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## Mitochondrial DNA Analysis Revealed Gondwanan Origin of The Common Evening Brown Butterfly, *Melanitis Leda* (Lepidoptera: Nymphalidae)

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**Abstract** The common evening brown butterfly, *Melanitis leda* is widely distributed in Africa, South Asia, South-East Asia and Australia. Here we report the partial sequence of cytochrome oxidase subunit I (COI) of *M. leda* isolated from Kerala, India. Comparison of the COI sequence of *M. leda* revealed close similarity to *M. leda* of Thailand, Madagascar and Tanzania than that of Papua New Guinea and Australia. The results indicate the common origin of the Common Evening Brown butterfly in Gondwana which later evolved into two clads with the geographical drifting of the countries. Genetically, *M. leda* of India is closer to those from Madagascar than that of Australia, depicting the rate of genetic variation of the insect has a strong co-relation with the time of its separation during the period of continental drift and the changes in environmental factors.

**Keyword:** Cytochrome oxidase, *Melanitis leda*, phylogeny, Gondwana

### Introduction

*Melanitis leda*, the Common Evening Brown butterfly seen flying at dusk, is widely distributed in Africa, South Asia and South-east Asia extending to parts of Australia. This leaf foliating insect is considered as a minor pest of paddy. Its larvae, the green-horned caterpillar, feed on the margin and tip of the leaves and remove the leaf tissue and leaf veins. The damage caused by the green-horned caterpillar is almost similar to that of grasshoppers and armyworms. Severe infestations of the larvae results in heavy chlorophyll loss

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due to the removal of leaf tissue and in a substantial loss of paddy yield (Heinrichs and Barrion, 2004).

Cladistics analysis of mitochondrial gene (COI) and two nuclear gene sequences revealed four major clades in Nymphalidae family (Wahlberg *et al.*, 2003). Murray and Prowell (2005) reported the phylogeny and evolutionary history of the neotropical Satyrine subtribe Euptychiina using the COI and elongation factor gene sequences. Gaikwad *et al.* (2012) studied the utility of DNA barcoding for the identification of nymphalids butterflies isolated from India. Here we report the partial sequence of COI gene of *M. leda* isolated from Kerala, India and review its possible origin and divergence.

### **Material and methods**

The adults of Common Evening Brown butterfly, *M. leda* were collected from the paddy fields of Kerala. The genomic DNA from the larvae was isolated using GeNei Ultrapure Mammalian Genomic DNA Prep Kit (Bangalore GeNei, Bangalore) as per the Manufacturer's instruction.

The COI gene was amplified using the forward primer with DNA sequence 5' CATTGGAGATGACCAAATTTATAATG -3' and reverse primer with DNA sequence 5'- TGAAATTAATCCAAATCCAGGTAAA-3'. The PCR reaction mixture consisted of 2 nanogram of genomic DNA (1 µl), 1 µl each forward and reverse primers at a concentration of 10 µM, 2.5 µl of dNTPs (2 mM), 2.5 µl 10X reaction buffer, 0.20 µl Taq polymerase (5 U/µl) and 16.8 µl H<sub>2</sub>O. The PCR profile consisted of an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 10 sec at 95°C, 10 sec at 55°C and 1 min at 72°C and ending with a final phase at 72°C for 3 min. The PCR product was column purified using UltraClean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the Manufacturer's instruction and sequenced from both ends using the Sanger's sequencing method at SciGenom Laboratories Ltd., Cochin.

The forward and reverse sequences were assembled by using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2>) after removing the forward and reverse primer sequences and the consensus was taken for the analysis. The phylogenetic genetic level identification of *M. leda* was done using BOLD Animal Identification System (<http://www.barcodinglife.com>) using sequence similarity and tree based identification (BOLD TaxonID Tree). The percentage of each nucleotide in the COI codon of *M. leda* was determined by MEGA5 software.

## Results

The PCR amplification of the COI sequence of *M. leda* isolated from Kerala, India yielded a single product of 563bp (GeneBank Accession No. KC433403). The nucleotide composition analysis revealed that the percentage of nucleotide present in the COI sequence of *M. leda* isolated from Kerala is identical with that of *M. leda* isolated from the different locations of India (GeneBank Accession No. GU012619, GU012624, GU012554) and Thailand (GeneBank Accession No. HQ962136, HQ962145, HQ962140). It showed +2% variation in the composition of nucleotide 'C' and -2% variation in codons third position compared to *M. phedima* (HQ962137, HQ962138).

The nucleotide sequence analysis of *M. leda* revealed the presence of conserved nucleotide sequences in specific locations. The nucleotide 'C' in the 14th, 38th and 218th positions, nucleotide 'G' in the 149th and 162th and nucleotide 'T' in 251th positions differentiate *M. leda* from most of the Lepidopteran species. The genetic similarity study showed that there is 97.19-100% similarity with the *M. leda* isolated from India and from the geographically isolated locations like Madagascar, Tanzania, Thailand, Kenya, Gabon Papua New Guinea and Australia. The COI sequence of *M. leda* isolated from Kerala showed 100-99.80% similarity with species of *M. leda* isolated from the other parts of India, Thailand, Madagascar and Tanzania. The *M. leda* isolated from Kerala has 94.57%, 94.38%, 92.13% and 91.76% similarity with *M. costantia*, *M. phedima*, *M. amabilis* and *M. zitenius* respectively (Fig. 1).

The phylogeny tree constructed (Fig. 2) by BOLD Animal Identification System (<http://www.barcodinglife.com>) clearly depicts the phylogenetic position of *M. leda* isolated from Kerala. The *M. leda* isolated from different parts of the world are arranged in 2 clads. The *M. leda* isolated from India, Madagascar, Tanzania, Thailand, Kenya, China and Gabon form one clad and the *M. leda* isolated from the Papua New Guinea and Australia form another clad.

## Discussion

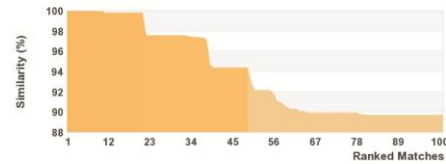
The genetic structure analysis of *M. leda* clearly reveals its phylogenetic status and origin. The variation in the COI sequence within the same species will be very low averaging 0.43% while congeneric species possess higher sequence divergence averaging 7.7% (Hebert *et al.*, 2009). *M. leda* isolated from Papua New Guinea and Australia showed higher variation (2.43-2.66%).

This high intraspecific variation is may be due to the geographical isolation of this population.

Identification Summary:

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Arthropoda	100
Class	Insecta	100
Order	Lepidoptera	100
Family	Nymphalidae	100
Genus	Melanitis	100

Similarity Scores of Top 100 Matches:

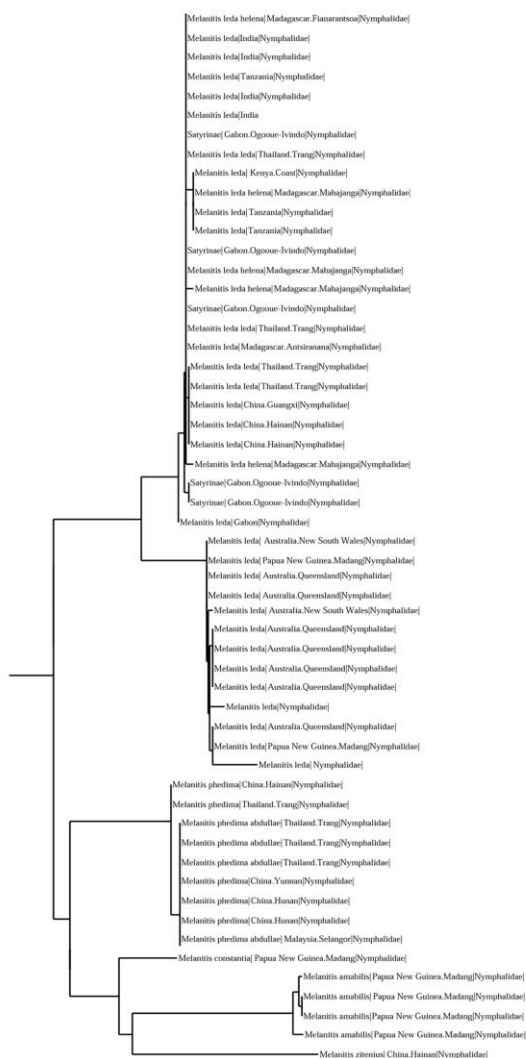


TOP 99 Matches:

Display option: Top 99

Phylum	Class	Order	Family	Genus	Species	Similarity (%)	Status
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	100	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	100	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	100	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	100	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	100	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledahelena</i>	100	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledahelena</i>	100	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledaleda</i>	100	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledaleda</i>	100	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledahelena</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledaleda</i>	99.81	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	99.81	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>ledahelena</i>	99.8	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.46	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.34	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.27	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>leda</i>	97.19	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>constantia</i>	94.57	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedimaabdullae</i>	94.38	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedimaabdullae</i>	94.38	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedimaabdullae</i>	94.38	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Published <a href="#">🔗</a>
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedima</i>	94.38	Early-Release
Arthropoda	Insecta	Lepidoptera	Nymphalidae	Melanitis	<i>phedimaabdullae</i>	94.38	Early-Release

Figure 1. Percentage of genetic similarity of *M. leda* isolated from Kerala, India with insects isolated from other locations (generated by BOLD Animal Identification System, <http://www.barcodinglife.com>)



**Figure 2.** A portion of BOLD TaxonID Tree showing the phylogenetic relationship of *M. leda* isolated from Kerala.

Before the evolution of the Asia, Africa and Australia, India was the part of supercontinent Gondwana, which includes Australia, Antarctica, South America, Newzealand, New Caledonia, New Guinea, Arabia, Iran, Africa and

Madagascar. The break-up of the supercontinent started in ~170 Million years ago (Ma), following the drifting of South America–Africa from Madagascar, India, Antarctica and Australian plate. About 132 Ma Australia–Antarctica began to drift away from India–Madagascar (Powell *et al.*, 1988; Muller *et al.*, 2000; Brown *et al.*, 2003) and 90–85 Ma Madagascar separated from Indian plate (Storey *et al.*, 1995; Torsvik *et al.*, 2000). New Guinea separated from Australia about 30Ma. The sequential breakup of the supercontinent Gondwana in different geographical time caused the successive division of an ancestral biota (Sanmartin&Ronquist, 2004). *M. leda* is distributed in Asia and Africa, Australia and Papua New Guinea (Dale, 1994, Heinrichs&Barrion, 2004). Interestingly most of the geographical areas of *M. leda* distribution were the part of Gondwana except some parts of Asia. The similarity between the fauna of India and Africa prove their common origin from Gondwanaland (Krause & Maas, 1990; Zardoya *et al.*, 1996; Cracraft, 2001; Cooper *et al.*, 2001; Biju&Bossuyt, 2003; Noonan &Chippindale, 2006). The genetic similarity analysis and geographical distribution of *M. leda* also indicate its origin from the supercontinent Gondwana. After the separation, India continued its rapid northward migration and colliding with southern Asia (Barron & Harrison, 1980; Lee &Lawver, 1995). Conti *et al.* (2002) argued that Crypteroniaceae are from Gondwanan origin and they reached in the tropical Asia by transportation from Gondwana on the Indian “raft”. Likewise the *M. leda* seen in China and Thailand may have come from the Indian subcontinent.

The Australian vegetation and climate substantially changed after its separation from Antarctica (Hill, 2004). The *M. leda* isolated from India showed more variation with the *M. leda* isolated from Papua New Guinea and Australia than Madagascar. This may be due to the early separation of Australian and New Guinea plates from the Indian plates than Madagascar plate and may be due to the changes in environmental factors of the Australian plate after its separation from the Antarctic plate. The drift between Madagascar plate and Indian plate occurred as a later event and therefore the *M. leda* isolated from the India and Madagascar showed close similarity. The results indicate that the Common Evening Brown, *M. leda* might have originated as early as the origin of the supercontinent Gondwana which later spread to the adjacent countries after the Gondwana separation.

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