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| http://www.philippe.cubry.com/wp-content/gallery/national-botanic-gardens/24062012-04742-irlande.jpg?uamfiletype=nggImage  **Herbarium and Molecular Laboratory PROTOCOL** |  |
| http://www.philippe.cubry.com/wp-content/gallery/national-botanic-gardens/24062012-04717-irlande.jpg?uamfiletype=nggImage | http://www.philippe.cubry.com/wp-content/gallery/national-botanic-gardens/24062012-04780-irlande.jpg?uamfiletype=nggImage |
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| 20/12/2012 | AMPLIFYING CHLOROPLAST MICROSATELLITES FOR ANALYSIS ON ABI 310 GENETIC ANALYZER |
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AMPLIFYING CHLOROPLAST MICROSATELLITES FOR ANALYSIS ON ABI 310 GENETIC ANALYZER

*List of contents:*

[1 Purpose 2](#_Toc343092469)

[2 Overview 2](#_Toc343092470)

[3 Safety Remarks 2](#_Toc343092471)

[4 MateriaL 2](#_Toc343092472)

[5 Reagents 2](#_Toc343092473)

[6 Procedure 2](#_Toc343092474)

[6.1 DNA preparation 3](#_Toc343092475)

[6.2 Primers 3](#_Toc343092476)

[6.3 Mix preparation 4](#_Toc343092477)

[6.3.1 Simplex experiment (for tests only) 4](#_Toc343092478)

[6.3.2 Multiplex experiment 5](#_Toc343092479)

[6.4 PCR program 5](#_Toc343092480)

[6.5 Preparing samples for the run on ABI 6](#_Toc343092481)

[6.6 Running on ABI310 genetic analyzer 6](#_Toc343092482)

[6.7 Analysis of the electropherogram 6](#_Toc343092483)

# Purpose

Amplifying universal chloroplast microsatellites markers for fast phylogeographic studies of plant species.

# Overview

Microsatellites are short DNA sequences (1-5 base pairs) tandem repeats. Universal primers have been developed to amplify chloroplast microsatellites by Polymerase Chain Reaction (PCR).

# Safety Remarks

Always wear gloves and lab coat. Pop4 is a neurotoxin and Formamide is toxic, be particularly careful handling those reagents.

# MateriaL

* Microtubes 0.5µl and caps
* Microplates 96 wells and caps
* Eppendorf Thermocycler or equivalent
* Microcentrifuge
* Septae for ABI 310 with specific microtubes or microplates and tray
* Micropipettes and tips

# Reagents

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Name | Security phrase | Toxicity | Personal Protection | Localisation |
| DNA |  |  | Gloves and coat | Personal |
| Fluorescently labelled forward primers (Work solution) | 3301-79-9 | Xi | Gloves and coat | Your PCR kit |
| Reverse primers (Work solution) |  |  | Gloves and coat | Your PCR kit |
| MyTaq DNA polymerase |  |  | Gloves and coat | Lab Freezer |
| Buffer MyTaq 5X |  |  | Gloves and coat | Lab Freezer |
| Autoclaved pure water |  |  | Gloves and coat | Your PCR kit/Lab freezer |
| HiDi Formamide | 75-12-7 | T | Gloves and coat, mask | Lab Freezer |
| Genescan GS-ROX500 |  |  | Gloves and coat | Lab Fridge |

# Procedure

## DNA preparation

DNA should be clean and of sufficient quality for PCR amplification. You should check quality and concentration of your DNA before anything else. Quality could be test by electrophoresis on an agarose gel and revelation using SYBR-Safe or other staining agent, concentration could be assessed by gel electrophoresis or spectrophotometry (Nanodrop or other equivalent device).

Dilute your DNA to 2-5ng/µl. Homogenisation of DNA concentration will facilitate accurate amplification of DNA fragments. Store your DNA in tubes or in plates in freezer (-20°C) for long time or in fridge (4°C) fort a short period (less than a week). If you store your DNA in plates, think about organization within the plate (always make a plan before making it)!

## Primers

6 primer pairs are available in the lab for amplification of universal chloroplast microsatellites: ccmp2, ccmp4, ccmp5, ccmp6, ccmp7 and ccmp10. Each of the forward primer is fluorescently labelled in order to be detected by the ABI310 Genetic Analyzer. Labels are as follow:

* Ccmp2 forward primer JOE (green)
* Ccmp10 forward primer FAM (blue)
* Ccmp4 forward primer TAMRA (yellow)
* Ccmp5 forward primer FAM (blue)
* Ccmp6 forward primer JOE (green)
* Ccmp7 forward primer TAMRA (yellow)

NB: relative fluorescence of TAMRA is about 4-time less intense than FAM and JOE, you may want to use more of TAMRA primer/product in relation to other dyes.

All fluorescently labelled forward primers and corresponding reverse primers are available at 100µM stock solution. Prepare a work solution at 10µM for each primer by diluting 10 times stock solution in water. You may want to prepare enough work solution for several PCR experiment. For example, preparing 200µl of work solution will be as follow: 20µl stock solution (100µM) + 180µl ultrapure (autoclaved) water. Store primers at -20°C.

## Mix preparation

This PCR is made in multiplex. Multiplex make possible to amplify several markers in the same PCR experiment. Up to 6-plex could be achieved. However it might be difficult to obtain reliable PCR for each primer pair. Tests are to be made for each species before enabling multiplex. PCR volume is 12.5µl. PCR experiment was adapted from Palmé et al, 2004???

### Simplex experiment (for tests only)

|  |  |  |
| --- | --- | --- |
| Name | Concentration required (1 reaction) | Quantity required (1 reaction) |
| Fluorescently labelled forward primer (10µM) | 0.2µM | 0.25µl |
| Reverse primer (10µM) | 0.2µM | 0.25µl |
| MyTaq Buffer mix (5X) | 1X | 2.5µl |
| MyTaq DNA polymerase | 1U | 0.2µl |
| Autoclaved water | Up to 12.5µl final reaction volume | 4.3µl |
| DNA (2ng/µl) | 10ng | 5µl |

You may want to test simplex on several samples (1 to 8) to ensure reliable amplification and non-overlapping of markers labelled with the same fluorescent dye (e.g. ccmp4 and ccpm7, ccmp2 and ccmp6, ccmp10 and ccmp5).

### Multiplex experiment

If tests revealed good amplification for each markers and no overlapping, you may want to try a multiplex design, allowing to amplify up to the 6 markers in one PCR experiment (you will save money and time). Allow to prepare a little more than needed (100 putative reactions for 96 real reactions).

Nota Bene: following primer concentrations are guidelines only and might be used as a starting point. Depending on your target species, you might want to run a test on a few genotypes and allow for adjust concentration. For example in Betulaceae (Alder, Corylus, Betula) optimal multiplex design use 0.4 µM each of forward and reverse primers for CCMP5, all others are the same as the following table.

|  |  |  |
| --- | --- | --- |
| Name | Concentration required (1 reaction) | Quantity required (1 reaction) |
| Ccmp2 labelled F primer (10µM) | 0.1µM | 0.125µl |
| Ccmp2 Reverse primer (10µM) | 0.1µM | 0.125µl |
| Ccmp10 labelled F primer (10µM) | 0.1µM | 0.125µl |
| Ccmp10 Reverse primer (10µM) | 0.1µM | 0.125µl |
| Ccmp4 labelled F primer (10µM) | 0.4µM | 0.5µl |
| Ccmp4 Reverse primer (10µM) | 0.4µM | 0.5µl |
| Ccmp5 labelled F primer (10µM) | 0.1µM | 0.125µl |
| Ccmp5 Reverse primer (10µM) | 0.1µM | 0.125µl |
| Ccmp6 labelled F primer (10µM) | 0.1µM | 0.125µl |
| Ccmp6 Reverse primer (10µM) | 0.1µM | 0.125µl |
| Ccmp7 labelled F primer (10µM) | 0.4µM | 0.5µl |
| Ccmp7 Reverse primer (10µM) | 0.4µM | 0.5µl |
| MyTaq Buffer mix (5X) | 1X | 2.5µl |
| MyTaq DNA polymerase | 1U | 0.2µl |
| Autoclaved water | Up to 12.5µl final reaction volume | 1.8µl |
| DNA (2ng/µl) | 10ng | 5µl |

## 

## PCR program

PCR program was adapted from Palmé et al, 2004???. It can be found as “ccmp” in the Eppendorf Thermocycler.

Initial denaturation/activation step: 95°C – 4 minutes

Then 35 cycles: Denaturation 95°C – 30 seconds

Hybridization 50°C – 45 seconds

Elongation 72°C – 75 seconds

Final elongation step: 72°C – 8 minutes

## Preparing samples for the run on ABI

PCR products have to be diluted in order to be accurately revealed by electrophoresis on the ABI310 Genetic Analyzer. Several dilutions should be tested in order to select the correct one. For example, for a 40-fold dilution, prepare a dilution at 1:40 (i.e. 1µl PCR product (multiplex) + 39µl autoclaved water) for each sample. If you use simplex products, pick 1µl of each product in FAM/JOE and 4µl of each product in TAMRA, complete to 40µl with water.

Prepare a mix of HiDi Formamide + Genescan GS-ROX500 size ladder at the following rates: for one sample 10µl Formamide + 0.3µl GS-ROX500. Prepare a little more than needed, for example count 50 for 48 samples or 100 for 96 samples. Dispense 10µl of the mix in 0.5microtubes or in the dedicated ABI plates for genetic analyzer.

Pick 1µl of the diluted PCR products and add them to the Formamide/Size ladder mix. Incubate for 3 minutes at 94°C and put on ice/freezer for at least 5 minutes.

In case of using microtubes, cut the caps. Place septae over the tubes/microplates. If using 96 well adapter, put the retainers over the septae and put the plate over the tray adapter.

NB: if running a small number of samples (less than 10), formamide should be replaced by ultrapure water.

## Running samples on ABI 310 genetic analyzer

Check if the genetic analyzer is set up correctly, typically verify buffer levels, POP4 level, capillary condition etc…

Create a genescan injection list on the ABI 310 collection software that states the samples to be used for electrophoresis.

Create a new genescan experiment and load the previously created injection list. Check run parameters are corrects, they should be:

* Temperature 60°C
* Module GS STR POP4 (1ml) A
* Injection 5 sec – 15kv (injection time should be increased if needed)
* Run 15kv
* Run time 24 minutes (to be adapted)

Launch the run.

## Analysis of the electropherogram

You can analyze the runs directly using Genescan/Genotyper on the Mac computer. You can also retrieve the run files on an USB key (FAT formatted). Before being able to open these files on a PC, you should add a “.fsa” extension to each file. You should then analyze the runs using dedicated PC software like Genemapper, STRAND or Genemarker.

## Data analysis

Once you have analyzed the gels, export the bin results to spreadsheet software (MS Excel or equivalent) and manage to format the result table for the suitable software. You can gain time by formatting the table for the “Create” software (Coombs et al, https://bcrc.bio.umass.edu/pedigreesoftware/node/2), which will allow you to export your result file in an extensive list of software format.