General recommendations for Tunicates Phylum

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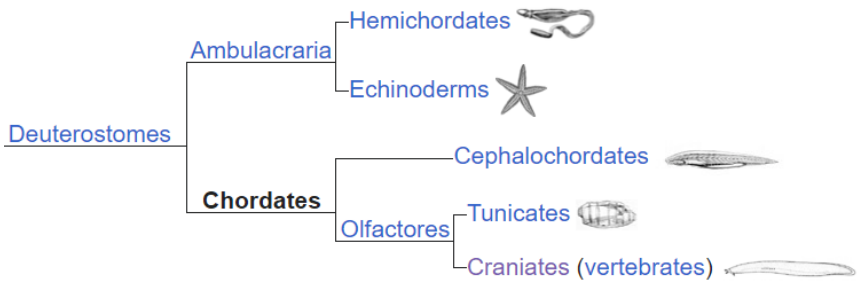
Tunicates constitute one of the subphyla of the phylum Chordata, alongside Cephalochordates and Vertebrates. The inclusion of tunicates within the phylum Chordata is characterized by the presence of a dorsal notochord in their larval forms. This characteristic disappears in sea squirts (ascidians) after the larva becomes fixed.

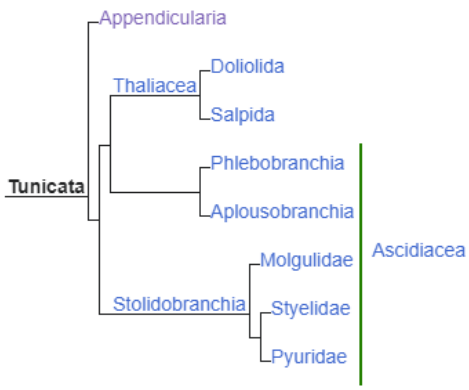


Tunicates are divided into three classes:

The class **Appendicularia** includes planktonic species that retain their dorsal cord in the adult stage.

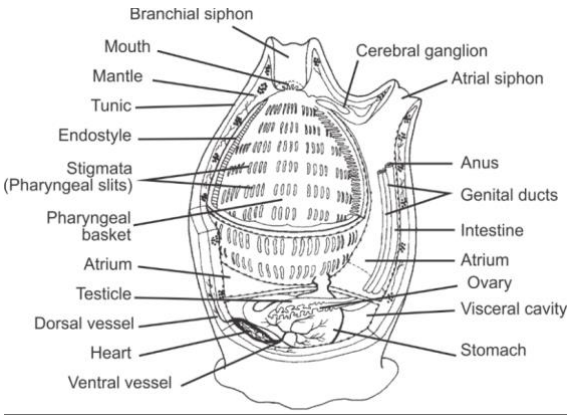
The class **Thaliacea** comprises the orders Doliolida, Pyrosomatida, and Salpida, which are also planktonic species.

The class **Ascidiacea** includes the orders Aplousobranchia, Phlebobranchia, and Stolidobranchia, which are exclusively sessile species.



Overview of Ascidians

Ascidians are sessile tunicates. They are exclusively marine, benthic, and sessile filter feeders, living either alone or in colonies. Approximately 3,000 species have been recorded worldwide, with around 350 species found in Europe.



Recognizing Ascidians in the Environment

Depending on the species, ascidians can be found either as solitary individuals or in colonies:

- **Solitary Forms:**

Each individual is independent, although some species may develop close to or even on top of one another. They have the characteristic "flask-like" shape with two siphons, one for inhaling and the other for exhaling, and they live attached by their base or on one side.

- **Colonial Forms ("Social Forms"):**

The individuals are connected by a stolon or develop in clusters. The thorax and abdomen of the zooids are not enclosed in a common tunic. These are clonal colonies.

- **Colonial Forms Grouped in a Common Cormus:**

These colonies can be fleshy, lobed, stalked, or encrusting. The zooids are enclosed within a common tunic called the cormus. The zooids measure approximately 1 to 20 mm, while the colonies can range from a few millimeters to several decimeters in size. The colonies develop by budding from a mother individual and are clonal colonies.

Where to Find Ascidians

Generally, each species is adapted to a specific environment—rock, algae, sand, etc. Some species are found in the intertidal zone, while others, which are subtidal, are observed only while diving.

In the intertidal zone, ascidians are found in places sheltered from heat, primarily protected from direct sunlight: under rocks, overhangs, or algae mats. In submerged areas, they live on (and under) rocks, on algae, on and in sand or other soft substrates, and on other organisms.

Sampling

Generally, each species is adapted to a specific environment—rock, algae, sand, etc. Some are found in the tidal zone, while others, being subtidal, are observed only during diving.

In the tidal zone, they are found in places sheltered from heat, mainly protected from direct sunlight: under rocks, under overhangs, or within algae mats.

In submerged areas, they live on (and under) rocks, on algae, on and in sand or other soft substrates, and on other organisms.

After sample arrival and sorting, organisms should be kept in in **running water** without exposing them to excessive heat, light and disturbance in general. Temporary use of the aquarium with air pump is possible if specimens cannot be processed in short times (especially for RNA extraction). At this step, specimens are identified with:

- sampling date
- station number
- name species / taxon
- "GENOME" label (to indicate that this specimen will follow the ATLASea cold chain)

After trawling or dredging, the specimen may be in poor condition, it must be processed as a priority.

The hand-collected specimen must be complete.

Photography

Always when possible take pictures of the specimen in the natural environment while sampling
Take pictures of the organisms upon arrival.
Ideally, images should be taken in the highest quality resolution (macro lens recommended) and where no voucher specimen parts are retained the pictures will serve as voucher and should include identifying features.
Specimen will be photographed in a glass container with sea water.
Water should be clean and changed between each specimen.
If possible, discuss with the taxonomist to find out the important morphological elements to see, and therefore to photograph.

Wait for the siphons to open to take the picture. Make as few movements and tremors as possible while taking photos so that the siphons don't close.

Take a general photo of the specimen or colony.

Take closer look of the specimen or colony.

For solitary ascidians **take picture of the ventral dissection** before the tissues are recovered for genomic samples. This can help in the posteriori identification.

(photo : Bay-Nouailhat)

With the specimen, one picture is taken with a **scale**, the **code identifier** (e.g. ATLASea QR code, specimen **MNHN-IT** barcode) and the station label.

Dissection for DNA barcoding and Genome Sequencing

Unitary or solitary ascidians

Solitary ascidians belong to different Acidiacea orders but they all share the same characteristics relative to sample processing. They are generally bigger in size and easier to manipulate than colonial ones, and each individual represents a single genetic entity. For this reason, always keep comparative material of the presumably same species as they are going to be irremediably damaged during the process.

/!\ **Never** squeeze ascidians to find the siphon, this can damage the specimen and sometimes identifications are made by observations of the internal organs after dissection.

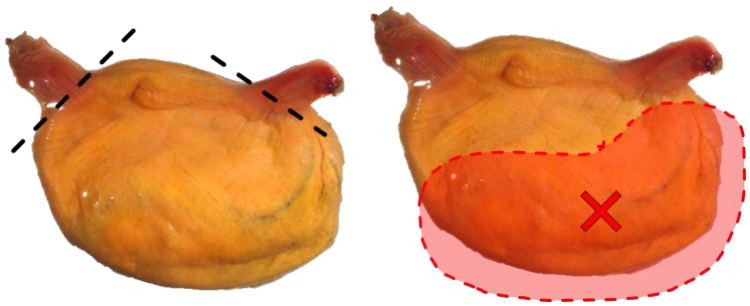
They are usually characterised by a thick tunic.

The tunic is mainly constituted by cellulose derivates resisting to protein digestion. It is also poor in DNA content. For these reasons, it has to be removed.

Depending on the species, the tunic can be heavily epiphyted:

1. Before removing the tunic, brush and eliminate most of the associated fauna, as it can be a source of contamination during dissection;
2. Remove the tunic as shown in the separate identification document;

3. **If the ID is (supposedly) known:**
    - Place the body mass on a sterile petri and cut with a scalpel the two siphons at their base;
    - Avoid taking tissues from the lower part of the body if not dissected (potential contaminations by gut or filtered particles, microbiota).

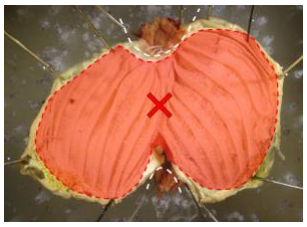


(photos : R. Virgili)

4. **If the ID is NOT known:**
    - Perform ventral dissection and identification;

(photos : R. Virgili)

- Cut the siphons at their base (white dashes line), trying to avoid the branchial sac and the gut (potential contaminations by gut, filtered particles, microbiota). In general, avoid any pigmented area, including the gonads. Gonads can be used in known species and groups with non-pigmented gonads (e.g. some Styelidae, Molgulidae);



(photos : R. Virgili)

- Avoid "gonads" when the species is not known. Some species can keep larvae and embryos in larval pouches similar to gonads. These represent actually different genetic entities and should be avoided;

5. Remove the internal tunic present in the siphon (siphon tunic). The external tunic goes down into the siphons and its covering epithelium is directly in contact with the exterior (potential source of contamination). The siphon tunic is much thinner than the external one and it is easy to remove. It looks like a thin coriaceous sheet, often perlaceous in colour, that can be peeled with the help of forceps;
6. Keep count of the isolated material. In small specimens, this could not be enough to cover all the required sub-samples for the following sequencing steps;
7. Once isolated, split and process the samples according to the organizer's requirements (number of replicates, weight in mg).
8. Depending on species and condition, tissue sampled may be the **mantle**, **gonoducts** (avoiding progeny) or **siphon**.

In large-bodied species with storage gonoducts (e.g. Ascidiidae, Ciona) it may be possible to collect substantial quantities of ***sperm*** *from the sperm duct (white, alongside rectum); if intending to use sperm from duct, maintain specimens in constant light to prevent spawning. Most unitary ascidians spawn eggs and sperm, but some (e.g. Corella eumyota, Dendrodoa grossularia, Asterocarpa humilis and a few other styelids) brood their sexual (generally outcrossed) progeny in the atrial cavity.* ***Avoid these progeny when gathering tissue for sequencing****.*

9. Dissect :

a. **10 pieces** (approx. **300 mg** each). Cut each piece into smaller before putting them in separate tubes (with unique identification labels) ⇒ for flash-freezing in liquid nitrogen.

10. Weight the tubes and scan the barcode on the log sheet.

Colonial ascidians

All three orders of Ascidiacea include colonial forms. Each colony, or system, is made by several small zooids, usually embedded in a common tunic matrix. A colony expanding asexually represents a single genetic entity.

Main challenge with these ascidians are gut and external contamination, as well as DNA yield.

Species creating flat colonies carry part of the substrate and debris after collection (potential external contamination).

Moreover, zooids are usually very small, and are almost impossible to isolate clean muscle tissue as for the solitary ascidians (potential contamination from gut and microbiota).

Colonies can be kept for longer time in aquarium for starving to reduce gut contamination, but this procedure is not recommended to avoid undesired RNA decay.

Blood can be extracted from the colonies and enriched for better yields, but this advanced technique will not be treated in this guide (see Sumner et al. 2023).

<u>In both cases the ID of the colony is known or not:</u>

1. Subsample part of the colony to be processed for morphology. This requires previous knowledge on morphological identification and fixation (see separate document);
2. Clean residual substrate and the associated fauna with a scalpel and place the cleaned colonies in a sterile petri dish;
3. As a rule of thumb, isolate around 20 zooids from the same system. In some groups (e.g. Polyclinidae, Holozoidae) zooids are sparse and embedded in a coarse matrix and they are easy to remove. The tunic can be cut with a scalpel. In other groups (e.g. Didemidae, Styelidae) getting completely rid of the tunic is unfeasible, and zooids may carry some tunic;
4. . Be careful with brooding species (e.g some Polyclinidae). Some species keeps embryos in specific compartments. These should be removed before extraction;
5. Extractions should be done on single zooids to avoid chimeras or mixing systems (Casso et al. 2019). However, to increase extraction yields, multiple zooids of the same system are commonly put in the same tube and extracted together (Salonna et al. 2021). Check the preferred extraction procedure and communicate with the organizers;
6. In some groups (the once called "social" ascidians), the zooids are exposed resembling solitary ascidians. These are connected by common stolons. Usually, zooids are larger than flat colonial ascidians and can be processed similarly so solitary ones (see previous section). Be careful to work on individual systems;
7. Once isolated, split and process the samples according to the organizer's requirements (number of replicates, weight in mg).
8. Weight the tubes and scan the barcode on the log sheet.

Backup/Biobanking:

1. Dissect at least 1 and up to 10 pieces in separate tubes (with unique identification labels).
2. 10 tubes by specimen
3. Tubes should be flash-frozen in a liquid nitrogen.

Voucher & Taxonomic Assignation samples:

Voucher will be storage at MNHN.

1. Keep the leftover organism, as many parts/tissues as possible or another individual from the same population and checked by a taxonomist as belonging to the same species.
2. Place the barcode **MNHN-IT** identifier and the station label with the organism in tube/container.
3. Put 75-80% ethanol in the tube/container. There must be 10 times the volumes of specimen in alcohol.
4. Some exceptions require storage in formalin.
5. Put the tube/container with the others specimens in the ATLASea barrels for shipment to the MNHN.

How to Preserve Ascidians for Dissection or Long-Term Conservation

Ascidians are very sensitive organisms that can contract strongly, making identification difficult. Throughout the process, care must be taken to minimize stress. This applies to all species, although solitary ascidians are generally less sensitive than colonial forms.

Before Collection:

If possible, take in-situ photographs. After the stress of collection and transport, ascidians will never display as fully as they do in their natural environment. For multiple collections, this also helps verify that the specimens are the same species. Take both overall and close-up photos to capture the siphons or individuals in a colony.

How to Collect:

Prioritize manual collection with support without touching the ascidian. Never squeeze the ascidian to expel water, avoid injuring it during collection, and do not expose submerged species to air. Place the sample in a numbered container. If the ascidian was photographed underwater, also photograph the sample container's number afterward. Specimens from dredging or shore collection should be placed in seawater as quickly as possible and then transferred to individual containers for relaxation and fixation.

Waiting for the Ascidian to Open Its Siphons:

Avoid any stress after collection: limit transport, avoid exposure to air, strong light, or heat. To encourage the opening of the siphons, the ascidian can be placed in an aquarium with circulating water or, if not possible, in a refrigerator to cool the water in the container. Once the ascidian is in the container for fixation, remove 20% of the seawater to make room for the anesthetic and fixative.

Wait for the siphons to open; a contracted ascidian will not open under the influence of the anesthetic.

Take Photographs (if not done in the natural environment).

Anesthesia:

The container should be emptied of 20% of the seawater to make room for the anesthetic and fixative. If not done previously, use a pipette to avoid moving the container, which could cause the siphons to close. Once the ascidian is relaxed, with open siphons, place a few pieces of menthol on the surface of the container holding the sample and seawater, and wait at least 20 minutes. Gently remove the menthol crystals (using a spoon), as they can be used for another ascidian, then add 2 to 3 drops of eugenol and wait at least 10 minutes before fixing the sample.

- **Other Anesthesia Techniques:**
  - ✓ **Cold Anesthesia**: Remove 30 to 50% of the water from the container. Relax with menthol for 1 to 2 hours, then place in the freezer for 24 hours. After 24 hours, pour formalin into the container, allowing it to diffuse during thawing.
  - ✓ **With Menthol Alone**: Wait about 1 to 2 hours before fixation; the ascidian is rarely fully anesthetized and reacts in the presence of the fixative.
  - ✓ **With Eugenol Alone**: The ascidian may contract when drops are added to the container, but the action is rapid, taking only a few minutes before fixation can proceed.
  - ✓ **With Cold Alone**: Place the container in the freezer until completely frozen, then pour in the fixative and let it thaw. Some species do not tolerate cold and may contract.

Fixing the Ascidians:

Ascidians are fixed with formaldehyde. It is important to protect yourself during this process by wearing a mask, gloves, and appropriate clothing. This operation should be performed under a fume hood or, if not available, outdoors. Place 7% formalin (38p/v) in the container with the ascidian and seawater; for large species, this should be repeated after 24 hours by replacing the initial solution with 7% formalin in seawater.

- **For Colonial Ascidians (Didemnidae)**: These species have calcareous spicules that are important for identification. Using regular formalin, which is very acidic, would destroy these spicules. Therefore, it is necessary to use buffered formaldehyde, which has a neutral pH. This formalin is often less concentrated (10p/v), but these species usually have very thin tunic tissues, so a 15% ratio should be added to the seawater. The fixation process can take several weeks, depending on the species.

Dissection for Identification

Most organs of ascidians are used for identification. Some species can be very similar, and only small details allow for distinction, such as the number of tentacles, rows of stigmata, or in some cases, the shape of the spinules surrounding the siphons. The criteria vary among groups, and for colonial species, the anatomy of the larva or the shape of the spicules are considered. A dissecting microscope or microscope is necessary for colonial species.

There are three morphologies:

- **Single-Part Form:** The branchial sac/pharynx occupies the entire body. The gonads, stomach, and intestinal loop are on the side.
- **Two-Part Form:** Thorax (with the branchial sac) and abdomen (stomach, gonads, intestinal loop).
- **Three-Part Form:** Thorax (with the branchial sac), abdomen (stomach, intestinal loop), and post-abdomen (gonad). In immature individuals, the post-abdomen may not be developed.

For Solitary (or Social) Ascidians:

- Cut the tunic along the ventral side from the buccal siphon to the atrial siphon.
- Similarly, cut the mantle and unfold the ascidian like opening a book.
- Pin it to the support.
- Take photographs of the opened individual (general view, details of the tentacles, pharynx, siphons, etc.).
- Carefully cut the branchial sac under the coronal arch to observe it separately.
- Take photographs of the individual (general view, details of the gonads, stomach, etc.).
- To observe the pharynx, stain with Masson's Hemalum.

For Colonial Ascidians:

- Cut the colony vertically.
- Extract the zooids from the common tunic.
- Stain them with Masson's Hemalum.
- Photograph the zooids.

References

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