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Original article

DNA Barcoding Reveals a Distinct Mediterranean Mitochondrial Lineage of *Lasiocampa tripolitania* from Libya Based on COI Sequences: Implications for Taxonomy, Conservation, and Forensic Identification

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ABSTRACT

DNA barcoding is widely used for organism identification because the mitochondrial cytochrome c oxidase subunit I gene, COI, usually separates closely related species and geographic lineages. In this study, we examined the COI barcode position of the Libyan moth Lasiocampa tripolitania using the GenBank sequence AM397633.1, reported from near Zuwara, Libya. The final aligned dataset included a 576 bp COI fragment, three closely related Mediterranean Lasiocampa sequences from Malta, 32 European sequences assigned to Lasiocampa quercus and Lasiocampa trifolii. Phylogenetic trees were reconstructed in MEGA 10 using Neighbor-Joining and Maximum Likelihood under the Tamura 3-parameter model with 1000 bootstrap replicates. Pairwise Tamura 3-parameter distances and ASAP delimitation were also used. The Libyan sequence clustered within a Mediterranean Lasiocampa lineage and was most closely related to Maltese Lasiocampa sequences, but it formed a distinct Libyan branch. Mean distance between Mediterranean Lasiocampa and European L. quercus was 0.163, while the distance from European L. trifolii was 0.119. These results support a distinct Mediterranean mitochondrial lineage, but broader sampling is required before formally finalizing the taxonomic revision.

Introduction

DNA barcoding is now one of the main tools used to identify species from short DNA sequences. The most common animal barcode is a fragment of the mitochondrial cytochrome c oxidase subunit I gene, usually written as COI or COX1. This marker is useful because it is found in most species, is easy to amplify in many groups, and often shows more variation between them than within species [1]. The original proposal of COI as a standard barcode was developed by Hebert and colleagues, and it has since been tested in many organism groups, including Lepidoptera [2, 3].

Moths and butterflies are especially suitable for DNA barcoding because many species are difficult to identify from morphology alone. Some species differ only in small wing-pattern characters, genital structures, larval characters, or geographic range. In old museum specimens, damaged samples, larval stages, or forensic traces, morphology may be incomplete or unavailable. For this reason, COI barcoding has become an important support tool for Lepidoptera taxonomy, biodiversity surveys, pest monitoring, conservation, and forensic identification [3-5]. Large Lepidoptera barcode studies have shown that COI can separate many species, although it should not replace morphology or broader genomic evidence in difficult cases [6].

The genus *Lasiocampa* belongs to the family Lasiocampidae, a group of moths that includes several European and Mediterranean species. The European taxa *Lasiocampa quercus* and *Lasiocampa trifolii* are well represented in barcode databases compared with many North African lineages. However, the North African and southern Mediterranean diversity of *Lasiocampa* remains less completely sampled than European diversity. This creates a common problem in DNA barcoding: a lineage may appear distinct, but the biological meaning of that distinctness depends on sampling density, correct identification of reference sequences, and agreement with morphology [5].

Taxonomic work on the *Lasiocampa trifolii* species group has shown that species boundaries in this complex are not always easy to define using morphology alone. Lewandowski and Fischer described *Lasiocampa tripolitania* as a new species from north-western Libya, with the holotype collected 19 km southeast of Zuwara, and they placed it within the broader *trifolii* species group. Their revision also noted that the male genitalia of *L. trifolii*, *L. josua*, and *L. tripolitania* do not provide constant diagnostic characters, meaning that external habitus and larval characters were important for species separation. Importantly, the same authors stated that modern analytical methods, including DNA analysis, would be needed to provide further evidence

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on the genetic and evolutionary relationships among these taxa. This makes COI DNA barcoding especially relevant for testing whether the Libyan *L. tripolitania* barcode represents a distinct mitochondrial lineage or falls within the variation of related Mediterranean and European *Lasiocampa* taxa [7].

Libya occupies an important geographic position in the central southern Mediterranean. Its northern coastal zone, including the Zuwara region, lies close to Malta, Sicily, Tunisia, and other Mediterranean biogeographic routes. For flying insects, historical and ecological connections across the Mediterranean may shape lineage structure. Coastal Libya may therefore contain lineages that are closely related to island or southern European populations, while still carrying unique local haplotypes. Such geographically structured mitochondrial variation is important in biodiversity and conservation studies because it may reveal evolutionary units that are not obvious from morphology alone [8-10].

The present study focuses on the GenBank COI sequence AM397633.1, labelled as *Lasiocampa tripolitania* from Libya near Zuwara. The study compares this Libyan barcode with other *Lasiocampa* sequences, and the central question is simple: does the Libyan *L. tripolitania* barcode fall inside European *L. quercus* or *L. trifolii*, or does it form a separate Mediterranean mitochondrial lineage?

This question has three main implications. First, it has taxonomic value. If the Libyan sequence is clearly separated from European *L. quercus* and *L. trifolii*, it supports the idea that North African *Lasiocampa* diversity may be underrepresented in current barcode libraries. Second, it has conservation value. A distinct Libyan mitochondrial branch may represent a geographically structured lineage that should be sampled and monitored, especially if local habitats are threatened [8, 9]. Third, it has forensic value. In forensic biology and wildlife forensics, species identification from DNA traces depends on reliable reference databases. DNA barcoding has already been used in forensic entomology to improve arthropod identification, especially when morphology is not enough [11, 12].

Methods

Sequence sampling

The focal sequence was *Lasiocampa tripolitania* AM397633.1 from Libya, near Zuwara. The analysed barcode region was 576 bp after alignment and trimming. Comparative sequences were selected from GenBank according to the uploaded result file and figure labels. The ingroup contained four Mediterranean *Lasiocampa* sequences, including the Libyan AM397633.1 sequence and three Maltese *Lasiocampa* sp. sequences: MW305916.1, MW305917.1, and MW305915.1. The European comparison dataset contained sequences assigned to *Lasiocampa quercus* and *Lasiocampa trifolii*. The outgroups were selected from related lasiocampid taxa, including *Macrothylacia rubi* and *Malacosoma neustria*. Public sequence repositories such as GenBank and BOLD are commonly used for this type of comparative DNA barcoding study, although correct taxonomic annotation remains essential.

The dataset, therefore, represented three main ingroup lineages: Mediterranean *Lasiocampa*, European *L. quercus*, and European *L. trifolii*. Outgroups were used to root or orient the broader topology and to test whether the ingroup sequences formed coherent clusters separate from non-*Lasiocampa* taxa. The use of outgroups is standard in phylogenetic analysis because it helps interpret the direction and relative placement of ingroup branches.

Alignment and sequence preparation

COI sequences were aligned as protein-coding mitochondrial barcode sequences. The final alignment length was 576 bp. Sequences were checked for obvious length problems, missing data, and unusual divergence. Because COI is a protein-coding mitochondrial gene, the alignment was treated as codon-aligned in principle, although the phylogenetic analyses were performed at the nucleotide level. Multiple sequence alignment is a key step before tree reconstruction because positional homology must be established before estimating genetic distances or phylogenetic relationships [13].

Phylogenetic analyses

Phylogenetic analyses were performed in MEGA 10. MEGA X/10 is widely used for molecular evolutionary analysis and includes model testing, genetic distance estimation, and tree reconstruction tools [14]. The Tamura 3-parameter model was selected for nucleotide substitution analysis. This model is suitable when transition/transversion bias and G+C content bias are relevant in mitochondrial DNA datasets [15].

Two tree-building methods were used. First, a Neighbor-Joining tree was reconstructed under the Tamura 3-parameter model. Neighbor-Joining is a distance-based method that builds a tree from pairwise genetic distances and is commonly used in DNA barcoding as a first view of sequence clustering. Second, a Maximum Likelihood tree was reconstructed using the same substitution model. Maximum Likelihood evaluates the probability of the observed sequence data under alternative trees and a specified model of sequence evolution. Bootstrap support was estimated with 1000 replicates for both analyses. Bootstrap analysis is a standard way to assess the repeatability of inferred clades under resampling of sites.

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Genetic distance estimation

Pairwise genetic distances were calculated in MEGA 10 using the Tamura 3-parameter model. Distances were summarized as mean within-lineage distances and mean between-lineage distances. Three lineages were compared: Mediterranean *Lasiocampa*, European *L. quercus*, and European *L. trifolii*. The uploaded MEGA tables provided the mean distance values and standard errors. Distance-based comparisons are widely used in DNA barcoding because they allow a simple evaluation of whether within-group distances are smaller than between-group distances.

ASAP delimitation

Species delimitation was further assessed using ASAPy, the graphical software implementation of ASAP**:** **Assemble Species by Automatic Partitioning**. ASAP is a distance-based method that groups barcode sequences into candidate species or molecular operational taxonomic units using pairwise distances [16]. The ASAP result was interpreted as a molecular delimitation tool, not as final taxonomic proof. This distinction is important because single-locus delimitation can detect structured mitochondrial lineages, but species status should ideally be tested with morphology, geography, nuclear markers, and additional specimens.

Results

Dataset composition

The final COI alignment contained 576 nucleotide positions. The ingroup consisted of Mediterranean *Lasiocampa*, European *Lasiocampa quercus*, and European *Lasiocampa trifolii*. The Mediterranean group included the Libyan *Lasiocampa Neustria235nia* AM397633.1 from near Zuwara and three Maltese *Lasiocampa* sp. Sequences. The outgroup sequences belonged to *Macrothylacia rubi* and *Malacosoma Neustria*.

The major result was consistent across analyses: the Libyan sequence did not cluster inside the main European *L. quercus* or *L. trifolii* groups. Instead, it was placed with the Maltese Mediterranean *Lasiocampa* sequences, forming a distinct Mediterranean barcode lineage. Similar agreement between distance-based and likelihood-based analyses is commonly treated as stronger support for a stable phylogenetic pattern than reliance on one method alone.

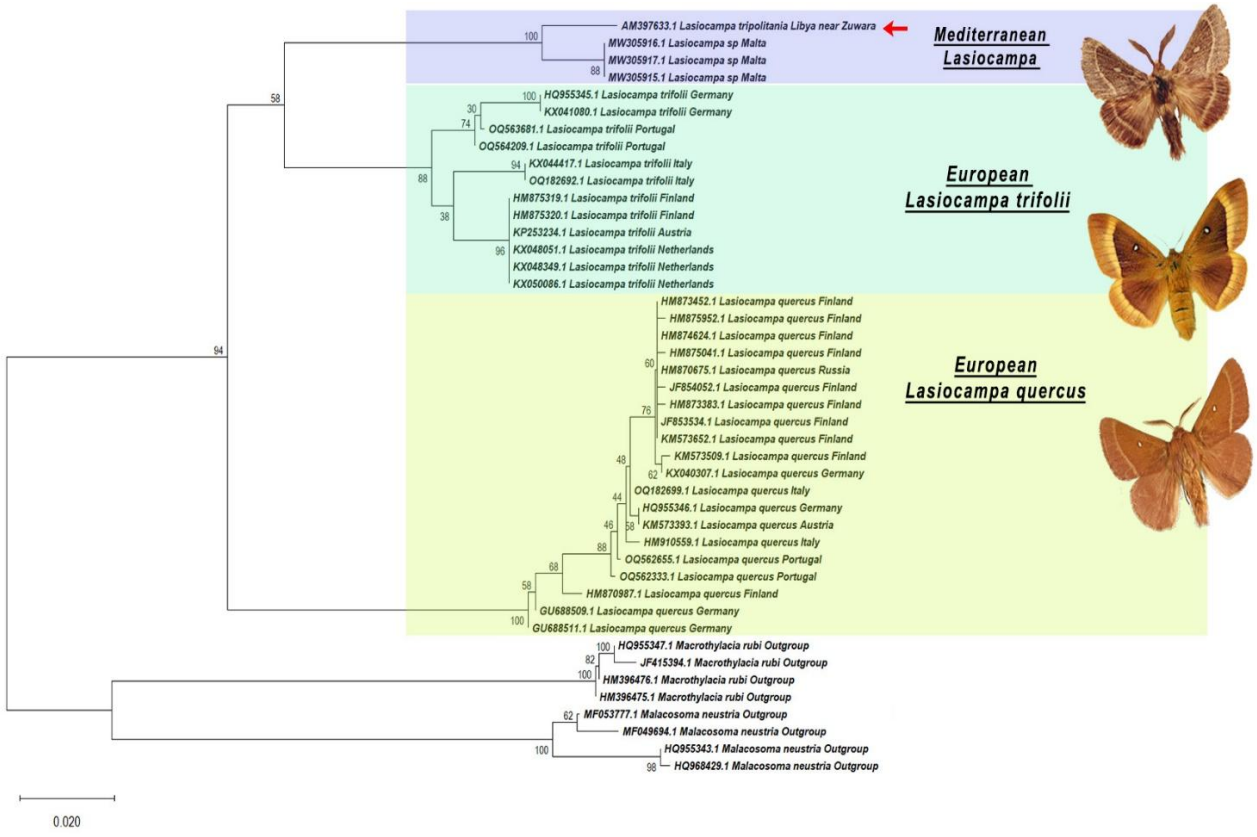

Figure 1. Neighbor-Joining phylogenetic tree of Mediterranean and European Lasiocampa COI sequences reconstructed in MEGA 10 using the Tamura 3-parameter model. Bootstrap values are based on 1000 replicates and are shown as percentages at nodes. The Libyan Lasiocampa Neustria235nia sequence AM397633.1 from near Zuwara clusters with

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Maltese Lasiocampa sp. Sequences, forming a Mediterranean lineage separate from European Lasiocampa quercus and European Lasiocampa trifolii. Outgroups include Macrothylacia rubi and Malacosoma Neustria.

Neighbor-Joining tree

The NJ tree (figure 1) separated the dataset into three main ingroup clusters. The first cluster contained the Mediterranean *Lasiocampa* sequences. Within this group, the Libyan AM397633.1 sequence was associated with Maltese *Lasiocampa* sp. sequences. The second cluster contained European *L. trifolii*, with sequences from Germany, Portugal, Italy, Finland, Austria, and the Netherlands. The third cluster contained European *L. quercus*, with sequences from Finland, Russia, Germany, Austria, Italy, and Portugal. The Mediterranean lineage was placed outside the main European *L. trifolii* and *L. quercus* clusters. The Libyan sequence formed a visible separate branch within the Mediterranean cluster rather than being identical in placement to the Maltese sequences.

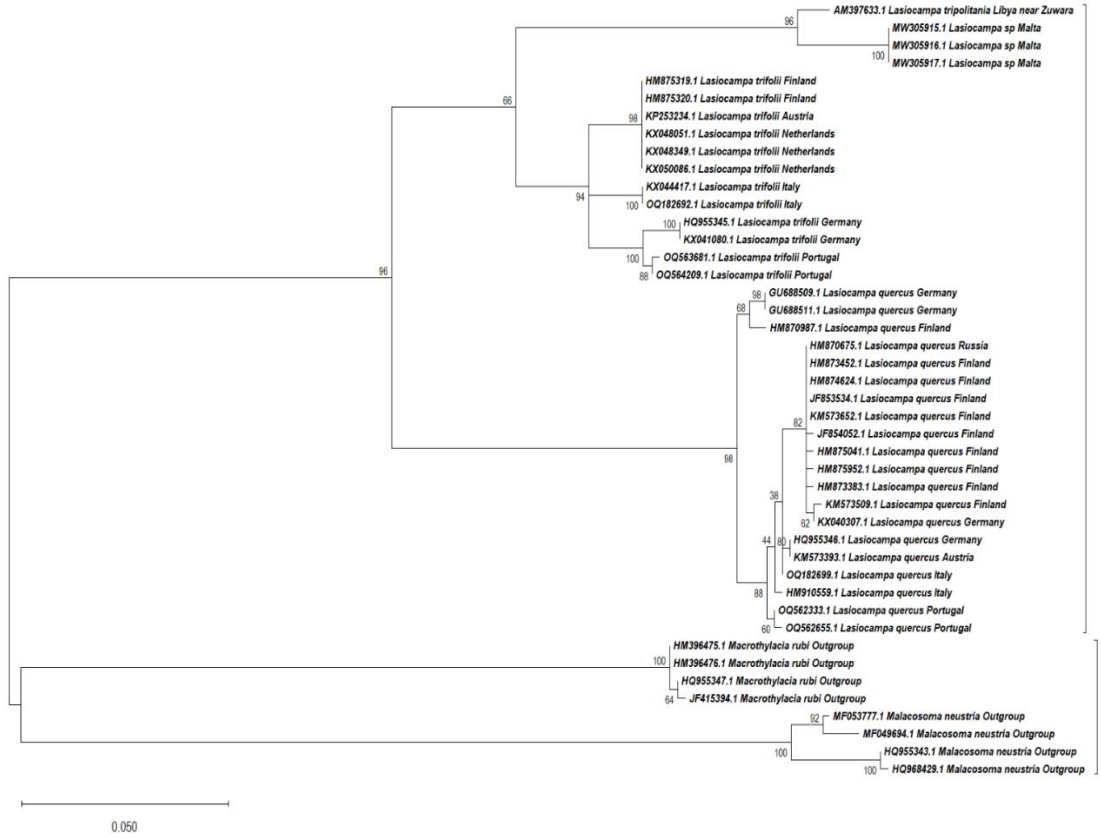

Figure 2. Maximum Likelihood phylogenetic tree of *Lasiocampa* COI sequences reconstructed in MEGA 10 under the Tamura 3-parameter model. Bootstrap values are based on 1000 replicates. The topology supports a Mediterranean *Lasiocampa* lineage containing the Libyan *Lasiocampa tripolitania* sequence and Maltese *Lasiocampa* sp. sequences. European *L. quercus* and *L. trifolii* form separate lineages, while *Macrothylacia rubi* and *Malacosoma neustria* are used as outgroups.

Maximum Likelihood tree:

The ML tree (Figure 2) supported the same broad pattern observed in the NJ tree. The Libyan AM397633.1 sequence grouped with the Maltese Mediterranean sequences and remained separated from the European *L. quercus* and *L. trifolii* clusters. The European *L. trifolii* sequences formed a compact group, although internal branches varied in bootstrap support. The European *L. quercus* group was larger and showed more internal subdivision.

The congruence between NJ and ML analyses indicates that the major placement of the Libyan barcode was not dependent on a single tree-building method. Both methods placed the Libyan sequence in the Mediterranean lineage. This is important because Neighbor-Joining and Maximum Likelihood use different assumptions and optimization procedures.

Table 1. Mean Tamura 3-parameter genetic distance within the three main Lasiocampa lineages. Values were calculated in MEGA 10 from the 576 bp COI alignment.

Lineage	Mean within-lineage distance	Standard error
Mediterranean *Lasiocampa*	0.0145	0.0055

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European *Lasiocampa quercus*	0.0105	0.0033
European *Lasiocampa trifolii*	0.0212	0.0048

Within-lineage genetic distances

The mean within-lineage distance (Table 1) for the Mediterranean Lasiocampa lineage was 0.0145 (standard error, 0.0055). Lasiocampa quercus had the lowest within-lineage distance with a mean value of 0.0105 (standard error, 0.0033). Lasiocampa trifolii had the highest within-lineage distance among the three groups, with a mean value of 0.0212 and a standard error of 0.0048.

Table 2. Mean Tamura 3-parameter genetic distances between the three main Lasiocampa lineages. The lower triangle shows mean genetic distances. The upper triangle shows standard errors from the MEGA 10 output.

Lineage	**Mediterranean *Lasiocampa***	**European *L. quercus***	**European *L. trifolii***
Mediterranean *Lasiocampa*	—	0.031 **SE**	0.025 SE
European *L. quercus*	0.163	—	0.033 SE
European *L. trifolii*	0.119	0.146	—

Between-lineage genetic distances:

In Table 2, the mean genetic distance between Mediterranean *Lasiocampa* and European *L. quercus* was 0.163. The mean distance between Mediterranean *Lasiocampa* and European *L. trifolii* was 0.119. The mean distance between European *L. quercus* and European *L. trifolii* was 0.146.

The smallest between-lineage distance involving the Mediterranean group was with European *L. trifolii* at 0.119, while the distance to European *L. quercus* was higher at 0.163. However, both values were much higher than the mean within-lineage distances.

ASAP delimitation

The ASAP delimitation (Figure 3) output supported the separation of major barcode groups. The Libyan sequence was placed near the Maltese Mediterranean sequences but showed a distinct Libyan branch relative to the Maltese branches. This result is consistent with the tree analyses: the Libyan barcode appears to be part of a Mediterranean *Lasiocampa* lineage, but not simply identical to the sampled Maltese haplotypes.

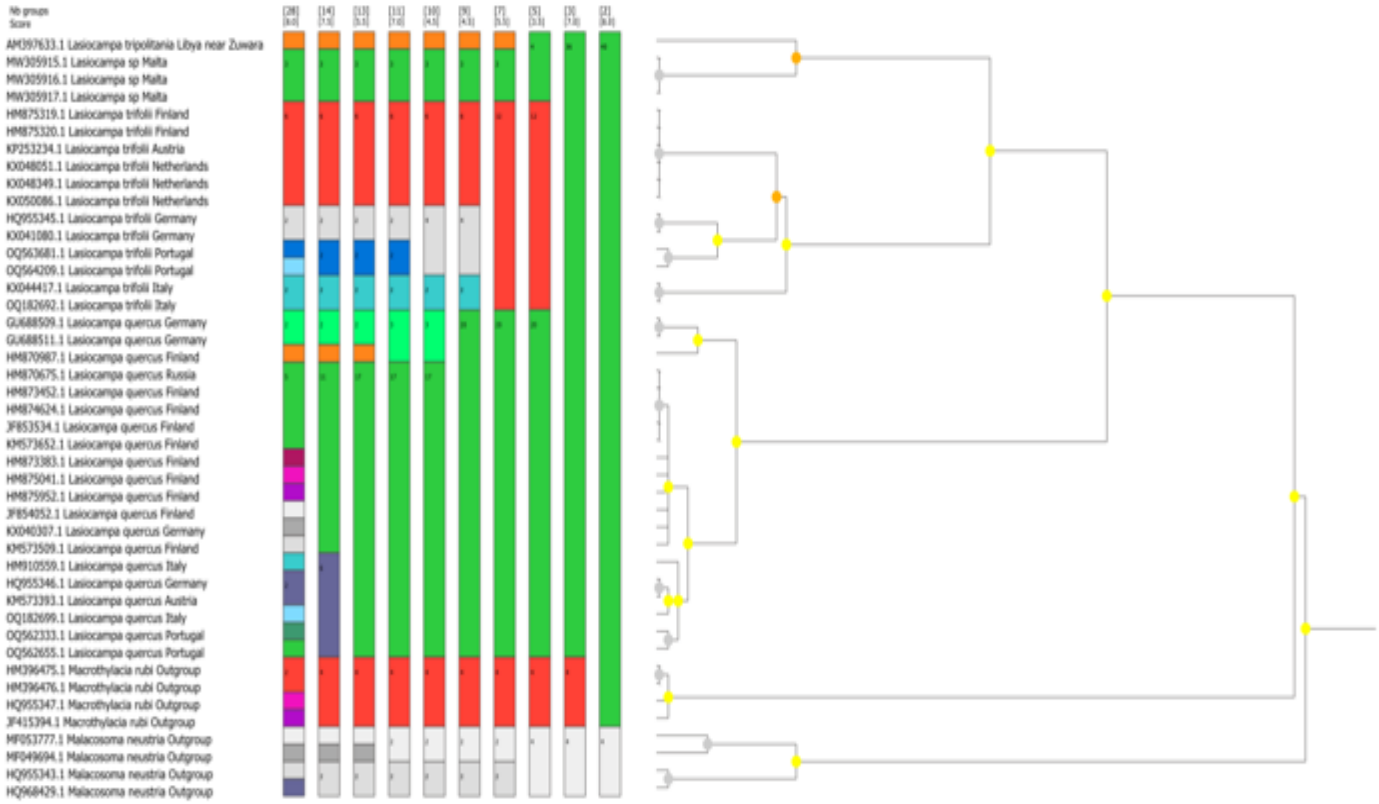

Figure 3. ASAP barcode delimitation output for the Lasiocampa COI dataset. The analysis supports separation of Mediterranean, European L. quercus, and European L. trifolii barcode partitions. The Libyan

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AM397633.1 sequence is associated with the Mediterranean group but is distinguished from the Maltese branches.

Discussion

The central finding of this study is that the Libyan *Lasiocampa tripolitania* COI sequence forms part of a distinct Mediterranean mitochondrial lineage. It is not embedded within the main European *L. quercus* cluster and is not placed inside the European *L. trifolii* cluster. Instead, its groups with Maltese *Lasiocampa* sp. sequences. This pattern suggests that the Libyan sequence shares a more recent common mitochondrial ancestry with the Maltese sequences than with the sampled European *L. quercus* or *L. trifolii* lineages. Such geographically structured mitochondrial patterns are common in animal phylogeography, although they do not always equal species boundaries [17, 18].

This finding is biologically plausible. Libya and Malta are both part of the Mediterranean biogeographic region. Historical dispersal, island-mainland connections, wind-assisted movement, or older population connections may explain why a Libyan barcode is closer to Maltese *Lasiocampa* sequences than to more northern European lineages. However, a COI tree can show mitochondrial relationships, but it does not by itself prove complete species boundaries [6, 19, 20].

Although the topology of phylogenetic trees in this study supports clearly close Mediterranean affinity but also suggests Libyan haplotype distinctness. In DNA barcoding studies, such clustering patterns are often interpreted together with genetic distance data and delimitation analyses, rather than from tree topology alone [2, 16, 21].

In addition, these results indicate that all three lineages were internally coherent at the COI level, although *L. trifolii* showed slightly greater internal mitochondrial variation than the Mediterranean *Lasiocampa* and *L. quercus* lineages. The Mediterranean lineage also showed low internal divergence, supporting its treatment as a closely related COI barcode group in the present dataset. However, the presence of a distinguishable Libyan branch within the Mediterranean group indicates that low average within-lineage distance does not exclude local haplotype differentiation. Therefore, the within-lineage distance results should be interpreted together with the NJ tree, ML tree, between-lineage distances, and ASAP delimitation analysis.

The distance results support the tree pattern. Mean within-lineage distances were low: 0.010 in Mediterranean *Lasiocampa*, 0.010 in *L. quercus*, and 0.020 in *L. trifolii*. In contrast, distances between lineages were much larger: 0.119 between Mediterranean *Lasiocampa* and *L. trifolii*, 0.163 between Mediterranean *Lasiocampa* and *L. quercus*, and 0.146 between *L. quercus* and *L. trifolii*. This clear difference between within-lineage and between-lineage distances is consistent with a barcode gap pattern [2, 22].

Still, barcode gaps can be affected by sampling. When few specimens are just been sequenced in the Database, within-group diversity may be underestimated and between-group distinctness may look stronger than it really is. This issue has been discussed in several DNA barcoding studies. Meyer and Paulay showed that sampling strongly affects barcode error estimates, while Wiemers and Fiedler showed that barcode gaps may shrink or disappear when taxa are sampled more densely [5, 21]. Therefore, the present result should be considered strong preliminary evidence for a distinct Mediterranean mitochondrial lineage, not final proof of species rank.

The Libyan barcode shows close affinity to the Maltese *Lasiocampa* sp. sequences. This is one of the most important findings. It suggests that the Mediterranean group may represent a regional lineage separate from the main European *L. quercus* and *L. trifolii* clusters. Although the Libyan sequence appears as a distinct branch, the Maltese sequences form nearby branches. This pattern can be described as a Libyan haplotype branch within a broader Mediterranean lineage.

In other words, the Libyan *L. tripolitania* barcode is closely related to Maltese Mediterranean *Lasiocampa* barcodes, but it is distinguishable from them in the analysed COI dataset. This cautious interpretation agrees with modern DNA taxonomy recommendations, which encourage barcode evidence to be treated as one component of species assessment rather than as a complete replacement for classical taxonomy [6, 20].

The present findings come in the same line as recent DNA barcode evidence from the Maltese Islands. Vella and his colleagues also reported five Maltese specimens identified as *Lasiocampa* sp., represented by three haplotypes, and these Maltese specimens were genetically closer to the same *L. tripolitania* than to *L. trifolii*. However, because the Maltese samples formed a separate cluster from *L. tripolitania*, they were conservatively retained as *Lasiocampa* sp. rather than being assigned directly to *L. tripolitania*. Together, these results support the presence of a central Mediterranean *Lasiocampa* mitochondrial complex that includes a Libyan branch and closely related Maltese branches, but they also confirm that broader geographic sampling, morphology, and additional genetic markers are still required before making formal taxonomic changes [23].

ASAP delimitation supported a distinctive Libyan branch against the Maltese branches, while still placing Libya logically within the same Mediterranean group. This agrees with the NJ and ML trees. ASAP is useful

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because it does not depend directly on a tree topology. Instead, it uses pairwise distances to propose barcode partitions [16].

However, ASAP and similar methods should be treated as hypothesis-generating tools. They can suggest candidate species or candidate lineages, but they cannot alone complete a taxonomic revision. Species delimitation is strongest when several lines of evidence agree: mitochondrial DNA, nuclear DNA, adult morphology, genital morphology, larval characters, ecology, distribution, and type material comparison.

For the present, our dataset, ASAP, supports the recognition of a distinct Libyan barcode branch. It does not, by itself, prove that *L. tripolitania* is a separate biological species from all related Mediterranean forms. The name *Lasiocampa tripolitania* may be valid, but this study provides mitochondrial support rather than a complete taxonomic diagnosis. This is especially important because mitochondrial introgression, incomplete lineage sorting, and species-level paraphyly can produce patterns that complicate species delimitation [17-19].

The Libyan sequence AM397633.1 is important because North African *Lasiocampa* diversity is poorly represented in public barcode datasets. When only European references are available, North African lineages may be incorrectly forced into European names or left unidentified. This is a common issue in molecular identification.

The present result suggests that Libya may contain a Mediterranean *Lasiocampa* lineage that is not adequately represented by European *L. quercus* or *L. trifolii*. This supports the need for more sampling in Libya, especially along the coastal zone and in habitats near Zuwara, Tripoli, the Jabal Nafusa region, and other northern localities. Additional sequences should include both males and females, multiple localities, and specimens identified by morphology. Broader geographic and taxonomic sampling is essential because barcode success and error rates depend strongly on the completeness of the reference dataset.

A future taxonomic study should compare Libyan specimens with Maltese, Tunisian, Sicilian, Italian, and broader Mediterranean material. It should also include nuclear markers because mitochondrial DNA follows only the maternal line. These limitations are well known in most organisms' phylogeography and barcoding [17, 18].

The Libyan barcode branch may have conservation value. A distinct mitochondrial lineage can indicate regional evolutionary history. Even if future work shows that the Libyan population is not a separate species, it may still represent a geographically important population unit. Conservation biology often recognizes such units because they preserve genetic diversity and local evolutionary potential [8].

This is especially important in North Africa, where insect diversity is less studied than in Europe. Habitat loss, coastal development, agriculture, pesticide use, climate change, and reduced vegetation connectivity can affect moth populations. If *L. tripolitania* depends on specific habitats or host plants, local decline could remove unique Libyan genetic diversity before it is properly studied. Cryptic or geographically restricted lineages are often overlooked in conservation planning when taxonomy is incomplete [9, 10].

This study also has forensic relevance. The COI barcoding is directly useful in forensic identification of biological traces. In forensic entomology, correct arthropod identification can be important when insects are associated with remains, crime scenes, transport routes, stored products, or environmental evidence. DNA barcoding can help when specimens are damaged, immature, partial, or morphologically difficult [11, 12, 24].

For *Lasiocampa*, forensic application may not be the same as in blow flies, which are directly used in post-mortem interval estimation. Instead, the value is broader: wildlife forensics, biodiversity crime, ecological trace evidence, geographic inference, and verification of unknown insect material. A validated Libyan barcode reference can help distinguish Libyan or Mediterranean *Lasiocampa* material from European species. Reference libraries are central to reliable DNA-based identification because the accuracy of an unknown match depends on database quality and taxonomic coverage.

However, forensic use requires strict standards. A single GenBank sequence is usually not enough for a forensic reference database. For forensic reliability, the database should include voucher specimens, photographs, expert morphological confirmation, collection locality, sequence chromatograms, and multiple individuals per locality. Public database errors can affect forensic interpretation, so reference libraries must be curated. This caution agrees with wider discussions of DNA-based identification and forensic entomology, where reference quality is a major requirement for reliable conclusions [11].

Conclusion

This study provides COI barcode evidence that the Libyan *Lasiocampa tripolitania* sequence AM397633.1 from near Zuwara belongs to a distinct Mediterranean mitochondrial lineage. Both NJ and ML analyses in MEGA 10 placed the Libyan barcode with Maltese *Lasiocampa* sequences, apart from European *Lasiocampa quercus* and *Lasiocampa trifolii*. Genetic distances supported this structure, with low within-lineage distances and much higher distances between Mediterranean and European lineages. ASAP delimitation also supported a distinctive Libyan branch within the Mediterranean group. The results are important for

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taxonomy, conservation, and forensic identification in Libya. They suggest that Libyan *Lasiocampa* diversity is underrepresented in current barcode libraries and may include regionally important mitochondrial lineages. However, because the Libyan evidence is based on one COI sequence, the findings should be treated as a strong preliminary barcode signal rather than a final taxonomic revision. Future work should include more Libyan specimens, Maltese and North African comparisons, nuclear markers, and detailed morphology. This cautious approach follows the broader recommendation that DNA barcoding should be integrated with classical taxonomy, population sampling, and independent genetic markers.

Conflict of interest
The authors declare that they have no conflict of interest.

Ethical approval
This study used publicly available GenBank sequence data and did not involve new animal collection, animal experimentation, or human participants.

Data availability
The focal Libyan COI sequence is available from GenBank under accession number AM397633.1. Comparative sequence accession numbers are shown in the phylogenetic figures.

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