Review:
Progress and potential of DNA barcoding for species identification of fish species

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Abstract. *Imtiaz A, Mohd Nor SA, Md. Naim D. 2017. Review: Progress and potential of DNA barcoding for species identification of fish species. Biodiversitas 18: 1394-1405.* DNA barcoding is a molecular technique to identify species by utilizing 600-800 base pairs genetic primer segments of mitochondrial gene cytochrome oxidase I. DNA barcoding has high potential to identify species into taxa, resolves ambiguousness in species identification, helps in accurate species identification, categorize species for conservation and also communize the information in the form of database system. The main challenge to this technique is regarding the use of barcoding information on 'biological species concept'. The extreme diversity of fish and their economic importance has made this group a major target of DNA barcoding. DNA barcoding can assign the status of known to unknown sample but it also has the ability to detect previously un-sampled species as distinct. In this review, we present an overview of DNA barcoding and introduce current advances and limitation of this promising technique.

Keywords: Cytochrome c oxidase I, barcode gap, DNA barcoding, fish diversity, NUMTS

INTRODUCTION

In modern society, the major environmental crisis is the loss of biodiversity and the decline is variable among different taxa. The issue has been highlighted at various levels. However, attempts to conserve biodiversity remained uncertain because the exact global baseline data is not available and the rate of loss is unknown, particularly in terms of background knowledge (Dirzo and Raven 2003). Although taxonomic history began since 250 years ago, there are still unknown numbers of species remain undiscovered and new species are waiting to be uncovered. Until now, there are only a few estimates about biodiversity have been published. Mora et al. (2011) reported in a study about globally unidentified biodiversity that 86% terrestrial species need their taxonomic identity whereas in marine biodiversity this number is extremely high (91% species). The standardized techniques and methods need to be developed for data acquisition of biodiversity in order to cope up the current biodiversity crisis. The importance of biodiversity in other fields of biology, i.e. ecology, evolutionary biology, molecular biology and biotechnology have been increased due to its crucial role in identifying specimens accurately (Simberloff et al. 2013). Being a most promising approach, 'DNA barcoding' is dominant among all identification techniques. Moreover, the advancement in biotechnology and taxonomic crisis helped in the creation of DNA barcoding technique.

DNA barcoding is helping taxonomists in terms of identification, discovery and genetically studying of specimens in order to achieve certain goals, i.e. knowledge about species diversity and level of variation among species. The challenge of DNA barcoding technique is not only trying to correctly identify species but also to make a standardized global reference library based on the identification of target specimen (Kerr et al. 2007). Several researchers recommended utilizing DNA barcoding techniques because it is cost effective, fast and authentic for species conservation (Li and Dao 2011). Using barcode data, stakeholders can collect conservation information, researchers can identify species without using the traditional method, taxonomists can resolve ambiguities in classification and policy makers can estimate the levels of conservation thus will depict proper management strategies for a species concerned (Francis et al. 2010).

The purpose of this review is to provide a critical overview of DNA barcoding and at what extent that this technique can realistically contribute to a practical identification of global biodiversity and biodiversity conservation efforts. We first address the scope of DNA barcoding as a standardized identification system, argue about the efficiency of DNA barcoding that depends on the use of integrative taxonomy, and assess the potential role of barcoding especially in the area of fisheries and most significantly in the conservation of known biodiversity.

OVERVIEW OF DNA BARCODING PROJECT

Short DNA sequences had been used for microbial species discrimination in early 80's (Kwok et al. 1984) and later had been tested on a variety of organisms (Chaves et

al. 2012). In 2003 it was the first time when Hebert et al. (2003) proposed that mitochondrial DNA cytochrome oxidase subunit I (COI) gene has the capability to serve as a barcode of all animals. DNA barcoding serves as a golden bullet for not only species identification, but also it delimits species boundaries. With this revolution in taxonomy, the Barcode of Life (BOL) project was launched to develop a globally accepted barcode system that is based on standard sequence of mitochondrial cytochrome oxidase I (COI) gene to identify eukaryotes and also enlist global biodiversity at a platform. Later in 2004, the Consortium for the Barcode of Life (CBOL) inaugurated this project. The aim of CBOL was to develop a standard protocol of DNA extraction, PCR and sequencing techniques, which aid the formation of a global DNA library. CBOL entered a new phase with the launch of iBOL (International Barcode of Life, http: //www.ibol.org) consisting of the collaboration of 26 countries that aimed to enlist eukaryotic biodiversity. In the initial few years, the iBOL concentrated mainly on a collection of maximum barcode sequences in a barcode library from all over the world to achieve the target of five million species barcodes. The iBOL also developed protocols, bioinformatics software with a huge data library. The purpose of these projects was to focus each animal group for making DNA barcode libraries. Some important project includes Fish-BOL (Fish barcode of life), Health BOL, Lepidoptera BOL, MarBOL (Marine Barcode of life), MBI (Mosquito Barcode of life), Mammalia Barcode of life campaign, Coral Reef Barcode of life, BeeBOL (Bee barcode of life) and CBOL fungal working group. Many countries have already collaborated to enlist their regional biodiversity and launched their own country's sub-projects i.e., Norway (NorBOL), Mexico (MexBOL), Japan (JBOLI) and Europe (ECBOL). Many regional based small projects on DNA barcoding are also under completion to identify unique biodiversity in small areas such as The Área de Conservación Guanacaste, a World Heritage Site in Costa Rica, which also combines barcoding project on insects (Hajibabaei et al. 2006; Janzen et al. 2009). Another example is the Moorea Bio Code Project, a comprehensive inventory of Moorea Island in French Polynesia that incorporates DNA barcoding (Check 2006). The DNA barcode from all projects was submitted to a database made by Canadian Center for DNA Barcoding (CCDB). BOLD is freely available to any researcher through the web page (http://www.boldsystems.og/index.php/Login/page?destination=MAS_Management_User Console).

DNA barcoding is the beginning of a revolution in species identification, in which can be accessed from the DNA libraries data record. BOLD provides information for basic identification by matching with the closely related individual, quality of sequences, as well as the quality of a core analysis by generating graphs of inter and intraspecific genetic distances, barcode gap, haplotype distributions and tree reconstructions (Ratnasingham and Hebert 2007).

AUTHENTICITY OF DNA BARCODING

DNA barcoding uses small regions of mitochondrial DNA that work as a barcode to amplify a gene. DNA sequencing and matching of unidentified sequence with the closely related individual in BOLD or NCBI libraries can be conducted within hours, so the response time depends greatly on available infrastructures, such as reference sequence or voucher specimen in NCBI and BOLD libraries. DNA barcoding is now well established; leads typically to accurate results and the DNA sequencing costs are low and constantly dropping. A major benefit of DNA-based analytical procedures is that they can be applied throughout the food supply chain, from whole specimens to trace samples (scales and fins), to highly processed and cooked fish products (Cawthorn et al. 2012). In addition, DNA analysis is use readily on not only fresh fish samples but also preserved historical material (bones and/or scales from museums).

The validity of the COI gene: For all or some species?

Mitochondrial Cytochrome c Oxidase subunit I (COI) is a mitochondrial DNA gene that codes a protein, which helps in cellular respiration. A 650 base pairs region of mitochondrial COI is considered as a universal barcode for animals because of its comparatively fast mutation rate over a relatively short time scale, has a high number of exons, high availability throughout cells and maternal inheritance (Steinke et al. 2009b). Due to these characteristic, the mitochondrial COI gene is a perfect universal barcode for species- level identification in sponges (Vargas et al. 2012), Platyhelminthes (Van Steenkiste et al. 2015), annelids (Achurra et al. 2013), molluscs (Layton et al. 2014), insects (Park et al. 2011), oysters (Zainal Abidin et al. 2016), echinoderms (Hemery et al. 2012), fish (Basheer et al. 2016), amphibians (Xia et al. 2012), reptiles (Hawlitschek et al. 2013), birds (Saitoh et al. 2015) and mammals (Alfonsi et al. 2013) (Table 1).

Although COI gene is considered as a universal barcode in animals, its potency is challenging in some protists, fungi, and plants (Schoch et al. 2012). In fungi, internal transcribed spacer (ITS) gene is more successful than COI gene to discriminate closely related taxa (Dentinger et al. 2010). Only a few published data are available on the successful of COI gene as a barcode in algae (Clarkston and Saunders 2010; Macaya and Zuccarello 2010). It has been reported that a universal plastid amplicon (UPA) works well as a barcode in algae instead of COI (Sherwood 2007). Furthermore, Saunder and Kucera (2010) reported that major advantage of UPA is its universality because this primer pair can reliably recover sequences from many groups of algae including green, red and brown marine macroalgae, diatoms, and also cyanobacteria (Sherwood and Presting 2007).

By the same token, the COI gene cannot be used as barcodes for some species of protists, which does not have mitochondrial DNA like anaerobic protists (Gómez 2014). Due to the ineffectiveness of the COI gene as a universal primer for protists, ribosomal subunits has been proposed to carry out that function well (Pawlowski et al. 2012). Moreover, low substitution rate, the high tendency of hybridization and polyploidy among closely related species of plants are the few factors that result in the failure of using mitochondrial COI gene as a universal primer in

plants (Kiewnick et al. 2014). Therefore, the combination of two alternative sequences in chloroplast DNA namely ribulose bisphosphate carboxylase and maturase K has been proposed as a standard barcode for the plant (Group et al. 2009).

DNA barcoding as an efficient tool for species identification

DNA barcoding is an advanced and efficient method for species identification. Various set of primers of mitochondrial gene for barcoding are available in published data. Researchers can also design primers by using different software, for example, PRiFi (Fredslund et al. 2005), BARCRAWL BARTAB (Frank 2009) and Primer-Blast (Ye et al. 2012). A good primer of a specific gene is basic key of success in PCR protocols. The translated sequences of unidentified samples after sequencing can be easily aligned by using various downloadable software or even online, such as ExPASy which is Sib information resource portal (Artimo et al. 2012) (www.expasy.org/genomics/sequence_alignment), MEGA version 6.06 (Tamura et al. 2013) (www.megasoftware.net)

and MAFFT version 7 (Katoh and Sandley 2013) (http://mafft.cbrc.jp/alignment/software).

The identification of a voucher specimen that is barcoded can also be confirmed by traditional taxonomy. However, traditional taxonomy fails to discriminate some species due to several conditions, for example, species that having various external body colorations especially when specimens are not fresh (Sriwattanarothai et al. 2010), museum preserved species (Hebert et al. 2013) and samples showing phenotypic plasticity (Weigand et al. 2011). Unlike the traditional taxonomy, DNA barcoding gives information on cryptic (Lim et al. 2016; Thiriet et al. 2016) and sibling species (Blanco-Bercial et al. 2014; Shao'e-Sun et al. 2016). This identification technique fully supports the improvement of animal classification as well as helps to sort out any ambiguity at the species level. However, DNA barcoding with the support of traditional taxonomy has the capability to identify species complexes within populations (Martínez-Aquino et al. 2009; Iwatsuki et al. 2015).

Table 1. Universality of mitochondrial COI gene as barcode in various groups of animals

Taxa name	No of taxa	% identified	Sampling location	Mitochondrial COI Primers	References
Fish	203 species	> 98%	Canada	FishF1, FishR1	Hebert et al. (2003)
Birds	643 species	> 98%	North America	BirdF1, BirdR1, FalcoFA, Vertebrate R1, BirdR2	Hebert et al. (2004)
Neo tropical bats	87 species	> 97%	Guyana	CVF1di, CVR1di, CVF1LFT1, CVR1LRt1, LepF1t1, LepR1t1	Clare et al. (2007)
Fish	94 species	>98%	Canada	16S, COI1, COI2, COI 3, M13	Ivanova et al. (2007)
Chondrichthyes	200 species	>98%	North Atlantic and Australasia	FishF1, FishR1, HCO2198	Ward et al. (2008)
Ornamental fish	201 species	>98%	Canada	FishF1, FishR1	Steinke et al. (2009a)
Birds	756 species	>98%	Iran	BirdF1, BirdR1, BirdR2, 16SAL, 16SBH	Aliabadian et al. (2009)
Fish	115 species	>98%	India	FishF1, FishR1	Lakra et al. (2011)
Marine fish	158 species	>98%	Japan	FishF1, FishR1	Zhang and Hanner (2011)
Freshwater fish	105 species	100%	Brazil	CFishF1t1, CFishR1t1, VF1LFt1, VR1LRt1	de Carvalho et al. (2011)
Oriental Black (Diptera)	41 species	>96%	Thailand	LCO1490, HCO2198	Pramual and Adler (2014)
Amphibians	82 species	100%	China	dgLCO, LCO1490, Chmf4, COI-C02, COI-C01, LEPF1, dgHCO, HCO2198, Chmr4, COI-C04, COI-C03, LEP-R1	Che et al. (2012)
Reptiles	>250 species	100%	Madagascar	RepCOIF, RepCOIR	Nagy et al. (2012)
Reptiles	27 species	100%	Comoros Archipelago	LCO, HCO	Hawlitschek et al. (2013)
Amphibians	251 species	>94%	Madagascar	dgLCO1490, dgHCO2198	Perl et al. (2014)
Hymenoptera	4118 species	>90%	Europe	As used by Ivanova et al. 2006 and deWaard et al. 2008	Schmidt et al. (2015)
Cyprinid	35 species	100%	China	FishF1, FishR1, FishF2, FishR2	Shen et al. (2016)

STANDARD LABORATORY PROCEDURE (SLP) OF DNA BARCODING

Barcoding is a worldwide project where hundreds of laboratories have been involved in enlisting the global biodiversity. Accordingly, new methodologies of DNA extraction and Polymerase Chain Reaction (PCR) (Handy et al. 2016) has been increasingly developed in parallel with the development of barcoding primers. To standardize the handling of DNA barcoding samples, BOLD has already made standard laboratory procedure (SLP) (Keele et al. 2013). In SLP (Fig 1) preferably fresh few millimeter-sized tissue is placed in 1.5mL Eppendorf tube. Fish Barcoding Method (Handy et al. 2016) which is also available online at BOLD website (http: //www.dnabarcoding101.org/files/using-dna-barcodes.pdf) does DNA extraction. Species-specific primer or universal DNA barcoding primers (based on animals group i.e. invertebrate, plant, mammal, etc.) was used for PCR amplification. Positive control band (a sample that is known to give a positive band for the chosen primers) is included in PCR as a positive control. A negative control (a mixture containing no DNA template) is also loaded in PCR mixture to ensure that the master mix does not contain contaminating DNA. The PCR amplification is analyzed by loading PCR-amplified products on an agarose gel electrophoresis with a standard marker (DNA of known base pair size), such as 1-kilo base pairs (kbp) DNA ladder

(i.e. First Base Laboratories, Sdn Bhd). If a bright band is present at the expected size, the PCR product will be purified. There were several purification kits are available in a market such as Mega quick spin of Intron Biotechnology Inc. After confirming a quality and quantity of the purified DNA, the PCR-amplified products were sent to a laboratory for sequencing.

Sequencing results can be analyzed by using National Centre of Biotechnology Information (NCBI) website (https: //www.ncbi.nlm.nih.gov) or BOLD website to determine the maximum identity of an organism with template sequences. BOLD compares unknown sequences with most closely resembling sequences available in the database. This database gives output in the form of resemblance percentage. The standard laboratory procedure is universally approved procedure but of course, for different organism modifications are required in order to obtain the best quality results. The DNA barcoding procedures totally fail if there is no barcode library to register barcoded samples (Ekrem et al. 2007). To develop barcode record, researchers required a species name, voucher specimen data (i.e. locality, date, repository of the specimen, photographs), a sequence data, PCR primers and trace files (sequencer's original outputs). It is estimated that sequencing can take place in less than 90 min at the cost of $2-5 per species, compared to several months of field work and a cost of at least $100 per specimen with morphological analyses (Stoeckle and Hebert 2008).
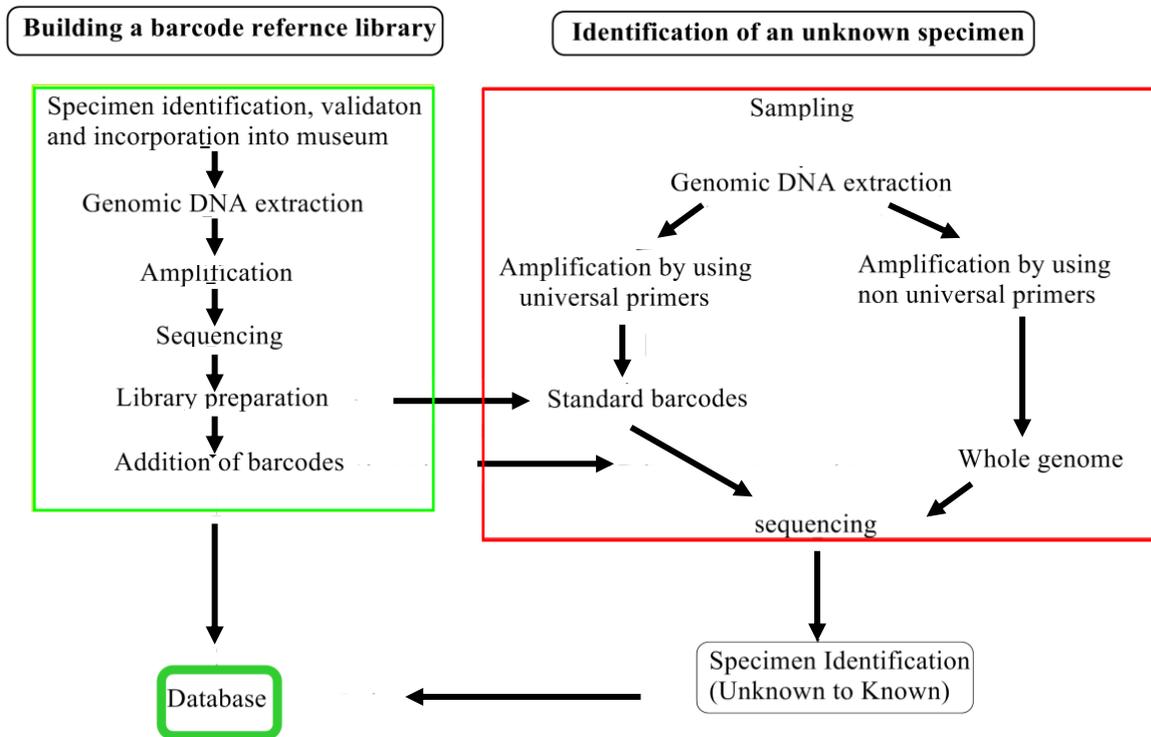

Figure 1. Overview of experimental procedures implemented for barcode libraries

USE OF LATEST TECHNIQUES IN DNA BARCODING

Real Time PCR

The real time PCR is the latest approach in DNA barcoding methods that is used to monitor the progress of PCR reaction in real time (Dias et al. 2016). At the same time, relatively small amount of PCR products cDNA or RNA can be quantified. One can start the PCR even with very small amount of nucleic acid and can quantify the end product quite accurately. Real time PCR technique is basically designed to maximize the amplification of barcode genes even in the presence of low-quality DNA (Doi et al. 2015). For this purpose a reporter molecule is used in PCR that binds to double stranded DNA that increases in number as PCR proceeds and produces fluorescence. The reporter molecules can be in the form of dye (i.e. SYBR Green) or sequence specific probes (Molecular Bacons or TaqMan Probes). The real time PCR is highly sensitive, easy to perform and cost effective, as no post PCR processing is needed that saves time and resources. Real time PCR is also called real time RT PCR that is used to convert DNA into RNA by an additional cycle of reverse transcription. Shokralla et al. (2015) used real time PCR based DNA barcode method as minibarcode system consisting127-314 base pair fragments of COI gene to identify all types of fish species used in fish products AND successfully identified 93.2% of samples upto species level. Feng et al. (2017) also recently identified four commercially important species of salmon and trout using real time PCR barcoding technique.

Microarrays

With the rise of next generation sequencing technologies and non-suitability of COI gene for design of oligonucleotide probes, it was believed that microarray technology is outdated. But developments in microarray technology (Microarray chips, mid-infrared chemical imaging) have refined the barcoding approach (Kochzius et al. 2010). Microarray chips are simple and can efficiently target specific group of species in a single assay. Mid-infrared chemical imaging (MICI) uses nanogold-silver augmented hybridized spots in microarrays and detects reflected substrate. These advanced methods of DNA barcoding have been applied in unknown species identification, point mutations, biodiversity assessment and food security (Trivedi et al. 2016). Handy et al. (2014) identified reported misidentification of seven catfish species in USA seafood market by using DNA microarrays with newly developed mid-infrared imaging detection method.

APPLICATIONS OF DNA BARCODING

With pits and falls of thirteen-year history, DNA barcoding project successfully not only identified a huge number of taxon but also addressed population and conservation issues in biodiversity. The unique features of DNA barcoding such as species identification, cryptic species and partial phylogenetic assessment is benefits to whole animal kingdom especially both basic and applied fisheries. Species identification methods using molecular data can help elucidate the relationships of morphologically variable individuals of the same species and individual's similarity in different developmental stages. In addition, in the features of typical non-barcode markers, the advantages of DNA barcoding include primer universality, the availability of information on a wide range of taxonomic groups, and its association with taxonomy.

DNA barcoding and species identification

Genetic identification of biodiversity is the necessity of time due to the presence of phenotypic similarities among neighboring species. Some organisms especially fish shows phenotypic plasticity with a change in its environment (Hutchings et al. 2007). DNA barcoding has the capability to identify not only in adult organisms but also at their early developmental stages. For example, Ko et al. (2013) used DNA barcoding technique to successfully identified 100 specimens of fish larvae with a success rate of > 65 percent at the species level. However, with an increase in taxonomic level the identity rate also increased up to > 85 Percent. Likewise, Naim et al. (2012) used COI gene to successfully identified approximately 60 individuals of mud crab into four species. Most recently, COI gene has been used to identify Ivory shell (Chiu et al. 2015), Atlantic goliath groupers (Damasceno et al. 2016) and yellowfin tuna (Higashi et al. 2016).

DNA barcoding and species delimiting boundaries

A great challenge associated with barcodes is to define species boundaries concepts. The literature on DNA barcoding studies suggests a different threshold approach in different taxa, as 2% difference in the DNA barcode of mammals implies that the samples are from different species (Cai et al. 2015). This value was considered as a divergence threshold and a standard cutoff value for species delimitation in fishes (Mabragaña et al. 2011). However, Ward et al. (2008) mentioned that 3% and 1% is the ideal threshold value for fish and birds respectively. These limitation values are based on the distribution of intra and inter-specific K2P genetic distance values in the approximately 172,000 species that have been barcoded (http: //www.boldsystems.org). It is important to set species delineation boundaries because, in homogenous populations, species identification is problematic. DNA barcoding can interpret a unique taxon as a separate species by recording intraspecific divergence which should be lower than interspecific threshold values.

DNA barcoding as a forensic tool

DNA samples can be used to check drug authenticity in forensic sciences (Carvalho et al. 2015). Forensically Informative Nucleotide Sequencing (FINS) was one of the earliest species diagnostic techniques for fish (Bartlett and Davidson 1992). In FINS the identity of an unidentified specimen is confirmed by comparing amplified sequence of

a mitochondrial COI gene fragment taken from the unknown sample to reference sequences in database library of known species. Such interrogative sequencing methodology used different types of genetic markers to identify a variety of meats (Pappalardo and Ferrito 2015). Barcoding provides a fast and reliable method to identify exotic species in a branch of biosecurity: one such example is the identification of the invasive North American bullfrog (*Rana catesbeiana*) found in water samples from ponds in France by using short mitochondrial fragments (Ficetola et al. 2008). Likewise, the Brazilian Forensic Government Programme reported that there is approximately 24 percent of seafood products has been mislabeled (Carvalho et al. 2015). Under those circumstances, biomedical research highlighted new aspects of DNA barcoding technique such as next-generation DNA sequencing, shotgun sequencing, transcriptomes and interactomes (Shendure and Ji 2008). All these techniques help in saving time and speeding up the process of collecting data that previously required a very extended period. DNA barcoding is not too difficult to handle as nonspecialized persons can participate in barcoding campaign by helping in samples collection from various geographical locations and uploading the pictures and other information on database system such as BOLD (http: //www.barcodinglife.org) for further taxonomic interpretation. This information will be used for forensic or biosecurity purposes as described above, henceforth, will encourage local participation in these matters. Such local involvement in barcoding project can lessen the field work of morphological taxonomists.

DNA barcoding and cryptic species

Fishes show significantly more divergence for COI gene at each hierarchical taxonomic level (Ward 2009). Barcodes have also complimented morphological studies in cases where taxa were easily misidentified due to the existence of cryptic species (Ward et al. 2008). If a sample from an unidentified specimen shows zero divergence from a previously identified specimen, or differs from it by only one or two base pairs, the probability is that it is the same species due to high similarity with the already identified specimen (>95%). If on the other hand, the unknown specimen is more than 2 percent divergent from the known specimen, it is very likely (probability greater than 95%) to be a different species. Khedkar et al. (2014) reported the possibility of sibling species of *Labeo dyocheilus* by analyzing inter and intraspecific divergence values. Hubert et al. (2012) also revealed cryptic fish diversity in coral reefs of Indo-Malay Philippines Archipelago. Hamaguchi et al. (2017) reported about cryptic diversity in flat oysters distributed in Japan.

DNA barcoding and population diversity

Mitochondrial DNA markers are not diploid because they are inherited from a single parent (maternal inheritance) so they are equally good for targeting population level studies (Avise 2004). Although the cytochrome oxidase I (COI) gene information from DNA barcoding is not sufficient to investigate population-level questions (Bazin et al. 2006), yet still it can be used to partially interpret the distribution of genomic diversity within taxa. Barcodes can give the status of species to an unknown specimen because they also consist information of genetic models (coalescent-model) based on population genetics (Abdo and Golding 2007). The COI gene provides information about genetic variation within a population of a single species and this information can help to deduce the phenomenon of migration as well as genetic drift in fish populations (Mohammad Geba et al. 2016). To demonstrate, Boonkusol et al. (2016) used mitochondrial COI gene sequences to access the genetic variability in snakehead fish of Thailand and deduced that genetic variation in the central river basin is due to fish dispersal by the flood. In the same fashion, the use of a combination of mitochondrial (COI) and nuclear genes (recombination activating gene-I (Rag I) and from nuclear alpha tropomyosin 'intron V') can fully address the question of population structure as done by Eytan and Helburg (2010) in Caribbean reef fish.

DNA barcoding and phylogenetic reconstruction

In barcoding, the information from an assemblage of species is in the form of genetic sequences, which is uploaded in a barcode library. However, the gene lengths from barcoding data are not sufficient to construct a deeper phylogenetic tree in resolving evolutionary relationship of organisms. Although the barcoding sequences have been used to construct Neighbour-Joining (NJ) tree, this barcode-based tree cannot be alternates of the phylogenetic tree. We strongly emphasize on the statement that DNA barcoding data can provide partial information about the phylogeny of species and can draw an outline for phylogeny that should be deeply analyzed by nuclear genes data. Faith and Williams (2005) argued that the most significant contribution of DNA barcoding to conservation efforts is its role in improving and speeding up phylogenetic diversity assessments. Lakra et al. (2011) successfully identified 115 marine fish species that clustered into 79 genera when NJ tree was constructed to see the phylogenetic relationship among collected samples. Ardura et al. (2010) identified 21 Amazonian species, with 100% bootstrap support was obtained at a specific level in the reconstructed NJ phylogenetic tree.

DNA barcoding and food safety

The global fish market is facing challenges of seafood authenticity and food safety, although identification keys and molecular approaches (DNA barcoding) have limitations in the identification of species. The market frauds regarding substitution of high priced fish with low-priced fish in fish products is very common. Wong and Hanner (2008) used barcoding technique in North American seafood to check barcoding aspects of traceability and their specimen data matched of >97 percent similarity with barcode library sequences. Twenty-five percent of the samples were potentially mislabeled, demonstrating that DNA barcodes are a powerful tool for identification of seafood to the species level. Likewise, Smith et al. (2008) detected ten families from smoked fish

products were mislabeled. Furthermore, Yan et al. (2016) identified *Alepes apercna*, which had surprisingly been labeled as *Rastrelliger brachysoma* by using COI gene in imported seafood products. Mislabeling in the fish market for economic benefits has become challenging. For this reason, the Food and Drug Administration Department in the United States has adopted DNA barcoding technique to reduce market frauds (Naaum and Hanner 2015).

DNA barcoding and biodiversity conservation

One of the main goals of DNA barcoding is a utilization of genetic data to boost conservation strategies, and indeed molecular tools were used in conservation biology at different levels of analysis (DeSalle and Amato 2004). Rapid biodiversity assessment is a research priority as species extinction/extirpation rate is high, and barcoding is contributing to biodiversity conservation in facilitating biodiversity assessment cheaply and quickly where financial resources are limited (Krishnamurthy and Francis 2012). The majority of described biodiversity is in developing countries, where resources for comprehensive biodiversity assessments are lacking. For conservation biology, genetic tools can aid in research as diverse as fine-scale management of coral reefs (Neigel et al. 2007) and regional management of fisheries (Swartz et al. 2008). DNA barcoding contributes to conservation policy in two important ways; 1) by speeding up local biodiversity assessments to prioritize conservation areas or evaluate the success of conservation actions, and 2) by providing information about evolutionary histories and phylogenetic diversity (Rubinoff 2006). Hubert et al. (2012) barcoded 2,276 specimens into 668 coral reef fish species and provided data to biodiversity conservationists for making conservation strategies as his findings remarkably suggested most of the coral reef diversity is accumulated in Indo-Malayan Archipelago. There have been several notable conservation successes using barcoding. For example, the rapid classification of 99% of 210 chondrichthyan species from 36 families from Australia (Ward et al. 2008) and the identification of larval and juvenile organisms for which morphological data is non-existent (Neigel et al. 2007), all of which have accelerated conservation priorities and policy response.

Barcoding prosecutes illegal trading of endangered species, even after organisms have been processed. For example, the illegal shark-fin trade is a significant threat to biodiversity in many coastal African countries. The barcoding can identify different varieties of shark, including those that were critically endangered and protected under international legislation (Swartz et al. 2008). Therefore, DNA barcoding in conjunction with a reference database potentially plays a key role in environmental law enforcement and conservation of species that are already studied (Williams et al. 2013).

LIMITATIONS IN DNA BARCODING

DNA barcoding has attracted significant attention in systematic studies. Yet, the project is controversial because

many systematists feel that the conceptual foundation of DNA barcoding remains weak (Waterton et al. 2013). Here are few limitations of DNA barcoding that have been addressed in several types of research.

Barcode gap

The "barcode gap" refers to the differences between mean intra and interspecific genetic sequence variability of COI sequences within the same genus of animals (Meyer and Paulay 2005) (Fig 2). Large barcoding gaps are routinely used to predict DNA barcoding success for the taxon under study (Clare et al. 2007; Freshwater et al. 2010; Keskln and Atar 2013). The overlapping of genetic divergence values within and between species recorded in histograms shows that there is no gap between inter and intraspecific divergences in samples species (Mayer and Paulay 2005). In this regard, the inter-generic variation and overlapping of differences within and between species distances are ruled (Virgilio et al. 2010). We support the statement of Collins and Cruickshan (2013) that barcoding gaps are currently incorrectly computed and that the values reported in the barcoding literature are misleading. Various authors have pointed out that the use of smallest interspecific distances would be more appropriate (Cognato 2006; Roe and Sperling 2007).

Proper interspecific distances measurements are important for distance-based identification techniques. DNA barcodes with unusually large distances to conspecific sequences are often used to predict the possible existence of cryptic species (Clare et al. 2007). One cannot address the new species by considering barcoding gap only but the cryptic species can be predicted by using smallest intraspecific value (Will and Rubinoff 2004).
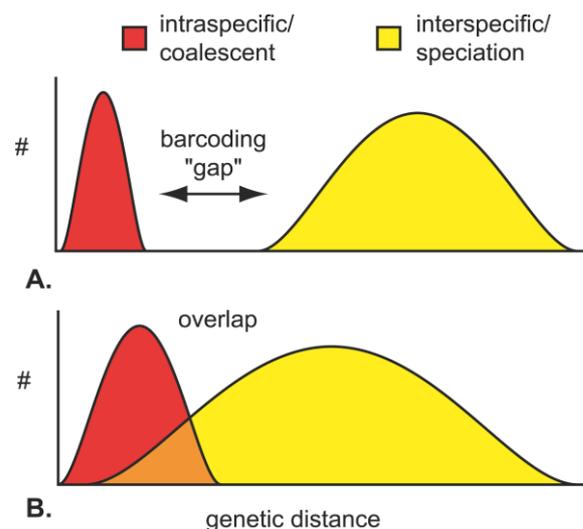

Figure 2. Barcode gap (Mayer and Paulay 2005). If there is no overlap between intraspecific and interspecific values as in A, it means the unknown sample has low threshed hold value all species are in their discrete distribution boundaries but if overlap is great as in B it means there are closely related taxa present and making this method problematic

Barcoding and phylogeny misinterpretation

Neighbor-Joining tree formation is easy to compute and is a part of standardized analytical procedure as used in various barcoding studies because NJ trees are fully resolved tree that is based on genetic distance matrix of similarity in sequences to create a clustering of taxon in the form of phenogram (Casiraghi et al. 2010). However, several authors have documented that phylogenetic approach to DNA barcoding is inappropriate (Goldstein and De Salle 2011). The NJ tree does not use cladistics sense as an identification system. NJ tree based identification can interpret wrong results about the identification of paraphyletic speciation and incomplete hierarchy (Lowenstein et al. 2010). Few species concepts require reciprocal monophyletic origin (Meier 2008) (Fig 3), and in any case, monophyly of mtDNA lineages can be an unrealistic scenario in many closely related groups (Zhang et al. 2012). It is important to note that a quantification of monophyly still remains a useful description of the data, and should be used but in conjunction with other methods. Problems mostly occur when NJ trees are the only analytical method presented (Little and Stevenson 2007). However, for the purpose of graphically summarizing the data, NJ trees can be considered appropriate.

For species identification, we suggest the mitochondrial plus nuclear gene combination with character based diagnostic methods should be used. Bayesian coalescent methods also promise statistical advantages, but may be too computationally inefficient in their current incarnations (Zhang et al. 2012).

NUMTS (Nuclear Mitochondrial Pseudo-genes)

Nuclear Mitochondrial Pseudo-genes (NUMTS) are non-active non-coding parts of mitochondrial DNA present in the nucleus of most eukaryotic organisms and can be easily amplified along with mitochondrial DNA even by using conserved universal primers (Song et al. 2008). The presence of co-amplification of NUMTS along with mitochondrial genes is problematic because DNA barcoding analysis can incorrectly overestimate the number of unique species. DNA barcoding is rapid and inexpensive technique of tagging species. However, the presence of NUMTS in COI amplified sequences can make DNA barcoding ambiguous (Buhay 2009). There are several techniques that can be used to control NUMTS such as (i) the use of muscle tissue as a source of DNA due to the presence of high concentration of mitochondrial DNA in those areas and (ii) amplification of longer fragments (750 bp) because barcode sequence is longer than NUMTS sequence (Pereira and Baker 2004). NUMTS is not only a major limitation of DNA barcoding, but also has significant taxonomic implications. Moulton et al. (2010) tried to identify Orthoptera species by using COI gene but the presence of NUMTS led to overestimation and misidentification of species. With attention to this matter, Song et al. (2008) suggested RT-PCR, long PCR, use of markers other than COI and choice of the mitochondrial rich region for extraction of DNA to avoid NUMTS. Similarly, BOLD also provides an opportunity to detect and delete NUMTS by analyzing sequences with Markov model during analysis.
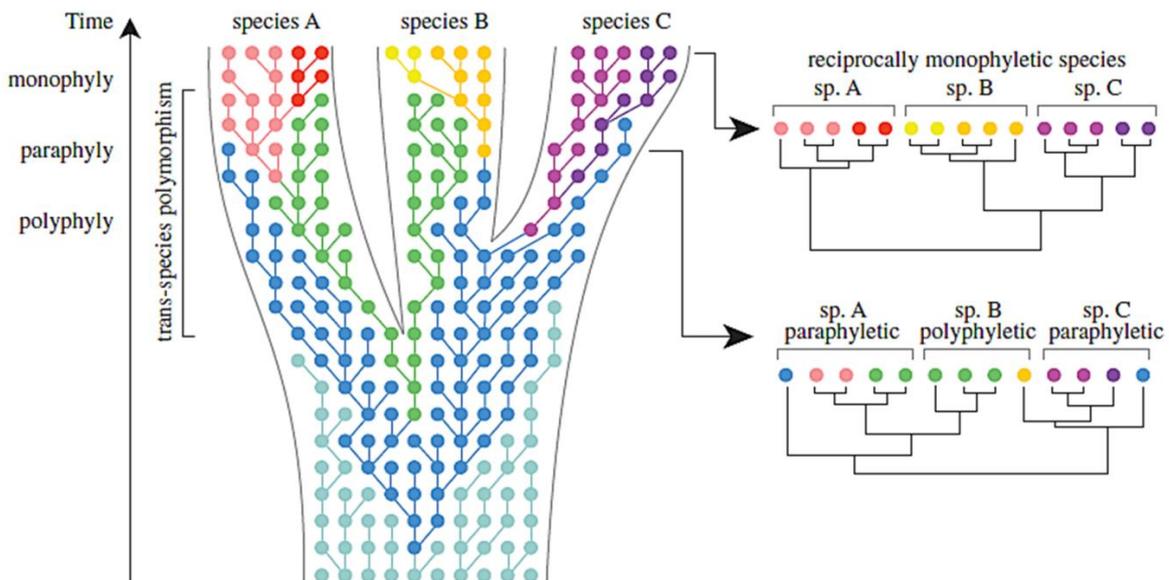

Figure 3. Differences among various types of phylogeny (Leliaert et al. 2014)

DISCUSSION

Although DNA barcoding is useful in classifying taxa, it is perhaps premature to suggest that DNA barcoding can offer a replacement for assessment of biodiversity and traditional taxonomy. Instead, we consider barcoding as an additional tool that links genetic data into existing studies. DNA barcodes have been particularly helpful in identifying cryptic diversity within species, especially when it is used in conjunction with other taxonomic methods such as morphology and ecology. DNA barcoding is worth capable of facilitating an integrative approach to species identification but with the involvement of traditional taxonomic studies. The role of DNA barcodes is positive to help in speeding up biodiversity assessments, however, integrative taxonomic methods are still required to address the 'taxonomic impediment' (Wilson 2000). There are three reasons for genetic barcoding should not be used alone for addressing the status of new species to a taxon as formerly described in this review; firstly, no standardized universal distance threshold has been identified for species delimitation, and thresholds vary between different taxa (Yassin et al. 2010). Secondly, discrimination of a closely related species is not an easy task by using a species definition based on only genetic distance data (Meier et al. 2006). Thirdly, for species identification, there is no universal identification technique, as species discovery requires a contribution from other branches of biology (Taylor and Harris 2012). DNA barcoding has the capability to discriminate species but it is quite unjustified to comment that DNA barcoding can replace traditional taxonomy. Additionally, barcoding research has limited success when identifying new species in unstudied groups (Meyer and Paulay 2005). For example, barcoding fails to identify recently isolated populations and reproductively isolated lineages of fish species that have undergone recent speciation (Zou et al. 2016). Even so, DNA barcoding has proved useful in enlisting global biodiversity by enhancing data availability for existing research problems (Rubinoff et al. 2006).

In summary, being taxonomist, we encourage DNA barcoding with a combination of traditional identification methods because it is an objective, quick, easy and replicable method to identify species. DNA barcoding has raised the profile of taxonomic research and contributed a valuable data for conservation of biodiversity. However, decisions for the conservation of a species concerned shall not be based only on DNA barcoding studies. The evidence-based approach to fundamental assessment about species, such as population size, species interaction with the environment and the possible effect of the ecosystem on species should be considered before jumped into conclusion. On the whole, DNA barcoding gives opportunity in various fields of biodiversity in a positive way and we think it will be regrettable to compromise on the efficiency of barcoding due to its presumed limitations.

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