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Research Article

Comparison of DNA Extraction Methods for the Detection of Canned Tuna Species with DNA Mini-Barcoding

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Tuna is susceptible to species mislabeling due to its high demand, quick rate of production, and wide range of price points. DNA barcoding, a sequencing-based technique, allows for the detection of species mislabeling by targeting a standardized region of DNA. A mitochondrial control region (CR) DNA barcode has been found to be capable of species discrimination for tuna, but it is challenging to recover the entire DNA fragment from canned tuna. While a short fragment of CR, referred to as a "mini-barcode," has shown some success with canned tuna species identification, more research is needed to improve identification rates. The objective of this study was to determine the optimal DNA extraction method for species identification of canned tuna using CR mini-barcoding. Four commercial DNA extraction kits were compared using a sample set of 24 different cans of tuna labeled as albacore, light tuna, skipjack, or yellowfin. All samples were tested in duplicate. The greatest success was found using the Qiagen DNeasy Blood and Tissue Kit and the Qiagen DNeasy *Mericon* Food Kit, which resulted in species identification for 42% of the samples. In comparison, the MP Biomedicals FastPrep-24 + Machery-Nagel NucleoSpin Tissue Kit resulted in species identification for 30% of the samples and the Qiagen DNeasy Blood and Tissue Kit + PowerClean Pro Cleanup Kit resulted in species identification for 21% of the samples. Overall, the top-performing DNA extraction methods for use with CR mini-barcoding of canned tuna products were determined to be the DNeasy Blood and Tissue Kit and the DNeasy *Mericon* Food Kit.

1. Introduction

Seafood is an important source of protein globally, with an estimated 178 million tonnes produced in 2020 [1]. Catches of tuna and tuna-like species have reached record highs in recent years, with 8.2 million tonnes harvested in 2019. Skipjack tuna (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) account for over half (55%) of the global tuna harvest [2]. Tuna is purchased and consumed in many forms, such as raw, canned, and dried. Canned tuna is among the top three seafood products consumed in the United States, with 205 million kg imported in 2020 [3, 4]. Tuna is highly susceptible to fraud because it is produced at such high quantities, is highly demanded, and exhibits a wide range of prices [5]. Furthermore, the morphological characteristics are removed during processing, making it visually difficult to identify the species within canned tuna [6]. The mislabeling of tuna species has been reported globally across a variety of products, including fresh, frozen, dried, and canned products [5–12].

In addition to the economic consequences of tuna species mislabeling, there are also potential health risks. For example, at-risk individuals, such as pregnant women and children, are advised to limit consuming fish with a high mercury content, including bigeye tuna (*Thunnus obesus*), and to limit consumption of albacore (*Thunnus alalunga*) and yellowfin tuna to one serving per week [13]. On the other hand, these at-risk consumers are encouraged to eat 2-3 servings per week of fish low in mercury, such as skipjack tuna. The mislabeling of bigeye tuna as skipjack, yellowfin, or albacore tuna could lead to elevated mercury exposure in at-risk individuals. In addition to substitution of one tuna

species for another, tuna has also been reported to be substituted with other types of fish such as escolar (*Lepidocybium flavobrunneum*) [10, 14]. Escolar, which contains gempylotoxin, is frequently mislabeled as "white tuna" at sushi restaurants [10, 14]. Tuna species mislabeling can also enable the sales of illegally caught fish and hinder conservation efforts, which could amplify the depletion of vulnerable or endangered stocks [5, 15]. For example, the mislabeling of the endangered southern bluefin tuna (*Thunnus maccoyii*) as yellowfin tuna or ahi (a vernacular name for yellowfin and bigeye tuna) has been reported previously [16, 17].

In the absence of morphological features, fish species are typically identified using DNA or protein-based techniques [18]. The U.S. Food and Drug Administration (FDA) utilizes DNA barcoding of a ~650 base pair (bp) region of the gene coding for cytochrome *c* oxidase subunit I (COI) for the regulatory identification of fish species [19]. However, the identification of species in canned products is challenging because the fish tissue is subjected to conditions of high temperature and pressure, causing the DNA to become highly fragmented [6]. In these instances, mini-barcodes targeting shorter regions of the DNA barcode (~150–300 bp) have been shown to be effective for species identification in canned products [5, 6, 20, 21]. While most canned fish species can be differentiated with COI mini-barcoding, this approach has been inadequate for the differentiation of closely related tuna species due to their high genetic similarity [20, 21]. The inability to reliably identify tuna species in commercial products combined with ambiguous labeling practices increases the vulnerability of these products to fraud.

The mitochondrial control region (CR) has been established as an effective genetic marker for the differentiation of closely related scombroid species, including tuna [22, 23] and mackerel [24, 25]. To enable the accurate identification of tuna species in canned products, a mini-barcoding system targeting a 236-bp fragment of the CR was developed by Mitchell and Hellberg [6]. While the system was successful in the identification of species in 43% of the products, the remaining samples failed to be identified. A subsequent study examining the use of the CR mini-barcoding system across a variety of tuna products reported identification rates of 100% with raw samples, 95% with dried samples, and 50% with canned samples [5]. Similarly, previous studies reported high identification success rates (100%) when using the CR mini-barcoding system with dried or raw tuna products [17, 26]. The reduced sequencing success associated with canned tuna products could be due to a number of factors, including the presence of highly degraded DNA, PCR inhibitors, and/or multiple tuna species. A previous study reported that the DNA extraction method and packing medium greatly influenced the quality and quantity of DNA isolated from canned tuna [27]. Because of the wide variety of packing media (e.g., oil, water, salt, and other ingredients) used in the industry, canned tuna may contain varying amounts of PCR inhibitors that can negatively impact the sensitivity of the PCR reaction [28]. Therefore, optimization of the DNA extraction method used in CR mini-barcoding may be required to improve success rates with canned products.

The objective of this study was to compare the following four different commercial DNA extraction kits for use in CR mini-barcoding of canned tuna: Qiagen DNeasy Blood and Tissue Kit (current method), Qiagen DNeasy Blood and Tissue Kit + Qiagen PowerClean Pro Cleanup Kit, MP Biomedicals FastPrep-24 + Machery-Nagel NucleoSpin Tissue Kit, and the Qiagen DNeasy *Mericon* Food Kit. These kits were selected due to their success in previous studies examining species identification of various processed seafood products [5, 21, 28, 29].

2. Materials and Methods

2.1. Sample Collection. A total of 24 cans of tuna were purchased from grocery stores ($n = 9$) across Orange County, CA, as well as from one online retailer. Four categories of canned fish products were targeted: light tuna ($n = 6$), albacore tuna ($n = 6$), skipjack tuna ($n = 6$), and yellowfin tuna ($n = 6$). Sampling was also designed to target an equal number of samples packed in water ($n = 12$) and in oil ($n = 12$). Following purchase, the entire contents of each can were transferred to a 24-oz Whirl-Pak bag (Nasco, Fort Akinson, WI) and hand-mixed for 60 sec [21]. Next, a sample of tissue (25–100 mg) was transferred to a sterile 1.5 mL microcentrifuge tube using sterile forceps, with the exact amount of tissue dependent on the DNA extraction method (see details below). Tissue samples were prepared in duplicate for each sample and stored at −80°C until DNA extraction.

2.2. Comparison of DNA Extraction Methods. Four different DNA extraction methods were compared in this study as follows: (1) the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), abbreviated as DNeasy, (2) the DNeasy Blood and Tissue Kit followed by the DNeasy PowerClean Pro Cleanup Kit (Qiagen), abbreviated as DNeasy + PC, (3) the MP FastPrep-24 Instrument (MP Biomedicals Inc., Irvine, CA, USA) combined with the NucleoSpin Tissue Kit (Machery-Nagel Inc., Allentown, PA, USA), abbreviated as MP + N, and (4) the DNeasy *Mericon* Food Kit (Qiagen), abbreviated as DNeasy MF. All the 24 canned tuna samples were extracted in duplicate for each method and each DNA extraction batch included a negative control with no sample tissue. Lysis was carried out using an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany) according to the times and temperatures designated by each extraction kit (detailed below). A shaking speed of 300 rpm was used unless specified otherwise by the manufacturer [5].

For the DNeasy and DNeasy + PC methods, the DNeasy Blood and Tissue Kit, the spin-column protocol was carried out according to the manufacturer's instructions, with an elution volume of 50 μL [5]. Lysis was performed at 56°C for 20 h with shaking at 300 rpm. For the DNeasy + PC method, the DNeasy PowerClean Pro Cleanup Kit was applied to samples after DNA was extracted with the DNeasy Blood and Tissue Kit. The DNeasy PowerClean Kit was carried out

according to the manufacturer's instructions, with the exception that AE buffer (Qiagen) was used to elute the DNA from the spin column [29].

For the MP + N method, 100 mg of the tissue was placed into an MP lysing tube "A" (MP Biomedicals Inc.) and homogenized using the MP FastPrep-24 Instrument (MP Biomedicals Inc.) at speed 6 for 40 sec [21]. Following homogenization, the NucleoSpin Tissue Kit, the standard protocol for the animal tissue was carried out on samples according to the manufacturer's instructions, with the exception that an elution volume of 50 μL was used. Lysis was performed at 56°C for 3 h, with shaking at 300 rpm. For the DNeasy MF method, the DNeasy *Mericon* Food Kit, the small fragment protocol was carried out according to the manufacturer's instructions, except that an elution volume of 50 μL was used. Lysis was carried out for 30 min at 60°C, with shaking at 1,000 rpm. Following DNA extraction, all samples were stored at −20°C until PCR amplification. The concentration and purity for each sample of DNA were measured with a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA).

2.3. PCR and DNA Sequencing. All samples underwent PCR for the CR mini-barcode using the primers and cycling conditions specified by Mitchell and Hellberg [6]. The reaction mixture for canned tuna fish samples included the following: 11.2 ml sterile H_2O, 17.5 ml Qiagen HotStarTaq Plus Master Mix (2X), 0.7 ml each of one 10-μM forward primer (CR_F) and two 10-μM reverse primers (CR_R1 and CR_R2) [6], and 4.2 ml of DNA template, for a total reaction volume of 35 μL. A nontemplate negative PCR control containing sterile water was included for all the PCR batches. Cycling conditions were as follows: 94°C for 15 min; 35 cycles of 94°C for 30 s, 49°C for 40 s, and 72°C for 1 min; and a final extension of 72°C for 10 min [6]. Thermocycling was performed using an Eppendorf Mastercycler nexus gradient thermal cycler and PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Precast 2.0% E-Gels with ethidium bromide (Invitrogen, Carlsbad, CA, USA) were used to confirm DNA amplification, with 20 ml PCR product loaded into each sample lane. The gels were run for 30 min with an E-Gel Simple Runner (Invitrogen). An E-Gel Imager (ThermoFisher Scientific) was used to visualize and photograph the results of gel electrophoresis.

2.4. DNA Sequencing and Species Identification. Confirmed PCR products underwent cleanup with ExoSAP-IT (Applied Biosystems, Santa Clara, CA, USA) following the manufacturer's instructions. Bidirectional sequencing was performed by Eurofins Genomics (Louisville, KY, USA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730xl DNA Analyzer (Applied Biosystems).

Geneious R7 (Biomatters, Ltd., Auckland, NZ) was used to assemble and edit raw sequencing data. The following quality control parameters were applied: sequences must have <2% ambiguities and be ≥180 bp in length (at least 76% of the target length). All consensus sequences passing quality control were searched against GenBank using the basic local alignment search tool (BLAST), the MegaBlast algorithm. The top species match was recorded and compared to the species labeled on the sample.

2.5. Statistical Analysis. DNA concentrations, A_{260}/A_{280} ratios, A_{260}/A_{230} ratios, sequence lengths (bp), sequence quality (% high quality bases), and sequence ambiguities (%) associated with samples extracted with the four different DNA extraction methods were compared using the Kruskal–Wallis H test with a significance level set at $p < 0.05$. Statistically significant results were compared using a post hoc Dunn's test with Bonferroni correction. DNA concentrations, A_{260}/A_{280} ratios, A_{260}/A_{230} ratios were also compared based on the packing medium (oil or water) using the Kruskal–Wallis H test with a significance level set at $p < 0.05$. The amplification and sequencing success associated with each DNA extraction method were compared using a Cochran's Q Test with a significance level set at $p < 0.05$. Significant results were further analyzed using the McNemar test. Amplification and sequencing success were compared based on the packing medium using a Pearson's chi-squared test. All statistical testing was performed using R studio version 4.3.0 [30].

3. Results and Discussion

3.1. DNA Concentration and Purity. As shown in Table 1, samples extracted using the DNeasy method had the highest average DNA concentration (35.32 ± 37.03 ng/μl) compared to samples extracted using the DNeasy + PC method (17.85 ± 8.17 ng/μl), DNeasy MF method (16.98 ± 8.43 ng/μl), and MP + N method (13.91 ± 25.67 ng/μl). All four extraction methods produced significantly different DNA concentration values except for DNeasy + PC and DNeasy MF, according to the Kruskal–Wallis H test with the post hoc Dunn's test ($p < 0.05$). The significantly higher DNA concentration obtained with the DNeasy method as compared to the DNeasy MF method is consistent with a prior study comparing DNA extraction kits for canned tuna [28]. Similarly, Zahn et al. [29] reported significantly higher DNA concentrations for shark cartilage samples extracted with the DNeasy method as compared to the DNeasy + PC method.

Samples extracted with the DNeasy MF method showed the highest A_{260}/A_{280} values (1.78 ± 0.33), followed by samples extracted with the DNeasy + PC method (1.55 ± 1.12) (Table 1). These values were significantly different according to the Kruskal–Wallis H test with the post hoc Dunn's test ($p < 0.05$). The A_{260}/A_{280} values for the samples extracted with the DNeasy method (1.20 ± 0.09) were significantly reduced ($p < 0.05$) as compared to samples extracted with the other three methods. An A_{260}/A_{280} ratio of ~1.8 is typically accepted as pure DNA, with lower values indicating the presence of contaminants that absorb strongly near 280 nm, such as proteins or phenol from the extraction process [31]. Low A_{260}/A_{280} values may also be caused by low concentrations (~10 ng/μL) of nucleic acids [31]. In contrast

Table 1: Comparison of DNA concentration and purity results obtained for canned tuna samples ($n = 24$) extracted in duplicate with four different DNA extraction methods.

	DNA concentration (ng/μl)	$A_{260/280}$	$A_{260/230}$
DNeasy Blood and Tissue Kit	35.32 ± 37.03^{a}	1.20 ± 0.09^{a}	0.83 ± 0.47^{a}
DNeasy Blood and Tissue Kit plus PowerClean Kit	17.85 ± 8.17^{b}	1.55 ± 1.12^{b}	0.98 ± 1.18^{a}
MP FastPrep plus NucleoSpin Tissue Kit	13.91 ± 25.67^{c}	1.33 ± 0.31^{b}	0.62 ± 0.51^{b}
DNeasy *Mericon* Food Kit	16.98 ± 8.43^{b}	1.78 ± 0.33^{c}	0.66 ± 0.22^{a}

[abc]A different superscript letter in the same column indicates a significant difference, according to a Kruskal–Wallis H test with the post hoc Dunn's test ($p < 0.05$). Values are expressed as the average ± standard deviation.

to the current study, a previous study examining the DNA extraction of shark cartilage pills reported no significant difference in A_{260}/A_{280} values for samples extracted with the DNeasy method and the DNeasy + PC method [29].

The A_{260}/A_{230} values, which have an optimal range of 2.0–2.2 [31], were used as a secondary means of determining DNA purity. Samples extracted with the DNeasy + PC method showed the highest average A_{260}/A_{230} values (0.98 ± 1.18), followed by samples extracted with the DNeasy method (0.83 ± 0.47); however, the results were not significantly different ($p > 0.05$). In comparison, Zahn et al. [29] reported significantly higher A_{260}/A_{230} values for shark cartilage samples extracted with the DNeasy + PC method as compared to the DNeasy method. As shown in Table 1, the MP + N method resulted in significantly lower A_{260}/A_{230} values (0.66 ± 0.22) when compared to the other three DNA extraction methods based on a Kruskal–Wallis H test with the post hoc Dunn's test ($p < 0.05$). Low A_{260}/A_{230} ratios indicate the presence of contaminants that absorb at 230 nm, such as proteins, guanidine HCL, EDTA, carbohydrates, lipids, salts, or phenols [32]. However, it is important to keep in mind that buffers used during the extraction process may interfere with the A_{260}/A_{230} values due to the presence of compounds such as guanidine HCL and salt.

The packing medium did not appear to impact the DNA concentrations, with similar results for samples packed in oil (21.17 ± 14.50 ng/μl) and water (21.35 ± 10.86 ng/μl). Furthermore, the average A_{260}/A_{280} values (1.45–1.48) and average A_{260}/A_{230} values (0.77–0.78) were similar for samples packed in water and oil. A Kruskal–Wallis H test revealed no significant differences ($p > 0.05$) when comparing the DNA concentration, A_{260}/A_{280} values, and A_{260}/A_{230} values between the two types of packing medium. In comparison, Chapela et al. [27] reported significantly increased DNA concentrations for canned tuna samples packed in oil as compared to brine, vinegar, and tomato, indicating a protective effect of oil and/or an inhibitory effect of packing media containing high salt or low pH.

3.2. Amplification and Sequencing Results. The results of amplification and sequencing success for all the samples extracted in duplicate with the four different DNA extraction methods are shown in Figure 1. Amplification and sequencing were considered successful if at least one of the duplicate extractions for a given sample resulted in a band in a gel or a species identification, respectively. Overall, the DNeasy MF method resulted in the greatest amplification success, with 13 out of 24 samples (54.2%) showing amplification for at least one of the duplicate extractions. Although the DNeasy method showed a slightly lower amplification rate (41.7%) compared to the DNeasy MF method, both methods showed equivalent sequencing success (41.7%). On the other hand, the MP + N and DNeasy + PC methods both showed reduced amplification (25.0–37.5%) and sequencing (20.8–29.2%) success. The amplification success rate for the DNeasy MF method was significantly greater than that found for the DNeasy + PC method, according to Cochran's Q test, with a McNemar post hoc test (adj. $p < 0.05$), while the results of sequencing success were not significantly different across the four extraction methods. In contrast to the findings of the current study, Zahn et al. [29] reported a slightly increased amplification rate for samples extracted with the DNeasy + PC method (52.3%) as compared to the DNeasy method (47.8%); however, the sequencing rates were not compared.

Interestingly, while the DNeasy MF Kit showed the greatest amplification rate, the consistency of amplification among duplicates (79.2%) was the lowest when compared to the other methods (Table 2). Samples were considered consistent if both replicates showed the same result for amplification. The highest amplification consistency rate was observed for samples extracted with the DNeasy + PC method (91.7%), followed by samples extracted with the DNeasy or MP + N methods (87.5%). These results are in agreement with Zahn et al. [29], which reported a higher amplification consistency rate for shark cartilage pills extracted with the DNeasy + PC method (95.5%) as compared to the DNeasy method (77.3%).

The sequencing quality parameters associated with samples extracted with each method are shown in Table 2. All four methods yielded average sequence lengths (228–234 bp) close to the target fragment length of 236 bp, with the DNeasy MF method resulting in the highest average sequence length (234 ± 2.2 bp); however, none of the DNA extraction methods yielded a significantly different target fragment length, according to a Kruskal–Wallis H test. The DNeasy + PC method yielded the greatest sequence quality, with $88.4 \pm 0.1\%$ high-quality bases (HQ), followed by the DNeasy method with $86.1 \pm 22\%$. The DNeasy MF method showed the lowest quality, at $74.3 \pm 0.24\%$ HQ; however, there were no significant differences in %HQ among the four extraction methods. Lastly, all methods resulted in sequences with a low percentage of ambiguities (<1%), with no significant differences among the four methods.

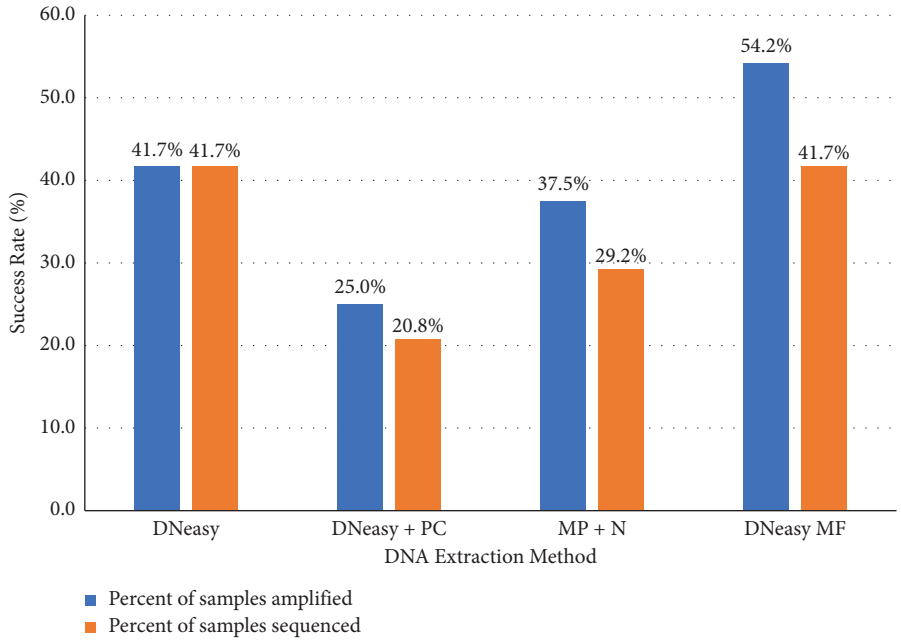

Figure 1: Amplification and sequencing results obtained for canned tuna samples ($n = 24$) extracted in duplicate with four different DNA extraction methods. Samples were considered successful if at least one of the duplicate extractions showed amplification/sequencing results. DNeasy = DNeasy Blood and Tissue Kit; DNeasy + PC = DNeasy Blood and Tissue Kit plus PowerClean Kit; MP + N = MP FastPrep plus NucleoSpin Tissue Kit; DNeasy MF = DNeasy *Mericon* Food Kit.

Table 2: PCR amplification consistency and sequencing quality parameters for 24 canned tuna samples extracted in duplicate with four different DNA extraction methods.

DNA extraction methods	Consistency of PCR amplification (%)	Number of samples sequenced	Sequencing quality parameters (ave ± stdev)		
			Sequence length (bp)	High quality bases (%)	Ambiguities (%)
DNeasy Blood and Tissue Kit	87.5	10	231 ± 11.53	86.1 ± 0.22	0.00 ± 0.00
DNeasy Blood and Tissue Kit plus PowerClean Kit	91.7	5	228 ± 12.90	88.4 ± 0.10	0.00 ± 0.01
MP FastPrep plus NucleoSpin Tissue Kit	87.5	7	232 ± 12.61	76.4 ± 0.27	0.01 ± 0.01
DNeasy *Mericon* Food Kit	79.2	10	234 ± 2.18	74.3 ± 0.24	0.00 ± 0.01

A sample was considered consistent if both duplicates showed the same result for amplification. There were no significant differences ($p > 0.05$) in the sequencing quality parameters across the four extraction methods, according to a Kruskal–Wallis H test.

Overall, the DNeasy and DNeasy MF methods showed the greatest performance associated with amplification and sequencing results. Although the DNeasy MF method showed reduced amplification consistency and greater amplification rates compared to the DNeasy method, the two methods showed equivalent sequencing success rates. In the case of the DNeasy method, the relatively high sequencing success may have been due to the increased DNA concentration as compared to the other methods. While the DNeasy MF method resulted in samples with a lower DNA concentration, the $A_{260/280}$ values for these samples were significantly higher than the other three methods, indicating greater purity.

3.3. Species identification. When the results of all the four extraction methods were combined, a total of 14 samples had sequences that passed quality control and were identified to the species level using BLAST. The tuna species identified in this study include albacore (*Thunnus alalunga*), yellowfin (*Thunnus albacares*), and bigeye (*Thunnus obesus*) (Table S1). Table 3 shows the sequencing success rates for the four categories of canned tuna tested (i.e., light tuna, albacore tuna, skipjack tuna, and yellowfin tuna). Samples labeled as albacore tuna showed the greatest sequencing success rate (100%), followed by those labeled as yellowfin tuna (83.3%). Samples labeled as light tuna (33.3%) and skipjack (16.7%) yielded the lowest success rate. Of note, two of the three light tuna/skipjack tuna samples that were successfully sequenced were found to be mislabeled (discussed below). Similar to the results of this study, prior research has also reported the greatest sequencing success for albacore and yellowfin tuna when using CR mini-barcoding [5, 6]. The reduced sequencing success for light tuna and skipjack tuna when tested with all the four DNA extraction methods may be due to a lack of primer binding during PCR. In order to improve sequencing success rates, future research should focus on optimizing PCR, including

Table 3: Sequencing success results obtained for the four different categories of canned tuna separated by DNA extraction methods.

DNA extraction methods	Sequencing success rate			
	Light tuna (%)	Albacore tuna (%)	Skipjack tuna (%)	Yellowfin tuna (%)
DNeasy Blood and Tissue Kit	16.7	83.3	0	66.7
DNeasy Blood and Tissue Kit plus PowerClean Kit	0	66.7	0	16.7
MP FastPrep plus NucleoSpin Tissue Kit	16.7[a]	66.7	16.7[b]	16.7
DNeasy *Nericon* Food Kit	0	100	16.7[b]	50
Results of all kits combined	33.3	100	16.7	83.3

[a]Labeled as "light tuna," sequencing identified as albacore (*Thunnus alalunga*). [b]Labeled as "skipjack tuna," sequencing identified as albacore (*T. alalunga*) and yellowfin (*Thunnus albacares*). A total of six samples were tested per canned tuna category. Samples were considered successful if at least one of the duplicate extractions showed sequencing results.

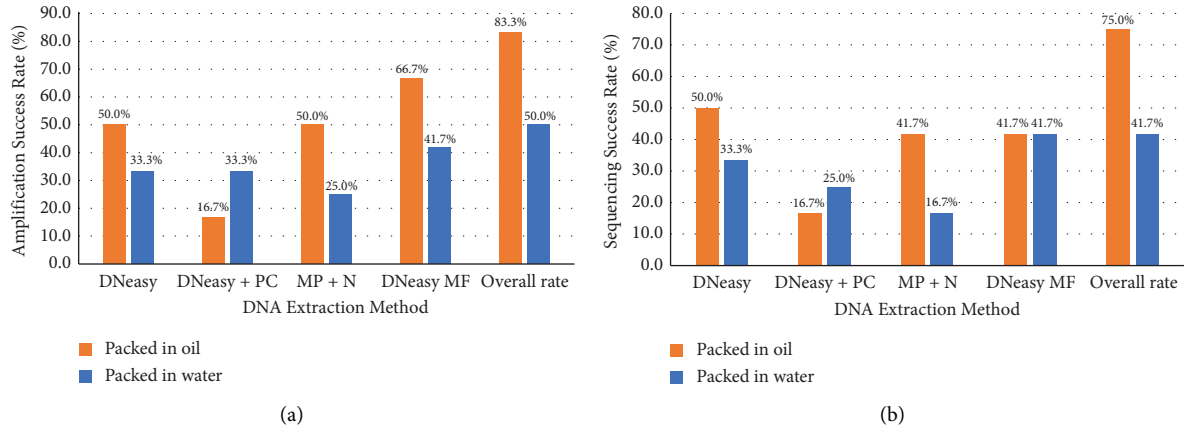

Figure 2: (a) Amplification success and (b) sequencing success rates for canned tuna samples ($n = 24$) extracted in duplicate packed in oil and water. Samples were considered successful if at least one of the duplicate extractions showed amplification results. DNeasy = DNeasy Blood and Tissue Kit; DNeasy + PC = DNeasy Blood and Tissue Kit plus PowerClean Kit; MP + N = MP FastPrep plus NucleoSpin Tissue Kit; DNeasy MF = DNeasy *Mericon* Food Kit.

the use of different primers for amplification of skipjack tuna and/or targeting a shorter DNA fragment. For example, a study analyzing next-generation sequencing to detect mixed species in canned tuna used a modified version of the forward primers designed by Mitchell and Hellberg [6] targeting a shortened amplicon length of 170 bp [33]. Furthermore, the choice of DNA polymerase should also be examined, as different polymerases have varying degrees of sensitivity to PCR inhibitors [34].

When comparing the packing medium (Figure 2(a)), tuna samples packed in oil (olive oil, soybean oil, vegetable oil, soya oil, or canola oil) had a higher overall amplification rate (83.3%) compared to samples packed in water (50.0%). The overall sequencing success rate (Figure 2(b)) was also higher for samples packed in oil (75.0%) compared to water (41.7%). However, the differences in amplification and sequencing rates were not significantly different ($p > 0.05$), according to a Pearson's chi-squared test. Similar to the current findings, previous research has also shown that canned tuna samples packed in oil yielded greater sequencing success as compared to samples packed in water [6].

3.4. Labeling Concerns. Of the 14 successfully sequenced canned tuna products, one was found to contain multiple species (T18) and another contained an unexpected species ((T04) (Table S1)). The product with multiple species was labeled as "skipjack tuna" but was found to contain both albacore and yellowfin tuna. The product with an unexpected species was labeled as "light tuna" but was found to contain albacore. The presence of albacore in a product labeled as "light tuna" is considered misleading because albacore is associated with the term "white tuna" [35]. Legally, the term "light tuna" applies to tuna with a Munsell value not greater than 5.3, while the term "white tuna" is reserved specifically for albacore and must not exceed a Munsell value of 6.3 (21 CFR 161.190 canned tuna). The presence of albacore tuna in a sample labeled as "light tuna" was also reported by Mitchell and Hellberg [6]. Both instances of mislabeling identified in the current study could cause potential harm to at-risk groups, such as women and children, because of differences in mercury levels between tuna species. Specifically, albacore and yellowfin are listed as a "good choices," whereas light tuna is considered a "great choice" [13]. At-risk individuals are advised to consume up to two servings of light tuna per week but only one serving of albacore or yellowfin per week [13].

3.5. Time, Cost, and Ease of Use Considerations. The price per sample for the four extraction methods ranged from $5.27 for the DNeasy method to $10.37 for the DNeasy + PC method (Table 4). The DNeasy + PC method requires the

Table 4: Comparison of labor, modifications, price, and time for the four DNA extraction kits used.

DNA extraction kits	Protocol	Manufacturer	Modifications	Ease of use	Hands-on technician time (h)	DNA extraction price per sample (USD)[a]	Estimated time (h) required to extract 15 samples
DNeasy Blood and Tissue Kit	Spin-column animal tissue	Qiagen	Elution volume decreased	Easy moderate	1	$5.27	21
DNeasy Blood and Tissue Kit plus PowerClean Kit	Spin-column animal tissue (DNeasy) + detailed (PowerClean)	Qiagen	Elution volume decreased; AE buffer used to elute	Moderate-difficult	2	$10.37	22
MP FastPrep plus NucleoSpin Tissue Kit	Standard protocol for animal tissue	MP-Biomedicals; Machery-Nagel	Speed 6 for 40 sec; elution volume decreased	Moderate	1.5	$6.01	4.5
DNeasy *Mericon* Food Kit	Small fragment (200 mg)	Qiagen	Elution volume decreased	Moderate	1.5	$5.83	2

[a]Price per sample is based on the list price for each kit (50 extractions per kit) at the manufacturer's website plus additional reagents required. Prices do not include the cost of instrumentation or consumables (e.g., pipet tips or extraction tubes).

successive use of two kits, which leads to an increase in price required to extract each sample. The time required to extract 15 samples ranged from 2 h (DNeasy MF) to 22 h (DNeasy + PC). A lysis incubation of 20 h, as well as additional wash steps from the PowerClean Pro Cleanup Kit both contributed to the length of time required to perform the DNeasy + PC method. The DNeasy MF method had the shortest lysis incubation time of 30 min, as well as no additional lysing or washing beyond the standard small fragment protocol. The ease of use for this kit, however, was rated moderate due to the need for chloroform, which requires additional safe handling procedures for the technician. While the incubation time of 20 h was longer than other methods, the DNeasy method was ranked as easy moderate because the steps following lysis were brief and did not require additional reagents besides ethanol. The MP + N method required homogenization using the MP FastPrep-24 instrument, which increased the amount of active laboratory time for the technician as well as the cost and materials needed. Between the four DNA extraction methods, the DNeasy method resulted in the greatest ease of use as well as the cheapest price per sample, while the DNeasy MF method required the shortest amount of time and was only slightly more expensive than the DNeasy method.

4. Conclusion

This study compared four DNA extraction methods with the goal of improving the identification of canned tuna species through CR mini-barcoding. The DNeasy and DNeasy MF methods led to the highest rate of species identification and were the two most inexpensive kits based on the price per sample. While the DNeasy method was relatively easy to carry out, additional optimization is suggested to determine whether a shorter lysis time can be implemented. Future studies should investigate the optimization of PCR and DNA sequencing to further improve species identification rates in canned tuna, including the use of a shorter amplicon mini-barcode target and/or next-generation sequencing. The combination of DNA barcoding with a species-specific approach, such as real-time PCR or multiplex PCR, may also help to improve species identification in these complex products.

Data Availability

The data used to support the findings of this study are included with the supplementary materials.

Conflicts of Interest

Emmi and Hellberg declare that there are no conflicts of interest. Fatusin was a student at Chapman University while conducting laboratory work for this project but has since been hired by Zymo Research, a biomedical company.

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Supplementary Materials

Table S1 provides sequence data and details on the tuna products collected for this project. (*Supplementary Materials*)

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