Lab protocols for DNA extraction and genotyping of SSR microsatellites for *Prunus mahaleb* (Rosaceae) seeds and *Quercus* acorns

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A. PROTOCOL FOR DNA EXTRACTION FROM LEAF TISSUE

A.1. **Reagents needed per sample**

Liquid nitrogen

480 μl Cheung buffer

120 μl Sarcosyl 5%

225 μl amonic acetate 10M

500 μl isopropanol

1.2 ml etanol 70%

200 μl TE

A.2. **Reagent preparation**

Cheung buffer			
Reagents	Stock concentration	Final concentration	Vol. (100 ml)
Tris-Hcl pH 8	1M	200 mM	20 ml
NaCl	5M	2 M	40 ml
EDTA	0.5 M	70 mM	14 ml
Sodium metabisulfite		20 mM	0.38 g
miliQ H_2O			26 ml

TE			
Chemicals	Stock	Final cc	Vol. (100 ml)
Tris-Hcl pH 8	1M	10 mM	1 ml
EDTA	0.5 M	1 mM	0.2 ml
H_2O miliQ			98.8 ml

Sarcosyl 5%	
Reagents	Vol. (50 ml)
N-Laurilsarcosina	2.5 g
H_2O miliQ	50 ml

A.3. **DNA extraction**

1. Take a sample equivalent to 150-300 mg of fresh weight (optimal yield for 200-250 mg) of the leaf tissue previously stored at –80ºC into a Sarstedt 72693 tube (2 ml, screw top) and add two steel beads (5 mm diameter).
2. Fill a container with liquid nitrogen and immerse the two blocks of the electric drill and up to 10 tubes containing the leaf tissue with the steel beads. You can work with a smaller number of tubes, but use always an even number of tubes. Be cautious: liquid nitrogen might damage you by contact, so it is recommended to use a face mask and gloves.
3. Take out the blocks and tubes from the liquid nitrogen with a pair of forceps. Introduce each tube into a hole in the block. Shake previously the tube in order to ensure that the steel beads easily move inside the tube. Each block consists on 5 holes and the tubes should be distributed so as to balance the total weight of the block.
4. Cover the blocks containing the tubes with their plastic tops and set them into the electric mill (Retsch MM 200) for 2 minutes 30 seconds at 30 Hz.
5. Take out the tubes from the block and keep them in ice while the remaining samples are homogeneized. If the extraction is not going to be carried on that moment, keep the homogenized sampled in liquid nitrogen.
6. Add 480 μl of Cheung's buffer. Shake the tube up with an orbital shaker to obtain a homogeneous mixture.
7. Add 120 μl if 5% sarcosyl
8. Incubate 30 minutes at 65ºC.
9. Centrifuge 15 minutes at 13000 rpm at room temperature.
10. Transfer 500 μl of the supernatant to a new tube (1.5 ml eppendorf tube). The steel beads can now be cleaned with bleach to be used next time.

11. Add 225 μl of ammonium acetate 10M and 500 μl of isopropanol. Mix and incubate at room temperature for 5 minutes.
12. Centrifuge at 13000 rpm for 15 minutes at room temperature.
13. Remove the supernatant carefully avoiding pellet resuspension. Spin down for 1 minute at 13000 rpm and remove remaining supernatant.
14. Add 1.2 ml of 70% etanol. Shake the tubes smoothly several times. If the extraction cannot be finished, samples can be stored at -20º C.
15. Centrifuge at 13000 rpm for 5 minutes at 4ºC.
16. Remove completely the supernatant as in step 13.
17. Dry at room temperature for 2 minutes.
18. Resuspend with 200 μl TE and shake with the orbital shaker smoothly.
19. Incubate at 65ºC with a smooth shake up to obtain a homogenous-clear solution (pellet should be completely dissolved). If necessary, add more TE in order to obtain a complete dissolution of the pellet.
20. Centrifuge for 5 minutes at maximum speed at 4ºC. Transfer the supernatant to a new tube and store at –20ºC.

A.4. **References**

Cheung WY, Hubert N, Landry BS (1993) A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and PCR analyses. *PCR Methods Appl*, **3**: 69-70.

B. PROTOCOL FOR DNA EXTRACTION FROM ENDOCARP TISSUE

B.1. **Reagent needed per endocarp**

Liquid nitrogen

400 µl Cheung buffer

100 µl Sarcosyl 5%

180 µl amonic acetate 10M

400 µl isopropanol

1.0 ml etanol 70%

80 µl TLE

B.2. **Reagent preparation**

Cheung buffer (see pg. 4)

TLE			
Reagents	Stock concentration	Final concentration	Vol. (100 ml)
Tris-Hcl pH 8	1M	10 mM	1 ml
EDTA	0.5 M	0.1 mM	0.02 ml
H_2O miliQ			98.98 ml

Sarcosyl 5% (see pg. 4)

B.3. **DNA extraction**

1. Split the seed open with small pliers and separate carefully the woody endocarp from the testa tissue and embryo tissue (see figure 3 in the accompanying document on general methods for a cross section view of a drupaceous fruit of *Prunus mahaleb*). The testa tissue is a thin layer covering the embryo, just beneath the endocarp; it is light-brownish color, very thin, and fully covers the whole embryo, which is lighter, yellowish color. Wash the pliers with 10 % bleach before next use to avoid tissue cross-contamination.
2. Transfer the endocarp to a Sarstedt 72693 tube (2 ml, screw top). Add two steel beads (5 mm diameter) into each tube.

3. Fill a container with liquid nitrogen and immerse the two blocks of the electric mill and up to10 tubes containing the leave tissue with the steel beads. You can work with a smaller number of tubes, but use always an even number of tubes. Be cautious: liquid nitrogen might damage you by contact, so it is recommended to use a face mask and gloves.
4. Take out the blocks and tubes from the liquid nitrogen with a pair of forceps. Introduce each tube into a hole in the block. Shake previously the tube in order to ensure that the steel beads easily move inside the tube. Each block consists of 5 holes and the tubes should be evenly distributed so as to balance the total weight of the block.
5. Cover the blocks containing the tubes with their plastic tops and set them into the electric mill (Retsch MM 200) for 2 minutes 30 seconds at 30 Hz.
6. Take out the tubes from the block and keep them in ice while the remaining samples are homogenized. If the endocarps are not fully powdered, repeat step 5 as many times as necessary. (If the extraction is not going to be carried on that moment, keep the homogenized sampled in liquid nitrogen).
7. Add 400 μl of Cheung's buffer. Shake the tube up to with an orbital shaker to obtain a homogeneous mixture.
8. Add 100 μl 5% sarcosyl.
9. Incubate 30 minutes at 65ºC.
10. Centrifuge 15 minutes at 13000 rpm at room temperature.
11. Transfer 400 μl of the supernatant to a new tube (1.5 ml eppendorf tube). The steel beads can now be cleaned with bleach to be used next time.
12. Add 180 μl of 10M ammonium acetate and 400 μl of isopropanol. Mix and incubate at room temperature for 5 minutes.
13. Centrifuge at 13000 rpm for 15 minutes at room temperature.

14. Remove the supernatant carefully, avoiding pellet resuspension. Spin down for 1 minute at 13000 rpm and remove the remaining supernatant by using a micropipete (P200).
15. Add 1.2 ml of etanol 70%. Shake the tubes smoothly several times. If the extraction cannot be finished, samples can be stored at -20º C.
16. Centrifuge at 13000 rpm for 5 minutes at 4ºC.
17. Remove completely the supernatant as in step 14.
18. Dry at room temperature for 2 minutes.
19. Resuspend with 100 μl with TLE and shake with the orbital shaker smoothly.
20. Incubate at 65ºC with a smooth shake up to obtain a homogenous and clear solution (pellet should be completely dissolved). If necessary, add more TLE in order to obtain a complete dissolution of the pellet.
21. Centrifuge for 5 minutes at maximum speed, at 4ºC. Transfer the supernatant to a new tube and store at –20ºC or at –80ºC for long term storage.

B.4. **References**

Cheung WY, Hubert N, Landry BS (1993) A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and PCR analyses. *PCR Methods Appl*, **3**: 69-70.

C. PROTOCOL FOR DNA EXTRACTION FROM PERICARP TISSUE

C.1. **Reagent needed per sample**

Liquid nitrogen

1000 μl CTAB-LiCL

750 μl Cloroformo-Isoamílico 24:1 (C-I)

500 μl isopropanol (isolation) + 500 μl isopropanol (resuspension)

3 ml etanol 70% (2 ml for isolation) + (1 ml for resuspension)

500 μl Tris-Hcl pH8 10 mM

50 μl Acetate sódico 3M pH 6.8

75 μl TLE

C.2. **Reagent preparation**

CTAB-LiCL buffer			
Reagents	Stock cocentration	Final concentration	Vol. (100 ml)
Tris-HCl pH8	1 M	10 mM	1 ml
EDTA pH8	0.5 M	20 mM	4 ml
NaCl	5 M	0.7 M	14 ml
LiCl	5 M	0.4 M	8 ml
CTAB (Hexadecyltrimethyl ammonium bromide)		1% CTAB	1 gr
1% PVP-40 (Polyvinylpyrrolidone)	PVP-360	1% PVP-40	1 gr
Destill water			65 ml
2% B-Mercaptoetanol (add just before use)			20μl

TLE (see p. 7)

C.3. **DNA extraction**

1. Separate the pericarp tissue from the other tissues of the acorn. Introduce 100-110 mg of pericarp tissue into a Sarstedt 72693 tube (2 ml, screw top) and add two steel beads (5 mm diameter).
2. Fill a container with liquid nitrogen and immerse the two blocks of the electric mill and up to10 tubes containing the leave tissue with the steel beads. You can work with a smaller number of tubes, but use always an even number of tubes. Be cautious: liquid nitrogen might damage you by contact, so it is recommended to used a face mask and gloves.
3. Take out the blocks and tubes from the liquid nitrogen with a pair of forceps. Introduce each tube into a hole in the block. Shake previously the tube in order to ensure that the steel beads easily move inside the tube. Each block consists of 5 holes and the tubes should be distribuited so as to balance the total weight of the block.
4. Cover the blocks containing the tubes with their plastic tops and set them into the electric mill (Retsch MM 200) for 2 minutes 30 seconds at 30 Hz. Repeat if necessary.
5. Take out the tubes from the block and keep them in ice while the remaining samples are homogenized.
6. Add 1000 μl of CTAB buffer.
7. Incubate at 65º C for 90 min with frequent agitation.
8. Centrifuge 10 minutes at 13000 rpm at room temperature.
9. Transfer the supernatant (750 μl aprox.) to a new tube (2.0 ml eppendrof tube). The steel beads can now be cleaned with bleach 10% to be used next time.
10. Add 750 μl of Chloroform-Isoamyl 24:1 (C-I) and mix.
11. Centrifuge at 13000 rpm for 10 minutes at 4ºC.

12. Transfer 600 μl of the upper aqueous phase to a new tube (2 ml). Add 600 μl of C-I. Mix smoothly to obtain a homogenous mixture.
13. Centrifuge 10 minutes at 13000 rpm at 4ºC.
14. Transfer 500 μl of the supernatant to a new tube (2 ml) and add 500 μl of isopropanol at –20ºC. Incubate at –20ºC overnight.
15. Centrifuge 15 minutes at 13000 rpm at room temperature. Remove the supernatant.
16. Wash twice with 1 ml ethanol 70%.
17. Centriguge at 13000 for 10 minutes at room temperature. Remove the supernatant.
18. Dry at room temperature for 5 minutes.
19. Resuspend the pellet in 500 μl of Tris-HCl pH8 10 mM. Shake smoothly in the orbital shaker and heat at 60ºC if necessary for until the total dissolution of the pellet.
20. Add 50 μl of 3M sodium acetate, pH 6.8, and 500 μl of isopropanol at –20ºC.
21. Centrifuge at 13000 rpm for 15 minutes at room temperature. Remove the supernatant.
22. Wash with 1 ml 70% etanol. Remove the supernatant completely.
23. Dry at room temperature for 5 minutes.
24. Resuspend in 75 μl 10 mM Tris-HCl, pH8 or TLE.

C.4. **References**

Ziegenhagen B, Liepelt, S., Kuhlenkamp,V. & Fladung, M. (2003) Molecular identification of individual oak and fir trees from maternal tissues of their fruits or seeds. Trees Structure and Function 17, 345-350.

D. PROTOCOL FOR DNA EXTRACTION FROM EMBRYO TISSUE

D.1. **Reagent needed per sample**

We used the GenElute Plant Genomic DNA kit

D.2. **Reagent preparation**

Reagents are provided ready-to-use.

D.3. **DNA extraction**

For one embryo:

1. Split the seed open and separate the embryo tissue from the rest of tissues carefully in a Petri dish.
2. Introduce the embryo into a tube (1.5 ml) and add sterile water. Leave for a few hours.
3. Separate the testa tissue (a fine mono-stratum tissue sourronding the embryo; see figure 3 in the accompanying document on general methods) from the embryo.
4. Introduce the embryo in a Sarstedt 72693 with one steel bead (5 mm diameter).
5. Homogenize the sample in an electric mill as in step 4-5 in p. 5 .Use the lysis solutions A and B prepared by SIGMA.
6. Follow the SIGMA protocol.

D.4. **References**

GenElute Plant Genomic DNA kit by SIGMA.

http://www. sigma-aldrich.com

E. PROTOCOL FOR DNA AMPLIFICATION

E.1. **PCR mix**

Reagents	**Stock CC**	**Final CC**	µl/20 µl
Bioline BUFFER	10x	1x	2
$MgCl_2$	50 mM	2 mM*	0.8
BSA	20 mg/ml	0.1 mg/ml	0.1
dNTPs	20 mM	0.25 mM	0.2
Primer F	10 µM	0.25 µM	0.5
Primer R	10 µM	0.25 µM	0.5
Taq	5 U/µl	0.5 U/ 20 µl	0.1
H_2O			10.8
DNA	15-25 ng/µl	75-125 ng	5 **

* This PCR mix was used for most of the primers, except for HexH03 and HexA05 which requiered 2.5 mM $MgCl_2$

** When DNA extraction yields a low amount of DNA, the DNA quantity can be increased up to 10 µl.

E.2. **PCR programs**

1. "*Touchdown 66/50* " PCR program*

Step	Temperature	Time
1- initial denaturalization	94 º C	2 min
2- denaturalization	92 º C	30 s
3- annealing	66 º C – 1ºC / cycle	30 s
4- extension	72 º C	30 s
5- repeated cycles	16 cycles of steps 2 to 4	
6- denaturalization	92 º C	30 s
7- annealing	50 º C	30 s
8- extension	72 º C	30 s
9- repeated cycles	18** cycles of steps 6 to 8	
10- final extension	72 º C	5 min
11- refrigeration	4 º C	indefinite

2. "*Touchdown 60/40* " PCR program (used for Fam 001)

Step	Temperature	Time
1- initial denaturalization	94 º C	2 min
2- denaturalization	92 º C	30 s
3- annealing	60 º C – 1ºC / cycle	30 s
4- extension	72 º C	30 s
5- repeated cycles	16 cycles of steps 2 to 4	
6- denaturalization	92 º C	30 s
7- annealing	44 º C	30 s
8- extension	72 º C	30 s
9- repeated cycles	18** cycles of steps 6 to 8	
10- final extension	72 º C	5 min
11- refrigeration	4 º C	indefinite

* The touchdowm 66/50 program was used for most of the primers. The alternative programs, used for some primers, are indicated below.

** The number of cycles can be extended up to 27, if necessary.

3. PCR program –44 (used for Ned-MC4 and HexHA05)

Step	Temperature	Time
1- initial denaturalization	94 º C	2 min
2- denaturalization	92 º C	30 s
3- annealing	44 º C	30 s
4- extension	72 º C	30 s
5- repeated cycles	44 cycles of steps 2 to 4	
6- final extension	72 º C	5 min
7- refrigeration	4 º C	indefinite

4. PCR program- 50 (used for HexH03)

Step	Temperature	Time
1- initial denaturalization	94 º C	2 min
2- denaturalization	92 º C	30 s
3- annealing	50 º C	30 s
4- extension	72 º C	30 s
5- repeated cycles	44 cycles of steps 2 to 4	
6- final extension	72 º C	5 min
7- refrigeration	4 º C	indefinite