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Comparison of genomic DNA extraction methods for *Colossoma macropomum* fish fin clippings for single nucleotide polymorphism genotyping

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ABSTRACT. Ideal DNA extraction techniques must be efficient in terms of time, labor, and costs, optimizing yield and quality of the DNA for the desired applications. We tested six DNA extraction methods: DNeasy® Blood & Tissue Kit, *Cetyltrimethylammonium bromide* (CTAB), Modified salting-out protocol (SA), Boiling Tissue, Proteinase K (PK) and Mini Kit Applied Biosystems, on fin clippings from tambaqui (*Colossoma macropomum*). DNA yield, purity, quality, and cost of each method were evaluated. The effectiveness of extraction was evaluated by PCR amplification and genotyping efficiency, repeatability and accuracy. DNA yield and purity were quantified using NanoDrop absorbance ratios. Cost was estimated in terms of time and material expenses. The results showed differences between the tested methods, with the PK method having the best performance, followed by SA and CTAB. PK was identified as the most economical and efficient technique in terms of time, cost, and scalability/potential automation, while also generating DNA of good quality for performing PCR amplification and SNP genotyping with tambaqui samples.

Key words: DNA Extraction; Fin clippings; Fish; PCR amplification; Proteinase K

INTRODUCTION

The varied uses of purified genomic DNA, ranging from research applications to clinical diagnosis and forensic investigations, makes DNA extraction procedures, with varying levels of throughput and overall quality and quantity, a routine step in molecular biology laboratories. Therefore, multiple DNA extraction methods for different biological materials have been developed and are widely used, although methodological bottlenecks still pose challenges, especially in high-throughput genotyping routine procedures (Schiebelhut et al., 2017). Ideal DNA extraction techniques should be efficient in terms of time, labor, and costs, while optimizing DNA yield and quality, and minimizing DNA degradation. Some of the widely utilized DNA extraction methods are based on the use of organic solvents, chaotropic agents, silica-based ionic binding and release, and salting out of non-nucleic acid substances resulting from proteinase digestion of biological samples. Many of these methods have evolved from time-consuming, single-sample procedures, to user-friendly microwell plate-based technologies adaptable to automated platforms (Ali et al., 2017).

Different positive and negative factors associated with each DNA extraction method can affect downstream molecular applications and the overall efficiency of routine lab operations. Phenol-chloroform-based methods are the most widely used however, they require the use of hazardous chemicals and are mostly restricted to single-tube processes. CTAB-based methods use cetyltrimethylammonium bromide as a chaotropic substance to precipitate DNA, and organic solvents in some steps (Tan and Yiap, 2019). Besides the use of hazardous substances, CTAB and phenol-chloroform extraction methods have been suitable for use across diverse organisms, including many challenging samples (Drábková et al., 2002; Anderson-carpenter et al., 2011; Sajali et al., 2018). Salting-out methods are inexpensive and use saturated sodium chloride solutions to remove non-nucleic acid contaminants after proteinase K digestion of tissue samples (Chacon-Cortes and Griffiths, 2014). These methods require considerable bench time, and scaling up for large-scale routine procedures involving automation based on microwell plate formats poses significant challenges (Schiebelhut et al., 2017). Although commercial solutions developed with silica-imbedded columns are usually relatively simple to perform and adaptable to high-throughput platforms, high costs per sample limit applications, especially in agrigenomics. Sample boiling methods are simple, inexpensive, and fast alternatives; however, these methods result in crude DNA extracts containing suspended impurities, which may result in enzymatic inhibition in subsequent applications and long-term DNA degradation, affecting downstream storage of processed DNA.

We performed this study to identify DNA extraction methodologies suitable for processing fish fin clippings, to maximize efficiency, while balancing time, cost, and quality and produce DNA suitable for SNP genotyping with Fluidigm SNPType® technology.

MATERIAL AND METHODS

Samples

Fin clippings from eight captive tambaqui (*Colossoma macropomum*), previously collected and stored in 90% Ethanol as part a large-scale, routine DNA testing study were

used. Each sample was processed to obtain multiple 6mm punches which were subsequently stored in 2.0mL tubes in 70% Ethanol until further processing.

DNA extraction methods

Fin clippings from each sample were processed with six different DNA isolation methods, according to published and commercially supplied protocols, as follows:

1. DNeasy® Blood & Tissue Kit (69504) – Qiagen (KQ): 180µL of lysis solution were added to 2mL tubes with two steel beads and one fin clip punch and homogenized in a Tissue Lyser equipment at 30Hz for 30 sec, followed by incubation in a heat block at 56°C for three hours after addition of 20µL of a proteinase K solution (20mg/mL). The subsequent steps were carried out as suggested by the supplier.

2. Cetyltrimethylammonium bromide (CTAB) (modified) (Boyce et al., 1989): 500µL of CTAB buffer (2% CTAB; 0.2% 2-mercaptoetanol; NaCl 1.4 M; Tris HCl 100 mM pH 8.0; EDTA 0.02 M) were added to 2mL tubes with two beads and one fin clip punch and homogenized in a Tissue Lyser equipment at 30Hz for 30 sec, followed by incubation in a heat block at 56°C for three hours after addition of 10uL of protease K (10mg/mL). After digestion, the solution was centrifuged at 14,000 rpm for 2 minutes and the upper aqueous phase was carefully transferred to a new microtube containing 500µL of chloroform: isoamyl alcohol (24:1) and vortexed for 10 sec. Samples were centrifuged at 14,000 rpm for 15 minutes at room temperature. The upper aqueous phase was pipetted avoiding disturbance of the debris between phases and transferred to a new tube containing 250µL of cold Isopropanol. Tubes were carefully mixed by inversion and kept at -20˚C for at least 30 minutes. DNA was pelleted by centrifugation at 13,000g for 10 minutes at room temperature. The supernatants were decanted off and pellets were washed by the addition of 700µL of 70% ethanol and centrifuged for 13,000g for 10minutes. Supernatants were removed by decantation and tubes left to dry open at room temperature. Pellets were suspended in 100µL TE and treated with 6µL RNase A (10mg/µL) and incubated at 37˚C for 30 minutes. DNA was stored at -20°C until required.

3. Modified extraction protocol with salt (SA) (Lopera-Barrero et al., 2008): 550µL of lysis buffer (50mM Tris-HCl pH 8.0, 50mM EDTA, 100mM NaCl, 1% SDS) were added to a 2mL tubes with two beads and one fin clip punch and homogenized in a Tissue Lyser equipment at 30Hz for 30 sec, followed by incubation in a heat block at 56°C for 30 min after addition of 1.4ug of proteinase K. After digestion, 600µL pf 5M NaCl were added to each sample, followed by mixing and centrifugation for 10 min at 12,000rpm. The upper phase was transferred to a new tube with 700µL of cold absolute ethanol and incubated at -20 ˚C for 2 hours for DNA precipitation. The DNA was pelleted by centrifugation at 13,000g for 10 minutes. The supernatants were carefully discarded, and pellets were washed by the addition of 700µL of 70% ethanol and centrifuged for 10 minutes. The supernatant was discarded again, and the pellet was resuspended in 100 µL of TE buffer.

4. Boiling Tissue (BT) modified (Valsecchi, 1998): One fin punch was added to 2mL tubes with two beads and 100µL of buffer (1% Triton-X100, 20mM Tris-HCl pH 8.0, 2mM EDTA) and homogenized in a Tissue Lyser equipment at 30Hz for 30 sec, followed by incubation at 96˚C for 10 min and centrifugation at 14,000rpm for 2 minutes. The supernatant was transferred to a new tube.

5. Proteinase K (PK) (DNA Preparation Techniques – URL in the Internet Resources session): One fin punch was added to 2mL tubes with 100µL of buffer (50mM KCl, 10mM Tris-HCl pH9.0, 0.1% Triton X-100, 0.4mg/mL of proteinase K). Samples were then incubated for 3 hours at 60˚C, and for 10 minutes at 94˚C to inactivate the proteinase K, followed by centrifugation at 14,000g for 15 minutes. The supernatant was transferred to a new tube.

6. Mini Kit Applied Biosystems (ref 4373872) (MK): 50µL of lysis buffer was added to 2mL tubes with one fin clip punch. Samples were vortexed and incubated at 95˚C for 3 min, followed by addition of 50µL of DNA stabilizing solution, mixing and centrifugation at 14,000g.

DNA quality evaluation

DNA yield and purity were only estimated for KQ, CTAB and SA. DNA yield by fin clip punch was measured using a Thermo Fisher NanoDrop 1000 spectrophotometer according to the manufacturer´s instructions. The integrity of extracted DNA was verified by electrophoresis of 2µL of the resulting solution from treatments KQ, CTAB and SA, and from 5µL of the resulting solution from treatments BT and PK, and 10µL from MK. Electroforesis was carried out in 1% agarose gels with 1x TAE at 180V and gel images captured with a ChemiDoc XRS+ system (Bio-Rad, USA) after 5 and 40 minutes.

Extraction methods performance

The performance analysis of each method was based on the methodology used by (Schiebelhut et al., 2017). Each criterion was scaled to 1, followed by computation of an overall average across all criteria, considering extraction efficacy (PCR amplification and genotyping efficiency), cost and time consumption.

Extraction efficacy estimated by PCR success

PCR reactions with the same four samples processed with each of the six tested methods were carried out using a tambaqui specific genomic primer (*CM_E2: F-GCTGCGTCTGCAAAACAATA / R-GTGGATGAGCGTAAGGCAAT*). The Qiagen Multiplex PCR Kit and Promega GoTaq DNA Polymerase were used to verify the effectiveness of amplification. PCR using the Qiagen PCR kit was performed according to the manufacturer's instructions while amplification with GoTaq was done with 10ng of DNA, 0.2µM of each of the primers, 200 µM dNTP, 1X buffer and 1 unit of GoTaq. Both PCR reactions were performed using a Veriti™ 96-Well Fast Thermal Cycler Applied Boisystens, in a 10ul final volume, with the following cycling parameters: 94°C/3min, 25 cycles of 94°C/30sec, 63°C/1min, 72°C/1min and a final step of 72°C/30min. PCR products were separated by electrophoresis in 1% agarose gel. DNA extraction efficacy was quantified as the proportion of successful samples with PCR amplification (positive amplification) divided by the total number of tested samples by method.

Extraction efficacy estimated by SNPType genotyping success

To test genotyping efficacy, 1.5µl of DNA solution from eight different individuals by method were tested with a tambaqui 96 SNP panel (TambaPlus®) on a Fluidigm EP1 platform. The Specific Target Amplification protocol (STA) was used to genotype SNPType assays, using 96.96 Integrated Fluidic Circuit format, according to the manufacturer's instructions. Samples from the KQ, CTAB, and SA extraction methods were initially diluted to 70ng/µL to standardize DNA amount. Samples from the PK, BT, and MK extraction methods were used without additional dilution. Each sample was genotyped twice to test concordance

between obtained genotypes. Genotyping accuracy was measured considering call rates and the correlation value between replicates of the same extraction method and correlation with the true genotypes, which were obtained previously with DNA samples processed using Qiagen method. Genotype calls were generated with Fluidigm SNP 4.1 genotyping analysis software with a minimum confidence limit of 98%.

Monetary and labor costs

Overall cost by method was calculated by sample considering local market values of necessary supplies, reagents, and kits in October 2020. Regarding labor time consumed by each method, time recorded in minutes, and number of steps required by each method were considered. The results were analyzed on a scale of 0 to 1. The values of call rate, correlation and PCR were scaled so that the highest values were assigned a value 1. The values of time, cost and stage were scaled to 1 and subtracted from 1. Finally, the protocol method with the lowest cost, time and number of steps was assigned a highest score representing the best performance.

RESULTS

DNA quality evaluation

The integrity of the DNA obtained with the six tested methods is shown in Figure 1. High-molecular weight genomic was observed in all samples processed with KQ, CTAB and SA, while varying levels of DNA fragmentation were also observed. High molecular weight residues as well as low molecular weight RNA can be observed in the wells and lower regions of the agarose gels, respectively, for PK, BT and MK. DNA concentrations and purity (A260/A280 and A230/A260) estimated for KQ, CTAB and SA methods are shown in Table S1 of the supplementary material.

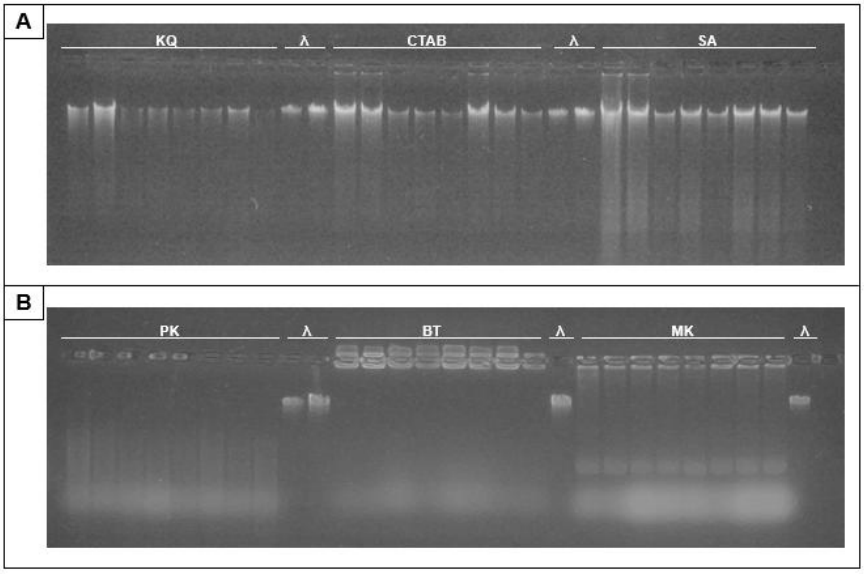

Figure 1. Agarose gel electrophoresis (1% agarose in 1xTAE buffer) of genomic DNA extracted from tambaqui fin clippings with six methods. Lanes: λ DNA (30ng and 60ng), KQ (DNeasy Blood & Tissue Kit - Qiagen); CTAB (cetyltrimethylammonium ammonium bromide); SA (Modified extraction protocol with salt); BT (boiling tissue); PK (Proteinase K); and MK (Applied Biosystem Mini Kit).

Extraction Method Performance

Extraction efficacy for PCR

The tambaqui specific DNA fragment was successfully amplified using DNA extracted with SA, CTAB, KQ and PK methods, considering results with both the Quiagen (2A) and Taq Promega (2B) amplification kits. Amplification was not successful with DNA extracted with BT and MK methods with any of the kits. PCR results contained a single band with 225 bp, as expected (Figure 2).

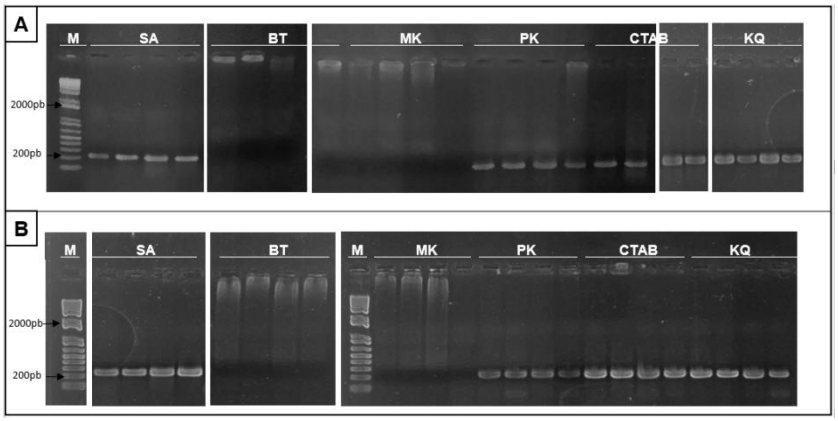

Figure 2. PCR results with tambaqui specific primers for CM_E2 using different DNA extraction methods. Amplification kits (A) Qiagen Multiplex PCR Kit and (B) Promega GoTaq DNA Polymerase. Lanes: M - ladder; SA - Modified extraction protocol with salt; BT - Boiling Tissue; MK - Applied Biosystem Mini Kit; PK - Proteinase K; CTAB - Cetyltrimethylammonium ammonium bromide; KQ - DNeasy Blood & Tissue Kit - Qiagen.

Extraction efficacy considering SNPType genotyping success

Genotyping performed with DNA extracted with KQ, CTAB, SA, PK and MK methods showed an average call rate (CR) >95% (Table 1). The BT method was the only one with a significantly lower CR (36%, Figure S1, supplementary material).

Table 1. Estimated values for all criteria used to calculate efficiency of the tested DNA extraction methods from tambaqui fin clipings.

Extraction method	Call rate	Repeatability*	Accuracy**	Time (min)	Cost (US$) by sample	No. of Steps	Number of amplified samples in PCR
KQ	0.92	0.83	0.84	188	4.40	14	8
CTAB	0.99	0.97	0.92	338	0.55	17	8
SA	1.00	1.00	0.99	181	0.22	13	8
BT	0.36	0.69	0.14	14	0.11	5	0
PK	1.00	0.99	1.00	205	0.15	4	8
MK	1.00	1.00	0.99	3	1.57	3	0

KQ: DNeasy® Blood & Tissue Kit Qiagen; CTAB: Cetyltrimethylammonium bromide; SA: Modified extraction protocol with salt; T: Boiling Tissue; PK: Proteinase K; MK: Mini Kit Applied Biosystems; *Pearson correlation between duplicates; **Pearson correlation with the correct genotypes

The BT method showed lower repeatability (69%) and accuracy (14%), followed by the KQ method, where observed values were 83 and 84%, respectively (Table 1). The PK and SA methods had a 100% correlation between replicates and 100 and 99% with the correct genotype, respectively.

Cost and labor consumption

The BT (US$ 0.11) and PK (US$ 0.15) extraction methods showed the lowest costs per sample, while the highest cost was observed in the KQ method (US$ 4.40) (Table 1). BT had the second-best result concerning time consumption (14min). CTAB does not have a high cost (US$ 0.55) however, it is the most time-consuming method (338 min). The number of steps for performing the extractions varied widely, with MK (3) and PK (4) requiring the fewest number of steps, and CTAB (17) and KQ (14) the greatest number of steps.

Table 2 shows the overall performance of each extraction method tested, listing call rate, accuracy, time, cost, numbers of steps, and amplification of the PCR, considering values on a scale of 0 (lowest) to 1 (highest). PK had the best overall average score (0.87), followed by SA (0.81) and MK (0.78). The first two methods showed satisfactory results in genotyping, PCR amplification and cost, while PCR with samples processed with MK did not work. Results are shown on a scale of 0 to 1 (best). Pearson Correlations between observed genotypes and true genotypes (accuracy) varied from 0.14 (BT) to 1.00 (PK).

Table 2. Overall performance of the extraction methods based on six criteria.

Method	SNPType Call rate	Accuracy	Time	Cost*	N° of Steps	PCR Performance	Average score
KQ	0.92	0.84	0.44	0.00	0.18	1.00	0.60
CTAB	0.99	0.92	0.00	0.87	0.00	1.00	0.68
SA	1.00	0.99	0.46	0.95	0.24	1.00	0.81
BT	0.36	0.14	0.96	0.97	0.71	0.00	0.55
PK	1.00	1.00	0.39	0.97	0.76	1.00	0.87
MK	1.00	0.99	0.99	0.64	0.82	0.00	0.78

*cost by method was calculated by sample considering local market values of all necessary supplies.

DISCUSSION

In the present study, a comparative analysis of six different methods of DNA extraction from fish fin clips was performed to identify the best method for SNP genotyping with Fluidigm SNPType® assays.

Low-cost, time-efficient and high-throughput sample processing methods for extraction of DNA are highly desirable in agriculture genomics applications. We found the best DNA extraction methodology for tambaqui fin clips among six different protocols, considering DNA yield, purity, and integrity, extraction efficiency (considering PCR amplification and SNP genotyping success), number of necessary steps, overall time consumption and cost. Considering all these criteria weighted uniformly, the proteinase K (PK) method, showed the best performance followed by SA and CTAB. The PK method was the most straightforward in terms of time consumption, number of required steps, and

laboratory supplies. In the PK protocol, DNA extraction occurs in a single tube and the resulting crude DNA extracts can be used directly in basic PCR amplification procedures, as in advanced SNP genotyping methods involving parallel amplification and fluorescence quantification, such as Fluidigm SNPType® assays performed in the 96.96 Integrated Fluidic Circuits.

DNA isolation is the first critical step for most molecular research. Several methods to isolate DNA from various animal tissues are available in the literature and commercially. However, most of these methods are time consuming and/or have high costs. The SA and CTAB methods are very laborious, require many pippeting and tube exchange steps, and could not be scaled-up to microplates, considering lab equipment available locally. Conversely, DNA extraction using the PK method requires few steps and is easily scalable to 96-well PCR plates. Thus, the possibility of using 8, 12 and even 96 multi-channel pipetters, and potentially even full automation (not tested), in the PK method is highly beneficial, reducing working time, minimizing human errors, and decreasing labor costs (Chacon-Cortes & Griffiths, 2014). The PK method does not use toxic or hazardous reagents, and although protocols based on chloroform and phenol, or CTAB, have been widely used in DNA extractions from fish tissues (Cawthorn et al., 2011; Awodiran & Afolabi, 2018; Xiong et al., 2019), significant disadvantages exist due to the toxicity associated with these chemicals.

The MK method generated satisfactory results, considering call rate, genotyping accuracy, number of required steps and time, however PCR amplification with CM_E2 primers failed, while overall cost was high. KQ was the most expensive method, and the results showed overall costs per sample to be 10 to 30 times lower when using the PK method in comparison to commercial kits MK and KB, respectively. Cawthorn et al. (2011) carried out a similar study when investigating the best method for extracting DNA from the muscle tissue of 28 different fish species. However, they reached a different conclusion, as the best method found was a commercial kit, with costs seven times higher than the cheapest method tested by them. In our study, we introduced process adaptations and fine adjustments that resulted in a positive balance between cost and obtained DNA quality and yield.

Among the five methods tested, the BT protocol had the lowest final average score. The extracted DNA did not show the minimal quality for SNPType® genotyping with the Fluidigm EP1 platform since call rates and accuracy were the lowest. In addition, DNA obtained with BT was not amplifiable by PCR with CM_E2 primers, and the method could not be scaled to PCR microplate format. In conclusion, the BT and MK methods were unsatisfactory in certain criteria and the KB kit was considered the most expensive. While SA and CTAB have a long execution and many steps, it is not possible to perform the extraction in PCR plates. PK was identified as the most economical and efficient technique in terms of time, cost, number of required steps, and scalability in 96-well format/automation, in addition to producing DNA with quality sufficiently good for performing SNP genotyping and PCR from tambaqui fin samples.

CONCLUSION

In this study we show that a simple DNA extraction process with proteinase K digestion from preserved fish fin samples is the most efficient for use in SNP genotyping

with Fluidigm SNPType assays in 96.96 Integrated Fluidic Circuits. The few reagents required by the PK method are commonly used in lab, greatly reducing operational and overall costs per sample. In the PK technique, the resulting crude extracts can be successfully used directly in further PCR-based applications. The PK method was the most straightforward in terms of time consumption, number of steps, and laboratory supply requirements, and can be carried-out in a single tube facilitating the scalability of routine lab procedures, while reducing cross-contamination risks.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ali N, Rampazzo RCP, Costa ADT and Krieger MA (2017). Current Nucleic Acid Extraction Methods and Their Implications to Point-of-Care Diagnostics. *Biomed. Res. Int.* 2017: 9306564.

Anderson-Carpenter LL, Mclachlan JS, Jackson ST, Kuch M, et al. (2011). Ancient DNA from lake sediments : Bridging the gap between paleoecology and genetics. *BMC Evol Biol.* 11: 30.

Awodiran MO and Afolabi O (2018). Genetic Diversity in Cultured and Wild Population of *Clarias gariepinus* (Burchell, 1822) in Nigeria Using Random Amplified Polymorphic DNA (RAPD) and Microsatellite DNA. *Fish Aquac. J.* 9(2): 1000247.

Boyce TM, Zwick ME and Aquadro CF (1989). Mitochondrial DNA in the bark weevils: Size, structure and heteroplasmy. *Genetics*. 123(4): 825-836.

Cawthorn D, Andrew H and Witthuhn RC (2011). Comparative study of different methods for the extraction of DNA from fish species commercially available in South Africa. *Food Control.* 22: 231-244.

Chacon-Cortes D and Griffiths LR (2014). Methods for extracting genomic DNA from whole blood samples : current perspectives. *J. Biorepos Sci. Appl. Med*. 2: 1-9.

Drábková L, Kirschner J and Vlcek C (2002). Comparison of Seven DNA Extraction and Amplification Protocols in Historical Herbarium Specimens of *Juncaceae*. *Plant Mol. Biol. Report*. 20: 161-175.

Lopera-Barrero NM, Povh JA, Ribeiro RP, Gomes PC, et al. (2008). Comparison of DNA extraction protocols of fish fin and larvae samples: Modified salt (NaCl) extraction. *Cienc. Investig. Agrar*. 35(1): 65-74.

Sajali N, Wong SC, Hanapi UK, Abu S, et al. (2018). The Challenges of DNA Extraction in Different Assorted Food Matrices : A Review. *J. Food Sci*. *83*(10): 2409–2414.

Schiebelhut LM, Abboud SS, Daglio LEG, Swift HF, et al. (2017). A comparison of DNA extraction methods for high-throughput DNA analyses. *Mol Ecol Resour*, 17(4): 721-729.

Tan SC and Yiap BC (2009). DNA, RNA, and Protein Extraction: The Past and The Present. *J. Biomed. Biotechnol.* 2009: 574398.

Valsecchi E (1998). Tissue boiling : a short-cut in DNA extraction for large-scale population screenings. *Mol Ecol.* 7: 1243-1245.

Xiong X, Huang M, Yuan F, Lu L, et al. (2019). Development and Validation of a Fast DNA Extraction Protocol for Fish Products. *Food Anal. Methods*. 12: 1998-2008.