Technical paper

A Comparison of Eight Methods for DNA Etraction from Processed Seafood Products

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DNA extraction is critical for seafood authentication based on DNA techniques. In this study, eight extraction methods, including commercial kit, from three types of seafood (quick frozen, roasted, and canned products) were compared in terms of DNA yield and purity. The quantity and quality of extracted DNA were evaluated using the ratio A260/A280. Quality was also evaluated by two pairs of primers that could amplify two different sizes of DNA fragments respectively. For quick frozen samples, the results showed that the top three methods for DNA yield were the SDS method, TIANGEN GMO food DNA Extraction Kit, and TIANamp Marine Animals DNA Kit. For roasted samples, the top two methods for DNA yield were the SDS and improved CTAB methods. For canned samples, the top two methods for DNA yield were the SDS and phenol/chloroform/isoamyl alcohol methods. The differences between these aforementioned methods and the remaining methods were highly significant ($P < 0.01$). The DNA extracted from roasted and canned seafood could not amplify the 650 bp but amplified the 358 bp target fragment. This study determined the proper DNA extraction method for three types of seafood to facilitate seafood authentication.

Keywords: seafood, authentication, DNA extraction, comparison, PCR

Introduction

In recent years, commercial seafood and processed fishery products have been widely distributed worldwide. A wide variety of seafood species is available in the global market, with more than 20,000 species of fish and seafood estimated to be used worldwide for human consumption. In their whole, unprocessed forms, these species can generally be identified morphologically. However, most seafood has been processed to some extent, such as roasting and canning, without morphological characteristics. Increased international trade, increased worldwide fish and seafood consumption, and various levels of supply and demand of certain species have led to cases of economic fraud in which one seafood species is illegally substituted for another (Civera, 2003).

Market surveys in Europe and North America have reported

seafood species substitution at a level of 15% to 43% (Espiñeira *et al.*, 2008a, 2008b, 2009a, 2009b, 2009c; Wong *et al.*, 2008), with an exceptionally high level of 75% for red snapper products (Marko *et al.*, 2004). Therefore, to enforce labeling regulations and to prevent product substitution, sensitive analytical methods are required for determining the species of a seafood product with no detectable external features (Gil, 2007; Mafra *et al.*, 2007).

DNA-based techniques are considered highly efficient for seafood authentication because of their remarkable stability, even for thermally processed seafood products (Comi *et al.*, 2004; Pepe *et al.*, 2005; Chapela *et al.*, 2007). The isolation of genomic DNA remains critical for DNA-based species identification methods, such as polymerase chain reaction (PCR). Various protocols have been developed for DNA extraction from aquatic species, including

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many commercially available kits. However, DNA isolation from food matrices for use in PCR is complicated because many ingredients in food act as PCR inhibitors, and the quantity and the quality of the extracted DNA are extremely sample dependent (Chapela *et al.*, 2007). The appropriate method for different types of samples produced by different processing techniques may differ. The aim of this study is to compare eight DNA extraction methods (including four commercial kits) in terms of DNA yield and quality when applied in three types of seafood with different processing techniques, such as quick frozen fish, roasted fillet, and canned fish.

Materials and Methods

(1) *Sample preparation* The roasted and canned codfish products were purchased at a local supermarket in Qingdao. Quick frozen codfish was purchased at a local fish market and taxonomically identified as *Gadus macrocephalus* with respect to external characters. The fish was stored at -80°C until analysis.

(2) *Sample pre-treatment* Oil and lipids were removed from roasted and canned fish by soaking fish muscles overnight in a mixture of chloroform/methanol/water (1:2:0.8, v/v). The defatted muscle was recovered by filtration and stored at -80°C until DNA extraction.

(3) DNA extraction

a) *Cetyl trimethylammonium bromide (CTAB) method* DNA was extracted according to the protocol described by Lipp *et al.* (1999) with minor adjustments. Briefly, 50 mg of homogeneous sample was added in 1 mL of CTAB buffer (20 g of CTAB/L, 1.4 M NaCl, 0.1 M Tris/HCl, and 20 mM EDTA), and the solution was mixed and incubated at 65°C for 30 min. The solution was then centrifuged for 10 min at 12,000 rpm. The upper layer was transferred to a tube containing 200 μL of chloroform. The mixture was mixed for 30 s and centrifuged for 10 min at 11,500 rpm. The supernatant was transferred into two volume parts of CTAB precipitation solution (5 g of CTAB/L and 0.04 M NaCl). The mixture was incubated for 60 min at room temperature and centrifuged for 5 min at 12,000 rpm. The precipitate was dissolved in 100 μL of NaCl (1.2 M) and added with 100 μL of chloroform. The mixture was mixed for 30 s and centrifuged for 10 min at 12,000 rpm. The upper layer was mixed with isopropyl alcohol (0.6 volume parts) and centrifuged again for 10 min at 11,500 rpm. The separated pellet (DNA) was redissolved in 100 μL of sterile, deionized water and frozen at -20°C until use.

b) *Improved CTAB (ICTAB) method* DNA was extracted according to the protocol described by Chapela *et al.* (2007) with minor adjustments. Briefly, 100 mg of sample was mixed with 500 μL of Buffer I (1.2% CTAB w/v, 10 mM EDTA-Na, 60 mM Tris-Cl, 0.8 M NaCl, pH 8) containing 0.5 μL of 3-mercapto-1,2-propanediol (1% v/v) and 2.5 μL of proteinase K (10 mg/mL). Samples were incubated at 65°C until they were digested completely and then centrifuged for 10 min at 12,000 rpm. The

supernatant was extracted with 500 μL of chloroform and centrifuged again for 30 min at 12,000 rpm. The upper phase was precipitated with 500 μL of chloroform and centrifuged for 5 min at 12,000 rpm. The supernatant was mixed with two volumes of Buffer II (1% CTAB w/v, 10 mM EDTA-Na, 50 mM Tris-Cl, pH 8) and centrifuged for 10 min at 12,000 rpm. The pellet was mixed with 400 μL of Buffer III (1 M NaCl, 10 mM Tris, 1 mM EDTA-Na, pH 8) and incubated at least 30 min at 65°C . Then, samples were cooled, and 400 μL of isopropanol was added to the samples. The mixture was incubated at room temperature for 10 min, and the samples were centrifuged for 10 min at 12,000 rpm. The pellet was dissolved in 100 μL of sterile, deionized water and frozen at -20°C until use.

c) *Phenol/chloroform/ isoamyl alcohol (PCI) method* DNA was extracted according to the protocol described by Sambrook *et al.* (1989) with minor adjustments. Briefly, 100 mg of sample was homogenized with 500 μL of lysis buffer (10 mM Tris-HCl, 100 mM EDTA, pH 8, 0.5% SDS, 100 $\mu\text{g}/\text{mL}$ proteinase K, and 20 $\mu\text{g}/\text{mL}$ RNase A). The mixture was incubated at 55°C with shaking until the sample was digested completely and then chilled on ice for 10 min. Following centrifugation at 12,000 rpm for 10 min, the supernatant was extracted twice with PCI (25:24:1, v/v) and once with chloroform, and then precipitated with ethanol using a 1/10 volume of 3 M sodium acetate at -20°C for 10 min. After centrifugation at 12,000 rpm for 10 min, the pellet was resuspended in 50 μL of sterile, deionized water and frozen at -20°C until use.

d) *SDS method* DNA was extracted according to the protocol described by Hsieh *et al.* (2001) with minor adjustments. Briefly, 100 mg of sample was homogenized with extraction buffer (50 mM Tris-HCl, pH 8, 0.1 M EDTA, 1% SDS, 0.2 M NaCl, and 100 $\mu\text{g}/\text{mL}$ proteinase K). The mixture was incubated at 55°C with shaking until the sample was digested completely. After incubation, tubes were placed on ice for 10 min and centrifuged at 12,000 rpm for 10 min. The supernatant was extracted twice with PCI (25:24:1, v/v) and once with chloroform, and then precipitated with ethanol at -20°C . After centrifugation at 12,000 rpm for 10 min, the pellet was resuspended in 100 μL of sterile, deionized water and frozen at -20°C until use.

e) *TIANGEN GMO food DNA Extraction Kit (Kit 1)* DNA was extracted following the manufacturer's protocol. Briefly, 500 μL of GMO1 buffer and 20 μL of proteinase K (20 mg/mL) were added to 100 mg of sample, vortexed vigorously for 1 min, and incubated at 56°C by occasionally inverting the tube until the sample was digested completely. We added 200 μL of GMO2 buffer to the mixture, which we vortexed for 1 min and incubated at room temperature for 10 min. The tube was centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to a new tube containing 0.7 volume parts of isopropyl alcohol. The tube was centrifuged at 12,000 rpm for 5 min. The DNA pellet was dissolved in 100 μL of sterile, deionized water and frozen at -20°C until use.

f) TIANGEN TIANamp Marine Animals DNA Kit (Kit 2) DNA was extracted following the manufacturer's protocol. Briefly, 30 mg of sample was added into 200 μ L of GA solution, vortexed vigorously for 15 s, and incubated for 5 min at room temperature. We added 20 μ L of proteinase K (20 mg/mL) to the solution and incubated the solution for 1 h at 55°C, with occasional inversion of the tube. When lysis was complete, 200 μ L of GB solution was added to the sample and incubated for 10 min at 70°C. We added 200 μ L of alcohol to the mixture, which was transferred to a column CB3 placed in a collection tube. The tube was centrifuged at 12,000 rpm for 30 s, the flow-through liquid was discarded, and the membrane of CB3 was washed twice with 700 μ L of wash solution PW. Finally, the column CB3 was placed in a new 1.5 mL tube, and DNA was eluted with 100 μ L of sterile, deionized water and frozen at -20°C until use.

g) Fermentas Genomic DNA purification Kit (Kit 3) DNA was extracted following the manufacturer's protocol. Briefly, the sample was pulverized in liquid nitrogen using a pestle. We resuspended 30 mg of powder with 200 μ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). We mixed 200 μ L of the sample with 400 μ L of lysis solution and incubated the mixture at 65°C for 5 min, with occasional inversion of the tube. The mixture was extracted with 600 μ L of chloroform and centrifuged at 10,000 rpm for 2 min. The upper aqueous phase was transferred to 800 μ L of precipitation solution, mixed gently at room temperature for 2 min, and centrifuged at 10,000 rpm for 2 min. The DNA pellet was completely dissolved in 100 μ L of NaCl solution. We added 300 μ L of cold ethanol to precipitate DNA for 10 min at -20°C. After centrifugation at 10,000 rpm for 4 min, the DNA pellet was dissolved in 100 μ L of sterile, deionized water and frozen at -20°C until use.

h) TOYOBO MagExtractor® Nucleic Acid Purification Kit (Kit 4) DNA was extracted following the manufacturer's protocol. Briefly, the sample was pulverized in liquid nitrogen using a pestle. We resuspended 10 mg of powder with 90 μ L of proteinase K buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA) and then added 5 μ L of proteinase K (10 mg/mL) and 5 μ L of 10% SDS to the mixture. The mixture was incubated at 55°C, with occasional inversion of the tube until the sample was digested completely. After centrifugation at 10,000 rpm for 5 min, the supernatant was transferred to 750 μ L of Lysis-Binding Solution and 40 μ L of magnetic beads. The mixture was vortexed vigorously for 10 min and centrifuged at 3,000 rpm for 1 min. The pellet (magnetic bead) was washed twice with 900 μ L of washing solution, centrifuged at 3,000 rpm for 1 min, added with 900 μ L of 70% alcohol, vortexed vigorously for 1 min, and centrifuged at 3,000 rpm for 1 min. The pellet was resuspended with 100 μ L of sterile, deionized water, vortexed vigorously for 10 min, centrifuged at 3,000 rpm for 1 min, collected the supernatant containing DNA into another tube, and frozen at -20°C until use.

(4) DNA quantification and purity Three types of samples

were extracted by each method repeatedly for six times. Extracted DNA was quantified by measuring the absorbance of the DNA extracts at 260 nm (1 optical density at 260 nm equals 50 μ g/mL DNA). Purity was verified by determining the ratio A260/A280 (Sambrook *et al.*, 1989).

(5) PCR amplification PCR was used to verify the suitability of the prepared DNA for molecular identification of the sample origin. The primers COI-1(5'-CAC AAA GAC ATT GGC ACC CT-3') and COI-2(5'-CCT CCT GCA GGG TCA AGA A-3') described by Iguchi *et al.* (2012) amplified the 650 bp fragment of the mitochondrial *cytochrome c oxidase subunit I* (COI) gene. The primers Cytb-1 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and Cytb-2 (5'-CCC TCA GAA TGA TAT TTG TCC TCA-3') described by Bartlett *et al.* (1991) amplified the 358 bp fragment of the mitochondrial *cytochrome b* gene. Each PCR analysis was performed in a volume of 25 μ L containing 17.3 μ L of distilled water, 2.5 μ L of 10 $\times$ PCR buffer, 1.0 μ L of dNTP Mix (2.5 mM), 1.0 μ L of each primer (10 mM), 0.2 μ L of 5 U/ μ L *Taq* DNA polymerase (Takara, Japan), and 2.0 μ L of template DNA containing approximately 5 ng of DNA. PCR consisted of an initial denaturation step at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 52°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification was checked by ultraviolet (UV) light after electrophoresis in 1.5% agarose gel (Biowest®, Spain) in the presence of GeneFinder™ nucleic acid stain (BioV Genetech Co., Ltd., China) and sequenced in an automated sequencer (3730XL ABI, Shanghai Sangon Co., Ltd., China).

All PCR experiments were repeated thrice, and a negative control sample (deionized water) was run with each test.

(6) Statistical analysis For each extraction, the results are presented as mean $\pm$ standard deviation ($n = 6$). All data were statistically treated with SPSS 13.0 (USA). A P value <0.05 was considered statistically significant, whereas a P value <0.01 was considered highly significant.

Results and Discussion

The authentication of fish and seafood species has become an important issue in the seafood industry (Rasmussen *et al.*, 2008). Many authentication methods for seafood have been developed based on proteins or DNA. Protein-based methods, such as isoelectric focusing (Ataman *et al.*, 2006), chromatographic techniques (Horstkotte *et al.*, 2003), and immunological techniques (Ochiai *et al.*, 2001), are of considerable value in certain instances. However, these methods are not suitable for routine analysis because proteins lose their biological characteristics or become degraded in processed foods (Rasmussen *et al.*, 2008).

The use of DNA-based methods for species detection has many advantages over protein-based methods, such as increased specificity, sensitivity, and reliable performance with highly processed samples. DNA has the potential to provide a greater amount of information because of the degeneracy of the genetic

Table 1. Comparison of DNA yield between three kinds of seafood extracted using eight methods

Extraction Method	Quick frozen codfish	Roasted codfish	Canned codfish
CTAB	0.316 ± 0.164 ^b	0.214 ± 0.067 ^b	0.184 ± 0.051 ^b
ICTAB	0.349 ± 0.168 ^b	0.480 ± 0.097 ^a	0.209 ± 0.065 ^b
PCI	0.597 ± 0.241 ^b	0.346 ± 0.065 ^b	0.419 ± 0.097 ^a
SDS	1.057 ± 0.114 ^a	0.505 ± 0.063 ^a	0.594 ± 0.058 ^a
Kit 1	1.223 ± 0.177 ^a	0.190 ± 0.021 ^b	0.033 ± 0.009 ^b
Kit 2	1.190 ± 0.217 ^a	0.278 ± 0.025 ^b	0.021 ± 0.008 ^b
Kit 3	0.312 ± 0.066 ^b	0.022 ± 0.004 ^b	0.171 ± 0.221 ^b
Kit 4	0.042 ± 0.002 ^b	0.009 ± 0.003 ^b	0.025 ± 0.002 ^b

Data are mean±standard deviation, n=6 in each case.

Different letters in the same column indicate significant different at P=0.01.

code and the existence of noncoding regions. Therefore, for seafood species identification in heat-processed matrices, a DNA method instead of protein analysis is preferable (Lockley *et al.*, 2000).

The first step in DNA-based species identification is the isolation of genomic DNA. However, the different ways seafood products are processed with different conditions may need different DNA extraction methods. The aim of this study is to determine whether differences exist in quantity and quality in the three kinds of seafood products using eight different DNA extraction methods.

The DNA yield data are shown in Table 1. We found statistically significant differences in both product type and extraction method, supporting the finding that DNA extraction depends both on the method used and the type of product.

For quick frozen codfish, a product which was freezed until the product temperature had reached −18°C or colder at the thermal center, and kept deep frozen during transportation, storage and distribution, the top three methods for DNA yield were the SDS method (1.057 µg/mg), Kit 1 (1.223 µg/mg), and Kit 2 (1.190 µg/mg). The differences between these three methods and the rest of the methods were highly significant ($P < 0.01$), but the differences between these three methods were not significant ($P > 0.05$). Kit 2 was the fastest method (30 min without digestion time), and SDS was the cheapest method with the best within-run and between-run precision among these three methods.

For roasted codfish, a product which process usually involved moderate temperature (40°C~45°C) for 60 min to 120 min, depending on the thickness of the fillet, and then roasted at high temperature (160°C~170°C) for 2 min, the top two methods for DNA yield were the SDS (0.505 µg/mg) and ICTAB methods (0.480 µg/mg). The differences between these two methods and the rest of the methods were highly significant ($P < 0.01$), but the differences between these two methods were not significant ($P > 0.05$). The SDS method (60 min without digestion time) was faster than the ICTAB method (90 min without digestion time), moreover, its within-run and between-run precision was better than ICTAB.

For canned codfish, a product which process usually involved

high temperature (115°C~121°C) with overpressure and the time varied from 15 min to 60 min, the top two methods for DNA yield were the SDS (0.594 µg/mg) and PCI methods (0.419 µg/mg). The differences between these two methods and the rest of the methods were highly significant ($P < 0.01$), whereas the differences between the two methods were significant ($P < 0.05$). The within-run and between-run precision of SDS method was also better than PCI methods.

In a previous study, the CTAB method was considered a standard for DNA extraction from seafood tissues (Espiñeira *et al.*, 2010). However, in our research, the DNA yield of CTAB method was lowest in the four manual methods for three kinds of seafood products; it was even lower than Kits 1 and 2 applied in quick frozen and roasted products. A similar situation was found in Besbes' research (2011). The discrepancy in the result could be related either to the sample type and/or to the CTAB concentration because an inappropriate concentration could form complexes with DNA in the presence of lipids (Krishnaswamy *et al.*, 2006). Moreover, DNA can condense with CTAB in an insoluble complex when the CTAB/DNA ratio is over 1.0 (Chatterjee *et al.*, 2002). Although the roasted and canned samples were defatted by the chloroform/methanol/water mixture, the residual lipid and the non-optimal CTAB concentration could have contributed to the low DNA yield.

The quality of the DNA extracted was evaluated using the A260/A280 ratio, with values close to 2.0, indicating good purity of DNA with little protein contaminants. Most of the methods tested in this study produced a good A260/A280 ratio, with values close to 2.0 (Table. 2). The lowest value (1.489), which was attributed to protein contamination, was obtained from canned codfish extracted with Kit 4.

The quality of the extracted DNA was measured by PCR with two pairs of primers, respectively. The PCR products were visualized by UV light after electrophoresis in a 1.5% agarose gel with the presence of GeneFinder™ nucleic acid stain. Amplification using primers COI-1/COI-2 for DNA extracted from quick frozen codfish with the eight methods all showed a sharp band at the expected size of 650 bp (Fig. 1). However, no band was

Table 2. Comparison of DNA purity from three kinds of seafood extracted using eight methods

Extraction Method	Quick frozen codfish	Roasted codfish	Canned codfish
CTAB	1.902 ± 0.064 ^b	2.084 ± 0.106 ^b	2.086 ± 0.139 ^b
ICTAB	1.929 ± 0.023 ^b	2.024 ± 0.011 ^b	1.889 ± 0.083 ^b
PCI	2.003 ± 0.032 ^b	1.840 ± 0.152 ^b	1.831 ± 0.049 ^b
SDS	2.010 ± 0.010 ^b	1.998 ± 0.017 ^b	1.912 ± 0.081 ^b
Kit 1	1.973 ± 0.037 ^b	2.009 ± 0.043 ^b	1.921 ± 0.050 ^b
Kit 2	2.053 ± 0.014 ^b	2.177 ± 0.158 ^b	1.843 ± 0.205 ^b
Kit 3	1.977 ± 0.023 ^b	2.320 ± 0.25 ^b	1.921 ± .0353 ^b
Kit 4	1.602 ± 0.016 ^a	1.514 ± 0.058 ^a	1.489 ± 0.057 ^a

Data are mean±standard deviation, n=6 in each case.

Different letters in the same column indicate significant different at P=0.01.

Fig. 1. PCR products from quick frozen codfish obtained with the COI-1/COI-2 primers (650 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit1-4; 9. Negative control)

Fig. 2. PCR products from roasted codfish obtained with the COI-1/COI-2 primers (650 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit1-4; 9. Negative control)

Fig. 3. PCR products from canned codfish obtained with the COI-1/COI-2 primers (650 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit1-4; 9. Negative control.)

Fig. 4. PCR products from quick frozen codfish obtained with the Cytb-1/Cytb-2 primers (358 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit1-4; 9. Negative control)

observed at the expected size for the DNA extracted from either roasted codfish or canned codfish with the eight methods using the same primer set (Fig. 2, Fig. 3). The amplification results using another primer set, Cytb-1/Cytb-2, showed a target band at the expected size of 358 bp for the three kinds of samples (Fig. 4-6). When sequenced, the positive amplicons proved to be fragments of

the COI gene and *Cyt b* gene of *G. macrocephalus*, respectively. No band was observed in the negative control.

Heat exposure can cause DNA fragmentation, and physical or chemical treatments during the process can cause DNA strands to break randomly (Peano *et al.*, 2004). The size of DNA extracted from cans ranged from less than 100 bp to 200 bp (Quinteiro *et al.*,

Fig. 5. PCR products from roasted codfish obtained with the Cytb-1/Cytb-2 primers (358 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit 1-4; 9. Negative control)

1998), even to 300 bp using some commercial kits for special products (Chapela *et al.*, 2007). In the present study, the DNA template extracted from roasted and canned samples could amplify a 358 bp fragment but failed to amplify a 650 bp fragment, indicating that the DNA from roasted and canned codfish was damaged. Furthermore, the DNA size in these seafood products was smaller than 650 bp, with parts of the fragments being larger than 358 bp. The different result on the fragment size in the aforementioned study of Quinteiro *et al.* (1998) and Chapela *et al.* (2007) may be partly caused by the sample tested in these studies being a different species, with different matrices and different processing conditions.

The extraction method and the type of seafood product significantly influence the quantity and quality of DNA. To determine the DNA yield and purity of quick frozen seafood products, the SDS method, TIANGEN GMO food DNA Extraction Kit, and TIANGEN TIANamp Marine Animals DNA Kit can be selected preferentially than other methods tested in this study. For roasted seafood, the SDS and ICTAB methods produced the highest DNA yields. For canned seafood, SDS and PCI methods should be chosen to determine DNA quantity. All the DNA samples extracted by the eight methods tested can be used for PCR using the proper primer set. Based on the kind of seafood product, a proper DNA extraction method should be selected in conjunction with an appropriate primer set target to the small DNA fragment to make an important contribution to seafood authentication.

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Fig. 6. PCR products from canned codfish obtained with the Cytb-1/Cytb-2 primers (358 bp) using eight extraction methods. (M. DNA marker; 1. CTAB; 2. ICTAB; 3. PCI; 4. SDS; 5-8. Kit 1-4; 9. Negative control)

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