Bioinformatics; DNA Sequencing of the New Zealand Forest Ringlet Butterfly (*Dodonidia helmsii*) Butler, 1884 for Conservation and Forensic Wildlife Efforts

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Abstract

The New Zealand forest ringlet butterfly, *Dodonidia helmsii* Butler, 1884, is an endemic and rare species vulnerable to habitat loss, climate change, and invasive species. Conservation efforts for this butterfly should increasingly incorporate genetic analyses to interpret its evolutionary relationships within the Satyrinae subfamily. This bioinformatics study analysed DNA sequencing of mitochondrial and nuclear markers—such as COI, EF-1α, and wgl—to investigate the genetic makeup of *D. helmsii* and its relatedness to close relatives, including *Erebiola butleri* Fereday, 1879 and *Percnodaimon merula* Hewitson, 1875. The COI marker, widely used in species identification due to its balanced conservation and variability, served as a key target for primer design. Primer suitability assessments indicated effective amplification in *D. helmsii* and *P. merula*, though limitations emerged with *E. butleri* due to sequence divergence at the primer binding site, underscoring the need for tailored primers in diverse taxa. Phylogenetic tree analysis demonstrated that *D. helmsii* shares a recent common ancestor with these related species, reinforcing its position within the Satyrinae subfamily.

This research highlights the value of mitochondrial and nuclear markers in constructing phylogenies and supporting conservation strategies for *D. helmsii*, paving the way for future studies to optimse primers for broader taxonomic applications across related species in the Satyrinae. This genetic data not only advances conservation efforts by clarifying evolutionary lineage but also holds potential applications in forensic wildlife investigations. DNA markers from *D. helmsii* can contribute to forensic wildlife databases, assisting in the identification and protection of this rare

species under conservation legislation. Ultimately, the more research contributions made to this area will only increase the valuable genetic tools to support the species' preservation and forensic identification of butterfly specimens in cases of illegal trafficking and habitat encroachment.

Introduction

Dodonidia helmsii Butler, 1884, commonly known as the New Zealand forest ringlet butterfly or Helms butterfly (Fig. 1), is a rare endemic species found primarily inhabiting native forests at higher altitudes, with its population mostly concentrated in the North Island. Characterised by its distinctive brown and orange patterns with eyespots, this species faces threats from habitat destruction, climate change, and invasive species (Wheatley 2017). Conservation efforts focus on habitat restoration and protective measures to support its declining population, however, genetic analysis of D. helmsii is critical for understanding its phylogenetic relationships and informing conservation and forensic efforts. Yang et al. (2020) provide insights into the use of genome- wide data for constructing phylogenies of butterfly species. By employing molecular markers and DNA sequencing, researchers can clarify evolutionary relationships across the butterfly family. This is especially important for species like *Dodonidia helmsii* due to its New Zealand threat classification of "At Risk, Relict" (Wheatley 2017).

Figure 1: Helms Butterfly (*Dodonidia helmsii*) at Pirongia Forest Park. Image: Beard, C. (2015). https://www.inaturalist.org/photos/1548651.

Beyond conservation, bioinformatics and DNA analysis play an increasing role in forensic wildlife cases. Genetic markers help identify endangered species in cases of illegal poaching, trafficking, and habitat destruction, providing evidence that can be used to enforce wildlife protection laws. In New Zealand, bioinformatics is particularly valuable for identifying at-risk species like *D. helmsii* that are or should be protected under conservation legislation. By establishing genetic markers for *D. helmsii* and closely related species, this study not only supports conservation strategies but also contributes to a genetic reference database. Such databases can aid forensic wildlife investigations by enabling authorities to verify species identities, helping to protect vulnerable species and deter illegal exploitation.

This study focuses on identifying suitable molecular markers, including COI, wgl, and EF-1α gene regions, to clarify phylogenetic relationships within Satyrinae and enhance the genetic tools available for both conservation and forensic purposes.

Related Species

Dodonidia helmsii is the only species of this genus, thus making literature availability slim. Closely related species of *D. helmsii* were identified through the Open Tree of Life (n.d.), which maps evolutionary relationships within the Satyrinae subfamily. Genera like *Taenaris* and *Physcopedaliodes* were found to be closely linked to *D. helmsii* through shared common ancestors. Identifying these related species is crucial for expanding genetic studies, as *D. helmsii* is understudied, and using information from its relatives allows for a broader exploration of evolutionary data, which can support further research. (Figure 2).

Some closely related species include:

i. *Erebiola butleri* (Butler's ringlet) Fereday, 1879 – Another New Zealand endemic butterfly, sharing similar habitats and characteristics.

ii. *Percnodaimon merula* (Black Mountain ringlet) Hewitson, 1875 – A butterfly native to New Zealand, closely related in terms of taxonomy.

iii. *Hallelesis halyma* (Fabricius, 1793) – A species from the Satyrinae subfamily, found in Africa.

iv. *Hallelesis asochis* (Hewitson, 1866) – Another Satyrinae species, contributing to the diversity within the subfamily and used for comparative phylogenetic studies.

These species are relevant for comparing genetic markers and evolutionary traits within the Satyrinae subfamily.

Gene Sequences and Primers

The NCBI Nucleotide database (n.d.) lists five gene sequences for *D. helmsii*, which include:

i. COI (Cytochrome c oxidase subunit I): (1,450 bp) A common mitochondrial gene widely used as a barcode for species identification. Its variability makes it suitable for differentiating closely related species.

ii. wgl (wingless gene): A nuclear gene associated with wing development, which can provide insights into evolutionary relationships within butterflies.

iii. EF1-alpha (Elongation factor-1 alpha): A nuclear gene involved in protein synthesis, useful for phylogenetic studies due to its conservation across species.

iv. RpS5 (Ribosomal protein S5): Part of the ribosomal machinery, this nuclear gene can contribute to understanding genetic relationships among species.

v. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase): An enzyme involved in glycolysis, which also serves as a housekeeping gene, providing a baseline for comparisons in genetic studies.

The primers used for these sequences were designed based on the study of the phylogeny of the Satyrinae subfamily (Peña et al. 2006) targeting mitochondrial and nuclear genes such as COI and EF-1α. These genes are commonly used in butterfly taxonomy due to their balance of variability and conservation. Peña et al. (2006) emphasises the utility of ribosomal genes (16S and 28S rDNA) alongside protein-coding genes in reconstructing phylogenetic relationships within the Satyrinae subfamily. This research highlights three well-supported clades within the Satyrini, demonstrating the effectiveness of these genetic markers for taxonomic resolution. This finding reinforces the rationale for utilising COI or EF-1α in analysing *D. helmsii* and suggests that similar methodologies can clarify its evolutionary relationships.

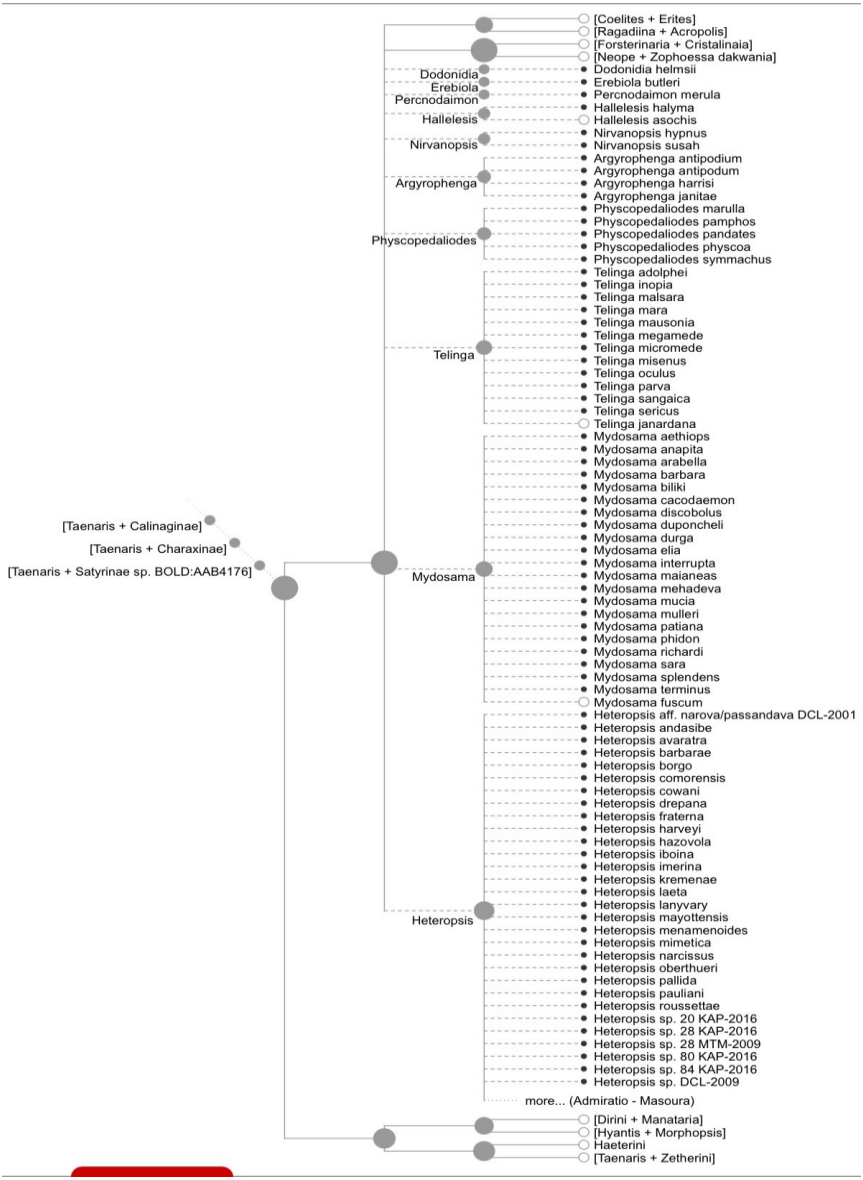

Figure 2: Phylogentic tree showing closely related species of *D. helmsii.* (Open Tree of Life).

The Nymphalidae molecular resource page (2015) discusses various aspects of molecular phylogenetics in the Nymphalidae family, including primer information and genetic markers used in studies. It focuses on the utility of mitochondrial and nuclear genes for resolving phylogenetic relationships within butterfly taxa. It highlights the use of mitochondrial genes like COI and various nuclear genes, including EF-1α and wgl.

Given the availability of these sequences, the COI gene emerges as a prime candidate for primer design and species identification. COI primers used in this report have been sourced from Wahlberg et al. (2016), and those selected will amplify regions of these genes to facilitate species identification and analysis of genetic diversity.

Suitability of Primers

The chosen primers are expected to be suitable for *D. helmsii* based on their successful application in other Lepidoptera and similar species within the Satyrinae. The utility of these primers for resolving phylogenetic relationships is demonstrated by their effectiveness in amplifying mitochondrial and nuclear DNA regions in closely related species.

Further support of primer use is given by Aduse-Poku et al. (2016) who discusses the sampling of *Hallelesis* species to study their phylogenetic relationships, using molecular markers such as mitochondrial DNA. This is relevant to DNA sequencing of *D. helmsii* because *Hallelesis* is closely related within the Satyrinae subfamily. The genetic data from *Hallelesis* species can serve as a comparative basis for *D. helmsii* sequencing. In the article, primers were used to amplify 10 molecular markers, including one mitochondrial gene (COI) and nine nuclear genes to study phylogenetic relationships in *Bicyclus* species, as well as in *Hallelesis* species. The study used primers designed by Wahlberg and Wheat (2008), making them relevant for comparison with *D. helmsii* sequencing, as both are part of the Satyrinae subfamily.

The primers designed for the COI region of *D. helmsii* were aligned with sequences from *D. helmsii* itself as well as two closely related species: *E. butleri and P. merula*, to assess their suitability across species within the Satyrinae subfamily. (Figure 3).

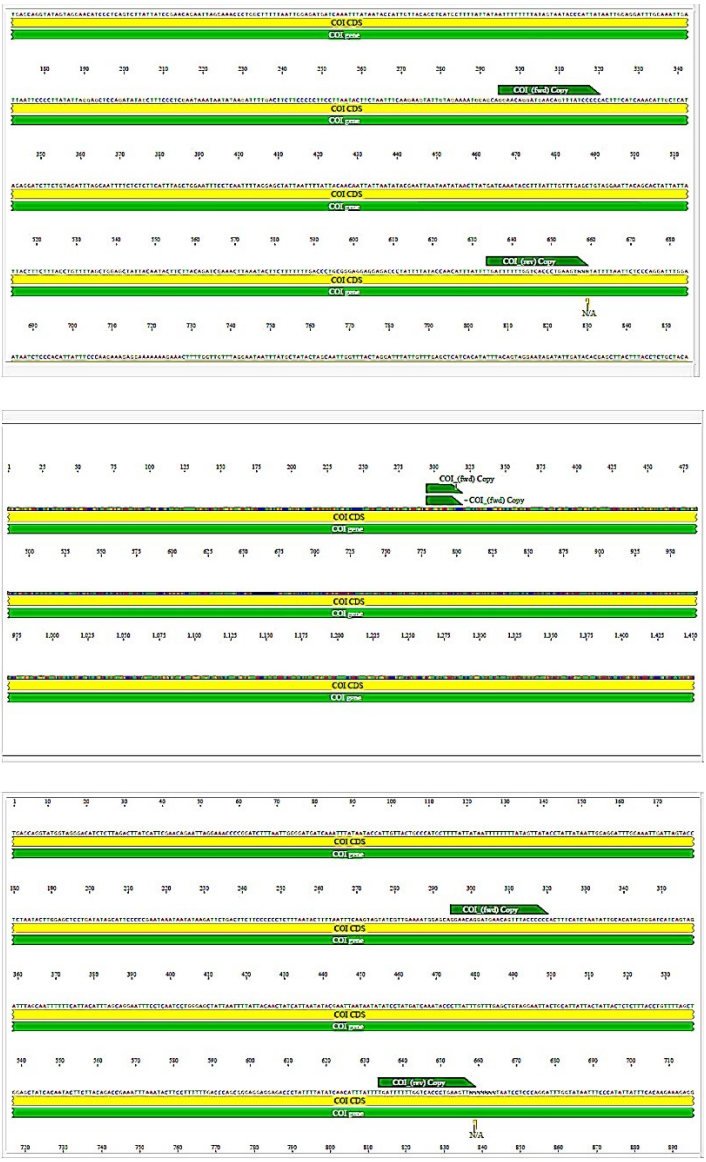

Figure 3: COI Primer Mapping of *D. helmsii* (top), *E. butleri* (middle), and *P. merula* (bottom) using Genious Prime.

- *Dodonidia helmsii:* The alignment of the forward and reverse primers to the COI gene of *D. helmsii* (voucher number NW123-15) showed perfect binding for both primers. The primers covered a critical portion of the COI gene, facilitating amplification for species identification and phylogenetic analysis. There were no mismatches or gaps, suggesting that the primers are highly specific and efficient for this species.
- *Erebiola butleri*: The forward primer aligned successfully to the COI region of *E. butleri*, but the reverse primer did not bind. This indicates sequence divergence in the reverse primer binding site between *D. helmsii* and *E. butleri*, potentially due to evolutionary divergence within the Satyrinae subfamily. The inability of the reverse primer to bind suggests that these primers may not be universally applicable across all related species without modification, particularly for *E. butleri*.
- *Percnodaimon merula:* Both the forward and reverse primers successfully aligned to the COI gene of *P. merula*. The primers showed no significant mismatches, indicating that this region is conserved between *D. helmsii* and *P. merula*. This suggests that the primers are suitable for amplification in *P. merula* and other closely related species where the COI gene sequence is more conserved.

The results demonstrate that the primers designed for *D. helmsii* are highly suitable for binding and amplifying the COI region in *D. helmsii* itself, as well as *P. merula*, but face limitations with *E. butleri*. These findings highlight several key aspects:

- Suitability for *D. helmsii*: The primers are highly effective for amplifying the COI gene in *D. helmsii*, suggesting they can be reliably used for DNA barcoding and phylogenetic studies within the species. The perfect alignment indicates no need for modification of the primers for future sequencing of this species.

- Divergence in *E. butleri*: The lack of reverse primer alignment in *E. butleri* indicates significant sequence divergence in the COI gene between *E. butleri* and *D. helmsii*. This suggests that the primers designed for *D. helmsii* may not be directly applicable to all related species in the Satyrinae subfamily, particularly in cases where evolutionary divergence has altered the sequence in key regions. Alternative strategies, such as designing a new reverse primer or using degenerate primers, may be necessary to accommodate this divergence.

- Conservation in *P. merula*: The successful alignment of both primers in *P. merula* shows that the COI gene region targeted by these primers is relatively conserved between *D. helmsii* and *P. merula*. This implies that these primers can be used for multi-species comparative studies, providing insights into phylogenetic relationships within the Satyrinae subfamily. The conservation between these two species makes these primers suitable for broader taxonomic studies.

Overall, the primers designed for the COI gene of *D. helmsii* are effective for use in both *D. helmsii* and *P. merula* but require modifications for broader applicability across other related species like *E. butleri*. The findings emphasise the importance of species-specific testing of primers in phylogenetic studies, particularly in subfamilies where genetic divergence may affect primer binding efficiency. Future studies may benefit from improving or redesigning the reverse primer for use in more divergent species within the Satyrinae subfamily.

Alignment Assessment

Within the provided phylogenetic tree, *D. helmsii* (DQ338822) serves as the reference species, with *E. butleri* (DQ338823) and *P. merula* (DQ338829) aligned for comparison (Figure 4). The branch lengths represent the genetic distance between the species, with values indicating how much they diverge. Shorter branch lengths between *E. butleri* and *D. helmsii* suggest a closer evolutionary relationship compared to *P. merula*. These species are all part of the Satyrinae subfamily, and their genetic proximity suggests a shared recent common ancestor.

DQ338823 (*E. butleri*) shows relatively shorter genetic distance to D. helmsii, reflecting greater similarity. DQ338829 (*P. merula*) exhibits slightly more divergence, indicating a more distant relationship but still within the same evolutionary lineage. Genetic distances like 0.0135, 0.0288, and others give us insights into the subtle genetic variations between these species, with *E. butleri* being the most closely aligned to *D. helmsii* based on the smaller distance metrics.

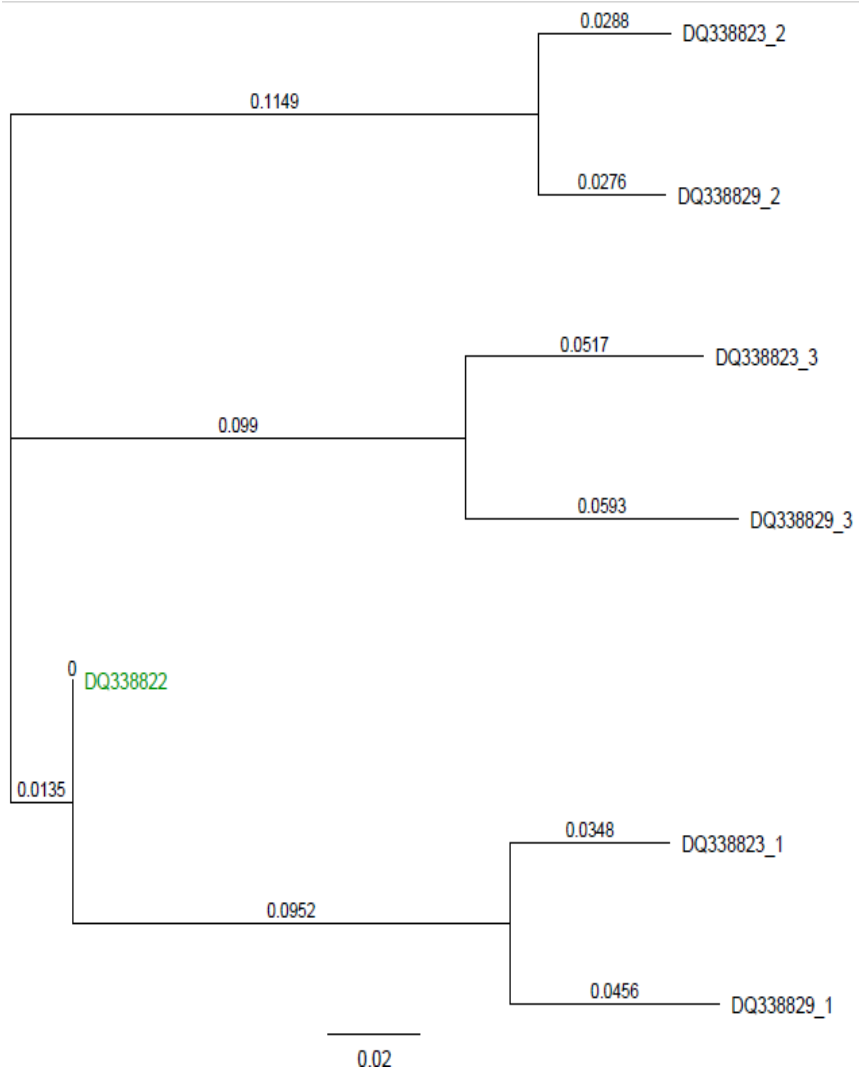

Figure 4: Phylogenetic Tree Alignment of *D. helmsii* (DQ338822) with *E. butleri* (DQ338823) and *P. merula* (DQ338829).

This tree supports the conclusion that these species are closely related members of the Satyrinae subfamily but still exhibit important genetic differences that may account for species-specific traits. The DNA markers used in the phylogenetic analysis, particularly the Cytochrome c oxidase I

gene are highly suitable for distinguishing *D. helmsii* from closely related species like *E. butleri* and *P. merula*. These markers are frequently used in species-level identification due to their balance of conservation and variability. The short branch lengths in the tree suggest that the markers effectively highlight subtle genetic differences, providing enough resolution to differentiate *D. helmsii* from its relatives.

Annealing Temperatures and Dimer Formation

In the Wahlberg et al. (2016) study from which the used primers were sourced, universal tails were added to all primers to facilitate sequencing, and a standard thermal cycling profile was used for PCR testing. The annealing temperature was set at 55°C across all primers, simplifying procedures for large-scale testing without optimising specific primer pairs. This choice of annealing temperature is a balance between primer specificity and binding efficiency.

For dimer formation, the lack of optimisation suggests a risk of primer-dimers, especially at the universal tail sites. However, the use of enzyme-based cleanup and successful sequencing indicates minimal dimer interference.

Conclusion

The bioinformatic study of *D. helmsii* using mitochondrial and nuclear DNA markers demonstrates the species' evolutionary relationships with closely related species like *E. butleri* and *P. merula*. The use of COI, and other markers in literature (such as EF- 1α and wgl) proved suitable for distinguishing *D. helmsii* from its relatives. However, species- specific divergence, particularly in *E. butleri*, indicates that primer optimisation may be necessary for broader applicability across the subfamily. Overall, the markers effectively capture genetic variability, offering valuable tools for species identification, phylogenetic studies, and conservation efforts.

Further genetic work on *D. helmsii* could prove valuable in a forensic wildlife context by enabling the identification of individuals or populations in cases of illegal wildlife trade or habitat destruction. Genetic markers, like the COI gene, can help distinguish this species from other butterflies, verifying if it has been illegally harvested or trafficked. Additionally, DNA analysis could assist in monitoring genetic diversity for conservation purposes, identifying poaching hotspots, or verifying compliance with conservation laws, helping enforce protections for this endangered species.

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