

Sequencing using capillary electrophoresis on the ABI Prism 3700

1. Subject

To do phylogenetic research on fungi we make use of sequences of the LSU, SSU and ITS1-5.8S-ITS2 region of the ribosomal gene. After alignment and phylogenetic tree constructions relationships among fungi can be established.

2. Principal

The sequences of fungi can vary considerably. Relationship at family or order level can be checked with SSU sequences. When relationships on family or order level have been established by SSU sequences, ITS sequences show the variability within a genus (up to species level). To obtain sequences of SSU and ITS regions a PCR has to be performed first to create the appropriate amplicon. After purification of this amplicon and performing a sequence-PCR with specific primers, the results can be analyzed using capillary electrophoresis.

3. Reagents and material

- 3.1 pipets
- 3.2 pipettips
- 3.3 BigDye terminator cycle sequencing RR mix (Applied Biosystems, 4303153)
- 3.4 PCR machine, adjustable to a rapid thermal ramp of 1°C/sec
- 3.5 96-well plate centrifuge (Sigma 3K15)
- 3.6 sephadex G-50 superfine (Amersham Pharmacia, 17-0041-01)
- 3.7 ultrapure water
- 3.8 column loader (MACL 09645)
- 3.9 multiscreen HV plate (MAHV N45)
- 3.10 aluminum foil cover (3M, 0012-02-01 or Costar, 6524AL)
- 3.11 DNA template or PCR product
- 3.12 microtiter plate 96-wells (Applied Biosystems, N801-0560)
- 3.13 primers
 - primer ITS1 (5'-TCCgTAggTgAACCTgCgg-3')
 - primer ITS2 (5'-gCTgCgTTCTTCATCgATgC-3')
 - primer ITS3 (5'-gCATCgATgAAgAACgCAgC-3')
 - primer ITS4 (5'-TCCTCCgCTTATTgATATgC-3')
 - primer ITS5 (5'-ggAAgTAAAAGTCgTAACAagg-3')
 - primer Oli 01 (5'-ATTACCgCggCTgCTggC-3')
 - primer Oli 02* (5'-CCgTCAATYYTTTTRAgTTT-3')
 - primer Oli 03* (5'-gACgggCggTgTgTRC-3')
 - primer Oli 04* (5'-CTggTTgATYCTgCCAgT-3')
 - primer Oli 05 (5'-gAAACTgCgAATggCTCATT-3')
 - primer Oli 06 (5'-AATgAgCCATTCgCAgTTTC-3')
 - primer Oli 07* (5'-AgggYTCgAAYCCggAgA-3')
 - primer Oli 08* (5'-TCTCAggCTCCYTCTCCgg-3')
 - primer Oli 09 (5'-CgCggTAATTCCAgCTCCA-3')
 - primer Oli 10* (5'-TTggYRAATgCTTTCgC-3')
 - primer Oli 11* (5'-TTRATCAAgAACgAAAgt-3')
 - primer Oli 12 (5'-AATTTgACTCAACACggg-3')
 - primer Oli 13 (5'-gggCATCACAgACCTgTTAT-3')
 - primer Oli 14 (5'-ATAACAggTCTgTgATgCCC-3')
 - primer Oli 15* (5'-TTTgYACACACCgCCCgTCg-3')

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primer Oli 16* (5'-CYgCAggTTCACCTACRg-3')
* [6.1]

- 3.14 200 µl eppendorf cups ultra thin (Biozym 179401)
- 3.15 Tris(hydroxymethyl)-aminomethane (Fluka 93362)
- 3.16 MgCl₂-6H₂O (Merck 5833)
- 3.17 HCl (Ferak 11448)

4. Solutions

- 4.1 Sequencing buffer 5x:
Dissolve 4.84 g Tris [3.15] and 0.2 g MgCl₂·6H₂O [3.16] in 80 ml ultrapure water. Adjust pH to 9.0 with 1N HCl [3.17]. Add ultrapure water to 100 ml.

5. Protocol

- 5.1 Purify PCR products using the GFX protocol (Protocol 12, p. 41).
Starting a sequencing PCR
- 5.3 Mix for X strains (X*2) µl BigDye RR mix [3.3], X µl primer [3.13] and (X*5) µl milliQ-water and X µl sequencing buffer [4.1, 8.2, 6.2].
- 5.4 Pipet 1 µl amplicon with the right concentration [6.8] into a ultra thin PCR vial [3.14]] on ice.
- 5.5 Pipet 9 µl mix [5.3] to every sample (reaction volume 10 µl).
- 5.6 Control: pipet 4 µl BigDye RR mix, 4 µl M13-primer and 2 µl pGEM 3Zf(+) in a PCR vial.
- 5.7 Place the vials in a PCR-machine (e.g. GeneAmp 9700) and run the following cycle 25x (don't perform a 'hot start'):

96°C	10 sec.
50°C	5 sec.
60°C	4 min.

Then cool to 4°C.

The rapid thermal ramp should be set to 1°C/sec. The GeneAmp 9700 should be run in 9600 mode.

Purifying sequencing products

- 5.8 Add sephadex G-50 superfine [3.6] to the column loader [3.8].
- 5.9 Remove excess of resin from the top of the column loader with the scraper.
- 5.10 Place multiscreen HV [3.9] plate upside-down on top of the column loader.
- 5.11 Invert both multiscreen HV plate and column loader without shifting.
- 5.12 Tap on top of the column loader or tap softly on the bench to release the resin.
- 5.13 Add 300 µl ultrapure water to each well to swell the resin.
- 5.14 Incubate at room temperature for 3 hours.
- 5.15 Place the multiscreen HV plate on top of a (used) standard 96-well microtiter plate.
- 5.16 Spin the assembly [5.15] at 910xg for 5 min. at 15°C to pack the columns [6.3].
- 5.17 Turn the plate and spin again at 910xg for 5 min. at 15°C
- 5.18 Add 100 µl ultrapure water to each well.
- 5.19 Spin the assembly [5.15] at 910xg for 5 min. at 15°C to pack the columns [6.3].
- 5.20 Turn the plate and spin again at 910xg for 5 min. at 15°C
- 5.21 Add the sequencing reaction [5.7] to the center of each well.
- 5.22 Place multiscreen HV on top of a clean standard 96-well microtiter plate [3.12].
- 5.23 Spin the assembly at 910xg for 5 min. at 15°C to collect purified sequencing products.
- 5.24 Turn the plate and spin again at 910xg for 5 min. at 15°C
- 5.25 Remove the multiscreen HV [6.4].
- 5.26 Seal the microtiter plate with aluminum foil or tape [3.10].
- 5.27 Mark the plate with a waterproof marker (e.g. on the foil cover).

Preparations for capillary electrophoresis

- 5.28 Store the samples at -20°C near the ABI Prism 3700 at the NIOB.
- 5.29 Go to <http://sequencing.niob.knaw.nl/> and login to fill in your data.

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6. Remarks

- 6.1 The concentration of the standard primers for sequencing should be 4 pmol/ μ l. When modified primers* are used the concentration should be more than 4 pmol/ μ l: these are Oli 03, 04, 08, 11 and 15: 8 pmol/ μ l; Oli 10 and 16: 16 pmol/ μ l; Oli 02 and 07: 32 pmol/ μ l.
- 6.2 Only one primer is used per reaction. To obtain the sequence of ITS1-5.8S-ITS2 at least two sequencing-PCRs have to be performed, one with primer ITS1 and one with primer ITS4. Unless there is an intron present, then extra reactions with primers ITS5 and ITS3 can improve readability of the sequence. To get a SSU sequence we need to perform more sequencing PCR reactions. Primers Oli 01, (Oli 02), Oli 05, (Oli 7, Oli 8), Oli 09, Oli10, Oli11, (Oli 12), Oli 13, Oli 14, (NS1) and NS24 are used. Primers between brackets are optional.
- 6.3 This should be done directly before use.
- 6.4 Don't throw away the multiscreen HV plate. The resin should be discarded when dry and the multiscreen HV plate cleaned for further use.
- 6.5 The EXCEL file can be found in directory I:\SequenceForm\empty template.xls
This form can be filled in before the sequencing products are loaded into the 96-wells microtiter plate. Then it can be used as a pipetting guide.
- 6.6 The name of the file (and the microtiter plate) is recommended to look like this: yyyymmddXX (yyyy:year; mm:month; dd:day; XX:your initials), e.g. 20010716BG.
- 6.7 Results of the capillary electrophoresis will be sent to you by email.
- 6.8 Important: check the concentration of the template before sequencing PCR. Concentrations of PCR products should be tested AFTER purification (Protocol 20, p. 65). Failure of the reactions is most often due to a wrong primer/template ratio (Sequencing troubleshooting, p. 69). More template is NOT improving the reaction! Not only will the reaction fail, but will also shorten the life span of the capillary array in the sequenator.

7. Literature



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8. Graphs and Tables

8.1 IUPAC/IUB ambiguity

R	=	{AG}	[puRine]
Y	=	{CT}	[pYrimidine]
M	=	{AC}	[aMino]
K	=	{GT}	[Keto]
S	=	{CG}	[Strong]
W	=	{AT}	[Weak]
H	=	{ACT}	[not G]
B	=	{CGT}	[not A]
V	=	{ACG}	[not T]
D	=	{AGT}	[not C]
N	=	{ACGT}	[unkNown]

8.2 Pipetting protocol to obtain a mastermix for sequencing-PCR using 1 µl template

	1 sample	X samples
BigDye terminator RR mix	2 µl ¹	(X*2) µl ¹
Primer (...)	1 µl	X µl
Sequencing buffer 5x	1 µl	X µl
milliQ ultrapure water	5 µl ¹	(X*5) µl

¹ For templates up to about 1000 bp 2 µl of BigDye should suffice. To sequence the SSU (>1000 bp) the amount of BigDye should be 4 µl and the amount of ultrapure water decreased with 2 µl.

8.3 Concentration of PCR product for sequencing

Length of PCR product in basepairs	Optimal concentration in ng/µl
100-200	1-3
200-500	3-10
500-1000	5-20
1000-2000	10-40
>2000	40-100

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