

**A short term scientific mission (STSM) within the COST863 (Euroberry Research:
From Genomics to sustainable production, quality and health).**

STSM Scientific Report 2008.

Title: Construction of Pools and Superpools for PCR analysis of a *Fragaria vesca* BAC library

Reference code: COST-STSM-863-03959

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Objective

The visit to IRTA Cabrils, Spain was undertaken to create a genetic resource for the analysis of a diploid *Fragaria* high molecular weight BAC library constructed at Clemson University, USA. The objective of the STSM was to create DNA pools of the *Fragaria* BAC library to allow the identification of individual BAC clones containing specific DNA markers using PCR. The 18,432 clones from the library were condensed into 48 plate pools and 80 row and column pools, to allow the identification of a single BAC clone from the library using PCR, without needing to employ radioactive labelling and DNA hybridisation techniques. The DNA pools and superpools that were successfully created and verified will permit the rapid analysis and identification of BAC clones from the library, saving time and labour costs over the techniques that were available before the STSM.

Materials and methods

Creation of BAC pools

Pools were created from a BAC library of the *Fragaria vesca* cultivar 'Ali Baba' (J. Davik *pers comm*) which contains 18,432 clones in 48 384-well plates. The 18,432 clones were condensed into 48 96-well plates (4,608 cultures) with each culture containing four adjacent clones from the original 384-well plates. The condensed cultures were initiated in 180 µl LB containing 12.5 µg ml Chloramphenicol. Plates

were incubated at 37°C in a shaker incubator rotating at 50 rpm for a minimum of 16 hours. The BACs were then pooled in three separate orientations from the initial 4,608 cultures. Firstly, all 96 cultures from each plate were pooled to create 48 plate pools. Then each column from sets of 12 plates (96 cultures in total) were pooled to create 48 column pools, and finally each row from sets of 12 plates (144 cultures in total) were pooled to create 32 row pools, creating 128 BAC pools in total. A total of 20 µl from each culture was mixed in each pool and the resulting pooled culture was mixed thoroughly using a 1000 µl pipette. Half of each pooled culture was then used to initiate two separate 5 ml aliquots of LB containing 12.5 µg/ml chloramphenicol, and these culture pools were grown for a minimum of 16 hours at 37°C, rotating at 250 rpm.

Once grown, 850 µl of the cultures was removed and 150 µl of glycerol was added to each aliquot. These were mixed thoroughly and were stored at -80°C. The remaining culture was centrifuged for 30 minutes at 3500 rpm to pellet the bacteria. The supernatant was then discarded and the BAC DNA from two replicates of each culture (256 minipreps in total) was extracted following the miniprep procedure described below.

Mini-prep BAC DNA extraction

The bacterial pellets and the small amount of LB left in each tube after the supernatant had been discarded were vortexed and transferred to 1.5 ml tubes. Then 200 µl of Solution I containing 50 mM glucose, 10 mM EDTA, 25 mM Tris (pH 8.0) and 5 mg/ml lysozyme were added to each tube. The tubes were then vortexed and incubated on ice for 5 minutes. Four hundred microlitres of Solution II containing 0.2 N NaOH and 1% SDS were added to each tube, the tubes were vortexed and returned to ice for a further 5 minutes. Finally, 300 µl of Solution III containing 3.0 M KAc (pH 4.8 – 5.5) was added to each of the tubes, which were then vortexed and transferred to -80°C for 15 minutes. The tubes were thawed at room temperature and centrifuged for 15 minutes at 12,000 g to pellet the precipitate, and then 750 µl of the supernatant was transferred to a fresh tube. The BAC DNA was precipitated by adding 450 µl of cold isopropanol and the tubes were centrifuged for 5 minutes at 14,000 g to pellet the DNA. The supernatant was removed from the tubes containing the DNA pellets by decanting and 500 µl of cold 70% ethanol was added to wash the pellets. The tubes were then centrifuged for 2 minutes at 14,000 g. The ethanol was removed by decanting and any remaining ethanol residue was removed by pipetting. The pellets were air dried and

40 µl of HPLC grade water was added to resuspend the pellets. A random selection of 36 BAC DNA minipreps were quantified from each replicate using a nanodrop spectrophotometer.

Plate, row and column pool testing

The plate, row and column pools were diluted 1:100 (~50 ng/µl DNA) for use in PCR. A set of 6 PCR-based markers were selected from six of the seven *Fragaria* linkage groups and PCR was performed using the following protocol. Reactions were performed in a final volume of 15 µl containing ~100 ng DNA, 1× PCR buffer, 3.0 mM MgCl₂, 0.2 mM each dNTP, 0.2 uM each primer and 0.5 U *Taq* polymerase. The following PCR conditions were used for all PCR: An initial denaturation step of 94°C for 5 minutes followed by 10 cycles of 94°C (30 sec), 55-50°C annealing temperature decreasing by 0.5°C per cycle (30 sec) and 72°C (30 sec) then by 25 cycles of 94°C (30 sec), 50°C (30 sec) and 72°C (30 sec), and a final elongation step of 72°C for 7 minutes. PCR products were electrophoresed on a 1.2% NEB agarose gel for 2 hours at 120 V and visualised over UV light after staining with ethidium bromide.

Positive BAC clone verification

Pools of four individual BACs giving positive amplification for a PCR marker were identified from plate, row and column pool PCRs. To verify the positive results and to identify which of the four BACs in each positive pool contained the marker locus, each of the four individual BACs was picked from the original 384-well plates and grown in 180 µl LB containing 12.5 µg/ml Chloramphenicol as described above. The colonies were grown for 20 hours after which 2 µl of culture was used for PCR using the primer pairs for the marker which was amplified in the plate, row and column pools, using the PCR conditions and parameters described above. The PCR products were then visualised by electrophoresis as described above.

Results

BAC pool DNA

The 128 plate, row and column pool cultures all grew successfully and BAC DNA was extracted from each. Quantification of the BAC DNA by nanodrop spectrophotometry showed a consistent yield in the region of 5 µg/µl for each of the pools and so the minipreps were diluted 1:100 for use in PCR. In total, DNA from eight

superpools, each containing six plate pools, 48 individual plate pools, 48 column pools and 32 row pools, were diluted and used for PCR.

Plate, row and column pool testing

Six PCR-based markers, CEL-1, EMFv021, EMFv164, EMFv183, EMFvi108 and UDF017, which were located to six of the seven linkage groups of the *Fragaria* reference map, were selected to test the BAC DNA pools and superpools. Each marker was tested in all of the plate row and column pools, regardless of whether a positive amplification was expected, to test for the presence of false negative and positive amplifications. All markers amplified at least one positive plate pool and for all positive plates, at least one positive row and column pool was identified. More than one positive row and column pool was identified in some cases when two or more corresponding plate pools gave a positive amplification, but in all cases, the same number of row and column pools gave positive amplifications, indicating different positive BACs had been identified in each case. Figure 1 shows the pattern of amplification for the plate, row and column pools that was observed for marker EMFv021. The number of positive row and column pools and the corresponding possible positive BACs are listed in Table 1.

Positive BAC clone verification

For each of the six markers tested, at least one set of row and column pools gave just a single positive plate amplification. These positive markers were selected for BAC clone verification, as each required the testing of just four individual BAC clones from the original library. Four BAC clones from one 384-well plate were tested for each of markers CEL-1, EMFv164, EMFv183 and UDF017, four clones from two plates were tested for marker EMFv021 and four clones from three plates were tested for marker EMFvi108. In all cases, a single positive clone (listed in Table 1) was identified from the four clones tested which contained the corresponding PCR marker.

Conclusions

The objectives of the STSM, namely the creation of pools and superpools from a diploid *Fragaria* BAC library was successfully completed during the four weeks spent at IRTA Cabrils. A set of 48 plate pools, 48 column and 32 row pools were created from 18,432 initial BAC clones and DNA was extracted from each of the 128 cultures.

The DNA was diluted for use in PCR and tested with six DNA markers previously mapped to six of the seven diploid *Fragaria* linkage groups. A set of positive BAC colonies was identified from the PCRs of the plate, row and column pools and these BACs were picked from the original library colonies and grown overnight to provide culture for use in PCR. Colony PCR was performed from the cultures using primer pairs for the six markers and in all cases, colonies positive for the markers were identified.

The results obtained show that plate, row and column pools were successfully created. This resource will be useful for further analysis of the *Fragaria* BAC library and a joint collaborative project has been planned between East Malling and IRTA Cabrils to further characterise and identify a set of BAC clones containing 70 molecular markers from the diploid *Fragaria* reference linkage map. The anchoring of these BAC clones to the *Fragaria* genetic map will provide the basis for the creation of a diploid *Fragaria* physical map.

Figures and Tables

Figure 1. A. The PCR products produced from 48 plate pools for marker EMFv021 showing six positive plates containing the marker. B. The PCR products produced from 48 column (tracks 1 and 2) and 32 row (tracks 3 and 4) pools for marker EMFv021, showing six positive column and six positive row pools containing the marker.

Table 1. The six PCR markers used to analyse the plate, row and column pools, the positive plates, rows and columns identified and the positive BAC clones identified for each marker

Figure 1A

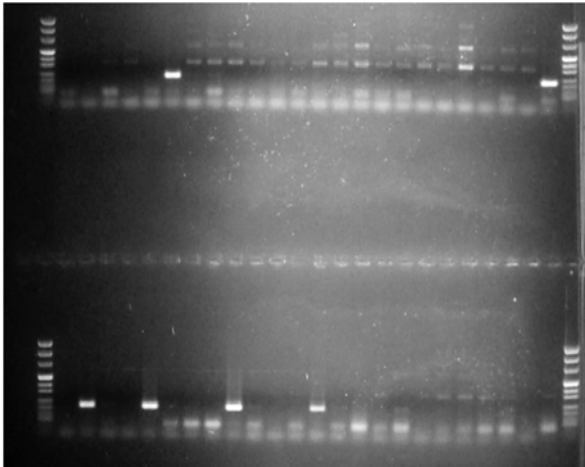


Figure 1B

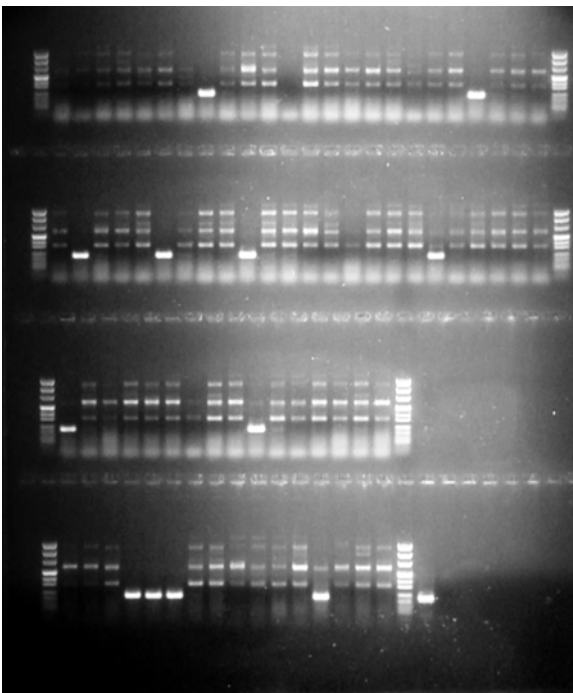


Table 1

Marker name	Plate number	Positive plates	Positive columns	Positive rows	Positive BACs identified
CEL-1	Plates 01-12	P4, P8	C4	R2, R4	
	Plates 13-24	P23	C8	R8	P3 H3
	Plates 25-36	P30, P34	C2	R4, R7	
	Plates 37-48	P41, P42, P44	C1, C7, C11	R2, R5, R6	
EMFv021	Plates 01-12	P6	C8	R1	P6 A15
	Plates 13-24	P24	C9	R2	P24 C18
	Plates 25-36	P26, P29, P33	C2, C6, C10	R4, R5, R6	
	Plates 37-48	P37	C7	R5	
EMFv164	Plates 01-12	P1, P8	C1	R2, R3	
	Plates 13-24	P14	C10	R6	P14 K20
	Plates 25-36				
	Plates 37-48				
EMFv183	Plates 01-12	P7	C11	R8	P7 P21
	Plates 13-24				
	Plates 25-36				
	Plates 37-48				
EMFvi108	Plates 01-12	P3	C2	R4	P3 H3
	Plates 13-24				
	Plates 25-36	P30	C1	R4	P30 G1
	Plates 37-48	P41	C2	R3	P41 E14
UDF017	Plates 01-12				
	Plates 13-24	P19	C5, C8	R1, R2	P19 B9
	Plates 25-36				
	Plates 37-48				