REVIEW

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DNA BARCODING: FOUNDATIONS AND APPLICATIONS FOR SOUTHEAST ASIAN FRESHWATER FISHES

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ABSTRACT

Identifying and delineating species are the primary tasks of taxonomy. Owing to the decreasing interest of the nations for taxonomy and the inventory of living beings, funds have been drastically decreasing during the last two decades for taxonomic studies. As a consequence, the worldwide pool of taxonomists has dramatically decreased. DNA barcoding, as an automated tool for species delineation and identification, proved to rejuvenate the field of taxonomy and open new perspectives in ecology and conservation. In the present review, we will discuss how DNA barcoding established as a new paradigm in taxonomy and how DNA barcoding has been recently integrated in taxonomic studies. We will further detail the potential applications for species identifications and discuss how DNA barcoding may positively impact the inventory and conservation of living beings, particularly in biodiversity hotspots. We emphasise the benefit of DNA barcoding for the conservation of Southeast Asian freshwater fishes.

Key words: biodiversity hotspots, DNA barcoding, integrative taxonomy, species delineation, species identification

INTRODUCTION

After three decades of inventorying living beings, Earth's biodiversity, which consist of not less than 10 millions of described species to date, is still poorly known and includes many species that carry potential economic and societal applications that are still to be revealed (Vernooy *et al.* 2010). Despite the importance of identifying species for either scientific or societal purposes, the interest of the nations in taxonomy and in pursuing the inventory of earth living beings has decreased since its earlier development during 18th century with Carl Linnaeus (Mallet & Willmott 2003). During the second conference of the parties of the Convention on Biological Diversity (CBD) held in Jakarta in 1995, the participant countries have explicitly formulated through the concept of taxonomic impediment, which epitomises the major concern raised by the worldwide community of taxonomists since the 90's about the increasing disinterest from governments and funding agencies for taxonomy. Unfortunately, several global initiatives such as the Global Taxonomic Initiative (GTI) launched in the context of the CBD early in 2002 failed to embrace a massive adhesion and to help reach the CBD goal to slow-down the pace of species loss by 2010 (www.cbd.int/cop). Several challenges prevented the emergence of a global project, which include the settlement of a universal information system in taxonomy and the digitisation of the collections in national museums, both calling for a more massive investment in taxonomy as a research priority by the nations (De-Carvalho *et al*. 2005, Agnarsson & Kuntner 2007, Godfray 2007). Another

challenge is caused by the lack of consensus on the morphological characters to be used by the community of taxonomists, a limit that was to be overcome by the use of DNA sequences due to the universality of the genetic code (Tautz *et al*. 2002, Blaxter 2003, Tautz *et al.* 2003, Hebert & Gregory 2005, Godfray 2006 & 2007). Moreover, the ease of access to sequencing facilities was expected by a large community to counterbalance the impact of the taxonomic impediment in conservation and basic biodiversity sciences (Vernooy *et al*. 2010).

Hebert and colleagues (2003) proposed to develop a new paradigm for species identification based on universal molecular markers (*i.e.* DNA barcoding) with enriched metadata to allow the sustainability and reproducibility of species identification based on DNA sequences. This approach opened new perspectives in taxonomy and conservation by enabling the development of automated molecular identifications that impacted fields as diversified as functional ecology (Smith *et al*. 2007), taxonomy (Hebert & Gregory 2005, Miller 2007, Smith *et al*. 2008), biogeography (Fouquet *et al*. 2007, Kerr *et al*. 2007, Hubert *et al*. 2012), conservation (Forest *et al*. 2007), wildlife forensics (Armstrong & Ball 2005, Wong & Hanner 2008, Holmes *et al*. 2009, Ardura *et al*. 2010, Floyd *et al*. 2010) and biodiversity socio-economics (Stribling 2006, Vernooy *et al*. 2010).

In front of the massive extinction rates at play in nature nowadays, identifying species is an important application of taxonomy and DNA-based taxonomy opened new perspectives (Ubaidillah & Sutrisno 2009, Sutrisno *et al*. 2013). The objectives of the present review are: (1) to present how DNA barcoding has emerged as a new paradigm for species identification, (2) to discuss how DNA barcoding complement taxonomy, (3) to discuss the potential benefits of using DNA barcoding for the inventory and conservation of Southeast Asia freshwater fishes.

Why DNA barcoding emerged as a new perspective in taxonomy?

Godfray (2002) stated that using morphological characters for species delineation do not reveal all the diversity of the world biodiversity because it is time-consuming, while funds and specialist taxonomists are few nowadays. Thus, screening phenotypes is often of limited use and molecular methods such as DNA barcoding may open new perspectives (Blaxter 2003). Quoting Mallet and Willmott (2003:59): “Biodiversity is in crisis, and taxonomy is now in vogue again…”. Then, quoting Shimura (2010) *in* Vernooy *et al.* (2010:1): “…The science of taxonomy is key to understanding and monitoring biodiversity…”. The last two quotes highlights that conservation biology is a discipline tightly related to the timeframe of taxonomic studies based on morphology (Wilson 2000, Fisher & Smith 2008 *in* Smith *et al*. 2008) and both taxonomist and ecologist are responsible of the identification of priority species for conservation plans (Smith *et al*. 2008). Radulovici *et al.* (2010) stated that species identification could be conducted quickly, accurately, and at low cost through molecular analysis.

The characterisation and documentation of biodiversity using phenotypes is currently bridled by several limits inherent to morphological characters. First, the morphological variation of a species often overlaps that of its sister taxa in nature (*i.e.* morphological characters are similar for some individuals belonging to different species), which can lead to incorrect identifications or species delineations if based on morphological characters only (Pfenninger *et al.* 2006). Second, the diagnostic morphological characters used for species identification are often defined on adults and may be of limited used for the identification of some ontogenetic stages (*e.g.* larval stages) or particular samples (*e.g.* fish fillet). By contrast, DNA-based identifications may be applied whatever the life stages under scrutiny or available biological materials for identification (Caterino & Tishechkin 2006, Pegg *et al.* 2006). Third, new species have been frequently detected using DNA-based methods, sometimes in the absence of diagnostic morphological characters to further discriminate them (*i.e.* cryptic species) (Hebert *et al*. 2004a, Witt *et al*. 2006, Smith *et al.* 2007).

Tautz *et al.* (2002) proposed that DNA might offer new perspectives in taxonomy (Hebert *et al.* 2003, Hebert *et al.* 2004b, Hebert & Gregory 2005). DNA barcoding is a fast, easy, relatively inexpensive approach that provides alternative solutions for species difficult to identify because of their morphological similarity (*i.e.* cryptic species). Cryptic species are actually separated by reproductive isolation or alternative geographic distribution but they lack morphological differences or exhibit conflicting individual assignment to the species level if based on morphological characters. Thus, DNA barcoding may help clarify the biological status of morphological variations within and among closely related species, and guide the detection of new diagnostic morphological characters.

DNA barcoding and taxonomy: how they complement each other

Species concept and the need for integrative taxonomy

The main purpose of taxonomy is to delineate species, to explore their boundaries and to develop the knowledge to further assign specimens to nominal species (Mallet & Willmott 2003, Seberg *et al.* 2003, Godfray 2007). Identifying and delineating species is a very important activity that has many applications as, for instance, the control of human pathogens, or identifying suitable biological control agent for agricultural pests (Godfray & Knapp 2004, Agnarsson & Kuntner 2007, Godfray 2007). Therefore, "Ideally, identification should be easy and efficient because different users, such as pharmacologists, physiologists, conservation biologists and ecologists, need to identify species…. (Dayrat 2005:408)".

From an historical perspective, several operational species concept have been proposed and applied:

1. Morphospecies concept (MSC)

According to this concept, the delineation of species is based on the morphological discrimination of specimens that lead to the recognition of morphospecies (Cain 1954 *in* Dayrat 2005). The variability of morphological characters likely affects this concept, but many species have been described based on this concept (Dayrat 2005).

2. Biological species concept (BSC)

Biological species includes interbreeding individuals that produce fertile offsprings. Reproductive isolation can be either based on: (i) isolation, that is the intrinsic reproductive isolation - the absence of interbreeding between heterospecific organisms based on intrinsic properties -, as opposed to extrinsic [geographic] barriers; or (ii) recognition, that is the shared specific mate recognition or the fertilisation system mechanisms by which conspecific organisms, or their gametes, recognise one another for mating and fertilisation (De-Queiroz 2007).

3. Phylogenetic species concept (PSC)

PSC is based on a phylogenetic property of species that is the monophyly (all individuals are derived from a common ancestor that shared derived character states). When considering genetic characteristics, these refer to all alleles of a given gene that are descended from a common ancestral allele, yet not being shared with those of other species (De-Queiroz 2007).

Integrative taxonomy or integrated concepts required unifying the properties of each concept for species delineation in order to cope with the diversity of speciation mechanisms and species properties in nature (De-Queiroz 2007). It is now acknowledged that the criteria for species recognition derived from the species concepts are not fundamental properties of the species but clues to be invoked for justifying hypotheses of species delineation (De-Queiroz 2007). Considering the diversity of the mechanisms leading to the emergence of new species, species can exhibit multiple combinations of the criteria defined by the MSC, BSC and PSC. In this context, combining evidences from different sources of biological characters may be expected to provide hypotheses of species delineation that are more robust than those based on a single source of evidence (Pante *et al.* 2015). This statement calls for an integrated assessment of independent evidence based on genomes, phenotypes and ecology (*e.g*. Smith *et al*. 2008). It has been recently proposed that the integration of DNA barcoding as a preliminary step during biodiversity inventories may help speed up the pace of species discovery by avoiding the time consuming sorting of specimens based on their morphological attributes. This approach, recently named as ‘turbo-taxonomy’, proved to open new perspective in taxonomy by streamlining the description of large number of species through the combination of DNA barcodes, concise morphological descriptions and high-resolution digital images (Butcher *et al.* 2012, Riedel *et al.* 2013).

Concepts behind DNA barcoding (repeatability and accessibility of the data)

DNA barcoding is a system designed to provide accurate, fast, and automatable species identification by using short and standardised gene regions as internal species tag (Hebert & Gregory 2005). DNA barcoding is an accessible method for anyone who wants to use molecular data for species identification either in basic or applied research related to health or medical purposes and even food security (Hebert & Gregory 2005). DNA barcodes data are easily accessible through BOLD (Ratnasingham & Hebert 2007), even for researcher focusing on zoogeography or phylogenetic reconstructions.

DNA barcode records are expected to follows the standards in BOLD (Fig. 1), as established by Ratnasingham and Hebert (2007) and Hubert *et al.* (2008), namely:

1. Name of species
2. Voucher data (catalog number and repository institution)
3. Collection data (collector, collection date and location with GPS coordinates)
4. Identifier (people who identified of specimen)
5. The order of sequence COI at least 500 bp
6. PCR primers used to generate amplicons
7. Traces file (raw electropherograms from sequencing analyses)

The data elements of a record in BOLD are separated into: (i) a specimen page that includes the information about the voucher specimen such as identifier, taxonomy, GPS data and photograph, catalog repository and museum catalog number, (ii) a sequence page that includes the DNA barcode sequence, PCR primers and trace files (Hubert *et al*. 2008).

Quoting Smith *et al*. (2008:12364) "…the barcoded specimens are vouchered in permanent collections for repeated iterative study and are linked through publicly accessible databases of host records and associated metadata…". Then, quoting Hebert and Gregory (2005:852) "…DNA barcoding allows a day to be envisioned when every curious mind, from professional biologists to schoolchildren, will have easy access to the names and biological attributes of any species on the planet". These two quotes highlight the repeatability and accessibility of DNA barcodes data in BOLD for universal applications involving species identification.

Benefits of DNA barcoding based on a mitochondrial gene

DNA barcoding rely on the COI mitochondrial gene that presents several advantages: (1) the mitochondrial genome is present in a large number of copies yielding substantial amounts of genomic DNA from a variety of extraction methods (Gemeinholzer *et al*. 2010, Weigt *et al*. 2012); (2) the high mutation rate and small effective population size make it often an informative genome about evolutionary patterns and processes; (3) multiple primers and new amplification techniques based on primers cocktail have been developed for the ease of amplification across metazoan lineages (*e.g.* Ivanova *et al*. 2007).

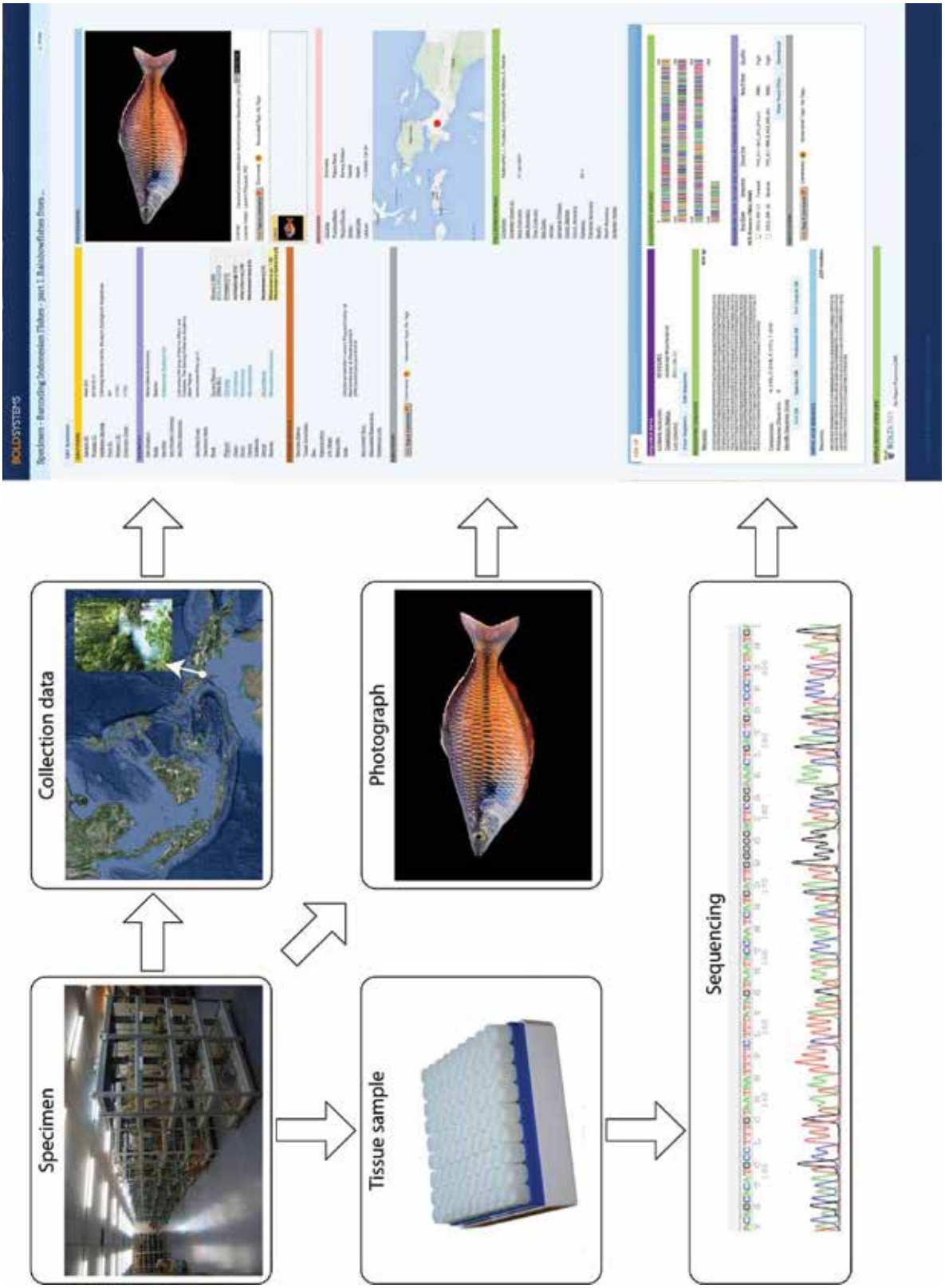

Figure 1. Structure of a specimen record in BOLD including details about voucher specimen, tissue sample in a bio-repository, collection data, specimen photograph and DNA barcode including primary data (*e.g.* trace files).

So far, results of DNA barcoding are mostly above 90% of accuracy for species identification that is, DNA barcodes have been estimated to match morphospecies in 90% of the species analysed, the 10% of failure resulting from the retention of ancestral polymorphism or introgressive hybridisation (Hubert *et al*. 2008, April *et al*. 2011, Pereira *et al*. 2013). This result highlights that DNA barcoding is a powerful tool for species identification (Pereira *et al*. 2013). For some vertebrate taxa, alternative mitochondrial markers have been frequently used in the past, such as 12S rRNA and 16S, thus successfully enabling species delineation in several groups such as amphibians (Matsui *et al*. 2012, Nishikawa *et al*. 2012). Although, quoting Smith *et al.* (2008:238) "In our preliminary sampling of Holarctic amphibians — we found that a single mitochondrial gene DNA barcode correctly identified 94% of species. Amplicons of the 5′ CO1 region are straightforward to generate using standard primers designed for insects and vertebrates, we experienced no more difficulty with amphibian samples than with insects (Hebert *et al*. 2004a, Smith *et al.* 2005), fishes (Ward *et al*. 2005), birds (Hebert *et al.* 2004b), or bats (Clare *et al*. 2007)". This result highlights that DNA barcoding may be successfully applied for the whole vertebrate fauna.

For a barcoding approach to species identification to succeed, however, within-species DNA sequences need to be more similar to one another than to sequences in different species. Several processes, such as pseudogenes ontogenesis, introgressive hybridisation, and retention of ancestral polymorphism pose potential difficulties in capturing species boundaries using mtDNA sequences (Funk & Omland 2003, Pamilo & Nei 1988, Zhang & Hewitt 1996). The detection of mixed genealogy between closely related species has been previously estimated to occur in nearly 20 percent of the cases in the wild (Funk & Omland 2003). Recent barcoding studies emphasised that this percentage can vary widely among phyla, yet species assignment failures typically do not exceed 5 to 10 percent in a large array of organisms (April *et al*. 2011, Hubert *et al*. 2012, Kerr *et al*. 2007). Nevertheless, distinguishing between introgressive hybridisation and the retention of ancestral polymorphism call for an integrative assessment of independent sources of evidence including nuclear DNA and phenotypes due to the maternal inherence of the mitochondrial genome (Funk & Omland 2003).

Applications of DNA barcoding in fish biology and conservation in Southeast Asia

Taxonomy and species delineation: cryptic diversity

During the last years, several DNA-based studies highlighted the limits of morphological characters to accurately delineate and uncovered new species as a substantial amount of cryptic diversity has been frequently described and fishes are no exception. Many examples have been described in Indo-Pacific coral reef fishes (Hubert *et al*. 2012), Indo-Malay Carangidae (Teleosteii: Perciformes) (Jaafar *et al*. 2012), flathead fishes (Scorpaeniformes: Platycephalidae) (Puckridge

et al. 2013) and gobies from the genus *Trimma* (Percomorpha, Gobiiformes) (Winterbottom *et al.* 2014). These studies have enlarged the growing body of evidence suggesting that cryptic diversity may be a much common trend than previously considered as cases of cryptic diversity have been detected in neotropical butterflies (Hebert *et al*. 2004a), ants of Madagascar (Smith *et al.* 2005), parasitoid flies and wasps from Central America (Smith *et al*. 2007, Smith *et al*. 2008). Worth mentioning, all these studies were based on the use of DNA barcoding as a first step during biodiversity inventories.

Identification of early life stages

The most prominent benefit of DNA barcoding for species identification lies in the ability to identify early stages that cannot be done by using morphological characters. DNA barcoding proved to be effective for identifying species in juvenile and larvae of *Lutjanus cyanopterus* in Caribbean beach (Victor *et al.* 2009), and mantis shrimp larvae from coral reefs in Kimbe Bay, Western Pacific, Papua New Guinea and Red Sea (Barber & Boyce 2006). Along the same line, Hubert and colleagues (2010) collected 46 larvae of Pacific coral reef fishes from the families Holocentridae and Acanturidae, 100% of which were identified to the species level through DNA barcoding (Hubert *et al.* 2010). Similarly, Ko and colleagues (2013) estimated, based on DNA barcoding, that less than 30% of marine fish larvae were accurately identified to the species level based on morphological characters (Ko *et al.* 2013). Later, Hubert and colleagues (2015) evidenced that on 1379 coral reef fish larvae sampled in the Pacific, 1264 samples were successfully amplified (92 %) and nearly 90 % can be identified to the species level through DNA barcodes.

Market substitution and the ornamental fish trade

Expensive fishes for consumption like *Tuna* are much appreciated worldwide. Substitutions with inexpensive fishes (less flavour, low in nutrients, readily available, low price) are tempting and *Tuna* fishes are no exception. After testing the identity of samples of "White Tuna Sushi" through DNA barcoding in North American market, Wong & Hanner (2008) detected that the sold fillet were derived from the fish *Oreochromis mossambicus* instead of *Thunnus alalunga* or "White tuna or Albacore tuna". For safety and economic reasons, the certification of appropriate labeling for fisheries products is required. Recent studies highlighted that DNA barcoding may be efficiently used for the regulation of the fisheries market and detection of market substitution (Ardura *et al.* 2010, Haye *et al.* 2012, Maralit *et al.* 2013, Cutarelli 2014).

Alternatively, ornamental fishes are much appreciated by the public as pet animals due to less space required space than the other domesticated animals; an aquarium of only 30 cm size can be readily used to enjoy ornamental fishes. Many tropical ornamental fishes display beautiful colours and are popular (Veiga *et al*. 2014), thus provide important sources of incomes. The international ornamental fish market has drastically increased during the recent years. This pressure

urges for the regulation of this market to protect native species and to promote more sustainable practices. Many ornamental fishes fall in the category of endemic and threatened in the IUCN Red List. Conservation of ornamental fishes is a concern in order to avoid their extinction in natural habitat (Raghavan *et al*. 2013). One important element of the regulation is the effective identification of the species. For this purpose, DNA barcoding has proven to meet the requirement as shown in the following studies that successfully identify species each in a promising success percentage: 98% of the 391 species of Indo-Pacific coral reef fishes analyzed by Steinke *et al.* (2009); 90–99% of the 172 cyprinid fish species examined by Collins *et al.* (2012); and 60% (6 species) of the 10 species of *Hyphessobrycon*, which were altogether represented by 158 specimens observed by Paz *et al*. (2014), were easily distinguishable by DNA barcoding: *H. bentosi, H. copelandi, H. eques, H. epicharis, H. pulchripinnis,* and *H. sweglesi.*

DNA barcoding enables to identify species for the purpose of certifying the labeling of consumed fisheries products for consumption as well as verifying the species identification in the export-import business of the international ornamental fish trade. Exporting countries can increase their incomes by improving the competitiveness of their fisheries products with the certification and the accurate labeling. Importing countries can significantly reduce the loss due to misidentification simply by using DNA barcoding (Steinke *et al.* 2009, Collins *et al.* 2012, Raghavan *et al*. 2013). Parties associated with the ornamental fish trade regulations are collectors, wholesalers and retailers, as well as regulatory control agencies, all of which will undoubtedly benefit from the identification services available from a comprehensive DNA barcoding framework (Steinke *et al.* 2009).

Perspectives

Biodiversity hotspots and the taxonomic impediment: the example of fishes

Biodiversity is not evenly distributed in the world, some parts of which have a higher number of endemic species that are impaired primarily by human activities. Such areas are classified as ”biodiversity hotspots” (Myers *et al*. 2000) and constitute absolute priorities for conservation purposes (Sechrest *et al*. 2002). To date, 25 hotspots are recognised worldwide based on the number of endemic species in plants and four groups of vertebrates including mammals, birds, reptiles and ampibia (Fig. 2). Among those 25 hotspots, two are present in Indonesia, namely Sundaland and Wallacea. In Sundaland, a total of 701 species of endemic vertebrates live in protected areas covering 90000 km^2, while it adds up to 529 species in 20415 km^2 correspondingly in Wallacea. For the four groups of vertebrate, Sundaland has a high percentage of endemic vertebrates species namely 2.6%, which is higher than Brazil, one of the highest biodiversity countries in the world with 2.1%. Globally, Sundaland is the third hotspot in terms of endemism, while the most diverse Amazonian hotspots is the fourth (Myers *et al.* 2000). The exponential

growth of human impacts on ecosystems has called considerable attention on the role of biodiversity in maintaining ecosystem services on which a growing human population depends. Ecosystems provide a wide variety of services including food resources (*e.g.* fisheries) and incomes (*e.g.* ecotourism) for millions of people, flood control (*e.g.* forest cover) and waste detoxification (*e.g.* nitrogen cycle). Recent meta-analyses of threats, however, evidenced that Indonesian hotspots are the world most endangered to date (Orme *et al*. 2005, Lamoureux *et al*. 2006, Hoffman *et al*. 2010).

Fishes account for more than 50% of entire vertebrate diversity with 32.900 fish species described worldwide (Nelson 2006, Froese & Pauly 2014). Kottelat & Whitten (1996) proposed biodiversity hotspots based of freshwater fish species endemism in Sundaland and Wallacea (Fig. 3). Kottelat & Whitten (1996) stated that Indonesia has the highest number of freshwater fish species among the Asian countries and second worldwide after Brazil with 1216 species (Froese & Pauly 2014). Endemic freshwater fish species in both Sundaland and Wallacea altogether (*i.e.* Sumatra, Borneo, Java and Sulawesi) add up to 243 species (Kottelat *et al*. 1993). Given the high endemic diversity and multiple threats on freshwater fishes, the accumulation of new studies is likely to increase the number of endemic species and provide clues for the definition of new biodiversity hotspots for fishes (*e.g.* Kadarusman *et al*. 2012).

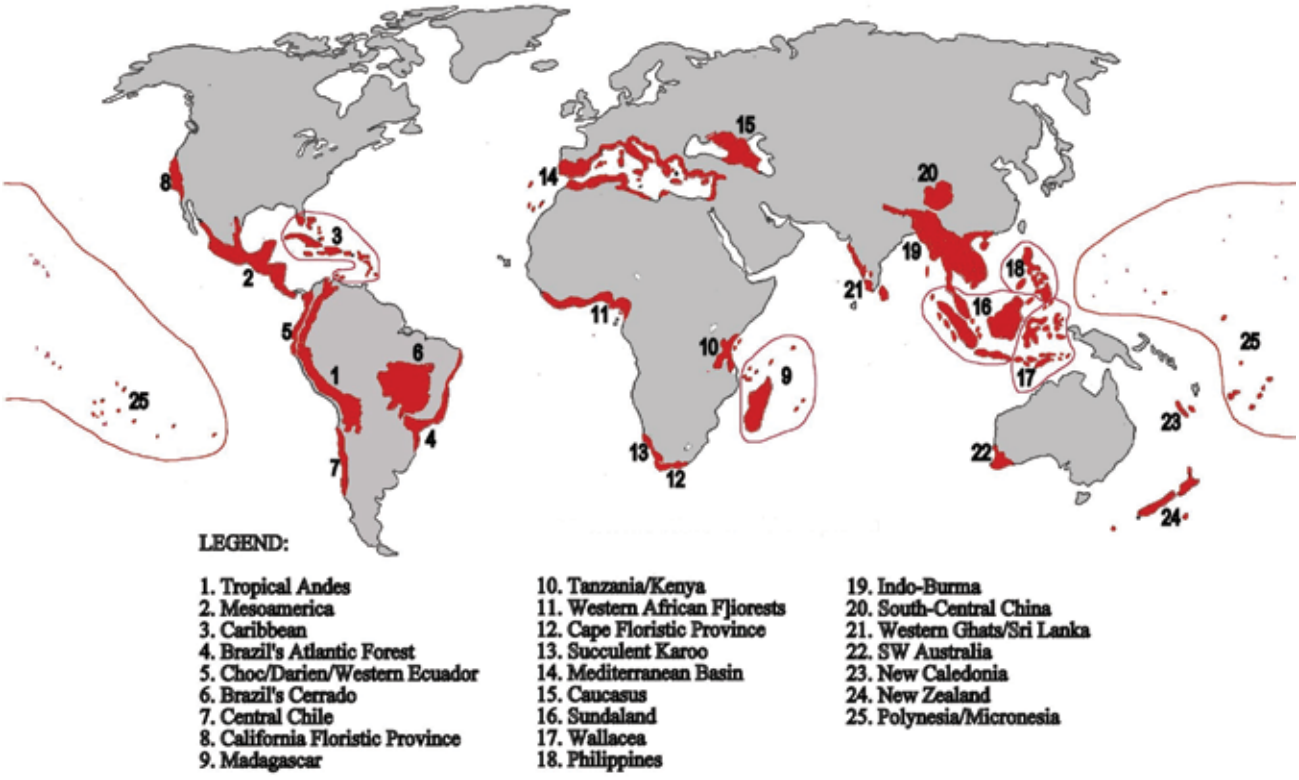

Figure 2. The 25 world hotspots (Myers 2000).

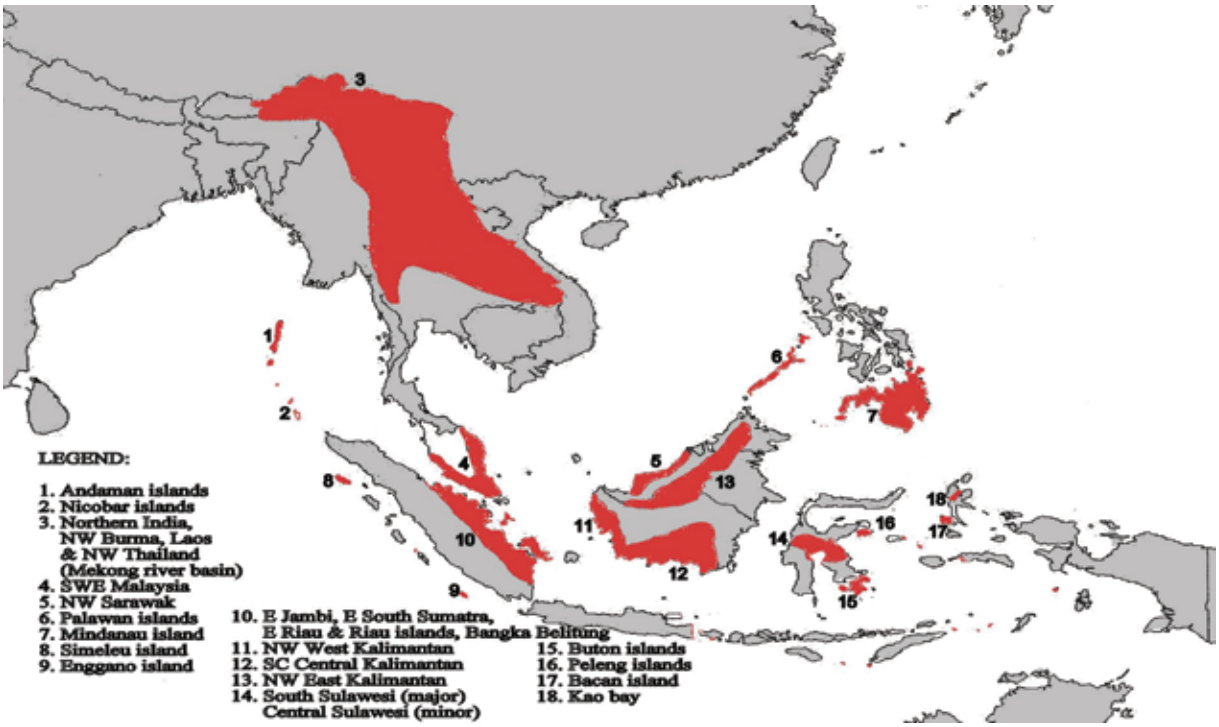

Figure 3. Hotspots based on the distribution of Sundaland freshwater fishes (source: modified of Kottelat & Whitten 1996).

How DNA barcoding can help for the conservation of the biodiversity hotspots

Fishes account for half of the vertebrate species described to date. Nevertheless, this estimation is likely to be underestimated as many fish species are still to be validated because the majority of the fish species described to date have been delineated using a traditional analyses of morphological characters, thus not accounting for the existence of cryptic taxa, and many areas are still to be explored. Although, many locations or habitats have not been categorized as biodiversity hotspots because of the paucity of ichthyological inventories in those regions that have experienced an increase of the anthropogenic pressures during the last decade (*e.g.* Papua). DNA barcoding, as an accurate tool for species delineation, has the potential to accelerate the pace of species description, particularly in remote and unexplored areas that may prove to constitute biodiversity hotspots (*e.g*. Kadarusman *et al*. 2012). Together with the morphological approach, DNA barcoding will help to validate and/or to delineate new fish species and to promote more taxonomic studies on fishes. Generally, biodiversity hotspots have been determined through the number of endemic species based on morphospecies concept, especially for species described before the 2000s. The joint use of DNA barcoding and morphology to delineate species may prove to be a solution for the appraisal of difficult cases such as cryptic species. Along the same line, biodiversity hotspots, such as Sundaland and Wallacea, may be prove to be even more important in terms of endemism if DNA barcoding, for instance, reveal a substantial amount of endemic cryptic species. Along the same line, a more detailed knowledge of the distribution and the concordance of the species range distributions among endemic species may also lead to the recognition of sub-regions within these majors biodiversity hotspots that are currently more endangered than others.

CONCLUSIONS

DNA barcoding, as a new component of biodiversity sciences and integrated with taxonomic routines, is expected to help delineate species more accurately and to open new perspective in the inventory and conservation of living beings. This is particularly evident in for the biodiversity hotspots where inventorying is still ongoing. Due to the properties of mitochondrial DNA, DNA barcoding can be readily used to identify specimens, whatever the life stages under scrutiny. Much of the hindrance toward the development of taxonomy lies in the cost and time needed to train new taxonomists based on morphological approaches. DNA barcoding currently offers an efficient solution to the taxonomic impediment. Therefore, DNA barcoding makes taxonomy more attractive to many scientists and students interested in learning taxonomy.

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