SHORT COMMUNICATION

Species identification of "padas" from fermented fish paste or "bagoong" using DNA barcodes

Altair B. Agmata, Angelli Marie Jacynth M. Asis, and Mudjekeewis D. Santos*

Genetic Fingerprinting Laboratory, National Fisheries Research and Development Institute, 101 Mother Ignacia St., Quezon City, 1103 Philippines

In the Philippines, juvenile/fry fisheries have always been an important industry. But, despite the benefits these fisheries can provide, they suffer from lack of proper management. The "padas" is one of the highly sought-after siganid fry resource, especially in Northern Luzon. However, the establishment of the true identity of "padas" has not been done and, consequently, the possibility of appropriate management action has been limited. Here, where we used DNA barcoding, we report that "padas" from a fermented fish paste "Cristal Sky Bagoong" bought from Pozorrubio, Pangasinan is composed of at least two species of *Siganus*, namely *S. fuscescens* and *S. spinus*. This information is crucial in raising awareness of which specific resources should be targeted by the fisheries industry and it provides an initiative towards evoking proper management efforts for the sustainability of juvenile fisheries. We also demonstrate that DNA barcoding is a powerful tool in identifying fish species even in fermented form.

KEYWORDS

DNA Barcoding, CO1, fermented fish products, juvenile fish, Northern Luzon

*Corresponding author
Email Address: mudjiesantos@yahoo.com
Submitted: April 18, 2013
Revised: August 20, 2013
Accepted: August 21, 2013
Published: November 22, 2013
Editor-in-charge: Eduardo A. Padlan

INTRODUCTION

Siganids are a well-known and highly esteemed commodity in the Western Pacific. In the Philippines, adult siganids are caught in large quantities and sold fresh or cut open and dried (Bagarinao et al. 1986), while juvenile siganids or "padas" are often used as raw material for fermented fish products such as fish pastes or "bagoong" (Lam 1974). "Bagoong" is a famous delicacy highly sought after both by locals and tourists alike. Among the variety of "bagoong" products existing locally, the "bagoong" from Pangasinan, popularly known as "Lingayen bagoong", is often regarded as the best "bagoong" in the Philippines. Given the high demand for the product and the fact that the raw material is cheap, seasonally plentiful, and easily captured with the use of simple fishing gear (Ruddle 1993), juvenile resources, including siganid fry, become highly vulnerable to overfishing (Bell 1999, Soliman et al. 2009). In view of this, proper management of fry fisheries is important, so as to avoid stock depletion which will be detrimental to the fisheries as a whole (Soliman et al. 2009).

Species identification is a crucial step in the implementation of fisheries management efforts since the effectiveness of conservation and protection relies on the correct identification of the resource to be conserved (Nwani et al. 2011). In the case of "padas", distinguishing the fry of the many species of *Siganus* spp. has been difficult and employing meristic and morphological identification is of little help (Bagarinao et al. 1986). Although it is already known that "padas" is siganid fry, no literature exists that clearly defines its exact identity at the species level.

Given the limitations of the conventional methods, DNA barcoding arises as a powerful tool in providing a clear and accurate determination of the true identity of the fish. Its foundation lies on the principle that species are generally well-defined by species-specific molecular tags, like the one derived from the mitochondrial cytochrome c oxidase 1 (CO1) gene which allows unambiguous identifications (Hebert et al. 2003, Hubert et al. 2008). DNA barcoding not only enables detection of mislabeled products, but also assists in managing fisheries for long term sustainability (Ward et al. 2009). Specifically in the Philippines, DNA barcoding has been used in the identification of juvenile tunas (Pedrosa-Gerasmio et al. 2012), in resolving identity issues regarding sardine species (Willette and Santos 2013, Willette et al. 2011), and in detecting mislabeled fish and fish by-products (Maralit et al. 2013).

In this study, we report that "padas" from a fermented fish paste "Cristal Sky Bagoong" bought from Pozorrubio, Pangasinan is composed of at least two species of *Siganus*, namely *S. fuscescens, and S. spinus*. Our results highlight the importance of species identification to help in formulating effective management and monitoring strategies in the country. By identifying the species of the fish in "padas", this study also raises our awareness of juvenile fishery resources. Further, our study shows that DNA barcoding is a powerful tool in identifying fish species even in fermented form.

Figure 1. "Padas" from fermented fish paste with brand name "Crytal Sky Bagoong" bought from Pozorrubio, Pangasinan.

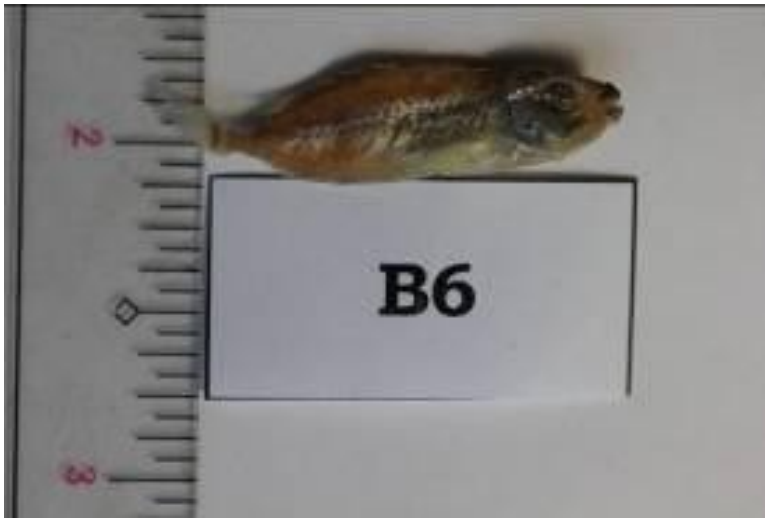

Figure 2. Representative image of "padas" sampled from the fermented fish paste product "Cristal Sky Bagoong" from Pozorrubio, Pangasinan"

METHODOLOGY

The "padas" sample came from one bottle of the fermented fish product labeled "Cristal Sky Bagoong", which was obtained from a highway vendor in Pozorrubio, Pangasinan (Figure 1). Muscle tissues of about 150 mg were obtained from 26 randomly selected samples, fixed in 95% ethanol, and stored at -20 °C until DNA extraction. DNA extraction was based on the method of Doyle and Doyle (1987) with modifications by Santos et al. (2010). The concentration of the DNA extracts was measured using a Nanophotoemeter, with concentrations ranging from 144 to148 ng/ul relative to the elusion buffer. An approximately 600bp fragment of the CO1 was amplified using PCR with primers C_FishF1t1 and C_FishR1t1 from Ivanova et al. (2007). The PCR amplicons were sent to Macrogen Korea for sequencing. Sequence analysis was done by the construction of a phylogenetic tree via MEGA 5 (Tamura et al. 2011), inferred using the Neighbor-Joining method (Saitou and Nei 1987) and the Maximum-Likelihood method (Kimura 1980), as well as by computing pairwise genetic distances using Kimura-2 parameters from sample sequences and reference sequences from GenBank. Generated CO1 sequences of "padas" were submitted to BOLD with the following accession numbers: B2 (BFPHL087-13), B3 (BFPHL088-13), B4 (BFPHL089-13), B5 (BFPHL090-13), B6 (BFPHL091-13), B7 (BFPHL092-13), B8 (BFPHL093-13), B9 (BFPHL094-13), B11 (BFPHL095-13), B12 (BFPHL096-13), B14 (BFPHL097-13), B16 (BFPHL098-13), B17 (BFPHL099-13), B18 (BFPHL100-13), B19 (BFPHL101-13), B21 (BFPHL102-13), B22 (BFPHL103-13), B23 (BFPHL104-13), B24 (BFPHL105-13), B25 (BFPHL106-13) and B26 (BFPHL107-13), and then compared with adult siganid sequences generated by NFRDI and submitted to BOLD with the following accession numbers: SFU01 (BFPHL108-13), SFU02

(BFPHL109-13) SFU03 (BFPHL110-13), SFU04 (BFPHL111-13), SGU02 (BFPHL112-13), SGU03 (BFPHL113-13), SGU04 (BFPHL114-13), SJA_01 (BFPHL115-13). *Naso unicornis* (JQ350131.1), belonging to the same order as siganids (perciformes), was used as an outgroup. Voucher CO1 EF609464.1, HQ149955.1, HQ149953.1, HQ149951.1, JQ432158.1, JQ432158, JQ432159.1, FJ584116.1, FJ584115.1, FJ584117.1, FJ584118.1, FJ584114.1, FJ584108.1, EU752210.1, EU752209.1, FJ84112.1, FJ84113.1, FJ84110.1, FJ237938.1, FJ237940.1, FJ237944.1, FJ237934.1, JN021253.1, JN021251.1, EU620492.1, EU620493.1, EU620485.1, and EU620484.1 were obtained from GenBank for further comparison.

RESULTS AND DISCUSSION

A total of 26 "padas" samples were processed for partial CO1 sequencing, 21 of which yielded high quality DNA sequences and were subsequently analyzed. This suggests that the DNA extraction methodology via CTAB extraction buffer (Doyle and Doyle (1987) with modifications by Santos et al. (2010)) that was used in this study is appropriate for extracting DNA from fermented samples and can thus be used for future studies with the same nature of sample source.

The CO1 sequences generated from the "padas" samples appeared to be identical with sequences of the same gene region in *S. fuscescens* and *S. spinus* (90.4% and 9.6% of the samples, respectively), based on data obtained from reference samples that are available in GenBank. Both the Neighbor-Joining and Maximum-Likelihood trees (Figures 2 and 3) showed a big cluster comprised of 19 "padas" samples and the *S. fuscescens* reference sequences. The second and much smaller clade contains the other two "padas" samples and the *S. spinus* reference sequences. Low values of intraspecific pairwise distances of 19 samples from pairing with *S. fuscescens* (average of 0.008) and two samples from pairing with *S. spinus* (0.016) was also observed (Table 1), which corroborates the results generated from the tree analysis. An additional analysis using the BOLD database Animal Identification also identified the samples B2 and B9 CO1 sequences as 100% and 98.11% identical with the CO1 sequences of *S. spinus*, respectively. These data suggest that the "padas" being sold in Pangasinan, particularly "Cristal Sky Bagoong", is made from these juvenile siganid species.

Fermented fish products like "bagoong" are made from inexpensive and abundant raw materials.

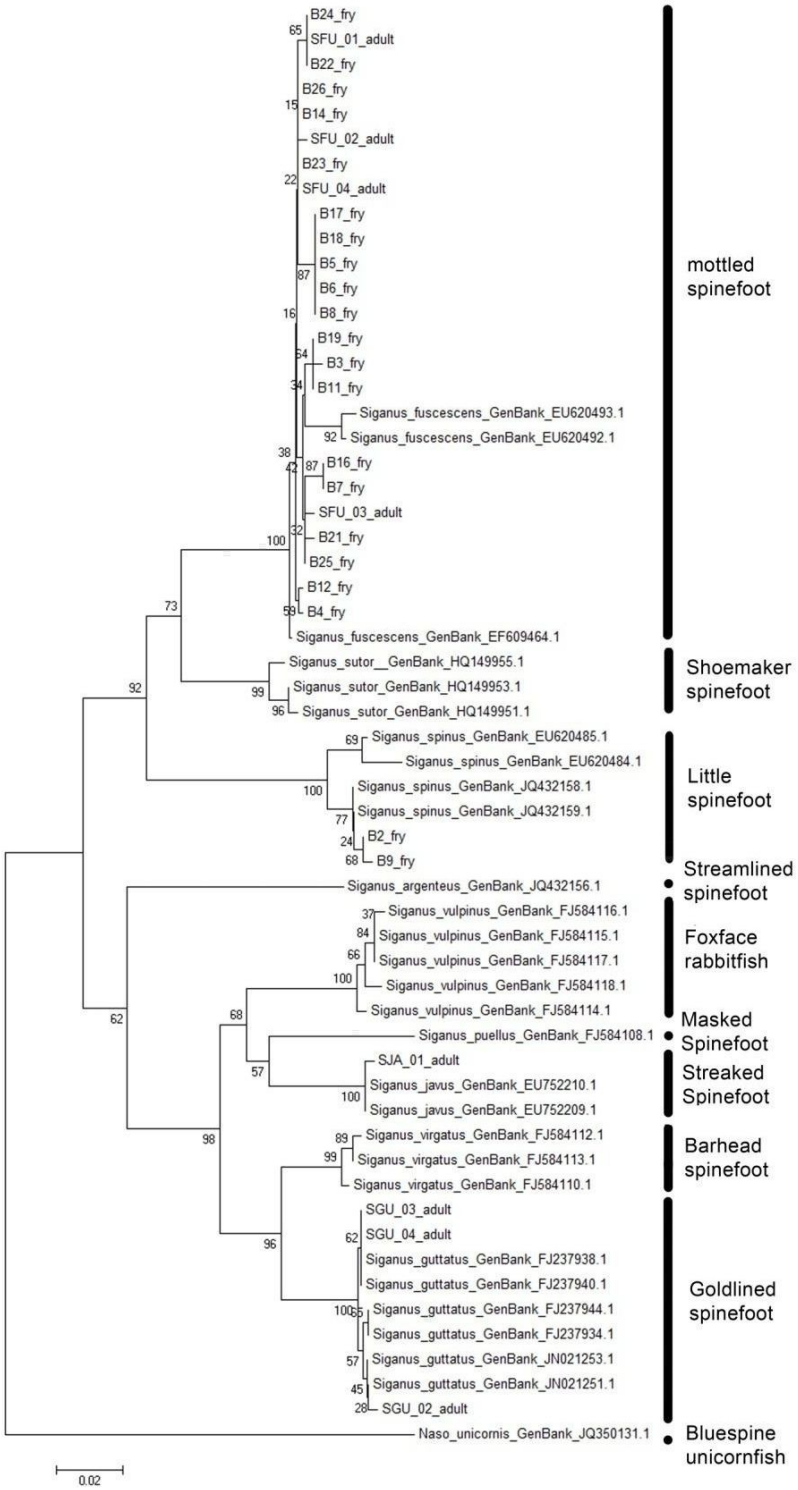

Figure 3. Neighbor-Joining Tree of "padas" CO1 sequences from "Cristal Sky Bagoong" samples and adult siganid samples from NFRDI and GenBank with corresponding common names using Kimura 2-parameter model.

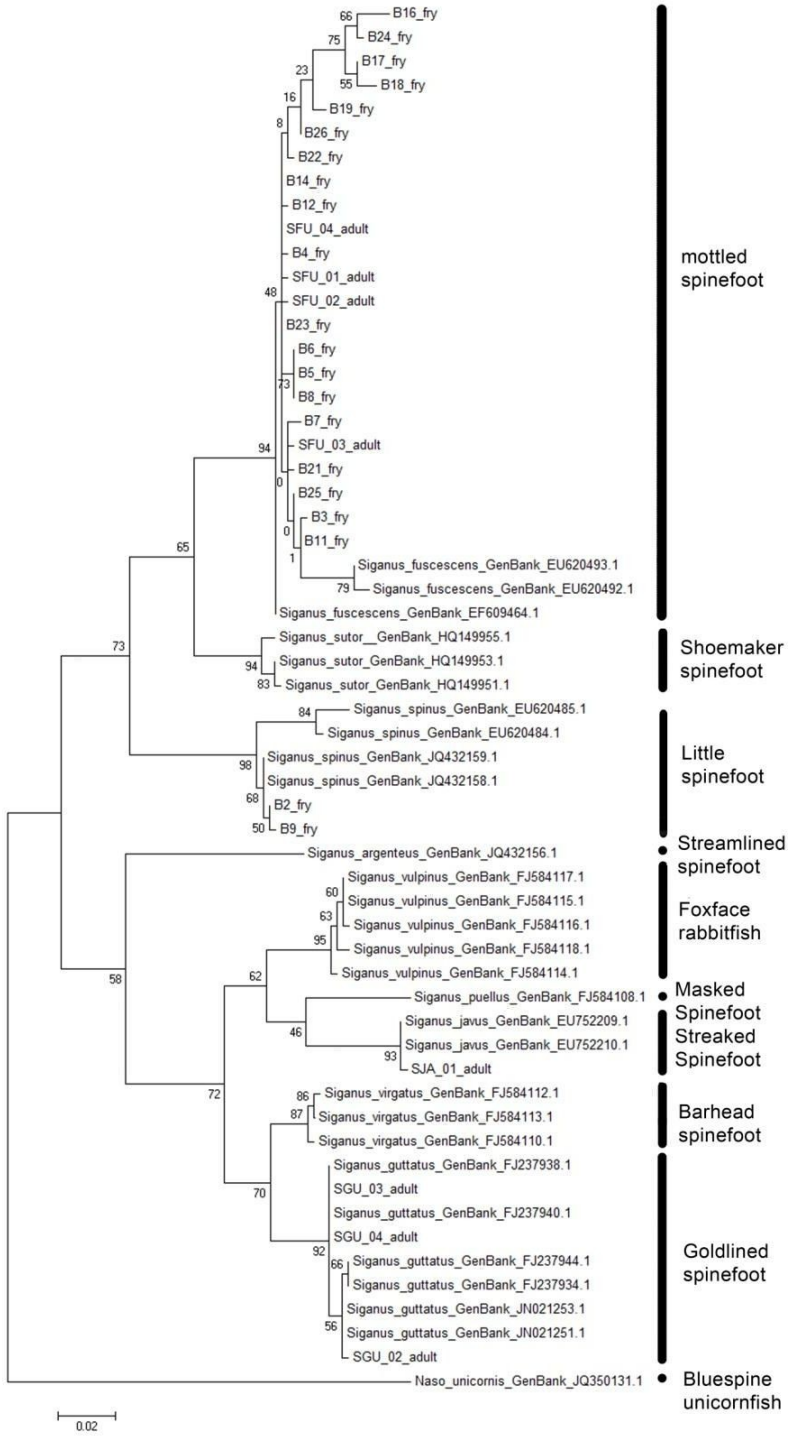

Figure 4. Maximum-Likelihood Tree of "padas" CO1 sequences from "Cristal Sky Bagoong" samples and adult siganid samples from NFRDI and GenBank with corresponding common names using Kimura 2-parameter model

These are small and of low economic value, but are seasonally abundant and are easily captured with rather simple gear (Ruddle 1993) so that they are readily available in large quantities. Juvenile species that are made into these products are therefore highly vulnerable to exploitation. Also, their capture method in the form of bagnets is fairly easy and inexpensive, hence making them very prone to overfishing. The results presented here have significant implications on issues regarding assessments of successful conservation efforts and the formulation of effective management strategies, as well as on issues regarding food labeling.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the National Fisheries Research and Development Institute (NFRDI) for giving the necessary funding for the study.

CONFLICT OF INTEREST

None.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Framing of the hypothesis and experimental set-up was done by Mudjekeewis D. Santos. Laboratory work, data analysis, and manuscript preparation were done by Altair B. Agmata, Angelli Marie Jacynth M. Asis, and Mudjekeewis D. Santos.

REFERENCES

Bagarinao TU, Solis NB, Villaver WR, Villaluz AC. Important fish and shrimp fry in Philippine coastal waters: identification, collection and handling. Aquaculture Extension Manual No. 10. Iloilo: SEAFDEC Aquaculture Department. 1986; p. 52.

Bell KNI. Overview of goby-fry fisheries. NAGA - the ICLARM quarterly 1999; 22(4):30-36.

Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 1987; 19:11-15

Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc R Soc Lond. B Biol. Sci 2003; 270:313–321.

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Watkinson D, Dumont P, Curry A, Bentzen P, Zhang J, April J, Bernatchez L. Identifying Canadian

Table 1. Mean values of pairwise genetic distances (expressed in percentage) within and among clades formed by Neighbor-Joining and Maximum-Likelihood tree analysis.

Species clades	Ave. within clades (%)	Ave. among clades (%)									
		S.fuscescens	*S.spinus*	*S.argenteus*	*S.sutor*	*S.guttatus*	*S.vulpinus*	*S.virgatus*	*S.puellus*	*S.javus*	*N.unicornis*
S.fuscescens	0.8	n/a									
S.spinus	1.6	11.2	n/a								
S.argenteus	n/a	13.9	15.7	n/a							
S.sutor	0.9	6.9	10.0	14.0	n/a						
S.guttatus	0.3	14.8	14.7	13.6	14.8	n/a					
S.vulpinus	0.7	14.5	17.3	13.6	14.9	8.7	n/a				
S.virgatus	0.5	14.5	14.6	11.5	14.2	4.6	7.8	n/a			
S.puellus	n/a	16.4	15.8	14.3	14.0	8.8	7.6	10.2	n/a		
S.javus	0.3	14.8	16.2	13.3	13.4	9.0	8.1	8.8	7.2	n/a	
N.unicornis	n/a	20.8	21.8	22.4	20.4	19.9	20.7	20.0	21.8	19.9	n/a

Freshwater Fishes through DNA Barcodes. PLoS ONE 2008; 3(6):e2490.doi:10.1371/journal.pone.0002490.

Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN. Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes 2007; 7:544-548.

Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980; 16:111-120.

Lam TJ. Siganids: Their Biology and Mariculture Potential. Aquaculture 1974; 3:325-354.

Maralit BA, Aguila RD, Ventolero MFH, Perez SKL, Willette DA, Santos MD. Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety. Food Control 2013; 33(1):119-125.

Nwani CD, Becker S, Braid HE, Ude EF, Okogwu OI, Hanner R. DNA Barcoding Discriminates Freshwater Fishes from Southeastern Nigeria and Provides River System-level Phylogeographic Resolution within Some Species. Mitochondrial DNA 2011; 22(Suppl. 1):43-51.

Pedrosa-GerasmioIR, Babaran RP, Santos MD. Discrimination of juvenile yellowfin (Thunnus albacares) and bigeye (T. obesus) tunas using mitochondrial DNA control region and liver morphology. PLoS ONE. 2012; 7(4): e35604.DOI 10.1371/journal.pone.0035604.

Ruddle K. The Availability and Supply of Fish for Fermentation in Southeast Asia. In: Lee C, KH Steinkraus, PJA Reilly, eds. Fish Fermentation Technology. Japan: United Nations University Press, 1993: pp. 62-63.

Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-425.

Santos MD, Lopez GV, Barut NC. A pilot study on the genetic variation of eastern little tuna (Euthynnus affinis) in Southeast Asia. Phil J Sci 2010; 139(1):43-50.

Soliman VS, Bobiles RU, Yamaoka K. Overfishing of three siganid species (Family: Siganidae) in Lagonoy Gulf, Philippines. Kurushio Science 2009; 2:145-150.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28(10):2731-2739.

Ward RD, Hanner R, Herbert PDN. The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol 2009; 74:329-356.

Willette DA, Santos MD. Correcting widespread misidentifications of the highly abundant and commercially important sardine species Sardinella lemuru, Bleeker, 1853, in the Philippines. Journal of Applied Ichthyology. 2013; 29(4):881-885.

Willette DA, Santos MD, Aragon MA. First report of the Taiwan sardinella Sardinella hualiensis (Clupeiformes: Clupeidae) in the Philippines. J Fish Biol 2011; 79:2087-2094.