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Needlepoint non-destructive internal tissue sampling for precious fish specimens

by

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Abstract. – In this paper, we describe a new non-damaging internal tissue sampling method for preserved collection of teleostean specimens. It was tested on freshwater pipefish, as external tissue sampling is made difficult by the lack of scales, the lack of pelvic fins, the atrophy of pectoral and anal fins. The internal tissue is detached by scratching the inside of the urogenital papilla with a fine metallic probe. 95% ethanol is injected using a fine syringe, and then sucked back into the syringe with the detached tissue. This protocol has been tested on 6 specimens from 5 species of pipefish. For each specimen DNA was extracted from the internal tissue, a caudal fin clip, and when possible, eggs sampled from the male brooding pouch. Partial *Cytochrome c oxydase I* (*COI*) was amplified and sequenced. For each specimen, the 582 bp long sequences obtained from the internal tissue, the fin clip and the eggs were identical. These results validate this non-damaging internal tissue sampling method, which leaves absolutely no trace on the specimen. Although this method was developed on pipefish, it could be applied to other teleostean, even precious museum collection specimens such as type specimens. The aim of this paper, using the example of freshwater pipefish, is to present this method, which aims at preserving precious collection specimens while still valorising them.

Key words
Teleostei
Syngnathidae
Non-destructive tissue sampling
Urogenital papilla
Syringe
Collection specimens

Résumé. – Échantillonnage interne non destructif à la seringue pour les spécimens précieux de poissons.

Dans cet article, nous décrivons une nouvelle méthode non destructrice de prélèvement de tissus internes pour les spécimens de collection de téléostéens. Elle a été testée sur des syngnathes d'eau douce, car l'échantillonnage de tissus externes est problématique dû à l'absence d'écailles, de nageoires pelviennes et à l'atrophie des nageoires pectorales et anale chez ces espèces. Les tissus internes sont détachés en grattant l'intérieur de la papille urogénitale à l'aide d'une sonde métallique. De l'éthanol à 95% est injecté avec une seringue, puis aspiré de nouveau dans la seringue avec les morceaux de tissus détachés. Ce protocole a été testé sur 6 spécimens appartenant à 5 espèces de syngnathes. Pour chaque spécimen, l'ADN a été extrait à partir des tissus internes, d'un morceau de nageoire caudale, et, le cas échéant, des œufs dans la poche à couvain des mâles. Une partie du gène codant pour la *Cytochrome c oxydase I* (*COI*) a été amplifiée et séquencée. Pour chaque spécimen, les séquences de 582 pb obtenues à partir des tissus internes, du morceau de nageoire caudale et des œufs étaient identiques. Ces résultats valident l'efficacité de notre méthode de prélèvement de tissus internes, qui n'abîme aucunement les spécimens. Bien que cette méthode ait été développée sur les syngnathes, elle pourrait être appliquée à d'autres téléostéens, y compris à des spécimens précieux comme les spécimens types. Le but de cet article, à partir de l'exemple des syngnathes d'eau douce, est de présenter cette méthode, qui vise à préserver les spécimens de collection tout en permettant leur valorisation.

INTRODUCTION

Nowadays, the study and the description of species are no longer only based on morphomeristic data. Taxonomists increasingly combine morphological and molecular data as well as, environmental, geographical or behavioural data to refine and improve species delineation (integrative taxonomy, Padial *et al*., 2010). For studies on teleosteans, fin clips are generally used for DNA extraction (Denys *et al*., 2014; Hamilton *et al*., 2017; Keith *et al*., 2017; Longo *et al*., 2017; Mennesson and Keith, 2017). However in many teleostean families such as, the Centriscidae, the Ostraciidae, the Liparidae, the Indostomidae, the Gasterosteidae, the Sternoptychidae, the Peristediidae and the Syngnathidae, external tissue sampling is tricky. In the case of pipefish (Syngnathidae), they lack pelvic fins, the pectoral fins and anal fins are atrophied and the dorsal and caudal fins cannot be sampled as they are used for the morphological description and identification of species (Dawson, 1986). Furthermore, Syngnathidae lack scales. Instead, the body is covered in dermal plates arranged to form a series of rings, also representing important morphomeristic characters. The gill openings and mouth are very small, so gill sampling is also ruled out. Thus, external tissue sampling on pipefish damages impor-

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tant morphological characters for species description. Museum curators usually refuse the demands from researchers to sample tissue in order to protect the collection, but working on collection specimens is often the only way to solve taxonomic issues. We therefore developed a new non-destructive sampling method. It samples internal tissue by retrieving it via the urogenital papilla using a fine syringe. This method was tested on 5 different freshwater pipefish species [20 to 30 species inhabit freshwater and have not been studied for over 35 years, so the taxonomy and nomenclature are unclear and solely based on morphological characters (Dawson, 1985; Kottelat, 2013; Miesen *et al.,* 2016); hence the urgency to find a way to study them]. To test it, we performed traditional sampling on caudal fin clips and, when possible, also sampled eggs from the male brooding pouch. Indeed, the male carries the eggs in a ventral pouch and takes care of them until they hatch (Dawson, 1985). DNA was extracted from these three types of sample (internal tissue, fin clips and eggs), and the partial *cytochrome c oxydase I* (*COI*) was amplified and sequenced. The sequences were compared and our non-damaging sampling method has proven to be effective. This new method is obviously not exclusively for the study of pipefish but can be used to study any other teleostean fish family. We just used pipefish as an example of the method's efficiency. The main added value is that it gives access to tissue samples from precious collection specimens, including type specimens, potentially leading to molecular data needed for taxonomic or phylogenetic studies.

MATERIAL AND METHODS

Samples

A total of 6 specimens was used. Individuals were caught in freshwater streams during field missions in Papua New Guinea (PNG) and the Solomon Islands between 2015 and 2018 as part of faunal surveys. The care and use of fish complied with the annex IV of the directive 2010/63/EU animal welfare laws, guidelines and policies, which have been approved by Papua New Guinea (PNG) and Solomon Island authorities (permit numbers: 018196 for PNG survey; EX2015/142 for the 2015 Solomon survey and EX2016/156 for the Solomon 2016 survey). Individuals were sampled using a DEKA 3000 electrofishing system (Gerätebau, Marsberg, Germany). Fish were euthanized using an overdose of clove essential oil (10%). Entire fish specimens were stored and preserved in 95% ethanol for molecular analysis. No surgical procedures were performed so fish weren't under any stress nor did they suffer. No experiment of any kind was performed on the fish.

Each specimen was morphologically determined according to Kottelat (2013). They belong to 5 currently valid species: *Coelonotus argulus* (Peters, 1855), *Coelonotus leiaspis* (Bleeker, 1854), *Oostethus brachyurus* (Bleeker, 1854), *Oostethus manadensis* (Bleeker, 1856) and *Lophocampus retzii* (Bleeker, 1856).

The standard length of each specimen was measured to the nearest tenth of a millimetre, using a digital *Mitutoyo* dial calliper. Out of the 6 specimens, 3 were females and 3 were males, 2 of which carried eggs in their pouch (Tab. I).

Tissue sampling

For each specimen, a caudal fin clip was preserved in 95% ethanol until DNA extraction. For brooding males, about 6 eggs (Fig. 1B) were sampled and preserved in 95% ethanol until DNA extraction. The internal tissue sampling followed a simple multi step protocol: first an intra-oral exploration probe (dental tool, Fig. 1A) inserted via the urogenital papilla (Fig. 1C) was used to scratch the inside of the specimen's body. With a 1-mL insulin syringe MYINJECTOR® U-100 (Fig. 1A), about 0.3 mL of 95% ethanol was injected through the urogenital papilla. Then, the injected ethanol was sucked back into the syringe in order to collect the tissue that had been detached during scratching (Fig. 1C-E). The contents of the syringe was placed in a 2 mL Eppendorf (final volume about 0.2-0.3 mL) and observed under a K-401L MOTIC© binocular to check for the presence of suspended matter in the liquid.

A total of 14 tissue samples (Tab. I) were obtained and the 14 Eppendorf tubes were stored in the fridge at 10°C.

Table I. – List of specimens used. MNHN ID is the collection number at the National Museum of Natural History of Paris. ID tag number was attributed to each specimen in the field. Standard length was measured to the nearest tenth of a millimetre. Fin and internal tissue were sampled for each individual, as well as eggs from the two brooding males (NA: not applicable). BOLD ID for sequence identification in FWSYN bold project.

MNHN ID	Tag ID	Species	Locality & date	Sex	Fin	Internal tissue	Egg	Standard length (mm)	BOLD ID
2019-0079	19169	*Coelonotus leiaspis* (Bleeker, 1854)	Papua New Guinea 2018	Female	✓	✓	NA	138.53	FWSYN001-19
2019-0080	14964	*Coelonotus argulus* (Peters, 1855)	Salomon 2016	Male	✓	✓	✓	113.23	FWSYN002-19
2019-0081	19066	*Lophocampus retzii* (Bleeker, 1856)	Papua New Guinea 2018	Female	✓	✓	NA	63.19	FWSYN003-19
2019-0082	19193	*Lophocampus retzii* (Bleeker, 1856)	Salomon 2015	Male	✓	✓	✓	110.72	FWSYN004-19
2019-0083	19058	*Oostethus manadensis* (Bleeker, 1856)	Papua New Guinea 2018	Male	✓	✓	NA	141.1	FWSYN005-19
2019-0084	17762	*Oostethus brachyurus* (Bleeker, 1854)	Papua New Guinea 2018	Female	✓	✓	NA	191.49	FWSYN006-19

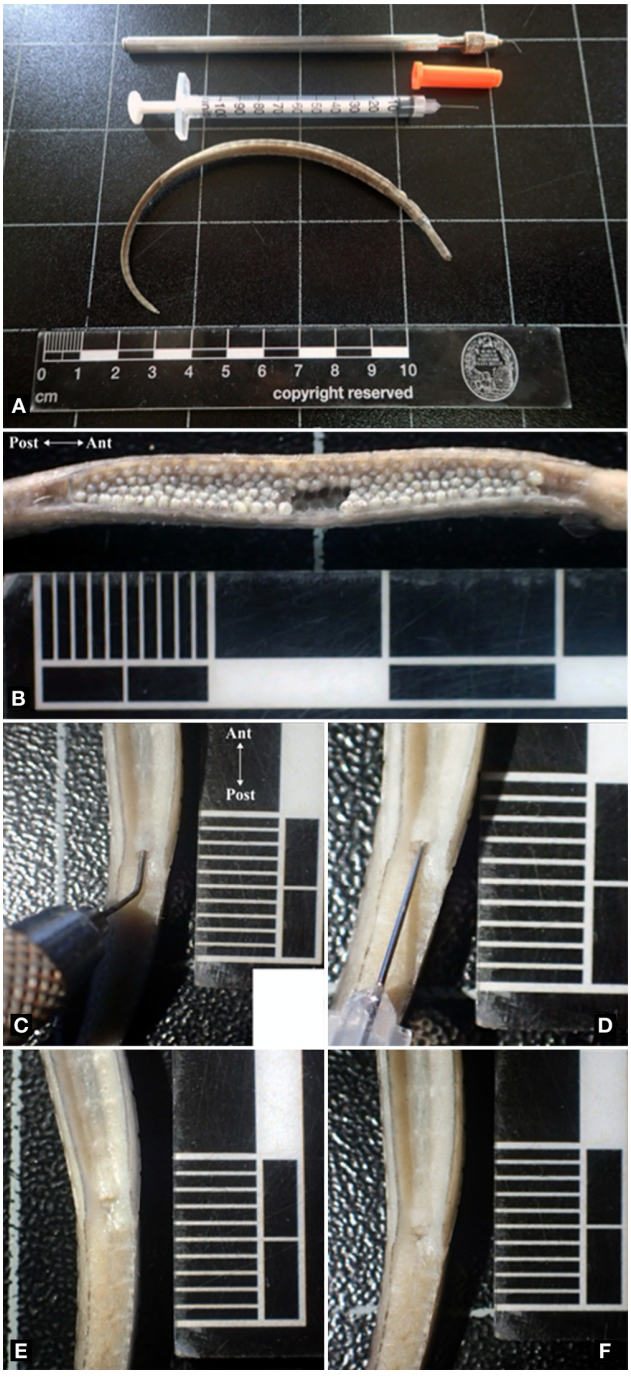

Figure 1. – Non-destructive tissue sampling. **A**: (from top to bottom) dental probe, 1 mL insulin syringe MYJECTOR ® U-100 and specimen 19058 (standard length: 141.1 mm), male *Oostethus manadensis*; **B**: View of the brooding pouch and eggs of specimen 14964 (standard length: 113.23 mm), *Coelonotus argulus*, male; missing eggs correspond to the sample for DNA extraction; **C**: Scratching the inside via the urogenital papilla with the dental probe; **D**: Internal tissue sampling using the syringe via the urogenital papilla; **E**: State of urogenital papilla before sampling; **F**: State of urogenital papilla after sampling. Photos B to F are ventral views of the specimens; the arrow in B gives the orientation of the specimen in photos A, B; the arrow is C gives the orientation of the specimen in photos C-F.

DNA extraction, quantification and amplification

The tubes containing the eggs and the internal tissue suspension were placed in a DNA concentrator (Thermo Scientific™ DNA120OP230) at room temperature at a speed of 1750 rpm for 1 hour to evaporate the ethanol. The tubes were then checked for the presence of dry tissue and egg pellets.

DNA extraction was performed on the fin clips, the eggs and the internal tissue pellets by following the CTAB (Cetyl trimethylammonium bromide) protocol described by Winnepenninckx *et al*. (1993). In addition to Winnpenminck's protocol 1 µL of RNA carrier was added to each tube during the nucleic acid precipitation step with isopropanol. The dry DNA pellets obtained at the end of the extraction protocol were resuspended in 25 µL of Tris-EDTA Buffer and stored at –20°C.

DNA quantification was performed for each of the 14 DNA extracts using a Qubit® 2.0 Fluorometer following the manufacturer instructions.

A mitochondrial fragment of the *COI* gene (650pb) was amplified using the tailed fish specific primers VF2-t1 5'TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC3'; FishF2-t1 5'TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC3'; FishR2-t1 5'CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA3' (Ward *et al*., 2005); FR1d-t15 'CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA3' (Ivanova *et al*., 2007). DNA was amplified by PCR in a final 20 µL volume containing 1 µL DMSO, 1 µL BSA, 1 µL of dNTP 6.6 µM, 0.15 µL of Qiagen Taq DNA polymerase, using 2 µL of the buffer provided by the manufacturer and 0.4 µL of each of the four primers at 10 pM; 2 µL of DNA extract was added. After a 2 min denaturation at 94°C, the PCR was run for 50 cycles (30 s, 94°C; 45 s, 54°C; 1 min, 72°C), with a 2-minute terminal elongation on a Bio-Rad T100™ Thermal Cycler. Successful PCRs were selected on ethidium-bromide stained agarose gels. Sanger sequencing was performed in both directions by a commercial company (Eurofins) (http://www.eurofins.fr) using M13 tail primers M13F (–21) 5'TGTAAAACGACGGCCAGT3'; M13R (–27) 5'CAGGAAACAGCTATGAC3' (Messing, 1983). Sequences were quality checked and aligned with Geneious 7.5.1 (http://www.geneious.com, Kearse *et al*., 2012). The percentage of divergence between sequences was calculated in Geneious 7.5.1. Sequences coming from the fin clip, the internal tissue and, when applicable, the eggs, of the same specimens were compared.

The sequences were used to blast-search the BOLD and ncbi nucleotide databases.

RESULTS

As shown on Fig. 1E and 1F, no external damage to the specimen was caused. DNA quantification results are shown in table II.

DNA was present in each sample (fin, internal tissue and egg). The quantity of DNA obtained from the eggs is a lot higher than from the fin and the internal tissue.

DNA amplification and sequencing was successful on all 14 samples, yielding a 582 bp alignment. Sequences were deposited in BOLD under the FWSYN project (Tab. I). The *COI* sequences are the same for each specimen whatever the tissue used (fin clip, internal tissue or eggs) (Tab. III). The blast search with the sequences generated in this study all matched with freshwater pipefish sequences.

DISCUSSION

The results show that sampling of internal tissue via the urogenital papilla is an effective method and provides the same results as the traditional sampling method (i.e. fin clip). For each individual the quantity of DNA obtained from the fin clips and the internal tissue is comparable. The quantity of DNA obtained from the eggs is a lot higher, but it is not surprising since DNA was extracted from about 6 eggs, thus form 6 embryos. The *COI* sequences obtained from fin clips, internal tissue, and even eggs are identical (Tab. III).

Syringes have been used to draw blood samples from freshly caught specimens in the field, but the blood is generally used for bacteriological studies (Zhang *et* al., 1994; Martinez *et al*., 1998; Renoux *et al*., 2017; Tarnecki *et al*., 2018). It could be used to extract DNA for taxonomic studies, but taking blood samples can be difficult (especially on small specimens), and they anyway contain multiple PCR inhibitors (Schrader *et al*., 2012). Additionally it is compulsory to have animal experimentation authorisation for blood sampling on live specimens. In the case of tropical insular freshwater fish sampling surveys, because of the conditions in which the surveys take place, blood samples can generally not be preserved properly. Indeed, when in the field in these remote areas, where there usually is no electricity, researchers can only reliably use 95% ethanol to preserve samples. However, the urogenital papilla method that we developed could be used on freshly caught specimens in the field, guaranteeing high quality tissue samples, and even a better preservation of the whole specimen as the ethanol preservative is directly injected in the ventral cavity.

Fish eggs have been used before for DNA extraction and species identification (Aranishi, 2006; Karaiskou *et al*., 2007; Weigt *et al*., 2012). Using the presence of brooding males, we also tested the use of eggs for DNA extraction and molecular species identification. Egg sampling also leaves diagnostic morphological characters undamaged, if there are enough eggs remaining for reference. The sequences obtained with the eggs are also identical to the sequence from the carrying male. However, the *COI* sequence belongs to the developing embryo inside the egg or to the egg tis-

Table II. – DNA quantification in µg/mL for each sample. IT: internal tissue.

Species	Tag ID	Fin	IT	Egg
Coelonotus leiaspis	19169	4.32	1.06	NA
Coelonotus argulus	14964	4.82	1.53	89.4
Lophocampus retzii	19066	1.23	5.04	NA
Lophocampus retzii	19193	5.64	1.35	71.6
Oostethus manadensis	19058	8.5	2.62	NA
Oostethus brachyurus	17762	3.52	2.46	NA

Table III. – Distance matrix (%) obtained for the 14 sequences. IT: Internal tissue sample; F: Fin clip; E: Eggs.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Oostethus manadensis* 19058 **IT**													
2	*Oostethus manadensis* 19058 **F**	**0**												
3	*Oostethus brachyurus* 17762 **IT**	18.7	18.7											
4	*Oostethus brachyurus* 17762 **F**	18.7	18.7	**0**										
5	*Lophocampus retzii* 19066 **IT**	18.4	18.4	17.7	17.7									
6	*Lophocampus retzii* 19066 **F**	18.4	18.4	17.7	17.7	**0**								
7	*Lophocampus retzii* 19193 **IT**	18.4	18.4	17.7	17.7	**0**	**0**							
8	*Lophocampus retzii* 19193 **F**	18.4	18.4	17.7	17.7	**0**	**0**	**0**						
9	*Lophocampus retzii* 19193 **E**	18.4	18.4	17.7	17.7	**0**	**0**	**0**	**0**					
10	*Coelonotus leiaspis* 19169 **IT**	18.4	18.4	17.7	17.7	17.4	17.4	17.4	17.4	17.4				
11	*Coelonotus leiaspis* 19169 **F**	22.0	22	19.1	19.1	17.4	17.4	17.4	17.4	17.4	**0**			
12	*Coelonotus argulus* 14964 **IT**	22.0	22	19.1	19.1	16.5	16.5	16.5	16.5	16.5	15.1	15.1		
13	*Coelonotus argulus* 14964 **F**	21.8	21.8	18.0	18.0	16.5	16.5	16.5	16.5	16.5	15.1	15.1	**0**	
14	*Coelonotus argulus* 14964 **E**	21.8	21.8	18.0	18.0	16.5	16.5	16.5	16.5	16.5	15.1	15.1	**0**	**0**

sue from the female, which laid the eggs. As the mitochondrial genome is maternally inherited, and because the female provides the egg tissue, the *COI* sequence obtained from the eggs is of maternal origin and not the sequence corresponding to the brooding male. In this study, the egg sequence is identical to the one of the carrying male. So, the egg sequence shows that the female with which the male specimen reproduced was genetically very similar, however differences in mitochondrial sequences between the eggs and the carrying male could highlight cases of hybridization, which are common in freshwater fish whether inter-specific or inter-generic (Dowling and DeMarais, 1993; Scribner *et al*., 2000; Denys *et al*., 2014). In these study cases, where several species of the same family are found in the same rivers and in the same habitat within the river, using the eggs might be an easy way to detect possible hybridization events, based on mitochondrial sequences only, whereas this normally needs to take into account both mitochondrial and nuclear sequences.

Specimens in museum collections represent an important source of morphological and molecular information and should be used to resolve taxonomic problems. The use of integrative taxonomy has led to the discovery of many new species, especially in groups where species delineation is blurry (Denys *et al*., 2014; Geiger *et al*., 2014) and integrating the data of voucher specimens is of crucial importance, especially old specimens, or even type specimens. The methods developed for ancient DNA extraction can be highly invasive, leading sometimes to the total destruction of specimens (Garrigos *et al*., 2013), which goes against the very principle of storing collections and museum curators often refuse any sampling request to avoid any damage to such specimens. In consequence, non-invasive tissue sampling methods have been developed for small fluid-preserved fish specimens, such as GuSCN (Guanidine-based) baths in which the specimen is soaked (Garrigos *et al*., 2013). This bathing protocol desquamates external tissue on which DNA amplification is then performed. But this approach was designed for mammalian hard tissues (bones, teeth, skin) (Rohland *et al*., 2004) and can be problematic for preserved teleostean. This method can cause external modifications of the specimens (dehydration and distortion of body), leading to the loss of important taxonomic characters such as the percentage of standard length measurements, body depth measurements or fin ray count due to extreme dehydration. With our method, the sampling of tissue is completely non-damaging and is applicable on freshly caught specimens as well as on recent or old fluid-preserved collection specimens, including type specimens. Museum curators could thus validate the use of this method on voucher specimens and give access to researchers to precious missing data to fulfil taxonomic revisions of various groups. Considering our results, the amount of tissue sampled by detaching cells from inside the body is sufficient material for the amplification of mitochondrial markers, as the DNA quantity obtained is comparable to that obtained with a fin clip. For old specimens, for which the DNA amplification may be challenging, internal specific primer pairs for the amplification of small gene fragments could be tested. In any case, for most taxonomic groups, a *COI* fragment of only about 100-150 bp is enough to place specimens with their conspecific in a phylogenetic tree (Garrigos *et al*., 2013).

CONCLUSION

The main advantage of the internal tissue sampling method is that it leaves absolutely no external trace of the sampling (Fig. 1F-G). This protocol thus allows obtaining molecular data without damaging the morphomeristic characters necessary for species identification and description. We have tested and developed it on freshwater pipefish, as they are particularly difficult to sample externally without damaging diagnostic morphological characters, but this method can obviously be used on any teleostean specimen preserved in museum collections. For instance, this protocol can be applied to retrieve tissue samples from precious specimens such as type specimens for which researchers are often denied the authorisation to sample because of their taxonomic importance. Species description is increasingly based on integrative taxonomy. The use of morphological characters is compulsory when describing a new species, but molecular data is now considered crucial for species delineation and taxonomic revision, especially in complex cases. The present sampling method gives access to good quality tissue samples for specimens is of high value for the collections, or when external tissue sampling is too damaging. This method will enable to use and valorise all Natural History Museum collections and will help the taxonomic revision of many teleostean families.

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