# *Original Research Article*

## 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

**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 DRR 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 DRR 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 **(**Chang *et al.,* (2016, Katara *et.al.,* 2017). 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 (Virmani and Van, 1988, Zhang *et al.,* 1997, Yao *et al.,* 1997). 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 (Xie, 2015). 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. MATERIAL 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.



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

Genomic DNA was isolated as per the protocol of Doyle and Doyle (1987). The PCR mix for one reaction (volume 20μl) contained 2μl DNA, sterile and Nano pure water 13.5μl, 10x assay buffer, 1μl dNTP, 0.5μl of each forward and reverse primer and 0.5μl Taq DNA polymerase. PCR amplification

was performed by denaturing at 94°C for 4 min, followed by 35 cycles of 94°C for 1min, 55°C for 1min, and extension for about 10 minutes at 72°C. Electrophoresis was done using 5% polyacrylamide gel for one hour at 199 volts and then stained with Ethidium bromide (10μg/10ml) for 40-45 minutes. 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% KI solution and observed under microscope. The pollen fertility was calculated using the following formula:

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

The pollen parents were classified as follows based on pollen fertility as proposed by Virmani *et al.,* (1997)

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

## 3. RESULTS AND DISCUSSION

4. As early as 1994, Yuan 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 (Fu *et.al* 2014). 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 (Hossain *et.al.,*2010). 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 (Khush, 1995). The frequency of restorers in *indica* types and *indica*/ tropical *japonica* derivatives is 40%, hence to have broader genetic diversity, Virmani and Ishkumar (2004) 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 simple sequence repeat markers (Sangeetha *et. al*., 2019). 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 **(**Sangeetha, 2019**)**. Kumar *et. al.,* (2017) 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.,* (2020) 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, Jin *et.al*., (2021) selected a single plant from $BC_3F_1$ (Tetep/Hopum$^4$ ) crosses, which showed >80% seed setting and crossed it 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'. In good combiners with partial restoration, through marker assisted backcross breeding, *Rf3* and *Rf4* genes were stacked into Mahalaxmi and Gayathri (Verma *et.al.,* 2021). 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). At this stage, in order to isolate and advance only desirable restorers to next generation, both test-cross and molecular screening were employed. 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 $F_1$s 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.

**5. Molecular screening for the presence of fertility restorer genes**

6. Fertility restoration of the WA-CMS system has been reported to be controlled by two nuclear genes: *Rf3* and *Rf4*, which are located on chromosomes 1 and 10, respectively (Zhang *et. al.,* 1997, Jing *et. al.,* 2001, Alavi *et. al.,* 2009). 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, DRR 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 (Singh *et.al.,* 2005, Suresh *et.al.,* 2012 and Revathi *et.al.,* 2013). The marker DRR RF3-10 has been used in earlier investigations by Katara *et. al*., (2017), Kumar *et. al*., (2017), Shidenur *et. al*., (2019) and Rashid *et. al*., (2019). RM6100 is the marker of choice for *Rf4* gene in many of the studies, some recent ones to quote be that of Madhuri *et. al*., (2019), Majid *et. al*., (2020), Surendar (2021) and Venkanna *et. al*., (2022). The results of molecular screening are depicted in Table 1 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.
7. The 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.,* (2016) also noticed that 31 lines out of 59 lines (52.54%) screened with the same set of markers did not carry both the genes.
8. 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 it showed score '1' for both the genes in the other selected plant. 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.

9. Both the plants in nine families namely 95-1, 134-3, 135-3, 135-5, 216-1, 295-4, 307-5, 314-3 and 411-5 exhibited amplification for DRR RF 3 -10 alone and in four families namely 211-5, 409-3, 409-5 and 447-5, both the plants showed amplification for RM6100 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.

Kumar *et. al.,* (2017) 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.,* (2019) also reported higher frequency of *Rf4* than *Rf3*. Out of 106 *indica* x tropical *japonica* derivatives (Sruthi *et. al*., 2020), 2% of genotypes were identified with three gene combinations (*Rf3*/*Rf4*/*S5*n), 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.

**Test cross evaluation**

10. 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 behaviour of the same pollinator to different CMS lines with same WA cytoplasmic source has been encountered (Virmani 1987; Salgotra *et al*. 2002). Since the study involved an early testing of lines under development ($F_4$ generation), one representative CMS line COMS 23A which is the female parent of popular hybrid CO 4 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 (Ikehashi and Araki, 1984; Virmani, 1997; Akhter *et. al.,* 2008; Reddy *et al*. 2014). Both pollen and spikelet fertility can be used to evaluate the fertility of $F_1$s in test cross nursery, but pollen fertility is reliable since several physiological and environmental factors influence spikelet fertility (Hossain *et.al.,* 2010, Seesang *et.al.,* 2014). 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 (Joshi *et al.,* 2007). Hence, pollen fertility was assessed in the present study to classify the restoration ability.

11. In molecular screening, 45 plants did not possess any of the fertility restorer genes and were rejected. Out of 105 test crosses attempted with COMS23A, 67 hybrids alone could be evaluated for their fertility reaction with adequate number of plants. The data presented in Table 2 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%.
12. 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.,* (1997), 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.,* (2016) in identifying suitable hybrids for North-East India and Singh *et.al.,* (2016) in classifying 36 hybrids synthesized using one CMS line Pusa 6A.
13. In some of the studies involving inter sub-specific crosses (Hossain *et.al.,* 2010; Vaithiyalingan and Nadarajan, 2010), the classification by Chaudhary *et. al.,* (1981) has been followed in which, plants with above 60% fertile pollen were grouped as fully fertile. So also, Hasan *et al.,* (2015) followed this classification in their inheritance studies on fertility restoration in ID type CMS lines and Kumar *et.al.,* (2017) in test crosses involving iso-cytoplasmic restorer lines in rice
14. As early as 1966, Jennings noticed wide variation for fertility restoration in many crosses of *indica* with *japonica*. He attributed high fertility of 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 by Eusebio *et. al.,* (2002). Huang *et. al.,* (2008) observed various degrees of fertility restoration including complete restoration in $F_1$s, when $F_5$ lines of Reimei (*rufipogon*) A/IR5032-6B-13-1 were test crossed with Zhen Shan 97A.
15. In test-crosses of 204 drought tolerant *indica* breeding lines with IR 58025A, Singh *et. al.,* (2021) 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.,* (2019) 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.,* (2017) identified 18 restorers, 17 partial restorers and remaining as partial maintainers from 38 test crosses based on pollen and spikelet fertility. Out of 31 test-crosses evaluated using two CMS lines CHAO1 and IR80151A, Seesang *et al* (2014) identified six restorers based on pollen fertility data. 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 (Singh *et.al.,* 2016).

**16. Correspondence between phenotypic and genotypic data and marker efficiency**

17. In this study, the frequency of restorers is high (49.25%) followed by partial restorers (29.85%) and remaining were partial maintainers. No complete maintainers could be observed. From Table 2, 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 while 10 plants had both *Rf3* and *Rf4* 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 DRR 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 DRR RF3-10 for *Rf3* gene was reported as 84% by Kumar *et.al.,* (2017) and 92% by Kiani *et al.,* (2015). For *Rf4* gene, the efficiency by marker RM6100 was reported as 92% by Suresh *et.al.,* (2012), 75% by Revathi *et. al.,* (2013), 97.4% by Kiani *et.al*., (2015) and 80% by Rajendra Prasad *et.al.,* (2017).
18. 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 58.4 to 78.7% and for six plants with both the genes was from 59.0 to 76.0%. Singh *et. al.,* (2016) observed that plants with pollen fertility ranging from 67 to 79% had spikelet fertility ranging from 80-90%. Ponnuswamy *et. al.,* (2020) 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%.
19. Using the same molecular markers, Shidenur *et.al.,* (2020) 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. 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 (Singh *et al.,* 2016), only seventeen genotypes were confirmed as effective restorers based on pollen and spikelet fertility data.

20. In partial maintainers, all were unique families and one plant 83-1-6 with both the genes expressed only 14.0% fertility. Singh *et. al.,* (2016) also observed that out of three parents with both the fertility restorer genes, only one parent exhibited complete restoration. Li *et.al.,* (1986) 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.,* (2016) observed six genotypes identified as restorers based on pollen and spikelet fertility percentage did not have *Rf4* and *Rf3* genes and thus modifiers play a role in fertility restoration as also emphasized by Bharaj *et.al.,* (1991).
21. 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, 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 in fertility restoration.
22. 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.,* (2010) 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 the materials and methods used. Shidenur*, et. al.,* (2020) 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 (Govindaraj and Virmani, 1988). 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.

**Table.1. 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 | |
|---|---|---|---|---|---|---|---|
| | | DRRF RF3-10 (*Rf3*) | RM 6100 (*Rf4*) | | | DRRF 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 |

Table 1 *contd…*

| Lane No. | CB174R/ Azucena $F_4$ families | Marker scoring | | Lane No. | CB174R/ Azucena $F_4$ families | Marker scoring | |
|---|---|---|---|---|---|---|---|
| | | DRRF RF3-10 (*Rf3*) | RM 6100 (*Rf4*) | | | DRRF RF3-10 (*Rf3*) | RM 6100 (*Rf4*) |
| 77 | 314-3-6 | 1 | 0 | 114 | 122-4-5 | 0 | 0 |
| 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 2. 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 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Rf 3* | *Rf4* | | | | | *Rf 3* | *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

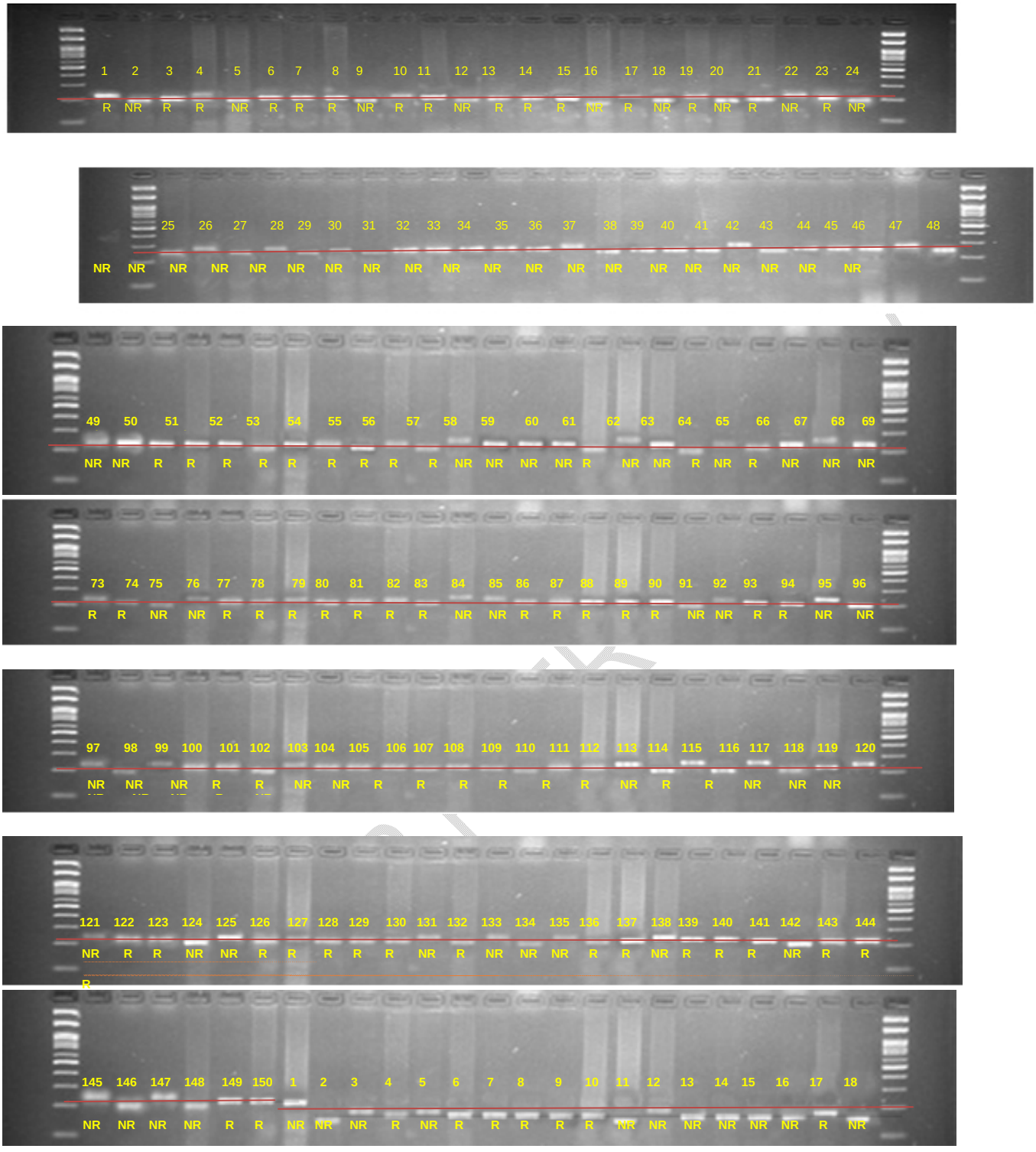


**Plate 1a. DRRF RF3-10 molecular marker amplification profile of 150 $F_4$ families (1-150) of CB174R/Azucena at 210bp and RM6100 marker amplification profile of 18 $F_4$ families of the same cross**

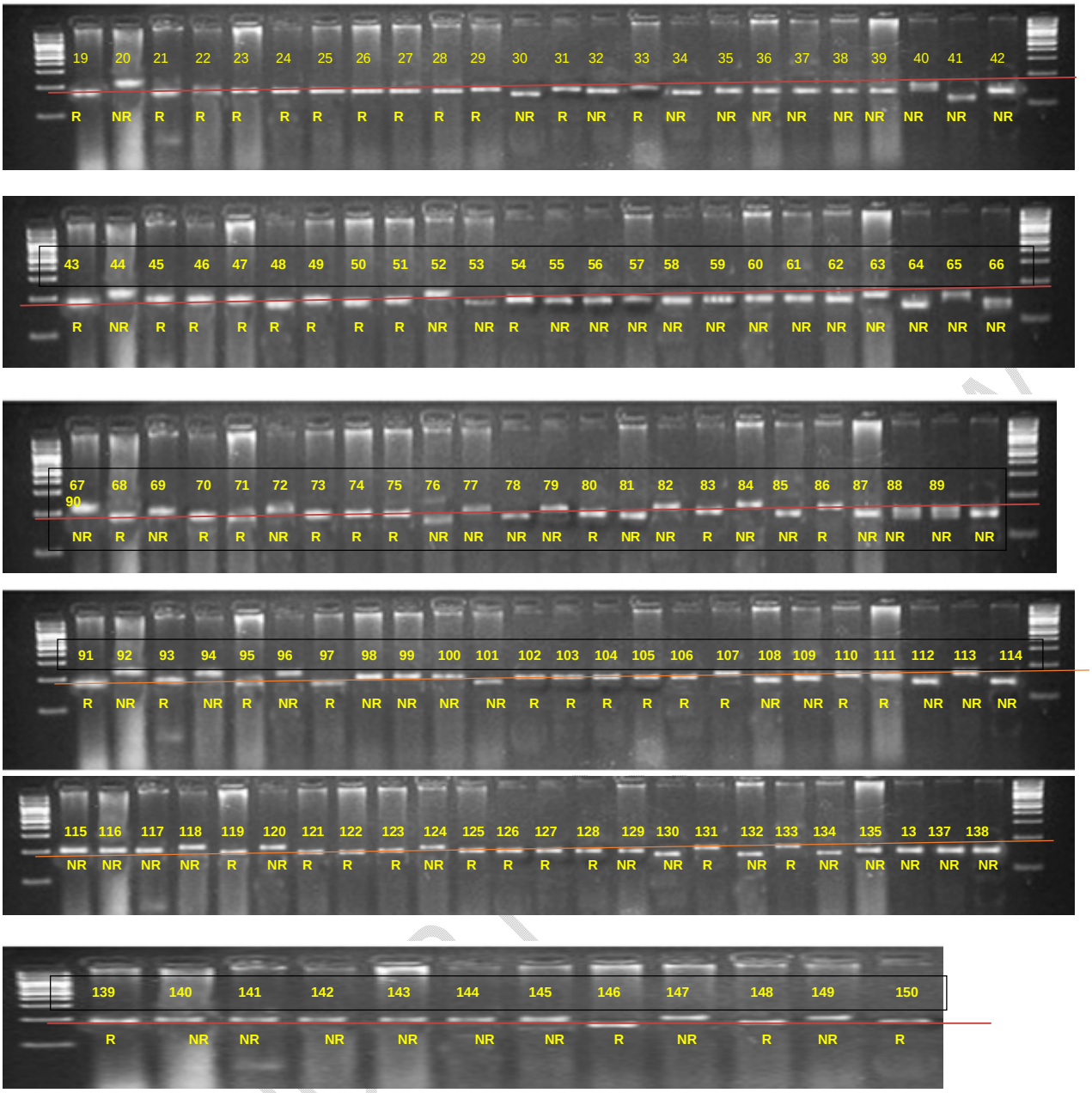


Plate 1b. RM6100 molecular marker amplification profile of 132 $F_4$ families (19-150) of CB174R/Azucena at 185bp

## 4. CONCLUSION

1. Based on the phenotypic and genotypic data, all the 33 plants that behaved as restorers and 13 plants with above 70.0% pollen fertility that showed amplification for *rf3* or *rf4* or both and based on molecular screening, 34 plants from 26 families (other plant in the family is included in 33 or 13 plants) which were suggested earlier for isolating restorers can be advanced to $f_5$ generation. thus, the present study using test cross performance and molecular screening has suggested to advance 80 $f_4$ plants out of 150 evaluated (53.3%), to next generation so as to develop inter sub-specific restorers for three-line hybrid rice.

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