Molecular diagnostics of *Dothistroma*

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cost

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Why molecular, DNA-focused diagnostics?

- Practical

any phase of fungal life cycle – no conidia needed

- Rapid

no need to wait till culture grows on agar medium

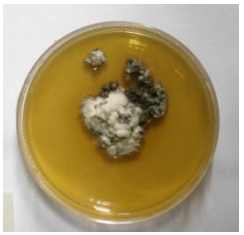
- Reproducible

if good laboratory practice (GLP) is followed, different laboratories get the same results.

- No previous experience with Dothistroma is necessary to identify the pathogen !

DNA extraction

Needles



Cultures

Homogenisation

Manual (mortar and pestle in liquid nitrogen)

Automatic in a mill

DNA extraction

Homogenous samples in microtubes (eppendorf tubes) should be processed immediately :

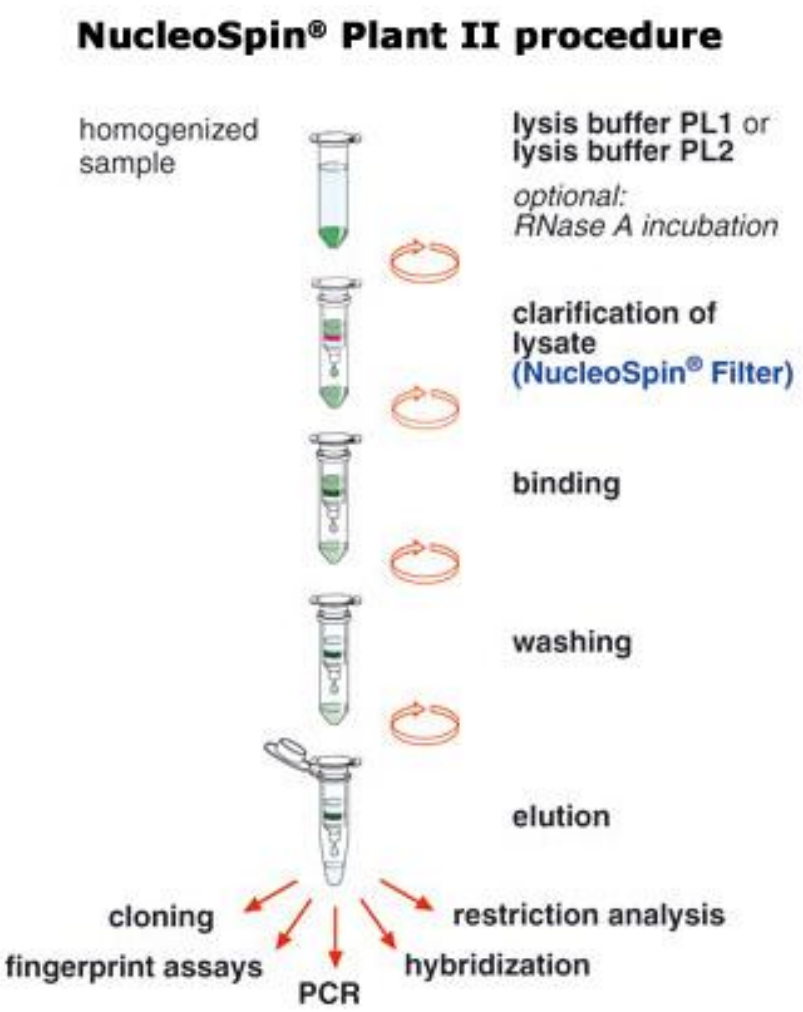
1) CTAB extraction, Phenol/chlorophorm extraction (hand made buffers)

1) Commertial extraction kits
   a) Kits with spin columns (DNA binds on a membrane during centrifugation)

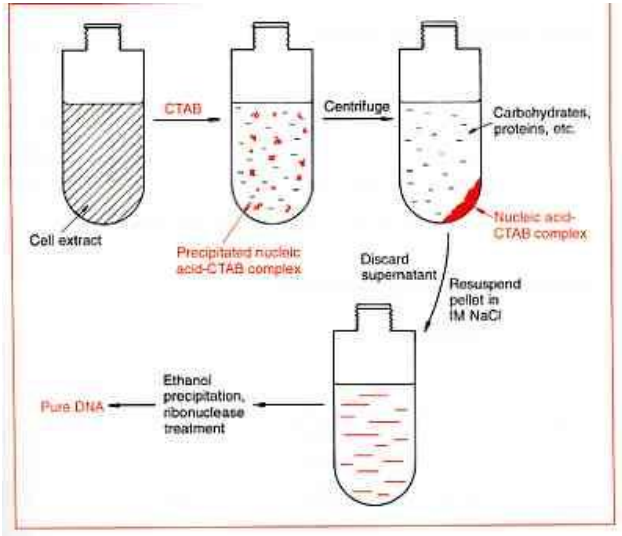
      Qiagen plant , Mo-Bio, Macherey Nagel, Sigma, Omega etc.

   a) Rapid kits: Prepman (AB), Phire Plant Direct PCR (Thermo Scientific)

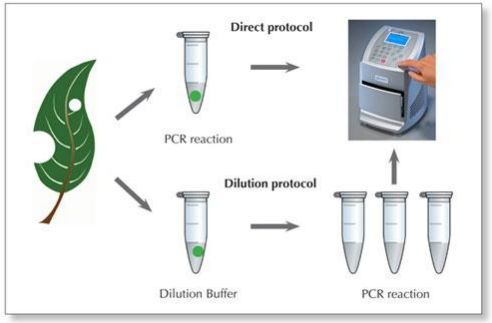
DNA extraction



Nucleospin Plant II – Macherey Nagel



CTAB protocol



Phire® Plant Direct PCR Kit

PCR – Polymerase chain reaction

- The enzymatic amplification of selected part of DNA (gene, non-coding region)
- The method relies on **thermal cycling**, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.
- **Primers** (short DNA fragments) containing sequences complementary to the target region along with a **DNA-polymerase** are key components to enable selective and repeated amplification.
- As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

PCR

Mixing of reaction components:

- Sterile distilled **H_2O**
- **PCR Buffer** – provides proper environment for DNA-polymerase to function.
- In some DNA-polymerases, $MgCl_2$ must be added
- **dNTPs** – nucleotides, the substrate for the enzyme
- **Primers** - provide short, dsDNA targets for DNA-polymerase*Taq* on both strands of DNA. Define the 5' and 3' ends of amplification target
- The forward and reverse primers are specific for each PCR.
- **DNA-polymerase**
- **Template DNA**- the DNA extracted from envirnmental sample (needle)or a fungal culture.

PCR

Reaction volume is 25 µl

Example of calculation:

H_2O – 19.5 µl
PCR Buffer 2.5x – 2.5 µl
Primer 1 (10 pmol/ µl) – 0.5 µl
Primer 2 (10 pmol/ µl) – 0.5 µl
dNTPs (10mM) – 0.5 µl
DNA-Polymerase (2 U/µl) – 0.5 µl
Template DNA – 1.0 µl

Total – 25.0 µl

The stock solution is premixed (without Template DNA) – so called Mastermix.

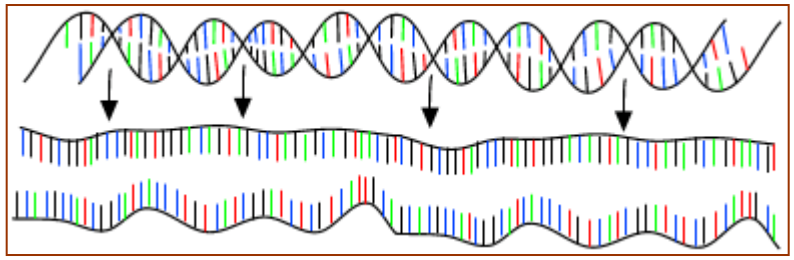
PCR tubes in strips

PCR tubes

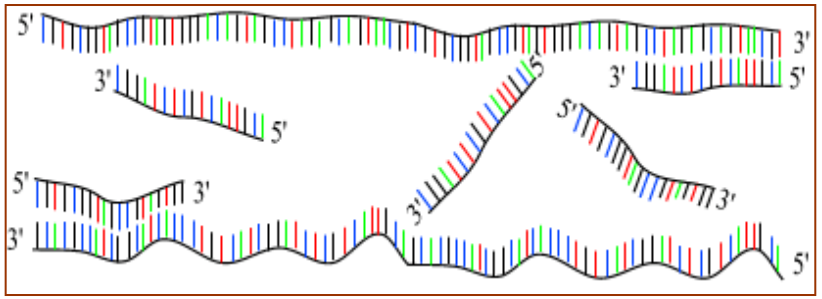
The Thermocyclers – the device enabling PCR

PCR – changing of temperatures

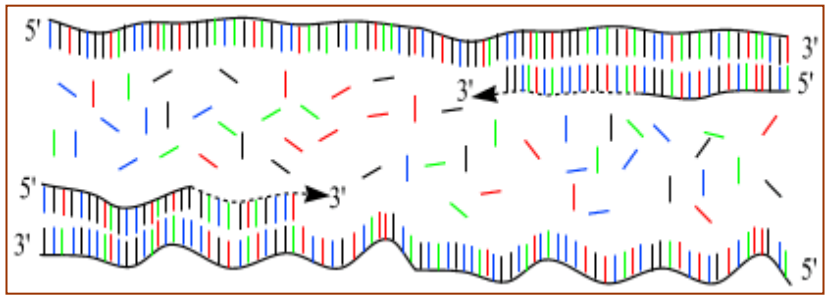
95 ºC – Denaturation. The hydrogen bonds are broken in the double stranded DNA, creating single strands of DNA that are susceptible to copying.



40-60 ºC – Annealing. The specific binging of primers to single strand DNA.



72 ºC – Extension. DNA-polymerase adds DNA nucleotides from 5'to 3', reading the template from 3' to 5' side, making two double stranded molecules from each one double stranded.

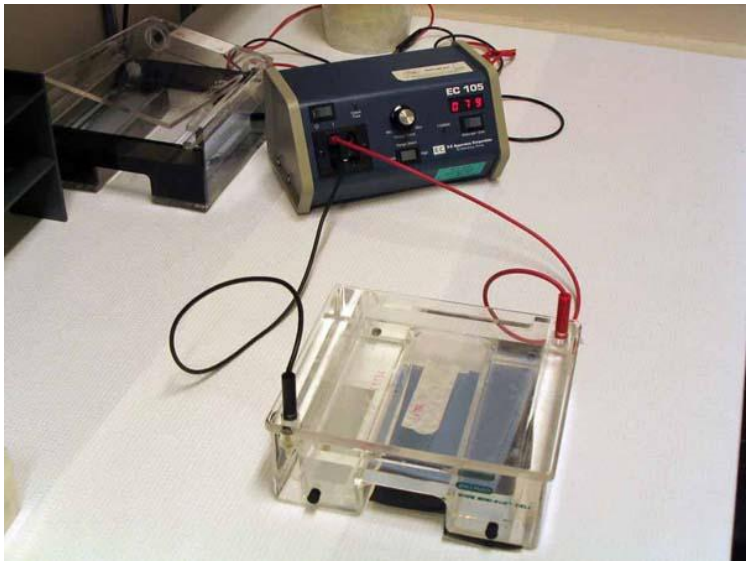


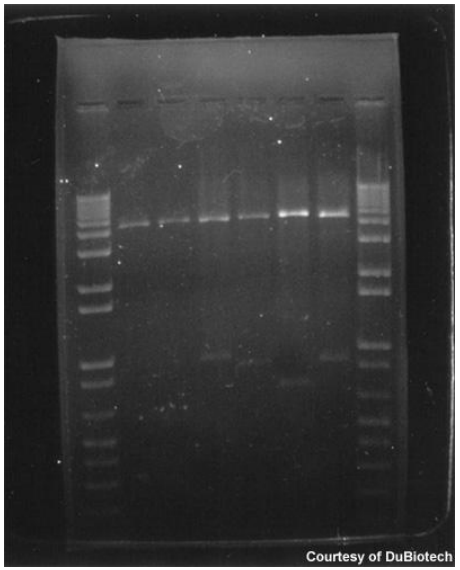
Electrophoresis

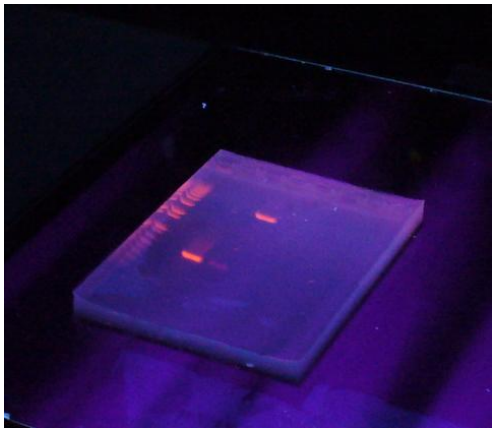
The motion of dispersed particles relative to a fluid under the influence of a spatially uniform electric field.
In electric field (**agarose** gel in TBE buffer), DNA fragments resulted in PCR are devided according to their lengths (in base pair numbers).

The length of a DNA fragment is compared to the known length of reference **mass ladder**.

DNA is staining in ethidium bromide (toxic!) or a non toxic alternative stain visible under UV light.







DNA sequencing

… ATATATAGGCAAGGAATCTCTATTATTAAATCATT…

Determining the order of the nucleotide bases—adenine, guanine, cytosine, and thymine—in a molecule of DNA.

Conservative DNA regions – constant sequence among all individui forming a species.

Applicable for identification of the pathogen – the conservative DNA regions are sequenced for taxonomic/identification applications:

ITS region of ribosomal RNA genes (ribosomal DNA).

Single copy protein genes:

beta-tubulin gene

translation elongation factor 1-alpha

When sequence of particular pathogen is known, can be subjected for development of species-specific primer for **PCR diagnostics**.

The diagnostics of Dothistroma

Ioos R. et al, (2010). Development, comparison, and validation of real-time and conventional PCR tools for the detection of the fungal pathogens causing brown spot and red band needle blights of pine. Phytopathology. 100(1):105-14.

Groenewald M. et al, (2007). Characterization and distribution of mating type genes in the dothistroma needle blight pathogens. Phytopathology. 97(7):825-34.

Pehl L. et al, (2004). Mycosphaerella-Nadelpilze der Kiefer: Identifikation durch ITS-RFLP-Muster [Mycosphaerella needle fungi: Identification by means of ITS-RFLP patterns]. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 56(10): 239-244.

Ioos (2010) – species specific PCR

- Development of rapid PCR metod discriminating *D. septosporum* and *D. pini*.
- Species-specific primers targeted single-copy genes: beta-tubulin and translation elongation factor 1-alpha

How does it work?

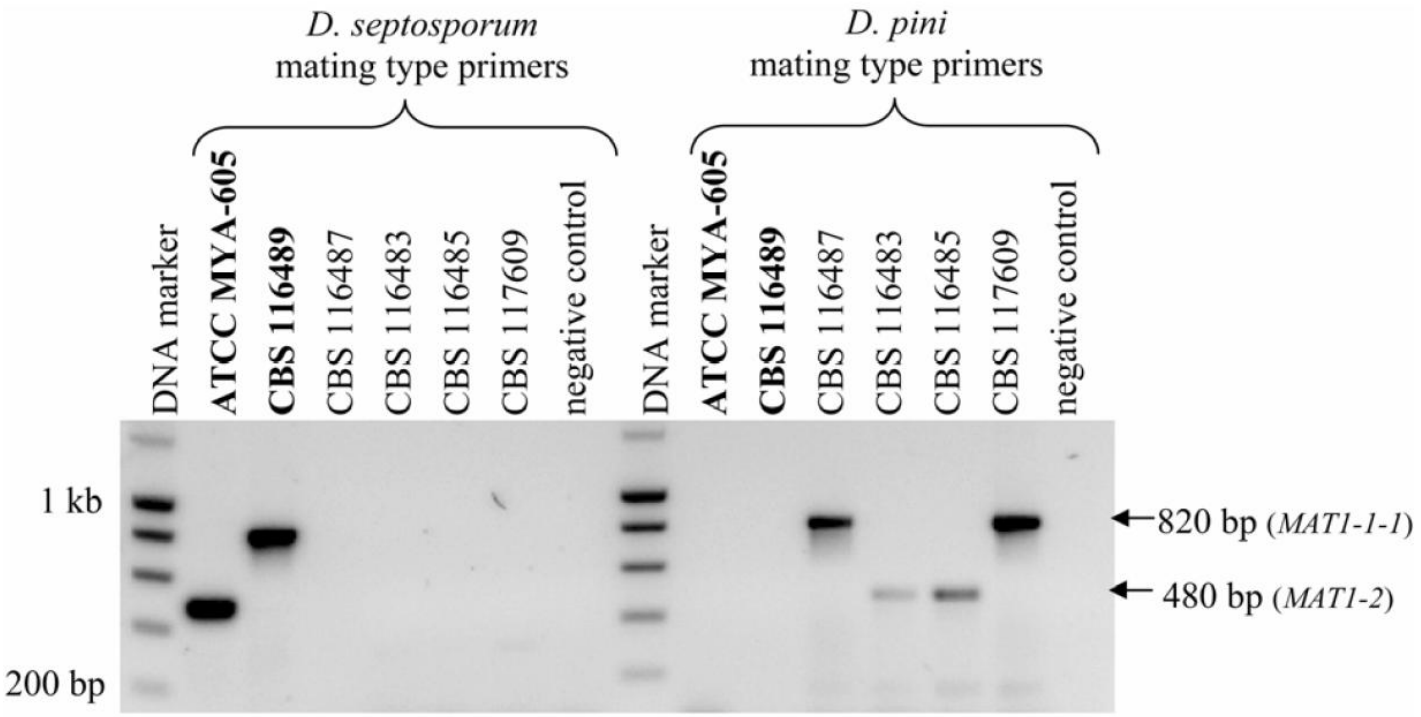
- PCR including specific primers for *D. septosporum* (DStub2-F/DStub2-R) amplifies only DNA of this fungus – the PCR product of expectable length is visible on agarose gel.
- The similar primers available for *D. pini* (Dptef-F/Dptef-R).

Ioos (2010) developed also primers for **quantitative PCR** (estimation of concentration of fungal DNA in particular sample).

No subject of our course

Groenewald (2007) – mating type genes

- The primers specific for either, D. septosporum and D. pini.
- The primers targeted mating types (MAT1, MAT2)
- Multiplex PCR (including primers for both species) can be used in the PCR, when mating types are screened.



Pehl (2004)- PCR+RFLP

Combination of PCR and RFLP was used for fungal identification

RFLP – specific cleavage of a particular DNA sequence by a **restriction enzyme (endonuclease) .**

The restriction patterns of ITS region of ribosomal RNA genes were investigated using various restriction enzymes.

Less useful then species-specific PCR – more time consuming, less specific.