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Catfish Species Identification Using Lab-On-Chip PCR-RFLP

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Lab-on-a-chip based Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technology was developed for the identification of seven catfish species including an Ictalurid hybrid. RFLP profiles of mitochondrial cytochrome b fragments digested by three different restriction endonucleases (*Dde*I, *Hae*III, and *Nla*III) were constructed from morphologically verified catfish samples on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). High sensitivity and ease of handling through end-point microchip-based capillary electrophoresis increased resolution and accuracy of DNA fragment sizing. RFLP profiles derived from a combination of all three enzymes produced consistent species-specific identification profiles. Observed restriction fragment patterns for *Clarias batrachus* and *Ictalurus punctatus* were similar, but both of these species could be consistently differentiated using a single band of the *Hae*III restriction site. Assay advantages due to shorter assay times, assay ease, and minimum usage of harmful solvents and chemicals when compared to traditional DNA barcoding were counterbalanced by the need to develop and optimize specific restriction digest profiles for all potential species of interest. With further development, this method may be utilized in testing of catfish products to ensure the enforcement of seafood labeling regulations.

Keywords: lab-on-chip, PCR-RFLP, catfish, species identification, seafood

INTRODUCTION

Catfish have been extensively marketed in both fresh and frozen forms and constitute the primary farm-raised fish in the United States. A rising market share for imported catfish has introduced mislabeling and fraudulent substitution of lower value species for others of higher price, as seen

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previously in other seafood sectors. Mislabeling has taken the form of substitution of Asian basa and tra catfish as Ictalurid catfish or through fraudulent mislabeling of country-of-origin, impacting the profitability of the U.S. catfish industry as well as raising public health concerns (Brambilla et al., 2009). Marketing of fish as processed fillet and seafood products has exacerbated the difficulties of species authentication because distinct morphological characteristics of the fish are removed during the processing stage (McDowell and Graves, 2002). Hence, the development of innovative, rapid, and low cost analytical platforms for species identification is of high importance to guarantee the authenticity and origin traceability of catfish along the seafood distribution chain from pond to plate.

Numerous protein-based analyses have been developed specifically for species authentication (Tepedino et al., 2001; Ochiai et al., 2001). However, these methods are only reliable for identification of raw fish and not applicable for processed fish products as the thermally labile proteins become irreversibly denatured by heat (Dooley et al., 2005). Alternatively, DNA-based techniques are a more efficient identification approach, largely because of their high thermal stability as well as their independence of cell types and age (Davidson, 1998; Bossier, 1999; Lockley and Bardsley, 2000).

Although DNA-based methods such as Real-time Polymerase Chain Reaction (Real-time PCR) and direct sequencing provide definitive species identification, their higher cost and/or longer assay times are drawbacks for their use as a robust and routine fish screening test (Meyer et al., 1995). Therefore, a relatively simple and inexpensive species discrimination technique such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) may be more appropriate for targeted, rapid species identification. In fact, PCR-RFLP mapping has been successfully applied in various fish species and fish products identification (Arahishi, 2005; Akasaki et al., 2006; Lin and Hwang, 2007; Cespedes et al., 1998; Hsieh et al., 2007). Briefly, this technique incorporates the analysis of specific DNA fragment profiles generated by digestion of PCR amplicons with selected restriction endonucleases to produce restriction fragment patterns through electrophoretic sorting. However, PCR-RFLP still relies on conventional gel electrophoresis for endpoint detection that can be time demanding and complicated by the production of variable results (Dooley et al., 2005). The complex DNA fingerprints and sizing variation has led to the development of microchip-based capillary electrophoresis technology, such as automated lab-on-chip electrophoretic systems to replace the traditional gel electrophoresis step (Fajardo et al., 2009; Panaro et al., 2000). The Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) is the first commercialized chip-based electrophoresis apparatus that utilizes capillary electrophoresis (CE) technology. This automated system involves DNA fragment separation by CE in miniaturized channels of the microfluidic chip followed by detection steps using laser-induced fluorescence (Vasilyeva et al., 2004). This user-friendly technology permits the generation of higher resolution PCR-RFLP profiles in comparison to conventional gel-based approaches (Uthayakumaran et al., 2005). Though the overall cost of this advanced device is fairly high, it may be more suitable for routine surveys compared to the real-time PCR method (Dooley and Garrett, 2001).

With the collaboration of Agilent Technologies, we developed PCR-RFLP profiles on the 2100 Bioanalyzer based on the mitochondrial cytochrome b gene to enable identification of seven catfish species (two Ictalurid species and an Ictalurid hybrid; three Clariid species; and two Pangasiid species) that are commercially important in the seafood industry. Sequences generated from the cytochrome b gene were also used as supporting analysis to confirm the competence of the PCR-RFLP technique. Overall, our results demonstrated that the lab-on-chip technique may be rapidly performed for catfish species authentication by regulatory agencies to enforce transnational laws and regulations, protecting the consumer against seafood fraud.

MATERIAL AND METHODS

Sample Collections

At least 12 individuals from each catfish species were morphologically identified by a fish taxonomist at the respective sources before the samples were obtained. All fin clips or tissue samples were preserved in 95% ethanol (1:10 w:v) upon collection. Details of specimens are presented in Table 1.

DNA Extraction

Total genomic DNA was isolated from fin clips or muscle tissues. In brief, 20 mg of samples were incubated overnight in digestion buffer (Liu et al., 1998) containing 100 ug/mL Proteinase K at 55°C. The subsequent extraction process was based on a slight modification of the Gentra Puregene Tissue Kit protocol (Qiagen, Valencia, CA, USA). Final concentration and purity of extracted DNA was estimated by means of an Ultrospec 1100 Pro spectrophotometer (GE Sciences, Piscataway, NJ, USA). DNA quality of the samples was examined by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

PCR Amplification

Amplification of the partial mitochondrial cytochrome *b* gene was carried out in a total volume of 25 μL containing 50 ng of the DNA template and components from the Fish Species ID Beta Kit (Agilent Technologies, Inc.) which consists of sterile water, 2 × PCR Master Mix, and a primer mix. The primer mix is constituted of the following pair of universal primers: L14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3') and H15149ad (5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3') which were described by Russell et al. (2000) and Wolf et al. (2000), respectively. The PCR cocktail (DNA template, sterile water, PCR Master Mix, and primer mix) was mixed according to the kit's manual, and the reaction was carried out in a PTC-200 DNA Engine Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) programmed as follows: 5 min at 95°C, 40 cycles of 30 s at 95°C, 30 s at 50°C, and 30 s at 72°C, and a final elongation step at 72°C for 7 min. For PCR quality assurance, each assay was accompanied by a negative control (no-template blank) and a positive control (*Oncorhynchus keta* reference specimen). PCR products were examined using the DNA1000 LabChip to confirm amplification.

TABLE 1
Catfish species used in the development of PCR-RLP profiles

Species name	*Common name*	*Sampling location*	*Sample size*
Ictalurus punctatus	Channel catfish	Auburn University, USA	21
Ictalurus furcatus	Blue catfish	Auburn University, USA	18
I. punctatus × *I. furcatus*	Hybrid catfish	Auburn University, USA	12
Clarias batrachus	Walking catfish	Nakhon Ratchasima Province, NE Thailand	15
Clarias gariepinus	African sharp-toothed catfish	Nakhon Ratchasima Province, NE Thailand	14
Clarias macrocephalus	Bighead catfish	Faculty of Fisheries, Kasetsart University, Thailand	12
Pangasius bocourti	Basa catfish	Yasothon Province, NE Thailand	14
Pangasianodon hypophthalmus	Swai or Sutchi catfish	Nakhon Ratchasima Province, NE Thailand	15

Restriction Digestion and PCR-RFLP Profiling

Unpurified PCR amplicons were subjected to three independent restriction reactions with *Dde*I, *Hae*III, and *Nla*III enzymes. The amplified fragments were digested for at least 2 h with 1 unit of each restriction endonuclease in a total volume of 5 μL at 37°C, and the reactions were inactivated by incubation at 65°C for 15 min.

PCR-RFLP profiles were visualized using DNA1000 LabChips (Agilent Technologies, Inc.) with the 2100 Bioanalyzer microchip capillary electrophoresis system. Both DNA1000 LabChips and reagents were prepared as required according to the manufacturer's instructions. Digested PCR products were mixed with 60 mM EDTA before being loaded on the microchip. RFLP fingerprints were obtained within 30 min and were analyzed using 2100 Expert software and compared to the restriction map generated from the sequence analysis.

Mitochondrial Cytochrome b Gene Sequencing

To confirm restriction fragment patterns, PCR products were sequenced bi-directionally with the same primers used for the PCR reaction. PCR amplicons were cleaned before the sequencing reaction following the Exo-SAP protocol as described in Dugan et al. (2002). Direct sequencing reactions were carried out using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresed on an ABI 3130xl Genetic Analyzer. Sequence Analysis Software version 5.2 (Applied Biosystems) was used to generate sequence tracefiles and contiguous read lengths.

Sequences were end-trimmed using the SeqMan program (DNASTAR, Madison, WI, USA). Based on the sequence similarity approach, specimen identification was also carried out by blasting the sequences against the GenBank database. Multiple sequence alignment was performed to create consensus sequences for each catfish species using the ClustalW program (Thompson et al., 1994). A phylogenetic tree was constructed from the species consensus sequence by the maximum parsimony (MP) algorithm (Nei and Kumar, 2000) along with bootstrapping analysis using MEGA 5.0 (Tamura et al., 2007). A distantly related catfish, *Cranoglanis bouderius*, was used as an outgroup for this MP tree. In addition, neighbor-joining (NJ) and maximum likelihood (ML) methods were performed to confirm the absence of clustering output variation between different phylogenetic reconstruction methods.

These sequences were also used to produce predicted RLFP patterns generated by the three enzymes. These restriction maps were constructed using the software NEBcutter (Vincze et al., 2003). A species identification flowchart was built to simplify the analysis process (Figure 1). All observed fragment sizes were rounded to account for fragment size variation.

RESULTS AND DISCUSSION

In order to detect commercial fraud in marketing of catfish products, the development of reliable, rapid traceability techniques is necessary. Therefore, an extension of the PCR amplification method using RFLP analysis that has previously proven successful for fish species authentication was used (Asensio Gil, 2007; Mackie et al., 1999). To improve the detection and resolution of the RFLP patterns, a highly sensitive, rapid (less than one working day), and feasible end-point detection method based on a capillary electrophoresis lab-on-chip device (2100 Bioanalyzer) was utilized to identify seven catfish species and an Ictalurid hybrid species. A complimentary sequence analysis on the cytochrome b region for each species studied was also conducted to compare the generated RFLP profiles. Catfish species were also confirmed by comparing the sequences against vouchered

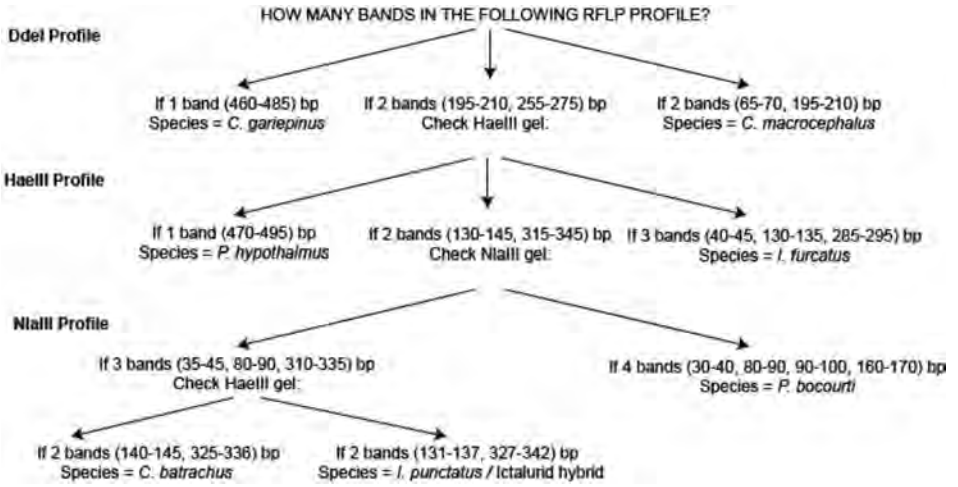

FIGURE 1 Species identification flowchart developed for seven catfish species (and an Ictalurid hybrid) based on the PCR-RFLP profiles generated by the Agilent 2100 Bioanalyzer.

entries in GenBank (Table 2). No ambiguities were present in the alignment of all the catfish species sequences.

The experimental restriction profiles revealed that some catfish species identification could be achieved using only a single endonuclease. Based on Table 3, *Pangasius bocourti* displayed a unique restriction RFLP pattern when digested with *Nla*III. Likewise, no other catfish species produced fragment profiles similar to that of *Ictalurus furcatus* when restricted with enzyme *Hae*III. However, a combination of three enzymes was required to produce species-specific RFLP profiles in order to distinguish the other five catfish species in the present study. For instance, the three Clariid species generated almost identical profiles from two enzymes, *Hae*III and *Nla*III, but were only differentiated following the restriction by *Dde*I. Digestion using the *Dde*I restriction enzyme yielded two fragments at size ranges of 196–208 and 261–275 bp from the PCR amplicons of *Clarias batrachus*, whereas PCR fragments of *Clarias gariepinus* were not cleaved due to the absence of the *Dde*I restriction site. Despite nearly identical patterns between *Pangasius bocourti* and *Pangasianodon hypopthalmus*, species identification was still possible

TABLE 2
Summary of identification matches based on each catfish species consensus sequence of cytochrome *b* region BLASTED against entries in the GenBank database.

Species studied	*GenBank (BLASTN) Species identification*	*% Max identity*
Ictalurus furcatus	*Ictalurus furcatus*	99
Ictalurus punctatus	*Ictalurus punctatus*	100
Hybrid (I. punctatus × I. furcatus)	*Ictalurus punctatus*	98
Clarias batrachus	*Clarias batrachus*	96
Clarias gariepinus	*Clarias gariepinus*	97
Clarias macrocephalus	*Clarias macrocephalus*	97
Pangasius bocourti	*Pangasius bocourti*	98
Pangasius hypophthalmus	*Pangasianodon hypophthalmus*	99

TABLE 3
Predicted and observed PCR-RFLP fragment sizes of catfish species generated with three different restriction endonucleases *Dde*I, *Hae*III, and *Nla*III used in this study

Species		Dde*I*	Hae*III*	Nla*III*
Clarias batrachus	E	164–165 266–272	138–144 292–293	30–36 85–86 315
	O	**196–208** 261–275	140–144 **325–336**	37–38 80–86 311–336
Clarias gariepinus	E	424–445	132–137 286–294	26–29 79–87 315
	O	460–484	141–143 **328–337**	37–39 84–87 322–333
Clarias macrocephalus	E	60 162–166 200–202	4 6 124–127 290–294	83–87 340–341
	O	65–69 * 198–207	* * 131–138 **320–335**	35–39 81–86 317–365
Pangasius bocourti	E	158–162 248–273	123–128 292–297	23–26 70 79–84 91 154
	O	**199–208** 265–274	133–138 332–344	37–39 * 82–86 95–102 160–168
Pangasianodon hypopthalmus	E	156–168 258–263	416–426	22–27 77–89 154 161
	O	**200–209** 256–268	471–495	38–40 81–86 156–166 170–177
Ictalurus furcatus	E	193–198 260–262	43 126–128 284–289	24–26 36 78–83 154 161
	O	201–207 264–271	43–45 132–135 286–293	* 37–39 84–87 157–163 166–172
Ictalurus punctatus	E	204 262–263	128–129 338	26–27 36 89 315
	O	199–208 263–274	131–137 327–342	* 36–39 84–89 316–333
Hybrid (*I. puncatus*)	E	197–199 261–267	127–130 331–338	25–28 36 82–84 314–315
	O	204–208 263–273	135–137 334–341	37–39 87–88 324–331

E: predicted fragment sizes were generated from *in silico* restriction analysis of cytochrome *b* sequences. O: observed sizes are the mean sizes obtained from analysis performed on at least 12 catfish individuals in each species. *Indicates small fragments which are not detected by the 2100 Bioanalyzer, while bold values indicate observed fragment sizes which are larger than the predicted fragment sizes.

due to comparison of the restriction patterns of *Hae*III. Two fragments were detected following *Hae*III digestion in *P. bocourti*, while amplification fragments of *P. hypopthalmus* remained undigested. Similarly, *C. batrachus* and *Ictalurus punctatus*, which shared highly similar restriction profiles, displayed a minor but consistent difference in *Hae*III (Table 3) which enabled them to be differentiated. Due to minimal differences in the profiles between these two particular species, an additional restriction enzyme digestion may be necessary to increase the discriminatory power of the assay, particularly if applied in a regulatory setting. As expected, both *I. punctatus* and the Ictalurid hybrid generated identical RFLP patterns due to the fact that the Ictalurid hybrid inherited the mtDNA cytochrome b gene from the maternal parent channel catfish (*I. punctatus*).

Predictions based on *in silico* restriction of generated sequences showed that *C. macrocephalus* produced DNA fragments of 4, 6, 124–127, 290–294 bp when digested with the *Hae*III enzyme. However, PCR-RFLP experimental results revealed only two cleaved fragments (Table 3). Similarly, the absence of these theoretically shorter fragments of ~25 bp was also observed in both *I. punctatus* and *I. furcatus* following restriction with the *Nla*III enzyme. This was likely because these small fragments were too weak to be detected by the Bioanalyzer 2100 due to poor fluorescence of

these small and low concentration fragments. Fragments smaller than 40 bp were inconsistently detected. These observations were concordant with the other documentation indicating the difficulties of the Bioanalyzer 2100 in resolving fragments which are close to the minimum sizing limits of approximately 25 bp (Dooley et al., 2005; Ogden and McEwing, 2008). Although these fragments were not observed as expected, the ability to identify catfish species were not affected.

The absence of a 70-bp band following *Nla*III digestion of *P. bocourti* was clearly problematic and possibly related to the flagged warning of "possible co-migration of 2 peaks" indicated by the 2100 Expert software. The loss of this fragment predicted in the *in silico* restriction patterns was possibly explained by the co-migration of larger fragments as a single band (Table 3). In contrast, the missing DNA fragments of approximately 162–166 bp following *Dde*I restriction of *C. macrocephalus* could not be interpreted clearly since there was no evidence of co-migration effects on these sites. Instead, the fragment patterns of approximately 65–69 and 198–207 bp were consistently present in all the individuals of *C. macrocephalus* digested with *Dde*I, indicating that this species could still be differentiated unambiguously.

The RFLP profiles were observed to be largely consistent in all the tested individuals for each catfish species in this study. In fact, the majority of the experimental RFLP patterns matched those that were predicted from simulated restriction digests using NEB cutter, except for the absent smaller ($<$ 25 bp) fragments. However, variation in the fragment sizes resolved by the 2100 Bioanalyzer was observed. Observed restriction patterns were often larger than expected by 10–40 bp based on *in silico* predictions. Indeed, in some cases, $>$10% variation was observed, especially in profiles with smaller fragment sizes where both absolute error and percentage error were large (Ogden and McEwing, 2008). This discrepancy was strongly apparent in fragments of Clariid species digested by *Hae*III as well as *Dde*I-digested fragments of Pangasiid species (Table 3), with one of the fragment sizes observed consistently larger than those expected by approximately 38 bp. For example, in *C. batrachus*, the expected fragment size was 292–293 bp, while corresponding observed fragments were 325–336 bp. It was initially assumed that these discrepancies were caused by technical errors in either sequencing or restriction analysis. However, after thorough comparison with the species-specific sequence entries from GenBank, a similar pattern was also seen for the *in silico* restriction of reference sequences for these Clariid and Pangasiid species. Reconfirmation through replication of PCR-RFLP and 2100 Bioanalyzer analysis has suggested that these larger fragments were not artifacts as the DNA sizing results remained consistent, returning the same profiles for each species in each replicate. These divergences may be explained by the level of variation among chips and machines which have been reported in earlier studies (Ogden and McEwing, 2008) or differences in digestion efficiency. Reproducibility testing of the 2100 Bioanalyzer results in catfish species identification conducted by other laboratories may be required. Despite these discrepancies, species-specific banding patterns for both expected and observed results were relatively concordant, permitting their reliable identification.

Overall results obtained from the restriction analysis of the mtDNA cytochrome b gene using three enzymes *Dde*I, *Hae*III, and *Nla*III were displayed in the computer-generated gel image in the 2100 Expert software (Figure 2). The RFLP fingerprints generated by these three enzymes complemented each other in the catfish species identification. Additionally, the species identification flowchart also serves as a supporting tool for gel image interpretation. The single band observed in the fragments of *C. gariepinus* digested by *Dde*I (Figure 2a) indicated the absence of a *Dde*I restriction site in this particular species. Discrimination of *Clarias gariepinus* from other catfish species could also be achieved with a combination of the three restriction enzymes, instead of depending solely on the absence of the *Dde*I site. Of note, accurate identification of other species needs to be facilitated by the electropherograms corresponding to each band (Figure 3) due to the slight variations in band intensity displayed in the gel image.

A species identification flowchart (Figure 1) was developed as a rapid mean for catfish species identification following the development of PCR-RFLP profiles on the 2100 Bioanalyzer. Species

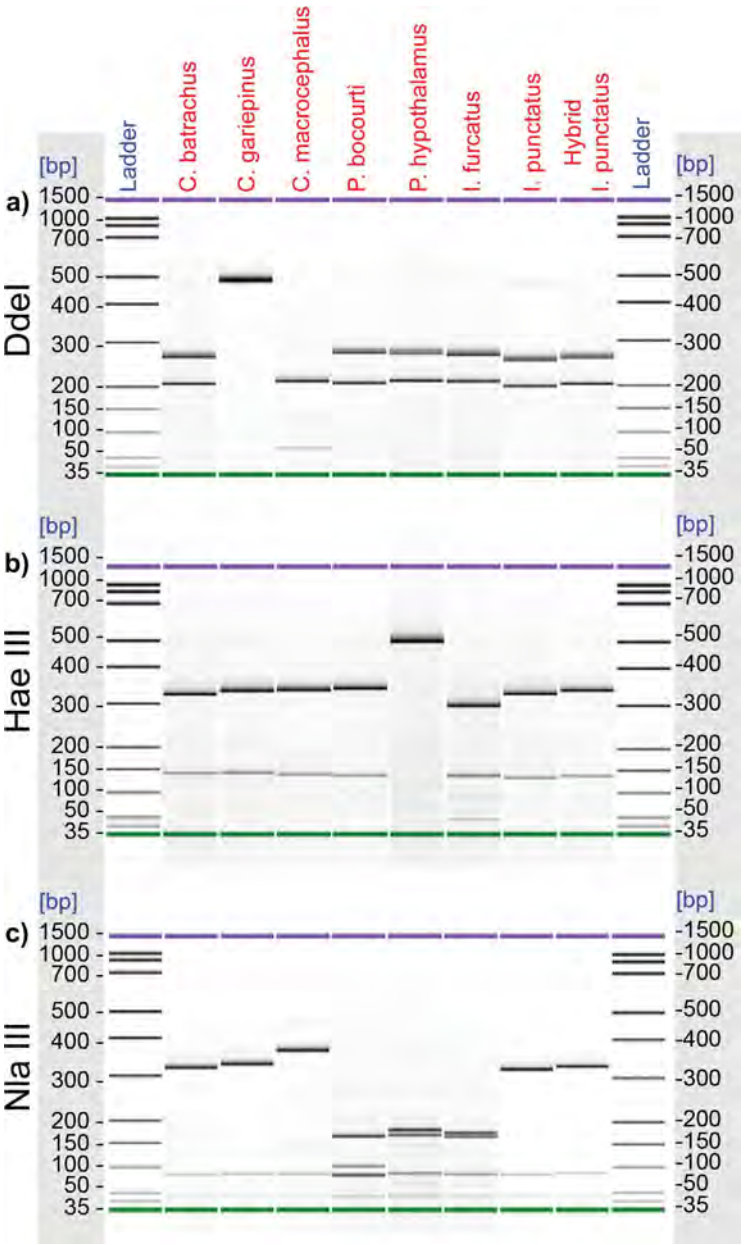

FIGURE 2 Computer generated restriction fragment length polymorphism (RFLP) gel image on the Agilent 2100 Bioanalyzer for cytochrome *b* fragments of catfish species. Each gel image consists of the following enzyme profiles: (a) *DdeI*, (b) *Hae*III, and (c) *Nla*III. Two lanes of size markers (15–1,500 bp) were incorporated into each profile.

diagnosis could be made by comparing the experimental banding patterns to the established reference profiles within the flowchart in a few basic steps which focus on the comparison between the banding patterns in the 2100 Bioanalyzer output and the key description in the flowchart.

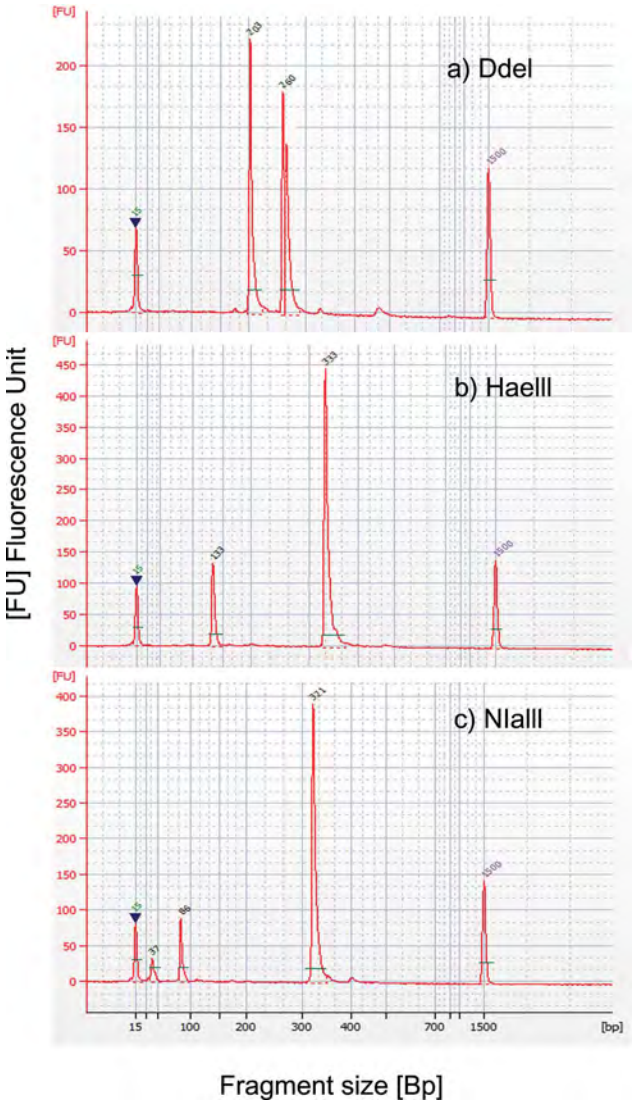

FIGURE 3 Electropherograms show the presence of species defining peaks (band size) for *Ictalurus punctatus* which corresponds to the RFLP profiles generated by three restriction endonucleases: (a) *Dde*I, (b) *Hae*III, and (c) *Nla*III.

To ensure the reliability of the PCR-RFLP technique, it is recommended that initial sequence analysis be performed on all species of interest for a given assay. Alternatively, if vouchered sequences for cytochrome *b* from species of interest currently exist in GenBank, these may be used. All the vouchered catfish specimens used in this study were sequenced. Sequences from at least five individuals were aligned to produce species-specific consensus sequences before the consensus sequences were compared against the GenBank database. All the samples revealed relatively high identity matches with GenBank entries in the range of 96–100% (Table 2). Although there were variations of fragment sizing between the expected restrictions profiles based on the *in silico* restriction analysis of sequences and the observed profiles generated by the 2100 Bioanalyzer, their restriction fragment patterns were still in agreement with each other. In fact, the cytochrome b gene sequences

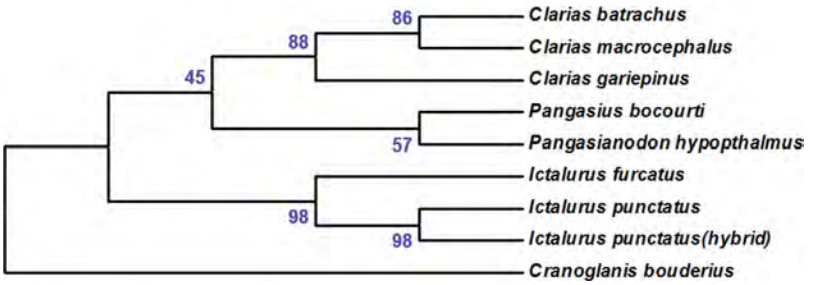

FIGURE 4 Phylogenetic consensus tree of seven catfish species (and an Ictalurid hybrid) constructed using the maximum parsimony (MP) method. Bootstrap values are shown next to the branches. The MP tree was built using the close-neighbor-interchange algorithm.

were aligned well within each species with an average sequence identity of 96%. Lower levels of sequence identity between our *Clarias* spp. samples and GenBank entries (Table 2) have previously been reported (Wong et al., 2011) and may reflect the use of *Clarias* hybrids in previous "vouchered" entries.

A phylogenetic tree was constructed from consensus sequences for each species to verify the presence of sufficient sequence variation between the catfish species studied. Phylogenetic trees were obtained from maximum parsimony (Figure 4) and neighbor-joining and maximum likelihood analysis (not shown) and produced similar clustering patterns. Tree topologies generated by the present study were similar to those of a previous study using cytochrome oxidase I (COI; Wong et al., 2011) where the catfish species were grouped into three distinct clades of families Ictaluridae, Pangasiidae, and Clariidae. Family Pangasiidae was more closely related to Ictalurid species compared to Clariid species, although the latter family was derived from the same geographical region as Pangasiid species. On the other hand, hybrid catfish with a maternal parent from *I. punctatus*, showed the expected result by forming a cohesive subclade with *I. punctatus*. Considering the intraspecific variations that can influence the reliability of PCR-RFLP analysis, all the replicated specimens were collected from different sampling locations.

The results obtained in this study show that the use of this system for catfish species identification is potentially feasible and offers several advantages over conventional gel-based electrophoresis. One of the advantages is the improved resolution of RFLP patterns, through the detection of smaller fragments that are undetectable on conventional gels. Furthermore, the small-sized LabChips are also easier to manipulate, requiring only a small amount of laboratory space. In addition, overall PCR and restriction enzyme costs could also be significantly reduced, due to the small volumes that are needed (Dooley et al., 2005; Spaniolas et al., 2006).

CONCLUSION

Our results from PCR-RFLP analysis of the mitochondrial cytochrome b gene were able to discriminate all of the studied catfish species. However, differences between *Clarias batrachus* and *Ictalurus punctatus*, while consistent, should be strengthened through the use of an additional restriction enzyme if the assay were to be utilized in a regulatory setting. Assay advantages due to shorter assay times and assay ease when compared to traditional DNA barcoding were counterbalanced by the need to develop and optimize specific restriction digest profiles for all potential species of interest. With further development, this method may be utilized in testing of catfish products to ensure the enforcement of seafood labeling regulations.

REFERENCES

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H., and Sato, S. 2006. Species identification and PCR-RFLP analysis of cytochrome b gene in cod fish (Order Gadiformes) products. J. Food Sci. 71(3): 190–195.

Arahishi, F. 2005. PCR-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. J. Agri. Food Chem. 53: 508–511.

Asensio Gil, L. 2007. PCR-based methods for fish and fishery products authentication. Trends Food Sci. Technol. 18: 558–566.

Bossier, P. 1999. Authentication of seafood products by DNA patterns. J. Food Sci. 64: 189–193.

Brambilla, I., Porto, G., & Tarozzi, A. (2009). Adjusting to Trade-Policy Changes in Export Markets: Evidence From U.S. Antidumping Duties on Vietnamese Catfish (Policy Research Working Paper WPS4990). Washington, DC: The World Bank Development Research Group Trade and Integration Team.

Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Sanz, B., Hernandez, P. E., and Martin, R. 1998. Identification of flatfish species using polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome b gene. J. Food Sci. 63: 206–209.

Davidson, W. S. 1998. PCR techniques. In: Analytical Methods of Food Authentication. Ashurst, P. R., and Dennis, M. J. (Eds.). London, United Kingdom: Blackie Academic and Professional. Pp. 182–203.

Dooley, J. J., and Garrett, S. D. 2001. Development of Meat Speciation Assays Using the Agilent 2100 Bioanalyzer (Agilent Technologies Application Note No. 5988-4069EN). Santa Clara, CA: Agilent Technologies.

Dooley, J. J., Sage, H. D., Brown, H. M., and Garrett, S. D. 2005. Improved fish species identification by use of lab-on-a-chip technology. Food Control 16: 601–607.

Dugan, K. A., Lawrence, H. S., Hares, D. R., Fisher, C. L., and Budowle, B. 2002. An improved method for post-PCR purification for mtDNA sequence analysis. J. Forensic Sci. 47: 811– 818.

Fajardo, V., Gonzalez, I., Dooley, J., Garret, S., Brown, H. M., Garcia, T., and Martín, R. 2009. Application of polymerase chain reaction–restriction fragment length polymorphism analysis and lab-on-a-chip capillary electrophoresis for the specific identification of game and domestic meats. J. Sci. Food Agri. 89: 843–847.

Hsieh, H. S., Chai, T., and Hwang, D. F. 2007. Using the PCReRFLP method to identify the species of different processed products of billfish meats. Food Control 18: 369–374.

Lin, W. F., and Hwang, D. F. 2007. Application of PCR-RFLP analysis on species identification of canned tuna. Food Control 18: 1050–1057.

Liu, Z., Nichols, A., Li, P., and Dunham, R. A. 1998. Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus)*, blue catfish (*I. furcatus*) and their F_1, F_2 and backcross hybrids. Mol. Gen. Genet. 258: 260–268.

Lockley, A. K., and Bardsley, R. G. 2000. DNA-based methods for food authentication. Trends Food Sci. Technol. 11: 67–77.

Mackie, I. M., Pryde, S. E., Gonzales-Sotelo, C., Medina, I., Pérez-Martín, R., Rey-Mendez, M., Quinteiro, J., and Rehbein, H. 1999. Challenges in the identification of species of canned fish. Trends Food Sci. Technol. 10: 9–14.

McDowell, J., and Graves, J. E. 2002. Nuclear and mitochondrial DNA markers for specific identification of istiophorid and xiphiid billfishes. Fish. B-NOAA 100: 537–544.

Meyer, R., Höfelein, C., Lüthy, J., and Candrian, U. 1995. Polymerase chain reaction-restriction fragment length polymorphism analysis: A simple method for species identification in food. J. Assoc. Off. Ana. Chem. 78: 1542–1551.

Nei, M., and Kumar, S. 2000. Molecular Evolution and Phylogenetics. New York, NY: Oxford University Press.

Ochiai, Y., Ochiai, L., Hashimoto, K., and Watabe, S. 2001. Quantitative estimation of dark muscle content in the mackerel meat paste and its products using antisera against myosin light chains. J. Food Sci. 66: 1301–1305.

Ogden, R., and McEwing, R. 2008. Adaptation of DNA Analysis Techniques for the Identification of Illegally Imported Bushmeat for Use on the Agilent 2100 Bioanalyser (Final Technical Report for Food Standards Agency United Kingdom Project Q01109). London, United Kingdom: Food Standards Agency. Pp. 1–20.

Panaro, N. J., Yuen, P. K., Sakazume, T., Fortina, P., Kricka, L. J., and Wilding, P. 2000. Evaluation of DNA fragments sizing and quantification by the Agilent 2100 Bioanalyzer. Clin. Chem. 46: 1851–1853.

Russell, V. J., Hold, G. L., Pryde, S. E., Rehbein, H., Quinteiro, J., Rey-Mendez, M., Sotelo, C. G., Perez- Martin, R. I., Santos, A. T., and Rosa, C. 2000. Use of restriction fragment length polymorphism to distinguish between salmon species. J. Agric. Food Chem. 48: 2184–2188.

Spaniolas, S., May, S. T., Bennett, M. J., and Tucker, G. A. 2006. Authentication of coffee by means of PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis. J. Agric. Food Chem. 54: 7466–7470.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596–1599.

Tepedino, V., Berrini, A., Borromeo, V., Gaggioli, D., Cantoni, C., Manzoni, P., and Secchi, C. 2001. Identification of commercial fish species belonging to the orders pleuronectiformes and gadiformes: Library of isoelectric focusing patterns. J. Assoc. Off. Ana. Chem. 84(5): 1600–1607.

Thompson, J. D, Higgins, D. G, and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Uthayakumaran, S., Batey, I. L., and Wrigley, C. W. 2005. On-the-spot identification of grain variety and wheat-quality type by lab-on-a-chip capillary electrophoresis. J. Cereal Sci. 41: 371–374.

Vasilyeva, E., Woodard, J., Taylor, F. R., Kretschmer, M., Fajardo, H., Lyubarskaya, Y., Kobayashi, K., Dingley, A., and Mhatre, R. 2004. Development of a chip-based capillary gel electrophoresis method for quantification of a half-antibody in immunoglobulin G4 samples. Electrophoresis 25(21–22): 3890–3896.

Vincze, T., Posfai, J., and Roberts, R. J. 2003. NEBcutter: A program to cleave DNA with restriction enzymes. Nucleic Acids Res. 31: 3688–3691.

Wolf, C., Burgener, M., Hubner, P., and Luthy, J. 2000. PCR-RFLP analysis of mitochondrial DNA: Differentiation of fish species. Lebensm. Wiss. Technol. 33: 144–150.

Wong, L. L., Peatman, E., Lu, J., Kucuktas, H., He, S., Zhou, C., Na-nakorn, U., and Liu, Z. 2011. DNA barcoding of catfish: Species authentication and phylogenetic assessment. PLoS ONE 6(3): e17812. doi:10.1371/journal.pone.0017812