APPENDIX S1. The SSR-patchwork protocol for SSR libraries.

LEGEND

ATTENTION

* *HINT*

REST

I. DNA extraction and quantification.

* *Before beginning the preparation, set the water bath to 60°C.*

1) Grind 100 mg of fresh leaf tissue to a fine powder in liquid nitrogen with a mortar and pestle. For algal culture, go directly to the next step.

2) Transfer the resulting powder to a new 2-mL tube and add 900 µL of 2× CTAB extraction buffer.

3) Mix vigorously and then incubate at 60°C for 30 min in a water bath with gentle agitation.

4) Add an equal volume of chloroform–isoamyl alcohol (24:1) and mix vigorously.

5) Centrifuge the sample at 7000 g for 5 min.

6) Recover the top aqueous layer and transfer into a new tube.

7) Repeat steps 4, 5, and 6.

If the aqueous phase is not clear, then repeat step 7.

8) Add 70% of cold isopropanol and mix gently by inverting the tube.

9) Place the sample at –20°C for 10 min.

10) Centrifuge the sample at 10,000 g at 4°C for 8 min and discard the supernatant.

11) Rinse with 70% EtOH and centrifuge at 10,000 g at 4°C for 5 min.

12) Discard the supernatant.

13) Dry the pellet and resuspend in 50 µL of sterile water.

14) Add 1 µL of (1 mg/mL) RNase A and mix the sample.

15) Incubate the sample for 1 h at 37°C.

16) Precipitate the DNA with 0.7 volumes of isopropanol and 0.1 volume of 3 M NaOAc, pH 5.2.

17) Place the sample at –20°C for 10 min.

18) Centrifuge the sample at 10,000 g at 4°C for 10 min to pellet the DNA.

19) Discard the supernatant.

20) Rinse with 70% EtOH and centrifuge at 10,000 g at 4°C for 5 min.

21) Discard the supernatant.

22) Dry the pellet and resuspend in 50 µL of sterile water.

23) Run 2 µL of DNA with Marker II (AppliChem) on a 0.8% agarose gel containing 0.5 µg/mL of ethidium bromide.

24) Run at 60 V for approximately 4 h.

25) Visualize the gel using an UV-transilluminator and estimate the concentration of DNA.

** Extraction should yield a high-molecular-weight band of DNA.*

II. Restriction enzyme digestion.

1) To digest genomic DNA with restriction enzymes, mix the following components in a 1.5-mL tube:

Component	**Quantity**
2 µg genomic DNA	x µL
10× buffer ReAct3 (Invitrogen)	6 µL
*Eco*RI (10 U/µL) (Invitrogen)	0.5 µL
*Mse*I (5 U/µL) (Invitrogen)	1 µL
Sterile water	x µL
Total volume	60 µL

Always add the restriction enzymes last, removing them from –20°C only when needed and immediately return the enzyme to –20°C after use.

2) Incubate the sample at 37°C for 2 h.

3) Inactivate the enzymes by heating the sample to 75°C for 15 min.

4) Precipitate the DNA with 2 volumes of 100% EtOH and 0.1 volume of 3 M NaOAc, pH 5.2.

5) Place the sample at –20°C for 10 min.

6) Centrifuge the sample at 10,000 g at 4°C for 10 min to pellet the DNA.

7) Discard the supernatant.

8) Rinse with 70% EtOH and centrifuge at 10,000 g at 4°C for 5 min.

9) Discard the supernatant.

10) Repeat steps 8 and 9.

11) Dry the pellet and resuspend in 15 µL of sterile water.

III. Size selection, gel extraction, and purification.

1) Run the precipitated digestion with a 100-bp DNA ladder (Promega) on a 1% agarose gel containing 0.5 µg/mL of ethidium bromide.

2) Run at 50 V for approximately 4 h.

Run until the markers are well separated.

3) Visualize the gel using an UV-transilluminator and cut a slice containing DNA fragments of 250–500 bp in size from the gel with a razor blade (Fig. 1).

Be sure to use a sterile scalpel and trim away as much agarose as possible.

4) Place the gel slice into a 2-mL tube.

5) Add 500 µL of 20 mM Tris-HCl (pH 8.0) with 1 mM EDTA (pH 8.0) to the slice of agarose.

6) Close the tube and mix by vortexing vigorously. Incubate for 5 min at 65°C.

7) During the incubation period, a special filtration column to elute the contents of the band must be prepared, according to the instructions at the end of protocol (see Filtration column).

8) After the incubation, transfer the sample to the filtration column and centrifuge at 10,000 g for 5 min.

9) Remove the pierced tube and add an equal volume of phenol to the eluate.

10) Vortex the mixture for 20 s and centrifuge at 7000 g for 10 min.

11) Recover the aqueous phase and transfer it into a new 1.5-mL tube.

12) Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the sample.

13) Vortex the mixture for 20 s and centrifuge at 7000 g for 10 min.

14) Recover the aqueous phase and transfer it into a new 1.5-mL tube.

15) Add an equal volume of chloroform:isoamyl alcohol (24:1).

16) Vortex the mixture for 20 s and centrifuge at 7000 g for 10 min.

17) Transfer the aqueous phase into a new 1.5-mL tube.

18) Add 2 volumes of 100% EtOH and 0.1 volume of 3 M NaOAc, pH 5.2.

Store at –20°C overnight. Alternatively, you can place the sample at –80°C for 15 min.

19) Centrifuge the sample at 10,000 g at 4°C for 10 min to pellet the DNA.

20) Discard the supernatant.

21) Rinse with 70% EtOH and centrifuge at 10,000 g for 5 min.

22) Discard the supernatant.

23) Repeat steps 21 and 22.

24) Dry the pellet and resuspend in 15 µL of sterile water.

IV. Adapter preparation and ligation.

Adap_*Eco*A: 5′-CTC gTA gAC TgC gTA CC-3′

Adap_*Eco*B: 5′-AAT Tgg TAC gCA gTC TAC-3′

Adap_*Mse*A: 5′-gAC gAT gAg TCC TgA g-3′

Adap_*Mse*B: 5′-TAC TCA ggA CTC AT-3′

1) For the preparation of the *Eco*RI-adapter (5 µM), in a 0.2-mL PCR tube, add:

Component	**Quantity**
Adap_*Eco*A, 100 µM	2 µL
Adap_*Eco*B, 100 µM	2 µL
Sterile water	36 µL
Total volume	40 µL

2) For the preparation of the *Mse*I-adapter (50 μM), in a 0.2-mL PCR tube, add:

Component	**Quantity**
Adap_*Mse*A, 100 μM	20 μL
Adap_*Mse*B, 100 μM	20 μL
Total volume	40 μL

3) Incubate the two PCR tubes in a thermocycler using the following protocol:

PCR PROGRAM = ADAPTERS		
Step	**Temperature**	**Time**
Hold 1	94°C	3 min
Hold 2	70°C	5 s
Hold 3	60°C	5 s
Hold 4	50°C	5 s
Hold 5	40°C	5 s
Hold 6	25°C	5 s
Hold 7	14°C	1 min
Hold 8	4°C	1 min

4) To perform the ligation of the adapters to restricted DNA, in a 0.2-mL PCR tube, add:

Component	**Quantity**
Digested DNA	7.5 μL
5× DNA ligase buffer (Invitrogen)	5 μL
5 μM *Eco*RI-adapter	3 μL
50 μM *Mse*I-adapter	3 μL
T4 ligase (5 U/μL) (Invitrogen)	0.4 μL
Sterile water	6.1 μL
Total volume	25 μL

⇨ *Always add T4 ligase last, removing it from –20°C only when needed and immediately return the enzyme to –20°C after use.*

5) Incubate the reaction at 24°C for 2 h.

V. First enrichment.

1) To increase the amount of DNA ligated with the adapters, perform a PCR reaction.

Pre_*Eco*-0: 5′-gAC TgC gTA CCA ATT C-3′

Pre_*Mse*-0: 5′-gAT gAg TCC TgA gTA A-3′

In a 0.2-mL tube add:

Component	**Quantity**
Restricted-ligated DNA	2.5–5 µL
10× *Taq* DNA polymerase buffer (DreamTaq, Fermentas)	2.5 µL
2.5 mM dNTPs (Promega)	2 µL
Primer Pre_*Eco*-0, 50 µM	0.125 µL
Primer Pre_*Mse*-0, 50 µM	0.125 µL
5 U/µL *Taq* DNA polymerase (DreamTaq, Fermentas)	0.25 µL
Sterile water	x µL
Total volume	25 µL

* *The 10× DreamTaq buffer contains 20 mM $MgCl_2$.*

Vortex to mix the solution and spin down.

2) Incubate in a thermocycler using the following protocol:

PCR PROGRAM = PRE1_ARR			
Step	**Temperature**	**Time**	**No. of cycles**
Extension	72°C	2 min	1
Denaturation	95°C	30 s	25
Annealing	50°C	1 min	

Extension	72°C	1 min	
Final extension	72°C	2 min	1

3) Run 4 µL of PCR product with 1 µL of a 100-bp ladder on a 1% agarose gel containing 0.5 µg/mL of ethidium bromide (Fig. 2).

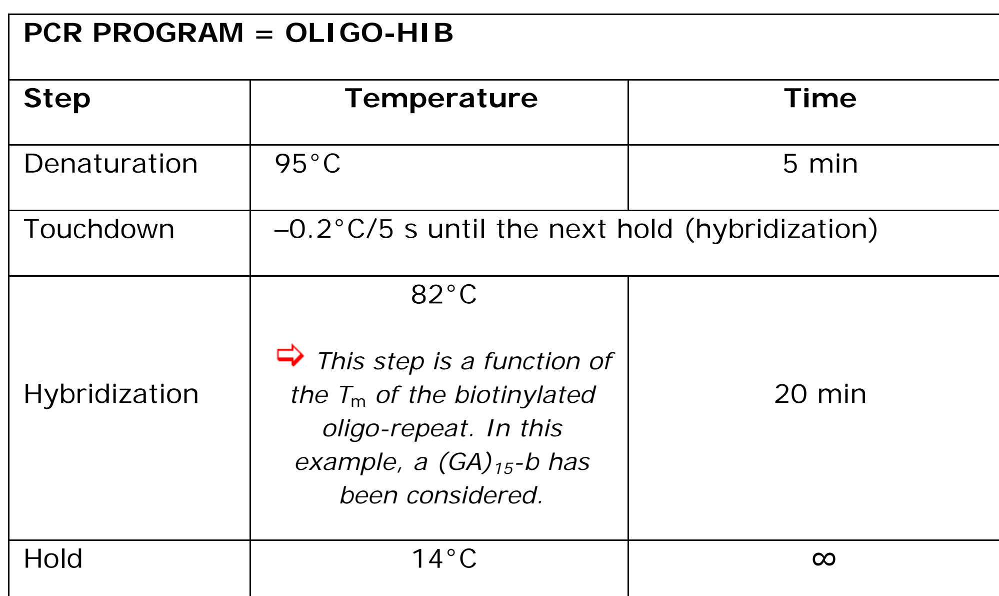
The smear of fragments should be visible and centered at approximately 500 bp.

VI. Preparation of the biotinylated oligo-repeat and hybridization.

1) In a 0.2-mL PCR tube, add:

Component	**Quantity**
250 ng PCR product of the first enrichment	x µL
500 ng biotinylated oligo-repeat (1 µg/µL) (e.g., GA_{15}-b)	0.5 µL
2× hybridization buffer	25 µL
Sterile water	x µL
Total volume	50 µL

2) Incubate the reaction in a thermocycler using the following protocol:



PCR PROGRAM = OLIGO-HIB		
Step	**Temperature**	**Time**
Denaturation	95°C	5 min
Touchdown	–0.2°C/5 s until the next hold (hybridization)	
Hybridization	82°C *This step is a function of the T_m of the biotinylated oligo-repeat. In this example, a $(GA)_{15}$-b has been considered.*	20 min
Hold	14°C	∞

VII. Preparation and VETREX Avidin D capture.

1) Place 40 μL of VETREX Avidin D into a 1.5-mL tube.

2) Centrifuge the sample at 12,000 g for 30 s and discard the supernatant.

3) Equilibrate the VETREX Avidin D by washing twice with 2 volumes of 1× TBS (500 μL for 1 min), centrifuge and discard the supernatant following each wash.

4) In a new 1.5-mL tube, mix the following:

Component	**Quantity**
2× TBS	500 μL
Biotinylated hybridized sample from the VI.2 procedure	50 μL
Sterile water	450 μL
Total volume	1000 μL

5) Add this solution to the VETREX Avidin D pellet, gently resuspend, and allow the reaction to bind for 1 h at room temperature with occasional mixing.

6) Centrifuge the binding reaction for 1 min at 12,000 g.

7) Carefully remove the supernatant without disturbing the VETREX Avidin D pellet.

** Retain the supernatant until binding of the biotinylated sample has been verified in step 8.*

8) Wash the VETREX Avidin D matrix with 1 mL of 1× TBS to remove any unbound molecules.

9) Pellet the matrix by centrifugation for 1 min at 12,000 g and discard the supernatant.

10) Repeat steps 8 and 9 two times.

11) Wash the VETREX Avidin D pellet with 500 μL of 1× TBS at 50°C.

12) Pellet the matrix by centrifugation for 1 min at 12,000 g and discard the supernatant.

13) Wash the VETREX Avidin D pellet with 500 μL of 1× TBS at 65°C.

14) Pellet the matrix by centrifugation for 1 min at 12,000 g and discard the supernatant.

15) Wash the VETREX Avidin D pellet with 500 μL of 0.1× TBS at 65°C.

16) Pellet the matrix by centrifugation for 1 min at 12,000 g and discard the supernatant.

17) Resuspend the VETREX Avidin D pellet in 250 µL of sterile water at 65°C for 30 min.

18) Centrifuge the sample for 1 min at 12,000 g and transfer the aqueous phase into a new 1.5-mL tube.

19) Add 2 volumes of 100% EtOH and 0.1 volume of 3 M NaOAc, pH 5.2. Place at –20°C for 20 min.

20) Centrifuge the sample at 11,000 g at 4°C for 8 min to precipitate the DNA.

21) Discard the supernatant.

22) Rinse the sample with 70% EtOH and centrifuge at 10,000 g for 5 min.

23) Discard the supernatant.

24) Repeat steps 22 and 23.

25) Dry the pellet and resuspend in 25 µL of sterile water.

Store the sample at 4°C overnight.

26) To remove any residual VETREX Avidin D matrix, vortex and centrifuge at 12,000 g for 2 min.

27) Transfer the aqueous phase into a new 1.5-mL tube.

VIII. Second enrichment and cloning.

1) To increase the selected DNA fragments, perform triplicate PCR reactions. For each PCR reaction, add the following:

Component	**Quantity**
Enriched-recovered DNA post Avidin D	5 µL
10× *Taq* DNA polymerase buffer (DreamTaq, Fermentas)	2.5 µL
2.5 mM dNTPs (Promega)	2 µL
Primer Pre_*Eco*-0, 50 µM	0.125 µL
Primer Pre_*Mse*-0, 50 µM	0.125 µL
5 U/µL *Taq* DNA polymerase (DreamTaq, Fermentas)	0.25 µL
Sterile water	15 µL

Total volume	25 µL

2) Incubate in a thermocycler using the following protocol:

PCR PROGRAM = PRE2_ARR			
Step	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95°C	30 s	1
Denaturation	95°C	30 s	30
Annealing	50°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	3 min	1

3) When the PCR reactions are completed, join all three PCR reactions in a unique tube.

4) Purify the PCR product with DNA Enzyme-free Isolation Spin Kit (AppliChem), according to the standard protocol recommended by the manufacturer, and resuspend in 30 µL of sterile water.

5) To estimate the concentration of purified product, run 2 µL on 1% agarose gel with Marker II (AppliChem).

6) For cloning, follow the protocol supplied using pMosBlue blunt-ended cloning kit (GE Healthcare) or any other comparable cloning kits on the market (e.g., CloneJET PCR Cloning Kit, Fermentas–Thermo Fisher Scientific).

7) To calculate the appropriate amount of PCR product (insert) to include in the ligation-cloning reaction, calculate the average of an insert between 250 and 500 bp (i.e., approximately 375 bp).

IX. Colony screening and sequencing.

1) The presence of an appropriate insert can directly be determined using colony PCR.

In this step, we were able to use a homemade *Taq* polymerase (NAP-*Taq*; De Castro, unpublished data).

2) Pick an individual colony from the plate containing the transformants using a sterile tip, place into a 0.2-mL PCR tube and resuspend in 20 µL of the PCR master mix. Pipet up and down to disperse the pellet.

3) Prepare a PCR master mix for the number of colonies analyzed plus one extra sample. For each 20-μL reaction, mix the following reagents:

Component	**Quantity**
10× *Taq* DNA polymerase buffer	2 μL
2.5 mM dNTPs (Promega)	1.8 μL
Plasmid forward primer, 50 μM	0.1 μL
Plasmid reverse primer, 50 μM	0.1 μL
5 U/μl *Taq* DNA polymerase (homemade NAP-*Taq*) ⇨ *Taq made in the authors' laboratory (De Castro, unpublished data)*	0.25 μL
Sterile water	15.75 μL
Total volume	20 μL

4) Incubate the reaction in a thermocycler using the following protocol:

PCR PROGRAM = Colony screening			
Step	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95°C	3 min	1
Denaturation	94°C	30 s	30
Annealing	T_m primers	30 s	
Extension	72°C	1 min	
Final extension	72°C	2 min	1

5) On a 1% agarose gel containing 0.5 μg/mL of ethidium bromide, run 2 μL of PCR product with a 100-bp ladder (Promega) and Marker II (AppliChem).

6) Run the sample at 80 V for approximately 30–40 min.

7) Visualize the gel using an UV-transilluminator and examine the PCR results.

8) Quantify the PCR product concentration and size for subsequent sequencing. Select the fragments larger than 350 bp.

* *Discard the fragments smaller than 350 bp because if a non-recombinant colony is picked, a sequence of 139 bp would be amplified.*

9) Proceed to sequencing, without purification of the PCR products. For each 10-μL sequencing reaction, mix the following reagents:

Component	**Quantity**
5× Sequencing Buffer, BigDye Terminator v.1.1, v.3.1 (Applied Biosystems)	1.75 μL
Plasmid forward primer, 6.4 μM (Macrogen)	0.5 μL
BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems)	0.3–0.4 μL
3–5 ng of positive PCR product	≤0.25 μL
Sterile water	x μL
Total volume	10 μL

Vortex the sample and centrifuge briefly.

10) Incubate the reaction in a thermocycler using the following protocol:

PCR PROGRAM = Sequencing			
Step	**Temperature**	**Time**	**No. of cycles**
Denaturation	96°C	10 s	25
Annealing	50°C	5 s	
Extension	60°C	4 min	

11) Perform a precipitation of the sequencing reactions by adding 2 volumes of 100% EtOH and 0.1 volume of 3 M NaOAc, pH 5.2. Place the reaction at –20°C for 10 min.

12) Centrifuge the sample at 11,000 g at 4°C for 8 min.

13) Discard the supernatant.

14) Rinse the sample with 70% EtOH and centrifuge at 11,000 g for 5 min.

15) Discard the supernatant.

16) Repeat steps 13 and 14.

17) Dry the pellet and resuspend in 13 μL of Hi-Di Formamide.

18) Denature the sample at 95°C for 3 min.

19) Load the samples into a 3130 Genetic Analyzer (Applied Biosystems, Life Technologies) or similar old-generation automated sequencer.

20) ... and good luck screening!

21) During the screening, discard the sequences without repeating. Perform the sequencing reaction again with the reverse primers on the positive sequences (with repeat).

22) Following DNA sequencing, the poly-linker vector and adapter sequences should be removed. Both strands are contiged and edited to ensure the accuracy of the sequencing. The sequences are now available to design primers.

23) Following editing, PCR primers are designed from the flanking DNA sequences using handmade methodology or with the free software available on the web (e.g., NCBI/ Primer-BLAST = http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

RECIPES

2× CTAB Extraction Buffer

CTAB	2% (w/v)
EDTA	20 mM
NaCl	1.4 M
Tris–HCl	100 mM (pH 8.0)
β-mercaptoethanol	0.2% (v/v)

Room temperature

2× Hybridization Buffer

NaH_2PO4	1 M (pH 7.4)
SDS	1% (w/v)

Room temperature

2× TBS (binding buffer)

Tris	200 mM (pH 7.5)
NaCl	300 mM

Room temperature

CHEMICALS AND SUPPLIES

Chemical Check List:

Agarose: AppliChem, A2114,0050.

Chloroform:Isoamylalcohol (24:1): AppliChem, A1935,0100.

CTAB: AppliChem, A0805,0100.

dATP: Promega, U1205.

dCTP: Promega, U1225.

dGTP: Promega, U1215.

DNA ladder 100-bp: BenchTop, Promega, G8291.

dTTP: Promega, U1235.

EDTA: AppliChem, A3145,0500.

Ethidium bromide: AppliChem, A1152,0025.

EtOH: AppliChem, A3693,1000PE.

Hi-Di Formamide: Applied Biosystems–Life Technologies, 4311320.

Isopropanol (2-Propanol): AppliChem, A3465,1000.

Marker II: AppliChem, A5223,0005.

NaCl: AppliChem, A4661,1000.

NaH_2PO4: AppliChem, A4229,0250.

NaOAc: AppliChem, A3802,0500.

Oligo or primer: Macrogen.

Phenol: AppliChem, A1624,0100.

Phenol:Chloroform:Isoamyl alcohol (25:24:1): AppliChem, A0889,0100.

SDS: AppliChem, A2263,0100.

Tris-HCl: AppliChem, A3452,0250.

VETREX Avidin D: Vector Laboratories, A2020.

β-mercaptoethanol: AppliChem, A4338,0100.

Enzyme Check list:

*Eco*RI: Invitrogen–Life Technologies, 15202.

*Mse*I: Invitrogen–Life Technologies, 15494.

RNase A: AppliChem, A2760.

Taq DNA Polymerase: DreamTaq DNA Polymerase: Fermentas–Thermo Fisher Scientific, EP0703; homemade NAP-*Taq* (De Castro et al., unpublished data).

T4 ligase: Invitrogen–Life Technologies, 15224.

Kit Check list:

BigDye Terminator v.3.1. Cycle Sequencing Kit: Applied Biosystems–Life Technologies, 4337454.

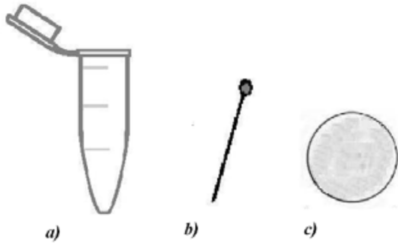
CloneJET PCR Cloning Kit: Fermentas–Thermo Fisher Scientific, K12341.

DNA Enzyme-free Isolation Spin Kit: AppliChem, A5266,0050.

pMOS*Blue* Blunt-ended Cloning Kit: GE Healthcare, RPN5110.

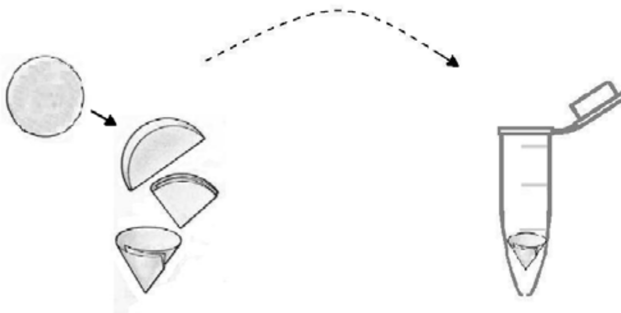
FILTRATION COLUMN

1. For construction of "special" filtration column, the following materials are necessary: (a) a 1.5-mL tube, (b) a sterile needle, and (c) a small piece of blotting paper with diameter of 1.5 cm.



2. Pierce the bottom of a 1.5-mL tube with a hot sterile needle. The hole must have a diameter of approximately 3 mm.

3. Using a small piece of blotting paper, create a small funnel and place it into pierced 1.5-mL tube.



4. Finally, insert the pierced tube into a new 1.5-mL tube.