Marteilia refringens detection and characterization by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

According to Le Roux et al. (2001)

1. Scope

This procedure explains a standard diagnostic test used for *Marteilia refringens* detection and characterization in flat oysters (e.g. *Ostrea edulis*) and mussels (*Mytilus edulis* and *M. galloprovincialis*) to confirm a previous histological or cytological diagnosis at the genus level. It allows a specific diagnosis between *Marteilia refringens* type O and *Marteilia refringens* type M.

2. References

Le Roux F., Lorenzo G., Peyret P., Audemard C., Figueras A., Vivarès C., Gouy M. & Berthe F., 2001. Molecular evidence for the existence of two species of Marteilia in Europe. *J. Eukaryot. Microbiol.* **48**, 4: 449-454.

OIE (2009). Manual of Diagnostic Tests for Aquatic Animals, section 2.4, Paris, France, (*web format of Manual of Diagnostic Tests*: http://www.oie.int/en/international-standard-setting/aquatic-manual/access-online)

3. Equipment and environmental conditions

This test requires the equipment and environmental conditions classically used for PCR assays:

- A closed hood equipped with an UV producing system to eliminate potential contaminations when preparing PCR mix
- Two complete sets of pipettes (2 µl; 20 µl; 200 µl and 1000 µl), the first one for DNA extraction, and the second one for PCR mix preparation.
- Three different pipettes: one pipette (2 µl) to dispense samples in PCR mix, one pipette (20µl) for BET sampling and another pipette (20 µl) to load PCR products in agarose gels
- Filter pipette tips (2 µl; 20 µl; 200 µl and 1000 µl) for DNA extraction, PCR mix preparation and sample dispensing
- Pipette tips (20 µl) to collect BET and to load amplification products in agarose gel
- A thermal cycler to perform amplifications
- A horizontal electrophoresis system for PCR products electrophoresis
- An UV table to observe PCR products after agarose gel electrophoresis
- A system to acquire pictures of the gels

Manipulator must wear a lab coat and gloves during all the different steps described bellow. Lab coat and gloves must be changed preferably after each main step: DNA extraction, preparation of PCR mix, sample dispensing, amplification and gel loading.

It is recommended to perform these different steps in different rooms. Amplification and gel loading / electrophoresis should particularly take place in a room separate from DNA extraction, PCR mix preparation and DNA dispensing.

4. Procedure

4.1. Sample preparation

DNA is extracted from a piece of digestive gland using QIAamp® DNA Mini Kit (QIAGEN) and following instructions for Tissue Protocol.

➔ **Carefully read the protocol given with the kit before starting DNA extraction**

1. Cut up to 25 mg of tissue into small pieces, place in a 1,5 ml microcentrifuge tube and add 180 µl of Buffer ATL
2. Add 20 µl Proteinase K, mix by vortexing and incubate at 56°C until the tissue is completely lysed (overnight). Vortex occasionally during incubation to disperse sample. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid.
3. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s and incubate at 70°C for 10 minutes. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid
4. Add 200 µl ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 s. Briefly centrifuge the 1,5 ml microcentrifuge tube to remove drops from the lid
5. Carefully apply the mixture from step 4 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the tube containing the filtrate.
6. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 10 000 rpm for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided in the kit) and discard the collection tube containing the filtrate.
7. Carefully open the QIAamp Spin Column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (14 000 rpm) for 3 min.
8. Place the QIAamp Spin Column in a new 2 ml collection tube (not provided in the kit) and discard the collection tube containing the filtrate. Centrifuge at full speed (14 000 rpm) for 1 min.
9. Place the QIAamp Spin Column in a clean 1,5 ml microcentrifuge tube (not provided in the kit) and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 50 µl of distilled water. Incubate 1 minute at room temperature and centrifuge at 10 000 rpm for 1 min.
10. Control the quality and efficacy of the extraction (for example by measuring the absorbance at 260 nm with a spectrophotometer or after electrophoresis in agarose gel).
11. Prepare dilutions of your samples in order to have a final DNA concentration of 100 ng/µl
12. DNA solutions are kept at 4°C until PCR analyses are performed

4.2. Polymerase Chain Reaction (PCR)

4.2.1. Reactives

- 10 X Buffer (provided with the Taq DNA Polymerase)
- $MgCl_2$ (provided with the DNA polymerase) (25 mM)
- Taq DNA Polymerase (Goldstar, Eurogentec) 5 U/µl
- dNTP Master Mix (20mM) must be diluted 10 fold (at 2mM) before use
- H_20 (free of DNA and RNA)

4.2.2. Primers

M2A 5'- CCG CAC ACG TTC TTC ACT CC - 3'
M3AS 5'- CTC GCG AGT TTC GAC AGA CG - 3'

(*corresponding to primers Pr4 and Pr5 in Le Roux* et al. *2001*)

4.2.3. PCR Mix

PCR mix for each tube is:

	Volume per tube	Final concentration
Buffer (10X)	5 µl	1X
$MgCl_2$ (25 mM)	5 µl	2,5 mM
dNTP (2mM)	5 µl	0,2 mM
M2A (100µM)	0,5 µl	1 µM
M3AS(100µM)	0,5 µl	1 µM
Taq polymérase (5U/µl)	0,5 µl	2,5 U
dH_2O	32,5 µl	

- 49 µl of this PCR mix is dispensed in each PCR tube
- 1 µl of extracted DNA (100 ng/µl) is added to each tube

Two types of control are used:

- **Negative** controls consist of dH_2O (1 µl for 49 µl of PCR Mix). They aim at detecting potential reactive contamination or working environment. One negative control should be included every 10 samples or after each batch of samples.
- **Positive** controls consist of DNA extracted from known highly infected oysters or mussels. They aim at checking the efficacy of the PCR reaction. One positive control should be included for each PCR analysis.

4.2.4. Amplification

Amplification cycles are performed in a thermal cycle apparatus (PTC-100 MJ Research, Inc.Perkin).

- Initial denaturation: 10 min at 94°C
- Amplification: 30 cycles (1 min at 94°C, 1 min at 55°C and 1 min at 72°C)
- Final elongation: 10 min at 72°C

4.2.5. Interpretation

A positive result is an amplicon of the appropriate size (412 bp) with all negative controls negative and all positive controls positive.

4.3. Restriction Fragment Length Polymorphism (RFLP)

4.3.1. Reactives

- 10 X Buffer (provided with the restriction enzyme)
- H_20 (free of DNA and RNA)
- *Hha*I (10 U/µl)

4.3.2. Digestion mix

Digestion mix for each tube is:

- 2 µl of the appropriate buffer
- 1 µl of enzyme
- 7 µl of dH_2O

↳ 10 µl of this digestion mix is dispensed in each tube
↳ 10 µl of PCR products are added to each tube

4.3.3. Digestion

Digestion is then performed by incubating samples for 1 hour at the temperature indicated by the manufacturer.

4.3.4. Interpretation

M2A / M3AS PCR products will be digested differently according to the type of *Marteilia refringens*.
Table below indicates expected restriction profiles:

	***Hha*I**
Marteilia refringens type M	157 bp + 156 bp + 68 bp + 31 bp
Marteilia refringens type O	226 bp + 156 bp + 31 bp

4.4. Electrophoresis

4.4.1. Reactives

- 50 X TAE (can be bought directly ready for use):
  - Tris base (40 mM) 242 g
  - glacial acetic acid (40 mM) 57,1 ml
  - $Na_2EDTA.2H_2O$ (1 mM) 18,61 g
  - dH_2O for 1 liter

  ↳ Ajust at pH 8
- Agarose gel:
  - 1 % for PCR products or 2% for RFLP products of agarose in 1X TAE

  ↳ Ethidium bromide (0,5 µg/ml) is added after cooling the gel.
- Loading blue dye:
  - Bromophenol blue 0,25 %
  - Cyanol xylene FF 0,25 %
  - Sucrose 40 %

  ↳ Keep at 4°C.

  ↳ Use diluted 6 times (2 µl of loading blue buffer for 10µl of PCR products).
- Molecular weight marker:
  - SmartLadder SF (Eurogentec): a ready-to-use molecular weight marker including 9 regularly spaced bands from 100 to 1000 bp.

4.4.2. Agarose gel preparation

1. Weight X g of agarose, add 100 x X ml of 1X TAE and heat until the mix is melted.
2. After cooling the solution, ethidium bromide is added (5 µl for 100 ml of agarose gel) and the solution is disposed in a specific mould equipped with combs (to make wells in the gel).
3. When gel is polymerised, combs are removed and the gel is placed in a horizontal electrophoresis system full of 1X TAE.
4. 10 µl of PCR products or 20 µl of RFLP products are mixed with 2 µl (for PCR products) or 4 µl (for RFLP products) of blue dye (6X) and disposed in the wells
5. One well is dedicated to the molecular weight marker (5 µl)
6. A voltage of 50 to 150 volts is applied during 30 min to 1 hour depending on the gel size and thickness
7. Gel is observed under UV.