COI GENE: A MOLECULAR MARKER OF SIGNIFICANCE FOR IDENTIFICATION OF BUTTERFLIES

Abstract

Identification of butterflies heavily relies on body colour, wing patterns and venation, and structure of male and female genitalia. However, it gets challenging in species which exhibit polymorphism, polyphenism, cryptic colourations, seasonal morphs, sexual dimorphism and other variations. This limits accurate representation of butterfly diversity. The advent of nuclear and mitochondrial molecular markers has not only facilitated identification of species, but has also made it more precise and rapid by resolving confusing features of species. Besides the identification of species, these markers have opened new vistas in the identification of higher taxa, understanding speciation, and comparison of allopatric and sympatric populations. One of the markers, mitochondrial cytochrome c oxidase subunit I, often referred to as COI, is a universal marker of significance across butterfly taxa. It is used alone or in unison with other mitochondrial and nuclear markers for taxonomic and population studies. The Barcode of Life database (BOLD Systems) represents an appreciable number of animal taxa, including butterflies, based on the COI marker. Though molecular markers also have their limitations, they have definitely strengthened taxonomy and conferred stability to nomenclature vis-à-vis classification.

Keywords: COI Gene, Butterflies, Taxonomy, Genetic Markers.

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I. INTRODUCTION

Butterflies are beautiful flier insects known for their vibrant colors and wing patterns. They have attracted the attention of people, particularly children, and those who appreciate beauties of nature. Scientists have been equally attracted to butterfly diversity and their role as pests of many wild plant species with commercial value, ornamental and horticultural plants, and agricultural crops. For their beautiful colours, wing patterns and their similarities, butterflies have fetched diverse common names; and many of them are more famous than the scientific names. However, for authentic identification, apart from general morphology and wing patterns, wing venation (Heppner, 2008) and more recently external genitalia (Powell, 2009) have been added to their taxonomic characterization. Taxonomic keys based on diagnostic features facilitate identification of higher and lower taxa of butterflies. Genitalic descriptions and their incorporation in keys is far from completion. The existence of polymorphism, cryptic colorations and polyphenism across various taxa of butterflies make identification a little difficult and also indicate the probable existence of species complexes.

Taxonomy – Identification of Taxa; Morphology to Molecular Basis: Taxonomy is an integral discipline in the biological sciences and its history can be traced back to Linnaeus, (1758). Identification of species has always been recognized as a foundation for diverse biological research - conservation, biodiversity and evolution (Dincă et al., 2021; Nneji et al., 2020; Purty & Chatterjee S, 2016; Hubert et al., 2015; Hebert et al., 2003). However, the correct identification of species is challenging to taxonomists. Besides, taxonomists have been confronted with the scope of the term species and the basis for delimiting populations (Blaxter, 2004).

From the times of Linnaeus, morphological characters have been the benchmark for identification and diagnosis of taxa (Wingert, 2022; Nneji et al., 2020; Hebert et al., 2003) While the reliance on such diagnostic morphological characters appears to offer a sense of objectivity for identification, taxonomists seldom agree on what should constitute diagnostic characters (Hubert et al., 2015). Recognition of such characters vary from taxa to taxa. For butterflies, presently we rely on wing patterns, wing venation, and structures of male and female external genitalia (Nneji et al., 2020; Pazhenkova & Lukhtanov, 2019; Lukhtanov & Tikhonov, 2015; Burns et al., 2008; Hebert et al., 2004). However, Ford (1946) had expressed his reservations on using wing venation for identification, citing that the overall wing venation remains fairly consistent across all butterfly species. Besides, traditional and classical taxonomy based on morphology is confronted with enormous challenges like prevalence of polymorphism, phenotypic plasticity, sexual dimorphism, organismal life stages (Hebert et al., 2003), and cryptic colourations (Gaikwad et al., 2012; Hebert et al., 2003). Additionally, the morpho-taxonomy requires expertise and is time consuming (Dong et al., 2021). Referring to original descriptions of the taxa and their revisions is gigantic; though framing of taxonomic keys has facilitated identification.

In view of the diverse limitations of morpho-taxonomy, two decades ago, Hebert et al., (2003), established the use of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene as a molecular marker for the identification of animals. The rationale for the preference of *COI*, as a molecular marker, was the universality of the genetic code and the rapidity of *COI* evolution; enabling easier delineation of even geographically separated populations (Hubert et al., 2015; Hebert et al., 2003). Since then, *COI* has been regarded as a universal

marker for species identification (Wingert, 2022). In Lepidoptera, it was empirically demonstrated that, on an average, a *COI* divergence exceeding 3% denotes that the organisms being compared are different species. This 3% threshold has been discovered in a majority of animal taxa (Shapoval et al., 2021; Singh et al., 2021; Nneji et al., 2020; Blaxter, 2004; Hebert et al., 2004). The validity of the threshold is predicated on whether interspecific sequence divergences exhibit a demonstrable gap from intraspecific sequence divergences (Purty & Chatterjee S, 2016; Wiemers & Fiedler, 2007). The use of such a diagnostic molecular sequence has come to be known as DNA barcoding and the genetic marker as a DNA barcode; and it has proven invaluable in identifying problematic butterfly species. By definition, a DNA barcode is a short genetic sequence, ideally a 650 base pair mitochondrial sequence, that is unique enough to identify an organism as a particular species (Dincă et al., 2021; Dong et al., 2021; Laiho & Ståhls, 2013; Wiemers & Fiedler, 2007; Brower, 2006). DNA amplification procedures like PCR are invaluable to DNA barcoding, therefore identification of species (Pentinsaari et al., 2016; Purty & Chatterjee S, 2016).

II. BUTTERFLY DIVERSITY AND IDENTIFICATION

Butterflies, alongside moths, comprise the insect order Lepidoptera, exhibiting the second highest diversity after the order Coleoptera among insects vis-à-vis animals (Dincă et al., 2021; Pentinsaari et al., 2016). Butterflies are regarded as important bioindicators in the evaluation of the effects of climate change, as well as taking part in plant pollination, and serving as important models in the study of evolution (Dincă et al., 2021; Haroon et al., 2014). This makes them some of the most widely studied groups of insects. Presently, butterflies are represented globally by over 20,000 species (Nneji et al., 2020); while India supports 1300 species (Nneji et al., 2020; Smetacek, 2017), which have been classified and named. Butterflies are grouped into one superfamily, Papilionoidea, which includes six families - Nymphalidae, Roidinidae, Papilionidae, Pieridae, Lycaenidae, and Hesperiidae (Varshney & Smetacek, 2015).

Since 2003 (Hebert et al.), there have been a significant number of taxonomic studies where molecular markers have been employed in the identification of butterflies (Lukhtanov & Tikhonov, 2015; De Mandal et al., 2014; Gaikwad et al., 2012; Hebert et al., 2004). Using the *COI* gene, Hebert et al., (2003) were able to accurately identify species of butterflies syncing with identifications arrived at through traditional taxonomy.

In recent times, there has been an increasing use of molecular markers in butterfly taxonomy in resolving cryptic species complexes, species with confusing variability in wing patterns, morphologically similar species complexes and species with high variability of male genitalia. Some of the notable cases are described to highlight the role of genetic markers in species resolution. Hebert et al., (2004) resolved a cryptic species complex of *Astrapes fulgerator* belonging to Hesperiidae family into 10 distinct species. Interestingly, there was no difference of male and female external genitalia in these different species; but the *COI* sequence divergence was too great to be attributed to one species. Similar results were found by Burns et al., (2008) in another cryptic species complex in *Perichares philetes*, a skipper butterfly widely distributed in the neotropics; and also, *Agrodiaetus* subgenus of *Polyommatus* (Lycaenidae family) by using karyotype and molecular markers. Lukhtanov & Tikhonov, (2015) were able to resolve a species complex *Agrodiaetus* by using *COI* and *COII* (cytochrome *c* oxidase subunit II gene) genetic markers. Prieto et al. (2020) resolved

species of the genus *Rhamma* by genetic markers as conclusions could not be drawn on the basis variability of wing patterns and other morphological features. Similarly, butterflies of the genus *Pseudophilotes* (Lycaenidae) were resolved by genetic markers as high variability in the genitalia of males prevailed both within and between species (Lukhtanov & Gagarina, 2022). There is practically an inexhaustible list of cases of butterflies where molecular tools have been used with profitable results.

The establishment and popularity of molecular markers for the identification of species is owed to the accuracy of DNA barcode technique and repeatability of results. The so-called barcode gap rule of no overlap between the intraspecific and interspecific sequence divergences imparts accuracy of identification. Based on the *COI* marker, Gaikwad et al., (2012) resolved 75% of the collected specimens of butterflies from western Ghats. The remainder of their specimens had an interspecific divergence of less than 3%, and even less for intraspecific divergence. Nneji et al. (2020) successfully delineated 90% of the butterfly species in Nigeria by DNA barcode. Dincă et al., (2021) reported 95% correct identification of specimens. However, Singh et al., (2021) reported an average intraspecific nucleotide divergence of 2.61% among 28 species of butterflies; and *Junonia* butterflies showing a rather low interspecific divergence, but with morphological distinctions. Together, these studies appear to offer a more objective basis for the identification and classification of butterflies by having a predefined gene divergence gap to look for when assigning specimens to particular species groups.

III. DIVERSITY OF GENETIC MARKERS

The mitochondrial (mt) genome sequences have long been regarded as ideal candidates for use in molecular taxonomy mainly because, unlike the nuclear genome sequences, they are highly conserved, with almost negligible genetic recombinations, evolve slowly, and the occurrence of maternal inheritance make it easier to track down organismal lineages (Dong et al., 2021; Purty & Chatterjee S, 2016; De Mandal et al., 2014; Hebert et al., 2003). In addition, a continuous coding frame (for the lack of introns – a prokaryotic feature) makes the mitochondrial genome advantageous in contrasting nucleotide sequences of different specimens (Purty & Chatterjee S, 2016; Hebert et al., 2003). We must recall the fact that 37 genes comprise the mitochondrial genome, out of which 13 genes, including *CO* gene series and *ND* gene series, *Cytb* and *ATPase,* are protein-coding and serve as DNA barcodes (Dong et al., 2021; De Mandal et al., 2014; Hebert et al., 2003). Besides, negligible DNA repairing mechanism enhances mutation rates tenfold in the mitochondrial genome as compared to nuclear genes, which further introduces variations among species to enable their prompt differentiation (De Mandal et al., 2014).

COI is recognized as the most prominent molecular marker for various reasons - easily demonstrating a barcode gap (percentage divergence), a near-universality of its primers across the animal kingdom, easier for amplification and enabling rapid identification of species (Singh et al., 2021; Purty & Chatterjee S, 2016; Gaikwad et al., 2012).The tissue for procuring genetic material (nuclear or mitochondrial) to be amplified (cloned; by plasmid vectors or PCR) for DNA barcode is commonly obtained from the hindleg of butterflies (Singh et al., 2021; Nneji et al., 2020) or thorax (Singh et al., 2021). Computational tools, such as Molecular Evolutionary Genetics Analysis (MEGA), have become invaluable as sequence (in FASTA format) alignment (blasting) tools that compare molecular sequences

from different specimens and measure genetic distances using models of choice, such as the Kimura 2-Parameter (Gaikwad et al., 2012). Thus, in trying to identify a specimen as belonging to one species or another, one need only to align the *COI* sequences of a known (submitted on public databases) and the unknown organism to score the divergence between the two, making it easier for taxonomists to identify organisms to the species level.

While *COI* is widely used, it is not the only candidate gene proposed for taxonomic studies. In fact, prior to the establishment of *COI* as a barcode of significance, researchers utilized the 12S and 16S ribosomal genes (rDNA) of the mitochondria for phylogenetic studies (Hebert et al., 2003). However, the defining challenges of these sequences were the frequency of insertions and deletions (indels), rendering them unideal for aligning different sequences, especially because indels lead to a shift in the open reading frame (Hebert et al., 2003). Despite this challenge, Hickson et al., (1996), Gerber et al., (2001), De Mandal et al., (2014) and Dong et al., (2021) reported the utilization of 12S and 16S rDNA in the identification of higher taxa; 12S for phyla and subphyla, and 16S for families and genera. This was a great development in the history of marker-based taxonomic conclusions.

Despite good results, identification based on a single marker has been challenged (Laiho & Ståhls, 2013; Hebert et al., 2003) for limitations of the application of threshold divergence. Therefore, there is increasing reliance on the use of more than one mitochondrial marker and also the use of nuclear barcodes in the identification of species. Laiho & Ståhls, (2013), utilized the mitochondrial ribosomal protein S2 (*RpS2*), alongside *COI*, in a study of butterflies belonging to the genus *Colias*, but it was reported that this region failed to demonstrate any utility as a DNA barcode. A shift from focusing solely on mitochondrial sequences led to the incorporation of nuclear DNA in the identification of organisms. Quek, (2022) utilized nuclear genes such as Carbamoylphosphate synthase (*CAD*), Elongation factor alpha (*EFI-alpha*), and Histone subunit 3 (*H3*) for identification purposes. Seraphim et al., (2018) used up to eight nuclear markers including the wingless (*wg*), arginine kinase (*AK*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes. An interesting study by Pazhenkova & Lukhtanov, (2019) that incorporated four nuclear sequences alongside *COI* in the study of the *Brenthis* genus of brush-footed butterflies, discovered that there was a discordance between the mitochondrial data and nuclear data. The nuclear data, however, were in accordance with morphological diagnostic characters. This led to the conclusion that for some genera, nuclear genes (*CAD*, *AK*, *wg*, and *Ca-ATPase*) were ideal identifiers.

IV. LIMITATIONS OF MOLECULAR MARKERS

Although molecular taxonomic tools have demonstrable utility to the identification of animal taxa, they also have their limitations as pointed out by many workers in the past. Brower, (2006) raised a philosophical challenge regarding the seemingly arbitrary choice of the interspecific sequence divergence threshold. It was further argued that molecular comparisons were predicated on comparing sequences already present in the public database (submitted by workers), and thus one could not rule out the possible existence of unknown organisms even more closely related to the unknown specimen. Similarly, Wiemers & Fiedler, (2007) also suggested that the apparent divergence gap of the DNA barcode can result from inadequate sampling across various taxa. These observations were made when very few genomic sequences, especially for insects, were available on public databases (De Mandal et al., 2014). Since then, a huge number of submissions have been made representing

major taxa across various animal groups, including butterflies. Seven years ago, the Barcode of Life database (BOLD Systems), a web platform dedicated to DNA barcoding, had nearly 5 million DNA barcodes (Pentinsaari et al., 2016). BOLD Systems has entered into partnerships with other public databases like iBOL, CBG, CCDB, GenBank, EOL and GBIF and at present (25.09.2023) has 14,324,971 specimens with barcodes. Of these Insects (251,456) and butterflies (104,865) account for appreciable submissions. Given the advancement in sequencing technologies, such as Next Generation Sequencing (NGS), the earlier criticisms regarding the availability of sequences stand largely addressed.

While the 3% threshold has been empirically demonstrated to be valid, its utility is fraught with limitations where species are clearly identified morphologically but have a sequence divergence below the threshold. Lukhtanov & Gagarina, (2022) demonstrated the same for four species of *Psuedophilotes,* which didn't meet the criterion of a barcode divergence gap, as opposed to three other species that were easily distinguished using the same method. Wiemers & Fiedler, (2007) further warned that the exclusive reliance on molecular markers could lead to false positives and false negatives. It was shown by them that allopatric conspecifics have a divergence above the threshold and reproductively isolated populations exhibiting divergences below the threshold. They further argued that butterfly taxa that are less than 4 million years old, such as *Agrodiaetus*, may not adhere to the established threshold owing to the amount of time needed for sequences to substantially diverge. The divergence threshold can also be violated in instances of rapid speciation events, as has been reported in some birds of North America where there has barely been any divergence in their mitochondrial genomes (Quek, 2022).

The choice of using regions of the mitochondrial genome as universal barcodes was predicated on its neutrality in the face of selection. However, Dong et al., (2021) elucidate that mitochondrial DNA can equally be under selection, wherein nucleotide variation impacts the fitness of the organism. Another challenge comes from instances of introgression as well as hybridization, which is now understood to occur more frequently than was previously thought (Shapoval et al., 2021; Purty & Chatterjee S, 2016). The same was held over 160 years ago, by Darwin, (1859) that there were no clear demarcations that could prevent two species from hybridizing. Additionally, the mitochondrial genome can fall victim of symbiotic infections, such as the *Wolbachia* strains of bacteria common in arthropods, which greatly influence its integrity (Dong et al., 2021).

V. CONCLUSION

In view of the limitations of both traditional taxonomy and DNA barcoding, the two taxonomic procedures are complimentary rather than mutually exclusive. The molecular markers have definitely revolutionized and enabled rapid taxonomic conclusions. These have equipped taxonomists to identify species at any stage of the development as barcodes remain the same. Morphological identity may seem to stand jeopardized in light of molecular marker consistency. Further, the 3% divergence rule as a gap to delineate species should be applied with caution. Rather, a 2% divergence if on an average is consistent in a taxon should also be accepted for taxonomic identification. In light of more and more submissions of DNA barcodes on public databases, the role of a single marker or several markers together; only mitochondrial or nuclear or in unison, furthers their universal utilization for butterflies. However, the success and increasing acceptance of molecular markers is not intended to

supplant traditional taxonomy. The requirement of voucher specimen for taxonomic treatment of any taxa highlights the role of traditional taxonomy and cannot be replaced. The thought that the complexity of biology lies in its lack of adherence to strict rules, as seen in the various exceptions to different established rules; and also, that nothing in science is ultimate, keeps all options open for scientists. Robust taxonomic studies definitely should employ both traditional and molecular tools for identifications and phylogenetics for all animal taxa, including butterflies.

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