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RESEARCH ARTICLE

MOLECULAR PHYLOGENY OF THE SOUTHERN BIRDWING BUTTERFLY, *TROIDES MINOS*, INDICATES ITS ORIGIN BEFORE THE RISE OF HIMALAYAS

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ABSTRACT

The Southern birdwing butterfly, *Troides minos* Cramer, 1779 is endemic to Western Ghats. This has many synapomorphic features with other members of *Troides* butterflies in Southern Asia. Here we report the partial sequence of mitochondrial cytochrome oxidase subunit I (COI) gene of *T. minos*, isolated from Northern Kerala (GenBank Accession Number: HQ424201.1). The sequence analysis of the COI DNA revealed that *Troides minos*, is closely related to *Troides aeacus* and *Troides helena* isolated from Indian subcontinent and they were evolved from the similar ancestors. The divergence of *Troides* butterflies of Asia began before the joining of Indian Plate with Eurasia and the rise of Himalayas. Molecular data the supports the existing view of origin and distribution of their ancestors from Northern hemisphere to the Indian subcontinent and Indonesia. The COI sequences of *T. minos* also demonstrate that their species diversification took place after the rise of Himalayas. The results of the present study also indicate the divergence of *Troides* butterflies of Indian subcontinent and endemism of *T. minos* in Western Ghats are associated with the loss of continuity in distribution of their ancestors as they become extinct in Deccan Plateau. The extinction of *Troides* population in Deccan Plateau was associated with the climatic changes resulted from the vicariance events like rise of Himalayas and disappearance of Tethys Sea.

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INTRODUCTION

Troides minos Cramer, 1779 commonly known as Southern birdwing butterfly or Malabar birdwing butterfly (Evans, 1927), comes under the genus *Troides* of family Papilionidae. The *T. minos* is endemic to Western Ghats comprising Goa, South Kanara, Coorg of Karnataka, throughout Kerala, Nilgiris and Palani Hills of Tamilnadu (Moore, 1858; Evans, 1927; Talbot, 1939; Wynter-Blyth, 1957; Kunte, 2005; Kehimkar, 2008). They are common along the south-western Peninsular India and characterised by large wing span and slow flight. Like other *Troides* butterflies, *T. minos* use plants of Aristolochiaceae family as their larval food.

The tribe *Troidini* involving Birdwing butterflies are distributed along India, South-East Asia, North-Western Australia, Madagascar, South America except Chile and South Mexico of North America. The Birdwing butterflies of India involve the genus *Triodes*, distributed along Western Ghats, North-Eastern Himalayas, Bihar, Assam, Nagaland, Sri Lanka, Andaman and Nicobar Islands (Collins and Morris, 1985). The Southern birdwing butterfly *T. minos* recently reported from Algar Hills of Eastern Ghats near to Madurai city, where they are not common but occasional visitors or migrants (Sharmila, 2014). *T. minos szechwanus* seen in Sichuan of Peoples Republic of China, is a subspecies of *T. minos* (Okano and

Okano, 1983). They are morphologically more similar to *T. minos* but geographically isolated. The *T. helena* Linn. is a related species of *T. minos* seen along Orissa, Bihar, North-Eastern India, Andaman and extend their presence to South-East Asia (Moore, 1858; Evans, 1927; Talbot, 1939; Kunte, 2005; Kehimkar, 2008).

The *T. minos* of Western Ghats was considered as *T. helena minos* a sub species of *T. helena*, seen along North-Eastern Himalayas (Talbot, 1939). It has been reported that *T. minos* is a subspecies of *T. aeacus* (Okano and Okano, 1983). The *T. minos* of Western Ghats is an isolated species from other members of the genus as they are separated from other related species by an extended land mass of Deccan Plateau more than 600 km wider in Peninsular India. The *T. darsius* Gray, 1852 of Sri Lanka is the species geographically more closely located to *T. minos* (Collins and Morris, 1985), but their molecular relationships are not known for want of genetic data.

The biogeographical distribution pattern of different swallow tail butterflies studied based on molecular data and their diversification revealed their distribution paths from their origin in Northern hemisphere near Siberia and their colonisation in new locations rather than vicariance events (Condamine et al., 2013). The exact phylogenetic position of *T.*

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minos is not described in earlier studies for want of a molecular data and hence this study.

MATERIALS AND METHODS

Collection of specimens and isolation of DNA

The *T. minos* butterflies were collected from Alakkode of Kannur District, a part of Western Ghats in North Kerala which is a part one of the 34 biodiversity hotspots of the world. The specimens were kept in the laboratory at -20°C , till genomic DNA isolation. The genomic DNA of the butterfly was isolated from one of the leg muscles, using 'Macherey-Nagel Nucleospin Tissue' kit (Macherey-Nagel, Duren, Germany) following the manufacturers' instructions.

PCR amplification of COI DNA

The PCR reaction mixture consist of 2 ng genomic DNA (1 μl), 1 μl each of forward and reverse primers (10 μM), 2.5 μl of dNTPs (2 mM), 2.5 μl reaction buffer (10X), 0.20 μl Taq polymerase (5 U/ μl) and 16.8 μl distilled H_2O .



Fig.1a Eggs lay on Aristolochia indica stem

The PCR temperature profile consisted of $95^{\circ}\text{C}/3$ minutes for initial denaturation followed by 45 cycles of $95^{\circ}\text{C}/10$ seconds, $50^{\circ}\text{C}/45$ seconds, $72^{\circ}\text{C}/45$ seconds and with a final extension of 72°C for 3 minutes. The PCR amplified product was column purified using Mo Bio Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the manufacturer's instructions. The purified product was sequenced from both ends with forward and reverse primers using the Sanger's sequencing method at SciGenom Labs, Cochin. The forward and reverse sequence was aligned and the consensus sequence was used for analysis. The phylogeny analysis was done using the NCBI nucleotide BLAST software (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>).

RESULTS AND DISCUSSION

The *T. minos* isolated is characterised with black dots at dorsal and ventral sides of yellow coloured abdomen (Fig.1c). The forewings are black coloured with white shades along their discocellular regions. The hind wings are bright yellow with black coloured veins and black markings at their margins. A black spot was observed between 5th and 6th discocellular veins of the hind wing (Fig.1d).



Fig.1b. Larvae feeding on Aristolochia indica leaf



Fig.1c. Adult Troides minos (dorsal view)



Fig.1d. Adult *T. minos* (ventral view) White coloured lining of discocellular veins, Black spot between 5th and 6th discocellular veins, Black spots at ventral side of abdomen

The partial sequence (GenBank accession No:HQ424201.1) of COI of *T. minos* showed 98% similarity to that of *T. aeacus* and 97% to that of *T. Helena*. The partial peptide sequences derived from the conceptual translation of the DNA also showed 97% similarity to that of both *T. aeacus* and *T. helena*. The partial COI DNA sequence of *T. minos* specimen isolate CUJ1 obtained in the present study is genetically distinct from that of *T. aeacus* and *T. helena*. Phylogenetic and molecular evolutionary analysis was conducted using NCBI nucleotide BLAST tool (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>). The COI partial sequences of *T. helena*, *T. aphrysus*, *T. haliphron*, *T. aeacus*, *T. prattorum*, and *T. staudingeri*, used for comparison are retrieved from the nucleotide database of NCBI (<http://www.ncbi.nlm.nih.gov/nucleotide/>). The tree analysis with Fast Minimum Evolution (Desper and Gascuel, 2004) and Neighbour joining methods (Saitou and Nei, 1987) yielded same type of tree topology.

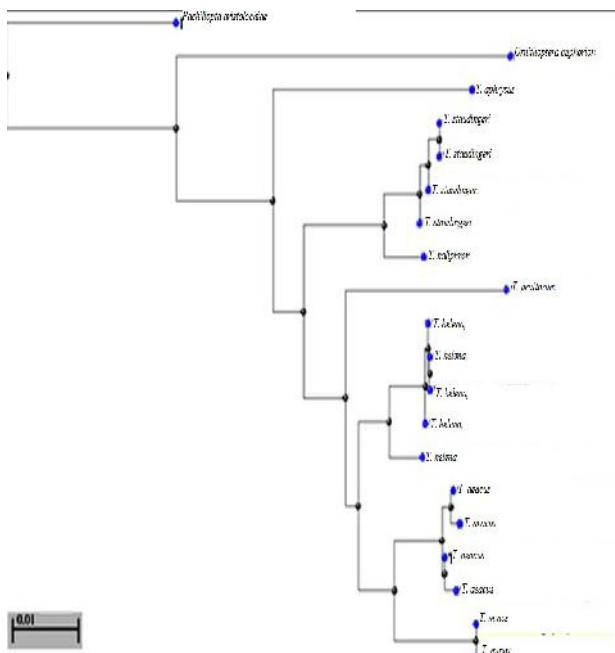


Fig.2. Evolutionary relationship of *Troides* butterflies, the phylogenetic tree was constructed by Fast Minimum Evolution method using the partial nucleotide sequence of COI gene (GenBank Accession Number: HQ424201.1). The *Ornithoptera euphorion* and *Pachilioptera aristolochiae* were two out groups used for rooting the tree. The tree also indicates that *T. helena* and *T. staudingeri* are early evolved than others. The *T. aeaacus* remains close to *T. minos* in the same clad and the *T. minos* is recently evolved. The tree is prepared by using online BLAST tool of NCBI (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>) with Fast Minimum Evolution method (Desper and Gascuel, 2004), and selecting maximum fraction of mismatched bases 0.75.

The tree diagram is constructed by comparing the partial COI sequences of *T. minos* endemic to Western Ghats (Collins and Morris, 1985) with the similar DNA sequences of *Troides* butterflies geographically separated from them with more than 600 km. The *T. aeaacus* is seen along North–Eastern India, Nepal, Myanmar, Peoples Republic of China, Thailand, Laos, Vietnam, Taiwan, Cambodia, Peninsular Malaysia, Indonesia and Sumatra (Collins and Morris, 1985). The *T. Helena*, is seen along Nepal, India, Bangladesh, Myanmar, Peninsular and Eastern Malaysia, Indonesia, Thailand, Laos, Kampuchea, Vietnam and Southern China. In India they are prominent along Sikkim, West Bengal, Odisha and in Andaman and Nicobar Islands (Collins and Morris, 1985).

The *T. helena*, *T. minos* and *T. aeaacus* are three species of *Troides* found in the India. The other sequences of *Troides* butterflies used in the present study are *T. pratorum*, an endemic species of Indonesia (Dixon, 1996), *T. haliphron* seen along South-West Sulawesi Island of Indonesia, *T. staudingeri* of Leti Island and Moa Island (Indonesia), Kisar island (Timor), Babar Island and Wetar Island and *T. amphrysus ruficollis* seen along Peninsular Malaya, Thailand and Myanmar (Collins and Morris, 1985) of the six evolutionary groups recognised in the tree diagram, the first group involve *T. minos* which is related to *T. aeaacus* in the same clad as they are monophyletic. *T. minos* and *T. aeaacus* are closely related to *T. helena* in the second clad. The tree diagram also showed *T. staudingeri* and *T. helena* are most early evolved among the eastern forms while *T. aeaacus* and *T. minos* are of recent origin.

CONCLUSION

The presence of *T. minos szechwanus* in Sichuan of Peoples Republic of China located North of Himalayas depicts that their ancestors are migrated from Siberia of North pole to Indian subcontinent before the rise of Himalayas. The ancestral *Troides* enjoy a continuous distribution from Peoples Republic of China to Indian subcontinent and it further extends to Sri Lanka.

Six phylogenetic groups in the phylogenetic tree constructed with BLAST using partial sequence of mitochondrial COI gene of *T. minos* revealed that they are geographically distinct in their distribution. This supports the existing view of Siberian origin of *Troides* ancestors and their distribution in the South – East Asia and their diversification into species with vicariance events and associated climate changes.

The phylogenetic tree also revealed two major groups of *Troides sp*: one occur in Indian subcontinent and the other in Indonesian Archipelago. The later vicariance events took place in Indian and Indonesian regions that affected the species diversification of these two groups. The rise of Himalayas and disappearance of Tethys Sea induce climate changes in Deccan Plateau. This leads to the extinction of *Troides* butterflies of Deccan Plateau and the above event also resulted in the loss of continuity in the distribution of *Troides* butterflies of Indian subcontinent, and their subsequent isolation and speciation. Similarly, the loss of land bridges and the increase in depth and distance of sea between Indonesian archipelagos isolated the *Troides* populations that lead to the speciation of Indonesian group. The first group was diversified into *T. helena*, *T. aeaacus*, *T. minos* and *T. pratorum* and the second group was diversified into *T. haliphron*, *T. staudingeri*, and *T. amphrysus*.

Genetically the *T. minos* has a close association with *T. aeaacus* which belonging to the same clad and are monophyletic; they remain as two allopartic species, the *T. minos* seen along Western Ghats and *T. aeaacus* seen along North Indian planes and North–Eastern Himalayas. The two species were geographically isolated by the extensive land masses of Deccan Plateau between them. The distribution of *T. minos* and *T. aeaacus* and their phylogenetic relationship provide evidence to the view of their speciation through the loss of continuity in distribution of their ancestors from Deccan Plateau and resulting isolation.

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