International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2014): 5.611

Cytochrome Oxidase Subunit I Gene Based Phylogenetic Description of Common Mormon Butterfly *Papilio polytes* (Lepidoptera: Papilionidae)

Akhilesh V. P.[1], Sebastian C. D.[2]

Molecular Biology Laboratory, Department of Zoology, University of Calicut, Kerala 673 635 India

Abstract: ***Lepidoptera is one of the largest orders of insects that include moths and butterflies. Most of the lepidopterans are morphologically similar, therefore the identification of these insect are tedious using morphotaxonomy and that is detrimental in designing a better strategy to control and conserve them. These are an extremely important group of 'model' organisms used to investigate many areas of biological research, including such diverse fields as navigation, pest control, embryology, mimicry, evolution, genetics, population dynamics and biodiversity conservation. Knowledge of lepidopteran genomics will create new methods of insect pest management and will contribute to sustainable agriculture, protection of the environment and the maintenance of biodiversity. In this study we have amplified cytochrome oxidase I gene of Papilio polytes for species identification and constructed phylogenetic tree for recognizing evolutionary relationship.***

Keywords: Molecular systematics, DNA barcoding, mitochondrial COI gene sequences

1. Introduction

Identification and preservation the genetic diversity is vital for Lepidoptera since it is one amongst the widespread and wide recognizable insect orders in the world. The strength of Lepidopteran genomics lies within the diversity of the group as a whole. Though there are clear insect models for genetic analysis (*Drosophila)* or disease vectors (*Anopheles)*, the Lepidoptera are rich in diverse model systems for a range of biological processes. Much of our knowledge of endocrinology, reproduction, behavior and immunity is derived from the studies of Lepidoptera [1]. They are important test case for the use of mitochondrial DNA in species identification. This is an important order among class Insecta that have appeared on endangered species list.

The genome of Lepidoptera is characterized by larger size and higher chromosome number, typically about 30. Genetic crosses are routinely accomplished in Lepidoptera. The GC content of Lepidopteran DNA is about 35-40%. The large body size, accessible genetics, and extreme diversity of Lepidopteran species are important experimental advantages. Identification at the molecular level is important since phenotypic variability and convergent evolution causes misidentification of many cryptic species, in addition sexual dimorphism causes more confusion to species identification [2]. To solve this problem in the taxonomy, recently a short nucleotide sequence of mitochondrial DNA (COI) is widely accepted as a marker for the accurate and easy identification of species. DNA sequences of the mitochondrial cytochrome oxidase I (COI) gene can serve as a DNA barcode for identifying all kinds of animals [3]. Phylogenetic analysis using COI gene sequences were extensively carried out by several workers in different group of organisms like southern house mosquito *Culex quinquefasciatus* [4], *Armigeres subalbatus* mosquito [5], green bottle fly *Lucilia sericata* [6], *Herpetogramma stultalis* [7], white backed plant hopper *Sogatella furcifera* [8], Asian honeybee *Apis cerana* [9] and lepidopteran species [10]. It is an important advancement in molecular biology for rapidly and cost-efficiently using a short reference sequence of DNA to help catalog and inventory biodiversity.

Classification of insect species is critical for both basic and applied research. The classification based on morphological features poses problems in many groups of insects because of their small size, morphological attributes that change as function of environment and prevalence of biotypes and species that cannot be easily differentiated by morphological criteria [11]. There have been many attempts to use techniques of molecular taxonomy to insects and these have yielded valuable result [12] [13].

Theory of the evolution of supergene which explains this mimicry studied in *Papilo polytes*, hence their identification at molecular level is very important. Besides, these lepidopteran insects serve as important model organisms for studies of genetics, physiology, development, ecology, evolutionary biology and insect-plant co-evolution [14]. Insect phylogenetic studies normally include use of morphological features as phenotypic characters and ontogenic stages, or pathways as developmental traits. In recent times, RNA, DNA, allozymes and amino acid sequences have been used to infer evolutionary relationships among insects as molecular markers [15]. Such studies are more important to rebuild the status of ancestral characters to provide an idea on divergence of insects and their homology [16] [17].

Morphological characters have a mosaic distribution and it is extremely difficult to find out phylogenetic indications from such variable data with a small number of functional characters [18]. In addition, phylogenetic analysis of species on the basis of morphological attributes may be faced with

problems because these features are variable with the environment and geographical locations. Molecular markers are more reliable in terms of the number of available taxonomic characters displaying an appropriate level of variability and thus they offer an alternate tool [3]. The present work reveals the partial mitochondrial COI gene sequences of *P. polytes* their genetic divergence and phylogenetic status.

2. Methodology

Collection and preservation

The *P. polytes* were collected from Botanical Garden of Calicut University (CUBG), Kerala using hand sweeping net. The sample were morphologically identified and placed in separate glassine envelope with 70% ethanol, assigned code number and stored at -20 ^{0} C as voucher specimen until further use.

DNA Extraction, Amplification and Sequencing

DNA was extracted from the leg piece of the specimen using phenol chloroform method [19]. The obtained DNA was amplified for COI gene using forward primer, 5'-GGTCAACAAA TCATAAAGATATTGG-3' and reverse primer 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'. The PCR reaction mixture consisted of 2 ng of genomic DNA (1µl), 0.5µl each forward and reverse primer with at a concentration of 5 µM, 0.5 µl $dNTP_S$ (2.5mM), 2.5 µl 10X reaction buffer, 0.5 µl Taq polymerase(5U/ µl) and 19.5 µl H_2O. The PCR profile consisted of an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 10s at 95°C, 30s at 55°C and 45s at 72°C and ending with a final phase of 72°C for 3 min. The PCR products were resolved on a 2% TAE- agarose gel, for confirmation of the target gene amplification. The PCR product was column purified using Fermentas, GeneJET PCR purification kit. The purified PCR product was sequenced using Sanger's method [20]. The obtained sequence was checked for its quality by examining chromatograms and the forward and reverse sequence were assembled using Clustal W. Sequence analysis and sample identification were done by inputting the trimmed sequence in NCBI's BLAST tool. Phylogenetic tree was then constructed using MEGA 6 software [21].

3. Result and Discussion

The present study was centered on *P. polytes* otherwise known as the Common Mormon butterfly (Figure 1) whose habitat ranges widely from Sri Lanka and India to Indo-China, S. Japan, the Philippines and Sunda Islands in SE Asia. Its caterpillars feed on Rutaceae plant varieties (*Citrus* and allied genera, as well as lemon and orange plants) and the butterfly is palatable to its predators like birds. The feminine kinds of this species derive protection from such predators with their resemblance to distantly related, chemically protected (toxic) *Pachliopta* butterflies, which experience eating evadance from birds. This type of resemblance is called Batesian mimicry, which is restricted in *P. polytes* to females, and the toxic species they resemble are called "models". Throughout its range, and in numerous subspecific variations, *P. polytes* has a single non-mimetic male form, with which cyrus, a male-like non-mimetic female form, co-occurs. Most populations also have up to two female forms that mimic locally available Pachliopta species. The female limited mimetic polymorphism reaches its apex in Sri Lanka and peninsular India where, in the subspecies *Papilio polytes romulus*, three female forms fly together: Form cyrus is male-like and non-mimetic form *polytes* (*stichius*) mimics *Pachliopta aristolochiae* and *Pachliopta pandiyana* (*Pachliopta jophon* in Sri Lanka), and form *Romulus* mimics *Pachliopta hector*.

Figure 1: *Papilio polytes*

In our studies, the resolution of phylogeny based on COI sequences reveals phylogenetic relationship of the species. DNA Sequences of good quality and length of 580 bp were generated in the present study. BLAST result concludes that COI gene sequence of *Papilio* species in this study was found to be novel. The evolutionary history of *Papilio polytes* is inferred using Neighbour joining method which shows clearly inter and intra species divergence (Figure 2). NJ clustering analysis showed that *Papilio polytes* from Calicut, Kerala belong to single monophyletic clade without any overlap, even though these species are separated by large geographic distances. The present results indicate that an identification system for insect life based on the COI gene will be highly effective. Although COI divergences appear too low to regularly enable species diagnosis within the insects, generic-level identifications remains a prospect.

The *P. polytes* found from Kerala is exhibiting 99% similarity with *P. polytes* from Pakistan (KC 158441) and China (HM 246458). Thus intra species nucleotide divergence between *P. polytes* calculated shows 1% divergence; here geographical barrier may act as evolutionary tool for the sequence divergence. Inter species divergence is found to be between 6-8%. The evolutionary divergence of *P. polytes* is given in the Table 1. Sequences generated in this study were submitted to GenBank, with Accession number KJ 636441 (*Papilio polytes*) and can be used as molecular barcode of this species. The present study on molecular evolutionary analysis using partial mitochondrial cytochrome oxidase subunit I (COI) gene sequence explicates phylogenetic relationships of *P. polytes*. The study suggests that the best phylogenetic inferences can be created through moderately divergent nucleotide data from mitogenomes, of which the COI gene is best studied.

Table 1: Evolutionary Divergence between related species on COI gene sequences of *Papilio polytes*

Species name with GenBank Accession number	*% of Divergence*
KC158441 *Papilio polytes*	1%
HM246458 *Papilio polytes*	1%
KF723532 *Papilio macilentus*	6%
HM246453 *Papilio memon*	6%
HM246457 *Papilio arcturus*	6%
AY457581 *Papilio protenor*	6%
KF404031 *Papilio aegeus*	7%
JQ982076 *Papilio hopponis*	7%
JQ982038 *Papilio arturus*	7%
JF681018 *Papilio bridge*	7%
KF226559 *Papilio helenus*	8%
KF401753 *Papilio fuscus*	8%
KC433408 *Papilio maackii*	8%
JQ982147 *Papilio syfanius*	8%

Reference

[1] N Koshy, KM Ponnuvel, RK Sinha SRH Qadri. Silkworm nucleotide databases - Current trends and future prospects. Bioinformation, **2(7)**: 308-310, 2009.

[2] C Mariavon, K Helena, P Maria, R Jouko. DNA barcoding: a tool for improved taxon identification and detection of species diversity. Biodiversty Conservation. **20**: 373–389, 2011.

[3] PDN Hebert, R Sujeevan, R Jeremy. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. **270**: 96-99, 2003.

[4] K Rukhsana, KS Ashitha, K Mashoor, CD Sebastian. Molecular phylogeny analysis of Southern house mosquito, *Culex quinquefasciatus* (Diptera: Culicidae) derived from mitochondrial DNA sequences. International Journal of Research, **1(5)**: 844-853, 2014.

[5] PU Bindu, CD Sebastian. Genetic structure of mitochondrial cytochrome oxidase subunit I gene ofthe mosquito, *Armigeres subalbatus.* International Journal of Research, **1(10)**: 49-56, 2014.

[6] KP Priya Bhaskaran, CD Sebastian. Molecular barcoding of green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) using COI gene sequences. Journal of Entomology and Zoology Studies, **3(1)**: 10-12, 2014.

[7] VP Akhilesh, CD Sebastian. Molecular barcoding and phylogeny analysis of *Herpetogramma stultalis* (Lepidoptera: Crambidae) using cytochrome oxidase subunit I gene sequence. International Journal of Advanced Life Sciences, **7**: 463-466, 2014.

[8] K Sreejith, CD Sebastian. Phylogenetic analysis and sequencing of the mitochondrial cytochrome oxidase subunit I (COI) of white backed plant hopper, *Sogatella furcifera* (Horvath). International Research Journal of Pharmacy, **5 (12)**: 887-890, 2014.

[9] K Rukhsana, VP Akhilesh, CD Sebastian. Deciphering the molecular phylogenetics of the Asian honey bee, *Apis cerana* (Hymenoptera: Apidae) and inferring the phylogeographical relationships using DNA barcoding, Journal of Entomology and Zoology Studies, **2(4)**: 218-220, 2014.

[10] E Pavana, CD Sebastian. Genetic diversity and phylogenetic analysis of lepidopteran species by molecular barcoding using COI gene sequences. International Journal of Science and Research, **3(5)**: 450-452, 2014.

[11] T Wei-Chih, Y Wen-Bin. DNA-Based Discrimination of Subspecies of Swallowtail Butterflies (Lepidoptera: Papilioninae) from Taiwan. *Zoological Studies*, **47**: 633-643, 2008.

[12] B Xiang, TD Kochar. Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Diptera). Genome, **34**: 306-311, 1991.

[13] J Tang, K Pruess, EW Cupp, TR Unnasch. Molecular phylogeny and typing of blackflies (Diptera: Simuliidae) that serve as vectors of human or bovine onchocerciasis, Med. Vet. Entomol., **10**: 228-234, 1996.

[14] AD Roe, SJ Weller, J Baixeras, J Brown, MP Cummings, DR Davis, M Horak, AY Kawahara, C Mitter, CS Parr, JC Regier, D Rubinoff, TJ Simonsen, N Wahlberg and A Zwick. Evolutionary framework for Lepidoptera model systems, in Genetics and Molecular biology of Lepidoptera, edited by M R Goldsmith & F Marec (CRC Press, Boca Raton, FL, USA), 1-24, 2010

[15] MR Goldsmith, F Marec. Molecular biology and genetics of the Lepidoptera. CRC Press, Boca Raton, FL, USA. 368 pp., 2010.

[16] PM Mabee. Developmental data and phylogenetic systematic: Evolution of the vertebrate limb, Integr. Comp. Biol., **40**: 789-800, 2000

[17] JC Regier, A Zwick, M Cummings, AY Kawahara, SP Cho. Toward reconstructing the evolution of advanced moths and butterflies (Lepidoptera: Ditrysia): An initial molecular study. BMC Evol. Biol., **9**: 280, 2009.

[18] VA Korneyev. Phylogenetic relationships among the families of the superfamily Tephritoidea; in Fruit flies (Tephritidae): Phylogeny and evolution of behavior, edited by M Aluja and A Norrbom. CRC Press, Boca Raton, FL, USA, 3-22. 1999.

[19] J Sambrook, EF Fritshi, T Miniatis. Molecular cloning: A laboratory manual 2nd edition. New York: Cold Spring Harbor Laboratory Press, 2001.

[20] Sanger, F. and Coulson, A. R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Journal of Molecular Biology*, **94 (3)**: 441–448, 1975.

[21] K Tamura, G Stecher, D Peterson, A Filipski, S Kumar. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, **30(12)**: 2725-2729, 2013.

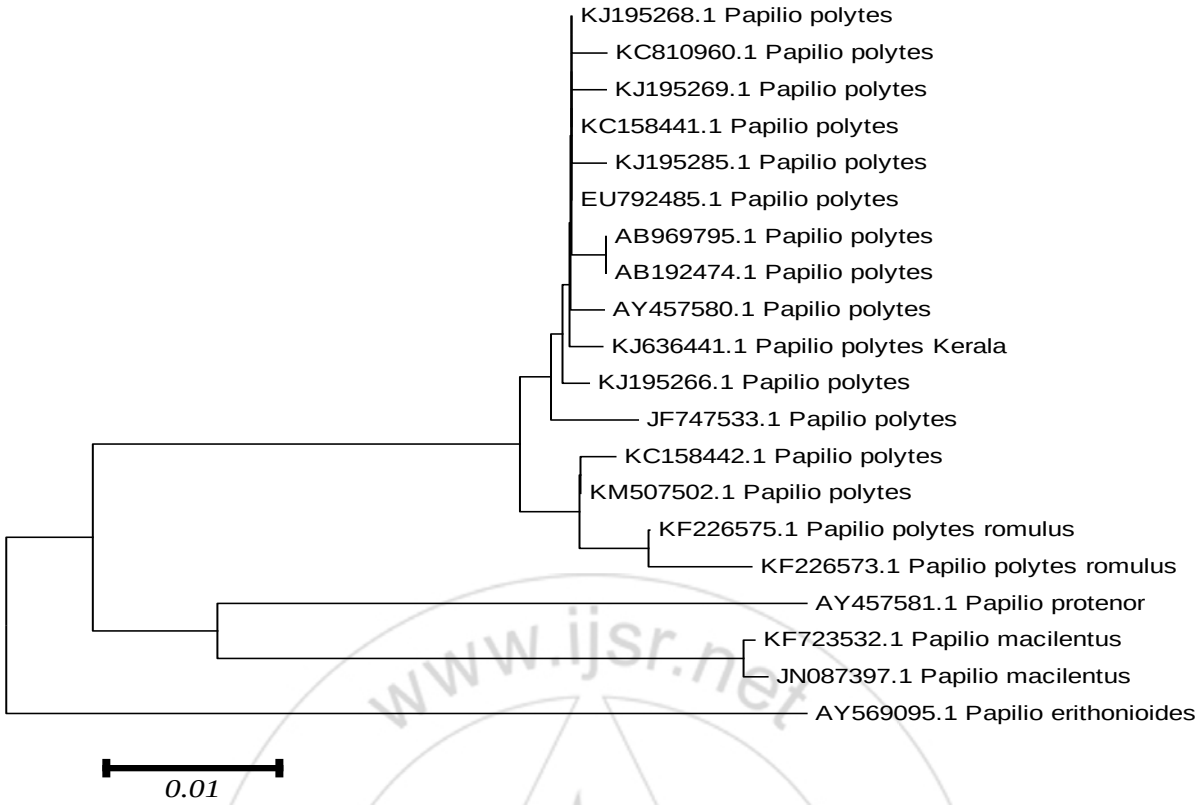

Figure 2: Phlogenetic status of Papilio polytes using neighbor joining method from NCBI