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Research Article

The Application of DNA Barcodes in the Authenticity Identification of Marine Fishes

Xueying Zhang, Qiong Jiang, Yanfei Din and Cheng Zhu
College of Life Science, China Jiliang University, Key Laboratory of Marine Food Quality and Hazard Controlling Technology of Zhejiang Province, National and Local United Engineering Lab of Quality Controlling Technology and Instrumentation for Marine Food, Hangzhou 310018, China

Abstract: Recently, the study about Marine fish identification is becoming a big challenge for us. Marine biodiversity is always to been underestimated for we can't know how enormous it is. And because of the boundedness, the existing traditional taxonomic methods have not enough ability to detect the mystery of ocean and we must seek for a new way to research how many kinds of fishes in ocean and what kind of the fish is. What's more, with the internationalization of market, not only taxonomy, but the seafood market chaos and plenty of issues about food safety also reflect the importance of Marine fish identification. In this context, a new identification tool ----DNA barcodes has appeared. DNA barcodes is a Molecular identification methods which through sequencing the mitochondrial genes of Cytochrome c Oxidase subunit I(COI) sequence and being compared with DNA barcodes database to rapid identification of the sample. In this study, we will introduce the principle, advantages and limitations of DNA barcodes and summarize the application of DNA barcodes in the aspect of Marine fish and seafood identification. All kinds of application research show that DNA barcodes has high resolution and can easily identify fishes to species level. So we have reason to believe that the DNA barcodes can been made full use of in the field of Marine fish authenticity identification though the research of predecessors. What's more, it benefits to enhance the effectiveness of food anti-counterfeit and can help to manage the seafood market more effectively.

Keywords: DNA barcodes, marine fish, species identification

INTRODUCTION

The sea is not only the most extensive in the world, but also the most mysterious to human beings. It covers about 360 million square kilometers total area, accounting for about 71% of the surface of the earth, but only 5% area of ocean has been understood. The Marine fish distributes from the poles to equator seas, coast to ocean, surface to kilometers depth. So we can say that it's the diversity of living environment created the diversity of sea creatures. If think about Marine diversity carefully, you will found the amount of ocean fishes will be a very large number. By February 2010, about 167817 to 229602 kinds of marine species have been recorded in WoRMS (the World Register of Marine Species, http://www.marinespecies.org), thus extrapolation of these numbers confirms estimates that ocean contain 10 million more species (Radulovici *et al*., 2010). Although the diversity of Marine fishes is lower to this number, we still can't underestimate it. Now 15304 species added to the catalog of Marine fish and this number will be about 20000 species in the eventually expect. To date, there are 210000 Marine life as we know, it is estimated that the actual quantity is more than 10 times of this number, namely 2.1 million (O'Dor, 2003). Facing of so many kinds of fish, undoubtedly, it is a very difficult issue to identify one by one by only using the traditional classification method. So, how can we find a way to identify them rapidly?

With the improvement of living standards and increasing demand to high quality food, Marine food have occupied an important place in the market for its rich nutritional value. Since the 1980s Japan first created artificial jellyfish silk, all kinds of bionic Marine food emerge in endlessly. At the same time those foods are popular with consumers for high similarity to native Marine products in aspect of appearance, taste, nutritional value, low price and so on, We can't ignore the hidden problems incurred by those characteristic, for example mixxing the false with the genuine in food market (Chen and Zhou, 2005). Currently we often hear about some vendors using cheap fish instead of expensive fish or other cheap

Corresponding Author: Cheng Zhu, College of Life Science, China Jiliang University, Key Laboratory of Marine Food Quality and Hazard Controlling Technology of Zhejiang Province, National and Local United Engineering Lab of Quality Controlling Technology and Instrumentation for Marine Food, Hangzhou 310018, China, Tel.: (0086571) 13606614936; Fax: (0086)0571-86914510

material mix in food to get huge benefits (Zhang *et al*., 2010). Such as the phenomenon of replace the high quality nature shark's fin with artificial shark's fin and the appear of false sea cucumber, jellyfish in recent years. In order to pursuit of similar taste, mouthfeel with the genuine, these food are always added all kinds of colloid and various additives, bringing serious harm to people's health. What's more, when food put into process, they loss their taxonomic character in the process of production since the diversity of the way of process, thus we can't use traditional taxonomy method to identify these material which have lost original form easily, this mean traditional taxonomy method is not suitable for seafood species identification, we are faced with a difficult in this area. In order to guarantee the safty in seafood market, a more effective, rapidly, convenient and different from traditional method identification methods have been in urgent need for seafood market. In this context, a new identification method, DNA barcode technology, has emerged. For understanding this technology comprehensively, this article study DNA barcode technology in the aspect of identification methods comparison, advantages, application, development status of DNA barcode and then provide the basis for whether this technology can be beneficial to the authenticity identification of marine fishes.

COMPARISON AMONG IDENTIFICATION METHODS

Although people have found many methods to achieve species identification rapidly, with the development of technology and the increasingly outstanding limitations which this taxonomic methods have, most of people not only focus on the traditional macroscopic taxonomic method, but also begin paying attention to micro level. Now taxonomic methods can be included in the followings:

Morphological identification: Morphological identification method is the first taxonomic method people master. It is a method that can identify and classify the species by observing and identifying the morphological characteristics. Morphological properties we commonly used, including morphology, quantity characteristics and biological body color of each part, etc. (Lv, 2012). We can realize rapidly the identification of various species intuitional through identifying the morphological characteristics of species, environmental distribution characteristics, or through the microscope. Thus morphological identification plays a very important position in the taxonomy. There is no doubt that this appraisal method is fast and convenient when the same time, many limitations can not be ignored.

Zemlak *et al*. (2009) selected 35 species of fish to conduct a molecular identification test, these fishes which distributing on coastal and offshore of the Indian Ocean have different living habits. Though this test they found that there has a bigger difference (average 5.10%) in a considerable part of the same fish which distributing on the sea of South Africa and Australia. So Zemlak *et al*. (2009) believed that the existing traditional classification systems underestimated the polymorphism of Marine fishes. They thought that there are at least a third of the 1000 kinds of fish in South Africa and Australia waters and fish should be divided into two taxa. What's more, there are some difficulties in morphological identification, which is widely distribution of monomorphic species in Marine systems and the existence of some sexual binary and phenotypic plasticity species (Radulovici *et al*., 2010).

The physical and chemical analysis method: The physical and chemical analysis method is a method based on identifying the differences of physical and chemical properties of some species composition and then distinguishing different species. Some special ingredient of species is usually choosed to test. Usually, the physical and chemical analysis method can be mainly included the followings: component analysis, protein analysis and immunological analysis, etc. (Lv, 2012).

Component analysis is a method that analysing the composition and content of the ingredients of species to achieve species identification. Chromatography and spectral method are commonly used (Zhou and Yuhua, 2009).

This method is identify species by analysing protein of samples. Commonly used methods include kjeldahl determination, High-Performance Liquid Chromatography (HPLC), capillary analysis, etc. (Wei *et al*., 2003).

Immunological analysis is based on the specificity of antigen-antibody binding reaction, it identifies species by observing reaction between the antigen (antibody) of the sample and the known specific antibodies (antigen). Among the immunological analysis method, ELISA (enzyme linked immunosorbent assay) is commonly used. When this method has high specificity, high sensitivity and simple operation, at the same time, because of the limitation of its specificity, the phenomenon of false positives and limitations in testing a variety of material things, this method can't identify species accurately and rapidly now (Wang *et al*., 2009).

The molecular taxonomy: It is the limitations of traditional methods, when PCR (polymerase chain reaction) and molecular biology techniques appeared, scientists start using molecular biology method to answer these problem that traditional taxonomy can't answer and begin to widely used these DNA-based molecular biology methods in the study of microbial, species diversity, etc. With the application and development of PCR technology in recent years, in the 1990s, standard molecular identification system has

been appeared and has been applied in the aspect of microbes. What's more, isomerase which coded by the same site of alleles as the first molecular marker has also been used in proving the model of population genetic diversity and used for the early molecular system research (Li *et al*., 2010).

At present, the mainly molecular biology methods apply in study of Marine fish species diversity is DNA fingerprint technology, including random amplified polymorphic DNA(RAPD), Restriction Fragment Length Polymorphism (RFLP), microsatellite DNA, Amplified Fragment Length Polymorphism (AFLP), Single Strand Conformation Olymorphism (SSCP), Denaturing High Performance Liquid Chromatography (DHPLC), etc. These technologies always amplify gene by PCR and thus identify by using various principle (Lv, 2012). For example, RFLP method use appropriate DNA restriction enzyme to process sample and then mapping specific segments by agarose gel electrophoresis. This method is characterized by simple operation, low cost, can be widely used in the identification of Marine organisms (Zhang *et al*., 2010).

DNA chips is also a common identification technology. There are many researchers used this method in Marine biological identification to realize the rapid identification of species. DNA chips fixed DNA fragment on the solid phase support and then come to a conclusion through cross-fertilizing with the known nucleic acid probe. Kochzius *et al*. (2008) aiming at 16 s rDNA sequence, produced a DNA chips that can identify 11 types of fish. The next, they plan to make 50 types of fish DNA chips. The advantage of this approach is can make one-time identification for large sample, however, it's too expensive.

DNA barcode, the proposed DNA-based project for species identification recently, is based on a short, standard and specific DNA fragments, which derive from organism, can be easily amplificated and represent this species for their distinctness. In 2003, Canada university of guelph professor Hebert *et al*. (2003) proposed can represent species with a single small fragments gene as a barcode, barcode global species, that is DNA barcode. And then we can scan and identify species rapidly by DNA barcode like scan commodity's barcode at supermarket. Hebert *et al*. (2003) is called the father of the DNA barcode proposed for the first time propose apply DNA barcode to species identification. With the development of molecular biology techniques, DNA barcode technology have get more and more attention over the years. Consortium for the Barcode of Life has associated with 40 countries, more than 140 research institutes by 2007. Nowadays, undoubtedly, DNA barcode technology has become a vital technology of ecology research (Yan and Jie, 2010).

The theoretical basis of DNA barcode is identifying species accurately and rapidly though a standard DNA fragments and it is a identification method by identifying the short sequences of genome of biological samples essentially. Kress *et al*. (2005) and Pierre *et al*. (2007) put forward the standard of ideal DNA barcode:

- Sufficient variability to distinguish different species and relatively conservative
- Must be a standard DNA area as much as possible to identify different taxa
- Enough system evolution information should be contained in target DNA region to locate species in the classification system
- Highly conservative primer design region to design universal primers
- Short enough target DNA region to the benefit of amplification of a part of degradation DNA

In general, a complete sequence length of barcode should be under 700 bp. There is a 692 bp length protein-coding genes sequence in Mitochondrial genes, that is Cytochrome Oxidase subunit I(COI). This gene have not introns and follow the matrilineal inheritance, result in a low rate of restructuring (Chaolun *et al*., 2011). Hebert *et al*. (2003) carried on a comparative analysis which compared mitochondrial cytochrome C oxidase subunit I (COI) gene sequence of the animal kingdom species, including vertebrates and invertebrates (a total of 11, 13, 320 species) and found that 98% of species genetic distance difference in the kind of 0~2% (except the coelenterate Cnidaria) and interspecific distance difference reached an average of 11.3%. What's more, many studies show that 97% of the fish can be identify to species level only by a 680bp COI sequences (Li *et al*., 2013). This shows that the COI sequences can be effective for accurate identification of species and can be widely used in ocean fish identification as the main DNA barcode.

The advantages of DNA barcode: In the development of DNA study, some critics have questioned whether DNA barcode technology can identify species effectively and accused it ignored the 'richness' inherent in traditional method. In order to verify the validity of the DNA barcode, Laurence *et al*. (2009) compared DNA barcode technology with morphological taxonomy in the matter of accuracy and properties diversity described by method, found morphological taxonomy does not work in some important cases. In these cases, we can see a effective complementary between DNA barcode and traditional morphological taxonomy is in urgent need.

The steps of identifying Marine fish by DNA barcode can mainly includes in the following:

- Extract the total DNA of fish for identification
- Amplify COI gene though PCR and sequence it
- Compared this sequence with known strands in the Gene Bank and then species of sample can be identified according to the homology

At present, the main advantages of DNA barcode can be showed in the following:

- DNA barcode technology use COI gene as a standard to identify species, hence it is not limited to the sample's form, can identify those species which embrace phenotypic plasticity accurately and avoid many drawbacks that morphological taxonomy have.
- DNA barcode has extensive database as the support, thus can convenient, quickly match the genes on the sample with gene databank, so as to more efficient to identify sample when faced with a large number of samples (Li *et al*., 2013).
- DNA is more stable than any other biological macromolecules such as protein in the process of production, thus can track food production process (Andrea *et al*., 2013).
- Unified standard of species identification is established to make data sharing more convenience and is benefit to avoid judge error due to subjective experience (Dupont *et al*., 2007).

Application of DNA barcode in Marine fish identification: In recent years, with the globalization of market, the scale of aquaculture industry is also expanding gradually. Under this background, DNA barcode technology play a more and more significant role in the fish classification. At first, in order to verify the reliability of DNA barcode technology, Rasmussen *et al*. (2009) widely captured more than 1000 salmon in North America to conduct DNA barcode identification, the results showed low intraspecies divergence (mean, 0.26%, range, 0.04-0.26%) and the mean congeneric divergence was 8.22% (range, 3.42-12.67%), 32-fold greater than intraspecies divergence and the minimum interspecies divergence was greater than the maximum intraspecies divergence, proved the feasibility of DNA barcode on the species identification. Li *et al*. (2011) studied DNA barcode for using in differentiating United States domestic catfish and imported catfish, found that the sequence of samples show highly consistency, the similarity is up to 98% on average. On these basis, we can found that DNA barcode technology has obvious advantages in identifying those fish which have difficult in recognizing their fuzzy morphological characteristics compared with other classification methods. Viswambharan *et al*. (2015) selected 11 kinds of goby fish which come from the west coast of India's to conduct DNA barcode identification and found the average genetic distance of species, genus and family, were 1.2, 22.2 and 25.3%, respectively show that DNA barcode can classify those small size, fuzzy morphology fish like prawn tiger fish effectively. In addition, DNA barcode technology also can be applied to the study of biological evolution. Radulovici *et al*. (2010) think people underestimate the Marine biodiversity in the level of molecular, genetic, time, space and so on, thereby they recommend mark fish by using DNA barcode technology for further study the diversity pattern and potential evolution process. What's more Li *et al*. (2011) estimate the genetic distance of a variety of catfish on the basis of results of DNA barcode identification calculated the various and thus map out the genetic evolutionary tree. All of these are beneficial to the research of fish genetic evolution.

The second, with the frequent appearance of food problems recently, people have pay more and more attention to the food problem. In order to manage the market more effectively, a quick and convenient method for product identification is in urgent need, especially to identify those food which processed by some complicated process. And the biggest advantage of DNA barcode is that it can identify food and not be affected by the sample form. Smith *et al*. (2008) have used DNA barcode technology for the identification of all kinds of smoked fish and success in identifying those smoked fish to the species level. Li *et al*. (2013) identified frozen fish, frozen fish fillet, grilled fish with DNA barcode and compared the results with morphological analysis results, showed that DNA barcode can effectively identify the kinds of fish food which have difficult in recognize their material and found the mistake of labeling "codfish" as "silver pout" in the market. To make better use of DNA barcode in seafood identification in the market, Eugene h. -k. Wong and Robert h. Hanner conducted a random selection and identified those 91 samples in DNA barcode. When they compare results with the database, they found the sequence's matching degree of 90 samples reached more than 97% and the surprising thing is 25% of the sample is wrong labeled (Andrea *et al*., 2013). What's more, DNA barcode technology can also label imported seafood, track trade chain, thus realize the efficient management of seafood market (Espiñeira *et al*., 2008). All of those shows that DNA barcode technology can realize a effective identification in food adulteration in the market, thus can make a signification contribute to food anti-counterfeit.

Development status:

The number of Marine fish's barcode: With the more and more outstanding function the DNA barcode technology show, the researchers of all countries pay a high attention to it. In 2006, in India, a DNA barcode plan about fish and Marine life have been open, 79 species and 37 family Indian Ocean fish, including carangidae, clupeidae, scombridae, grouper, drum fish, must sparidae, catfish, are first established a DNA barcode (Lakra *et al*., 2011). According to the data from BOLD (Barcode of Life Data systems), there are 228436 fish COI sequences in the fish Barcode library. What's more, with the widely application of the DNA barcode, about 500000 species have been included in the barcode plan to this day (Li *et al*., 2010). According

to the report, on August 18, 2014, our country build a key project, that is "The build of China's offshore Marine biological DNA barcode repository". This project will choose representative Marine organisms to obtain DNA barcode sequences and thus strive to build a resource sharing platform for the DNA barcode database on the basis of accurate morphological identification (Yu, 2014).

Some special problems on classifying marine fish: When people identify fish by using DNA barcode at the same time, some technical problems and obstacles also appeared. One of main limitation of DNA barcode is that it will become a big challenge to establish a sequence database for all kinds of fish since there are many different kinds of Marine fish in the ocean. In order to make contribution to this challenge, Ezequiel *et al*. (2011) made DNA barcode for 125 species of fish in Argentina, at test the recognition of this technology the same time, they also successfully use this technique to solve some ownership problem for those species which classification is not clear on this area.

Li *et al*. (2013) also found that DNA barcode technology is suitable only for identifying those fish products which component contain a kind of meat. Because there will make test purpose ambiguity, operation red tape and increase the cost of testing to identify those fish products which contain a variety of meat. In addition, there will bring some DNA damage to fish products through all kinds of process, especially high temperature, high pressure or deep fry process, so as to bring some difficult to DNA extraction. What's more, the application of some food additives also damage to DNA extraction. Therefore, DNA barcode technology is only applicable to identify those fish products which have mild conditions process and single meat composition.

In addition, the researchers also found that some species suitable for different gene to make DNA barcode, thus COI standard may can't suitable for all species. Additional, some problems about mitochondrial genes such as uniparental inheritance, heterogeneity, introgression and so on also wait to be solved (Li *et al*., 2010).

The future research direction: In the face of large number, a wide variety of ocean fish, in order to make DNA barcode to identify Marine life rapidly, the researchers established a DNA barcode index database (the reference libraries of DNA barcodes, RLDB) and the supplement and complement of this database will become a significance research content in the future. In order to test the inconsistent result caused by uncertainty classification or faulty operation, Filipe *et al*. (2012) suggest apply a hierarchy which composed of cross validation to put confidence interval on DNA barcode identification which based on RLDB and applied this new hierarchy to Portugal fish identification. However, this kind of hierarchical RLDB is effective only in worldwide. In order to make new data of DNA barcode combined with existing database, Knebelsberger *et al*. (2014) use BIN (BOLDs barcode index number) and rank system to prove the reliability of 85% of Nordic shelf fish's DNA barcode identification result. In order to realize the higher value of database, Francisco *et al*. (2014) suggest perfect other molecular markers technology to obtain more rich information on related genes, thus can combined with DNA barcode technology to study biological differentiation. In short, the improvement of the DNA barcode database is still need the further research.

CONCLUSION

As a new identification technology, DNA barcode technology is not limited to samples' shape, it identify species by amplifying, sequencing, comparing the COI gene of samples convenient and fast. Recently, with the perfection of the DNA barcode database, a large number of research findings show that species matching rate of DNA barcodes can reach up to 97% and have been applied to authenticity identification of marine fishes successfully. However, with some limitations of this technology like bad purposiveness when face with mixed sample, or DNA damage caused by harsh processing method, a combination between DNA barcode technology and traditional taxonomy is in urgent need to build a perfect identification system and then revise some mistakes about classification of marine fishes. By those works a good seafood market will be built. What's more, with the diversification of seafood processing method, we have reason to believe that DNA barcode technology has great potential to contribute to identify authenticity of marine fishes.

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