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Molecular Marker and Test Cross Information Aid Selective Advancement of F_4 Generation of CB174R/Azucena: An inter Sub-specific Cross in Rice for Restorer Development

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The present investigation was aimed to develop restorer lines for three-line hybrid rice using *indica*/tropical *japonica* derivatives to exploit the inter sub-specific heterosis. From 75 F_4 families of CB174R/Azucena, two plants were randomly selected and screened using simple sequence repeat markers DRRM-RF 3 -10 for *Rf3* gene and RM6100 for *Rf4* gene. One hundred and five plants possessing either or both of the genes were test crossed with CMS line COMS 23A. In 67 hybrids evaluated, the mean pollen fertility ranged from 97.3% (CB174R/Azucena 177-4-9) to 13.7% (CB174R/Azucena 13-2-4). The frequency of restorers was high (49.25%) followed by partial restorers (29.85%) and partial maintainers (20.90%). The selection efficiency for DRRM- RF3-10 and RM6100 markers were 75.75% and 54.54% respectively. Segregation for fertility restorer genes and pollen fertility among individual plants within a family was witnessed from molecular and phenotypic data. Based on phenotypic and marker information, it was concluded to advance 53.3% of plants to F_5 generation to isolate stable restorer lines that can be exploited in future to produce highly heterotic three-line hybrids in rice.

Keywords: Rice; tropical japonica; pollen fertility; Rf genes; restorer; fertility restoration; test-cross.

1. INTRODUCTION

The cytoplasmic genic male sterility (CMS) that fails to yield functional pollen is found suitable for hybrid seed production in many crops. In self-pollinated crops like rice also, the male sterility and fertility restorer system has made a revolution in rice production as first witnessed in China and subsequently adopted by many countries including India. The CMS system also suffers from many bottlenecks and one among them is the availability of narrow genetic resources that can be utilized as effective restorers and maintainers [1,2]. The rice hybrids released in India and most of the Asian countries are based on the wild-abortive CMS system in which the fertility is restored by *Rf3* (located on chromosome 1) and *Rf4* (located on chromosome 10) genes [3,4]. In-*indica* based CMS systems, the exploitable level of heterosis over the inbred varieties is only 15-20% which emphasizes the need to diversify the restorer lines. The concept of heterotic pools has much relevance in hybrid breeding [5].

In the course of development of new restorer lines, it is a routine procedure to test their restoration ability by regular test cross performance and molecular screening. With these backgrounds, using *indica* restorer line, CB 174R which is the parent of hybrid CO 4 and a tropical *japonica* upland rice Azucena, inter sub-specific crosses were effected and new restorer lines are being developed by recombinant selection. Testing the fertility restoration ability of lines under development (F_4) is important for breeders to decide about rejection of undesired lines. This will help in reducing the burden of handling huge genetic materials and also help in conserving other resources. Hence, screening using molecular markers linked to fertility restorer genes *Rf3* and *Rf4* of Wild abortive CMS line and test cross performance were employed in the present investigation to evaluate the worthiness of F_4 breeding lines which are derivatives of *indica* and tropical *japonica*.

2. MATERIALS AND METHODS

This experiment was carried out at Paddy Breeding Station, Department of Rice, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore. The F_4 generation of CB174R/Azucena comprising of 75 families was sown during February 2021. Two plants per family were selected at random and young disease-free leaf samples were collected from the field and stored at -20°C until further use. Molecular screening was done with two SSR markers namely DRR RF 3 -10 for *Rf3* gene and RM6100 for *Rf4* gene in 150 single plants. The sequence details of markers are furnished below.

Table 1. Sequence details of markers

S. No.	Primer Name	Forward sequence	Reverse sequence	Annealing temperature ˚C	Chromo some location
1	DRRM RF3 -10	TCACCTCTTCCTGCTTCGAC	CTCCACCAGTGCAGGTTTTT	55	1
2	RM6100	TTCCCTGCAAGATTCTAGCTACACC	TGTTCGTCGACCAAGAACTCAGG	55	10

Genomic DNA was isolated as per the protocol of Doyle and Doyle [6]. The cocktail for one reaction (volume 10μl) contained 1μl DNA, PCR master mix 3.5μl, Nano pure water 4.5μl, 10x assay buffer, 1μl dNTP, 0.5μl of each forward and reverse primer. The smART Prime 2x PCR master mix 1.25mL was used, which consisted of Taq DNA Polymerase (0.0125 U/μL), Reaction buffer 1mM $Mgcl_2$, 0.1 mM of each dNTPs and 1.25 mL Nuclease free water. PCR amplification (Thermo scientific) was performed by initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 45 seconds, 55°C for 1min, 72°C for 2 minutes, extension for about 10 minutes at 72°C and infinity retrieval at 4°C. Ethidium bromide was added to gel cast @10μg/10ml and electrophoresis was done using 2.5% polyacrylamide gel for one to one and half hours at 110 volts. The gel was visualized on a UV- transilluminator. Based on molecular screening data, plants with either or both restorer genes were selected as male parents. At the time of flowering, crosses were attempted with CMS line COMS23A belonging to WA cytoplasm, which is the female line of popular hybrid CO 4 and set seeds collected. The hybrids were evaluated for their test cross performance during September 2021. The recommended crop agronomy was followed to have a healthy crop stand. During flowering, spikelets from panicles of three plants were individually squashed, stained with 1% I_2-KI solution, and three microscopic fields were observed. Well filled plumpy and round pollen grains were counted as fertile and others as sterile. The pollen fertility was calculated using the following formula [7]:

$$\text{Pollen fertility (\%)} = \frac{\text{No.of.fertile pollen grains}}{\text{Total number of pollen grains}} \times 100$$

The pollen parents were classified as follows based on pollen fertility [8]

Table 2. Categorical distribution based on Pollen fertility

Category	Pollen fertility (%)
Maintainers	0-1
Partial Maintainers	1.1-50
Partial Restorers	50.1-80
Restorers	>80

Analysis of variance was done for pollen fertility using "R" software to affirm the variability.

3. RESULTS AND DISCUSSION

As early as 1994, Yuan [9] reported that hybrids between *indica* and *japonica* exhibited 30-40% yield heterosis over the best *indica/indica* hybrid. Among *indica* and *japonica* subspecies, the hybrids between *indica* varieties is of higher magnitude than between *japonica* varieties. Inter-sub-specific hybrids displayed higher heterosis than intra-subspecific crosses in rice [10]. Because of fertility and grain quality issues in such wide crosses between *indica* and *japonica*, development of their hybrid derivatives as new plant type restorers is a solution to realize heterosis [11]. Alternately, utilization of tropical *japonicas* in *indica*-based hybrids offers wider diversity from the *japonica* base for improving the agronomic superiority and thereby improving the heterosis in rice [12].

The frequency of restorers in *indica* types and *indical* tropical *japonica* derivatives is 40%, hence to have broader genetic diversity, Virmani and Ishkumar [13] suggested that development of intermediate lines possessing new plant type traits would be more useful in restorer breeding. It is important that the parents chosen should have adequate genetic diversity to isolate desirable recombinants with fertility restoration ability. Accordingly, both the parents chosen for our crossing programme namely CB 174R and Azucena are genetically diverse and fell in different clusters for both agronomic traits and using simple sequence repeat markers [14].

New restorer lines are developed from crosses between either both parents with fertility restorer genes or at least one restorer parent followed by a recombinant selection from F_2 till homozygosity is achieved. During the course of line development, it is a practice in hybrid programs to advance superior plants with fertility restoration ability at later generations. Both the parents used in the present study possess the fertility restorer genes *Rf3* and *Rf4* for Wild-abortive cytoplasm screened in an earlier study [15].

In the present investigation, emphasis was given for the traits spikelet fertility, plant height and high single plant yield to advance single plants from F_2 to F_4 generation (unpublished). Kumar et al. [16] developed 100 iso-cytoplasmic restorers from F_2 of top hybrids in India, progeny advancement from F_3 upto F_6 was done based on desirable traits like panicle exsertion, good spikelet fertility and yield in a restorer.

Ponnuswamy et al. [17] crossed phenotypically superior lines of BC_2F_4 and BC_1F_5 generations of Swarna × KMR3R with two CMS lines namely APMS 6A and CRMS 32A to develop experimental rice hybrids. Recently, while developing a novel CMS and fertility restorer system from Tetep, a single plant from BC_3F_1 (Tetep/Hopum^4) crosses, which showed >80% seed setting was selected [18] and crossed with Hopum A to develop restorer lines. The F_1 was selfed and carried forward upto F_4. One line that showed complete restoration of fertility against Hopum A was selected and named as 'Hopum R'.

The conventional method of classifying the pollinator parents as restorers, partials, and maintainers is by hybridizing them with stable CMS line (s) and examining the F_1s for their fertility behavior in test cross nursery. The method consumes time, labour and field resources but the results are accurate On the other hand, simple sequence repeat markers linked with the gene of interest offers lot of benefits in terms of saving in time and resources. The correspondence between phenotypic and marker data is a concern unless the marker efficiency is high.

3.1 Molecular Screening for the Presence of Fertility Restorer Genes

Simple sequence repeat markers linked to fertility restorer genes or gene based functional markers have been employed by many workers to screen their breeding materials. However, the choice of markers varies in different studies. In the present study, DRRM RF3-10, a gene based SSR marker for *Rf3* and gene linked marker RM6100 for *Rf4* were employed. These two markers were already validated as tightly linked with fertility restoration of WA cytoplasm [19-21]. The marker DRRM RF3-10 has been used in earlier investigations [2,16,22,23]. RM6100 is the marker of choice for *Rf4* gene in many of the studies, some recent ones to quote are [24-27].

The present population of F_4 consisted of selections from 75 F_3 families constituted from 40 F_2 individuals, of which nine F_2 plants *viz.,* 83, 95, 134, 169, 209, 295, 307, 314 and 411 had three families each and the rest had either one or two families. Family-wise scrutiny of data revealed that families 61-5, 209-2, 209-3, 264-5, 347-1, 443-4, 453-2 and 453-4 did not show amplification for any of the genes in both their selected plants in F_4. So also, in 22 families namely 13-2, 13-3, 44-4, 51-3, 61-2, 122-4, 134-2, 144-4, 157-1, 162-4, 162-5, 169-3, 169-5, 173-3, 211-4, 216-3, 307-4, 314-1, 327-3, 327-4, 443-1 and 447-4, one plant is devoid of both the genes and the other plant showed amplification for only one of the two genes. Hence, these 30 families out of 75 (40.0%) are likely to have more of non-restorer plants and need not be pursued further. Singh et al. [28] also noticed that 31 lines out of 59 lines (52.54%) screened with the same set of markers did not carry both the genes.

On the other extreme, three families *viz.,* 83-1, 295-3 and 403-5 showed amplification for both the markers in both the selected plants. In sixteen families namely 53-1, 83-3, 122-2, 134-1, 144-5, 161-3, 169-4, 177-4, 264-1, 295-1, 307-2, 314-5, 317-1, 326-1, 411-1 and 411-3, one plant had both the genes and the other plant had one of the two genes. Seven families namely 95-3, 95-5, 160-4, 209-5, 281-5, 317-2 and 403-4 showed scores of zero for both the genes in one selected plant, while in other related plant it showed score '1' for both the genes. These 26 families can be focused for isolating restorer lines with both *Rf3* and *Rf4* genes. Selection and advancement of desirable plants from these families to F_5 are likely to yield good restorer lines.

In nine families namely 95-1, 134-3, 135-3, 135-5, 216-1, 295-4, 307-5, 314-3 and 411-5 both the plants possessed *Rf3* alone while four families namely 211-5, 409-3, 409-5 and 447-5, had *Rf4* alone. In the rest of the six families 53-4, 83-5, 96-1, 366-1, 399-3 and 450-2, one plant possessed *Rf3* alone and the other plant had *Rf4* alone. Selection of plants from these families may result in plants with either *Rf3* or *Rf4*. Thus, segregation for fertility restorer genes among individual plants within a family is witnessed from molecular data.

The results of molecular screening are depicted in Table 4 and Plates 1a and 1b. Out of 150 plants screened, DRRM RF3 10 showed amplification at 210bp in 42 plants (28.0%), while RM6100 had shown amplification at 185bp in 34 plants (22.67%) indicating the probable presence of restorer genes *Rf3* and *Rf4* respectively. Twenty-nine plants (19.33%) expressed amplification for both the markers, thus indicating the probability of occurrence of both the genes, while 45 plants (30.0%) did not show amplification for both the markers.

Kumar et al. [16] noticed the frequency of iso-cytoplasmic restorer lines carrying only Rf4 genes to be the highest (40%) followed by the frequency of lines carrying both Rf3 and Rf4 genes (22%). He concluded that *Rf3* had synergistic effect on fertility restoration. Shidenur et al. [22] also reported higher frequency of Rf4 than Rf3. Out of 106 indica x tropical japonica derivatives 2% of genotypes were identified with three gene combinations (Rf3/Rf4/S5n), 15% were identified with both *Rf3* and *Rf4*, 14% possessed only *Rf4*, 13% were observed to be completely devoid of any of the genes tested through marker analysis [29].

3.2 Test Cross Evaluation

Numerous studies have generally shown that the genomic background plays a crucial role in fertility restoration in hybrids and there is a possibility of interaction with modifiers. Differential restoration behavior of the same pollinator to different CMS lines with same WA cytoplasmic source has been encountered. Since the study involved an early testing of lines under development (F_4 generation), one representative CMS line COMS 23A was chosen for testing the restorability. Traditionally, crossing the test genotypes with CMS lines has been reported as a standard procedure to identify maintainer and restorer lines [30,8,31,32]. Both pollen and spikelet fertility can be used to evaluate the fertility of F_1s in test cross nursery, but pollen fertility is reliable since several physiological and environmental factors influence spikelet fertility [11,33,34]. Even biotic factors like earhead bugs influence spikelet fertility and no conclusive decisions can be made. Sometimes, lesser pollen fertility tends to provide higher seed set due to the ability of single fertile pollen to fertilize a spikelet [35]. Hence, pollen fertility was assessed in the present study to classify the restoration ability.

Different workers have adopted different classes for concluding at fertility restoration in hybrids based on pollen fertility. As per the classification of Virmani et al. [8], parents producing >80% pollen fertility in hybrids were classified as restorers which has been adopted in the present study. This has been followed by Singh et al. [36] in identifying suitable hybrids for North-East India and Singh et al. [28] in classifying 36 hybrids synthesized using one CMS line Pusa 6A.

In some of the studies involving inter sub-specific crosses Hossain et al. [11]; Vaithiyalingam and Nadarajan, [37], the classification by Chaudhary et al. [38] has been followed in which, plants with above 60% fertile pollen were grouped as fully fertile. So also, Hasan et al. [39] followed this classification in their inheritance studies on fertility restoration in ID type CMS lines and Kumar et al. [16] in test crosses involving iso-cytoplasmic restorer lines in rice

In molecular screening, the present study revealed the absence of fertility restorer genes in 45 plants which were rejected. Out of 105 test crosses attempted with COMS23A, 67 hybrids alone could be evaluated with adequate number of plants.

Table 3. Analysis of variance for pollen fertility

Source	DF	MS	PR> Fvalue
Replications	1	0.69	0.2368
F_4 lines	66	1236.67	<2e -16***
Residuals	66	0.49	

The data presented in Table 5 showed a wide range of mean pollen fertility from 97.3% (CB174R/Azucena 177-4-9) to 13.7% (CB174R/Azucena 13-2-4) in 67 hybrids. As per the classification followed, 33 parents were identified as effective restorers with fertility of hybrids ranging from 97.3 to 79.95% (CB174R/Azucena-317-2-2). Twenty male parents behaved as partial restorers with pollen fertility ranging from 78.9% (CB174R/Azucena-144-4-3) to 52.0% (CB174R/Azucena-314-3-7). The rest of the hybrids (14 nos.) behaved as partial maintainers with pollen fertility ranging from 49.9% (CB174R/Azucena 95-1-7) to 13.7%.

As early as 1966, Jennings [40] noticed wide variation for fertility restoration in many crosses of *indica* with *japonica*. He attributed high fertility of a cross due to the presence of a wide compatibility gene or restorer gene in the cultivar. Male lines from Thailand and India showed lower frequency of restorers (34% and 41%) in analysis of 19,330 test crosses) [41]. Huang et al. [42] observed various degrees of fertility restoration including complete restoration in F_1s, when F_5 lines of Reimei (*rufipogon*) A/IR5032-6B-13-1 were test crossed with Zhen Shan 97A.

In test-crosses of 204 drought tolerant *indica* breeding lines with IR 58025A, Singh et al. [43] identified 24.02% restorers, 26.96% partial maintainers and 30.88% partial restorers based on spikelet fertility data. In *indica* background, out of 65 test crosses generated using one CMS

line IR79156A, Parimala et al. [44] identified 28 restorers, 20 partial restorers, 14 partial maintainers and three maintainers based on pollen and spikelet fertility. Using the same CMS line, Prasad et al. [45] identified 18 restorers, 17 partial restorers and remaining as partial maintainers from 38 test crosses based on pollen and spikelet fertility. From thirty six pollen parents, nine genotypes (25%) were classified as restorers, 11 as partial restorers (30.6%), and four as partial maintainers (11.1%) for the CMS line Pusa 6A [28]. Out of 31 test-crosses evaluated using two CMS lines CHAO1 and IR80151A, Seesang et al. [33] identified six restorers based on pollen fertility data.

3.3 Correspondence between Phenotypic and Genotypic Data and Marker Efficiency

In this study, the frequency of restorers is high (49.25%) followed by partial restorers (29.85%) and partial maintainers. No complete maintainers could be observed. From Table 5, it could be inferred that out of 33 restorers that produced hybrids with pollen fertility above 80%, 15 possessed *Rf3* alone, eight plants had *Rf4* alone and 10 plants had both the genes. The fertility of hybrids for the presence of three categories of genes *viz., Rf3* alone, *Rf4* alone and both ranged from 85.24 to 96.7%, 85.6 to 94.2% and 79.95 to 97.3% respectively. Thus the marker efficiency for DRRM RF3-10 (*Rf3*) in identifying plants with fertile pollen among 33 restorers is 75.75% and that for RM6100 (*Rf4*) is 54.54%. The selection efficiency for the marker DRRM RF3-10 for *Rf3* gene was reported as 84% [16] and 92% [46]. For *Rf4* gene, the efficiency by marker RM6100 was reported as 92% [20], 75% [21], 97.4% [46] and 80% [45].

In partial restorers, the range of pollen fertility for six plants possessing *Rf3* alone was from 52.5 to 78.9%, for eight plants with *Rf4* alone was from 58.4 to 78.7% and for six plants with both the genes was from 59.0 to 76.0%. Singh et al. [28] observed that plants with pollen fertility ranging from 67 to 79% had spikelet fertility ranging from 80-90%. Ponnuswamy et al. [17] observed that plants with 69.8 to 70.4% pollen fertility yielded plants with 77.5 to 75.6% spikelet fertility. So plants with above 70% pollen fertility may have >80% spikelet fertility. Thus there may be amplification for the genes *Rf3* and (or) *Rf4* even in plants falling short of present standards (80% pollen fertility). In that case, additionally, 13 plants out of 20 from partial restorers can be considered as restorers and the marker selection efficiency for *Rf3* will be 71.74% and *Rf4* will be 60.86%.

Using the same molecular markers, Shidenur et al. [47] identified 42 New Plant Type restorers derived from tropical japonica and crossed them with Pusa 6A and found hybrids with varying levels of spikelet fertility restoration. Ten restorers with both *Rf3* and *Rf4* alleles in the homozygous state produced hybrids with above 75.1% fertile grains. Eight hybrids, where restorer carried only the F allele of *Rf3* exhibited spikelet fertility that ranged between 20.8% and 52.9% and they putatively attributed the sterility observed among *Rf3* carriers to the relative restoration efficiency of *Rf3* locus. In our study, Twenty-one hybrids with the *Rf4* gene alone showed fertility range of 55.2 to 86.1%. Thus differences in level of fertility restoration was observed between the hybrids implying that the lines carrying a particular restorer gene even in the similar background of female parent imparts restoration of varying degrees which can be identified only in a test cross. In molecular screening of 28 genotypes identified to carry *Rf4* genes, only seventeen genotypes were confirmed as effective restorers based on pollen and spikelet fertility data [28].

In partial maintainer category, all were unique families and one plant 83-1-6 with both the genes expressed only 14.0% fertility in our study. Singh et al. [28] also observed that out of three parents with both the fertility restorer genes, only one parent exhibited complete restoration. Li et al. [48] reported that restorers with strong restoration ability have two major genes along with modifer genes and a restorer with semi-restoring ability have either one of the two major genes. In another work, Singh et al. [28] observed six genotypes as restorers based on pollen and spikelet fertility percentage but did not have Rf4 and Rf3 genes and thus modifiers play a role in fertility restoration as also emphasized by Bharaj et al. [49].

Within family variations were observed in the present study between the two selected plants for fertility restoration and the families to quote are 134-1, 211-5 and 403-5 with partial restoration and restoration; families 144-5 and 399-3 with partial maintenance and partial restoration and one family 135-5 with partial maintenance and restoration. On the contrary,

Table 4. Molecular marker screening of F_4 families of CB174R/Azucena for fertility restorer genes

Lane No.	CB174R/ Azucena F_4 families	Marker scoring		Lane No.	CB174R/ Azucena F_4 families	Marker scoring	
		DRRM RF3-10 (*Rf3*)	RM 6100 (*Rf4*)			DRRM RF3-10 (*Rf3*)	RM 6100 (*Rf4*)
1	144-4-3	1	0	39	453-4-6	0	0
2	144-4-6	0	0	40	453-4-7	0	0
3	144-5-1	1	0	41	347-1-4	0	0
4	144-5-2	1	1	42	347-1-7	0	0
5	403-4-4	0	0	43	211-4-2	0	1
6	403-4-9	1	1	44	211-4-7	0	0
7	403-5-4	1	1	45	211-5-1	0	1
8	403-5-9	1	1	46	211-5-4	0	1
9	307-2-2	0	1	47	447-4-1	0	1
10	307-2-6	1	1	48	447-4-9	0	0
11	307-4-8	1	0	49	447-5-3	0	1
12	307-4-9	0	0	50	447-5-9	0	1
13	216-1-1	1	0	51	411-1-2	1	1
14	216-1-7	1	0	52	411-1-3	1	0
15	216-3-6	1	0	53	411-3-3	1	0
16	216-3-8	0	0	54	411-3-4	1	1
17	281-5-6	1	1	55	411-5-2	1	0
18	281-5-7	0	0	56	411-5-3	1	0
19	160-4-5	1	1	57	307-5-9	1	0
20	160-4-9	0	0	58	307-5-10	1	0
21	161-3-4	1	1	59	443-1-1	1	0
22	161-3-9	0	1	60	443-1-7	0	0
23	326-1-6	1	1	61	443-4-1	0	0
24	326-1-7	0	1	62	443-4-10	0	0
25	409-3-1	0	1	63	157-1-4	0	0
26	409-3-3	0	1	64	157-1-5	1	0
27	409-5-1	0	1	65	264-5-4	0	0
28	409-5-6	0	1	66	264-5-10	0	0
29	327-3-4	0	1	67	96-1-1	1	0
30	327-3-5	0	0	68	96-1-4	0	1
31	327-4-3	0	1	69	450-2-2	1	0
32	327-4-10	0	0	70	450-2-5	0	1
33	61-2-1	0	1	71	169-3-4	0	1
34	61-2-5	0	0	72	169-3-10	0	0
35	61-5-3	0	0	73	169-4-5	0	1
36	61-5-7	0	0	74	169-4-6	1	1
37	453-2-9	0	0	75	314-1-1	0	1
38	453-2-10	0	0	76	314-1-2	0	0
77	314-3-6	1	0	114	122-4-5	0	0

Lane No.	CB174R/ Azucena F_4 families	Marker scoring		Lane No.	CB174R/ Azucena F_4 families	Marker scoring	
		DRRM RF3-10 (*Rf3*)	RM 6100 (*Rf4*)			DRRM RF3-10 (*Rf3*)	RM 6100 (*Rf4*)
78	314-3-7	1	0	115	209-2-4	0	0
79	314-5-7	1	0	116	209-2-6	0	0
80	314-5-10	1	1	117	209-3-5	0	0
81	95-1-7	1	0	118	209-3-6	0	0
82	95-1-8	1	0	119	209-5-1	1	1
83	95-3-1	1	1	120	209-5-2	0	0
84	95-3-2	0	0	121	317-1-7	0	1
85	95-5-6	0	0	122	317-1-9	1	1
86	95-5-9	1	1	123	317-2-2	1	1
87	135-3-4	1	0	124	317-2-4	0	0
88	135-3-5	1	0	125	295-1-9	0	1
89	135-5-1	1	0	126	295-1-10	1	1
90	135-5-2	1	0	127	295-3-2	1	1
91	169-5-4	0	1	128	295-3-3	1	1
92	169-5-5	0	0	129	295-4-5	1	0
93	53-1-2	1	1	130	295-4-8	1	0
94	53-1-3	1	0	131	399-3-6	0	1
95	53-4-1	0	1	132	399-3-8	1	0
96	53-4-2	1	0	133	51-3-3	0	1
97	13-2-4	0	1	134	51-3-4	0	0
98	13-2-5	0	0	135	162-4-2	0	0
99	13-3-2	0	0	136	162-4-5	1	0
100	13-3-4	1	0	137	162-5-8	1	0
101	366-1-7	1	0	138	162-5-10	0	0
102	366-1-8	0	1	139	134-1-3	1	1
103	264-1-4	0	1	140	134-1-4	1	0
104	264-1-5	1	1	141	134-2-5	1	0
105	83-1-5	1	1	142	134-2-8	0	0
106	83-1-6	1	1	143	134-3-2	1	0
107	83-3-6	1	1	144	134-3-5	1	0
108	83-3-8	1	0	145	44-4-3	0	0
109	83-5-1	1	0	146	44-4-7	0	1
110	83-5-4	0	1	147	173-3-1	0	0
111	122-2-4	1	1	148	173-3-2	0	1
112	122-2-5	1	0	149	177-4-4	1	0
113	122-4-2	0	1	150	177-4-9	1	1

Table 5. Correspondence between phenotypic and genotypic data for fertility restoration in hybrids of COMS23A/ CB174R×Azucena F_4 progenies

S. No.	Hybrids of COMS23A/ CB174R× Azucena F_4 progenies	Mean Pollen Fertility (%)	Fertility reaction as per test cross	Molecular scoring		S. No.	Hybrids of COMS23A/ CB174R× Azucena F_4 progenies	Mean Pollen Fertility (%)	Fertility reaction as per test cross	Molecular scoring	
				Rf3	*Rf4*					*Rf3*	*Rf4*
1	177-4-9	97.3	R	1	1	34	144-4-3	78.9	PR	1	0
2	122-2-4	96.9	R	1	1	35	211-5-4	78.6	PR	0	1
3	216-1-1	96.7	R	1	0	36	409-5-1	78.7	PR	0	1
4	13-3-4	95.0	R	1	0	37	162-5-8	76.4	PR	1	0
5	314-5-7	94.95	R	1	0	38	53-1-2	76.0	PR	1	1
6	134-3-2	94.6	R	1	0	39	326-1-6	75.3	PR	1	1
7	409-3-3	94.2	R	0	1	40	122-4-2	75.0	PR	0	1
8	61-2-1	94.0	R	0	1	41	161-3-4	75.0	PR	1	1
9	83-5-1	93.9	R	1	0	42	409-5-6	74.7	PR	0	1
10	403-4-9	92.6	R	1	1	43	403-5-9	73.5	PR	1	1
11	211-5-1	92.3	R	0	1	44	83-3-8	72.6	PR	1	0
12	411-1-2	91.1	R	1	1	45	144-5-2	72.5	PR	1	1
13	411-5-2	89.8	R	1	0	46	96-1-4	72.1	PR	0	1
14	450-2-5	89.3	R	0	1	47	264-1-4	62.3	PR	0	1
15	216-3-6	89.2	R	1	0	48	161-3-9	61.3	PR	0	1
16	307-5-10	89.2	R	1	0	49	134-1-3	59.0	PR	1	1
17	216-1-7	88.2	R	1	0	50	317-1-7	58.4	PR	0	1
18	447-5-9	88.1	R	0	1	51	399-3-8	57.1	PR	1	0
19	327-3-4	88.0	R	0	1	52	314-3-6	52.5	PR	1	0
20	134-1-4	88.0	R	1	0	53	314-3-7	52.0	PR	1	0
21	169-4-5	87.2	R	0	1	54	95-1-7	49.9	PM	1	0
22	411-1-3	86.2	R	1	0	55	307-4-8	49.0	PM	1	0
23	169-4-6	86.2	R	1	1	56	144-5-1	48.5	PM	1	0
24	134-2-5	86.0	R	1	0	57	51-3-3	45.2	PM	0	1
25	135-5-2	86.0	R	1	0	58	53-4-2	36.0	PM	1	0
26	211-4-2	85.6	R	0	1	59	169-3-4	25.7	PM	0	1
27	177-4-4	85.24	R	1	0	60	162-4-5	24.5	PM	1	0
28	96-1-1	83.2	R	1	0	61	399-3-6	21.4	PM	0	1
29	307-2-6	80.55	R	1	1	62	443-1-1	18.9	PM	1	0
30	403-5-4	80.12	R	1	1	63	447-4-1	19.3	PM	0	1
31	295-3-3	80.02	R	1	1	64	173-3-2	16.5	PM	0	1
32	295-3-2	80.0	R	1	1	65	135-5-1	16.3	PM	1	0
33	317-2-2	79.95	R	1	1	66	83-1-6	14.0	PM	1	1
						67	13-2-4	13.7	PM	0	1

R- Restorer, PR- Partial restorer, PM- Partial maintainer

families showing similar behaviour for fertility restoration were 169-4, 177-4, 216-1, 295-3 and 411-1 and they showed amplification for *Rf3* or *Rf4* or both. The reasons may be due to unstabilized breeding lines used as pollen parents and due to presence of modifiers governing fertility restoration.

Using three *indica/japonica* derivative restorers (P1277-100, P1266-89, and P1266-8) and three 'WA'-type cytoplasmic male sterile lines (Pusa 3A, Pusa 5A, and Pusa 6A), Hossain et al. [11] concluded that two or three major genes govern the fertility restoration, with epistatic interactions that differed from cross to cross. The number of nuclear genes controlling fertility restoration was also dependant on the materials and methods used. Shidenur et al. [46] studied 31 tropical japonica-derived Rf gene-carrying rice hybrids. The pollen fertility was five times higher among Rf4 hybrids than that of hybrids carrying Rf3 alone. Likewise, spikelet fertility among Rf4 hybrids was twice higher than that of *Rf3* hybrids. These results emphasize that one of the genes governing fertility restoration appeared to be stronger in action than the other [50]. From their study it was concluded that the *Rf4* gene is essential either alone or in combination with *Rf3* for fertility restoration to achieve enhanced grain yield in WA-CMS-based hybrids.

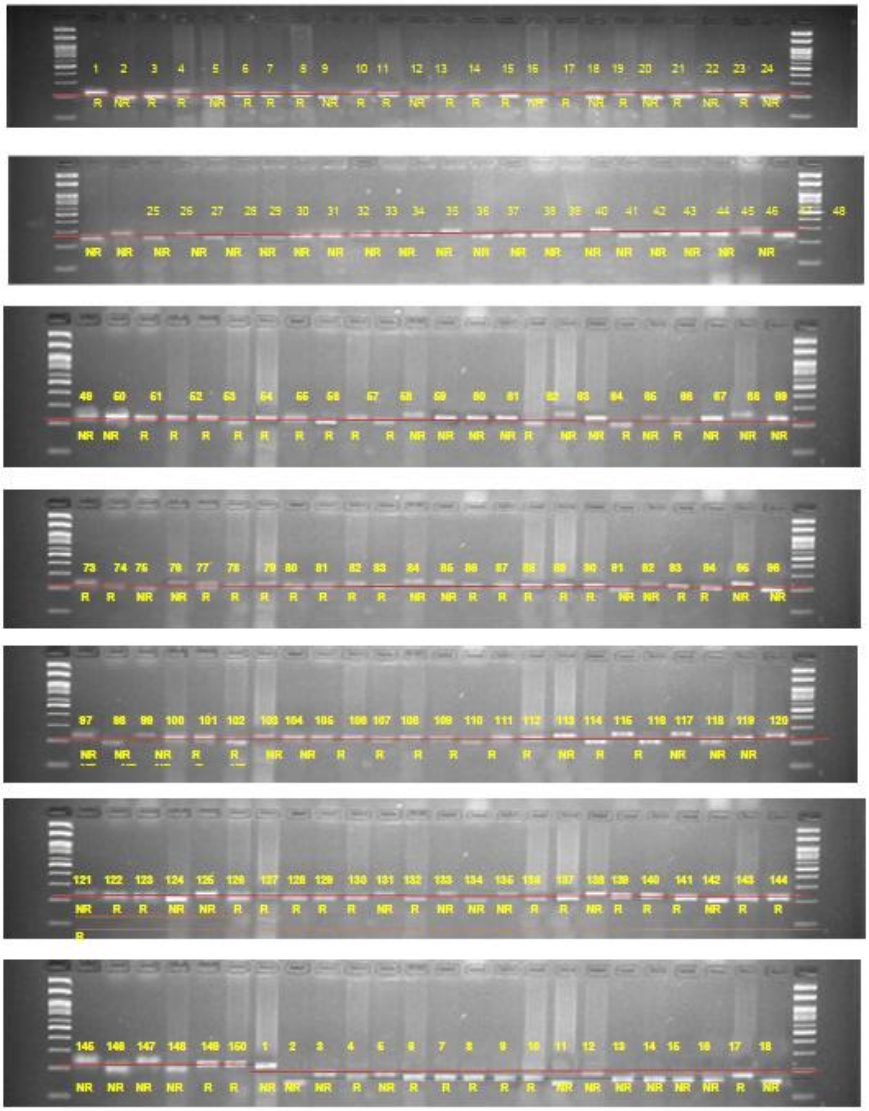

Plate 1a. DRRM RF3-10 molecular marker amplification profile of 150 F_4 families (1-150) of CB174R/Azucena at 210bp and RM6100 marker amplification profile of 18 F_4 families of the same cross

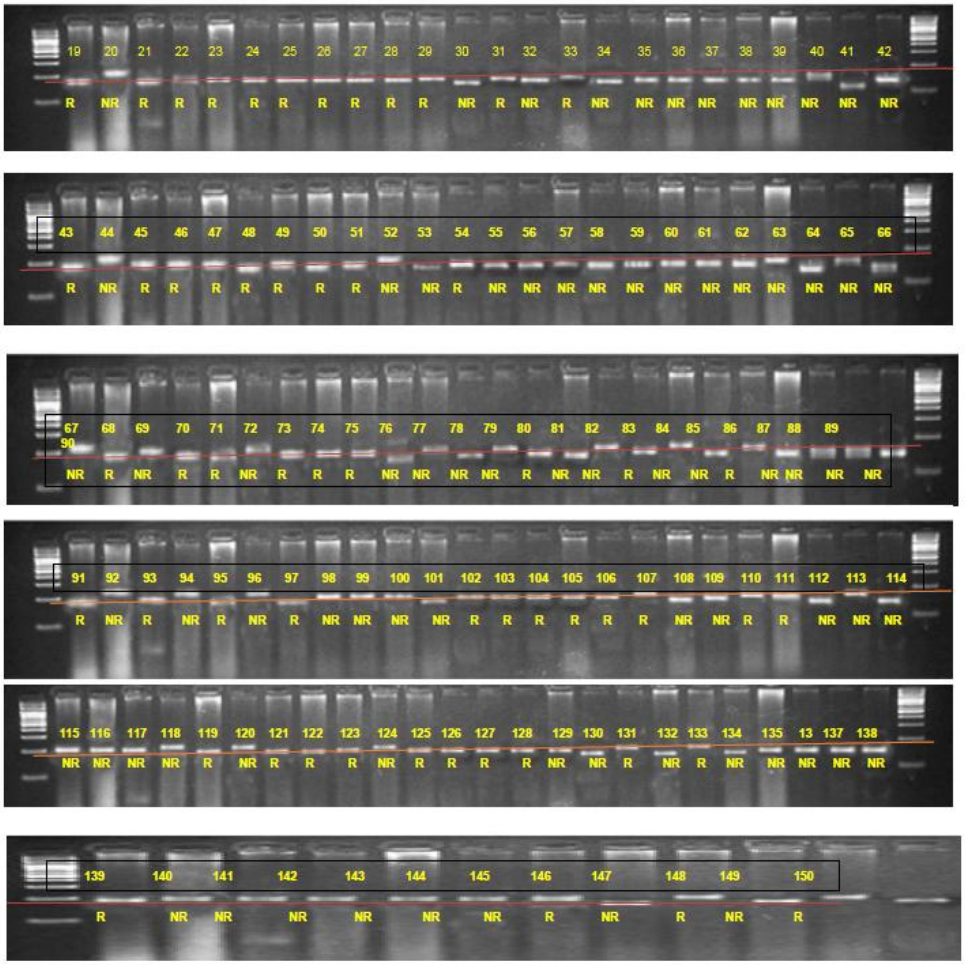

Plate 1b. RM6100 molecular marker amplification profile of 132 F_4 families (19-150) of CB174R/Azucena at 185bp

4. CONCLUSION

The information gained from the present study is that, plants that can be advanced to F_5 generation include 33 plants based on phenotypic and genotypic data; 13 plants with more than 70% pollen fertility and having either *Rf3, Rf4* or both and 34 plants from 26 families (other plant in the family is included in 33 or 13 plants). Altogether, based on test cross performance and molecular screening, totally 80 F_4 plants (53.3%) out of 150 evaluated, can be advanced to next generation to develop inter sub-specific restorer lines for three-line hybrid rice.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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