

# An Optimized Protocol for Myxosporidia (Cnidaria: Myxosporea) DNA Extraction for Molecular Studies

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## Abstract

Myxosporidia constitute a major group of fish parasites which have a significant negative impact on wild and cultured fish. The use of DNA in Myxosporidia studies has progressed rapidly over the last twenty years, especially in their identification and characterization as well as determination of species diversity and investigation of their evolutionary relationships. Extraction and isolation of pure and high quality DNA are essential for any molecular study, but constitute a challenge for many laboratories especially in low and middle income countries. Myxosporidia plasmodia filled with mature myxospores were isolated from different tissues of *Labeo batesii* Boulenger, 1911. DNA from myxosporidia myxospores were extracted using a Livak optimized DNAs extraction protocol. Four particular phases of the original protocol were optimized. Yield and absorbance ratios of extracted DNA were determined using spectrophotometer. DNA samples were used as template for the amplification of the 18S rDNA region and amplicons resolved on 1.5% agarose gel for determination of fragment sizes and purity evaluation. The concentration of extracted DNA from all Myxosporidia species ranged from 4.6 to 26 ng/μl with purity indices ranging from 1.88 to 2.12. We successfully amplified the 1050 bp DNA fragment as targeted. The intensity, thickness and clarity of the bands were evidences of non-degradation of DNA. The optimized Livak protocol is simple, low-cost and manageable. Regarding the quantity, purity and quality of extracted DNA, the optimized Livak protocol is highly recommended for Myxosporidia studies.

## Keywords

DNA Extraction, Livak Protocol, Optimization, Myxosporidia, Parasites

## 1. Introduction

Myxosporidia constitute a group of microscopic metazoan parasites [1], best known for the infections they cause in freshwater and marine fish [2]. To date, about 2400 species of Myxosporidia have been describe worldwide [3] with 280 in Africa [4]. Although their importance as fish parasites is well recognized in Africa, the taxonomy of the majority of Myxosporidia species found on the continent has been based solely on morphological and morphometric characteristics of their myxospores, host and organ or tissue specificity [5].

Approximately 500 DNA sequences originating from Myxosporidia species around the world are deposited in NCBI database with less than 30 from African species [3]. DNA sequence can provide more detailed information not only on the differentiation of myxosporidia species, but also on the diagnosis of economically important species. In addition, the availability of DNA sequences allows a phylogenetic comparison of different species and an investigation of the evolutionary relationships between them [3].

Extraction and isolation of pure and high quality DNA are essential steps for any molecular study [6]. Extensive literature survey clearly indicates that various methodologies have been used to isolate genomic DNA from Myxozoans myxospores [7]-[14]. However, most of these DNA isolation protocols have been reported using commercial kits. Because these commercial kits are very costly, DNA isolation is a challenge for many laboratories in low-incomes countries especially in Africa.

An ideal DNA extraction technique should not only optimize the DNA yield or minimize DNA degradation and contaminant, but it must also be suitable in terms of cost and supplies. Therefore, to extend the molecular and phylogenetic studies of Myxosporidia species to unfunded laboratories in low-incomes countries, it is necessary to establish an efficient and inexpensive DNA extraction protocol for this group of parasites.

Livak [15] has developed a simple, manageable, low cost and efficient protocol for DNA extraction from *Drosophila melanogaster* Meigen [16]. In the best of our knowledge, no report is available on the use of this method to isolate DNA from myxosporidia.

In the present work, we have optimized the Livak protocol to extract high quality and free of contaminant genomic DNA from Myxosporidia myxospores.

## 2. Methods

### 2.1. Sample Collection and Morphological Examination of Myxospores

Fresh specimens of *Labeo batesii* Boulenger [17] were sampled in river Makombè at Nkondjock (4°35' - 5°N, 10° - 10°23'E, Cameroon, Central Africa) from May 2017 to July 2018. Captured fish were transported in cool boxes from the field to the Laboratory of Parasitology and Ecology of the University of Yaoundé I. Once in the laboratory, the fish firstly underwent a macroscopic ex-

amination (eyes, fins, operculum, scales, skin) and a microscopic examination for the presence of Myxosporidia plasmodia using the Olympus BO61 binocular stereoscope. After dissection of a fish specimen, internal organs such as gills, heart, liver, kidneys, spleen, gallbladder, gonads, intestine and urethra were screened for the presence of plasmodia. When found, plasmodia were crushed between glass slides and coverslips, and identified using a light microscope (IVYMEN, objective 100X) according to Lom and Arthur [18]. The remaining myxospores from a ruptured cyst were preserved in 100% ethanol at -20°C for DNA extraction.

## 2.2. DNA Extraction

This part of the work was carried out at the Centre for Research in Infectious Diseases (CRID), which has a state-of-the-art technical platform for molecular analysis.

The remaining myxospores from each ruptured cyst were separately ground in 25 µl LIVAK grind buffer (0.08 M NaCl, 0.16 M Sucrose, 0.12 M Tris, 0.05 M EDTA, 0.5% SDS, 100 ml sterilise water) follow by the addition of 75 µl of the same buffer to rinse the pestle after grinding. Then, the homogenate was incubated for 30 min at 65°C in a bain-marie. After, a quick spin, 14 µl of 8 M K-acetate was added to each tube before incubation on ice for 30 minutes. The mixture was further centrifuged at 13,500 rpm for 20 min. The supernatant was transferred into a new tube and double volume of absolute ethanol was added to it. The samples were centrifuged at 13,500 rpm and kept for incubation at 4°C for 15 min to favour DNA precipitation. The supernatant was then removed and discarded. The DNA pellet was washed with 100 µl of cold ethanol 70% v/v and dried at room temperature during 60 minutes. Once dried, the pellet was suspended in 20 µl H<sub>2</sub>O and incubates at 65°C for 10 min.

## 2.3. Quantification and Visualisation of DNA

DNA concentration and purity were determined using NanoDrop Lite Spectrophotometer (Thermo Fischer scientific). The absorbance quotient (A<sub>260</sub>/A<sub>280</sub>) provided an estimation of DNA purity. To verify the quality of the extracted DNA, 3 µl of each isolated DNA sample were separated using an agarose gel 1.5%. The gel was stained using midori green (Dutscher/Genetics), visualized and photographed under ultraviolet light.

## 2.4. PCR Amplification

A fragment of 18S rDNA was amplified using forward (MC5F: 5'-CCTGAGAAACGGCTACCACATCCA-3') and reverse (MC3R: 5'-GATTAGCCTGACAGATCACTCCACGA-3') primers [19]. Briefly, 1.5 µl of extracted DNA were used as template in a mix containing 200 nM of each primer using One Taq Quick-Load (BioLabs inc.) in 15 µl of final volume. A Bioer gene touch Thermocycler (Dutscher) was used with a program including an ini-

tial denaturation step at 95°C for 5 minutes, followed by 35 cycles of: a: denaturation at 95°C for 60 seconds, annealing temperature at 60°C for 60 seconds, and an extension at 72°C for 90 seconds. A final extension step at 72°C for 5 minutes to terminate the amplification ends the process [20]. The amplified products were visualized on 1.5% agarose gel.

### 3. Results

#### 3.1. Plasmodia Content and Morphological Examination of Spores

Based on morphology and metric features of the Myxospores, three species of *Myxobolus* were differentiated from three different tissues of *Labeo batesii*. These *Myxobolus* species were arbitrarily named *Myxobolus* sp.1, *Myxobolus* sp.2 and *Myxobolus* sp.3. Out of the 454 fishes specimens examined, 138 (30.39%) harboured plasmodia of *Myxobolus* sp.1 on the secondary gill lamellae, 81 (17.84%) carried plasmodia of *Myxobolus* sp.2 on primary gill lamellae while 356 (78.41%) harboured the plasmodia of *Myxobolus* sp.3 within the muscle.

#### 3.2. DNA Yield and Purity

DNA yield from different *Myxobolus* species are presented in **Table 1**. The concentrations varied between 4.6 ng/μl and 26 ng/μl. The absorbance ratio of the extracted DNA measured at wavelengths of 260/280 nm and ranged from 1.88 to 2.12 are presented in **Table 1**. Three of the four samples presented an absorbance ratio < 1.8 whereas one sample has a ratio of >2.0.

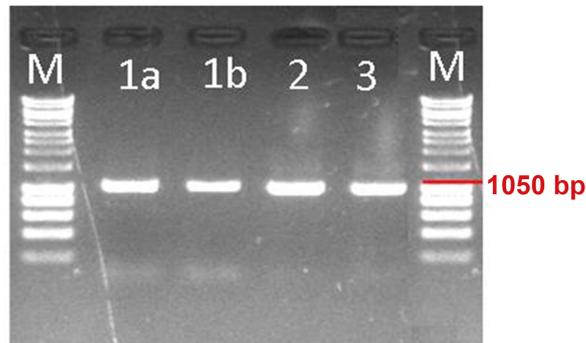
#### 3.3. PCR Amplification

The DNA extracts were successfully used to amplify 18S rDNA fragment gene for the three *Myxobolus* species. The amplified PCR products size of the four DNA samples was of 1050 bp as shown in **Figure 1**. The amplification procedure was repeated successfully with other extracted samples using the same optimized Livak DNA extraction protocol (unpublished data). As observed in **Figure 1**, the bands are well defined in 1.5% agarose gel. The sharpness of these bands showing that there was no sign of degraded DNA during preparation is shown in **Figure 1**. The amplicons of these 4 isolates samples were used to generate sequence data for the 18S rDNA region (unpublished data).

**Table 1.** Concentration and ratio A260/280 of the DNA samples extracted using Livak modified protocol.

Samples	Ratio of absorbance (260/280)	DNA yield (ng/μl)
1a	2.12	4.6
1b	1.97	7.8
2	1.98	8.3
3	1.88	26

1a & 1b: *Myxobolus* sp.1; 2: *Myxobolus* sp.2; 3: *Myxobolus* sp.3; ng: nanogram; μl: microlitre, DNA: Deoxyribonucleic acid.



**Figure 1.** Agarose gel (1.5%) photo showing amplified 18S rDNA gene of four samples using MC5F/MC3R primers. bp: base pairs; M: size marker; 1a & 1b: *Myxobolus* sp.1; 2: *Myxobolus* sp.2; 3: *Myxobolus* sp.3.

#### 4. Discussion

To date, a wide array of different DNA extraction protocols are in use for Myxosporidia studies. The traditional Phenol-Chloroform method for DNA extraction, have been experience by many authors on Myxosporidia [7] [21] [22]. This extraction method involves the use of lysis STE buffer for suspension of spores before DNA extraction. The preparation of lysis buffer requires proteinase K which is very expensive. Moreover, proteinase K in high concentration provides a risk of DNA degradation [23]. Timely availability and expensive price of commercial kits are limiting factors for molecular study in many of the developing countries [6]. The most common commercial kits used in Myxosporidia studies are DNAeasy Blood & Tissue Kit (Qiagen, USA) [10], QIAamp DNA mini Kit (Qiagen, Germany) [13], Qiagen DNAeasy Tissue Kit (Qiagen) [8], Purelink Genomic Mini Kit (Invitrogen) [12], Fast DNA kit (MP Biomedicals LLC) [9], General All Gen kit [14], TIANamp Genomic DNA kit [11]. A 50 reactions preparation of these commercial DNA extraction kits cost from \$168 for the cheapest kit (DNAeasy Blood & Tissue Kit) to \$568 for the most expensive one (TIANamp Genomic DNA kit). Moreover, they are not easily accessible for purchase and therefore cannot be used by most researchers in underdeveloped countries whose research activities are not always funded.

The present study attempts to propose a less expensive and more accessible method for extracting Myxosporidia DNA. So, the simple and manageable protocol developed by Livak [15] for *Drosophila melanogaster* DNA extraction was used as starting point and adapted to Myxosporidia. The reagents components of the Livak buffer that cost less than \$10 for a 50 reactions preparation are easily accessible and always available. The formulation of the Livak buffer can easily be modified contrary to those of the commercial kits. Four particular phases in the Livak protocol were optimized:

- the buffer was prepared in accordance with the original procedure but to avoid any DNA loss, the buffer (100  $\mu$ l) was added in two phases: firstly (25  $\mu$ l) to grind the plasmodia and secondly (75  $\mu$ l) to rinse the pestle after grinding;

- to avoid any dislodging of the DNA pellet, the pellet was washed only one time using 100 µl of cold 70% ethanol instead of two times;
- the DNA pellet was suspended in 20 µl H<sub>2</sub>O instead of 40 µl of restriction enzyme buffer proposed by Livak [15]. Use of water in this step is vital for further molecular techniques such as PCR application, RFLP and sequencing [24] [25];
- the final incubation at 65°C for 10 min (step lacking in Livak [15] protocol) allowed the total dissolution of DNA pellet and the destruction of any DNases that may be present [26].

When spooled out of solution, the DNA shows on agarose gel some white, thick and intact bands with no visible coloration. Furthermore, The A<sub>260/280</sub> ratio of the DNA from three out of the four Myxosporidia samples used ranged from 1.88 to 1.98 indicating that the isolated DNA is free from protein and RNA contamination [27] [28] and could be used for further molecular and bioinformatics investigations.

## 5. Conclusion

The present study provides the first report on the use of an optimized Livak protocol for Myxosporidian DNA extraction. The method is simple, manageable, low cost and efficient for genomic DNA isolation from Myxosporidia myxospores. The amount, purity and quality of the DNA extracted are suitable for molecular studies. This optimized Livak protocol, highly recommended for molecular studies of Myxosporidia, can also be extended for DNA extraction of other fish parasites as well in many laboratories especially in low and middle income countries.

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## Authors' Contribution

L. F. G. B., F. D. B. and T. F. B. drafted the proposal, L. F. G. B., F. D. B. and W. M. J. participated in the field work and laboratory analysis, L. F. G. B., F. D. B. and T. F. B. participated in the data analysis and interpretation, F. A. and W. M. J. contributed to the correction of the final draft of the manuscript. All authors read, corrected and approved the final manuscript.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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