in all parental pairs

The frequency of inheritance of PP traits in each progeny from the experimental plots assigned to the corresponding parental pairs (as numbered in col. 1 of **Table 4**) was estimated as the maximum number of offspring in F1 progeny showing any one of the 5 traits, from which CPF was estimated using eq. 1.Among the 25 pairs, 21 progeny lines showed a very high (≥ 80%) frequency of inheritance from PP.

A sufficiently wide time window is logically conducive to successful cross pollination. Thus, the CPF is expected to be associated with the length of EED. When the proportion of inheritance of PP traits is plotted against EED, a definitive trend of CPF escalating with EED is evident, which can best described as a remarkably strong ($p < 0.00001$) binomial function relationship % Inheritance = c1 + c1 EED + c2 EED^2, where c1 = —73.77, c2 = 5.89, c3 = — 0.05 (**Fig. 4**). The high frequency of inheritance of PP traits implies a high CPF, corroborating our previous finding[17].

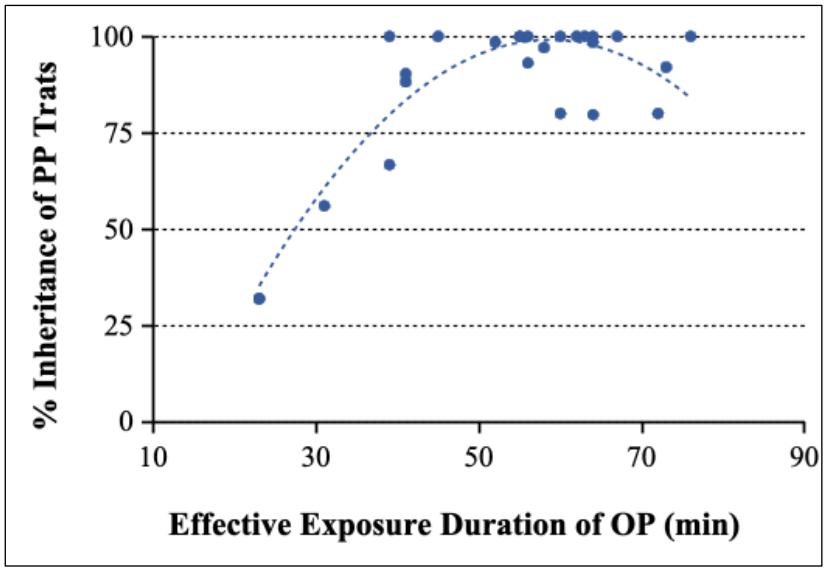

Fig. 4: The Association of the Frequency of PP Trait Inheritance with EED of the OP, Best Described as a Polynomial Function: % Inheritance = 5.89 EED — 0.05 EED^2 —73.77 (df = 24, R^2 = 0.805, t = 9.71, p <0.00001).

Discussion and Conclusion

In the trivial case of zero- or near-zero overlap of FED between the PP and OP (EED ≈ 0), CPF ≈ 0; and this justifies the reports of zero or near-zero CPF in most of the previous cross pollination experiments (ref. **1-9, 12-15**). In our previous experiment[17], one of the two parental pairs had an EED of ca. 10 min, and showed CPF = 0. In that same experiment, the FED overlap of 80 min was associated with CPF >80% -- a result that confirms our previous findings. The long duration of FED overlap does not imply that the pollen remain viable for more than 10 min, or that the same floret continually sheds pollen for a long time until the FCT of the floret. Either of this is impossible, as rice pollen is reported to die after 9 min. On the contrary, the ovary of an open floret remains receptive to viable pollen released from other florets that open successively later. Of course, if flowers remain open for too long, pollen decay within 9 min after anther dehiscence, so that longer (>50 min) EED may be associated with a lesser inheritance of OP traits.

A part of the design of the our present study was reciprocal crossing between two landraces (**Fig. 1**), to examine whether the pollen flow from PP to OP remains effectively the same between any pair of landraces. This trial shows at least two pairs yielded a strikingly asymmetric result: In the experimental plot B1, the number of fertile grains of the F1 progeny was 54, whereas the F1 progeny of the reciprocal cross in Plot B2 produced no viable offspring (100% F1 sterility). Likewise, the cross between the pair grown in Plot B3 produced 66 offspring, whereas the reciprocal cross between the same landraces grown in Plot B4 produced only sterile grains (**Table 3**). The proportion of sterile grains in the panicles of the F1 progeny in our study

seems to be associated with the EED of the crossing parents (**Fig. 2**), but the physiological reason is unclear; this association might also be a statistical artefact. Genetic interactions of the parental genotypes is a more likely factor resulting in a high frequency of F1 sterility, as reported in several studies (summarized in ref. [29] and ref. [30]). The low pollen fertility has been recorded mostly in *Oryza sativa* ssp. *indica* x *O. Sativa* ssp. *japonica* crosses, but also in some inter-varietal *indica* x *indica* crosses,[30],[31] where the sterility genes determined by allelic interaction seem to be of wide occurrence. Different combinations of the *HSA1a* and *HSA1b* genes in *indica* rice are currently known to confer sterility in F1 and F2 hybrids .[30], [32]

If indeed a proportion of grain sterility is a consequence of cross pollination itself, all the flowers of the OP plants in both Plot B2 and Plot B4 were cross pollinated (CPF =100). It is also reasonable to argue that crosses between specific cultivar genotype are likely to result in variable proportions of sterile grains in the F1 hybrids, as depicted in **Fig. 2**. This implies that in each of the crosses examined in this study, the actual frequency of cross pollination, CPF = max $[n(c_i)/N]$ (for fertile grains) + % sterile grains in the OP's panicle, in which case the actual CPF > max $[n(c_i)/N]$. Nonetheless, given the fact that the mechanism of the production of grain sterility in our genetic material is unknown, we presume that the only tangible evidence of cross pollination is max $[n(c_i)/N]$ as depicted in eq. 1. Even without considering the contribution of cross pollination to the production of sterile seeds in at least two pairs in our study, our study presents a convincing empirical evidence of considerably high CPF between *indica* landraces, as long as the overlap of FED between PP and OP flowers is of sufficiently long (>20 min) duration.

The unusually high frequency of CPF and gene flow reported here is in sharp contradiction to the received wisdom of 'less than 2% CPF'. The high frequency of inheritance of PP traits by the progeny documented in this study establishes the factual possibility of high CPF between landraces in field conditions, corroborating our earlier finding [17]. **Fig. 4** also confirms the assertion made in ref. [17] that longer EED is conducive to a greater frequency of cross pollination between neighbouring landraces.

The results of this study seems to be conclusive of the actuality of high CPF in rice landraces under circumstances of FED overlap between the parental cultivars, and indicates the necessity of further exploration into the genetic and physiologial mechanisms of sterile grain production from crosses between certain landraces. The latter investigation may consolidate our findings to establish that contrary to the received wisdom in the mainstream rice research, the outcrossing frequency between neighbouring landraces in field conditions is much higher than 2%. These findings are of profound importance in both hybridization experiments and in efforts to maintain genetic purity of rice cultivars.

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Data Availability

Phenological data of the landraces examined in this study are posted available in Harvard Dataverse.[33] All other data are freely available upon reasonable request.

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Conflicts of interest

Authors declare that they have no conflicts of interest.

Supplementary Material

Table S1: Experimental Design Matrix Showing Crosses between 15 Landraces as Pollen Parents and 20 Landraces as Ovary Parent. The numbers in the cells indicate the number of replications per pair.

	Aman 2020							**Boro 2021**							
PP ► ***OP*** ▼	C34	K131	L59	M120	M121	Q11	T05	AA19	B103	H01	J33	M91	M94	N01	S72
H19	1														
M63	2														
M84		2													
S20		1													
K92			2												
C44				2											
H40				1											
Q11					1										
DD16						2									
* S87						1									
K169							1								
S87							1								
B103								1							
* AA19									1						
* M94										1					
M91											1				
J33												1			
H01													1		
S72														1	
N01															1

OP landraces marked by an asterix (*) denote lost/ discarded data of their progeny.