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Emerging trends in DNA markers and their applications in Aquatic biodiversity with an emphasis on mitochondrial markers

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Abstract
Biodiversity is essential for maintaining ecosystem services and each species performs a particular function in an ecosystem. It constitutes a unique global heritage and its conservation and utilization is of immediate concern. The increasing loss of aquatic biodiversity globally has led the researchers to intensify their efforts to produce a census of all aquatic biodiversity and to modernize the taxonomy. It is not feasible to catalogue the vast diversity by traditional methods based on morphological description so the researchers adopt analytical molecular technologies as an alternative to fill the gaps in phylogeny. In this direction, DNA markers have revolutionized the analytical power necessary to explore the genetic diversity. The present article collates information on all types of molecular markers with their principles, important advantage and disadvantages with respect to aquatic biodiversity studies. Among them mitochondrial DNA markers are being used extensively in aquatic biodiversity and conservation studies particularly in tracking invasive species, detection of cryptic species and identification of various life history stages. Focus has been given on mtDNA based markers such as DNA Barcoding, mini-DNA Barcoding and environmental DNA (eDNA) based meta-DNA Barcoding technologies useful for aquatic biodiversity studies.

Keywords: aquatic biodiversity, cytochrome oxidase I (COI), DNA barcoding, environmental DNA (eDNA), molecular markers

1. Introduction
Aquatic organisms have been the integral part of aquatic biodiversity and human beings have long been dependent on these resources for their food, health, environmental security and other commercial use [1, 2]. Biodiversity constitutes a unique global heritage and its protection and conservation is of immediate concern [3]. Each species performs a particular function in an ecosystem. Aquatic biodiversity in marine and freshwater ecosystems is enormous and the quantity of explored diversity is far less than the actual diversity. The ancient ancestry extending into the past for more than 500 million years has allowed a vast span of time for evolutionary divergence and for the origin and extinction of major phyletic lines. Moreover, freshwater biodiversity has been experiencing alarming decline due to over exploitation of biota, habitat loss, anthropogenic pollution and other factors [4]. As a result, several organisms were listed as endangered or threatened [5]. The phylogenic lineages based on the evolutionary relationships are still highly debatable inspite of repeated and continuing investigations using classical taxonomy. This scenario forced the researchers to adopt analytical molecular technologies as an alternative to fill the gaps in phylogeny and exploring the aquatic biodiversity. In this direction, DNA markers, genome mapping, microarrays and sequencing are proved to be the most relevant technologies.

Molecular markers are potential tools to identify the genetic distinctiveness of individuals, populations or species. These markers have revolutionized the analytical strength necessary to investigate the genetic diversity. The development of DNA-based genetic markers created a huge progress in genetic studies. With these DNA markers, it is possible to observe and exploit genetic variation in the entire genome. Popular DNA markers in the aquatic biodiversity studies include: Allozymes, mtDNA (Mitochondrial DNA) markers, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), Microsatellites, SNP (Single Nucleotide Polymorphism) and EST (Expressed Sequence Tags) markers [6]. Selection of appropriate genetic markers to assess the genetic diversity is the primary concern for any application in the field of aquatic biodiversity. Of late, mtDNA markers, especially DNA Barcoding and environmental DNA (eDNA) technologies are being gained greater attention from scientists with broad applications. Different types of markers used for aquatic biodiversity studies are depicted in Fig.1.

In the process of evolution, every organism undergoes some genetic change at different magnitudes in reproductively isolated populations. Those changes enhance the capability of organisms to adapt to changing environment and are necessary for survival of the species. At the DNA level, genetic variation is mainly due to: base substitutions (Single nucleotide polymorphisms, SNPs); insertions or deletions of nucleotide sequences (Indels) within a locus; and the rearrangement of DNA segments around a specific locus. The present article collates information on molecular DNA markers with reference to their principles, potential power, applications and disadvantages in aquatic biodiversity studies. Emphasis has been given on recent technological advances in mtDNA based markers such as DNA Barcoding, mini-DNA Barcoding and environmental DNA (eDNA) based meta-DNA Barcoding technology.

2. Molecular Markers
2.1 Allozyme Markers
Allozymes were among the earliest markers used in 1980's. These are the variant forms of an enzyme that are coded by different alleles at the same locus [7]. They proved to be efficient both individually as well as in combination with other markers (RFLP, microsatellites or mitochondrial markers) for genetic stock assessment and revealing the genetic bottlenecks in various geographical regions [8].
They are well known for estimating genetic differentiation and reproductive isolation, analysis of mating patterns coupled with Hardy-Weinberg pattern [9] which helps in determining the origin of an individual from a sample whether from a vast, randomly mating population with equilibrium genotype frequencies or from group of genetically distinct units. Allozymes revolutionized the studies on spawning aggregations by showing the homogeneity among heterogenous spawn both morphologically and life stage-wise over large geographic distances [10]. Allozymes are not efficient with heterozygote deficiencies due to null (enzymatically inactive) alleles.

2.2 Microsatellite Markers
Microsatellites or Variable Number of Tandem Repeats (VNTR) are sequence repeats that range in size from 1 to 6 base pairs. Microsatellite loci typically exhibit elevated levels of length mutation facilitating high levels of heterozygosity in fish (ranging from 24-90%) which helps in stock identification [11]. By using this technique, it is possible to compare the genetic diversity and population structure between wild and cultured stocks [12, 13] which is useful for the researchers to improve species genetically by selective breeding and to design appropriate management guidelines for the conservation of a particular species. They provide valuable inferences while analyzing the genetic inheritance in the inter hybrid population [14] and parentage analysis [15]. Survey on genomic sequencing of channel catfish showed that microsatellites were found to represent 2.58% of the catfish genome [16]. In most fish species, dinucleotide $(AC)_n$ repeats are the most abundant forms of microsatellites. The disadvantage lies in the identification of microsatellite locus for its sequencing and it needs lot of investment and effort for primer designing. The mutation rates and patterns are also difficult to understand [17].

2.3 RAPD (Random Amplified Polymorphic DNA)
RAPD markers gain advantage with their arbitrary primers in several population study applications [18, 19]. RAPD can detect high levels of DNA polymorphism. The technique detects coding as well as non-coding DNA sequences, and most of the informative polymorphic sequences derived from repetitive (non-coding) DNA sequences in the genome. RAPD markers have been widely used for species and strain identification in fishes and mollusks, genetic diversity and rate of gene flow in fish and analysis of genetic impact by environmental stressors [20]. The major weakness of RAPD is its dominant mode of inheritance. Presence of paralogous PCR product (different DNA regions which have the same lengths and thus appear to be a single locus) limited the application of this marker.

2.4 RFLP (Restriction Fragment Length Polymorphism)
The RFLP technique is mainly based on DNA fragment length differences after digested genomic DNA with one or more restriction enzyme. Earlier these fragments were analyzed using Southern blot analysis by specific probes. Later, with the increasing number of "universal primers" developed from the flanking regions of fragments, it is replaced with PCR.
RFLP is a co-dominant marker. It unable to detect point mutations, low polymorphic rates at most loci and can detect only large shifts in DNA fragment sizes. RFLP requires previous genetic information. Often this information is not available for many fish or other aquaculture species. Future use of RFLP will be to focus on analysis of Single Nucleotide Polymorphisms (SNP) residing within restriction sites.

2.5 AFLP (Amplified Fragment Length Polymorphism)
AFLP is a PCR-based, multi-locus fingerprinting technique that combines the strengths of RFLP and RAPD. Like RAPDs, it does not require any prior molecular information and thus applicable even to less studied fish species. By using AFLP, Co-dominant scoring is possible for well-characterized families. AFLP markers have been extensively used for genetic improvement programmes [21], genetic comparison of cultured and wild populations [22] and other applications. The major weakness of AFLP marker is its dominant nature of inheritance. Also, it requires special equipment such as automated gene sequencers for electrophoretic analysis of fluorescent labels. Traditional electrophoretic methods can also be employed, but they require the use of radioactive labels or special staining techniques such as silver staining.

2.6 SNP (Single Nucleotide Polymorphism)
Single nucleotide polymorphism (SNP) describes polymorphisms caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus [23]. The PIC of SNP's is not as high as multi-allele microsatellites, but this limitation is balanced by their great abundance. The SNP's are inherited as co-dominant markers. Development of SNP-based genetic maps provides deep insights into genome-wide linkage study of sex determination and identification of loci on various chromosomes responsible for sex determination. SNP-based experiments showed direct evidence for polygenic sex determination in zebra fish [24]. SNP's applicability to non-model organisms based on an annotated sequence of a model organism is a recently discovered application [25].

2.7 EST markers (Expressed Sequence Tags)
Expressed sequence tags (ESTs) are single-pass sequences generated from random sequencing of cDNA (complementary DNA) clones. It offers a rapid and valuable first look at the genes expressed in specific tissues under specific physiological conditions, or during specific developmental stages. ESTs are useful for the development of cDNA microarrays that allow analysis of differentially expressed genes to be determined in a systematic way, in addition to their great value in genome mapping. Recent development of radiation hybrid panels for widely cultivable species is a very good option for the application of EST markers in aquaculture [26].

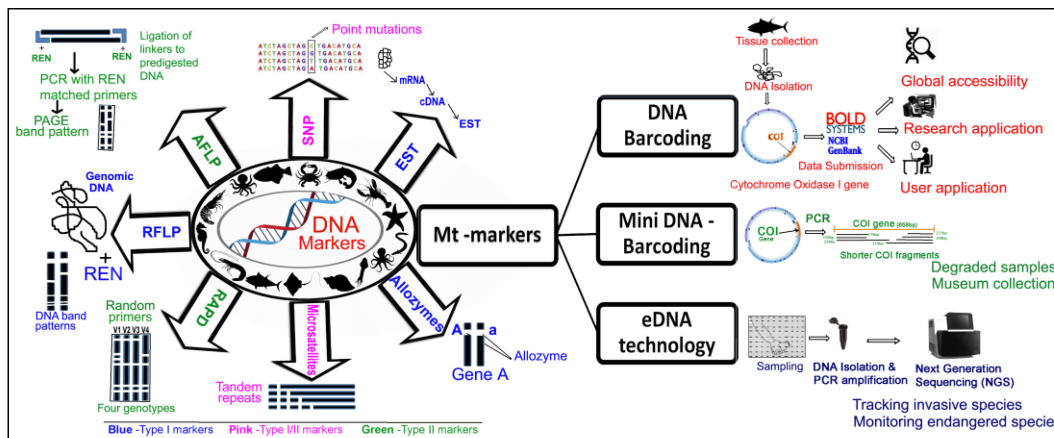

Fig 1: Different types of markers used for aquatic biodiversity studies

3. Mitochondrial DNA markers (mtDNA)

These are strictly maternal inherited markers that contain faster mutation rate than the nuclear DNA [27]. The recent application of mitochondrial markers is the identification of cryptic species and alien species. mtDNA markers have emerged as one of the major tools for integrative taxonomy. mtDNA markers are useful for exploring the relationships among closely related species and for the identification of brood stocks. Different parts of the mitochondrial genome are known to evolve at different rates. Almost the entire mtDNA molecule is transcribed except for about 1-kb control region (D-loop) where replication and transcription of the molecule is initiated. In general, non-coding segments like D-loop exhibit elevated levels of variation relative to coding sequences such as cytochrome b (*Cyt b*) gene. Inventorying the "universal primers" elevated the use of these markers greatly in the field of aquatic biodiversity studies.

Non-Mendelian inheritance and relatively minor proportion of mtDNA to total genome are the disadvantages for mtDNA markers. Gender biased inheritance (maternal) was also identified as another limitation to the validity of using mtDNA for genetic studies. Instances like back mutations (sites that have already undergone substitution are returned to their state), parallel substitutions (mutations occur at the same site in independent lineages), and rate of heterogeneity or mutational hot spots (large differences in the rate at which some sites undergo mutation when compared to other sites in the same region) also misleads the properties of mitochondrial markers.

3.1 DNA Barcoding

Before 2000s, various discrete units of mitochondrial genome were used in various applications without consistency among the scientific community. However, with the advent of DNA Barcoding technology using Cytochrome Oxidase I gene (COI/ Cox) by Noble laureate, PDN Hebert (2003), all the scientists come under one umbrella. This COI based technology is being used extensively in aquatic diversity studies for the identification of species [28, 29], to examine phylogenetic relations, strains and hybrids [30] as well as for species delimitation. This technology has solved many challenges in the field of classical taxonomy. It is proved as a successful tool for revealing cryptic species [31], calculating genetic divergence within and among the species [29], identification of Short Range Endemics (SRE's) [32], etc. Moreover, it plays a crucial role in rapid documentation of α-taxonomy for several precious faunal groups before they lost [30, 33] and accelerate the rate of species discovery [34]. However, the application of barcoding technology in taxonomy faced some controversies among scientists.

Availability of universal primers facilitate the broad application of technology across all hierarchical levels of many groups of aquatic invertebrates [35] and vertebrates [36] including marine organisms [37]. Many scientists across the globe initiated several projects, campaigns, databases and libraries to create online platform for those who work on different groups of organisms (Table 1). Food traceability is a recently emerged application of DNA Barcoding where it is more beneficial for quality assessment of aquatic products by controlling food fraudery activities, species substitutions and food authentication [38, 39].

Table 1: Different databases and initiatives working for DNA Barcoding of various fauna

S. No.	Database	Covered organisms	Origin Country	Reference
1	NCBI-GenBank	All fauna	USA	https://www.ncbi.nlm.nih.gov/genbank/
2	BOLD v4	All animal fauna	Canada	http://www.boldsystems.org/
3	FISH-BOL	All fish species	Multiple	http://www.fishbol.org/
4	CBOL Fungal working group	Fungal fauna	USA	http://www.fungalbarcoding.org/
5	Bee-BOL	Bee species	USA	http://www.ibol.org/phase1/about-us/campaigns/
6	Coral reef barcode of life	Coral reef fauna	USA	http://www.barcodeoflife.org-/content/community/projects
7	ECBOL	All fauna	Multiple	http://ibol.org/june-2-4/
8	HealthBOL	Voctor fauna	-	http://www.healthbol.org/
9	Lepidoptera Barcode of Life	Butterfly and Moth species	Germany	http://lepbarcoding.org

10	MarBOL	Marine species	-	http://www.marinebarcoding.org/
11	MBI	Mosquito species	USA	http://barcodeoflife.ning.com/group/mosquitobarcoding
12	Q-BOL	Plant pathogenic organisms	Netherlands	https://www.qbol.org/en/qbol.htm
13	SharkBOL	Sharks	-	-
14	SpongeBOL	Sponges	USA	http://www.palaeontologie.geo.uni-muenchen.de/SBP/
15	TrichopteraBOL	Trichopterans	Canada	http://trichopterabol.org/

High polymorphism due to rapid mutation rate in COI resulting from the lack of DNA repair mechanism during replication in the course of evolution is an advantage for using mtDNA marker in wide range of populations. The main pull backs associated with DNA barcoding are Pseudogenes (NUMTs) [40] and maternal inheritance.

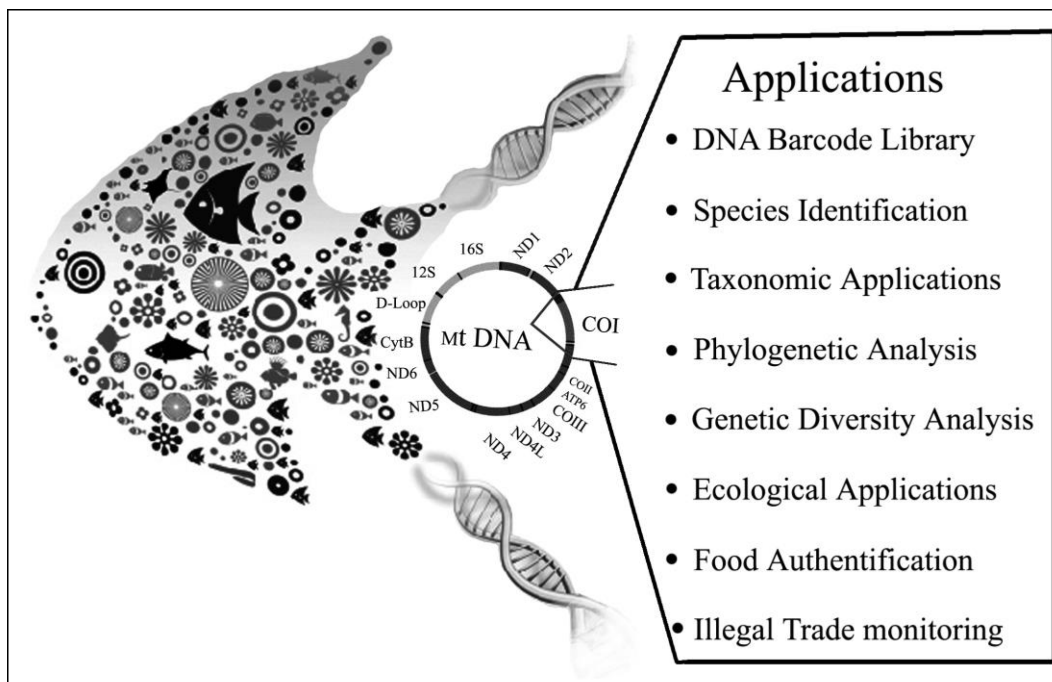

Fig 2: Applications of COI based DNA Barcoding

3.2 Mini DNA Barcoding

Mini DNA Barcoding is useful for degraded DNA samples and to identify the processed aquatic products [41]. It focuses the analysis of shorter DNA fragments (e.g. 100-200 bp) within the full-length barcode. It is proved to be effective in obtaining DNA sequence information from specimens containing degraded DNA [42]. Even short fragments of COI can be effectively used for species identification. With the advent of new sequencing technologies, this method gains much importance in determining the species composition of environmental samples [43], revealing prey-predatory relations, and identification of processed parts [44].

3.3 DNA meta Barcoding using environmental DNA (eDNA)

eDNA refers to the genetic material obtained from the environment which is in the form of whole microbial cells or shed off from multicellular organisms *via* metabolic waste, damaged tissue or sloughed skin cells. eDNA technology is based on the DNA released into environment in various forms like feces or excrements, fish slime, scrapped-off tissue cells and cells released after the death or decay of organism. It can be done even with low quantities of DNA. This technique is being widely used in determining the presence or absence of an organism in a particular aquatic environment [45, 46] which is critical to ecological management and conservation biology. It also estimates the abundance and biomass of a species in a particular environment [47], creating the distributional maps [48, 49], determining the feeding habits of endangered species [50] and finding the prey-predatory relationships.

eDNA monitoring has several advantages like increased sensitivity towards species detection, lower cost, extraction of DNA from multiple resources at a time and the possibility of targeting several taxa during amplification. Generation of sequencing data from environmental samples using eDNA is affordable. The major ecological applications of eDNA technology include tracking of invasive species and monitoring endangered species. eDNA technology helps in identifying the source species for whale meat, sturgeon eggs, shark fins and other high valued (and imperiled) species which are subjected to illegal trade. It is possible to track the presence of invasive species in an ecosystem before it gets established and cause harm to it [51]. It can be applicable from the pond ecosystem level [52] to the ocean ecosystem [53]. Its application is expanded to many groups of animals including crustaceans [54] mollusks [55], fishes [56] and amphibians [51]. Sometimes it requires past data for the analysis of eDNA. It is also possible to characterize diet contents of invasive species

which helps in risk assessment of an ecosystem. Even small samples are sufficient for reliable identification.

The main drawbacks of eDNA technology are: it is hard to obtain tissue samples of a single species from the whole sample; DNA fragments of interest often degrade faster in days to weeks beyond the level of detection in contemporary aquatic and marine ecosystems; false-positive or false-negative detection rates [57]; primer bias and variable eDNA concentrations; and expensive to adapt advanced PCR equipment (qPCR, ddPCR).

Furthermore, different sets of marker combinations strengthen our ability in fishery management, rehabilitation and conservation of various species which are endemic and endangered [58] due to destructive fishing practices and trade, over exploitation, habitat loss and restrictive distribution [59] or even due to marine regression during glacial epoch [60]. Allozyme and mitochondrial marker based studies revealed low levels of polymorphism, which pulls our discrimination power to assess stock structuring of freshwater fish. At this juncture microsatellites are proved to be the high polymorphic genetic marker than others [61]. However, when dealing with highly divergent groups, microsatellites are less informative [62]. Of late, various online and software analysis tools like e.g. MEGA V.7.0 [63], Dna SP v6 [64], POPGENE v1.32, Arlequin, MrBayes, XLSTAT, GenALEx etc. are available with fastest analytical power and giving reliable result for population data. Geneticists, conservationists and planners would take a strategic approach towards biodiversity monitoring by using molecular markers for sustainable management of aquatic biodiversity.

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