

Sequencing using capillary electrophoresis on the ABI Prism 310

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 other primers, the template can be sequenced using capillary electrophoresis.

3. Reagents and material

- 3.1 appropriate amplicon 15-45 ng
- 3.2 200 µl eppendorfcups ultra thin (Biozym 179401)
- 3.3 primers ITS1-ITS5:
 - primer ITS1 (5'-TCCgTAggTgAACCTgCgg-3')
 - primer ITS2 (5'-gCTgCgTTCTTCATCgATgC-3')
 - primer ITS3 (5'-gCATCgATgAAgAACgCAgC-3')
 - primer ITS4 (5'-TCCTCCgCTTATTgATATgC-3')
 - primer ITS5 (5'-ggAAgTAAAgTCgTAACAagg-3')
- 3.4 primers Oli 01-Oli 16:
 - primer Oli 01 (5'-ATTACCgCggCTgCTggC-3')
 - primer Oli 02* (5'-CCgTCAATTYTTTTRAgTTT-3')
 - primer Oli 03* (5'-gACgggCggTgTgTRC-3')
 - primer Oli 04* (5'-CTggTTgATYCTgCCAgT-3')
 - primer Oli 05 (5'-gAAACTgCgAATggCTCATT-3')
 - primer Oli 06 (5'-AATgAgCCATTcCgCAgTTTC-3')
 - primer Oli 07* (5'-AgggYTCgAYYCCggAgA-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')
 - primer Oli 16* (5'-CYgCAggTTCACCTACRg-3')
- * [6.1]
- 3.5 pipets
- 3.6 sterile pipettips
- 3.7 DNA sequencing kit (Applied Biosystems 4303152)
- 3.8 MilliQ water
- 3.9 1.5 ml eppendorfcups (Sarstedt 72.690)
- 3.10 GeneAmp 9700 or other PCR-machine suitable for 200 µl eppendorfcups
- 3.11 Na-acetate, CH₃COONa-3H₂O (Brocades)

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- 3.12 ethanol 70% and 96%
- 3.13 centrifuge (Eppendorf 5417R)
- 3.14 ice
- 3.15 TSR-buffer (Applied Biosystems 401674)
- 3.16 Genetic Analyzer 0.5 ml vials (Applied Biosystems 401957) with silicon septa (Applied Biosystems 401956)
- 3.17 ultrapure water (MilliQ)

4. Solutions

- 4.1 3M sodiumacetate pH 4.6:
Dissolve 20.4 g CH₃COONa-3H₂O [3.11] in 50 ml milliQ-water [3.8]. Adjust pH to 4.6. Aliquot the solution in eppendorfcups. Store at -20°C.
- 4.2 Ethanol 70%:
Mix 700 ml ethanol 96% with ultrapure water to a volume of 960 ml.

5. Protocol

- 5.1 To obtain ITS sequences a PCR is performed using primers V9G and LS266 and primers Oli 04 and Oli 16 to sequence SSU (Protocol 7, p. 25).
- 5.2 Purify the amplicon with GFX columns (Protocol 12, p. 41) or Microspin (Protocol 10, p. 37).
Starting a sequencing PCR
- 5.3 Mix for X strains (X*4) µl BigDye RR mix [3.7], X µl primer [6.3] and (X*3) µl milliQ-water [8.2].
- 5.4 Pipet 2 µl amplicon with a concentration of 8-25 ng/µl [8.3] into a ultra thin PCR vial [3.2] on ice.
- 5.5 Pipet 8 µl mix [5.17] to every sample.
- 5.6 Controle: pipet 4 µl BigDye RR mix, 4 µl M13-primer [3.7] and 2 µl pGEM 3Zf(+) in a PCR vial [3.2].
- 5.7 Place the vials in a PCR-machine (e.g. Amplitron or GeneAmp 9700) and run the following cycle 25x:

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.

Precipitate DNA fragments

- 5.8 Pipet 1 µl sodiumacetate [4.1] in a 1.5 ml eppendorfcup [3.9].
- 5.9 Add 10 µl (total amount) of reaction product [5.7].
- 5.10 Add 25 µl ethanol 96% [3.12].
- 5.11 Incubate 5-10 min. on ice.
- 5.12 Centrifuge 20 min. at full speed [3.13].
- 5.13 Discard supernatant carefully not touching the pellet [6.2].
- 5.14 Pipet directly 250 µl ethanol 70% to the pellet.
- 5.15 Centrifuge 20 min. at full speed.
- 5.16 Discard supernatant carefully [5.13].
- 5.17 Air dry the pellet until it is absolutely dry.

Preparations for capillary sequencing

- 5.18 Pipet 12 µl TSR-buffer [3.15] to the dry pellet [5.17].
- 5.19 Transfer the resuspended amplicon into a genetic analyzer vial [3.16] and close it tightly with a septum [3.16] by turning the septum a little bit.
- 5.20 Incubate 2 min. at 95°C and snap cool on ice (immediately).
- 5.21 Store the prepared samples for sequencing in the refrigerator.
- 5.22 The samples will be run by the ABI Prism operator and the sequences and ABI files will be distributed on Zip100 disks or can be copied via FTP from the sequencer to the appropriate user directory.

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6. Remarks

- 6.1 When modified primers are used the concentration should be more than 4 pmol/μl: these are Oli 03, 04, 08, 11 and 15: 8 pmol/μl; Oli 10 and 16: 16 pmol/μl; Oli 02 and 07: 32 pmol/μl.
- 6.2 Beware! The pellet should NOT dry out. Loose label eventually present will bind to the amplicon. This bond is irreversible and will give problems during sequencing.
- 6.3 Only one primer is used per reaction. To obtain the sequence of ITS1-5.8S-ITS2 two sequencing-PCRs have to be performed, one with primer ITS5 and one with primer ITS4. Unless there is an intron present, then primer ITS1-3 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 7, Oli 8, Oli 09, Oli10, Oli11, Oli 12, NS1 and NS24 are used.

7. Literature

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

	1 sample	X samples
BigDye terminator RR mix [3.7]	4 μl	(X*4) μl
Primer (...) 4 pmol/μl [3.3, 3.4]	1 μl	X μl
milliQ ultrapuur water [3.17]	3 μl	(X*3) 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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