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Working

Collect of Amoebophrya parasite (free-living stage) for genomic and transcriptomic analyses

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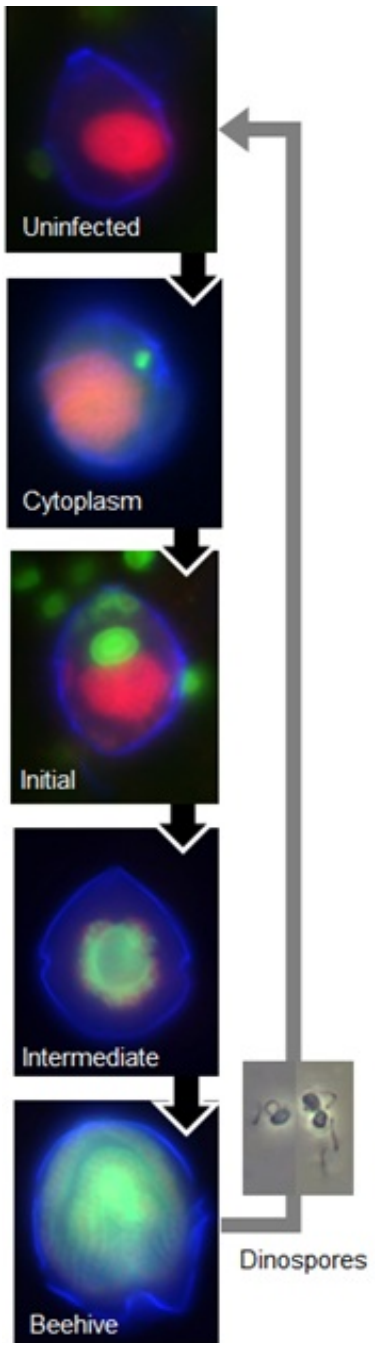
ABSTRACT

Amoebophrya (Syndiniales or MALVII, Alveolata) is an eukaryotic parasite which infect and kill its host.

Parasitic cultures are therefore particularly rich in bacteria which develop by degrading the organic matter released during the infection.



Photos: Catharina Alves-de-Souza & Laure Guillou

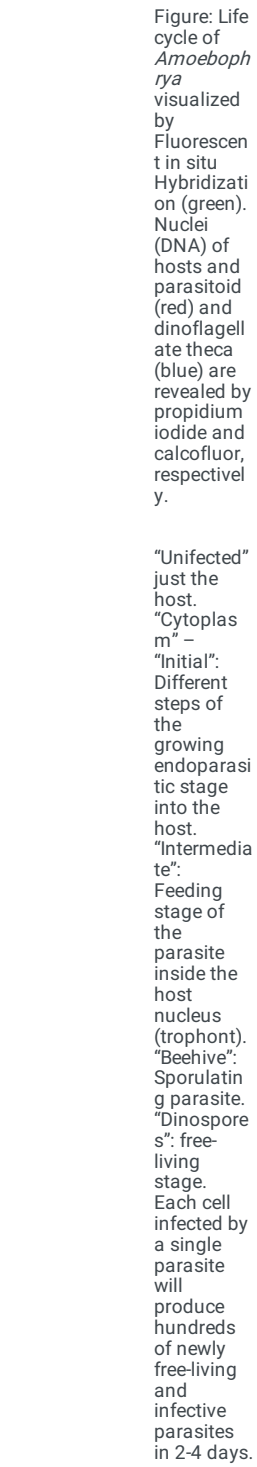


Figure: Life cycle of *Amoebophrya* visualized by Fluorescent in situ Hybridization (green). Nuclei (DNA) of hosts and parasitoid (red) and dinoflagellate theca (blue) are revealed by propidium iodide and calcofluor, respectively.

"Unifected" just the host. "Cytoplasm" – "Initial": Different steps of the growing endoparasitic stage into the host. "Intermediate": Feeding stage of the parasite inside the host nucleus (trophont). "Beehive": Sporulating parasite. "Dinospores": free-living stage. Each cell infected by a single parasite will produce hundreds of newly free-living and infective parasites in 2-4 days.

Strains of hosts and parasites come from the Penzé estuary (North-West of France, English Channel, 48°37'N; 3°56'W). A culture of the non-toxic dinoflagellate *Scrippsiella acuminata* (previously known as *S. trochoidea*, Kretschmann et al. in 2015) was previously established from the germination of a single cyst collected in 2005 from sediment. A culture of the non-toxic dinoflagellates *Heterocapsa*

triquetra was established after isolation of a single vegetative cell using a glass micropipette from water collected on July 6th 2007 at 27 of salinity. A monoclonal strain was subsequently obtained of each culture after the re-isolation of a single vegetative cell (names of strains: ST147 for *S. acuminata* and HT150 for *H. triquetra*). From natural samples, *Amoebophrya*-like parasites infecting dinoflagellates were detected by their natural bright green autofluorescence using an epifluorescence microscope (BX51, Olympus) equipped with the U-MWB2 cube (450- to 480-nm excitation, 500-nm emission, Coats and Bockstahler 1994).

Two reference strains (belonging to MALVII-Clade 2, nomenclature after Guillou et al. 2008) were selected :

- A25 : a specialist parasite belonging to sub-clade 1 originated from Penzé. A single infected *Scrippsiella sp*. cell in the late-stage of infection was picked from water sample collected on June 15th 2009 and incubated into exponentially growing *S. acuminata* (primary host ST147). This strain is currently maintained in culture with the host ST147 and was purified 6 times by isolating one infected host in late-stage replace into 5 ml exponentially growing healthy host strain into 24 well plates.This parasite is additionally maintained concomitantly with ST161 (*S. acuminata*) since the 26th of March 2012 .

- A120 : a generalist parasite belonging to sub-clade 4 originated from Penzé. A single infected *Heterocapsa triquetra* cell in late-stage of infection was picked from water sample collected on June 13th 2011 and incubated into exponentially growing *H. triquetra* (primary host HT150). This strain is currently maintained in culture with the host HT150 (parasite is named A120HT) and was purified 3 times (same protocol as described previously). It is also maintained with *S. acuminata* ST147 (parasite is named A120ST) since the 23th of April 2012.After a series of cycles in their primary hosts, the strain maintain in *H. triquetra* was successfully transferred and maintained in *S. acuminata* (ST147).

All strains have been deposited at the Roscoff Culture Collection, http://roscoff-culture-collection.org/, with the following ID numbers: ST147=RCC1627, HT150= RCC3596, A25= RCC4383, A120= RCC4398).

Principle / objective:

- We should synchronise the infection of the *Scrippsiella* hosts to have a mass production of dinospores at the same time
- We should remove bacteria on the beginning of the infection and limit their growth after
- We should collect the free-living stage of the parasite (dinospores) without hosts cells or large cells debris released from hosts (especially genomic material)
- We should extract genomic DNA for genomic analyses
- We should extract total RNA without residual DNA for transcriptomic analyses

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

protocol EB-BM-MO-009

MATERIALS TEXT

Flasks :

Flacons T-150 CytoOne ®, treated, ventilated Green – Starlab - Ref CC7682-4815

Flacons T-225 CytoOne®, treated, ventilated – Starlab – Ref CC7682-4822

Flacons T-75 CytoOne®, non-treated , non-ventilated – Starlab – Ref CC7672-4175

Filtration system : Nalgene PSF 250ml diameter 47mm

Erlenmeyer 250 and 500ml

Tweezer Millipore

Filters Nylon 10µm diameter 47mm - Millipore – NY1004700

Filters PC Whatman 10µm diameter 47mm – VWR – Ref 515-2089

Filters PC Whatman 5µm diameter 47mm – VWR – Ref 515-2087

Filters PC Whatman 3µm diameter 47mm – VWR – Ref 515-2086

Filters PC nuclepore Whatman 0.6µm diameter 47mm – VWR – Ref 515-2082

PNS 100x (Penicillin 5000units, Neomycin 10mg, Streptomycin 5mg) – Sigma – Ref P4083

Guillard's (F/2) Marine Water Enrichment Solution 50 × - Sigma – Ref G9903

Glutaraldehyde solution grade II 25% – Sigma – Ref G6257-10X10ML

Pluronic –Sigma – Ref P5556-100ml

SyBrGreen – Life technologies – Ref S7585

SAFETY WARNINGS

1 Host and parasite cultures

- Media

Infected and uninfected host cultures were maintained in F/2 medium (Guillard's Marine Water Enrichment Solution, Sigma), using filtered and autoclaved natural seawater from the Penzé estuary collected in June one year before at the 27 of salinity and stored at dark. After preparation, the medium was complemented with 5% (v/v) soil extract (Starr and Zeikus 1993). A final filtration using a 0.22 µm pore size filter was processed under sterile conditions.

- Stock cultures conditions

All stock cultures were maintained at 19°C and on a L:D cycle of 12:12 h at 80 µEinstein m2 s−1. Stock culture of the host is produced using non-ventilated flasks by transferring the host culture into fresh medium (ratio 1:1) every 3-4 days. Stock cultures of the parasite were propagated using 10mL culture flasks, by transferring 300 µL of infected host culture into 3mL of exponentially growing uninfected hosts every 3-4 days.

- *Scrippsiella acuminata* ST 147 Host culture

Inoculate the host culture (J3-J4) in fresh media in ventilated flasks by transferring 3/4 of media and 1/4 of host growing exponentially. Transferring should be done every Monday and Friday to maintain and increase the host culture volume.
Incubate at 21°C, light 12:12.

- Parasite *Amoebophrya* culture

Inoculate the parasite culture (J3-J4) by transferring 1/10 of parasite culture in host J3-J4 culture.
Seeding every Monday and Friday to maintain and increase the culture volume.
Incubate at 21°C, light 12:12.

2 Samples for cytometry

Take 1ml of each flask for each step (T0: Host and dinospores, J1 before and after filtration, J4 before and after filtration) and place in cryotubes.
Prepare a mix 10: 1 glutaraldehyde / pluronic
Fix sample with 1% glutaraldehyde/pluronic (10µl for 1 ml sample).
Incubate 5 minutes at 4°C in dark then place cryotubes at -80°C.
Samples will be analysed by flux cytometer to determine *Scrippsiella* concentrations (natural fluorescence), dinospores and bacteria concentrations (after the DNA staining by SyBr Green).

3 First step : Filtration of dinospores and initiation of the culture (T0)

This step should be adapted to the growth of the mother culture strains. It was realized 72h after initial inoculation for A25, 76h after for A120.

- Microscopic observation

Cultures are observed by epifluorescence inverted microscopy, under a450nm blue filter.
Host stocks: *Scrippsiella* culture must to be dense and still dividing (exponentially growing).
Parasite stocks: Dinospores must be abundant and freshly liberated in order to keep their virulence intact (still swimming)

- Filtration on PC 5µm filter

Install sterile filtration funnels on vacuum flasks.
Place a PC 5µm filter on the funnel and close the system with the suitable clamp.
Filtrate *Amoebophrya* culture by gravity (max 150ml per filter).
When the filter is under saturation, it should be removed (and destroy by autoclaving) and replaced by a new filter.
Check the absence of host cells using inverted microscope (facultative).
Several filtration devices could be used simultaneously. Pool all filtrates to homogenize.

- Initiation of infections (T0)

In a 600ml ventilated flask, add 300ml of host culture together with 150ml of the previous filtrate (parasite) (ratio 1 vol dinospores : 2 vol host).
Incubate at 21°C, light 12:12.

4 Second step : Physical removal of bacteria by 10µm filtration (~J1)

Bacteria and dinospores have more or less similar size (picoplanktonic size), and cannot be easily separated by filtration. Infected hosts can be physically separated from bacteria by filtration, but we should wait longer enough to ensure that all host cells have been infected.

This step was realized:
26h after mixing host and dinospores in A25
15h after mixing host and dinospores in A120

Filtration should be done at earlier stages of infection (later stages are more fragile and host membrane can disrupt during filtration).

- Microscopic observation

Microscopic observations using epifluorescence inverted microscope (450nm blue filter) can ensure that
1) most dinospores disappeared (supposedly because they initiated infections),
2) parasites cannot be still visible (not develop enough to have strong autofluoresecnce, thus at the beginning of the infection).

- First 10µm Filtration

Prepare some 200ml flasks with fresh media for rinsing.

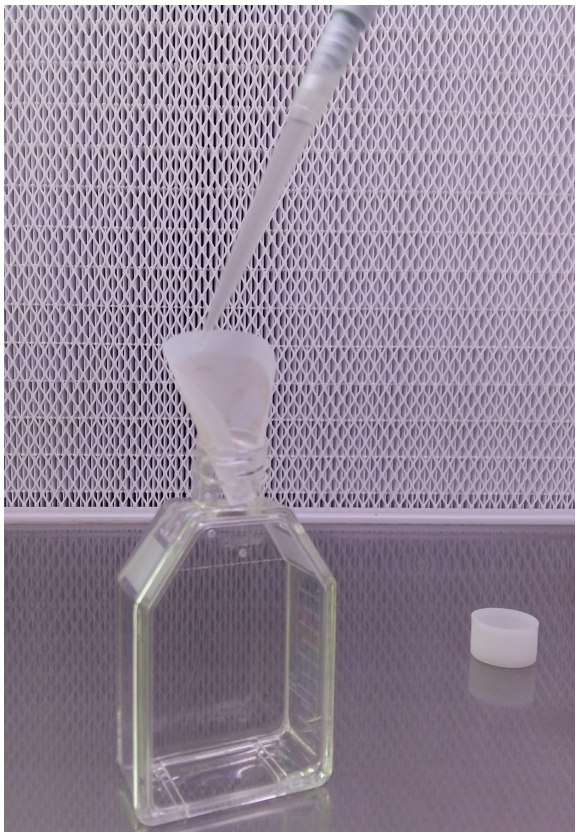
Install sterile filtration funnels on vacuum flasks.
Place a Nylon 10µm filter on the funnel and close the system with the suitable clamp.
Filtrate culture by gravity (max 125ml per filter).

Picture : Filtration on Nylon 10µ filter with a Nalgen fitration system

Using an ethanol cleaned tweezer, remove the filter and place it on the opening of a 50ml centrifuge tube.

Pipette fresh medium using a P1000 micropipette to remove host cells from the filter.

Rinse the filter in the centrifuge tube.



Picture : Rinsing of the filter with fresh media to remove infected host cell and put them in culture

When all host cells have been removed from the filter, throw it into an autoclave bag.
Observe the filtrate using inverted microscope in order to check that there are not too many host cells in the filtrate (optional).When the filter is cleared of the cells, throw it into an autoclave bag.
Observe the filtrate using inverted microscope in order to check that there are not too many host cells in the filtrate (optional).

- Second 10µm Filtration

Prepare some 200ml flasks with fresh media for rinsing.

Install sterile filtration funnels on vacuum flasks.
Place a Nylon 10µm filter on the funnel and close the system with the suitable clamp.
Realize a 2nd filtration by gravity of the « infected » host cells obtained previously.
Add fresh medium to rinse the tube and transfer in the filtration system to ensure that all cells have been collected.
Add approximately 100ml fresh medium on each funnel to remove bacteria physically. If saturation occurs, split your sample in two and use another filtration system.

Using an ethanol cleaned tweezer, remove the filter and place it on the opening of a 50ml centrifuge tube.
Pipette fresh medium using a P1000 micropipette.
Rinse the filter in the centrifuge tube.
When all host cells have been removed from the filter, throw it into an autoclave bag.

Put 350ml fresh medium in each 600ml ventilated flask.
Add the 30 ml containing « infected » and cleaned *Scrippsiella* cells in each of these flasks.
Complete the volume to 400ml final volume using fresh medium.

- Adding of antibiotic (PNS 100x)

In order to inhibit the growth of the bacteria in the culture, a cocktail of antibiotics is used (PNS at a final concentration of 5x).
Add 20ml PNS 100x to 400ml culture.
Incubate at 21°C, light 12:12

Be careful: PNS is stored at -20°C (100ml bottle or 15ml aliquots): Do not forget to place antibiotic at 4°C at the beginning of the experiment.

5 Purification of the parasite culture (~J4)

This step is realized 3 days after the bacteria removal.

- Microscopic observation

Observe the culture using epifluorescence inverted microscope (450nm blue filter) to check the presence and abundance of dinospores and the low presence of host cells.

- Filtration on 3µm filter

Install sterile filtration funnels on vacuum flasks.
Place a PC 3µm filter on the funnel and close the system with the suitable clamp.
Filtrate culture by gravity (max 100ml per filter).

Be careful, 1 filter per system should be used. Use a new sterile system for each new filtration to avoid contamination.
For one flask containing 420 ml, 4 filtration systems are required. Filtrate should be pooled at the end in a container.

When filtrations are ended, distribute 2 x 3ml of each filtrate in a 6 wells plate for microscopic observations.
Check the absence of host cells in the filtrate using inverted microscope. If a single host cell is observed, the sample should be filtrated again using a new sterile system. This step is important to prevent contamination by host genomic material (dinoflagellates have large genome size).

Pool all filtrates coming from the same initial flask together.

6 Collect of the parasite free fraction (~J4) for genomic approaches

Using PSM:

Install sterile filtration funnels on vacuum flasks.
Place a PC 0.6µm filter on the funnel and close the system with the suitable clamp.
Spread the filtrate into 2 filtration systems (approximately 200ml per filter).
Filter by gravity, helping by a hand pump if needed.

The 1st filter is placed in 15ml Falcon correctly labelled tube (the tube is placed on ice while the 2nd filtration ends)
Place le 2nd filter in the same 15ml Falcon tube.
Then flash freeze the falcon tube and place at -80°C.

NB1: in the case where the filtrations take longer time (max 30 minutes) , it is preferable to put a filter per tube and flash freeze each sample as soon as they terminated.

NB2: dinospores can be also centrifuged in 50ml Falcon tubes at 11000rpm 20 minutes 18°C.

7 Collect of the parasite free fraction (~J4) for transcriptomic approaches

Using chemical hood:

Add 4ml of trizol in 15ml falcon tubes.
Wrap them in aluminium foil to keep them in the dark and place it on ice.
Install sterile filtration funnels on vacuum flasks.

Place a PC 0.6µm filter on the funnel and close the system with the suitable clamp.
Split the filtrate into 2 and use separate filtration systems (approximately 200ml per filter).
Filter using a vacuum pump (<100 mm Hg) to avoid the host cells break (degradation of RNA).
Stop the filtration just before the filters let dry.

Fold the filters in 4.
Place it in a tube containing trizol.
Wait 1-2 minutes by mixing by hand so that the cells are all in trizol.
Then flash freeze the falcon tube (5 min) and place at -80°C.

NB: It is important to process the two filtrations in less than 15 minutes to avoid RNA degradation.