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Lucrative potentials of mitochondrial DNA: A laconic review accentuating particularly blow flies beyond forensic importance

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Abstract
Extensive survey of molecular research on animals, including insects, has brought forward remunerative scope of mitochondrial DNA (mtDNA). Among the insects, blow flies are consensually known for their forensic importance. This review is a compendious effort to bring forward other latent potentials of mitochondrial DNA, specifically of Calliphoridae, concerning vivid fields beyond its application in detecting post mortem interval (PMI) alone**.** Mitochondrial DNA acts as a key to unlock various unsolved mysteries regarding identification of fragmented, small, incomplete, fossilized or archive museum specimens; tracing evolutionary history; evaluating biodiversity; monitoring mortality dynamics during epidemics or mass death toll; detecting endangered species; resolve misidentification and monophyletic or paraphyletic issues and population studies. In this review an attempt has been made to pinpoint the most prolific regions of mitochondrial DNA for the purpose of systematics and bring to light the positive and negative potentials of mtDNA.

Keywords: mtDNA, nDNA, COI, COII, PMI, ETC, Numt, Calliphoridae, Diptera

1. Introduction
Man has always been prying to apprehend and learn about the bewitching and stupefying splendors of nature. Among his multifarious endeavors, the most enamoring is to unlock the mystery of origin and evolution of life, hence deciphering the interrelationships among various organisms. For interpreting phylogeny, identification is the foremost step. A wide gamut of fields have been alleviating taxonomist to unravel the veiled inscrutability of colossal biodiversity. Anatomical approach, cytological approach and even behavioral study of various organisms have been instrumental in systematics, but not very diligent and till long, it remained an unwieldy field. However, molecular approach has proved to be productive in efficiently taming, these walloping attributes of nature.

Use of mitochondrial DNA (mtDNA) to solve the maze of ancestral history has emerged as panacea for most metazoans. mtDNA serves to be more elucidative and easy to work with than nuclear DNA, as it is more abundant i.e.500-1000 copies per cell, no or rear recombination, higher rate of mutation and maternal inheritance. Sir Bryan Sykes, in his book The Seven Daughters of Eve (2001), quoted mitochondrial DNA accumulates mutations at the rate of approximately one every 3,500 years [1]. Thus, one can easily calculate the time elapsed since the origin of a species and in what stage of evolution it currently exists can be easily determined. Thus, ushers the way back to its ancestral history.

Within the diverse forms of creatures, what surprise us the most are the vivid, proficient, cosmopolitan and astute creatures, called the insects. Among these, the beckoning blow flies (magnificent creatures depicting beautiful gaudy sheen) present exhilarant subject of study. Blow flies are well known as carrion flies, bluebottles, free bottles, or cluster flies and belong to family Calliphoridae. Approximately 1,100 species are known worldwide, under 18 genera [2]. They are economically, ecologically and anthropologically quite significant as they act as forensic detectives; nature`s scavenger (feed on bodies, serum oozing out from body, dung, garbage, excreta etc.); hold aesthetic value; cause myiasis (the infestation of live vertebrates by flies); have medical importance (Maggot therapy); are good pollinators (feeds on nectar, honey dew and other sweet liquid and are vectors of diseases [3].

Valuable morphological and developmental work pertaining to these blow flies, both implying to entomotoxicology and forensic entomology has been undertaken in North West India [4].

Through this review, the author attempts to bring forward the immense scope and strength of molecular biology which could undermine the prevalent myth of mendacity of molecular approach.

Mitochondrial DNA versus Nuclear DNA

MtDNA clout over nuclear DNA is well advocated in evolutionary studies [5]. It has relatively fast mutation rate; higher copy number; is gene-dense circular molecule of ~16kbp; is polyploid; it`s genes lack introns, and intergenic regions have only a few nucleotides; shows maternal inheritance and almost no recombination, thus suits as the best devise to trace phylogeny [6, 7]. Phylogenetic relationships based on mtDNA have been quite successful [8].

However, in some cases it is observed that nuclear genes outplay mitochondrial genes in phylogeny tracement on a per-site basis [9, 10]. Also 16S and 18S rDNA have worked out well in some cases [11]. Mitochondrial gene sequences possess certain attributes that make them less convienient for deciphering older divergences in phylogenetic tree inference [12]. These include biases in base frequency [13], strand-influenced inversions [14], and inheritance through a single linkage group [15]. But in contempt of these flaws, mtDNA stills holds up high as for as independent estimator of phylogeny is concerned which is clearly indexed in the follow up columns.

Characteristics of DNA barcode

The doctrine of using a short region of~ 648 bp within COI (cytochrome oxidase subunit 1) gene for identification of different species in the same way as a supermarket scanner uses universal product code to identify products, budded as a crackerjack innovative notion in the mind of Dr. Paul Hebert`s research group at the University of Guelph [16]. It has been chosen as a fleckless marker by a Consortium for the Barcode of Life, because its size and structure is conserved in all aerobic organisms [17, 18]. it can explain homoplasy and convergence of phenotypes; in comparison to CO II it has higher % variable region, it is a large protein coding gene i.e. code for 511 amino acids, so calls for easier alignment; highly conserved and variable regions are closely affiliated; works for all life stages; this gene is very vital for the smooth working of ETC (Electron Transport Chain) as codes for the terminal catalyst in the respiratory chain; unaltered by sexual dimorphism and environmental induced phenotypic variation; is very well studied at the biochemical level; is suitable as a molecular marker for the taxonomic and evolutionary studies of insects; it is a short sequence region for which primers are very well known and so easily amplified and sequenced [19] and serves best for detecting cryptic species and microorganisms [20, 21].

Success of barcode in identifying and conserving biodiversity

MtDNA is a prudent and effective tool in augmenting the conservation planning and research activities for many organisms through species delineation and identification. There are ample evidences to prove that DNA barcodes are cogent in differentiating and identifying various organisms belonging to the animal kingdom. In case of mammals, barcode has worked to crack certain difficult codes in bats, rodents, primates and other insectivores [22]. DNA barcodes have uncluttered many new doors in ecology, diversity, and the taxonomy of fishes in various regions, worldwide, for instance, in Australia [23]. Central America [24] and Canada [25] but as far as amphibians and reptiles are concerned, the trend thrusts valuable anecdote [26]. While relying to aves, for korean birds [27] and North American birds. It too has shown great success. Pertaining to invertebrates, specifically insects, 1300 lepidopterans were barcoded [28]. Overall estimation of different insect orders successful with barcode comprize of 67% lepidopterans, 12.6% Hymenoptera, Diptera 7.6%, Coleoptera 2.8%, Hemiptera 2.4%, Orthoptera 0.6% and the rest 27orders 6.2% [29].

Thanks to the blessings of latest headlong evolutionary softwares (MEGA, MEGA1, MEGA 3, MEGA 4, Clustal Omega, PHYLIP) that has made it possible to indoctrinate this no way puzzle of evolutionary inter relationships and origins and studying genetic diseases [30].

Accent of mitochondrial DNA in evolutionary studies pertaining to blow flies

The first clue for the presence of mtDNA was fetched during the electron microscopic studies and biochemical assays carried out on mitochondria [31]. The use of mt DNA in systematics and population studies established its hegemony [32]. There are barn doors of examples to focus the enormous potential of COI gene in family calliphoridae. Its first relevance pertaining to blow flies dates back to the use of RFLP for mtDNA [33]. Application of COI gene in forensics was a major commitment [34]. RAPD and mt DNA regions other than COI and COII [35].

It has proved immensely valuable in the molecular study of Calliphoridae, worldwide.

Relevant work contributed in the field of mtDNA of Calliphoridae in various regions of the world in the last decade	Author and year
Molecular barcoding of green bottle fly, Lucilia sericata (Diptera: Calliphoridae) using COI gene sequences in India	Priya and Sebastian, 2015[36]
A 454 sequencing approach to dipteran mitochondrial genome research	Ramakodi *et al.*, 2015 [37]
. 16S rRNA: A reliable gene for DNA amplification from old and fragmented samples of Forensically important Blow flies.(Diptera: Calliphoridae) in Indias	Khullar and Singh, 2014 [38]
Genetic variations of Chrysomya megacephala populations in Malaysia (Diptera: Calliphoridae)	Chong *et al.*, 2014 [39]
Molecular characterization of mitochondrial DNA sequences of Calliphorid flies (Calliphoridae: Diptera) in India	Bajpai *et al.*, 2013 [40]
Identification of forensically important Lucilia in USA	DeBry *et al.*, 2013 [41]
Use of 307 bp Cyt b gene of calliphoridae in Iberian Peninsula	GilArriortua *et al.*,2013 [42]
Molecular identification of Malaysian Chrysomya megacephala (Fabricius) and Chrysomya rufifacies (Macquart) using life stage specific mitochondrial DNA.	Kavitha *et al.*, 2013 [43]
DNA typing of Calliphorids collected from human corpses in Malaysia	Kavitha *et al.*, 2013 [44]
Identification of blow flies in Southeastern Nebraska	Samarakoon *et al.*, 2013 [45]
Use of mammalian DNA derived from blow flies for cataloguing biodiversity	Calvignac -Spencer *et al*., 2013 [46]
Beyond Barcoding: A mitochondrial genomics approach to molecular phylogenetics and diagnostics of	Nelson *et al.*, 2012 [47]

blowflies (Diptera: Calliphoridae)	
The utility of Mitochondrial DNA fragments genetic identification of forensically important sarcophagid flies (Diptera: Sarcophagidae) in China	Guo *et al.,* 2012 [48]
Using SNP (Single Nucleotide Polymorphism) in Calliphoridae	Sze *et al.,* 2012 [49]
Use of 278-bp region of the COI gene in 75 Calliphorid specimens collected from 23 locations in China	Liu *et al.,* 2010 [50]
Mitochondrial DNA-based identification of some forensically important blowflies in Thailand	Kanok *et al*., 2010 [51]
Molecular studies on calliphoridae of Germany	Reibe *et al.,* 2009 [52]
mt DNA seuenced for Lucilia in South Africa	Tourle *et al.,* 2009 [53]
Use of 304 bp fragment of COI gene for DNA typing of 50 Chrysomyinae specimens in France	Desmyter and Gosselin, 2008 [54]
Use of 1588 bp of COI gene of 8 Calliphoridae species in Taiwan for DNA typing	Chen *et al.,* 2004 [55]
Sequencing of the mitochondrial genes CO1, CO2, ND4 and ND4L for 34 species of blowflies in Australia	Wallman *et al.,* 2005 [56]
Sequencing of mitochondrial genome 15,837bp of the blowfly Chrysomya chloropyga (Diptera: Calliphoridae using the shotgun approach.	Junqueira *et al*., 2004 [57]
Sequencing of mitochondrial DNA (mtDNA) CO1 gene as the prospective basis of a diagnostic technique of three species of Calliphorid (blow flies) commonly associated with corpses in western Australia.	Harvey *et al.,* 2003 [58]

Reliable genes for the purpose of taxonomy

Based on various studies conducted till date, we attempt to categorize certain genes and their fragments to be consequential, while others to be less lucrative for taxonomic purpose. Critical appraisal concerning both mitochondrial and nuclear DNA reveals that in most cases mtDNA has shown to be thriving [59]. Most frequently used phylogeneticaly informative mitochondrial genes include COI, COII, Cyt b, 12S rRNA, 16S rRNA, tRNA, NADH4 and NADH5 [61, 62, 63]. The most intermittently used gene is COI which is immensely informative and best for predicting divergence. It has both conserved as well as variable and hyper variable regions within a short fragment. However for accreditation and removal of anomalies, complete mitochondrial genome sequencing is recommended [64]. D-loop (control region) is least recommended as it shows immensely fast rate of mutations and thus give felonious results [65]. Based on elucidative survey, arbitrary potentials of the genes that have served the best till date can be predicted. For interpreting phylogeny at higher taxon as well as at specie and population levels; complete mtDNA is the best, followed by complete CO1 gene, then 650bp barcode region and finally COII gene and cytb gene. While, 12SrRNA and 16SrRNA show less average pair wise sequence divergence and thus, are not advised for predicting intra specific relationships, but serves good upto specie level identification. D-loop shows maximum pair wise divergence due to very high mutation rate and thus best for studying diseases (cancer etc.) but not recommended for phylogenetic purposes [66].

Flaws in mitochondrial DNA approach and their corrections

No doubt, mtDNA is a magnificent tool, but this field is also not exempted from flaws, and some indecorums are associated with it. Numts (nuclear mitochondrial DNA), Hetroplasmy, introgression and easy horizontal gene transfer are some of them but these limitations do have amelioration, which still maintains the paramountcy of mtDNA.

Numts [67] are mitochondrial pseudogene copies that got integrated in nuclear DNA in the past. These if mistakenly sequenced, may lead to felonious results and overestimation of species. However, numts can be easily detected, avoided and removed. Numts creep in, if the research work is carried out recklessly and without an expert vision. Thus, though numts are considered as limitations relating to the use of mtDNA, it in reality, is merely a manual error at the end of researcher and can be eliminated and minimized. Various methods are counseled for the purpose of avoiding numts like; the use of exclusive mtDNA extraction protocols, use of larger PCR products, use of specific primers, RT-PCR, RFLP- PCR and primer walking [68].

However, in coming times, numts may also be considered as a blessing in disguise. They may prove a valuable phylogenetic informative tool, as they are actually archive forms of mtDNA preserved as fossils in the womb of nuclear DNA. So, probably these might have clandestinely stored some secrets of our past form of mt DNA and may add potentially to our current knowledge about ancestry in future.

Barcoding works poorly with closely-related species [69]. The frequency with which mtDNA creeps between species (due to hybridization), is much more than of nDNA. This introgression results in complicated and vague results, thus may sometimes mess up the Qphylogenetic relations. It is due to homoplasy via mutational saturation and generally converges with results for other species [70]. It may result in incongruity between dendrograms based on morphological traits or those based on nDNA (nuclear DNA) sequences with those based entirely on mtDNA basis. Barcoding cannot positively identify Protocalliphora [71]. So it is recommended to use nuclear DNA along with mtDNA for generating infallible phylogenetic trees and to avoid discrepancies [72].

It's ironical to quote, but evidences mirror the fact that, mtDNA and specifically complete COI gene are best for specie level identification and also for intra specific differentiation. The approach proves inaccurate, when our research pivots on a small fragment of mtDNA. A short fragment is convenient, easy to amplify and sequence which promotes its rampant use. However, if adequate mtDNA fragment length and proper gene is chosen, it deposes all the problems [73]. It has been proved indubitable for approximately 65,000 species worldwide. mtDNA differs little within a species and generally does not overlap between species [74].We know that mtDNA shows high rate of mutation, but still depict little variation. This can result only in two ways either by natural selection or by genetic drift implying to population bottleneck [75]. This thus, also relate to a new concept in population studies and speciation concept.

Mitochondrial DNA for healthy life and speciation

Mitochondria are vital organelle for survival of any eukaryotic organism, so maintaining its integrity is a must. They are exposed to free radical so, they ought to encounter and accumulate copious somatic mutations with age, which leads to diseases and aging. It has a conglomerate of repair proteins and other pathways to affray DNA mutations. These mtDNA repair pathways are mediated by enzymes that are encoded by nuclear genes [76]. There is a benevolent association of nuclear and mitochondrial genes which is viscid. The protein enzyme units that constitute the Electron Transport Chain, comprise of subunits that are in turn coded by both nuclear as well as mtDNA.

Biological species concept i.e. reproductive isolation between

two different organisms based on sexual dimorphism, ecological preferences and hybrid sterility is widely accepted, however it is often observed that this reproductive barrier is not imperforate and there occurs gene movement between different species through hybridization. It is speculated with some strong backing of evidences that when mutation take place in mtDNA and their occurs discordance in the mitochondrial and nuclear coded subunits it leads to electron leakage to an extent that it interferes with the development, fertility and fitness of organisms, thus causing hybrid breakdown and leading to reproductive isolation [77]. There has to be an intimate compatibility between nuclear and mitochondrial genes for interbreeding to occur. In the absence of intimate compatibility, respiration and fertility are badly affected of the hybrid offspring due to introgression, thus with time this incompatibility leads to speciation [78]. For instance, COI complex is composed of 13 subunits, out of which 10 subunits are encoded by nuclear genes and 3 by mitochondrial. If the subunits of cytochrome oxidase show incompatibility, then it may lead to failure of normal working of ETC. Change in nuclear genes occurs in every generation via parental recombination, but mitochondrial DNA shows maternal inheritance. Still for proper functioning changes in one genome have to be compensated by changes in other genes. This also explains for high mutation rate in mitochondrial DNA to cope with nuclear changes during each generation.

Blow flies as unintended indicators of animal biodiversity

mtDNA could be deemed as a tool for future DNA-based evaluation of the biodiversity, specifically wild. Customary methods for assessing the biodiversity are tedious and time consuming which include studying and following foot prints of animals, evaluating their traces like feaces, droppings, study of skin moulds, feathers and hair, their nesting and resting sites, remote sensing and environmental DNA analysis [79]. Next generation sequencing of pool of blow flies, collected from carrion have proved to be very effective in detecting mammalian diversity predominantly in the vicinity, and have also been quite advantageous for deciphering amphibian and bird diversity [80]. Other haematophagous animals like blood sucking leeches detect only terrestrial biodiversity, [81] while insects like mosquites show host specificity [82] and tse tse flies can't detect small bodied mammals. Thus, for a good reason, mitochondrial DNA of Calliphoridae in coming times can prove peerless in explicating biodiversity. They nib on almost all dead organisms including mammals, amphibians, reptiles and birds both, terrestrial, arboreal and sometimes even aquatic, for they are omophagous sroungers. Thus, they prove to be valuable in detecting even rare and endangered species e.g. detection of one of the rarest antelopes (*Cephalophus jentinki*) by retrieving mammalian DNA from blow flies in tropical habitats of C^ote d'Ivoire and Madagascar.

Longitudinal fly sampling may be used to monitor mortality dynamics

When an organism dies in wild or stray and remains unattended for long it offers a feast for various scavengers and decomposers who then lavishly enjoy and multiply on the corpse. During an epidemic or large scale animal death, a humongous luncheon is opened for free visitors and the predominant among the nibblers are the blow flies. They are endowed with such powers that they can hint some hidden aspects of mortality dynamics. Blow fly derived DNA can be staunched for directed species monitoring. For instance**,** the gorilla (*Gorilla gorilla*) carnage caused by the Zaire strain of Ebola virus (ZEBOV) in Gabon and Congo in 2002–2003 was a result of group to group transmission gauged by longitudinal fly sampling [83]. Also it is one of the most valuable tools in forensics for detecting crime (murder) in humans [84].

Rearrangements in mitochondrial genes: indicator of parasitic mode of life

There exist many evidences that elucidate some sort of association of mitochondrial gene rearrangement with adoption of parasitic mode of life. For instance, in Hymenoptera [85] rearrangements of tRNA and proteincoding genes in the thrips and the psocopterans ; in nematodes via a special recombination mechanism that leads to gene inversions [86]; atp6 and nad1genes in wallaby louse [87]; Diptera, 3 species of mosquitoes [88]. The probable cause and mechanism behind this seems to be that mitochondrial DNA is prone to high rate of nucleotide substitution due to exposure to free radicals, thus witness high rate of mutations at initiation and termination sites of the mitochondrial genome. This may further lead to errors in replication, which in turn results in gene rearrangements by dulication, deletion, double strand break leading to illegitimate recombination or intramitochondrial recombination [89, 90]. It has been observed that mitochondrial genes specifically D-loop control region [91] shows high probability of mutations, thus hinting parasitic mode of life for blow flies. It also ushers path for future research on gene rearrangement differences in myasis causing and non myasis causing species of blow flies.

Progressive evolution and origin of myiasis using mitochondrial DNA

Ectoparasites generally are of three major types; obligate parasites, facultative parasites and saprophagous. Some calliphorids causing myiasis are studied using mitochondrial DNA [92, 93]. Studies have also been attempted on injury inducible gene in *Lucilia* [94]. Evolution of myiasis has also been attempted [95, 96]. Study on mtDNA serves to figure out the time of origin of myiasis in blow flies. It is anticipated that invading a living body when it is about to die or is highly critical and on the verge of death is called myiasis and it is a form of adaptation for evading competition by conquering a body before other parasites attack. Thus it is a smart way to safeguard its survival and propagation of next generation on the body as, they would be pioneers on the corpse, once the organism dies. Analyzing the mitochondrial genes COI and COII and 28S rRNA nuclear gene indicate that myiasis causing flies show multiple independent origins and their prevalence in particular zoogeographic regions relate their origin in the cretaceous period.

Resolve misidentification issues and monophyly and paraphyly issues

There are sufficient examples to quote the importance of mtDNA for resolving disconcerted misidentification issues. There can be some sort of discrepancies based on phylogenetic trees made on morphological traits; also misidentification of cryptic species is a common affair as it needs taxonomic expertise. For instance the phylogenetic tree constructed for subfamily Calliphorinae based on COI gene coincides with previously described sequences and taxonomic views for the synonymies genera *Aldrichina* and *Eucalliphora* [97]. Genus *Triceratopyga* also appears to be a synonym for the genus *Calliphora*. It has been valuable for tracing phylogeny i.e. Chrysominae is monophyletic [98]; Paraphyly in Hawaiian hybrid blowfly and Korean Lucliinae fly. Molecular identification using COI gene along with ITS (Internal Transcribed Spacer) of rDNA helped in resolving

misidentification of Calliphoridae specimens and also depicted monophyly.

Conclusion

A lot of pros and cons have been about the credibility of mitochondrial DNA as a tool for identification and phylogenetic analysis. Considerable amount of debate smokes dubious phrases both in favour and against the efficiency of mitochondrial DNA, specifically that of barcode. But it will not be an exaggeration to admit that this approach is based on hereditary units (molecular differences), which is unique to an organism. In fact, all the traits including morphological, anatomical, developmental and behavioral, which taxonomy gambles on, are ultimately governed by DNA. So it seems to be a much convenient, fast, coherent and genuine approach for identification and phylogeny when, considered along with traditional taxonomical approach. One can never deny the importance of morphotaxonomy, but in cases of fragmented, difficult, forensic and doubtful specimens, molecular approach proves wonders. The diminishing natural resources and depriving existing niches, alarms the urgency for earthy conservation deceit. The use of mtDNA and public access to these databases through Web portals will solve the quandary.

It is often misunderstood and feared as a felonious field which aims at replacing and demolishing the existing traditional taxonomy. Remit the recalcitrance in quoting that, when classical taxonomists have shown a lot of generosity adopting and integrating anatomy, cytology and behavior studies as a part of taxonomy, then, why do they hesitate in accepting molecular approach as one of its off shoots, which would in turn, aid in accomplishing its ultimate aim of classifying the vast biodiversity. At this point, we can conclude that mtDNA is definitely an anointed tool that tends to unlock mysteries, catalogue voluminous biodiversity and intensify our efforts to tame and conserve biodiversity but incontestably endorsed under the parenthood of traditional taxonomists. It is also recommended that phylogenies deciphered entirely on the basis of mtDNA must be further certified using nuclear genes for flawless authentic results.

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