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# DNA Barcoding of Lepidoptera Species from the Maltese Islands: New and Additional Records, with an Insight into Endemic Diversity

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**Abstract:** This work presents the first outcomes resulting from a DNA barcode reference library of lepidopteran species from Malta. The library presented here was constructed from the specimens collected between 2015 and 2019 and covers the genetic barcodes of 146 species (ca. 25% of lepidopterous Maltese fauna), including four newly recorded Lepidoptera species from the Maltese islands: *Apatema baixerasi*, *Bostra dipectinialis*, *Oiketicoides lutea*, and *Phereoeca praecox*. The DNA reference barcode library constructed during this study was analyzed in conjunction with publicly available DNA barcodes and used to assess the ability of the local DNA barcodes to discriminate species. Results showed that each species occupies a different BOLD BIN; therefore, DNA barcoding was able to discriminate between the studied species. Our data led to the formation of 12 new BOLD BINs—that is, OTUs that were identified during this work—while nearly 46% of the barcodes generated during this study were never recorded on conspecifics, further indicating the uniqueness of genetic diversity on these central Mediterranean islands. The outcomes of this study highlight the integrative taxonomic approach, where molecular taxonomy plays an important role for biodiversity investigation in its entirety.

**Keywords:** biodiversity conservation; endemism; genetic diversity; Malta; new records



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## 1. Introduction

Biodiversity conservation heavily relies on the use of appropriate tools for characterizing and monitoring various components of biological diversity. Frequently, these conservation efforts are limited by a lack of basic ecological information and efficient large-scale monitoring tools. One of the drawbacks of such assessments is that the morphological identification of species, especially for arthropods, often requires taxonomists with experience and specialization in specific taxa [1]. Additionally, morphological keys are frequently gender specific or life-stage specific, such as the one specifically for adult males, which lacks the taxonomic keys for immature or female specimens [2–5]. Moreover, several descriptions are based on few individuals and may include characters that exhibit phenotypic plasticity [4], while they may miss cryptic complexes [5,6]. To overcome these limitations, one of the most effective tools for species identification is DNA barcoding [2,7–9], which has the potential to accelerate taxonomic workflows while enabling the sorting of specimens into operational taxonomic units (OTUs) [10,11]. This standardized technique allows for rapid species identification and has valuable applications in improving biodiversity monitoring, both in terms of efficiency and accuracy in a wide range of taxa and ecosystems [12–19]. It also allows for the accurate genetic identification of alien species for timely mitigation of biological invasions [20–22].

Molecular taxonomy and its applications are dependent on comprehensive molecular data, which at times require the use of multiple genes, including both mitochondrial and nuclear sequences, to better comprehend the underlying genetic diversity and divergence

between species [15,23–26]. Therefore, while there is no single universal tool to delimitate all species, the use of COI for several animal taxa, including arthropods [15], is a widely accepted tool for the construction of DNA barcode libraries of taxonomically verified specimens, augmenting the data available for this gene [27–29]. Even though there has been an increase in initiatives to barcode the diversity of life [30], the goal of sequencing all species still requires considerable efforts to be achieved, especially for taxonomically diverse groups, such as insects that contain an estimated 5.5 million species [31], of which half a million are estimated to be Lepidoptera [32]. Currently, close to one third of the estimated number of Lepidoptera species have been described, with just over 1000 new species added and around 200 species names synonymized annually, with the majority of the additions covering micro-moths [32,33]. Additionally, on BOLD, there are nearly 1,670,000 Lepidoptera specimens that have been barcoded, with the publicly available data covering 76,298 species, which form 123,775 barcode index numbers (BINs) [34]. These BINs are part of an automated system that clusters DNA sequences algorithmically using refined single linkage (RESL) analysis, generating a unique identifier, known as a BIN, for each out [35]. Therefore, as these BINs cluster OTUs according to their DNA barcode, they assist in finding species boundaries and allow for improvements in taxonomic revisions and biodiversity assessments [35,36].

As with other taxa, in recent years, taxonomic additions and changes have been characterized by molecular phylogenetic analyses updating classification with the discovery of new species [9,14–16,37–40]. Taxonomic rearrangements involving certain taxonomic levels have been elevated to higher ranks, while others have been reconsidered to be synonyms [16]. These changes may be more frequently encountered in Lepidoptera due to the high degree of non-monophyly noted at species level associated with morphological misidentifications or subjectivity in species delimitation [41].

The identification of species is essential for defining the ecological functions of organisms within ecosystems; therefore, additions to the existing DNA barcode libraries are of the utmost urgency. Lepidoptera species are important pollinators, with moths being the major nocturnal pollinators of flowers [42,43], and therefore being of economic importance. However, various other species within this group feature among the increasing alien and invasive species, negatively affecting host plants and economic growth [44–46]. Data on the species richness, abundance, and spatiotemporal distributions of lepidopterans are frequently used in evaluating the quality of ecosystems [47,48]. Their utility as bioindicators comes from the fact that these species are sensitive to environmental changes, with the general trends showing that most native populations are adversely affected. Species and population numbers decline with increases in anthropogenic activities and the impacts of climate change, urbanization, insecticides and other chemical pollutants, light pollution, and the presence of alien plant species [43,47–49]. At the same time, other species such as *Zeuzera pyrina*, a woodborer species that is considered to be a pest to several trees [50] is found to be expanding in its abundance with increasing episodes of drought [51]. Within this scenario, lepidopteran studies from Malta are important, as this 316 $km^2$ central Mediterranean archipelago is the most densely populated country in Europe [52], and consequently, its natural habitats are constantly exposed to human activities. Local knowledge on the diversity of Lepidoptera, both at species and genetic levels, provide the required tools for accurate and efficient monitoring. Malta is estimated to host around 600 Lepidoptera species [53–55].

This study uses molecular taxonomy to produce a local DNA reference library for the Lepidoptera species found in Malta, and to increase the data on the barcodes found in international reference DNA databases. This is intended to allow for better taxonomic resolution that considers genetic diversity from a central Mediterranean archipelago that is distant from mainland Europe (80 km south of Sicily) and northern Africa (285 km from the Tunisian coast).

## 2. Materials and Methods

### *2.1. Specimen Collection and Morphological Identification*

The lepidopteran tissue samples collected from 374 specimens undertaken by the Conservation Biology Research Group at the University of Malta between 2015 and 2019 were used in this study. The specimen tissues were collected using insect nets or captured during the night using UV light traps set in the field between May and October of each sampling year. The specimens were collected (Figure 1) from various habitat types, including urban and rural areas, across the islands of Malta and Gozo from the central Mediterranean (geographical coordinates of the islands: Malta 35.917973 N 14.409943 E; and Gozo 36.044399 N 14.251222 E). The specimen tissues were individually stored in labelled sample bottles and placed at −20 °C on the same day as collection until further processing. Each specimen was photographed and morphologically identified to the lowest taxonomic level following [53,54,56–61], before sampling the tissues for genetic analyses. Morphologically identified species were checked for their occurrence in Malta using the Fauna Europaea database portal [55] and the published literature [59,61–66]. The collection of protected Lepidoptera specimens was conducted under permits NP0095/16 and NP0271/17, issued by the Environment and Resource Authority (Malta).

### *2.2. DNA Extraction, Amplification, and Sequencing*

Genomic DNA was extracted from a leg of the collected specimens using the GF-1 Tissue DNA Extraction Kit (Vivantis, Shah Alam, Malaysia), following the manufacturer's manual. PCR amplification of the standard DNA barcode region, mitochondrial cytochrome c oxidase subunit I gene (COI), was carried out using LCO1490/HCO2198 [67] and LepF1/LepR1 [38], appended with the universal M13 oligonucleotide tails. Amplification reactions were carried out following Mifsud et al. [68]. The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide to confirm amplification and estimate concentration. PCR products were then purified and sequenced using both the forward and reverse primers via an ABI3730XL sequencer.

### *2.3. Molecular Identification*

The quality checks, editing, and alignments of the resulting DNA sequences were conducted using Geneious v. 11.1.2 [69]. DNA barcode sequences were aligned using MUSCLE [70], primer nucleotide sequences were removed, and chromatograms were checked for the presence of double peaks, stop codons, and frameshifts, which could indicate the amplification of nuclear mitochondrial (NUMT) pseudogenes. None of the DNA sequences showed evidence of pseudogenes.

All new DNA barcodes were searched against the NCBI GenBank® database (GenBank, https://www.ncbi.nlm.nih.gov/genbank, accessed on 25th November 2022 [71]) nucleotide collection (nr/nt) using BLASTn v 2.9.0 [72,73], and against the species-level barcode records available at the Barcode of Life Data System (BOLD, http://www.boldsystems.org, accessed on 25th November 2022 [34]) using the Species Level Barcode Records within the identification portal system. Sequences were assigned to the BINs by the RESL algorithm, as implemented in BOLD [34,35]. Data related to each BIN, including the average and maximum intra-BIN p-distance and the minimum p-distance to the nearest neighboring BIN, as estimated through BOLD [34], were recorded.

Some cases were further investigated, using genetic sequences from GenBank and BOLD, with regard to the phylogenetic pattern of the specific taxa. In these cases, the sequences were aligned using MUSCLE [70]. The model of best fit, as identified by jModel [74], was used while constructing phylogenetic trees using Bayesian inference. This was estimated via MrBayes v3.2 [75,76] and used $8 \times 10^6$ generations with a sampling frequency of every 2000 generations and a burn-in of 25% to allow for the log-likelihood scores to stabilize. These phylogenetic trees allowed for better visualization of clusters to evaluate species delimitation using DNA barcodes. In some instances, as indicated in the results

section, the BOLD TaxonID Tree within BOLD was used to visualize divergence, which includes data that are not publicly available.


**Figure 1.** Images showing examples of the diversity of species that form part of the current data set. (**A**): *Lasiommata megera* (MW305918); (**B**): *Papilio machaon* (MW305956); (**C**): *Acherontia atropos* (MW305743); (**D**): *Pechipogo plumigeralis* (MW305957); (**E**): *Utetheisa pulchella* (MW306031). (**A**–**C**) are locally protected species. Photos by Denis Magro.

## 3. Results

### *3.1. Taxonomic Coverage and General Overview*

This study represents the first DNA barcode reference library of lepidopteran species from Malta, with a COI barcode dataset obtained from 374 specimens representing a total of 146 species belonging to 23 families: Autostichidae (1 species); Blastobasidae (1 species); Cosmopteriddidae (2 species); Cossidae (1 species); Crambidae (16 species); Erebidae (19 species); Gelechiidae (8 species); Geometridae (18 species); Lycaenidae (1 species); Lasiocampidae (2 species); Momphidae (1 species); Noctuidae (30 species); Nymphalidae (4 species); Papilionidae (1 species); Pieridae (3 species); Plutellidae (1 species); Psychidae (2 species); Pterophoridae (5 species); Pyralidae (16 species); Sesiidae (1 species); Sphingidae (3 species); Tineidae (3 species); and Tortricidae (7 species) (Table 1). This dataset represents around 25% of the currently known Maltese Lepidoptera species [55] and contributes to the knowledge of 147 species. The family represented by the largest sample number is the Noctuidae, which accounts for 26.5% ($n$ = 100) of the total collected spec-

imens, while 13 families are represented by one or two species. The newly amplified data did not include any insertions, deletions, or stop codons, thus indicating that these sequences represent functional mitochondrial COI sequences.

**Table 1.** A list of the species analyzed ([F] first records for Malta; [E] endemic species), including the number of specimens per species (*n*), number of haplotypic variants per species (H) (with the superscript indicating the number of newly identified haplotypes), the barcode index number ([N] indicates a new BIN that contains only current sequences), and associated data obtained from BOLD. [nB = number of sequences in BIN; AvD = average p-distance within BIN; MxD = maximum p-distance within BIN; DNN = distance to nearest neighbor; NN BIN = nearest neighbor BIN; NN taxonomy = species assigned to nearest neighbor BIN; nBN = number of sequences in nearest neighbor BIN; NN AvD = average p-distance within nearest neighbor's BIN; NN MxD = maximum p-distance within the nearest neighbor's BIN]. BOLD data presented here was last accessed on 25th November 2022.

| FAMILY<br>Species | *n* | H | BIN<br>BOLD: | nB | AvD (%) | MxD (%) | DNN (%) | NN BIN<br>BOLD: | NN Taxonomy | nBN | NN AvD (%) | NN MxD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AUTOSTICHIDAE** | | | | | | | | | | | | |
| *Apatema baixerasi* [F] | 1 | $1^{1}$ | AAV4815 | 10 | 0.56 | 0.98 | 4.04 | ADR6916 | *Apatema* sp. | 4 | 0.14 | 0.33 |
| **BLASTOBASIDAE** | | | | | | | | | | | | |
| *Blastobasis phycidella* | 3 | $3^{3}$ | AAF0414 | 69 | 0.57 | 2.43 | 2.97 | AAZ8649 | *Blastobasis* sp. | 2 | 0.46 | 0.46 |
| **COSMOPTERIGIDAE** | | | | | | | | | | | | |
| *Bifascioides leucomelanella* | 1 | $1^{1}$ | ABA4555 | 18 | 0.27 | 0.84 | 2.41 | ADU3943 | Lepidoptera sp. | 1 | - | - |
| *Pyroderces argyrogrammos* | 3 | $3^{3}$ | AAQ0242 | 114 | 0.78 | 3.05 | 6.46 | AEJ1178 | Lepidoptera sp. | 1 | - | - |
| **COSSIDAE** | | | | | | | | | | | | |
| *Zeuzera pyrina* | 2 | $1^{2}$ | AET9156 [N] | 2 | 0.00 | 0.00 | 1.93 | ADC8403 | *Zeuzera* sp. | 1 | - | - |
| **CRAMBIDAE** | | | | | | | | | | | | |
| *Agriphila trabeatellus* | 2 | $2^{2}$ | ACA9410 | 9 | 0.70 | 1.77 | 8.26 | ABA4409 | *Catoptria confusellus* | 3 | 0.27 | 0.33 |
| *Ancylolomia pectinatellus* | 1 | $1^{1}$ | ACA9335 | 5 | 0.49 | 0.67 | 7.87 | ACI1349 | Crambidae sp. | 2 | 0.15 | 0.15 |
| *Antigastra catalaunalis* | 3 | $3^{2}$ | AAE6976 | 38 | 0.61 | 1.53 | 3.22 | AAP5696 | *Antigastra catalaunalis* | 1 | - | - |
| *Aporodes floralis* | 3 | $1^{0}$ | AAN7323 | 40 | 0.13 | 0.59 | 2.62 | AAV4122 | *Aporodes floralis* | 6 | 0.65 | 1.12 |
| *Dolicharthria bruguieralis* | 1 | $1^{1}$ | AED1102 | 2 | 0.16 | 0.16 | 1.28 | AAO3560 | *Dolicharthria bruguieralis* | 10 | 0.12 | 0.48 |
| *Duponchelia fovealis* | 5 | $3^{2}$ | AAD9727 | 106 | 0.16 | 1.99 | 2.25 | ACR2019 | *Duponchelia fovealis* | 9 | 0.36 | 0.80 |
| *Euchromius cambridgei* | 4 | $2^{2}$ | ABY3890 | 12 | 1.24 | 2.18 | 7.21 | ADZ9070 | Phycitinae sp. | 1 | - | - |
| *Euchromius ocellea* | 8 | $4^{2}$ | AAA5671 | 231 | 0.37 | 1.77 | 3.19 | ABA8488 | *Euchromius* sp. | 1 | - | - |
| *Evergestis* sp. | 2 | $2^{2}$ | AEU3700 [N] | 2 | 0.16 | 0.16 | 2.73 | ADL3576 | *Evergestis isatidalis* | 3 | 0.54 | 0.80 |
| *Hellula undalis* | 5 | $4^{1}$ | AAC8519 | 78 | 0.48 | 1.61 | 3.45 | AAE6944 | *Hellula rogatalis* | 54 | 0.06 | 0.55 |
| *Herpetogramma licarsisalis* | 1 | $1^{1}$ | AAA3965 | 292 | 0.14 | 1.77 | 3.98 | AAA3967 | *Herpetogramma licarsisalis* | 38 | 0.06 | 0.32 |
| *Nomophila noctuella* | 3 | $1^{0}$ | AAA7880 | 319 | 0.46 | 3.75 | 3.86 | AAB5466 | *Nomophila corticalis* | 123 | 0.35 | 1.12 |
| *Palpita vitrealis* | 1 | $1^{0}$ | AAC1043 | 100 | 0.72 | 2.57 | 2.51 | AAB0733 | *Palpita margaritacea* | 51 | 0.18 | 0.64 |
| *Spoladea recurvalis* | 1 | $1^{0}$ | AAA3666 | 364 | 0.72 | 3.19 | 6.18 | ABA0182 | *Scoparia paracycla* | 1 | - | - |
| *Udea ferrugalis* | 3 | $2^{0}$ | AAC3729 | 104 | 0.64 | 3.04 | 3.47 | ABA1630 | *Udea stellata* | 1 | - | - |
| *Uresiphita gilvata* | 1 | $1^{0}$ | ACF5204 | 43 | 0.50 | 1.77 | 1.10 | AAA3568 | *Uresiphita ornithopteralis* | 170 | 0.15 | 1.02 |
| **EREBIDAE** | | | | | | | | | | | | |
| *Clytie illunaris* | 1 | $1^{1}$ | AAK5589 | 18 | 0.25 | 0.65 | 1.12 | AEH3335 | *Clytie* sp. | 3 | 0.11 | 0.16 |
| *Cymbalophora pudica* | 3 | $2^{2}$ | AAG6227 | 12 | 0.74 | 1.50 | 4.81 | ABZ5736 | *Turuptiana obliqua* | 1 | - | - |
| *Dysauxes famula* | 2 | $2^{1}$ | AAM0427 | 24 | 0.24 | 0.80 | 1.12 | ACF0669 | *Dysauxes famula* | 8 | 0.53 | 0.96 |
| *Eilema caniola* | 2 | $1^{0}$ | AAF6264 | 71 | 0.78 | 2.73 | 4.19 | AAA4503 | *Manulea bicolor* | 198 | 0.30 | 2.68 |
| *Eublemma ostrina* | 1 | $1^{0}$ | AAG1829 | 34 | 0.56 | 2.25 | 2.39 | ABW0690 | *Eublemma staudingeri* | 22 | 0.06 | 0.37 |
| *Eublemma parva* | 5 | $3^{1}$ | AAM5884 | 43 | 0.25 | 0.64 | 3.52 | ACL9149 | *Eublemma saldaitis* | 1 | - | - |
| *Eublemma scitula* | 1 | $1^{1}$ | ACD0717 | 6 | 0.62 | 1.77 | 4.33 | ACN9797 | Noctuidae sp. | 1 | - | - |
| *Eublemma* sp. | 1 | $1^{1}$ | AAL4752 | 13 | 0.81 | 1.77 | 1.12 | ACL7422 | *Eublemma parva* | 4 | 0.48 | 0.80 |
| *Hypena lividalis* | 3 | $2^{0}$ | AAE1121 | 69 | 0.19 | 1.15 | 5.93 | AAA2868 | *Chytolita morbidalis* | 187 | 0.70 | 1.92 |
| *Hypena obsitalis* | 10 | $5^{3}$ | AAK3686 | 23 | 0.12 | 0.39 | 3.19 | ACF0234 | *Hypena sordidula* | 17 | 0.16 | 0.65 |
| *Metachrostis velocior* | 3 | $2^{1}$ | AAH6931 | 8 | 0.51 | 1.25 | 1.44 | ACK1973 | *Metachrostis dardouini* | 6 | 0.05 | 0.16 |
| *Metachrostis velox* | 2 | $2^{1}$ | AAH6930 | 14 | 0.45 | 0.98 | 1.77 | ACK1973 | *Metachrostis dardouini* | 6 | 0.05 | 0.16 |
| *Nodaria nodosalis* | 1 | $1^{0}$ | AAK3749 | 50 | 0.76 | 3.00 | 3.10 | AAD1694 | *Simplicia cornicalis* | 50 | 0.25 | 2.17 |
| *Ophiusa tirhaca* | 1 | $1^{0}$ | ABZ7648 | 21 | 0.40 | 1.18 | 1.77 | ABZ4334 | *Ophiusa* sp. | 22 | 0.05 | 0.36 |
| *Orgyia trigotephras* | 1 | $1^{1}$ | AAM0804 | 4 | 1.03 | 1.44 | 4.17 | ACB6683 | *Orgyia* sp. | 4 | 0.00 | 0.00 |
| *Pechipogo plumigeralis* | 1 | $1^{0}$ | AAI4196 | 22 | 0.05 | 0.32 | 3.37 | AAA2868 | *Chytolita morbidalis* | 187 | 0.70 | 1.92 |
| *Phragmatobia fuliginosa* | 4 | $1^{0}$ | AAA6178 | 94 | 0.59 | 2.25 | 2.25 | AAN2564 | *Phragmatobia fuliginosa* | 2 | 0.36 | 0.36 |
| *Utetheisa pulchella* | 1 | $1^{0}$ | AAF0098 | 61 | 0.17 | 0.64 | 1.42 | ACT3042 | *Utetheisa elata* | 3 | 0.82 | 1.22 |
| *Zebeeba falsalis* | 6 | $1^{0}$ | AAJ9181 | 26 | 0.98 | 2.57 | 8.01 | AAN6974 | *Elaphria* sp. | 1 | - | - |

**Table 1.** *Cont.*

| FAMILY Species | *n* | H | BIN BOLD: | nB | AvD (%) | MxD (%) | DNN (%) | NN BIN BOLD: | NN Taxonomy | nBN | NN AvD (%) | NN MxD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **GELECHIIDAE** | | | | | | | | | | | | |
| *Agonopterix olusatri* | 1 | 1 [1] | ABW7168 | 15 | 0.52 | 1.61 | 1.93 | ADF2495 | *Agonopterix* sp. | 5 | 0.10 | 0.16 |
| *Agonopterix subpropinquella* | 1 | 1 [1] | AER7434 [N] | 1 | - | - | 1.93 | AAZ9000 | *Agonopterix subpropinquella* | 13 | 0.05 | 0.38 |
| *Aproaerema* sp. | 1 | 1 [1] | AET5627 [N] | 1 | - | - | 1.77 | AEA1472 | *Aproaerema* sp. | 5 | 0.00 | 0.00 |
| *Ornativalva plutelliformis* | 1 | 1 [1] | ABW9166 | 11 | 0.65 | 1.77 | 2.49 | ABX8241 | *Ornativalva plutelliformis* | 4 | 0.33 | 0.66 |
| *Phthorimaea operculella* | 4 | 1 [0] | AEL8356 | 95 | 0.18 | 4.31 | 5.54 | AED9067 | Phthorimaea sp. | 1 | - | - |
| *Platyedra subcinerea* | 6 | 5 [4] | AAD8749 | 49 | 0.54 | 1.50 | 5.36 | AAU3620 | *Pexicopia* sp. | 2 | 0.00 | 0.00 |
| *Ptocheuusa paupella* | 1 | 1 [0] | AAV2188 | 18 | 0.17 | 0.82 | 3.21 | ACW2460 | *Ptocheuusa paupella* | 7 | 0.57 | 1.29 |
| *Tuta absoluta* | 1 | 1 [0] | AAJ8033 | 973 | 0.04 | 1.80 | - | - | - | - | - | - |
| **GEOMETRIDAE** | | | | | | | | | | | | |
| *Charissa variegata* | 5 | 4 [4] | AAC1039 | 35 | 1.62 | 3.49 | 2.75 | AAC4341 | *Charissa subtaurica* | 18 | 0.35 | 1.50 |
| *Cyclophora puppillaria* | 1 | 1 [0] | AAB2523 | 60 | 0.03 | 0.65 | 3.17 | ACF3607 | *Cyclophora albipunctata* | 36 | 0.43 | 1.44 |
| *Epirrhoe alternata* | 2 | 1 [0] | ACE4142 | 134 | 0.63 | 2.81 | 1.02 | AAA3371 | *Epirrhoe alternata* | 85 | 0.85 | 2.09 |
| *Eucrostes indigenata* | 2 | 2 [2] | AAC6469 | 16 | 0.37 | 1.16 | 3.60 | ADF4899 | *Eucrostes* sp. | 1 | - | - |
| *Eupithecia centaureata* | 2 | 2 [2] | ACE9420 | 67 | 1.76 | 4.33 | 6.06 | ACJ9495 | *Eupithecia* sp. | 1 | - | - |
| *Gymnoscelis rufifasciata* | 1 | 1 [1] | AAA7404 | 97 | 0.74 | 2.20 | 2.74 | ADL3671 | *Gymnoscelis rufifasciata* | 2 | 0.00 | 0.00 |
| *Idaea elongaria* | 1 | 1 [1] | AAA8985 | 7 | 0.26 | 0.55 | 2.57 | ACK1747 | *Idaea elongaria* | 1 | - | - |
| *Idaea fractilineata* | 4 | 3 [2] | AAK4252 | 8 | 0.34 | 0.97 | 2.85 | ACM9078 | *Idaea purpurariata* | 5 | 0.00 | 0.00 |
| *Idaea obsoletaria* | 1 | 1 [1] | AAB4939 | 6 | 0.11 | 0.33 | 2.30 | ACE4926 | *Idaea obsoletaria* | 4 | 0.40 | 0.71 |
| *Idaea seriata* | 3 | 1 [1] | AAA9645 | 56 | 0.13 | 0.71 | 1.92 | ABZ4137 | *Idaea seriata* | 6 | 0.10 | 0.31 |
| *Isturgia pulinda* | 1 | 1 [0] | AAA6139 | 95 | 0.40 | 1.34 | 3.50 | AAU7783 | *Isturgia exerraria* | 2 | 0.00 | 0.00 |
| *Menophra japygiaria* | 5 | 1 [0] | AAB6706 | 42 | 0.41 | 2.64 | 2.66 | AAC8802 | *Menophra berenicidaria* | 13 | 0.18 | 0.75 |
| *Phaiogramma etruscaria* | 1 | 1 [0] | ABY4065 | 42 | 0.20 | 0.67 | 1.12 | ACW6537 | *Phaiogramma etruscaria* | 9 | 0.17 | 0.32 |
| *Phaiogramma faustinata* | 1 | 1 [0] | AAB4914 | 82 | 0.68 | 2.67 | 1.04 | ACW6536 | *Phaiogramma stibolepida* | 14 | 0.64 | 1.44 |
| *Rhodometra sacraria* | 6 | 3 [1] | AAA8983 | 138 | 1.11 | 5.41 | 5.92 | AAQ1498 | *Rhodometra sacraria* | 3 | 1.47 | 2.25 |
| *Scopula imitaria* | 2 | 1 [0] | AAB6665 | 56 | 0.23 | 1.44 | 1.80 | ABZ6950 | *Scopula imitaria syriacaria* | 5 | 0.00 | 0.00 |
| *Scopula minorata* | 1 | 1 [1] | AAA9357 | 125 | 0.94 | 3.86 | 2.12 | AEO1263 | *Scopula* sp. | 1 | - | - |
| *Xanthorhoe disjunctaria* | 1 | 1 [0] | ABY6341 | 27 | 0.45 | 1.15 | 1.77 | AET6043 | *Xanthorhoe sardisjuncta* | 11 | 0.23 | 0.64 |
| **LASIOCAMPIDAE** | | | | | | | | | | | | |
| *Gastropacha quercifolia* | 1 | 1 [1] | AAF4844 | 94 | 0.48 | 1.54 | 8.67 | AAI7018 | *Gastropacha sikkima* | 50 | 1.08 | 2.09 |
| *Lasiocampa* sp. | 5 | 3 [3] | AES9600 [N] | 3 | 0.00 | 0.00 | 1.93 | AAW9949 | *Lasiocampa tripolitania* | 3 | 0.20 | 0.31 |
| **LYCAENIDAE** | | | | | | | | | | | | |
| *Polyommatus celina* | 4 | 3 [1] | AAA3304 | 275 | 0.92 | 2.41 | 3.71 | AAA3303 | *Polyommatus erotides* | 976 | 1.28 | 3.88 |
| **MOMPHIDAE** | | | | | | | | | | | | |
| *Mompha subbistrigella* | 1 | 1 [1] | AAD0702 | 71 | 0.30 | 1.44 | 3.61 | ADB9986 | *Mompha glaucella* | 3 | 0.10 | 0.15 |
| **NOCTUIDAE** | | | | | | | | | | | | |
| *Acontia lucida* | 3 | 2 [1] | AAD6258 | 38 | 0.25 | 1.01 | 5.22 | ABV2194 | Lepidoptera sp. | 4 | 0.15 | 0.31 |
| *Agrotis biconica* | 1 | 1 [0] | AAE4276 | 36 | 0.67 | 1.51 | 2.09 | ABZ5220 | *Agrotis munda* | 28 | 0.28 | 1.02 |
| *Agrotis ipsilon* | 3 | 2 [0] | AAA3364 | 336 | 0.31 | 1.94 | 1.00 | ACE7272 | *Agrotis infusa* | 120 | 0.02 | 0.37 |
| *Agrotis lata* | 4 | 4 [2] | ACE7288 | 11 | 0.29 | 0.80 | 1.28 | AEH3853 | *Agrotis lata* | 1 | - | - |
| *Agrotis puta* | 5 | 1 [0] | AAB9164 | 79 | 0.09 | 0.64 | 2.19 | AAB9165 | Lepidoptera sp. | 10 | 0.31 | 0.64 |
| *Agrotis segetum* | 3 | 2 [1] | AAC3884 | 172 | 0.17 | 1.69 | 2.32 | AAB9113 | *Agrotis exclamationis* | 90 | 0.07 | 0.80 |
| *Agrotis trux* | 14 | 6 [6] | AET6510 | 14 | 0.19 | 0.50 | 1.12 | AAM0539 | *Agrotis trux* | 13 | 0.98 | 2.09 |
| *Anarta trifolii* | 1 | 1 [0] | ABZ1428 | 201 | 0.58 | 3.33 | 1.71 | AAA9985 | *Anarta columbica* | 71 | 0.13 | 1.07 |
| *Autographa gamma* | 3 | 2 [1] | AAB4345 | 626 | 0.03 | 2.02 | 2.17 | AAB2628 | *Autographa californica* | 110 | 0.12 | 0.67 |
| *Callopistria latreillei* | 1 | 1 [1] | AAP2182 | 47 | 0.28 | 1.28 | 3.24 | AAN8804 | *Callopistria* sp. | 2 | 0.00 | 0.00 |
| *Caradrina clavipalpis* | 3 | 1 [0] | AAB6999 | 83 | 0.36 | 2.17 | 2.41 | ABZ7109 | *Caradrina selini* | 48 | 0.13 | 0.64 |
| *Caradrina flava* | 2 | 1 [0] | AAK4908 | 8 | 0.12 | 0.48 | 3.21 | AET1610 | Lepidoptera sp. | 117 | 0.03 | 0.64 |
| *Caradrina flavirena* | 6 | 1 [0] | AAB7000 | 95 | 0.67 | 2.73 | 1.18 | ADB8712 | *Caradrina flavirena* | 9 | 0.04 | 0.16 |
| *Chrysodeixis chalcites* | 3 | 1 [0] | AAB3384 | 354 | 0.56 | 3.16 | 3.05 | AAG0704 | *Chrysodeixis kebea* | 6 | 0.33 | 0.61 |
| *Condica viscosa* | 1 | 1 [0] | AAN1812 | 13 | 0.34 | 0.72 | 2.09 | ADU4648 | Noctuidae sp. | 2 | 0.00 | 0.00 |
| *Cryphia algae* | 5 | 2 [2] | AAD6780 | 10 | 0.40 | 1.28 | 1.28 | AAD6780 | Lepidoptera sp. | 65 | 0.30 | 0.96 |
| *Hadena sancta* | 2 | 1 [1] | AAY8457 | 10 | 0.48 | 1.04 | 2.09 | ABY4816 | *Hadena ruetimeyeri* | 2 | 1.07 | 1.07 |
| *Heliothis peltigera* | 5 | 3 [0] | AAC6990 | 60 | 0.14 | 0.64 | 3.53 | AAV6844 | *Heliothis saskai* | 2 | 0.00 | 0.00 |
| *Leucania putrescens vallettai* [E] | 2 | 2 [2] | AER7912 [N] | 2 | 0.34 | 0.34 | 2.18 | AAK9298 | *Leucania putrescens* | 6 | 0.61 | 0.96 |
| *Mythimna sicula* | 1 | 1 [1] | AAF8181 | 53 | 0.49 | 1.13 | 2.75 | ABX0055 | *Mythimna opaca* | 2 | 0.32 | 0.32 |
| *Mythimna unipuncta* | 1 | 1 [0] | AAA2482 | 555 | 0.51 | 4.41 | 2.08 | ACG2559 | *Mythimna unipuncta* | 3 | 0.00 | 0.00 |
| *Noctua pronuba* | 2 | 2 [0] | AAA2632 | 321 | 0.22 | 1.61 | 3.69 | AAD0229 | *Noctua interjecta* | 56 | 0.41 | 1.46 |
| *Nyctobrya segunai* [E] | 4 | 4 [4] | AET0743 [N] | 4 | 0.53 | 0.68 | 1.68 | AAN0805 | *Cryphia muralis* | 8 | 0.21 | 0.50 |
| *Pseudozarba bipartita* | 6 | 6 [6] | AAE4331 | 48 | 1.15 | 3.28 | 3.17 | AAE8111 | *Pseudozarba orthopetes* | 24 | 0.54 | 1.93 |
| *Spodoptera cilium* | 1 | 1 [0] | AAC8279 | 79 | 0.15 | 0.99 | 2.11 | ACE3456 | *Spodoptera depravata* | 71 | 0.13 | 0.80 |
| *Spodoptera exigua* | 8 | 3 [0] | AAA6644 | 632 | 0.38 | 3.37 | 2.60 | ADB9075 | *Spodoptera exigua* | 1 | - | - |
| *Synthymia fixa* | 3 | 1 [0] | AAN0137 | 3 | 0.00 | 0.00 | 1.12 | AES6312 | *Synthymia fixa* | 6 | 0.27 | 0.80 |
| *Trichoplusia ni* | 1 | 1 [0] | AAC3410 | 50 | 0.02 | 0.35 | 4.01 | AAC3409 | *Trichoplusia ni* | 86 | 0.19 | 2.57 |
| *Tyta luctuosa* | 5 | 2 [0] | AAD5088 | 39 | 0.27 | 0.80 | 5.35 | AEI5594 | *Epharmottomena tenera* | 1 | - | - |
| *Xylena exsoleta* | 1 | 1 [1] | AAE4735 | 20 | 0.54 | 1.12 | 4.00 | ACD6521 | *Xylena formosa* | 9 | 0.79 | 1.61 |

**Table 1.** *Cont.*

| FAMILY Species | *n* | H | BIN BOLD: | nB | AvD (%) | MxD (%) | DNN (%) | NN BIN BOLD: | NN Taxonomy | nBN | NN AvD (%) | NN MxD (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NYMPHALIDAE** | | | | | | | | | | | | |
| *Coenonympha pamphilus* | 1 | 1 [0] | AAA7351 | 305 | 0.15 | 1.38 | 1.25 | ADJ7308 | *Coenonympha pamphilus* | 42 | 0.39 | 1.28 |
| *Danaus chrysippus* | 1 | 1 [0] | ABX5122 | 215 | 0.59 | 2.75 | 1.58 | AAB3216 | *Danaus chrysippus* | 22 | 0.02 | 0.20 |
| *Lasiommata megera* | 1 | 1 [0] | AAB0123 | 342 | 0.46 | 1.26 | 1.12 | ACE4512 | *Lasiommata paramegaera* | 55 | 0.05 | 0.50 |
| *Vanessa atalanta* | 2 | 2 [0] | AAA8638 | 271 | 0.22 | 2.71 | 3.85 | AAE5211 | *Antanartia abyssinica* | 22 | 0.13 | 0.61 |
| **PAPILIONIDAE** | | | | | | | | | | | | |
| *Papilio machaon* | 3 | 2 [0] | AAA5810 | 440 | 1.01 | 3.58 | 1.77 | ABZ2147 | *Papilio machaon* | 2 | 0.12 | 0.12 |
| **PIERIDAE** | | | | | | | | | | | | |
| *Colias croceus* | 2 | 2 [0] | ABZ3039 | 440 | 0.06 | 1.38 | 1.72 | ACF0844 | *Colias pelidne* | 115 | 1.16 | 2.57 |
| *Pieris brassicae* | 1 | 1 [1] | AAB0552 | 405 | 0.64 | 4.25 | 2.13 | ACN0735 | *Pieris brassicae* | 1 | - | - |
| *Pieris rapae* | 1 | 1 [1] | AAA2224 | 904 | 0.46 | 3.37 | 3.10 | AAB3783 | *Pieris mannii* | 128 | 0.24 | 0.80 |
| **PLUTELLIDAE** | | | | | | | | | | | | |
| *Plutella xylostella* | 3 | 3 [1] | AAA1513 | 3792 | 0.77 | 4.34 | 6.57 | AAC6876 | *Plutella australiana* | 121 | 0.06 | 0.62 |
| **PSYCHIDAE** | | | | | | | | | | | | |
| *Oiketicoides lutea* [F] | 4 | 3 [3] | AAM0038 | 6 | 2.09 | 3.35 | 7.50 | ABU9696 | *Oiketicoides* sp. | 1 | - | - |
| *Penestoglossa dardoinella* | 3 | 1 [1] | AEU4296 [N] | 3 | 0.00 | 0.00 | 1.28 | AAL3705 | *Penestoglossa dardoinella* | 12 | 0.03 | 0.17 |
| **PTEROPHORIDAE** | | | | | | | | | | | | |
| *Agdistis frankeniae* | 1 | 1 [1] | AED1693 [N] | 1 | - | - | 3.37 | ABV2042 | Lepidoptera sp. | 2 | 0.16 | 0.16 |
| *Emmelina monodactyla* | 2 | 1 [0] | ACE4862 | 110 | 0.08 | 0.70 | 1.34 | AAA3882 | *Emmelina monodactyla* | 111 | 0.45 | 1.77 |
| *Merrifieldia malacodactylus* | 1 | 1 [0] | ACS6787 | 17 | 0.28 | 0.64 | 4.49 | ADZ0299 | Pterophoridae sp. | 1 | - | - |
| *Pterophoridae* sp. | 1 | 1 [1] | ADZ0387 | 2 | 0.18 | 0.18 | 6.31 | AAV5270 | *Procapperia linariae* | 11 | 0.75 | 1.31 |
| *Stenoptilia* sp. | 1 | 1 [1] | ABW6859 | 7 | 0.12 | 0.32 | 4.65 | ACS3431 | *Stenoptilia* sp. | 6 | 0.80 | 2.14 |
| **PYRALIDAE** | | | | | | | | | | | | |
| *Aglossa caprealis* | 1 | 1 [1] | ACY8691 | 3 | 1.07 | 1.91 | 6.26 | ADR4870 | *Aglossa* sp. | 1 | - | - |
| *Apomyelois ceratoniae* | 2 | 2 [0] | AAU4812 | 114 | 0.43 | 1.44 | 3.00 | ACR0358 | *Cadra* sp. | 5 | 0.32 | 0.80 |
| *Bostra dipectinialis* [F] | 7 | 1 [0] | AAU4121 | 11 | 0.30 | 0.81 | 2.09 | AEO2408 | *Bostra* sp. | 15 | 0.76 | 1.61 |
| *Cadra abstersella* | 7 | 1 [0] | AAW5130 | 22 | 0.20 | 1.30 | 5.54 | AAB9605 | *Cadra cautella* | 167 | 1.24 | 4.21 |
| *Cadra cautella* | 2 | 2 [1] | AAB9605 | 167 | 1.24 | 4.21 | 3.04 | ADV8858 | *Cadra* sp. | 1 | - | - |
| *Cadra figulilella* | 6 | 2 [0] | AAZ9283 | 81 | 0.20 | 1.62 | 1.61 | ADS7823 | *Cadra* sp. | 7 | 0.41 | 0.80 |
| *Ceutholopha isidis* | 2 | 2 [1] | ABA4962 | 28 | 0.19 | 0.68 | 2.45 | ABA4962 | *Ceutholopha petalocosma* | 42 | 0.24 | 0.75 |
| *Ephestia elutella* | 1 | 1 [0] | AAC6157 | 57 | 0.76 | 1.61 | 4.15 | AAD1430 | *Ephestia parasitella* | 79 | 0.72 | 2.57 |
| *Lamoria anella* | 11 | 3 [3] | ACY8237 | 26 | 0.83 | 1.93 | 4.94 | AAY8816 | *Lamoria anella* | 2 | 0.48 | 0.48 |
| *Oxybia transversella* | 1 | 1 [1] | ACA9658 | 13 | 1.12 | 2.57 | 5.78 | AAB9775 | *Salebriaria roseopunctella* | 115 | 1.16 | 2.59 |
| *Phycita diaphana* | 1 | 1 [1] | ACA9652 | 8 | 0.32 | 0.80 | 5.78 | ACB7132 | Phycitinae sp. | 2 | 0.00 | 0.00 |
| *Phycitodes saxicola* | 2 | 1 [1] | AAD9531 | 42 | 0.63 | 2.02 | 6.04 | ABX8977 | *Phycitodes* sp. | 3 | 0.31 | 0.46 |
| *Plodia interpunctella* | 2 | 1 [0] | AAB2462 | 101 | 0.52 | 4.05 | 6.96 | ADG1988 | Pyralidae sp. | 1 | - | - |
| *Psorosa dahliella* | 1 | 1 [0] | ACA9753 | 3 | 0.55 | 0.84 | 2.09 | AEF6784 | *Psorosa ferrugatella* | 3 | 0.00 | 0.00 |
| *Pyralis farinalis* | 2 | 2 [2] | AAB3316 | 57 | 0.40 | 2.70 | 2.41 | AAY8728 | *Pyralis farinalis* | 4 | 0.19 | 0.32 |
| *Stemmatophora brunnealis* | 3 | 2 [1] | AAV6933 | 13 | 0.28 | 0.65 | 5.16 | AEO2608 | *Stemmatophora brunnealis* | 1 | - | - |
| **SESIIDAE** | | | | | | | | | | | | |
| *Bembecia albanensis* | 1 | 1 [1] | AAM2453 | 8 | 1.22 | 2.09 | 4.09 | ABX3895 | *Bembecia albanensis* | 1 | - | - |
| **SPHINGIDAE** | | | | | | | | | | | | |
| *Acherontia atropos* | 1 | 1 [0] | AAB7886 | 41 | 0.06 | 0.93 | 4.97 | AAD2845 | *Acherontia styx* | 24 | 0.28 | 1.39 |
| *Agrius convolvuli* | 1 | 1 [0] | AAA2393 | 162 | 0.60 | 2.09 | 3.32 | AAA2392 | *Agrius convolvuli* | 158 | 0.23 | 2.82 |
| *Hippotion celerio* | 2 | 2 [0] | ABZ5722 | 28 | 0.19 | 0.64 | 1.10 | ACE8834 | Sphingidae sp. | 18 | 0.10 | 0.33 |
| **TINEIDAE** | | | | | | | | | | | | |
| *Niditinea fuscella* | 1 | 1 [1] | AAF3430 | 59 | 0.90 | 2.87 | 6.37 | AAG3681 | *Niditinea truncicolella* | 5 | 0.12 | 0.32 |
| *Phereoeca praecox* [F] | 3 | 3 [2] | AAU1282 | 33 | 0.06 | 0.66 | 3.75 | AAH8518 | *Phereoeca uterella* | 11 | 0.63 | 2.43 |
| *Tinea murariella* | 1 | 1 [0] | AAE7470 | 8 | 0.19 | 0.93 | 2.90 | AEI9096 | *Tinea translucens* | 1 | - | - |
| **TORTRICIDAE** | | | | | | | | | | | | |
| *Aethes* sp. | 3 | 2 [2] | AEU4088 [N] | 2 | 0.39 | 0.39 | 3.53 | AAP7561 | *Aethes* sp. | 26 | 0.50 | 1.44 |
| *Cacoecimorpha pronubana* | 2 | 1 [1] | AAD3477 | 33 | 0.26 | 0.96 | 1.96 | ACS9337 | *Cacoecimorpha pronubana* | 2 | 0.00 | 0.00 |
| *Clepsis* sp. | 2 | 2 [2] | AED2423 [N] | 2 | 0.50 | 0.50 | 3.34 | ACT3810 | *Clepsis consimilana* | 4 | 0.08 | 0.16 |
| *Eucosma* sp. | 4 | 1 [0] | ACT0042 | 5 | 0.71 | 1.34 | 2.10 | AAB4296 | *Eucosma* sp. | 83 | 0.26 | 1.66 |
| *Lobesia botrana* | 2 | 2 [1] | ACH2178 | 77 | 0.57 | 1.70 | 4.24 | AAC9385 | *Lobesia reliquana* | 37 | 0.11 | 0.64 |
| *Pseudococcyx tessulatana* | 1 | 1 [0] | ACT0606 | 8 | 0.14 | 0.34 | 7.70 | AAH4831 | *Retinia sabiniana* | 3 | 0.10 | 0.15 |
| *Selania capparidana* | 1 | 1 [1] | AET4374 [N] | 1 | - | - | 2.50 | ABA4981 | *Selania* sp. | 1 | - | - |

All the species identified in this study were distinguishable from each other through their DNA barcodes, with each species being assigned to a different BOLD BIN (Table 1). Within our data set, the maximum intraspecific p-distance (MxD) noted was 1.83% for

both Charissa variegata and Lamoria anella (Supplementary Material Table S1). When considering all the data present in each of the analyzed BOLD BIN, the average p-distance (AvD) within the BINs ranged from 0% to 1.78% (overall mean 0.47% ± 0.35%), with the MxD within BINs reaching 4.41% (overall mean 1.65% ± 1.11%). The distance from the nearest neighboring BIN (DNN) varied between 1.00% and 8.67% (mean 3.24% ± 1.78%). In all instances, the AvD was smaller than the DNN, while in 18% of the taxa, the MxD was larger than the DNN.

### *3.2. Endemic Diversity*

Even though most species have been barcoded in other studies, and our specimens were grouped with conspecifics in their respective BOLD BINs, it was nonetheless noted that 46% of the barcodes generated here, accounting for 54% of the haplotypes, have never been recorded in conspecifics, revealing uniqueness of genetic diversity in these central Mediterranean islands (Table 1 and Supplementary Material Table S1). Additionally, our data cover 12 new BOLD BINs, therefore contributing newly barcoded OTUs to the existing literature. Apart from the newly barcoded species, such as *Agdistis frankeniae* and *Selania capparidana* (BOLD:AED1693 and BOLD:AET4374), these new OTUs include the two endemic noctuid moth species *Leucania putrescens vallettai* and *Nyctobrya segunai* (BOLD:AER7912 and BOLD:AET0743), and other species that formed new OTUs different from their conspecifics found elsewhere, such as *Agonopterix subpropinquella*, *Penestoglossa dardoinella*, and *Zeuzera pyrina* (BOLD:AER7434, BOLD:AEU4296, and BOLD:AET9156).

#### 3.2.1. Noctuidae: *Leucania putrescens vallettai* Boursin, 1952

The first endemic species covered in this study was *Leucania putrescens vallettai*, which is represented by two specimens that differ from each other by 2 bp (99.7% pairwise identity). Although this taxon is considered to be a subspecies, the level of genetic distance from *Leucania putrescens* is 2.2%, surpassing the threshold that is usually quoted for delimiting species [7,8,16,77–79], while the Barcode Index Number System places *L. putrescens vallettai* in the unique BIN of BOLD:AER7912. The BI analysis using publicly available data showed that these two taxa formed distinct, non-overlapping clusters (Figure 2), with a similar outcome noted when using the BOLD TaxonID Tree (data not shown), which includes more private data on *L. putrescens*. This level of genetic divergence is also corroborated by clear morphological differences between the two [80], indicating that the endemic subspecies represent a taxon that may be promoted to species level.

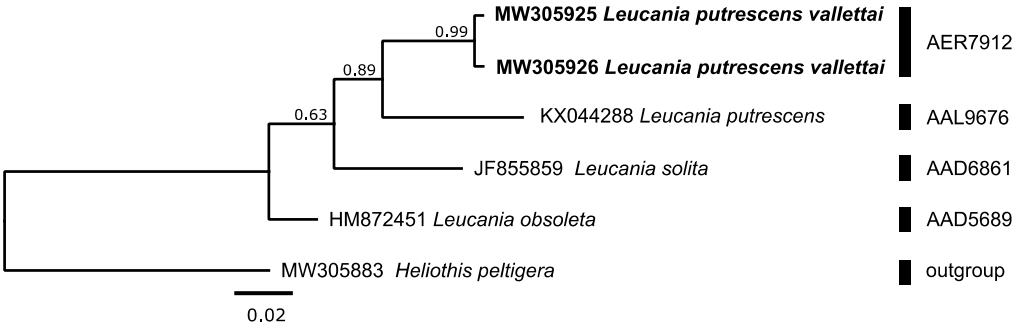


**Figure 2.** Bayesian inference phylogram showing the genetic relationship between the *Leucania putrescens vallettai* from the current study (in bold) and other species of *Leucania*, using publicly available data. Numbers at nodes indicate Bayesian posterior probabilities.

#### 3.2.2. Noctuidae: *Nyctobrya segunai* Fibiger, Steiner, & Ronkay, 2009

Four specimens of *N. segunai* were identified during this study, with each having a unique haplotype and 99.0% identical nucleotide positions, with a mean pairwise identity of 99.5%. These data represent the first barcodes for *N. segunai*, which diverges from its closest related species, *Nyctobrya muralis*, by at least 1.8% using BOLD data and 3.3% using GenBank data. Phylogenetic analysis shows that, genetically, *N. segunai* forms a

monophyletic clade within the paraphyletic *N. muralis*. The BI tree using both publicly available data (Figure 3) and BOLD TaxonID Tree (data not shown) indicates the presence of multiple clades for *N. muralis*, highlighting the need for taxonomic revisions of possibly undescribed cryptic species within the *N. muralis* complex.

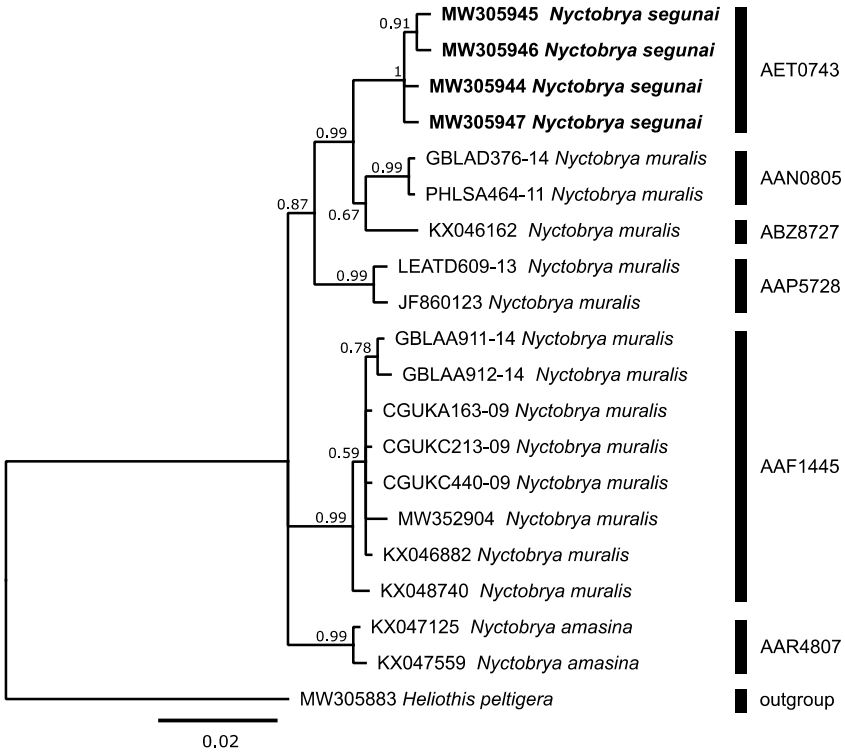


**Figure 3.** Bayesian inference phylogram showing the genetic relationship between the *Nyctobrya segunai* from the current study (in bold) and other *Nyctobrya* species, using publicly available data. Numbers at nodes indicate Bayesian posterior probabilities.

#### 3.2.3. *Lasiocampa* sp.

In this study, we collected five specimens of *Lasiocampa* sp., represented by three haplotypes which differ from each other by a maximum of 2 bp. Genetic data indicates that all of these specimens belong to the same species, and when checked on BOLD, they were all clustered into the same cluster: BOLD:AES9600. This BIN is solely composed of our specimens, with its closest neighboring BIN being that of *Lasiocampa tripolitania* (BOLD:AAW9949) (Figure 4), a recently described species found in northern Africa, specifically in Tunisia, Libya, and Egypt [57]. A similar outcome was observed using the BOLD TaxonID Tree (data not shown), which presents outcomes using private data on *Lasiocampa* species, including *L. tripolitania* and *Lasiocampa terreni*. Lewandowski and Fischer [57,81] have indicated that the *Lasiocampa* species found in Malta is the subspecies *Lasiocampa trifolii mauritanica* (Staudinger, 1891), a subspecies also found in North-West Africa and on the island of Lampedusa in Italy. Although we cannot exclude the presence of the latter subspecies in Malta, especially given that there are records of *L. trifolii* documented by other local authors [53,63], yet the specimens we collected are genetically more closely related to *L. tripolitania* than to *L. trifolii*. Given that our specimens formed a separate cluster to *L. tripolitania*, we have kept our records here as *Lasiocampa* sp., highlighting that this genus requires more studies to better understand the genetic diversity of the various subspecies present in the central Mediterranean.

### *3.3. New Additions to the Entomofauna of Malta*

Four of the species identified in this study are new records for the Maltese islands. These include *Apatema baixerasi*, *Bostra dipectinialis*, *Oiketicoides lutea*, and *Phereoeca praecox*.

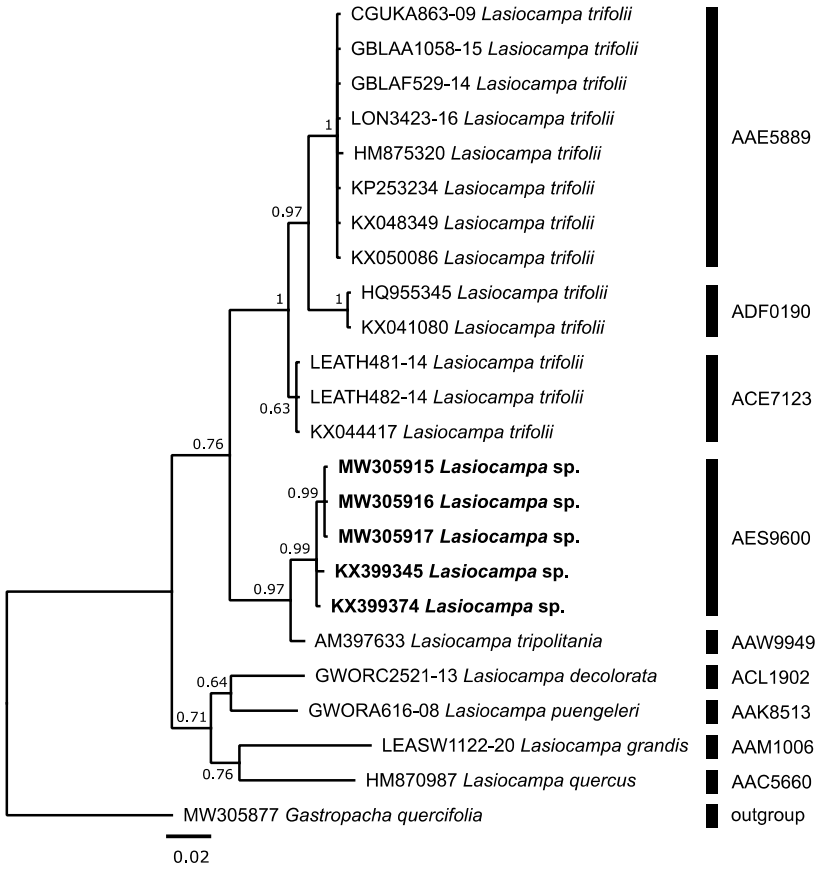


**Figure 4.** Bayesian inference phylogram showing the genetic relationship between the *Lasiocampa* sp. analyzed in the current study (in bold) and other *Lasiocampa* species, using publicly available data. Numbers at nodes indicate Bayesian posterior probabilities.

#### 3.3.1. Autostichidae: *Apatema baixerasi* Vives, 2001

In this study, we encountered one specimen of *A. baixerasi*. This specimen was collected on 16 May 2017 in Mtaħleb, Rabat at night, using a UV light trap. The area where it was found consists of a cliff site garigue area, in a rupestrial habitat.

In Malta, there are records of *Apatema mediopallidum* [55], which is genetically paraphyletic compared to *A. baixerasi*. The two species genetically differ from each other by more than 7% (Figure 5). The former species is represented by sequences that cluster in multiple BOLD BINs (BOLD:AAJ1446, BOLD:AAU3743, and BOLD:ADF1474), while the sequences of the latter only cluster in BOLD:AAV4815.

#### 3.3.2. Psychidae: *Oiketicoides lutea* (Staudinger, 1870)

In this study, we encountered four specimens of *O. lutea*. These specimens were collected in Fawwara, Siggiewi on 28 August 2018 from a quarry with a cliff-like habitat.

The four specimens analyzed here formed three distinct haplotypes, with 99.4% identical nucleotide positions and a mean pairwise identity of 99.6%, and they were all clustered into BOLD:AAM0038 (Figure 6) which is the only BIN that represents this species. The p-distance to the nearest neighboring BIN was more than 7.5%, with the closest neighbors being *Oiketicoides* sp. and *Oiketicoides tedaldii*, with the BINs BOLD:ABU9696 and BOLD:ABA9698, respectively (Table 1; Figure 6). Previous studies have indicated the occurrence of *O. tedaldii* in Malta [53,82], with this study presenting data on the occurrence of another species for this genus.

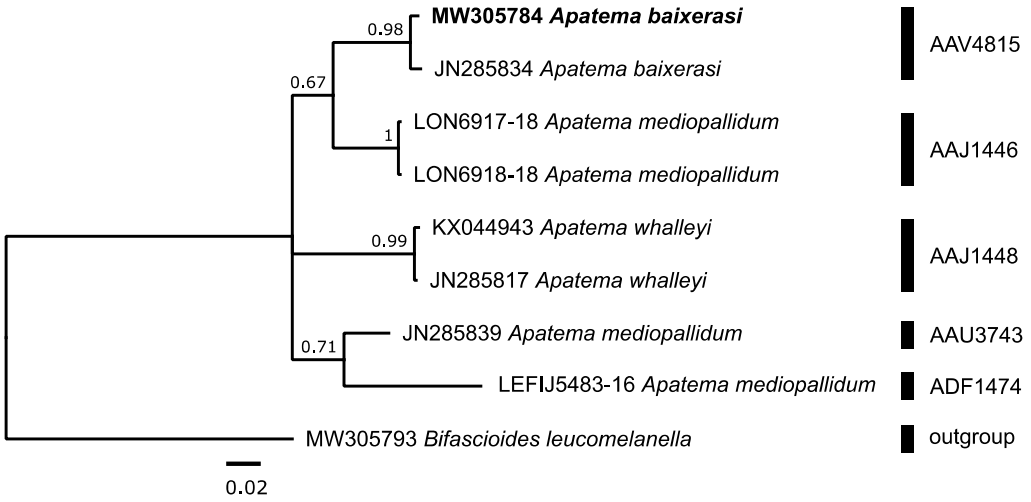


**Figure 5.** Bayesian inference phylogram showing the genetic relationship between the newly recorded *Apatema baixerasi* specimen from Malta (in bold) and other *Apatema* species, using publicly available data. Numbers at nodes indicate Bayesian posterior probabilities.

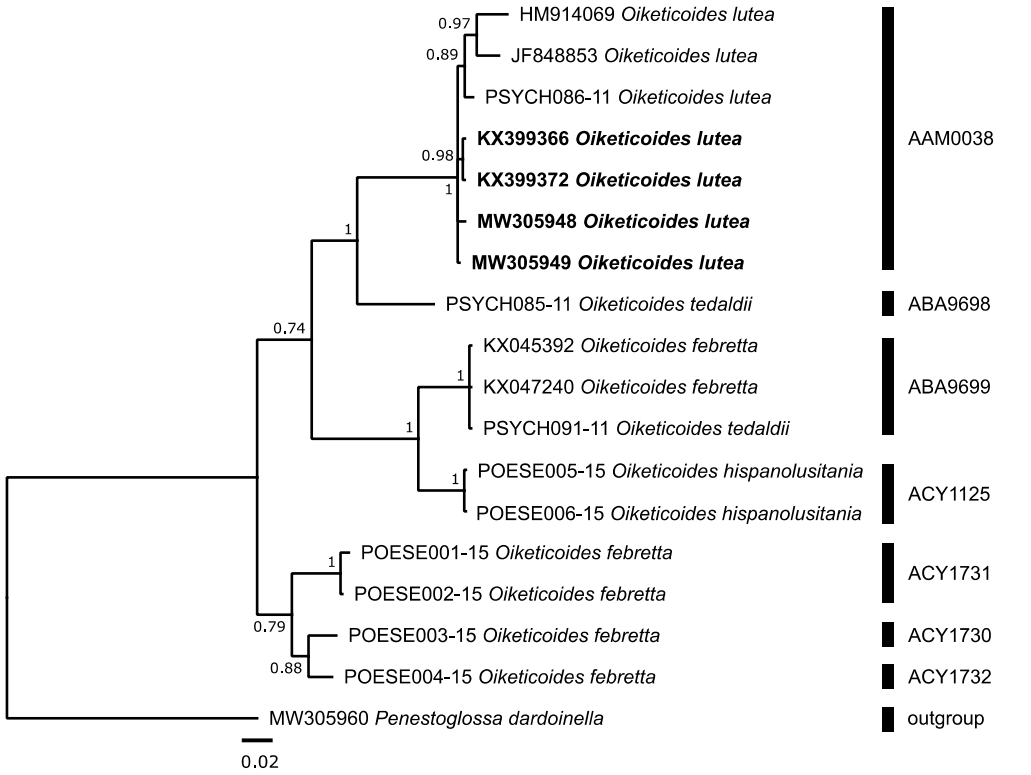


**Figure 6.** Bayesian inference phylogram showing the genetic relationship between the newly recorded *Oiketicoides lutea* specimens from Malta (in bold) and other publicly available *Oiketicoides* species data. Numbers at nodes indicate Bayesian posterior probabilities.

### 3.3.3. Pyralidae: *Bostra dipectinialis* Hampson, 1906

Seven specimens of *B. dipectinialis* were encountered in this study. These specimens were collected in Msida on 14 August 2017 (urban habitat), and in Mtarfa on 8 August 2018 (small pine tree woodland).

The seven specimens analyzed here all had the same haplotype and clustered into BOLD:AAU4121, which is the only BIN that represents this species (Figure 7). The BOLD TaxonID Tree (data not shown) indicates that this BIN contains private data on the same species based on specimens collected from Sicily, which completely match the Maltese

specimens, and specimens from Ethiopia, which differ by less than 1% from the ones presented here. Previous studies based on morphology indicate the presence of *Bostra obsoletalis* in Malta [63]; thus, this study presents data on the occurrence of the second species of this genus on these islands.

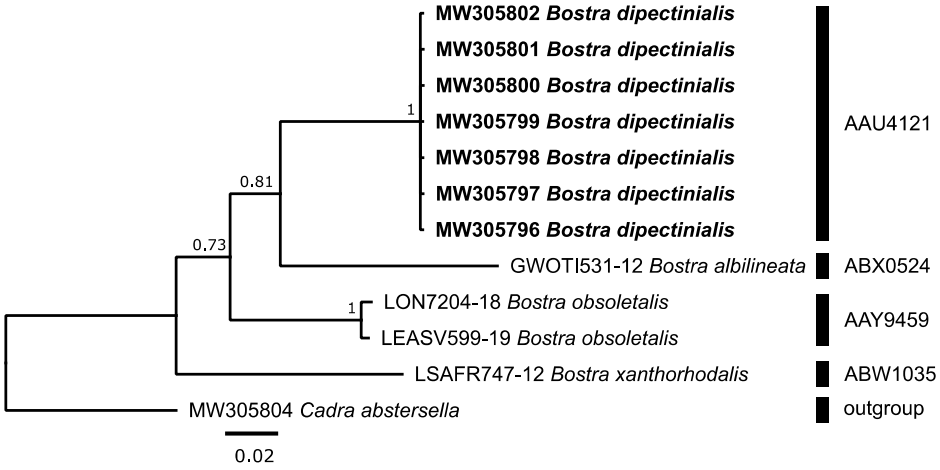


**Figure 7.** Bayesian inference phylogram showing the genetic relationship between the newly recorded *Bostra dipectinialis* specimens from Malta (in bold) and other publicly available *Bostra* species data. Numbers at nodes indicate Bayesian posterior probabilities.

3.3.4. Tineidae: *Phereoeca praecox* (Gozmany & Vari, 1973)

During this study, we found three specimens of *P. praecox*. These specimens were collected in Msida on 24 September 2015 and 14 May 2018, and in Attard on 5 May 2018. In all cases, the samples were collected within urban dwellings.

Each of the analyzed specimens had a distinct haplotype, with that were 99.6% identical. These specimens were clustered within BOLD:AAU1282 (Figure 8), with the nearest neighboring BIN being BOLD:AAH8518,which is composed of *Phereoeca uterella*. This species of case-bearing moth is becoming increasingly common in several countries, where they are usually associated with households, warehouses, and storage rooms [83,84]. The current three records represent the first records of this species in Malta.

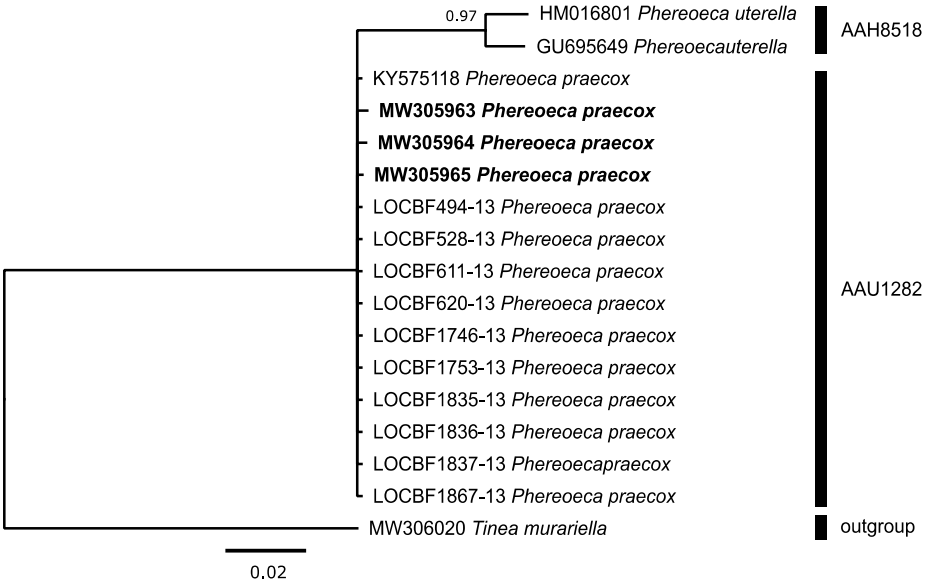


**Figure 8.** Bayesian inference phylogram showing the genetic relationship between the newly recorded *Phereoeca praecox* specimens from Malta (in bold) and other *Phereoeca praecox* specimens using publicly available data. Numbers at nodes indicate Bayesian posterior probabilities.

## 4. Discussion

The current study provides a better understanding of the inter- and intraspecific variation of Lepidoptera species from the Maltese islands. Using DNA barcoding, we took the first steps towards a comprehensive Maltese collection of DNA reference sequences for this order, recoding genetic data for around 25% of the locally known Lepidoptera species and capturing new first records, while highlighting species and OTUs that may require taxonomic revisions and identifying genetic diversity.

It is well known that the identification of Lepidoptera species is at times biased by the morphological characters chosen for delimitating a species, and consequently, a significant number of formal descriptions are considered to be synonyms, while the lack of standardized methods frequently leads to misidentifications [32,33]. In this scenario, DNA barcoding is an added tool that is used to support, refine, or challenge taxonomic descriptions.

In this study, we confirmed the presence of four newly recorded species for the Maltese islands. These include *A. baixerasi*, *B. dipectinialis*, *O. lutea*, and *P. praecox* (Figures 5–8). The close *morphological* resemblance of these species with conspecifics could have led to misidentifications, such as the presence of *A. mediopallidum* and *A. baixerasi*. One such recent genetic study from the Canary Islands confirmed that the presence of *A. mediopallidum* on this archipelago was based on misidentifications [77]. Additionally, in the case of *A. baixerasi*, the recent description may have led to this taxon being overlooked in other studies relying solely on morphological characters. In our study, the presence of these species was observed through morphology and confirmed genetically through comparisons with conspecifics. Such additions to the local entomofauna show the importance of molecular taxonomy in biodiversity research and monitoring [68,85].

Molecular taxonomy has led to the recording of the first barcodes for two endemic species, *L. putrescens vallettai* and *N. segunai*, and the development of the first phylogenetic analyses for them. The latter was found to be a species within the paraphyletic *N. muralis* complex (Figure 3), with a 1.8% distance from the closest *N. muralis* clade. *L. putrescens vallettai* exhibits clear morphological differences [80] and a high level of genetic variation from *L. putrescens*, leading to the formation of a unique BOLD BIN, indicating that this endemic subspecies reveals enough differences to be considered for promotion to species level. Apart from the described endemics, the results also show that several other species were clustered in a different BOLD BIN from their conspecifics (Table 1). One such example is *Lasiocampa* sp., which differs from *L. trifolii* by more than 5%, and from *L. tripolitania* by around 2% (Figure 4). Moreover, we have found a high proportion of unique barcodes that differ by a few base pairs from the conspecifics collected from other countries (Table 1), showing that some local populations have diverged from other populations found on mainland southern Europe and northern Africa.

Mediterranean islands have been characterized by various biogeographical changes, including sea-level changes and the intermittent connectivity of between islands and with the mainland during glacial periods [86]. These past events and the region's geography have shaped this area into a biodiversity hotspot, where different islands have unique faunal and floral assemblages [87–89], with isolation being the driving force towards the diversification of species and subspecies [89]. Discoveries of new species and the large number of unique DNA barcodes clearly demonstrates that the entomofauna of Malta deserves further attention for complete biodiversity inventories. Lack of knowledge on island fauna and the presence of endemic species, which are geographically very restricted, are considered crucial issues for island biodiversity conservation [90]. The number of unique haplotypes noted indicates that the Lepidoptera species in Malta may have low immigration probabilities, forming isolated populations from the mainland, in which case, as reported in other Mediterranean islands, local extinctions may not only mean the loss of this allopatric diversification. However, this also highlights the possible unlikeliness of recolonization [87].

In this respect, genetic and genomic data are essential additional tools for identifying species and indicating the degree of intraspecific divergence across a geographical range,

allowing for a better understanding of the management and conservation needs of insular biodiversity [91]. Studies on other Mediterranean islands show that old mature forests, with the highest levels of environmental stability, have the highest abundance and number of lepidopteran species, including endemic species [92]. Consequently, the protection of endemic biodiversity is tightly linked to the preservation of native natural habitats, which are highly threatened by climate change, fragmentation, urbanization, and other landscape modifications, including plantations of non-native flora and the introduction of alien fauna. The latter is facilitated by globalization and the multifaceted transportation of diverse goods and merchandise onto these islands [49,93–95].

## 5. Conclusions

Molecular taxonomy of Maltese Lepidoptera has led to the identification of new records, also unravelling aspects related to the taxonomic rank of several species. This comprehensive DNA barcode library of Maltese Lepidoptera can be applied to monitor and regulate potential introductions of pest species, biodiversity inventories, ecosystem biomonitoring, and conservation.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121090/s1, Table S1: A list of the species analysed ($^{F}$ first records for Malta; $^{E}$ endemic species), including the number of specimens per species (n), haplotypic variants per species (H), the mean percentage distance within species (AvD), the maximum percentage distance within species (MxD), the accession numbers, the percentage similarity to the closest BOLD match (matches < 100% indicate that the haplotypic variant was recorded for the first time).

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