

Genomic DNA Extraction and Genotyping of *Dictyochloropsis* Green Algae Strains

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[Abstract] *Dictyochloropsis* is an ecologically important genus of free-living and symbiotic green algae. Representatives of this genus are horizontally transmitted among several fungi of the family Lobariaceae, thus forming photobiont-mediated guilds. This protocol is suitable for extracting DNA from algal cultures and lichen samples and for genotyping seven unlinked *Dictyochloropsis reticulata* microsatellite markers in a single PCR multiplex.

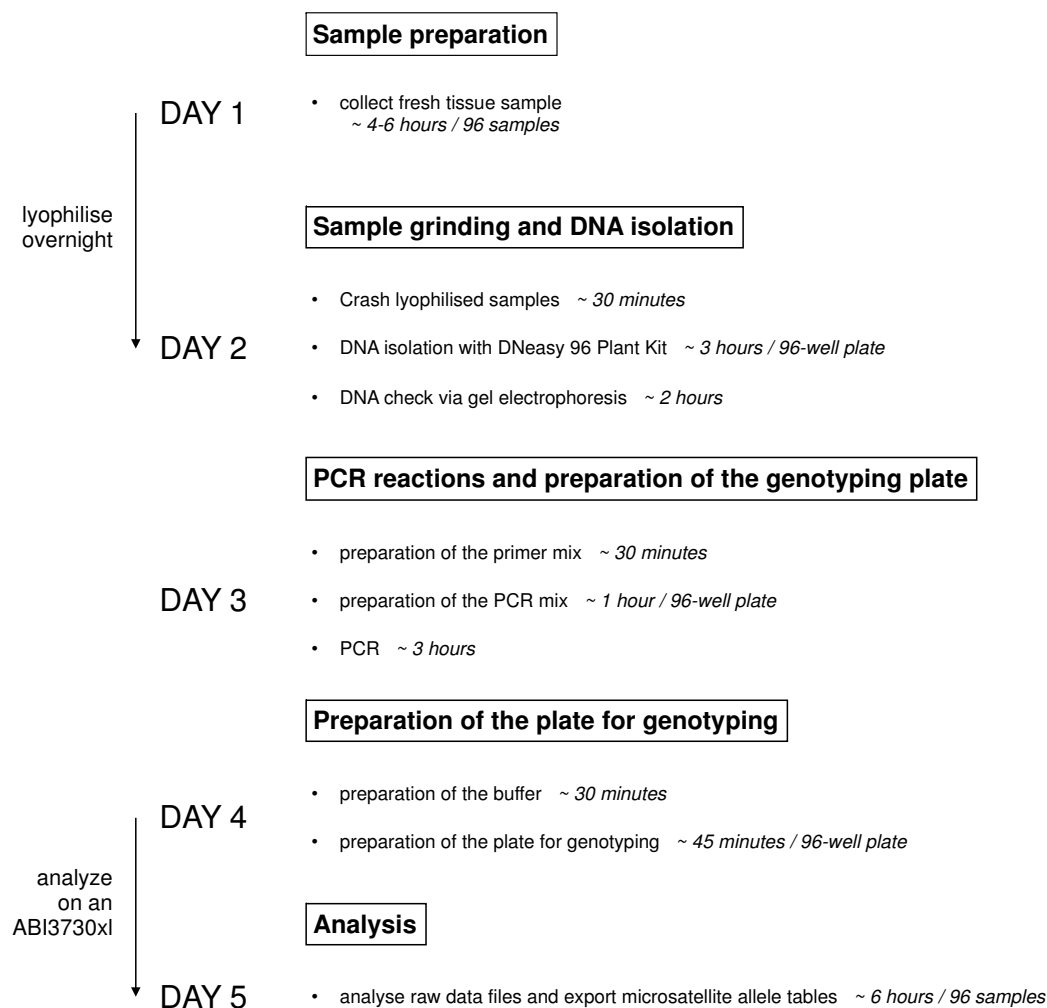


Figure 1. Schematic representation of the analysis pipeline

Materials and Reagents

1. Qiagen Type-it Microsatellite PCR kit (QIAGEN, catalog number: 206243)
2. 1x TE buffer (10 mM Tris, bring to pH 8.0 with HCl, 1 mM EDTA)
3. GelRed (Biotium, catalog number: 41003)
4. 1 kb DNA ladder (GeneRuler 1 kb DNA Ladder) (Thermo Fisher Scientific, catalog number: SM0313)
5. Nuclease-free water
6. Pure molecular biology grade ethanol (96–100%)
7. 2 ml tubes
8. DNeasy 96 Plant Kit (QIAGEN, catalog number: 69181)
9. Hi-Di™ formamide (Life Technologies, catalog number: 4311320)
10. GeneScan 500 LIZ size standard (Life Technologies, catalog number: 4322682)
11. Ice
12. Glucose (BD Biosciences, catalog number: 215510)
13. Proteose pepton (BD Biosciences, catalog number: 212230)
14. Agar (BD Biosciences, catalog number: 214883)
15. 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Life Technologies, Gibco®, catalog number: 11330032)
16. Laboratory wipes (e.g., KimWipes, Kimberly-Clark, catalog number: TW31KWPBX)
17. 4 mm steel balls (e.g., Spex Certiprep, catalog number: 12145950)
18. Pipette tips
19. Pipette (0.1-2 µl), pipette tips
20. Processing plate: 96-well plate (e.g., MicroAmp Optical 96-Well Reaction Plate) and 96-well plate (Septa)
21. 0.22 µm sterile polyethersulfon syringe filter (Merck Millipore, catalog number: P/N SLMP025SS)
22. Algal medium (see Recipes)
23. Algal culture medium preparation for growing *Dictyochloropsis* and other trebouxiophycean algal strains (see Recipes)
24. Vitamin solution (see Recipes)

Equipment

1. Multi-channel pipettes (0.1-10, 10-100, 100-1000 µl), with extended tips
2. Microcentrifuge with rotor for 2 ml tubes
3. Centrifuge for 96-well plates
4. BioRad Gel casting tray, running tray, power pack *etc.* (Bio-Rad Laboratories, catalog number: 164-0305)

5. Incubator (65 °C)
6. Freezer or cold room at -20 °C
7. PCR Thermal cycler
8. ABI3130/ 3130xl/3730/3730xl DNA Analyzer (Applied Biosystems)
9. Ball Mill MM 400 (Retsch®, catalog number: 20.745.0001)
10. Lyophilizer (e.g., FreeZone 4.5 Liter Benchtop Freeze Dry System, Labconco, catalog number: 7750021)

Software

1. GeneMapper® (Software v4.1, Applied Biosystems, catalog number: 4366925)

Procedure

A. Sample preparation (Day 1)

1. Take up to 10 mg fresh tissue sample (approximate wet weight) or 5 mm diameter in size of algal culture (Beck *et al.*, 1998) / lichen thallus in 2 ml tube (see Figure 2).
2. Add 2 steel balls in each tube.
3. Cover the open tubes with soft tissue paper.
4. Cool samples in -20 °C freezer for at least 6 h.
5. Fit the tubes in the lyophilizer and lyophilize overnight.

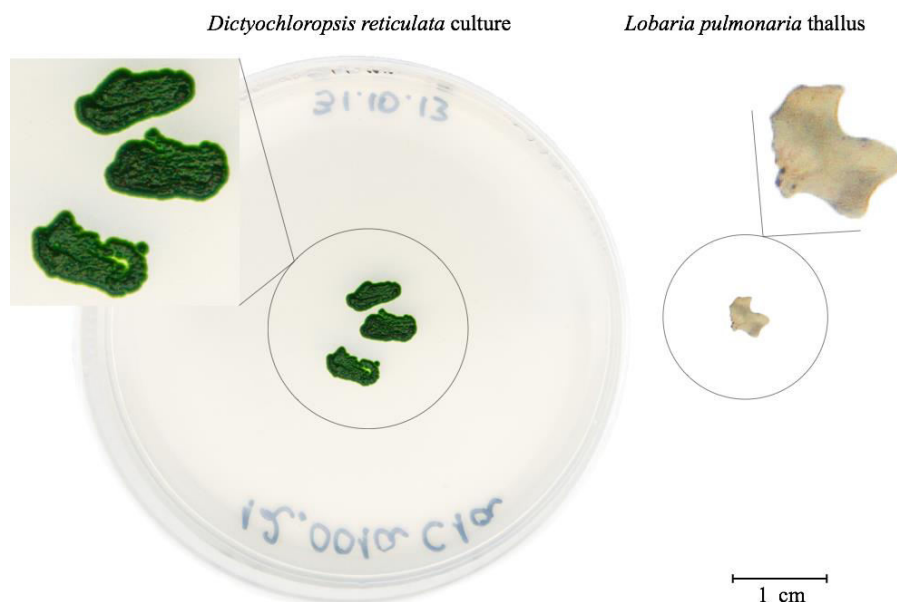


Figure 2. Example of samples used for DNA extraction

B. Sample grinding and DNA isolation (Day 2)

1. Close the tubes.
2. Crash the lyophilized samples using the ball mill MM 400 at 30 Hz for 30 sec without buffer at ambient temperature.
3. Spin down for a few seconds the crashed sample before proceeding with DNA isolation.
4. For DNA isolation follow DNeasy 96 Plant Kit, Qiagen protocol (Plant Handbook 10/2012, <http://www.qiagen.com>, pages 31-34).

Notes:

- a. For buffers AW1 and AW2, before using for the first time, add the appropriate amount of ethanol as indicated on the bottle to obtain a working solution.
 - b. Preheat Buffer AP1 to 65 °C.
5. Elute the purified DNA from the DNeasy spin column using Buffer AE twice (2 x 50 µl).
Note: Smaller or larger elution volumes can be used for more or less concentrated products. To ensure complete elution, 40 µl should be the minimum elution volume.

C. DNA check

1. Prepare 1% agarose gel with 1x TAE buffer. Add 0.5 µl GelRed for 100 ml gel.
2. Load 2 µl DNA.
3. Add 1 kb or 100 bp ladder for reference.
4. Run the gel electrophoresis.
5. Check DNA for quality and quantity in gel doc system (Figure 3).

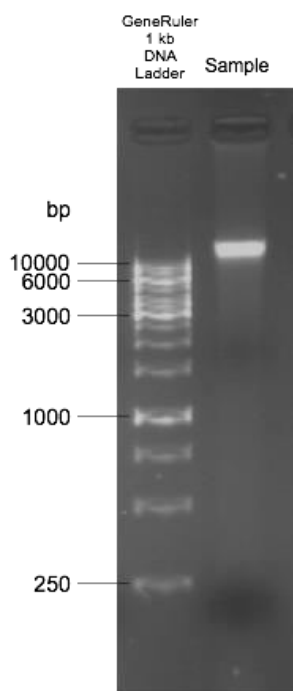


Figure 3. Example of good quality genomic DNA of *Dictyochloropsis reticulata* run on a 1% w/v agarose gel

D. PCR reactions and preparation of the genotyping plate (Day 3)

7x Primer mix

- a. First prepare 100 μ M primer stocks.
- b. Mix all primers according to Tables 1 and 2 to get 1ml primer-mix (sufficient for 10x 96-sample PCR plate).

Table 1. Primer sequences and labeling of the microsatellite Multiplex 1 specific to *Dictyochloropsis reticulata* (Dal Grande *et al.*, 2009; Dal Grande *et al.*, 2014)

Algal Loci	Locus GenBank	Primer Sequence (5'-3')	Label
LPh1	FJ754261	F: GTCTCAGGTGACCACTTGATTG R: GCAATGGATATGATGCTTGTTT	VIC
LPh2	FJ754262	F: GACAGCTGTTCCAGTGCATC R: GCAGAGGAAGTGCATGACG	FAM
LPh3	FJ754263	F: TGCAGTAGGTGTCATATGTGT R: GAAGGCGCATCTTGATATAC	NED
LPh4	FJ754264	F: GTGGTGGTACAACATGCTCA R: ACGACCACGTGGGATATCTA	NED
LPh5	FJ754265	F: TGGTGTAGTAAGAATCGGCATC R: GTGTATGTCGGCCCAATAA	PET
LPh6	FJ754266	F: GAATCCTGCCTGCCTACAAG R: AGCAACCCATTTCAACCAAC	FAM
LPh7	FJ754267	F: TGTGACAGGTGAAACACCAA R: TATGGTCCCTCATGGCAAAT	VIC

Table 2. 7x primer mix preparation

Concentration of normalized primer stocks: 100 μ M (100 pmol/ μ l)

Each primer 20 μ l (30 μ l for LPh7)

TE buffer 700 μ l

Total volume 1 ml

E. PCR reaction mix

1. Thaw template DNA, RNase-free water, the primer mix and the 2x Type-it Microsatellite PCR Master Mix, if stored at -20 °C.
2. Mix the solutions completely before use.
3. Prepare a reaction mix according to Table 3.

Note: The reaction mix contains all the components required for multiplex PCR except the template DNA. Prepare a volume of reaction mix 10% greater than that required volume for the total number of reactions to be performed.

4. Mix the reaction mix thoroughly and dispense appropriate volumes into PCR tubes or plates.

Note: Mix gently by pipetting the reaction mix up and down a few times. It is not necessary to keep samples on ice during reaction setup.

Table 3. PCR components of the microsatellite multiplex (total volume = 10 μ l)

PCR component	Multiplex 1 (10 μ l)
Multiplex PCR Master Mix ^a	5 μ l (1x)
Primer mix	1 μ l (0.2 μ M of each primer, 0.3 μ M for LPh7)
DNA template	1 μ l (1–10 ng)
RNase-free water	3 μ l

F. PCR program

1. Step 1: 95 °C for 5 min (initial activation)
2. Step 2: 95 °C for 30 sec (denaturation)
3. Step 3: 62 °C for 90 sec (annealing)
4. Step 4: 72 °C for 60 sec (extension)
5. Step 5: go to step F2, repeat 24 times
6. Step 6: 60 °C for 30 min (final extension)
7. Store PCR products in -20 °C, until further processing.

G. Preparation of the plate for genotyping (Day 4)

1. Dilute the PCR products 1: 10 with RNase-free water.
2. Combine 1 μ l of diluted PCR product with a buffer containing 9 μ l of a denaturing agent (Hi-Di™ Formamide) and 0.5 μ l LIZ500 size standard.
3. Note: For one 96 well plate prepare buffer for 105 samples.
4. Centrifuge the plates briefly (5 sec) at 1,500 RCF.
5. Prepare table of samples.
Note: Fill any blank sample-well with nuclease-free water.
6. Analyze on an ABI3730xl.

H. Analysis (Day 5)

Import the raw data files generated by the sequencer to the computer with GeneMapper software.

Genotyping in GeneMapper

1. Select 'Create new project' from the 'File' menu.

2. Import your samples.
3. Create marker panel and bin set.

Analysis parameters

4. Choose 'Microsatellite default' as table setting and 'Microsatellite analysis method' as the analysis method.
5. Set the panel for the 7x-primer mix.
6. Set size standard as LIZ500 and exclude the 35- and 250-bp peaks.
7. Analyze (see Figure 4).

Note: If some samples fail to match size standards, go to edit size standards and override size. If this does not solve the issue, there might be some problem with the sample or the analysis run.

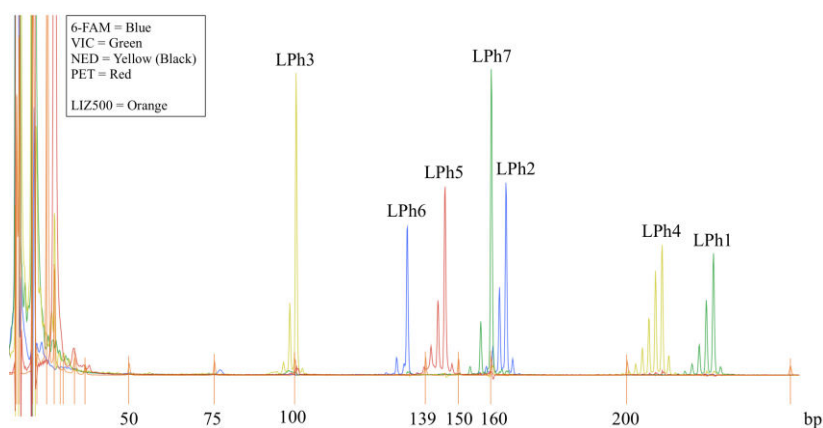


Figure 4. Example of the 7x-multiplex microsatellite electropherogram for one sample of *Dictyochloropsis reticulata*

Representative data

1. A simple, representative example of data that indicates what type of results to expect (Table 4).

Table 4. Example of a 7x-multiplex microsatellite allele table

Sample	Population	LPh1	LPh2	LPh3	LPh4	LPh5	LPh6	LPh7
1	Pop1	262	157	108	191	143	121	170
2	Pop2	260	151	138	176	156	121	190
3	PopN	148	159	122	181	140	116	152

Note: Numbers indicate the relative size of each microsatellite allele in base pairs.

Recipes

1. Algal medium

Ingredients for the preparation of algal culture medium

a. Macronutrients (g/400 ml)

NaCl	1	(42.7 mM)
CaCl ₂ ·2H ₂ O	1	(17.0 mM)
KNO ₃	20	(495 mM)
MgSO ₄ ·7H ₂ O	3	(30.4 mM)
(NH ₄) ₂ ·HPO ₄	10	(189 mM)

b. Micronutrients (g/500 ml)

KOH	15	(535 mM)
EDTA:	25	(171 mM)
FeSO ₄ ·7H ₂ O	2.49	(17.9 mM)
H ₃ BO ₃	5.52	(179 mM)
ZnSO ₄ ·7H ₂ O	4.41	(30.7 mM)
MnCl ₂ ·4H ₂ O	0.72	(7.28 mM)
NaMoO ₄	0.36	(3.50 mM)
CuSO ₄ ·5H ₂ O	0.79	(6.33 mM)
Co(NO ₃) ₂ ·6 H ₂ O	0.25	(1.72 mM)

2. Algal culture medium preparation for growing *Dictyochloropsis* and other trebouxiophycean algal strains

- For one liter of medium, take 10 ml of macronutrients and 1 ml of micronutrients, add 0.715 g HEPES buffer (final concentration 3 mM) and fill up to 1,000 ml with distilled water.
- Adjust pH to 5.5 with HCl.
- 20 g agar may be added.
- Autoclave and add 1 ml sterile vitamin solution after cooling down to at least 60 °C. Sterilize the vitamin solution by filtration using a 0.22 µm sterile polyethersulfon syringe filter.

Note: To receive higher amounts of algal cells 1.5% glucose and 1% proteose pepton can be added.

3. Vitamin solution

Thiamine	0.1 g/100 ml	(3.77 mM)
Biotin	2.5 mg/100 ml	(0.10 mM)
Vitamin B12	1.5 mg/100 ml	(9.50 µM)

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