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DNA BARCODING OF *Danaus chrysippus* FROM JHARKHAND (INDIA)

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Aim. To investigate the morphoanatomical characteristics and genetic diversity of *Danaus chrysippus* across diverse ecological landscapes of Jharkhand, India, using an integrative morpho-molecular approach.

Materials and Methods. Specimens were collected from nine sites across eight districts of Jharkhand. Morphoanatomical features, including wing patterns and genital structures, were examined using standard taxonomic procedures. Genomic DNA was extracted, and mitochondrial cytochrome c oxidase subunit I (COI) gene sequences were amplified and analyzed. Sequence data were submitted to DDBJ (accession IDs LC818942 to LC877969). Species identity was confirmed using BLASTn, and phylogenetic analyses were conducted in MEGA X using Maximum Likelihood (ML), Neighbor-Joining (NJ), and Minimum Evolution (ME) methods.

Results. Morphological observations confirmed characteristic traits such as brush-footed forelegs, sexually dimorphic hindwing markings, and distinct male and female genital structures. CO1 sequences (509–698 bp) exhibited an AT-rich composition (mean A+T ≈ 69.9%). Pairwise genetic distances among most isolates were low (0.0000–0.0197), indicating high genetic uniformity. However, the Dumri isolate (LC877967) showed marked divergence (~0.14–0.146), suggesting the presence of an Evolutionarily Significant Unit (ESU). Phylogenetic analyses consistently grouped all other isolates into a single clade, while the Dumri isolate formed a distinct lineage.

Conclusions. The study demonstrates the effectiveness of DNA barcoding in detecting genetic variation within *D. chrysippus* populations. The distinctiveness of the Dumri isolate highlights its potential evolutionary and conservation significance. These findings provide a baseline for future monitoring of butterfly genetic diversity in ecologically sensitive regions of Jharkhand.

Keywords: *Danaus chrysippus*, Morphomolecular, morphoanatomical, distance matrix, phylogenetic tree, BLAST.

Butterflies are not only a bounty and beauty of nature but also a component of biodiversity that supports human life through valuable ecosystem services and serves as a reliable bioindicator of environmental conditions in the habitat. The conservation policies and principles rely more on indicator species [1]. A good indicator species can be easily handled, identified, and comfortably monitored, sensitive to environmental changes, and readily visible to the naked eye, thereby capable of providing clear early signals [2, 3]. The butterflies serve as a good bioindicator of vegetational cover, specific plant groups, land-use patterns, and human-induced alterations. Butterflies are an indicator group of species and are among the best insect groups for detecting human-induced environmental

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changes [4–6]. According to Warren et al. [7]. Owing to some specialty butterflies, in contrast to other pollinator insects, more suitable group for biomonitoring. It has been argued that butterflies are good bioindicators of plants, birds, beetles, and Hymenoptera [5, 8–10]. In terrestrial ecosystems, Lepidopterans have recently been identified as a dominant and the most indicative insect taxon compared to other insect orders [11]. For butterflies and other pollinators, species richness and abundance are determined by the availability and abundance of floral and plant resources in the habitat [12, 13]. Currently, trends in butterfly observation are being extended to a wider range of pollinators, thereby saving a great deal of monitoring effort. However, according to Leone et al. [14], despite numerous studies on butterflies as bioindicators, further research is needed to exploit their quality better. Lack of proper taxonomic identification may lead to misleading results in biomonitoring.

The tropic of cancer crosses the Indian state of Jharkhand. The majority of Jharkhand's districts lie within the Chota Nagpur Plateau, including those in both North and South Chotanagpur Divisions. The 9 sampling sites for the collection of *Danaus chrysippus* are located in the Ranchi, Lohardaga, Gumla, Khunti, Seraikela Kharsawan, West Singhbhum, East Singhbhum, and Simdega districts of Jharkhand state. Situated in the eastern region of the Deccan Plateau, the Chota Nagpur Plateau is home to the following districts. The rapid development and unchecked deforestation have led to a rise in the region's average temperature in recent years [15, 16]. The perusal of available literature about butterflies of this region indicates a high paucity of information on the proper taxonomy of butterflies. In fact, the literature review [15–26] revealed a significant lacuna in taxonomic studies. Given the standardization of butterfly species as a potential bioindicator, we are the first to conduct studies aimed at taxonomically classifying the region's butterfly species. Morpho-anatomical taxonomic studies require a high level of expertise, which, when coupled with the morpho-molecular approach, makes the task easier, though it requires experimental precision. Keeping the above apprehensions in view, the present communication deals in detail with morpho-molecular studies of *Danaus chrysippus* samples collected from different (nine) sites of Jharkhand (India).

Materials and Methods

Butterfly Sampling

Sampling Sites

In our first-phase sampling drive, a total of 9 sampling sites distributed across 8 districts of the Jharkhand state, India (Table 1–2; Fig. 1), were surveyed for specimens of *Danaus chrysippus* as part of a two-phase sampling project for the species. The remaining districts in the state will be surveyed in the second phase. All of the sampling sites mentioned in this work lie south of the Tropic of Cancer in the state.

The spatial distribution of the sampling sites exhibited significant variation (Table 1). The shortest distance recorded was between Pansua Dam (West Singhbhum) and Angrabari Temple (Khunti) at approximately 49.59 km. In contrast, the farthest was between Patratoli (Ranchi) and Belma Dam (West Singhbhum), separated by a substantial 166.01 km. Other notable distances include the 148.29 km gap between Ranchi and Burudi Dam, and the 216.87 km between Gumla and East Singhbhum — the latter being the longest distance between any two sites.

The land use types across sites (Table 2) ranged from forest/agriculture and forest/urban fringe to industrial/urban and forest/mining. For instance, Patratoli in Ranchi is nestled at a forest-urban interface with sal and bamboo vegetation, alongside increasing urban development. Similarly, Nandini Dam in Lohardaga and Angrabari Temple in Khunti are dominantly forest-agriculture landscapes, characterized by deciduous cover and terraced farming, reflecting traditional land-use practices.

Sites like Jampani in Simdega presented a more agriculture-dominant matrix, with visible tribal farming and patches of secondary forest. In contrast, the Pansua and Belma dams in West Singhbhum were located in a blend of mining and forested areas. At the same time, the Burudi Dam in East Singhbhum was distinctly industrial, with coal pits and steel-related infrastructure forming the backdrop. Notably, Chandil Dam in Seraikela-Kharsawan stood at a confluence of mining activity and forest, making it a unique edge habitat.

The ecological heterogeneity among these sampling sites reflects a mosaic landscape, where *Danaus chrysippus* populations navigate through varied pressures — from urban expansion and industrialization to traditional and shifting agricultural practices. This gradient of habitat types and the varying inter-

Table 1. **Distance Matrix in Km between the Sampling sites of** *Danaus chrysippus*

Sampling sites		1	2	3	4	5	6	7	8	9
Patratoli, Ranchi	1		51.28	47.35	100.98	134.12	91.17	148.29	87.79	166.01
Nandini Dam, Lohardaga	2	51.28		54.37	54.35	105.96	102.33	189.21	130.45	185.73
Angrabari Temple, Khunti	3	47.35	54.37		78.66	91	49.59	139.83	85.2	131.88
Dumri, Gumla	4	100.98	54.35	78.66		67.65	109.86	216.87	163.86	189.84
Jampani, Simdega	5	134.12	105.96	91	67.65		87.26	200.57	161.51	147.49
Pansua Dam, W. Singhbhum	6	91.17	102.33	49.59	109.86	87.26		113.97	76.78	83.71
Near Burudi Dam, E. Singhbhum	7	148.29	189.21	139.83	216.87	200.57	113.97		60.51	89.98
Chandil Dam, Seraikela Kharsawan	8	87.79	130.45	85.2	163.86	161.51	76.78	60.51		104.34
Belma Dam, W. Singhbhum	9	166.01	185.73	131.88	189.84	147.49	83.71	89.98	104.34	

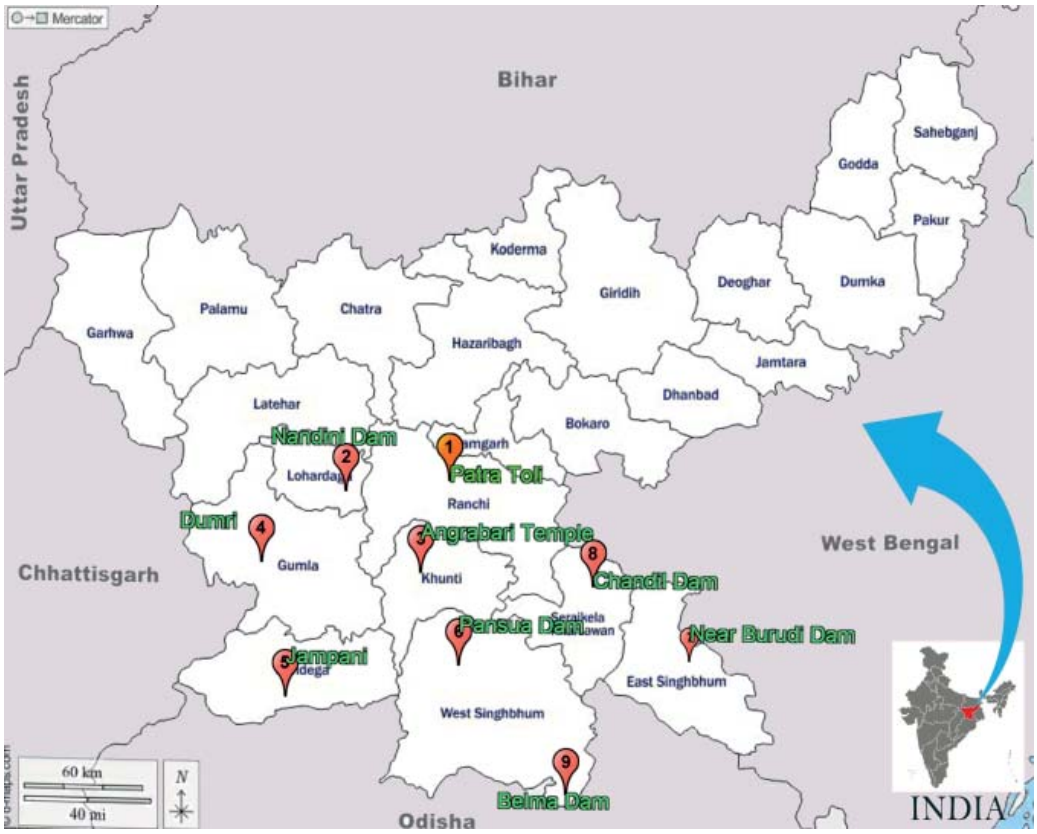

Fig. 1. **Map of Jharkhand showing the distribution of 9 sampling sites of** *Danaus chrysippus* **among the 8 districts of Jharkhand State, India**

Table 2. **The Land use type and Dominant patterns along the Sampling Sites of** *Danaus chrysippus*

Point	Sampling sites	GPS coordinates	Elevation	Land Use Type	Dominant Patterns
1	Patratoli, Ranchi	23.440839 N 85.334628 E	632.4 m	Forest/Urban fringe	Sal/bamboo forests, expanding urban pockets
2	Nandini Dam, Lohardaga	23.38976310 N 84.83592780 E	683.2 m	Forest/Agriculture	Deciduous forest, terraced rice/pulse farming
3	Angrabari Temple, Khunti	23.03192160 N 85.19963536 E	591.5 m	Forest/Agriculture	Mixed forest, interspersed cropland
4	Dumri, Gumla	23.06996160 N 84.43316114 E	827.3 m	Forest/Agriculture	Slash-and-burn farming, natural woods
5	Jampani, Simdega	22.46939092 N 84.55372406 E	324.2 m	Agriculture/Forest	Tribal farming, secondary forests
6	Pansua Dam, W. Singhbhum	22.61902621 N 85.38658956 E	352.3 m	Forest/Mining	Bamboo forests near mining sites
7	Near Burudi Dam, E. Singhbhum	22.64092505 N 86.49495244 E	163.9 m	Industrial/Urban	Dense steel-industry zone, coal pits
8	Chandil Dam, Seraikela Kharsawan	22.97483703 N 86.02843976 E	214.8 m	Forest/Mining	Forested with mining and transport infrastructure
9	Belma Dam, W. Singhbhum	22.03923865 N 85.90796710 E	426.7 m	Forest/Agriculture	Upland forests and valley farmlands

site distances also imply potential influences on the butterfly's dispersal dynamics, gene flow, and population structure, topics I aim to explore in further analyses.

Several butterflies, except five (from each sampling site), were captured and released after preliminary identification was done by examining the traditional morphological characteristics of wings, genitalia, locale, and other information as per the butterfly identification keys [27, 28]. Of the five captured samples, three were preserved in 70% alcohol; of these, two were for anatomical studies and one for DNA (deoxyribonucleic acid) extraction, PCR (Polymerase Chain Reaction) amplification, and sequencing. The left 2 specimens were preserved in small paper envelopes after being sacrificed under the influence of ethyl acetate fumes in an insect-killing jar and brought to the laboratory. These specimens were relaxed, pinned, and submitted to the ICRI (Insect Collection, Record and Identification), Entomology section, Department of Zoology, St. Xavier's College, Ranchi, with voucher number (Table 3).

Morphological investigations

Considering morphological indications, the sex and color pattern of each sample were noted, examined, and recorded. Based on the arrangement of markings on the butterfly's rear wings, sex was determined. The females have only three black spots on the hind wings, but the males have an additional large black spot surrounded by white [27, 28]. Following Owen et al.'s descriptions [29].

Anatomical investigations

For the study of male and female genitalia, the technique of Mal et al. [30] was slightly modified. A specimen's abdomen was separated from the body and boiled in 10% potassium hydroxide for six minutes. Afterward, the abdomen was dissected from the lateral side using forceps and a fine-tipped scalpel while being examined under a dissecting microscope. After the genitalia and abdomen were separated, they were cleaned with tap water and inspected under a dissecting microscope for confirmation. To facilitate further analysis, genital material was stored in microvials in 70% ethyl alcohol with a drop of glycerin. The terminology for genital investigations was adopted from Winter [31] and Klots [32].

Table 3. **9 sampling sites distributed among 8 districts of Jharkhand with the voucher number of representative samples submitted to ICRI (Insect Collection Record and Identification, Department of Zoology, St. Xavier's College, Ranchi), with their respective GPS coordinates**

SL. No.	District	Place Name	Voucher No.
1	Ranchi	Patra Toli	SXCRAN-ENT-0424-S14
2	Lohardaga	Nandini Dam	SXCRAN-ENT-180525-S14H
3	Khunti	Angrabari Temple	SXCRAN-ENT-220525-S14G
4	Gumla	Dumri	SXCRAN-ENT-120425-S14F
5	Simdega	Jampani	SXCRAN-ENT-100625-S14E
6	West Singhbhum	Pansua Dam	SXCRAN-ENT-100525-S14D
7	East Singhbhum	Near Burudi Dam	SXCRAN-ENT-240425-S14C
8	Seraikela Kharsawan	Chandil Dam	SXCRAN-ENT-280325-S14B
9	West Singhbhum	Belma Dam	SXCRAN-ENT-250425-S14A

DNA extraction, PCR amplification and sequencing

A specimen of a butterfly's foreleg was used to isolate DNA. Using a 1.0% agarose gel, the quality was assessed. Only one high-molecular-weight DNA band was observed. Using forward and reverse primers, mitochondrial cytochrome c oxidase subunit I (COI) gene fragments were amplified. On an agarose gel, a single discrete PCR amplicon band was seen. After that, impurities were removed by purifying the PCR amplicon. The PCR amplicon's sequencing reaction was performed using forward and reverse primers on an ABI 3730xl Genetic Analyzer equipped with a BDT v3.1 Cycle sequencing kit [33].

Molecular sequence analysis tools

Following the Sangner sequencing method, discrete PCR amplicon (s) were obtained from each sample. The obtained PCR amplicon(s) were compared with data present in nucleotide databases using the BLAST (Basic Local Alignment Search Tool) program based at NCBI (National Center for Biotechnology Information). The nucleotide blast (BLASTn) option was used. BLASTn compares the nucleotide query (our sequence) with the subject sequence (data present in databases). BLASTn was performed with the following parameters: Search Set: standard database; Program selection: highly similar sequences (megablast). Megablast is used for sequence identification and intra-species comparison [34].

Submission of nucleotide sequence to the GenBank

Based on the BLASTn results, the butterfly specimens were identified as *Danaus chrysippus*, and the COI nucleotide sequences were submitted to DDBJ (DNA Data Bank of Japan), yielding accession numbers (Table 2). The full definitions of our CO1 sequences submitted to DDBJ can be viewed by searching the accession IDs (Table 2) on NCBI's website or by scanning the QR codes provided in Fig. 2.

Distance matrix and Phylogenetic tree

All 9 sequences obtained from *Danaus chrysippus* samples were used to construct distance matrices and phylogenetic trees employing the Maximum Likelihood method [35], the Neighbor-Joining method [36], and the Minimum Evolution method [37] to infer evolutionary relationships. The evolutionary distances, which are expressed in units of substitution of bases per site, were calculated using the Maximum Composite Likelihood method [38]. For every pair of sequences, pairwise deletion was performed at unclear positions. This analysis involved 9 nucleotide sequences. The final dataset contained 949 positions. These analyses were performed using MEGA X (Molecular Evolutionary Genetics Analysis) software, version 10.2.6, build 10210527-x86_64 (Windows 11), which was used to perform evolutionary analyses [34, 39].

Results and Discussion

More than 100 butterfly species are on the verge of extinction in India [40]. Over the past two decades, governments have

Table 4. **Accession IDs of all sequences from *Danaus chrysippus* submitted to DDBJ**

Sl. No.	Sampling Sites (District: Site Name)	Sampling sites denoted in map	Accession id
	Ranchi: Patra Toli	1	LC818942
	Lohardaga: Nandini Dam	2	LC877969
	Khunti: Angrabari Temple	3	LC877968
	Gumla: Dumri	4	LC877967
	Simdega: Jampani	5	LC877966
	West Singhbhum: Pansua Dam	6	LC877965
	East Singhbhum: Near Burudi Dam	7	LC877964
	Seraikela Kharsawan: Chandil Dam	8	LC877963
	West Singhbhum: Belma Dam	9	LC877962

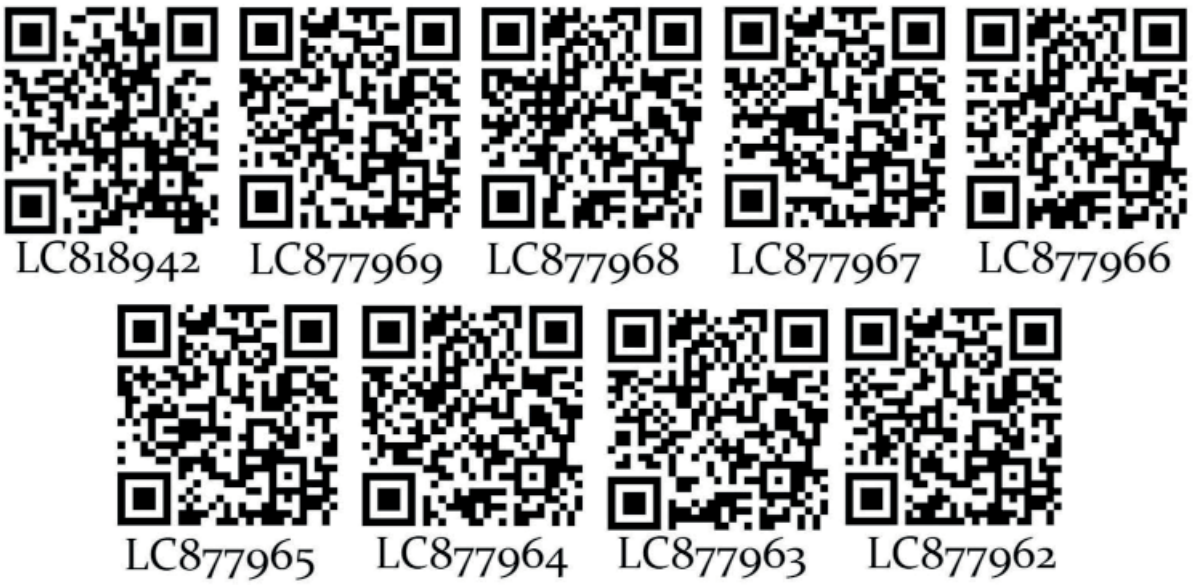

Fig. 2. **QR codes for viewing the full definition of the sequences from the NCBI website**

also begun to take an interest in butterfly conservation because of their importance in maintaining critical ecosystem services. The butterflies maintain the ecosystem by acting as pollinators, prey, biological pest control, inducing genetic variation in plants, and enhancing environmental beauty. However, the butterfly population is declining rapidly. Carlton and Ravichandiran [41] observed that microclimatic changes are causing a decline in butterfly populations. A similar trend was highlighted by Ghazanfar et al. in their review on butterflies and their contribution to the ecosystem [42]. These facts suggest that conservation measures for butterflies need to be emphasized.

Even though the butterflies' importance is not an unheard tale, works related to their conservation are limited to hobby collections and some very superficial studies focused on preparing their checklist and publishing coffee table books in India, in general, and Jharkhand, in particular. A review of the literature reveals that the butterflies of the Indian state of Jharkhand are poorly studied. Over the last decade, various authors [15–22] have studied the butterflies of Jharkhand. However, these studies lack taxonomic studies and are limited to inventory preparation, preliminary reports, and initial listings of the butterflies.

Keeping the above facts in mind, and a motive to contribute significant information for conservation of butterflies, we surveyed and studied samples of *Danaus chrysippus* collected from 9 different sites in the Indian state of Jharkhand, spread across 8 different districts, for morphological, anatomical, and molecular features, which will be helpful in studies related to conservation activities of the species in Jharkhand as well as in India.

Morpho-anatomical description

The species belongs to the family Nymphalidae (Rafinesque, 1815) of the order Lepidoptera (Linnaeus,1758) [43].

Nymphalidae (Rafinesque, 1815)

The forelegs of members of the family Nymphalidae are never fully developed, may be reduced or functionless as walking legs in either males or females, or in both. They may be covered with long, dense scales that form brush-like stubs; hence, they are commonly called 'brush-footed' butterflies or 'four-footed' butterflies [27].

DIAGNOSIS

Danaus (Kluk, 1780)

1804, *Danaida* Latreille, Type-species: *Danaus Plexippus* Linnaeus, by monotypy [44].

1806, *Limnas* H bner, Augsburg. Type-species: *Papilio chrysippus* Linnaeus, by monotypy. Placed on the Official List of Rejected and Invalid Names in Zoology; Opinion 278, 1954. *Opinions and Declarations Rendered by the International Commission on Zoological Nomenclature* 6: 137-177 [45].

1807, *Danais* Latreille, Type-species: *Danaus Plexippus* Linnaeus, by monotypy [46].

1809, *Danaus* Latreille. Parisiis & Argentorati. Type-species: *Danaus Plexippus* Linnaeus, by subsequent designation [47].

1872, *Festivus* Crotch, Type-species: *Danaus plexippus* Linnaeus, by original designation [48].

1937, *Panlymnas* Bryk's Gravenhage. Type-species: *Papilio chrysippus* Linnaeus. Replacement name for *Limnas* H bner [49].

Colour

The head is black with white scale tufts, the antennae and proboscis are black, the maxillary palpi are covered in white and black scales, the thorax is black with white spots, the fore and hind wings are tawny, and the belly is yellow. These features are consistent with those of Mal et al. [30].

Shape

Head round, antennae slender without scales, gradually clavate slightly club-shaped, eyes large, maxillary palpi well developed, slightly compressed, proboscis long and highly coiled, slightly compressed, proboscis long and highly coiled; forewings simple, terminal margins never dentate or caudate, hindwings with terminal margin never dentate, cell closed, with patches of modified scales around the cell; legs with tibia as long as the femur with long hairs, in females wide and club-shaped, with four segments, all firmly joined together; abdomen slender. These features are consistent with those of Mal et al. [30].

Chrysippus

Wings yellowish-brown, forewings with asubapical white band.

1758, *Papilio chrysippus* Linnaeus

1862, *Danais chrysippus* Linnaeus. Trimen, 1862c. [Referable to subspecies *orientis* (Aurivillius, 1909)] [50]

1764, *Danais chrysippus* (Linnaeus, 1764). Trimen & Bowker, 1887. [Referable to subspecies *orientis* (Aurivillius, 1909)] [51]

1953a, *Danaus chrysippus* Linnaeus. Swanepoel, 1953. [Referable to subspecies *orientis* (Aurivillius, 1909)] [52]

1758, *Danaus chrysippus* (Linnaeus, 1758). Dickson & Kroon, 1978. [Referable to subspecies *orientis* (Aurivillius, 1909)] [53]

1758, *Danaus anosia chrysippus* (Linnaeus, 1758). Pringle *et al*., 1994: 47. [Referable to subspecies *orientis* (Aurivillius, 1909)] [54]

Coloration

The head is black with black spots that have tufts of white scales; the eyes are brownish black; the antennae are black; the maxillary palpi is covered in large black and white scales; the thorax is black with white spots; the forewings are tawny with ground color that makes the upperside brighter than the underside; half of the apical part is black with white bands; the costal margin is black with black spots; the hind wings are tawny with black spots that are semicircular in shape; in the male, there is one scent-producing white spot in the post discal area that has a thick black border; the abdomen is yellowish [30].

Shape

Head: Head round; antennae short, eyes large, maxillary palpi well developed; second segment larger than first and third segment; massive, heavily coiled proboscis.

Fig. 3. **Photograph of the Upper side of the specimen of Dc *Danaus chrysippus* submitted to the ICRI (Insect Collection, Record and Identification), Entomology section, Department of Zoology, St. Xavier's College, Ranchi, with voucher number SXCRAN-ENT-0424-S14**

Fig. 4. **Photograph of the Underside of the specimen of dc *Danaus chrysippus* submitted to the ICRI (Insect Collection, Record and Identification), Entomology section, Department of Zoology, St. Xavier's College, Ranchi, with voucher number SXCRAN-ENT-0424-S14**

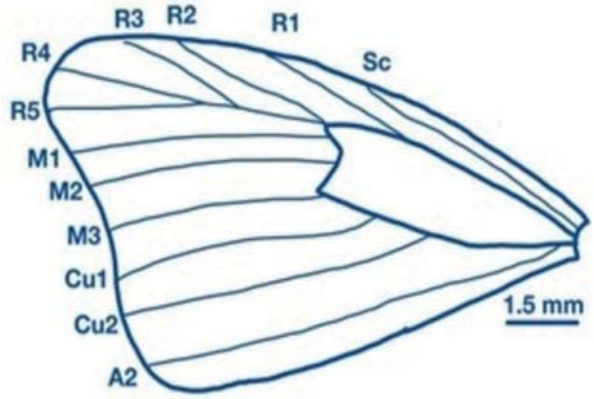

Fig. 5. **Venation in the forewings of *Danaus chrysippus***

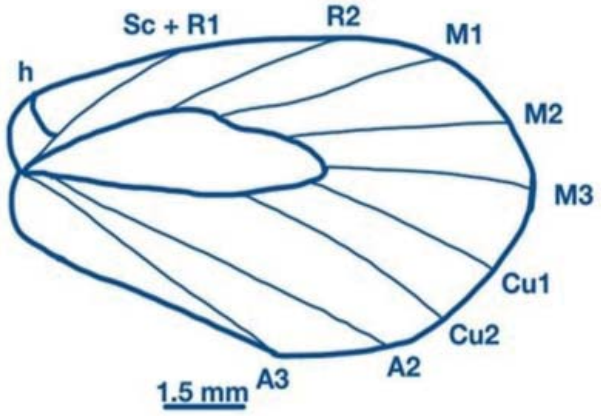

Fig. 6. **Venation in the hindwings of *Danaus chrysippus***

Forewing

Triangular, slightly longer than hindwing, costa widely arched; apex round, termen slightly concave, discoidal cell closed, elongated, well over half length of wing; veins, Sc (Subcosta) arises from the axillary of the wing, ending at the middle of the costal margin, R (Radial) arises next to and parallel to Sc, at distally forks into R1 and Rs, later being divided into R2, R3, R4 and R5, R2 and R3 ending before apex of the cell, R4 ending at the apex of the cell, R5 ending on the terminal margin, M1 begins from upper angle of discal cell, M2 begins from middle of the discal cell, M3 begins from lower angle of the discal cell, Cu1 and Cu2 begin separately from discal cell for an unequal distance, A2 arises from axillary region, separately from the distal cell up to the tornus of the wing.

Hindwings

Pear shaped, costa straight, apex round, termen sinuated, dorsum straight, discoidal cell closed, more than half length of the wings; veins, Sc +R1 arises from the axillary region, short and separated from R1, humeral (h) curve toward the proximal region of the costal margin begins from Sc+R1 and Rs, M1 originates from upper angle of the discal cell, M2 origiantes from middle of discal cell,

M3 originates from lower angle of cell, Cu1 parallel to Cu2, two veins A2 and A3 arise from the axillary of the wing. A2 ending at the tornus of the wing, A3 ending at the dorsal margin.

Abdomen

Abdomen yellowish orange; a pair of protrusible brushes of hairs at the abdomen.

Anatomical investigations

Male Genitalia

Uncus sclerotized, curved, slender distally, tegumen broad; juxta plate-like, bilobed; saccus V-shaped, without saccular process; valva broad, outwardly curved, distally cone-shaped lobe with small hair; aedeagus broad, rod-like, dorsoventrally curved, dorsally a pair of hex-dentate thecal appendage, ventrally membranous lobe.

Female Genitalia

Papillae anal small, triangular, beset with small scales, apophysis posterior large, thorn-like, apophysis anterior reduced, ductus bursae short, wide, corpus bursa large, balloon-like with a pair of plate-like sclerotized cornute.

Specimens examined are *Danaus chrysippus* (Kluk, 1804)

All over the Afrotropical Region, which includes Guinea, Cameroon, Equatorial Guinea, Gabon, Congo, Angola, Central African Republic, Democratic Republic of Congo, Sudan, Uganda, Ethiopia, Somalia, Kenya, Tanzania, Malawi, Zambia, Mozambique, Zimbabwe, Botswana, Namibia, South Africa, Lesotho, Swaziland, Arabia (Yemen (including Socotra), Saudi Arabia, Oman, United Arab Emirates), Madagascar, Comoro Islands, Mauritius, Rodriques, Reunion, Bourbon, Seychelles, Aldabra, St Helena, Cape Verde Islands **[55]**. Extralimitally (as the nominate subspecies) in Palestine, Lebanon, Turkey, Cyprus, Malta, Greece, Italy, Spain, Corsica, Sardinia, Tunisia, Algeria, Morocco, Canary Islands, Arabia, Egypt; Borneo; Malay Peninsula (Kedah & Langkawi Island); Thailand, Burma, Sri Lanka; Andaman & Nicobar Islands [56]. From Great Britain (Jersey) (Long, 2016).

In India **[56],** the species has been reported from Ranchi, Jamtara, and Gumla in the state of Jharkhand **[**15–22, 58**]**.

Biology

Habitat

A range of environments, from sea level to high mountains, except dense forests. During the dry season, even dense primary forest can be momentarily penetrated (Larsen, 2005) **[59].**

Habit

Both sexes frequently graze on flowers, and males will occasionally mud-puddle; males are rarely drawn to carnivore feces. Males are frequently observed absorbing pyrrolizidine alkaloids from suitable plant material (e.g., *Heliotropium* species) **[59]**, making the Plain Tiger unappealing to vertebrate predators, resulting in their delayed flight.

Life cycle

Females deposit eggs singly on the underside of the larval-fed plant leaves **[60].** Plain tiger eggs measure around 1.7 mm in length and 0.5 mm in width. When first laid, it is white, but over time, it turns brown. The egg is ridged and dome-shaped. Depending on the temperature, the egg will hatch in 3 to 5

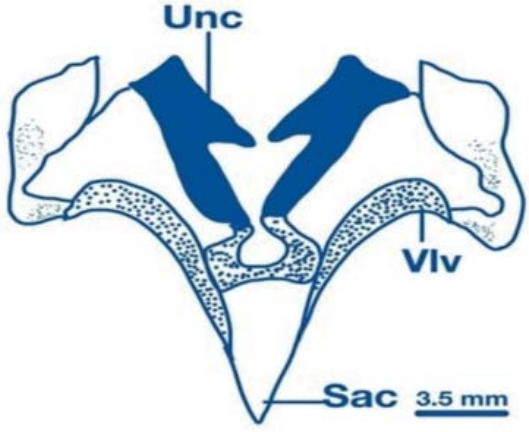

Fig. 7. **Male genitalia of *Danaus chrysippus***

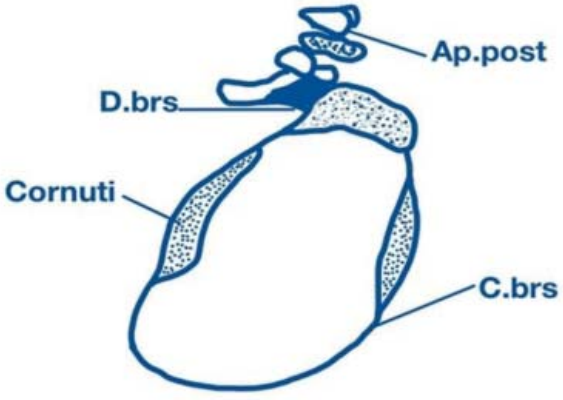

Fig. 8. **Female genitalia of *Danaus chrysippus***

days. The caterpillar passes through five instar stages. The first instar has a black head and a white body. It is 4 mm long. The second instar has a body that is mostly grey, with horizontal black-and-yellow stripes about 8 mm long. The length of the third, fourth, and fifth instars, respectively, is approximately 14 mm, 25 mm, and 36 mm. Twelve to twenty days can pass during the larval stage **[61].**

Food Source

Adult plain tiger butterflies obtain nectar from various flowering plants — *Antigonon leptopus, Asystasia gangetica, Catharanthus roseus, Cyanthilium cinereum, Heliotropium indicum, Lantana camara, Tecoma stans, Tridax procumbens, Eucalyptus conglobate, Goodenia maideniana, Ptilotisobovatus* **[61].** They primarily consume plants in the genus *Asclepias* (milkweeds). Milkweeds contain certain toxic compounds, which are fed upon by *Danaus chrysippus*; thus, the plain-tiger is unpalatable to most predators (Batesian mimicry). As a result, its coloration is widely mimicked by various butterfly species **[28].**

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The morpho-anatomical characters observed are in accordance with the features of the insect previously described by Kunte **[27],** Mal et al. **[30];** Smetacek **[28],** and Ackery and Vane-Wright **[62].**

Based on the morphological and anatomical characters listed previously in this article, the butterfly specimens were identified as *Danaus* (Kluk, 1780) *chrysippus* (Linnaeus, 1758).

Morphomolecular description

DNA Extraction, PCR amplification, and Nucleotide Sequencing

Discrete PCR amplicons ranging from 509 to 698 bp of the COI region were obtained using specific primers. The obtained sequences are listed below in FASTA format.

1. Sample 1: Collected from Patratoli, District Ranchi

>LC818942_Dc_Patratoli

TGCCCGTTTTGGGATGTGAGCAGGAATAG-
TAGGAACATCTTTAAGTCTTTTAATTC-
GAACAGAATTAGGAACACCGGGATCTC-
TAATTGGAGATGATCAAATTTATA-
ATACTATTGTAACAGCTCATGCTTT-
TATTATAATTTTTTTTTATAGTTATAC-
CAATTATAATTGGAGGATTCGGAAATT-
GATTAGTACCTTTAATATTAGGAGCCCCT-
GATATAGCTTTCCCACGAATAAATA-
ATATAAGATTTTGACTTTTACCCCCAT-
CATTAATATTACTAATTTCAAGAAGAATT-
GTAGAAAATGGAGCAGGAACAGGAT-
GAACGGTTTACCCCCCCCTTTCATCTA-
ATATTGCCCATAGAGGATCTTCTGTAG-
ATTTAGCTATTTTTTCTTTACATTTAGCTG-
GAATTTCTTCTATTTTAGGAGCTATTA-
ATTTTATTACTACAATTTTAAATATAC-
GAATTAATAATATATCATTTGAT-
CAAATATCTTTATTTGTTTGAGCAGTAG-
GTATCACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGGGCAATTAC-
CATACTTCTTACTGACCGAAATCTAAA-
CACTTCATTTTTCGATCCTGCTGGAGGAG-
GAGATCCAATTTTATATCAACATTTATTTT-
GATTTTTTGGTCATCCAGAAGTTTATATTT-
TATTTTTACCGGGGAAGG

2. Sample 2: Collected from Nandini Dam, District Lohardaga

>LC877969_Dc_Nandini Dam

ATTAGGAACACCAGGATCTCTAATTG-
GAGATGATCAAATTTATAATACTATT-
GTAACAGCTCATGCTTTTATTATA-
ATTTTTTTTATAGTTATACCAAT-
TATAATTGGAGGATTCGGAAATT-
GATTAGTACCTTTAATATTAGGAGCTCC-
TGATATAGCTTTCCCACGAATAAATA-
ATATGAGATTTTGACTTTTACCCCCATCAT-
TAATATTACTAATTTCAAGAAGAATT-
GTAGAAAATGGAGCAGGAACAGGAT-
GAACGGTTTACCCCCCCCTTTCATCTA-
ATATTGCCCATAGAGGATCTTCTGTAG-
ATTTAGCTATTTTTTCTTTACATTTAGCTG-
GAATTTCTTCTATTTTAGGAGCTATTAATTT-
TATTACTACAATTTTAAATATACGAAT-
TAATAATATATCATTTGATCAAATATCTT-
TATTTGTTTGAGCAGTAGGTATTA-
CAGCTCTTCTTTTATTACTATCATTACCT-
GTTTTAGCTGGGGCAATTACCATACTTC

3. Sample 3: Collected from Angrabari Temple, District Khunti

>LC877968_Dc_Khunti

CGAAAATGACTTTTTTCTACAAAT-
CATAAGGATATTGGCACTATATATTT-

TATTTTTGGAATTTGAGCAGGAATAG-
TAGGAACATCTTTAAGTCTTTTAATTC-
GAACAGAATTAGGAACACCAGGATCTC-
TAATTGGAGATGATCAAATTTATAATAC-
TATTGTAACAGCTCATGCTTTTATTATA-
ATTTTTTTTATAGTTATACCAATTATA-
ATTGGAGGATTCGGAAATTGATTAG-
TACCTTTAATATTAGGAGCTCCTGA-
TATAGCTTTCCCACGAATAAATAATATA-
AGATTTTGACTTTTACCCCCATCATTA-
ATATTACTAATTTCAAGAAGAATTG-
TAGAAAATGGAGCAGGAACAGGAT-
GAACGGTTTACCCCCCCCTTTCATCTA-
ATATTGCCCATAGAGGATCTTCTGTA-
GATTTAGCTATTTTTTTCTTTACATT-
TAGCTGGAATTTCTTCTATTTTAGGAGC-
TATTAATTTTATTACTACAATTTTA-
AATATACGAATTAATAATATATCATTT-
GATCAAATATCTTTATTTGTTTGAGCAG-
TAGGTATTACAGCTCTTCTTTTATTAC-
TATCATTACCTGTTTTAGCTGGGGCAAT-
TACCATACTTCTTACTGACCGAAATCTA-
AATACTTCATTTTTCGATCCTGCTGGAG-
GAGGAGATCCAATTTTATATCAACATT-
TATTT

4. **Sample 4: Collected from Dumri, Gumla**

>LC877967_Dc_Dumri

ATTTGGGCAGGAATAGTAGGAA-
CATCTCTTAGACTATTAATTCGAACT-
GAATTAGGTAATCCAGGTTCTTTA-
ATTGGAGATGATCAAATTTATAATAC-
TATTGTTACAGCTCATGCTTTTATTATA-
ATTTTCTTCATAGTTATACCTATTATA-
ATTGGAGGATTTGGTAATTGATTAG-
TACCTCTAATACTAGGAGCTCCCC-
GATATAGCTTTCCCCCGAATAAATA-
ATATAAGTTTTTGACTTCTTCCCCCATCTT-
TAATTTTATTAATTTCTAGTAGAATTG-
TAGAAAATGGAGCAGGAACAGGATGAA-
CAGTTTATCCCCCACTTTCATCTAATATT-
GCCCATAGAGGATCCTCGGTTGATT-
TAGCTATTTTTTCATTACATTTAGCTG-
GAATTTCATCAATTTTAGGAGCTATTA-
ATTTTATTACTACAATTATTAATATACGAG-
TAAATAGTTTATCTTTTGATCAAATACCTT-
TATTTGTTTGATCAGTAGGTATTACT-
GCCCTTCTTTTATTACTTTCTTTACCAGTTT-
TAGCCGGAGCTATTACTATATTATTAA-
CAGACCGAAATTTAAATACTTCATTTTTT-
GATCCTGCAGGAGGAGGAGATCCTATTTA

5. **Sample 5: Collected from Jampani, Simdega**

>LC877966_Dc_Jampani

CCACGAATAAATAATATAAGATTTT-
GACTTTTACCCCCATCATTAATATTAC-
TAATTTCAAGAAGAATTGTAGAAAATG-
GAGCAGGAACAGGATGAACGGTT-
TACCCCCCCCCTTTCATCTAATATT-
GCCCATAGAGGATCTTCTGTAGAT-
TTAGCTATTTTTTCTTTACATTTAGCTG-
GAATTTCTTCTATTTTAGGAGCTATTA-
ATTTTATTACTACAATTTTAAATATAC-
GAATTAATAATATATCATTTGAT-
CAAATATCTTTATTTGTTTGAGCAGTAG-
GTATTACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGAGCAAT-
TACCATACTTCTTACTGACCGAAATCTA-
AATACTTCATTTTTCGATCCTGCTGGAG-
GAGGAGATCCAATTTTATATCAACATT-
TATTTTGATTTTTTGGTCATCCAGAAGTT-
TATATTTTAATTTTACCAGGCTTTGGGATA-
ATTTCCCATATTATTAGTCAAGAAAGAG-
GAAAAAAAGAAACTTTTGGATCACTAG-
GAATAATTTATGCAATAATAGCAATTG-
GATTATTAGGATTTATTGTATGAGCTCAT-
CATATATTTACAGTTGGAATAGATATT-
GACACTCGAGCTTATTTTACTTCCGCGAC-
TATAATTATTGCTGTACCAACAGGTATTA-
AAA

6. **Sample 6: Collected from Pansua Dam, West Singhbhum**

>LC877965_Dc_Pansua Dam

TAAAGATATTGGCACTATATATTT-
TATTTTTGGAATTTGAGCAGGAATAG-
TAGGAACATCTTTAAGTCTTTTAATTC-
GAACAGAATTAGGAACACCGGGATCTC-
TAATTGGAGATGATCAAATTTATA-
ATACTATTGTAACAGCTCATGCTTT-
TATTATAATTTTTTTTATAGTTATAC-
CAATTATAATTGGAGGATTCGGAAATT-
GATTAGTACCTTTAATATTAGGAGCCCCT-
GATATAGCTTTCCCACGAATAAATA-
ATATAAGATTTTGACTTTTACCCCCAT-
CATTAATATTACTAATTTCAAGAAGAATT-
GTAGAAAATGGAGCAGGAACAGGAT-
GAACGGTTTACCCCCCCCTTTCATCTA-
ATATTGCCCATAGAGGATCTTCTGTA-
GATTTAGCTATTTTTTCTTTACATT-
TAGCTGGAATTTCTTCTATTTTAGGAGC-
TATCAATTTTATTACTACAATTTTA-
AATATACGAATTAATAATATATCATTT-
GATCAAATATCTTTATTTGTTTGAGCAG-
TAGGTATCACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGGGCAATTAC-
CATACTTCTTACTGACCGAAATCTA

7. **Sample 7: Collected from Burudi Dam, East Singhbhum**

>LC877964_Dc_Burudi_Dam

ATTTGAGCAGGAATAGTAGGAA-
CATCTTTAAGTCTTTTAATTCGAA-
CAGAATTAGGAACACCGGGATCTCTA-
ATTGGAGATGATCAAATTTATAATAC-

TATTGTAACAGCTCATGCTTTTATTATA-
ATTTTTTTTATAGTTATACCAATTATA-
ATTGGAGGATTCGGAAATTGATTAG-
TACCTTTAATATTAGGAGCCCCT-
GATATAGCTTTCCCACGAATAAATA-
ATATAAGATTTTGACTTTTACCCCCAT-
CATTAATATTACTAATTTCAAGAAGAATT-
GTAGAAAATGGAGCAGGAACAGGAT-
GAACGGTTTACCCCCCCCTTTCATCTA-
ATATTGCCCATAGAGGATCTTCTGTAG-
ATTTAGCTATTTTTTCTTTACATTTAGCTG-
GAATTTCTTCTATTTTAGGAGCTATTA-
ATTTTATTACTACAATTTTAAATATAC-
GAATTAATAATATATCATTTGAT-
CAAATATCTTTATTTGTTTGAGCAGTAG-
GTATCACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGGGCAATTAC-
CATACTTCTTACTGACCGAAATCTAAA-
CACTTCATTTTTCGATCCTGCTGGAGGAG-
GAGATCCAATTTTATATCAACATTTATTTT-
GATTTTTTGGTCA

8. **Sample 8: Collected from Chandil Dam, Seraikela Kharsawan**

>LC877963_Dc_Chandil_Dam

CACTATATATTTTATTTTTGGGATTT-
GAGCAGGAATAGTAGGAACATCTTTA-
AGTCTTTTAATTCGAACAGAATTAG-
GAACACCAGGATCTCTAATTGGA-
GATGATCAAATTTATAATACTATT-
GTAACAGCTCATGCTTTTATTATA-
ATTTTTTTTATAGTTATACCAATTATA-
ATTGGAGGATTCGGAAATTGATTAG-
TACCTTTAATATTAGGAGCTCCT-
GATATAGCTTTCCCACGAATAAATA-
ATATAAGATTTTGACTTTTACCCCCAT-
CATTAATATTACTAATTTCAAGAAG-
AATTGTAGAAAATGGAGCAGGAACAG-
GATGAACGGTTTACCCCCCCCTTTCATC-
TAATATTGCCCATAGAGGATCTTCTG-
TAGATTTAGCTATTTTTTCTTTACATT-
TAGCTGGAATTTCTTCTATTTTAGGAGC-
TATTAATTTTATTACTACAATTTTA-
AATATACGAATTAATAATATATCATTT-
GATCAAATATCTTTATTTGTTTGAGCAG-
TAGGTATTACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGGGCAATTAC-
CATACTTCTTACTGACCGAAATCTAAA-
TACTTCATTTTTCGATCCTGCTGGAGGAG-
GAGATCCAATTTTATATCAACATTTATTT

9. **Sample 9: Collected from Belma Dam, West Singhbhum**

>LC877962_Dc_Belma Dam

CACTATATATTTTATTTTTGGAATTT-
GAGCAGGAATAGTAGGAACATCTTTA-
AGTCTTTTAATTCGAACAGAATTAG-
GAACACCGGGATCTCTAATTGGA-
GATGATCAAATTTATAATACTATT-
GTAACAGCTCATGCTTTTATTATA-
ATTTTTTTTATAGTTATACCAATTATA-
ATTGGAGGATTCGGAAATTGATTAG-
TACCTTTAATATTAGGAGCCCCCT-
GATATAGCTTTCCCACGAATAAATA-
ATATAAGATTTTGACTTTTACCCCCAT-
CATTAATATTACTAATTTCAAGAAG-
AATTGTAGAAAATGGAGCAGGAACAG-
GATGAACGGTTTACCCCCCCCTTTCATC-
TAATATTGCCCATAGAGGATCTTCTG-
TAGATTTAGCTATTTTTTCTTTACATT-
TAGCTGGAATTTCTTCTATTTTAGGAGC-
TATTAATTTTATTACTACAATTTTA-
AATATACGAATTAATAATATATCATTT-
GATCAAATATCTTTATTTGTTTGAGCAG-
TAGGTATCACAGCTCTTCTTTTATTACTAT-
CATTACCTGTTTTAGCTGGGGCAATTAC-
CATACTTCTTACTGACCGAAATCTAAA-
CACTTCATTTTTCGATCCTGCTGGAGGAG-
GAGATCCAATTTTATATCAACATTTATTT

Table 5. **Nucleotide base composition (%) of COI sequences obtained from different specimens of *Danaus chrysippus***

Accession No.	Sample	T(U)	C	A	G	Total
LC877962	Dc Belma Dam	38.6	15.8	31.3	14.3	658
LC877963	Dc Chandil Dam	39.1	15.8	31.3	14.3	658
LC877964	Dc Burudi Dam	38.5	15.8	31.1	14.6	650
LC877965	Dc Pansua Dam	38.4	15.4	31.8	14.4	610
LC877966	Dc Jampani	38.2	15.4	32.1	14.3	676
LC877967	Dc Dumri	39.5	15.8	29.8	14.8	620
LC877968	Dc Khunti	38.8	15.2	31.8	14.2	696
LC877969	Dc Nandini Dam	39.3	15.5	31.2	14.3	509
LC818942	Dc Patratoli	38.4	15.8	30.2	15.6	698
Average		38.7	15.6	31.2	14.5	641.7

Table 3 exhibits data on nucleotide base composition (%T(U), C, A, G) for different isolates of *Danaus chrysippus*. This kind of analysis is often performed in molecular phylogenetics and genomics to assess variations in the nucleotide content of DNA sequences.

From the data, the average percentages of thymine (T/U), cytosine (C), adenine (A), and guanine (G) across all samples are approximately 38.7%, 15.6%, 31.2%, and 14.5%, respectively. These results suggest that the genomes of the analyzed isolates are AT-rich, a trait common to many organellar genomes. The higher AT content is a notable feature often observed in mitochondrial DNA due to replication bias and selective pressure favoring AT substitutions over time (Jermiin et al., 2008) [63].

The average sequence length is approximately 642 nucleotides per isolate, which is typical for short gene markers commonly used for barcoding, such as COI or partial ribosomal DNA markers (Hebert et al., 2003) [64].

The close consistency across the isolates implies that the samples may be closely related, possibly different strains or variants of the same species found across different sites. Slight variations in T(U) content — as observed with LC877968 (38.8%) and LC877969 (39.3%) — could reflect microevolutionary changes driven by local environmental conditions (Nei & Kumar, 2000) [65].

Base composition is also an important factor to consider when performing phylogenetic analyses. Nucleotide bias can cause systematic errors in phylogenetic inference if not corrected with appropriate evolutionary models (Tamura et al., 2004) **[38].** Hence, before generating trees, models such as GTR (General Time Reversible) with site-rate heterogeneity (GTR+G) are often recommended (Yang, 1994) [66].

The resultant nucleotide sequences (query) were compared with the nucleotide databases using the BLASTn program based at NCBI. The BLASTn search settings were — Search Set: Standard database; program selection: Highly Similar sequences (megablast). The megablast is used for sequence identification and intraspecies comparison [67, 68]. All 9 COI sequences of *Danaus chrysippus* were used to construct a distance matrix and a phylogenetic tree.

Distance Matrix and Phylogenetic Tree

The COI nucleotide sequences from 9 strains of *Danaus chrysippus* were used to prepare a distance matrix using the MEGA X software. Distance matrices are used in phylogeny as a non-parametric distance method. These distances are then reconciled to construct a phylogenetic tree. Distance matrix methods of phylogenetic analysis explicitly rely on a measure of 'genetic distances' between the sequences being studied [69].

The nucleotide sequences obtained from the 9 specimens of *Danaus chrysippus* were aligned using CLUSTAL-W alignment in MEGA X, and a distance matrix and phylogenetic tree were constructed. The bootstrap method was employed to test the phylogeny [36].

Table 4 presents a pairwise distance matrix of different isolates obtained from several sampling sites from distinct geographic regions (Belma Dam, Chandil Dam, Burudi Dam, Pansua Dam, Khunti, Nandini Dam, Dumri, Jampani, and Patratoli). Pairwise genetic distances, often estimated using a substitution model such as Kimura-2-Parameter (K2P) or Tamura-Nei, provide a useful metric for inferring genetic divergence between DNA sequences and assessing their evolutionary relationships [65].

In this case, the isolates have been labeled as A to I for analysis: A: LC877962_Dc_Belma_Dam; B: LC877963_Dc_Chandil_Dam; C: LC877964_Dc_Burudi_Dam; D: LC877965_Dc_Pansua_Dam; E: LC877966_Dc_Jampani; F: LC877967_Dc_Dumri; G: LC877968_Dc_Khunti; H: LC877969_Dc_Nandini_Dam; I: LC818942_Dc_Patratoli.

Intra-group Similarity

Notably, the pairwise distances between most isolates — except isolate F (Dumri) — remain very small. Values across most pairs range between 0.00000 and 0.01970, indicating a very low level of sequence divergence among those groups. Specifically:

- The distance between Belma Dam (A) and Burudi Dam (C) is 0.00000, suggesting these two isolates may be identical or extremely closely related.
- Distances between Chandil Dam (B) and other isolates, such as Jampani (E) (0.00237), Khunti (G) (0.00152), and Nandini Dam (H) (0.00197), also indicate a very tight cluster of genetically similar isolates.

Such low sequence divergence may imply recent common ancestry or limited genetic variation across these geographical isolates, possibly owing to gene flow, common environmental selection pressures, or a relatively short evolutionary timescale since divergence [70].

Genetic Outlier

The isolate LC877967_Dc_Dumri (F) is a notable outlier in this analysis. Its

Table 6. **Estimates of Evolutionary divergence between sequences of *Danaus chrysippus* collected from different parts of the Jharkhand state of India**

Specimens		A	B	C	D	E	F	G	H	I
LC877962_Dc_Belma_Dam	A		0.00767	0.00000	0.00168	0.00715	0.14303	0.00613	0.00793	0.01099
LC877963_Dc_Chandil_Dam	B	0.00767		0.00634	0.00844	0.00237	0.13337	0.00152	0.00197	0.01581
LC877964_Dc_Burudi_Dam	C	0.00000	0.00634		0.00174	0.00692	0.14303	0.00634	0.00793	0.00154
LC877965_Dc_Pansua_Dam	D	0.00168	0.00844	0.00174		0.00835	0.14551	0.00828	0.00994	0.01391
LC877966_Dc_Jampani	E	0.00715	0.00237	0.00692	0.00835		0.14081	0.00237	0.00580	0.01970
LC877967_Dc_Dumri	F	0.14303	0.13337	0.14303	0.14551	0.14081		0.13337	0.13217	0.14578
LC877968_Dc_Khunti	G	0.00613	0.00152	0.00634	0.00828	0.00237	0.13337		0.00197	0.01741
LC877969_Dc_Nandini_Dam	H	0.00793	0.00197	0.00793	0.00994	0.00580	0.13217	0.00197		0.00793
LC818942_Dc_Patratoli	I	0.01099	0.01581	0.00154	0.01391	0.01970	0.14578	0.01741	0.00793	

pairwise distances with all other isolates average ~0.133–0.146, which is markedly higher than all other comparisons. This indicates that Dumri isolate (F) is highly divergent at the genetic level. Such substantial divergence could imply that:

• Dumri isolate may represent a highly differentiated lineage.

• It could originate from a geographically or ecologically isolated region where gene flow is limited.

• Alternatively, technical factors (e.g., sequencing errors, contamination) could also produce artificially long branch lengths, although careful validation usually minimizes this risk. This degree of genetic isolation, as observed in Dumri (F), is often a strong indicator of allopatric speciation or long-term separation from other populations [71]. In a conservation and management context, this could highlight the Dumri isolate as a distinct genetic unit that deserves special attention.

The observed genetic distances will produce a phylogenetic tree in which Dumri (F) forms a separate, basal branch relative to the tight cluster of remaining isolates. Given distances approaching 0.14–0.146, Dumri may emerge as a distinct species or an evolutionary significant unit (ESU) on the tree. The remaining isolates will likely group into a single well-supported clade with very short branch lengths, reflecting their close genetic affinity.

Such results underscore the potential for regional genetic homogeneity across the different dam populations except for one markedly divergent isolate [72].

Conservation biologists often use genetic distance data to identify management units (MUs) **or** evolutionary significant units (ESUs) within species [73]. The exceptionally high divergence of the Dumri isolate warrants its recognition as an ESU or as a candidate for more intensive monitoring. Maintaining its genetic integrity is vital to preserving the taxon's overall genetic diversity.

Meanwhile, the minimal divergence across other isolates suggests that a unified conservation strategy is adequate for those populations, as they share a common gene pool. However, ongoing monitoring is essential as further sampling might reveal finer-scale differences or unrecognized substructure.

Molecular phylogenetics plays a crucial role in understanding the evolutionary history of taxa, allowing us to infer relationships and ancestry among species or isolates from genetic data. Figures 9, 10, and 11 show the evolutionary relationships of various isolates of *Danaus chrysippus*, derived using the Maximum Likelihood, Neighbor-Joining, and Minimum Evolution tree-building algorithms, respectively. Together, they provide complementary insights into genetic divergence and evolutionary patterns across the sampled taxa.

Evolutionary Analysis by Maximum Likelihood

Maximum Likelihood (ML) is a statistical approach that constructs a phylogenetic tree by evaluating the likelihood of the observed sequence data under a particular evolutionary model. The resulting tree shown in Figure 9 places isolates into clusters that maximize the probability of observing the given data under a defined evolutionary model, often a substitution model such as Jukes-Cantor, Kimura 2-parameter, or General Time-Reversible (GTR) [74]. ML is computationally intensive as it searches across a large number of possible trees. However, this approach is robust and widely considered one of the most accurate methods for inferring phylogeny.

In the ML tree (Figure 9), the isolates from Burudi Dam **and** Patratoli cluster together with a very short branch length, suggesting that these two isolates share a recent common ancestor and minimal genetic divergence. Interestingly, the isolates from Jampani and Dumri also cluster as sister taxa, sharing a close evolutionary path. This indicates that the genetic variation among these isolates is very small, suggesting either very recent divergence or ongoing gene flow. Other isolates, such as those from Pansua Dam and Khunti, also show similar close relationships. Overall, the ML tree reveals a well-supported, hierarchical structure of the isolates that matches biogeographical expectations, separating the populations into distinct clades and sub-clades based on genetic similarity.

Evolutionary Analysis by Neighbor-Joining

Neighbor-Joining (NJ) is a distance-based algorithm that constructs a tree by iteratively joining pairs of taxa that minimize the overall branch length at each stage [36]. The resulting tree is displayed in Figure 10. NJ is computationally faster than ML and is often one of the first methods used to obtain a quick approximation of relationships. Unlike ML, it does not require explicit substitution models, although a distance matrix is usually calculated using some evolutionary model.

In the NJ tree (Fig. 10), we see a similar broad pattern as in the ML tree (Fig. 10), with the same pairs (e.g., Burudi Dam–Patratoli, Jampani–Dumri) clustering together. However, the branch lengths and topology of some intermediate nodes vary. NJ may produce slightly different relationships among distantly related taxa; for instance, the isolate from Chandil Dam appears to branch earlier than some other clades, which could be due to its slightly higher pairwise distance. This reflects a characteristic of NJ — it provides a good summary of distances but may not capture all the fine details that a likelihood-based method can.

Still, the NJ tree is especially valuable for large datasets or preliminary analyses. Its main strength lies in its efficiency and minimal assumptions, making it a useful complementary tool to ML and Minimum Evolution analyses.

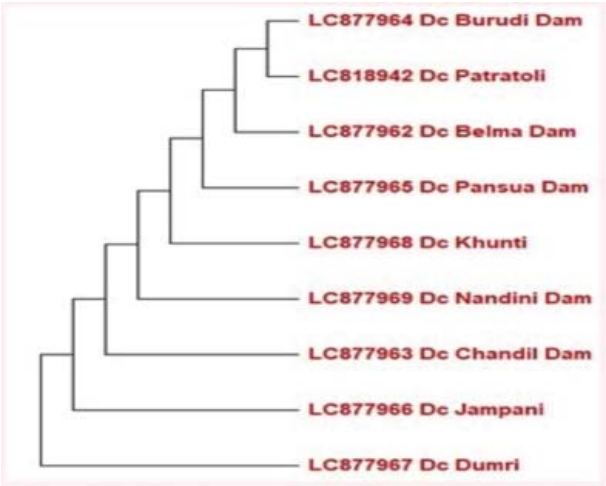

Fig. 9. **Evolutionary analysis by the Maximum Likelihood method**

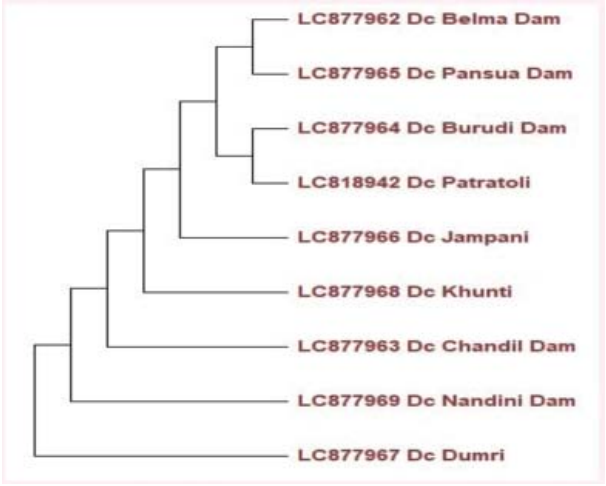

***Fig. 10*. Evolutionary relationships of taxa following Neighbour joining method**

Evolutionary Analysis by Minimum Evolution

Minimum Evolution (ME) aims to produce the tree that minimizes the sum of branch lengths across all taxa [37]. Figure 11 depicts the resulting ME tree for the isolates. The ME algorithm is related to NJ but includes an optimization procedure that further adjusts branch lengths to achieve the shortest overall evolutionary path. Essentially, ME looks for the most parsimonious explanation for the observed data.

In the ME tree (Figure 11), topological structure closely follows that of the NJ tree, which is expected, given that the NJ tree is often a good starting point for ME optimization. Again, Burudi Dam–Patratoli and Jampani–Dumri cluster together with high support. The clade containing Pansua Dam, Khunti, and Nandini Dam isolates also remains stable across all three trees, supporting the idea that these isolates share a relatively more recent common ancestor.

The analyses collectively illustrate the evolutionary relationships among *Danaus chrysippus* isolates. Despite the differences in algorithms, all three methods — ML, NJ, and ME — yielded broadly consistent groupings. Minor differences in the topology reflect the underlying assumptions and computational strategies of each algorithm.

In particular, ML yielded the most statistically robust tree under a specified evolutionary model, NJ provided a fast, straightforward estimate of evolutionary distances, and ME optimized branch lengths to produce a more parsimonious tree. Together, these trees underscore the genetic relatedness between isolates such as those from Burudi Dam and Patratoli, and highlight broader geographic clusters comprising isolates from Jampani, Dumri, Chandil Dam, and others.

Overall, the phylogenetic trees derived here are valuable tools for understanding evolutionary patterns, guiding further sampling strategies, and informing conservation or management practices across these aquatic ecosystems. Future work could incorporate more isolates, additional gene markers, and model-testing approaches to refine these phylogenies further and assess their support using bootstrap or Bayesian posterior probability values.

In this integrative morpho-anatomical and DNA barcoding study of *Danaus chrysippus*, we undertook sampling across nine ecologically diverse sites spanning eight districts of Jharkhand, India.

The distance matrix indicated considerable spatial variation, with inter-site distances ranging from approximately 49.59 km (between Angrabari Temple, Khunti and Pansua Dam, West Singhbhum) to a maximum of 216.87 km (between Dumri, Gumla and Burudi Dam, East Singhbhum). Despite these distances, the genetic distance matrix revealed minimal nucleotide divergence across most sampling sites (ranging from 0.0000 to 0.0197), underscoring a high degree of genetic homogeneity likely facilitated by the species' strong dispersal ability and wide habitat tolerance.

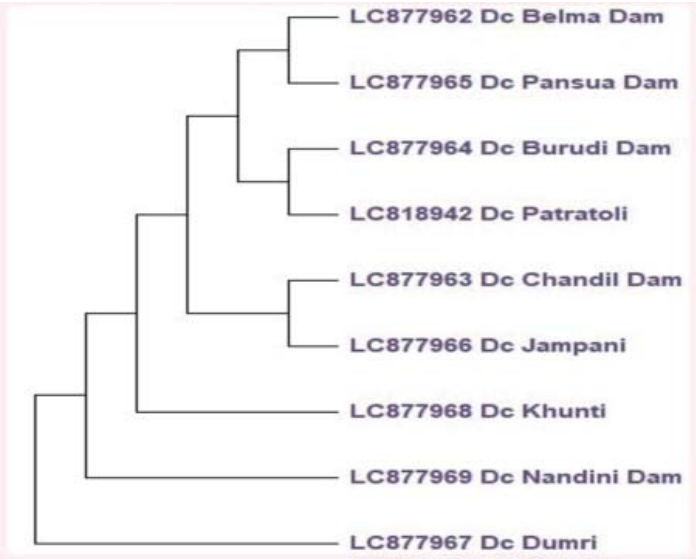

Fig. 11. **Evolutionary relationships of taxa following the Minimum Evolution Method**

However, Dumri (Gumla) emerged as a genetic outlier. Situated at an elevation of 827.3 meters, it is the highest among all sampling locations. Its COI sequence exhibited a pronounced divergence from all other populations, with genetic distances ranging from 0.132 to 0.146. This divergence was consistently reflected across all three phylogenetic reconstructions — Maximum Likelihood, Neighbor-Joining, and Minimum Evolution — in which the Dumri isolate formed a distinct, basal clade. This pattern provides strong evidence for considering the Dumri population as an Evolutionarily Significant Unit (ESU), potentially shaped by ecological or geographic isolation.

In contrast, locations such as Burudi Dam (East Singhbhum) and Patratoli (Ranchi), despite being over 140 km apart and differing in land-use types (industrial vs. urban-forest fringe), exhibited no genetic differentiation (distance = 0.0000). This likely indicates high levels of gene flow across human-modified landscapes, possibly facilitated by continuous floral corridors or butterfly mobility.

The role of elevation as a structuring factor was also evident. Populations from higher elevations — particularly Dumri (827.3 m), Lohardaga (683.2 m), and Ranchi (632.4 m) — demonstrated broader genetic distances when compared with lowland sites like Burudi (163.9 m) and Chandil (214.8 m). This suggests that altitudinal gradients might influence gene flow and could act as barriers to dispersal, thereby promoting microevolutionary divergence.

Land use analysis provided further ecological context. Sites varied from urban-industrial landscapes (e.g., East Singhbhum) and mining-dominated regions (e.g., Seraikela-Kharsawan, West Singhbhum) to agro-forestry mosaics (e.g., Khunti, Simdega) and relatively pristine forest habitats (e.g., Dumri). The genetic cohesion among most populations, despite ecological heterogeneity, highlights the adaptive plasticity of *D. chrysippus*—a trait likely contributing to its resilience and widespread distribution.

Collectively, the integration of morphological, molecular, spatial, and ecological data reveals a nuanced population structure: one that is largely homogeneous and interconnected, yet punctuated by local divergence in isolated, high-elevation, or less disturbed regions. The Dumri population, in particular, stands out as a candidate **for** special conservation attention due to its distinct evolutionary lineage.

This study reinforces the utility of DNA barcoding not only for taxonomic verification but also for detecting cryptic divergence across landscapes with varying human and natural influences. It also underscores the need for landscape-level conservation strategies that balance the preservation of genetic diversity with monitoring of habitat change, particularly in environmentally sensitive regions like the Chotanagpur Plateau.

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ШТРИХ-КОДУВАННЯ ДНК *Danaus chrysippus* З РАЙОНІВ ДЖАРКХАНДА (ІНДІЯ)

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Мета. Дослідити морфоанатомічні характеристики та генетичне різноманіття *Danaus chrysippus* у різноманітних екологічних ландшафтах Джаркханда (Індія), використовуючи інтегративний морфомолекулярний підхід.

Матеріали й методи. Зразки були зібрані з дев'яти місць у восьми районах Джаркханда. Морфоанатомічні особливості, включаючи візерунки крил та структури статевих органів, були досліджені за допомогою стандартних таксономічних процедур. Геномну ДНК було екстраговано, а послідовності генів мітохондріальної субодиниці I (CO1) були ампліфіковані та проаналізовані. Дані послідовностей були надіслані до DDBJ (ідентифікатори доступу LC818942 — LC877969). Видову ідентичність було підтверджено за допомогою BLASTn, а філогенетичні аналізи були проведені за допомогою програмного забезпечення MEGA X з використанням методів максимальної правдоподібності (ML), об'єднання сусідів (NJ) та мінімальної еволюції (ME).

Результати. Морфологічні спостереження підтвердили характерні риси, такі як кистоподібні передні лапи, статево диморфні мітки на задніх крилах та чіткі структури статевих органів чоловічих та жіночих особин. Послідовності CO1 (509–698 п.н.) демонстрували склад, багатий на AT (середнє значення A+T ≈ 69,9%). Попарні генетичні відстані між більшістю ізолятів були низькими (0,0000–0,0197), що вказує на високу генетичну однорідність. Однак ізолят Dumri (LC877967) показав помітну дивергенцію (~0,140–0,146), що свідчить про наявність еволюційно значущої одиниці (ESU). Філогенетичний аналіз послідовно групував усі інші ізоляти в одну кладу, тоді як ізолят Dum ri утворив окрему лінію.

Висновки. Дослідження демонструє ефективність ДНК-баркодування у виявленні генетичної варіації в популяціях *D. chrysippus*. Відмінність ізоляту Dumri підкреслює його потенційну еволюційну та природоохоронну значущість. Ці результати забезпечують основу для майбутнього моніторингу генетичного різноманіття метеликів в екологічно чутливих регіонах Джаркханда.

Ключові слова: морфомолекулярний, морфоанатомічний, матриця відстаней, філогенетичне дерево, BLAST.

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