

Protocol for collecting Killifish and other freshwater fish for diversity surveys

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Introduction

Many fish enthusiasts and amateur ichthyologists have been conducting collecting trips and making big collecting efforts in the past decades contributing to the knowledge of fishes. Nevertheless, there is not a standardised collecting protocol which can lead to deficient data availability or to poor preservation of specimens. Therefore, here we present a collecting protocol, mainly focused on preserving specimens for taxonomic studies, providing new records and obtaining tissue samples for molecular analysis.

Planning

The first step on any collecting expedition starts long before the collection with the planning of the trip. Good planning is key to any successful sampling trip. You should ask other people that have visited the area before and gather all the information available of the place you are visiting – roads, possible threats, accommodations. Try to detect which areas have already been explored and which areas are more promising. Be sure you go at the right time of the year. For example, the rainy season if you are intending to collect annual fish, as you don't want to find the ponds dry. If there are local researchers in the area, we strongly recommend that you contact them. Maybe you can go collecting together or, if not, they can provide you with useful data.

Collecting permits

In general, to go collecting in a country you need permission from the local authorities. You should contact them a long time in advance and ask what the regulations are and how to obtain the permits to collect fish, transport fish, transport tissue samples and export fish and tissue samples. Acquiring collecting permits generally requires presenting a detailed plan of what you want to achieve, what you expect to collect and where, if you know.

You are generally committed to present a final report with the results of your expedition. These are very important for local authorities as it is data they can use to manage and protect the fishes and their environment as that information is generally scarce. Also it is strongly recommended to deposit some specimens in a local fish collection in the country of origin of the fish. This sometimes is mandatory but, if not, we would suggest it as local collections are key factors for the local authorities to protect nature, generate conservation policies and wildlife management and to promote scientific studies in the country of origin. Local collections are one of the main ways of assessing local diversity and design adequate management and conservation programs for local governments, NGO and researchers so it is important to collaborate with them.

It is also worth noting that going to collect fish without permissions may result in the confiscation of your fish and equipment, fines and even penal consequences, so we strongly recommend that you obtain all pertaining permits before going to collect, aside from the importance of collaborating with local authorities to help them protect the wildlife.

Health

Be aware of local diseases, especially when travelling to tropical areas where you can contract malaria, yellow fever, chikungunya, etc. Take all precautions before travelling. In some cases you may need to take some pills or be vaccinated prior to departure.

What to take on a collecting trip?

Of course, besides adequate clothes for the place you are visiting, you should take some specific things to preserve fish and to take tissue samples:

- A field notebook in which you will register all data regarding the environments, locations and any relevant observations. This is very important. All important collectors have one. Some have been even published – Darwin’s, for example.
- Nets: For seasonal killifish which generally live in highly vegetated habitats, the best nets are generally hand nets and what is called a ‘frame net’ which consists on a rectangular frame with a net stretched across it.
- A camera: Good pictures of live specimens immediately after collection are very useful for taxonomic studies. We recommend that you take a camera with



Different types of nets for collecting fish in shallow water.

a good macro function and a good sensor. External flashes are a good option if available, but if not, a camera with a low f value (aperture) is recommended. High megapixels resolution will allow you to crop or scale the picture later in publishing software without noticeable reduction in image quality.

- A tripod: Sometimes a tripod helps you to remain still and get better pictures, reducing blur.
- A photography aquarium. The use of photography aquaria in the field is very important to get on-site pictures of adequate quality. These aquaria are generally narrow and deep with a moveable glass panel inside that allows you to immobilise fish at the front to get it parallel to the focus plane so most or all the fish is in focus. Use two clips to hold the glass panel in the desired position (see page 91). Herbert Axelrod’s design (published in his Mini-Atlas) is very effective: two panes of 8" x 8.5", two panes of 2" x 8", one of 2" x 8.5" and one pane of 8" x 8". 2 mm glass is adequate for this size tank.

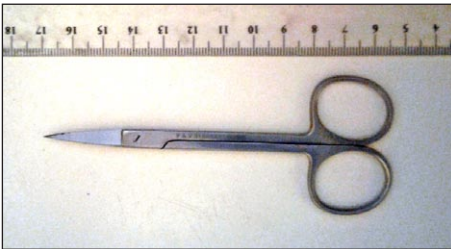
- A collapsable table and a bench. You will need somewhere to put the photography aquarium and to sit, if possible, to make work more comfortable.
- An 18% grey background. It can be a plastic laminate or similar to put behind the photography aquarium.

Fish preservation:

For this, you will need jars with a good lid that closes hermetically. In the field plastic jars are more practical. You will also require alcohol in the form of 96% ethanol (in general you can buy this in a pharmacy so you wont need to bring it on the plane, but check before you travel). Finally, a trusty pencil.

Tissue sampling:

For this you will need Eppendorf tubes, small pointed scissors, labels (these can be printed before travelling), 99.6% ethanol (also known as ‘absolute alcohol’), a pipette, scotch tape and a pencil and paper.



Small scissors for careful dissection.

What to register in the field?

You can make printed labels containing information and put them inside the jars, and duplicated, pasted outside the jar. Write with pencil, as it is not washed out by alcohol. Also copy the information in

your field notebook. Mark the number of the jar or collecting locality on the top of the lid with a permanent marker and in the jar. The following is the most important data to register in the field:

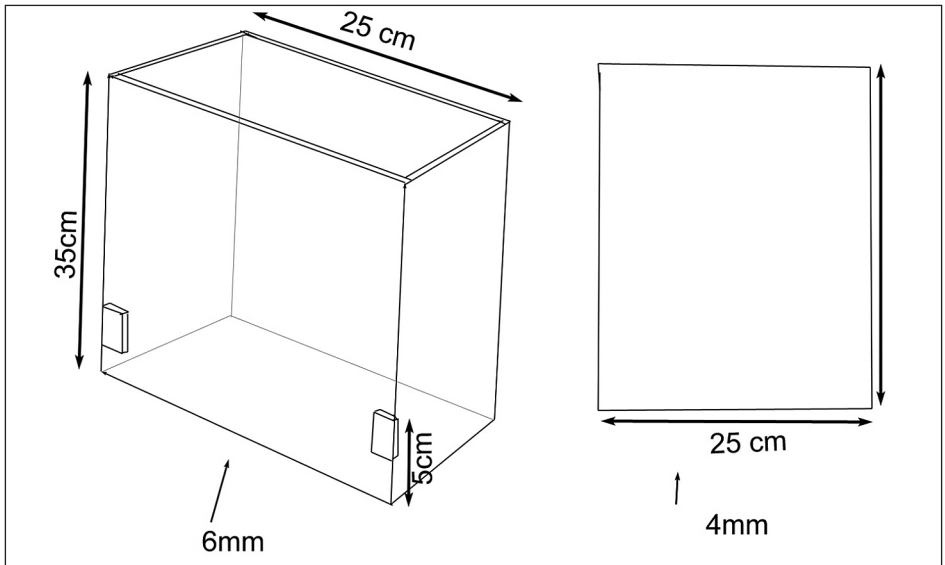
- Collecting point number (it is useful to identify each collecting point with a number).
- Locality (with GPS coordinates if available, otherwise enough information for others to find the location again).
- Drainage (the source of the body of water where the fish are collected).
- Date.
- Collectors names.
- Observations (depth, areas of the location, soil type, plants).
- Water parameters (pH, temperature, hardness, tds, conductivity)

Additional observations can be written in the field notebook.

How to photograph fish?

Pictures of live fish just after collection are extremely important since many species are differentiated by live coloration and markings.

A photography aquarium is mandatory for this purpose as described earlier. You can use some low doses of anesthetics (clove oil or menthol, for example) to reduce fish activity. Although for ‘artistic’ pictures you can use some green background (like the grass or vegetation surrounding you in the



Schematics for a photography aquarium. These can be cheaply constructed from 6 mm glass from a hardware store using marine silicone. Careful packing will be needed to ensure it doesn't break in transit. The thicker the glass, the tougher the tank will be.



Pablo Calvinño photographing fish in the field with a photography aquarium. Note the piece of glass to restrain the fish, limiting its movement so that it will remain in focus.

field) a grey background is better for scientific purposes to get a clean picture of the fish. Always be sure that the background is out of focus, as a blurred backdrop enhances the clarity of your photographic subject (the fish). Avoid introducing plants and other objects to the aquarium as they can make the water turbid and can also affect the focus point of the camera. We recommend, when possible, to take some clear water with you for the photography aquarium as sometimes the water in the collecting location can be turbid, reducing the quality of your photos.

It is recommended to practice some fish photography at home before going to the field to be sure that all these parameters can be easily managed and you don't waste time in the field that could be used to collect fish.

Animal euthanasia

There are several described methods to euthanise fish before tissue sampling and preservation of the specimens. It is most important to try to preserve the fish in the field. A detailed work on fish euthanasia by Neiffer, D. L., & Stamper, M. A. (2009) should be consulted. You can use some of the following options depending on the availability:

- Clove oil: Dilute at a ratio of 1:9 in ethanol, apply at 0.4 ml per litre to sedate. 2 ml per litre for euthanasia.
- Menthol: Dilute menthol pills in water and add to a receptacle containing the fish until no responses or ventilation is observed.
- Cold: In the field you can put the fish in bags with water in an icebox with ice. This is generally difficult in the field, as it

is difficult to store ice in transit, but it is another option to consider.

- Benzocaine: Dissolve 0.5g benzocaine in 42 ml ethanol. Add this to 5 litres of water from the collecting locality. This yields a benzocaine concentration of 100 mg/l, which is the recommended dose for fish euthanasia.
- 2-phenoxyethanol at 0.1%. Immerse the fish in this solution for a couple of minutes until no responses are observed and no ventilation occurs.
- Other possible anesthetics to be used are tricaine methane sulfonate (MS222), lidocaine, eugenol and ketamine among others.

How to take tissue samples?

Tissue samples are meant to be used in molecular analyses, that is for constructing phylogenies based on molecular markers (DNA sequences), or sometimes for genetic analyses.

Immediately after fish die, you have to take the tissue samples (before fixation in formalin). Always take the samples from the right side of the specimens, as counts and measurements for taxonomic studies are always taken from the left side. With the pointed scissors, you can take a fin clip or a piece of tissue from the dorsal portion of the fish and introduce it in one eppendorf previously filled with absolute ethanol (use the pipette for this). Put a small piece of paper with an identification number in the eppendorf and then put the same number on the fish as a tag (voucher). You can attach it to the fish with fine string, or you can put each voucher specimen in separate

jars with the paper and ID number inside. After sampling each fish, you have to immerse the scissors in alcohol and ignite it to sterilise them, eliminating potential cross contamination among the samples.

Eventually, if you have many small specimens, you can put a couple of fish in a jar with absolute ethanol. Please note that you do not need to tissue sample all fish, the number of specimens depends on the study, but five specimens are generally enough.

How to preserve collected specimens?

After euthanasia and tissue sample, fix (preserve) the specimens in the jars. The best way to preserve fish is using formaldehyde. It usually comes as formalin (a solution of 40% formaldehyde with water and sometimes stabilisers). Use a solution of 1 part formalin to 9 parts of water. You can prepare it in the field using water from the collecting location. Fish should not occupy more than approximately 20% of the jar volume. The specimens have to remain in formalin for between 7 and 10 days (more time for bigger fish) and then be transferred to water for one day to eliminate the rest of formalin, before being preserved in 70% ethanol. To prepare this, use a ratio of 1/3 tap water to 2/3 regular alcohol (96% ethanol).

WE WANT YOUR FISH'S DNA!

Killi-Data's Molecular Platform has been in operation since 2016. We have amassed over 150 samples, held in collection by myself, Andy Patel and Frans Vermeulen. Already this collection has proven useful to science, supplying samples to *Kryptolebias*, *Nothobranchius* and *Simpsonichthys* molecular phylogenetics projects.

For taxonomic studies, 30 adult specimens are a relatively representative sample. Be sure you have at least 10 males if possible.

If you do not have formalin available in the field you can preserve specimens directly in 70% ethanol. If this is not available either, buy a strong white alcoholic drink, such as rum or vodka, and put the fish in it. Write in your field notebook and on the labels what you used, for example: 'fixed with vodka'.

If you use this protocol or have any comments about it you can give us feedback so that we can improve the protocol in the future.

References

Neiffer, D. L., & Stamper, M. A. (2009). Fish sedation, anesthesia, analgesia, and euthanasia: considerations, methods, and types of drugs. *ILAR journal*, 50(4), 343–360.

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We would like to thank members of the BKA (and other societies) for donating fish. We still have many species to bring into the collection and look forward to continued support. It is notable that our collection still lacks *Aphyosemion australe* and several other common species. We also need lampeyes and *Aphanius*. It is possible for any-