Molecular identification of marine invertebrate larvae: the example of bivalves

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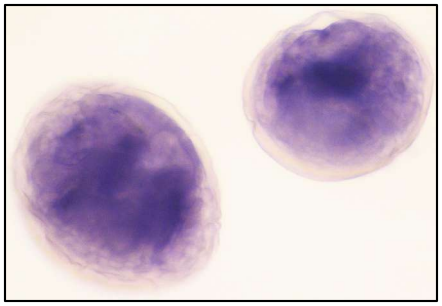
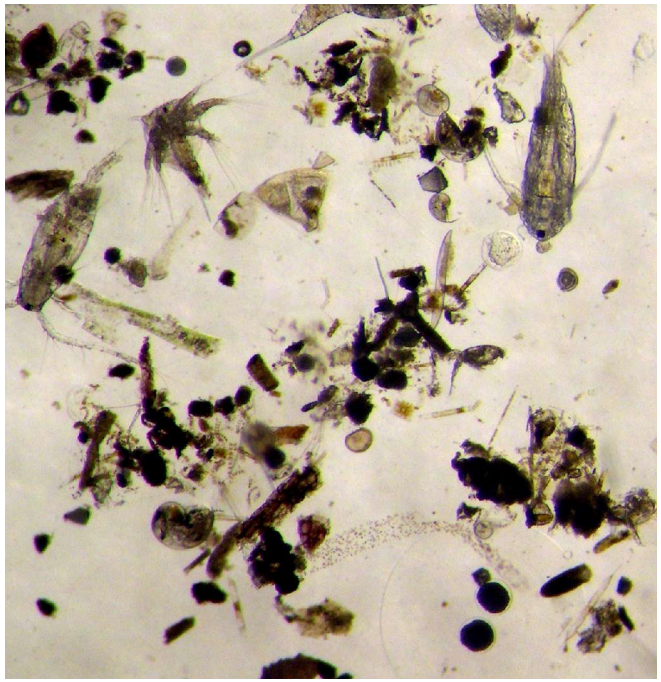




Several constraints for identification

- Small size (100 µm – few mm)

- Early developmental stages morphologically similar for closely-related sp

- Find reliable diagnostic characters, stable whatever the developmental stage and the environmental conditions

- Huge number of samples

Methods for identification

- **Morphology-based methods (including automatic image analysis)**
- **Molecule-based methods**
  - **Immunological methods**
  - **Isoenzymes**
  - **DNA-based methods**

DNA-based methods

Possible through PCR allowing to process low amounts of target-DNA, then working with individual larvae

- ***Advantages***
  - ✓ **Invariable whatever the age, development stage and physiological state**
  - ✓ **Method development possible from adult individuals**
  - ✓ **A lot of sequences are available in databases**

- ***Drawbacks***
  - ✓ **Very sensitive to fixation (hypersaline buffers, ethanol, liquid nitrogen)**
  - ✓ **Larval DNA extraction: critical step (but facultative)**

DNA-based methods: numerous methods exist

- **Hybridization on nylon membrane**
  e.g. Medeiros-Bergen et al. (1995) Limnol Oceanogr 40:1225-1235

- **PCR-RFLP**
  e.g. Comtet et al. (2000) Limnol Oceanogr 45:1655-1661

- **PCR-RAPD**

- **Microsatellites**
  e.g. Morgan & Rogers (2001) Mar Biol 139:967-973

- **Multiplex PCR**
  e.g. Hare et al. (2000) Mar Biol 137:953-961; Larsen et al. (2005) Mar Biol 146:1119-1129

The method depends on the objective

✓ **Diversity, multispecific approach**

« *Barcoding* » (sequencing of a species-specific gene)

✓ **Population dynamics of one or a few species**

***In situ* hybridisation on the whole larva**

Larval identification through sequencing (barcode approach)

- **Target sequences**

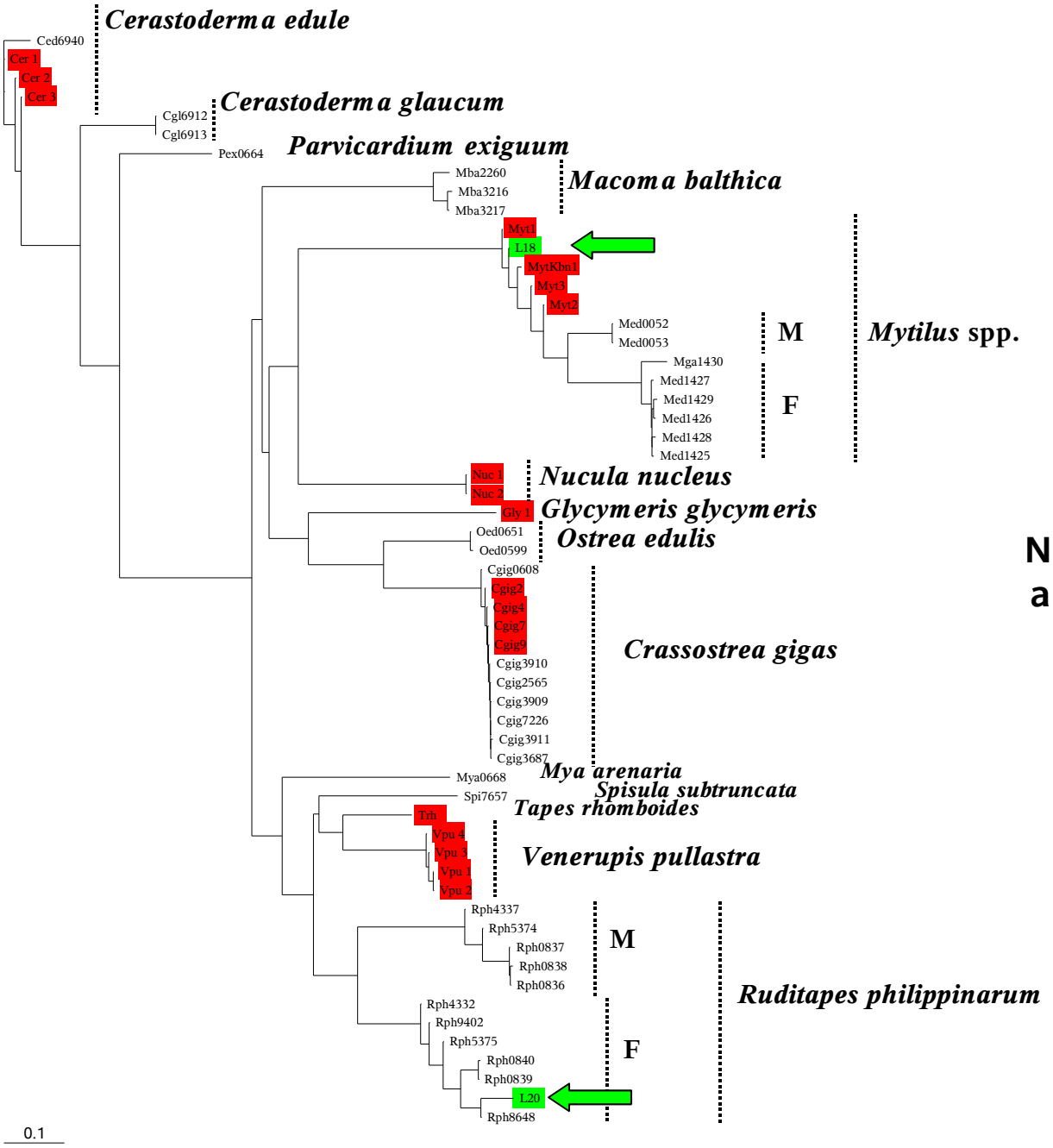
Need for a sequence (gene) variable enough to find interspecific polymorphism but conserved to reduce intraspecific polymorphism

- *mitochondrial DNA*
COI (THE gene of the consortium *Barcoding of life*)

- *ribosomal DNA*
18S rDNA gene

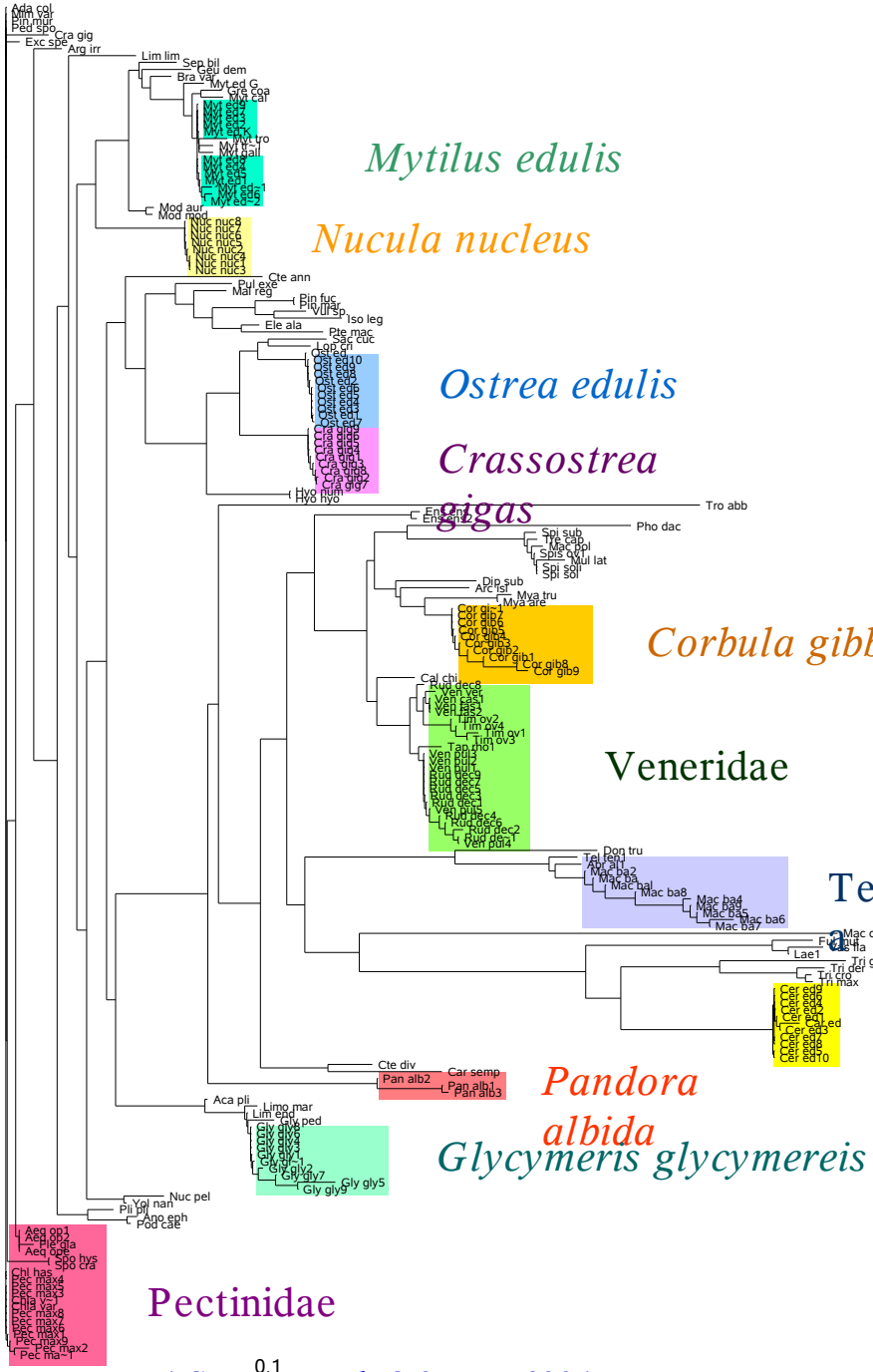
- **reference sequences = sequences from adults + databases**

- **PCR (universal primers) and sequencing of larval DNA**



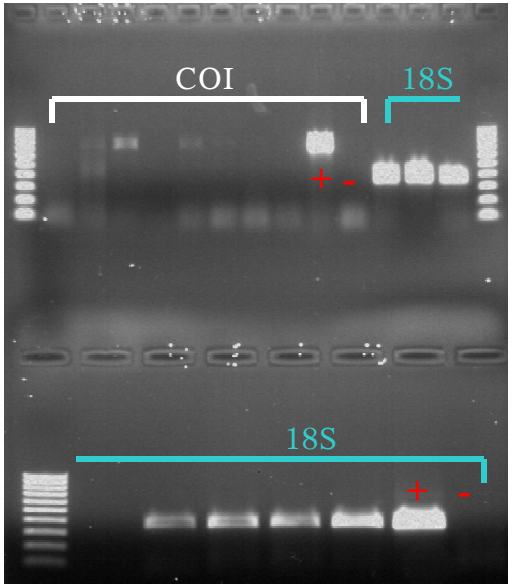
Neighbor-Joining tree from a 760 bp portion of the COI gene

Larvae collected in the bay of Morlaix



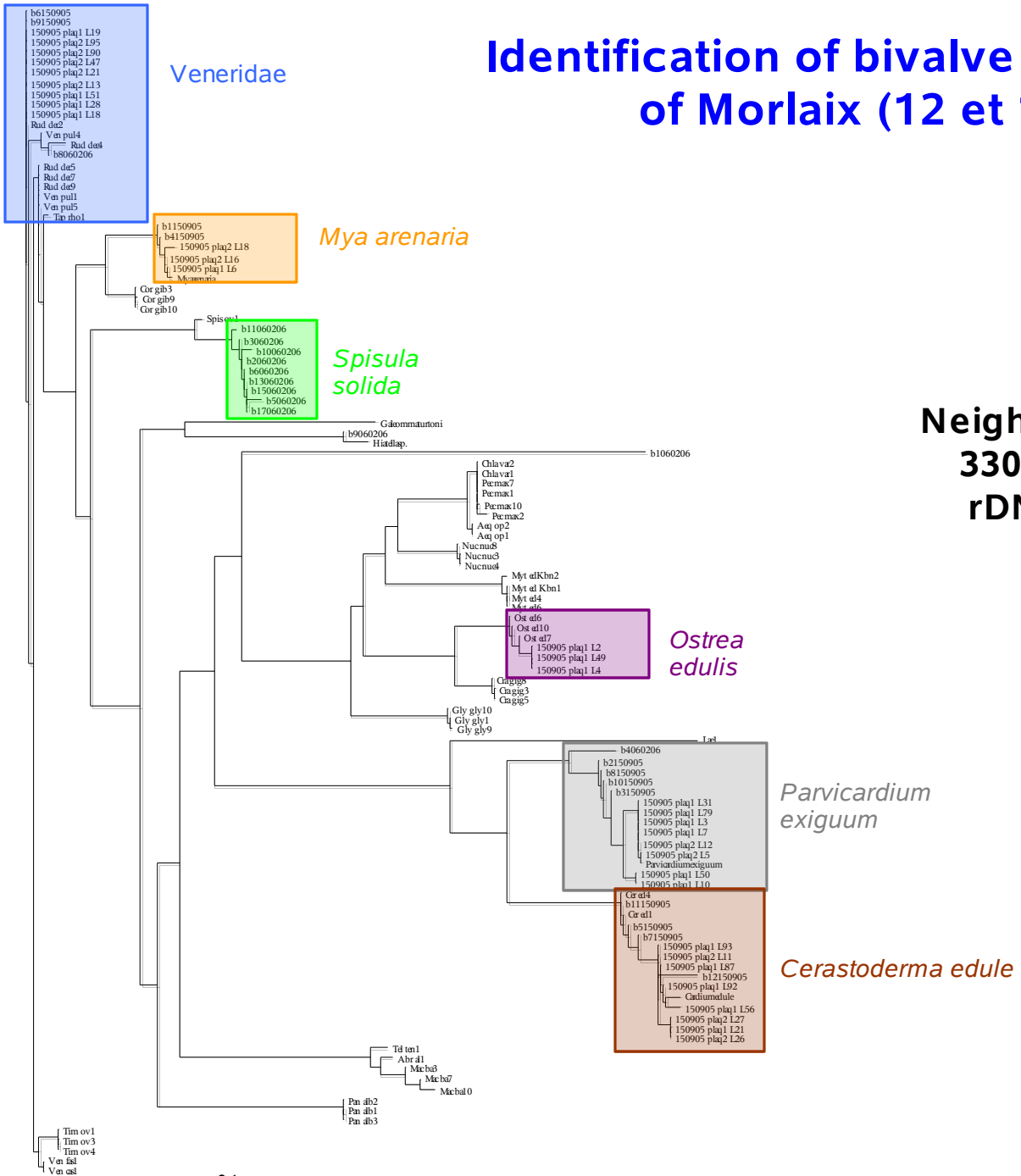
Neighbor-Joining tree from a 330 bp portion of the 18S rDNA gene – adults only

Amplification of the target sequences in larvae collected in the bay of Morlaix



Better amplification of the 18S rDNA gene
- Number of copies ?
- Specificity of the primers ?

Identification of bivalve larvae from the bay of Morlaix (12 et 19/07/05, 15/09/05 et 06/02/06)



Neighbor-Joining tree from a 330 bp portion of the 18S rDNA gene – adults and larvae

BUT…

415 larvae (fresh material)

190 positive PCR results for 18S (46%)

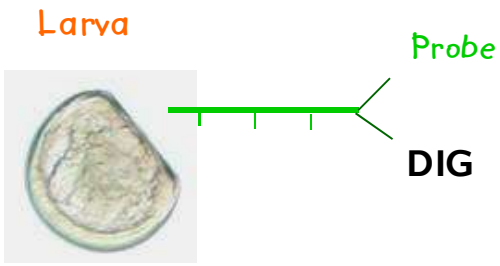
125 18S sequences (30%)

…/…

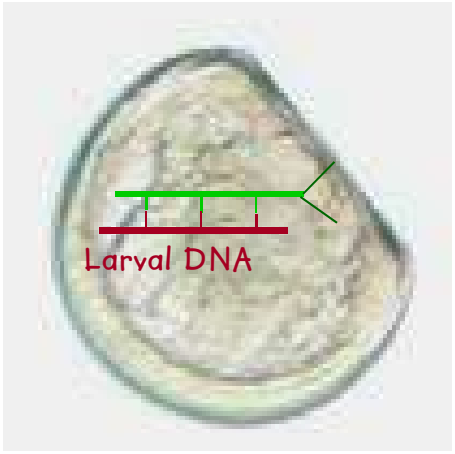
Development of a protocol of *in situ* hybridisation on the whole larva

✓ Target sequence: 18S rDNA

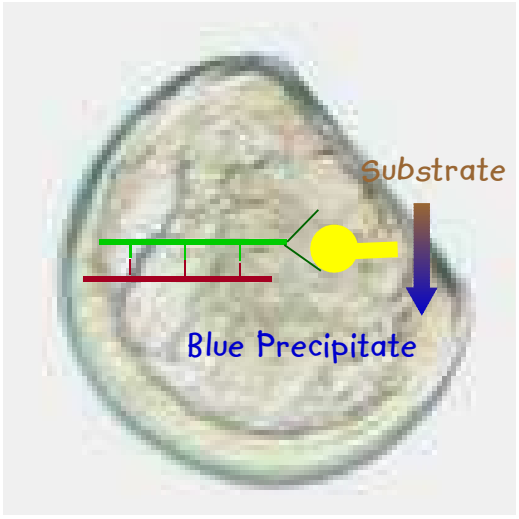
✓ Use of oligonucleotide 18S rDNA probes modified with digoxigenin, with colorimetric detection



Penetration of the probe within the larval cells



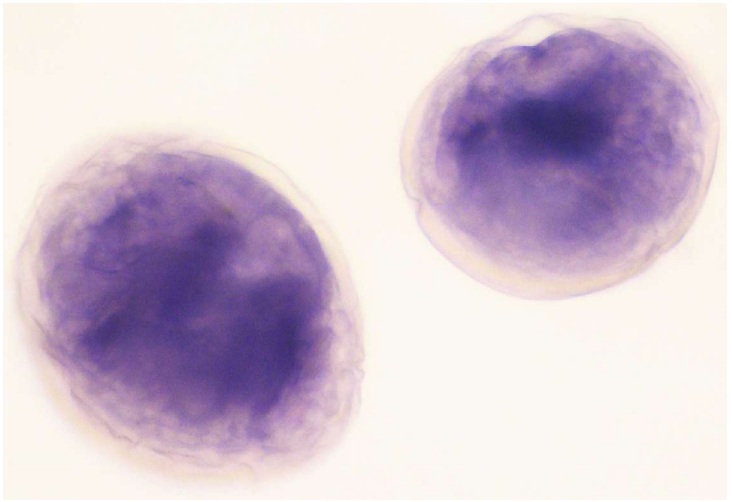
Specific hybridisation



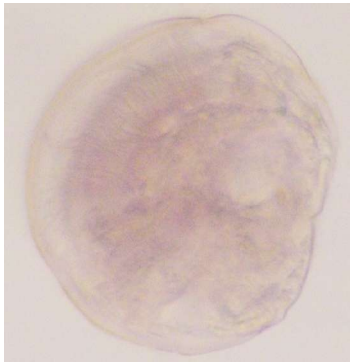
Colorimetric reaction

➢ Test of the method by using universal 18S probes and negative controls

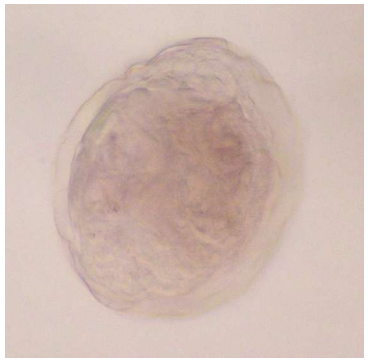
Universal eukaryote probe



Negative probe



No probe



➢ Design of 12 specific probes from a database of 196 18S partial sequences from adult bivalves

Species-specific

- *Cerastoderma edule*
- *Corbula gibba*
- *Crassostrea gigas*
- *Macoma balthica*
- *Ostrea edulis*
- *Pandora albida*
- *Tellina tenuis*

Genus-specific

- *Glycymeris*
- *Mytilus*
- *Nucula*

Family-specific

- Pectinidae
- Veneridae

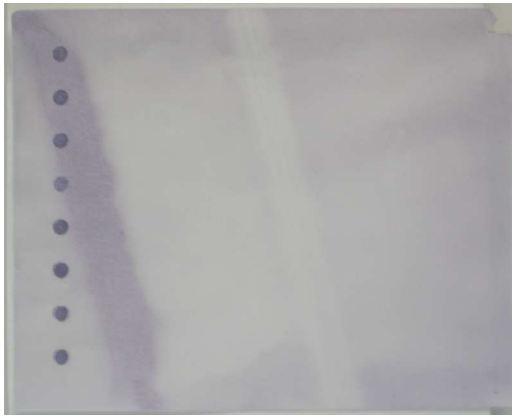
➢ Specificity tests of 9 probes through dot-blots

Total 18S PCR products from 77 adult individuals (18 species)

Myt ed	Nuc nuc	Ost ed	Cra gig	Ven pul	Tap rho	Mac bal	Cer ed	Gly gly	Pec max	Tel ten
Myt ed	Nuc nuc	Ost ed	Cra gig	Ven pul	Rud dec	Mac bal	Cer ed	Gly gly	Pec max	Cor gib
Myt ed	Nuc nuc	Ost ed	Cra gig	Ven pul	Rud dec	Mac bal	Cer ed	Gly gly	Pec max	Lae cra
Myt ed	Nuc nuc	Ost ed	Cra gig	Ven pul	Rud dec	Mac bal	Cer ed	Gly gly	Pec max	Abr alb
Myt ed	Nuc nuc	Ost ed	Cra gig	Ven pul	Rud dec	Mac bal	Cer ed	Gly gly	Pec max	Spi ov
Myt ed		Ost ed	Cra gig	Tim ov	Rud dec	Mac bal	Cer ed	Gly gly	Pec max	
Myt ed		Ost ed	Cra gig	Tim ov		Mac bal	Cer ed	Gly gly	Chl var	
Myt ed		Ost ed		Tim ov				Gly gly	Chl var	

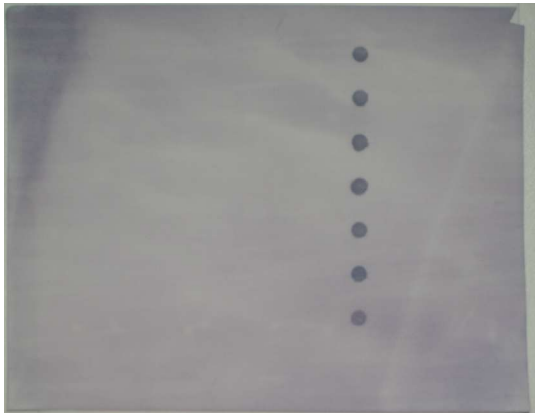
Some examples

***Mytilus*-specific probe**



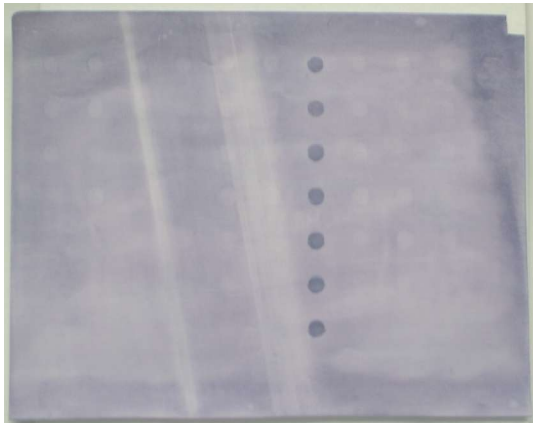
Mytilus edulis

***Cerastoderma edule*-specific probe**



Cerastoderma edule

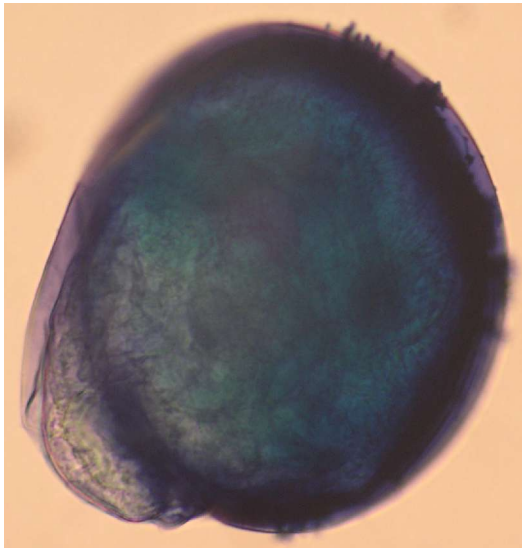
***Macoma balthica*-specific probe**



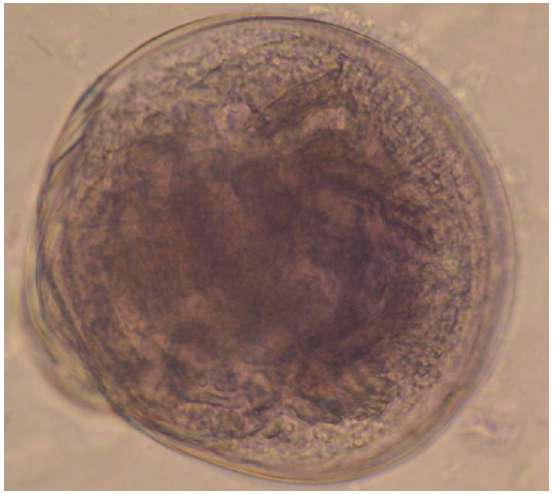
Macoma balthica

➢ Test of the specific probes on whole larvae of known species (obtained from laboratory fertilization)

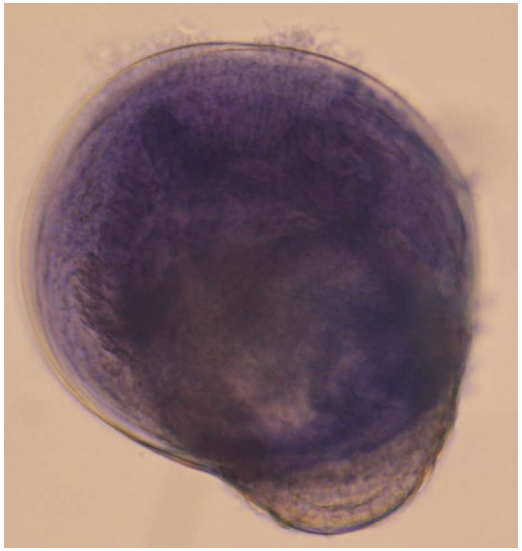
- ***Crassostrea gigas*** **larvae**



Universal probe

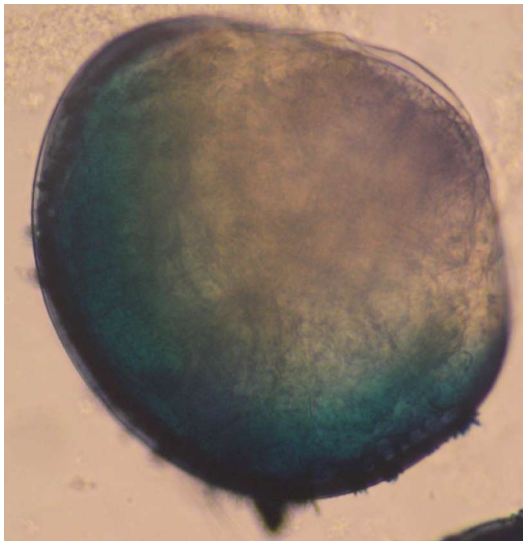


Negative probe

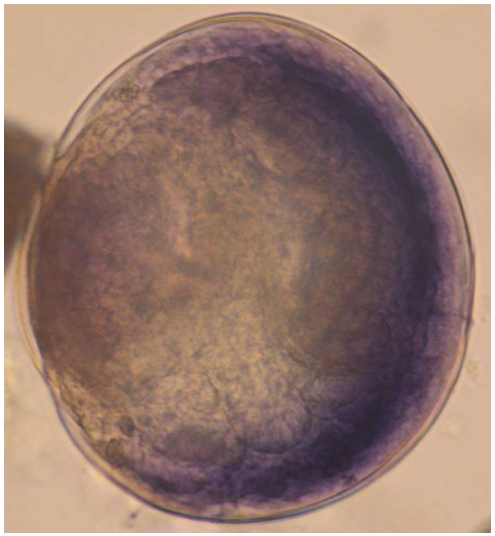


***C. gigas*-specific probe**

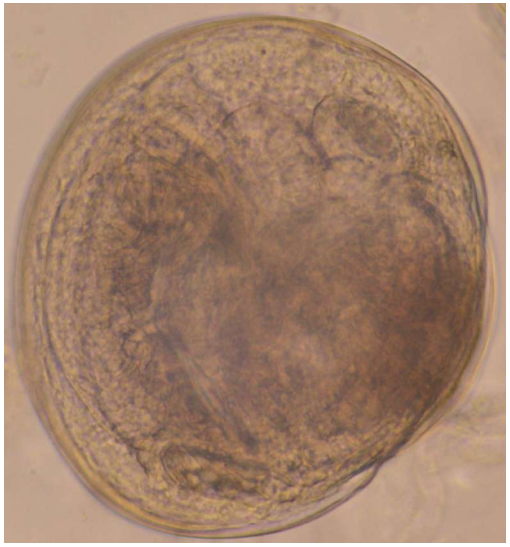
- ***Mytilus edulis*** **larvae**



Universal probe

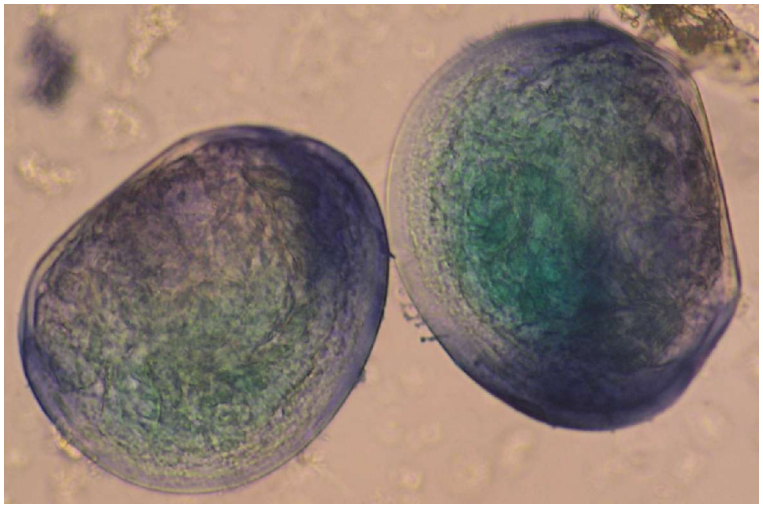


***Mytilus*-specific probe**

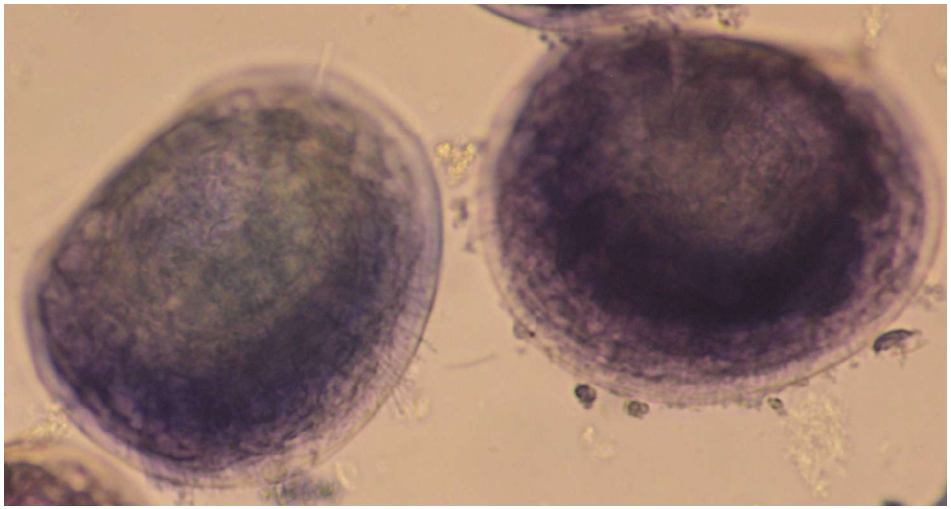


Negative probe

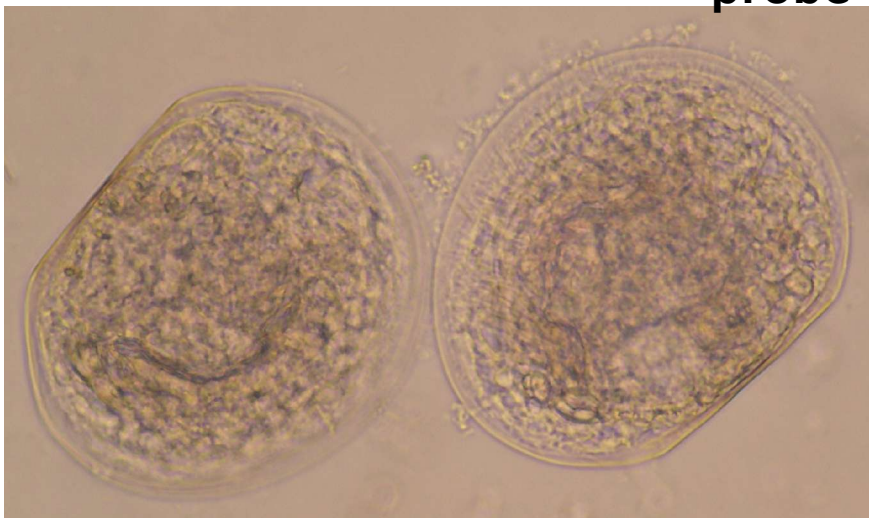
- ***Pecten maximus*** **larvae**



Universal probe



Pectinidae-specific probe



Negative probe

➢ Tests on field-collected larvae