

A COMPARATIVE STUDY OF FOUR DNA EXTRACTION PROTOCOLS FROM ADDUCTOR MUSCLE IN GOLDEN MUSSEL (*Limnoperna fortunei*)

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ABSTRACT

The golden mussel, *Limnoperna fortunei*, is a mollusk native to Southeast Asia and a highly invasive species in South American countries such as Brazil, Uruguay, and Argentina. In order to better understand the biological behavior of the species and develop alternative control methods, genetic studies involving the optimization of DNA isolation procedures are of utmost importance. The objective of the present study was to develop a simple, reproducible, free of contaminants, and cheap protocol to extract DNA from *L. fortunei* using the adductor muscle of the mussel as the source. Four DNA extraction protocols were compared: extraction with SDS and proteinase K (P1); extraction with SDS, proteinase K and phenol (P2); TRIzol extraction (P3); and NaCl, SDS and RNase extraction (P4). DNA concentration ($\text{ng } \mu\text{L}^{-1}$) and purity (at 260/280 nm) were measured using a spectrophotometer. DNA purity and amplification were verified by electrophoresis and PCR, respectively. P1 resulted in samples with low DNA concentrations or without any DNA, as revealed by the quantification and purity analysis; P2 had low efficiency, given the absence of DNA in most of the samples subjected to electrophoresis. On the other hand, P3 showed contamination with proteins, as indicated by an absorbance of <1.8 and by the low-quality electrophoresis results. Finally, P4 resulted in well-defined bands, absorbance between 1.8 and 2.0, and successful amplification by PCR. In conclusion, the extraction protocol P4 is a practical, fast, free of contaminants, and efficient method for the isolation of *L. fortunei* DNA.

Key words: molecular biology; mollusk; NaCl; phenol/chloroform; Proteinase K; SDS; TRIzol.

ESTUDO COMPARATIVO DE QUATRO PROTOCOLOS DE EXTRAÇÃO DE DNA DO MÚSCULO ADUTOR EM MEXILHÃO DOURADO (*Limnoperna fortunei*)

RESUMO

O mexilhão dourado, *Limnoperna fortunei* é um molusco originário do sudeste da Ásia, altamente invasor em países Sul-Americanos como Brasil, Uruguai e Argentina. Para compreender melhor o comportamento biológico da espécie e criar alternativas de controle é indispensável a realização de estudos genéticos, onde a otimização dos procedimentos de isolamento do DNA é fundamental. O objetivo desse estudo foi obter um protocolo simples, reproduzível, não contaminante e barato para a extração do DNA de *L. fortunei*. Foram comparados quatro protocolos experimentais de extração de DNA, utilizando como material biológico o músculo adutor: extração por SDS e proteinase K (P1), extração por SDS, proteinase K e fenol (P2), extração por Trizol (P3) e extração por NaCl (P4). A quantificação ($\text{ng } \mu\text{L}^{-1}$) e a pureza (260/280 nm) do DNA foram obtidas por espectrofotometria. A integridade e a amplificação do DNA foram verificadas através de eletroforese e PCR, respectivamente. P1 demonstrou baixas concentrações e ausência de DNA nas amostras, identificado pela quantificação e teste de integridade. P2 apresentou baixa eficácia, visualizada pela ausência de DNA na maioria das amostras na eletroforese. Por outro lado, P3 exibiu sinais de contaminação por proteínas, identificado pela razão de absorvância <1.8 e pela baixa qualidade da eletroforese. Finalmente, P4 mostrou um padrão na formação das bandas, absorvância entre 1,8 – 2,0 e sucesso na amplificação pela PCR. Conclui-se que o protocolo de extração P4 mostrou-se como um método prático, rápido, não contaminante e eficiente para obtenção do DNA de *L. fortunei*.

Palavras-chave: biologia molecular; fenol/clorofórmio; NaCl; molusco; SDS/Proteinase K; Trizol.

INTRODUCTION

The golden mussel (*Limnoperna fortunei*), originally from Southeast Asia (BARBOSA and MELO, 2009), presents physiological and ecological characteristics propitious to fast and efficient proliferation in fresh water (DARRIGRAN and PASTORINO, 1995), resulting in its high invasiveness (MONTALTO and EZCURRA DE DRAGO, 2003).

The first occurrence of *L. fortunei* in Brazil was registered in the delta of the Jacuíno River in 1998 (MANSUR *et al.*, 2003). Since then, it has caused serious damage to water collection systems, river navigation, and tourism (AGUDO-PADRÓN, 2008). Incrustation on galvanized screens was observed in fish farming systems, hindering water renewal, oxygenation, and the elimination of residues toxic to the animals. It also interferes with tank operation and durability, exposing the fish to constant surface wounds.

Additional studies on the variability and genetic structure of *L. fortunei* are required in order to increase the available information regarding this mollusk. Genetic studies represent an important approach to understanding the establishment of the species in ecosystems (LOPES *et al.*, 2014), and they provide information that might contribute to the development of new technologies for controlling mollusk populations (OLIVEIRA *et al.*, 2014). However, in the case of *L. fortunei*, genetic studies are still scarce and do not allow for a scientific confrontation of the results observed in recent studies (GHABOOLI *et al.*, 2013; PAOLUCCI *et al.*, 2014; ULIANO-SILVA *et al.*, 2016).

One of the limiting factors for these genetic analyses is the lack of a DNA extraction protocol that is reproducible and species-specific. Commercial kits can be used for the extraction of DNA from *L. fortunei* (ULIANO-SILVA *et al.*, 2016); however, these are often expensive, especially in the analysis of a large number of samples. Given the importance of genetic studies on *L. fortunei*, it is necessary to develop protocols that enable easy DNA manipulation, that are accessible and, above all, give satisfactory results adequate for the type of sample in question. In this context, the objective of the present study was to develop a simple, reproducible, free of contaminants, and cheap protocol for the extraction of *L. fortunei* DNA.

METHODS

L. fortunei samples were collected at fish farms with net cages, located in the reservoirs of Canoas I (22° 56' 25.63" S, 50° 24' 49.86" W), Rosana (22° 39' 25.20" S, 52° 46' 52.78" W), and Capivara (22° 41' 17.16" S, 51° 17' 51.30" W), supplied with water from the Paranapanema River, an affluent of the Paraná River, in the state of Paraná, Brazil.

The material was collected during fish removal, by extracting the mussels from the tank screens. The samples were immediately washed with water from the corresponding reservoir in order to remove all material attached to the surface. Thereafter, they were placed in thermo-boxes containing ice to kill them and then immediately transported to the laboratory, where they were

stored in a freezer at -20 °C until the analysis. All procedures were carried out taking care to avoid the transportation of larvae or living adults, thus preventing the contamination of other areas.

The analyses were performed in the NEPAG and Animal Virology Laboratories, both at the State University of Londrina (UEL). The adductor muscle (responsible for shell closure) was used as the sample for DNA extraction. After identification, the muscle was removed by sectioning at the insertion point using a blade. Four protocols routinely used in laboratories that manipulate DNA were tested. For each protocol, four mussels from each fish farm were analyzed, amounting to 12 samples per protocol (48 samples in total).

Protocol 1 (P1) – Extraction using SDS and proteinase K

The muscle samples were grinded, diluted in 500 µL PBS, homogenized, and centrifuged at 3.000 rpm for 5 min, yielding 500 µL of supernatant, to which 10 µL proteinase K and 50 µL of 10% SDS were added. The samples were then homogenized and incubated in a thermoblock at 56 °C for 30 min, followed by centrifugation at 3.000 rpm for 5 min. The resulting supernatant was stored at -20 °C.

At the time of extraction, 500 µL lysis buffer (Tris/HCl, Triton, Guanidine isothiocyanate, EDTA) and 25 µL hydrated silica were added to the supernatant. The samples were homogenized using a vortex at room temperature for 30 min, followed by centrifugation at 3.000 rpm for another 5 min. The supernatant obtained was transferred to a flask containing NaOH, to which 500 µL lysis buffer (Tris/HCl, Guanidine isothiocyanate) was added, with subsequent homogenization and centrifugation. This stage was repeated twice. After that, 1 mL cold 70% ethanol was added to the supernatant, which was homogenized using a vortex and discarded. This step was repeated twice. Subsequently, 1 mL cold acetone was added, and, after homogenization, the supernatant was discarded, while the pellet consisting of silica was dried in a water bath at 56 °C for 15 min. DPEC (diethyl pyrocarbonate) water (50 mL) was added to the pellet, followed by homogenization to prevent the silica from adhering to the wall of the tube. The samples were immediately placed in a water bath at 56 °C for 15 min, before the final homogenization using a vortex and centrifugation at 13.000 rpm for 4 min. The supernatant obtained by this step was placed in a 50 µL microtube and stored at -20 °C.

Protocol 2 (P2) – Extraction using SDS, proteinase K, and phenol/chloroform

The muscle sample was grinded, diluted in 500 µL PBS, homogenized, and centrifuged at 3.000 rpm for 5 min, yielding 500 µL of supernatant, to which 10 µL proteinase K and 50 µL 10% SDS were added. The samples were then homogenized and incubated in a thermoblock at 56 °C for 30 min, with subsequent centrifugation at 3.000 rpm for 30 s. After that, 500 µL of phenol/chloroform/isoamyl alcohol was added to the samples, which were homogenized using a vortex and placed in a water

bath at 56 °C for 15 min. The samples were again homogenized and centrifuged at 3.000 rpm for 10 min, with the supernatant being transferred to a new microtube and stored at 4 °C.

Subsequently, 500 µL lysis buffer (Tris/HCl, Triton, Guanidine isothiocyanate, EDTA) and 25 µL hydrated silica were added to the supernatant, followed by homogenization using a vortex at room temperature for 30 min and centrifugation at 3.000 rpm for 5 min. The supernatant was transferred to a flask containing NaOH, to which 500 µL lysis buffer (Tris/HCl, Guanidine isothiocyanate) was added before being homogenized and centrifuged anew (this step was repeated twice). After that, 1 mL cold 70% ethanol was added to the supernatant, which was homogenized using a vortex and discarded. This step was repeated twice before addition of 1 mL cold acetone (P.A.). After homogenization, the supernatant was discarded, and the silica pellet was dried in a water bath at 56 °C for 15 min. DPEC water (50 µL) was added to the pellet, and the samples were immediately homogenized to prevent the silica from adhering to the wall of the tube, before being placed in a water bath at 56 °C for 15 min. A final homogenization was carried out using a vortex, followed by centrifugation at 13.000 rpm for 4 min. The resulting supernatant was placed in a 50 µL microtube and stored at -20 °C.

Protocol 3 (P3) – Extraction with TRIzol

TRIzol (750 µL) was added to the macerated muscle sample, homogenized using a vortex, and incubated at room temperature for 5 min. After that, 200 µL chloroform was added, followed by homogenization for 15 s and incubation at room temperature for 15 min. Subsequently, the samples were centrifuged at 12.000 rpm at 4 °C for 15 min, with the supernatant being collected and transferred to a new microtube. Propanol (500 µL) was then added to the samples, which were then homogenized, incubated at room temperature for 10 min, and centrifuged again at 12.000 rpm, at 4 °C for 10 min. The resulting supernatant was removed by inversion, and the pellet was left to air-dry at room temperature. Subsequently, 1 mL of 75% ethanol was added to the pellet, followed by homogenization and centrifugation at 7.500 rpm, at 4 °C for 5 min. The supernatant was removed by drying in a thermoblock. After that, 30 µL DPEC water was added to the tubes, which were then incubated in a water bath at 56 °C for 10 min. Finally, the samples were spun, and the supernatant was collected into new microtubes, which were then stored at -20 °C.

Protocol 4 (P4) – Extraction using NaCl, SDS, and RNase

This protocol was based on the methodology described by LOPERA-BARRERO *et al.* (2008), with modifications. Initially, the muscle samples were washed with absolute ethanol, transferred to new microtubes, and kept at room temperature for 10 min for the residual ethanol to evaporate. Subsequently, 700 µL lysis buffer, 50 µL of 20% SDS, and 15 µL proteinase K (200 µL mL⁻¹) were

added to the samples, followed by homogenization by inverting the tube, which was then placed in a water bath at 50 °C for 17 h.

In the next step, 700 µL of 5 M NaCl was added to the samples, which were homogenized by inverting the tube and centrifuged at 12.000 rpm for 10 min. The supernatant (800 µL) was collected into a new microtube, 700 µL cold absolute ethanol was added, and the tube was homogenized by inverting, before being stored in a freezer at -20 °C for 2 h. After that, the samples were centrifuged at 12.000 rpm for 10 min, the ethanol in the supernatant was immediately discarded, and the pellet was air-dried at room temperature for 30 min. Subsequently, 35 µL Tris/EDTA (TE) and 5 µL RNase (30 µg mL⁻¹) were added to the tubes, which were then placed in a water bath at 37 °C for 40 min. Finally, the samples were stored at -20 °C.

DNA was quantified using the SLIPQ 026 - Quantificador L-Quant (Scienlabor, Ribeirão Preto, SP, Brazil), using 2 µL of each DNA sample and TE as the blank. The purity of the samples was assessed via the ratio of absorbance values measured at 260 and 280 nm. The DNA concentration of each sample was adjusted to 30 ng µL⁻¹ using TE.

DNA purity was verified by horizontal electrophoresis on a 1% agarose gel (1.6 g agarose, 160 mL of 1X TBE, and 13 µL Sybr Safe (Life Technologies, São Paulo, SP, Brazil)). Each DNA sample (12 µL) was added to a separate well, and 2 µL bromophenol blue was added to each well. The gel was run at 100 V for 60 min. The gel was then photographed using a UVP transilluminator (Upland, CA, USA) coupled to a camera.

The microsatellite primer Lf04 (GenBank access: HQ843811) (ZHAN *et al.*, 2012) was used in the PCR. The reactions were performed in a final volume of 15 µL, consisting of 7.1 µL ultrapure water, 1.5 µL of 1X Tris-KCl Buffer (20 mM Tris-HCl at pH 8.4 and 50 mM KCl), 0.6 µL of 2X MgCl₂, 2.4 µL dNTP (0.4 mM), 0.6 µL forward primer (0.4 mM), 0.6 µL reverse primer (0.4 mM), 0.2 µL of one-unit Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 2 µL template DNA.

The reactions were carried out in a Veriti® thermocycler (Applied Biosystems, Austin, TX, USA) with the following cycles: 94 °C for 5 min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; and 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 3% agarose gel, carried out in 1X TBE buffer and run at 60 V for 4 h. Gel images were captured using a transilluminator coupled to a camera.

The obtained concentration (ng µL⁻¹) and absorbance values (260/280 nm) were subjected to analysis of variance (ANOVA). The normality of the distribution of residuals was tested and confirmed by a p value > 0.05 on the Lilliefors (Kolmogorov-Smirnov) normality test. The Tukey test was used for comparison of averages when the p-value was <0.05.

RESULTS

Quantification and purity of DNA

Due to the low concentrations or the absence of DNA extracted using P1, it was considered unsuitable for the extraction of *L. fortunei* DNA and therefore excluded from the statistical analyses.

Compared to the other protocols, the mean DNA concentration ($\text{ng } \mu\text{L}^{-1}$) was the highest ($p < 0.05$) when P4 was utilized, indicating larger amounts of DNA in the samples. The mean absorbance (260/280 nm) values ranged from 1.587 (in P3) to 1.857 (in P4) (Table 1).

The purity analysis using gel electrophoresis (Figure 1) showed an absence of bands in samples extracted using the protocol P1,

confirming this protocol as unusable. P2, in turn, showed low efficiency, since only two of 12 samples (approximately 17%) showed well-defined bands. On the other hand, P3 showed signs of contamination by proteins, RNA, or solvents, visible by the smeared bands on the gel. Finally, it was confirmed that P4 yielded the best band patterns, without contamination, evident by the absence of smearing and by the well-defined fragments (Figure 1).

DNA Amplification

The PCR performed on the samples extracted using P4, using the Lf04 primer, yielded a satisfactory amplification pattern, with the formation of microsatellite alleles being clearly distinguishable on the gel (Figure 2).

Table 1. Means and standard deviations (SD) of DNA concentrations and absorbances at 260/280 nm using the extraction protocols P2, P3, and P4.

Protocols	DNA concentration ($\text{ng } \mu\text{L}^{-1}$)		Absorbance (260/280 nm)	
	Mean	SD	Mean	SD
P2	198.155 a	111.435	1.793 a	0.282
P3	136.584 a	47.804	1.587 b	0.274
P4	328.83 b	126.151	1.850 a	0.119
p value	0.0002		0.0258	

Letters indicate significant difference between Protocols P2, P3, and P4 by Tukey's test at $p < 0.05$. P2: Extraction using SDS, proteinase K, and phenol; P3: Extraction with TRIzol; P4: Extraction using NaCl, SDS, and RNase.

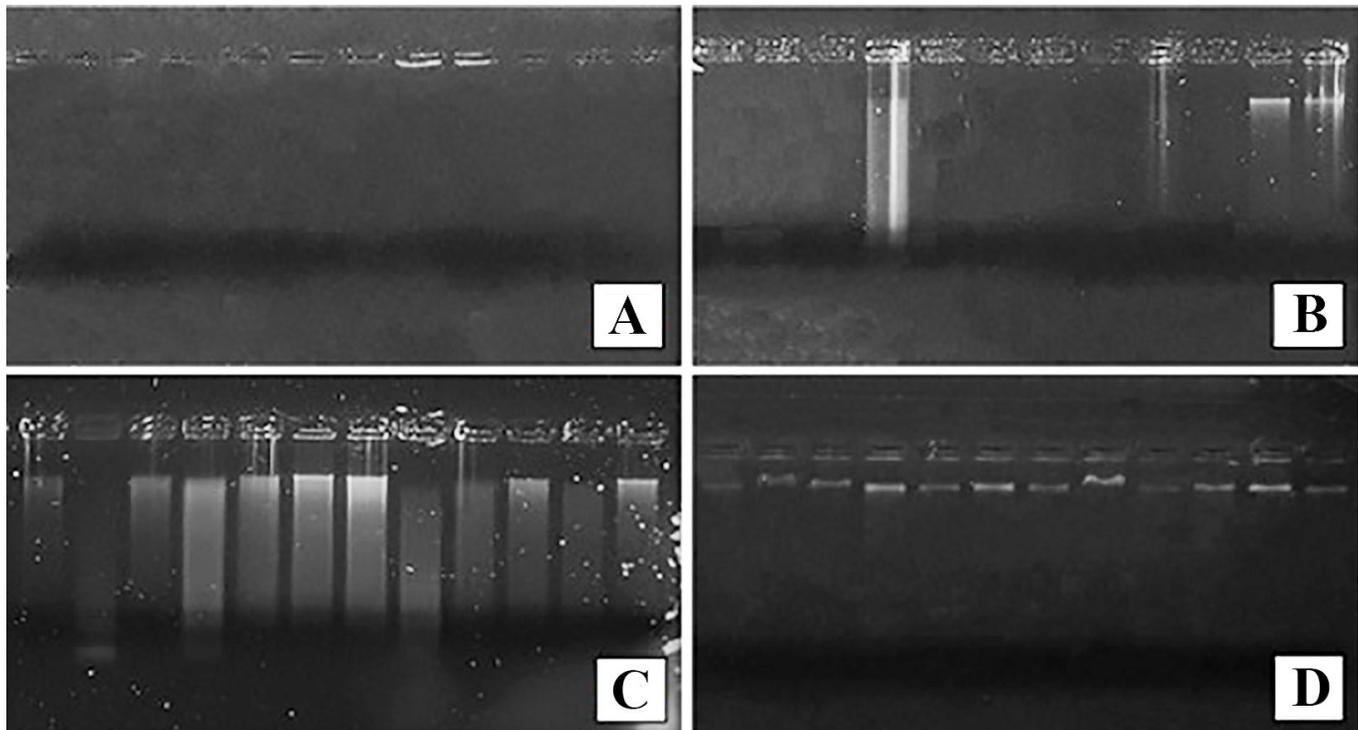


Figure 1. One-percent agarose gel electrophoresis of the 12 DNA samples extracted from the adductor muscle of *L. fortunei* using the four extraction protocols. P1 (A); P2 (B); P3 (C); and P4 (D). P1: Extraction using SDS and proteinase K; P2: Extraction using SDS, proteinase K, and phenol; P3: Extraction with TRIzol; P4: Extraction using NaCl, SDS, and RNase.

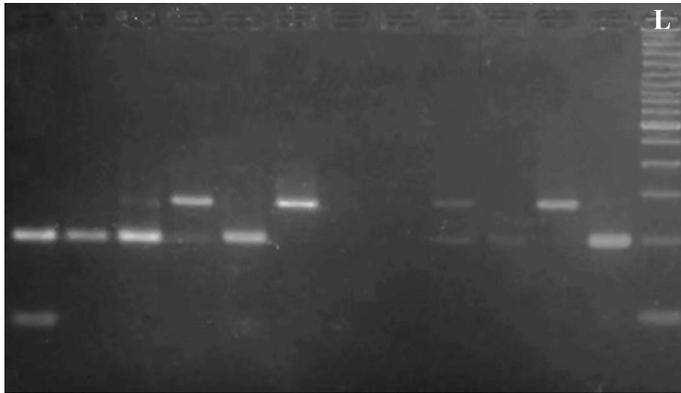


Figure 2. Amplification of the 12 samples from the extraction using P4, using Lf04, which has a microsatellite molecular marker, as the primer. L = 100 bp ladder. P4: Extraction using NaCl, SDS, and RNase.

DISCUSSION

The adductor muscle has proven to be a simple sampling source, as well as allowing for the extraction of DNA of a sufficient quantity and quality. This tissue was also used by ENDO *et al.* (2009), GHABOOLI *et al.* (2013), and ZHAN *et al.* (2012), corroborating its efficacy in the extraction of DNA from *L. fortunei*.

Through the quantity of DNA and proteins (using the 260/280 nm ratio), it is possible to evaluate the quality of DNA (VIGLIARI BONDOLI *et al.*, 2016; LEE *et al.*, 2017), with the values that range between 1.8 and 2.0 reflecting the presence of DNA of good quality with a low level of contamination (ZARZOSO-LACOSTE *et al.*, 2013; HEALEY *et al.*, 2014). In this context, P2 and P3 did not show satisfactory results for DNA extraction, possibly due to contamination with organic solvents such as phenols (HEALEY *et al.*, 2014) used in P2, or with proteins, characterized by the smearing during the electrophoresis and by absorbance values below 1.8 (LOPERA-BARRERO *et al.*, 2008; ZARZOSO-LACOSTE *et al.*, 2013; GHATAK *et al.*, 2013), as seen when P3 was used. On the other hand, in P4, the absorbance values remained within the ideal range (1.8-2.0), demonstrating its efficiency regarding the purity of extracted DNA, as confirmed by well-defined bands on the agarose gel. These results also confirm that there was no degradation in the transport process, since all individuals were collected from the same sites and used in all protocols.

A factor that enabled the use of P4 was the presence of proteinase K and RNase. The first is a proteolytic enzyme highly reactive at various conditions of pH, detergents (SDS), and buffers (KRISTJÁNSSON *et al.*, 1999), being widely used to degrade various proteases (KUMAR SHUKLA and RAO, 2013), including some that could cause damage to DNA. Treatment with RNase, in turn, enables the production of high quality DNA samples after precipitation (HEALEY *et al.*, 2014), since the presence of RNA might interfere with precise DNA amplification (WASKO *et al.*, 2003). LOPERA-BARRERO *et al.* (2008) observed that the use

of RNase reduces the smearing on gels caused by the presence of RNA, in turn yielding a purer product with well-defined bands. However, it should be noted that the use of these enzymes does not ensure a successful extraction, since extraction with neither P1 nor P2, both including proteinase K treatment, gave satisfactory results. Nevertheless, two samples extracted with P2 had well-defined bands on the gel, indicating that this protocol could be improved and made usable for DNA extraction. In P3, in turn, the fact that these enzymes, primarily proteinase K, were not used was probably responsible for the smearing observed on the gel and for the absorbance values below 1.8, characteristic of protein contamination. Further tests are therefore needed to improve these two extraction protocols.

According to MESQUITA *et al.* (2001), concentration, purity, and integrity of the extracted DNA depend on several factors, and greatly affect the success of subsequent procedures such as PCR. Considering the concentration, purity, and integrity of our DNA samples, obtained by gel electrophoresis, we can conclude that P4 yielded results characteristic of a satisfactory extraction. This was corroborated by amplification with the microsatellite primer Lf04, which showed high resolution in the visualization of alleles in *L. fortunei*.

Finally, the optimization of DNA extraction protocols is fundamental for the elimination of traces of contaminants that could be detrimental for the PCR and for simultaneously increasing the sensitivity of the detection of genetic material (ZARZOSO-LACOSTE *et al.*, 2012). Therefore, extraction methodologies that maximize the number of extracted samples and promote band patterns that maintain the purity and integrity, are recommended in population genetics analyses. In this context, through our study, we developed a simple, cost-effective, and fast method of DNA extraction based on the use of NaCl, proteinase K, and RNase that enables quality isolation applicable on multiple samples of *L. fortunei*.

CONCLUSION

The extraction protocol using NaCl, SDS, and RNase is a practical, fast, free of contaminants, and efficient method for the isolation of *L. fortunei* DNA.

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