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# Effectiveness of DNA Barcoding Libraries Boosted through Taxonomy: the Case of a Neglected Taxon within an Underexplored Region

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In recent decades, taxonomy has been significantly improved by integrating molecular techniques with classical morphological methods, leading to the discovery of cryptic species. On the other hand, molecular datasets by themselves are ineffective in several types of research without basic taxonomic studies, as the ecological and biological roles of a given species cannot be determined without an accurate name. DNA barcoding libraries are widely used as identification tools by non-specialists to overcome the taxonomic impediment, but they fail when basic taxonomic studies are insufficient and faunistic inventories are lacking. South European microlepidoptera are poorly studied, with the exception of a few families such as Depressariidae. We tested the effectiveness of the DNA barcoding library for this family to identify 174 specimens collected in southern Italy, where faunistic studies are very limited. All specimens were successfully barcoded, and 95% of them were assigned to 47 species, 43 of which correspond to a Barcode Index Number (BIN). Four additional species shared a BIN but were still clearly separated into different clusters at within-BIN resolution. Only seven specimens belonging to four BINs remain unnamed, and *ad hoc* studies are needed to clarify their status. The regional fauna was enriched by 37 species, three of which are new for the Italian mainland and 21 for peninsular Italy, demonstrating the usefulness of the DNA barcoding library in assessing local diversity and overcoming the taxonomic impediment. Improving taxonomic studies is crucial for utilizing molecular datasets to depict ongoing macroecological dynamics, highlight species richness trends, and identify changes in species assemblages.

**Keywords:** Taxonomic impediment, Biodiversity, Microlepidoptera, Depressariinae, Italy

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## BACKGROUND

The accurate identification of species represents a fundamental pillar on which further research in the fields of ecology, genetics, and conservation can be built. Traditional taxonomic methods based on morphology have some limitations, as they can be prone to subjectivity and may be ineffective in case of cryptic species (Pires and Marinoni 2010; Vacher et al. 2017). Molecular techniques have often been integrated into taxonomic studies, contributing to the recognition of cryptic species even in well-studied geographic context (Hebert et al. 2004; Smith et al. 2005; Mutanen et al. 2013; Scalercio et al. 2021). Molecular analyses have also contributed to reassigning species rank to previously synonymized taxa, reducing the subjectivity of morphology-based conclusions (Veijalainen et al. 2011; Infusino et al. 2018; Govi et al. 2022). Furthermore, advances in molecular biology, particularly in DNA sequencing, have significantly enhanced biodiversity studies by enabling species identification through genetic markers (Hebert et al. 2003; Smith et al. 2005; Dai et al. 2012; Hausmann et al. 2013; Vacher et al. 2017).

The most commonly used molecular technique for species identification is DNA barcoding, a method that involves sequencing a specific mitochondrial DNA region and comparing it with sequences stored in a reference database. Species identification is achieved by matching the obtained sequence to those of known species already in the database, which are assigned to a given Barcode Index Number (BIN) (Hebert et al. 2003). This technique has been proven to be very effective in species detection for most of studied taxa, with identification rates exceeding 90% (Hausmann et al. 2013; Pentinsaari et al. 2014; Raupach et al. 2014; Hawlitschek et al. 2016). Consequently, DNA barcoding reference libraries have been built for several animal taxa and geographic areas, enabling even non -specialists to recognize challenging species. However, BIN sharing and high intraspecific mitochondrial diversity (Hausmann et al. 2013; Schmidt et al. 2015; Huemer and Wieser 2023), always suggest the need for cross-validation of species identification through morphology.

Libraries should always be based on robust taxonomic studies of voucher specimens to reliably link the correct nomenclature to a sequence. Misidentifications and taxonomic gaps, particularly in overlooked geographic areas, strongly reduce the effectiveness of libraries (Meyer and Paulay 2005; Collins and Cruickshank 2013; Kjærandsen 2022). For example, in Greece, a Linnaean name cannot be assigned to 20% of the BINs recovered for microlepidoptera, leading the authors to stress the need to supplement the already advanced European barcode library of Lepidoptera with taxa from the Mediterranean that have not yet sequenced (Huemer and Mutanen 2022). The use of traditional taxonomic knowledge in combination with genetic techniques known as Integrative Taxonomy, is crucial for the accurate identification of species, as neither approach can be considered exclusive (Pires and Marinoni 2010). Together, they provide a more complete and reliable understanding of biodiversity. Thanks to this approach, knowledge of European fauna has significantly increased in recent years, including animal groups such as microlepidoptera, a large and heterogeneous lepidopteran group for which knowledge gaps still exist. A significant exception concerns the Depressariidae family, for which several contributions have been recently produced (e.g. Buchner 2015; Buchner et al. 2017; Corley and Buchner 2018; Corley and Ferreira 2019; Corley et al. 2019), contributing to the publication of the Depressariidae of Europe (Buchner and Corley 2024).

The Depressariidae family (sensu Heikkilä et al. 2014) is composed of about 2,300 species worldwide. Over the last few decades, the number of species belonging to the Italian fauna has increased from 100 (Baldizzone et al. 1995) to 119 (Buchner and Corley 2024), excluding some subfamilies of questioned systematic position, such as Peleopodinae, Hypercalliinae, Ethmiinae, and Oditinae. Data concerning the distribution of Depressariidae at the national level are, in most cases, included in papers that also cover other taxa (Huemer 2011; Pinzari et al. 2015; Scalercio 2016), while specific studies have been scarce and have focused on the northern part of the territory (Fiumi 2016). Before this study, the fauna of Depressariidae (sensu Heikkilä et al. 2014) in the Calabria region consisted of only 10 species (Scalercio et al. 2015; Baldizzone and Scalercio 2018; Leonetti et al. 2018) belonging to the subfamilies Peleopodinae, Hypercalliinae, Ethmiinae, Oditinae, and Depressarinae.

The main goal of this study was to test the hypothesis that a taxonomically improved DNA barcoding library can be effectively utilized to investigate the fauna of an underexplored region. Depressariidae were chosen as a model due to the availability of a DNA barcoding library recently ehnanced by basic taxonomic studies, as well as the large number of specimens collected by the authors in an area where faunistic knowledge are limited. Meanwhile, this study also contributed to improving the molecular library of this taxon and expanding the knowledge of the regional fauna.

## MATERIALS AND METHODS

### Moth sampling

During the last decade, we investigated the lepidopteran fauna of the Calabria region through several light trapping sessions, primarily conducted in forested habitats (chestnut woodlots, from about 500 up to 850 metres of altitude: Greco et al. 2018; beech forest from about 1000 up to 1650 metres of altitude: Infusino and Scalercio 2018; maple forest from about 1300 up to 1600 metres: Greco et al. 2019; silver fir forest from about 800 up to 1100 metres of altitude: Ienco et al. 2020; various thermophilus oaks from 90 up to 600 metres of altitude: Scalercio 2022; black alder woodlots from about 1100 up to 1350 meters of altitude: La Cava et al. 2023; suburban area at 250 meters of altitude: Zucco and Scalercio 2023; olive grove at about 200 meters of altitude: Zucco et al. 2024). The focus of these surveys was the community composition of Macrolepidoptera, for which quantitative data were collected, although Depressariidae and other microlepidoptera were also retained to the best of our possibilities. The sampling primarily involved the use of UV LED light traps (Infusino et al. 2017), which were set up before sunset and switched off after sunrise. Usually, monitoring was conducted monthly for one year, but see cited literature for additional details. Occasionally, specimens were individually collected during daytime and kept alive in tubes until they reached the laboratory. The specimens are preserved in the research collection of the Faunistic Management and Forest Biodiversity lab, Research Centre for Forestry and Wood, Rende, Italy, where the initial stage of sample preparation for sequencing was also carried out.

**DNA barcoding**

This study involves a short fragment of the cytochrome *c* oxidase subunit I (*COI*) gene, which is commonly used for DNA barcoding. One or more legs from each of the 174 specimens were placed in a separate 96-well microplate. 30 µl of ethanol were added in every well to avoid the involuntary leg shifting in the wrong one. Each box accommodated 95 leg specimens, with the 96th well reserved for negative control. All samples were sent to the Canadian Centre for DNA Barcoding (CCDB) for DNA extraction, amplification, and sequencing, following standard CCDB protocols (Ivanova and Grainger 2012a; Ivanova and Grainger 2012b). Depressariidae were analysed as part of the Barcoding Calabrian Lepidoptera Project (BCLEP). The mitochondrial DNA (mtDNA) sequences, along with specimen data such as images, GPS coordinates, exact site, collector(s), and assigned Barcode Index Numbers (BINs), were stored in The Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007) in the public dataset DS-DEPREITS

(Depressariidae of South Italy). Specimens were initially identified through sequence comparison using the BOLD Identification Engine, followed by morphological analysis of wing patterns and genitalia. To assess the levels of intra- and interspecific variation in the DNA barcode fragments, we calculated *p-distance* models of nucleotide substitution using the analytical tools available in BOLD Systems v. 4.0 (http://www.boldsystems.org). To visualize the divergence patterns in COI among BIN sharing species, we constructed a neighbor-joining tree (Saitou and Nei 1987) under the *p-distance* model using Mega 10.0.5 (Tamura et al. 2021). Node confidence was evaluated using the bootstrapping method with 500 replicates.

## RESULTS

### DNA barcoding

A DNA barcode sequence was recovered from all the studied specimens. The fragments ranged from 645 to 682 bp, all of which were useful for subsequent analyses. 166 specimens were attributed to 46 already available BINs, while 8 belonged to 5 unique, *i.e.*, newly discovered, BINs which were in labeled "YES" in table 1. Molecular analyses allowed us to identify at species level the 95% of the barcoded specimens, remaining only the 5% in need of further taxonomic investigations (Table 1).

**Table 1.** Taxonomy, DNA barcoding and faunistic relevance data of studied Depressariidae. Each row corresponds to a different species or to a different Barcoding Index Number (BIN). N: number of specimens barcoded in this study; NN: Nearest Neighbor

| Taxon | N | BIN | Unique BIN | intra-BIN variability | | Distance from NN (*p*-dist) | Faunistic relevance |
|---|---|---|---|---|---|---|---|
| | | | | Average distance (*p*-dist) | Maximum distance (*p*-dist) | | |
| Depressariinae | | | | | | | |
| *Agonopterix* | 2 | BOLD:AFT9394 | YES | 0% | 0% | 1.12% | Unknown |
| *Agonopterix* | 3 | BOLD:AFR9579 | YES | 0.11% | 0.16% | 4.46% | Unknown |
| *Agonopterix adspersella* (Kollar, 1832) | 5 | BOLD:ABY6929 | NO | 0.22% | 0.65% | 1.12% | New for the region |
| *Agonopterix alstroemeriana* (Clerck, 1759) | 2 | BOLD:AAC0206 | NO | 3.92% | 7.33% | 2.2% | New for South Italy |
| *Agonopterix arenella* ([Denis & Schiffermüller], 1775) | 2 | BOLD:AAC6982 | NO | 0.29% | 1.75% | 3.01% | New for South Italy |
| *Agonopterix aspersella* (Constant, 1888) | 1 | BOLD:ACX8920 | NO | 0.45% | 0.8% | 3.04% | New for South Italy |
| *Agonopterix assimilella* (Treitschke, 1832) | 13 | BOLD:AAJ7526 | NO | 0.39% | 1.35% | 2.77% | New for the region |
| *Agonopterix astrantiae* (Heinemann, 1870) | 4 | BOLD:AAJ1481 | NO | 0.91% | 2.25% | 2.56% | New for South Italy |
| *Agonopterix atomella* ([Denis & Schiffermüller], 1775) | 2 | BOLD:AAC0206 | NO | 3.92% | 7.33% | 2.2% | New for the region |
| *Agonopterix carduncelli* Corley, 2017 | 2 | BOLD:AAC0206 | NO | 3.92% | 7.33% | 2.2% | New for Italian mainland |
| *Agonopterix chironiella* (Constant, 1893) | 1 | BOLD:ACE2899 | NO | 0.98% | 1.93% | 1.12% | No |
| *Agonopterix ciliella* (Stainton, 1849) | 1 | BOLD:ACF5176 | NO | 0.6% | 1.67% | 1.25% | New for South Italy |
| *Agonopterix doronicella* (Wocke, 1849) | 2 | BOLD:ABW9282 | NO | 0.76% | 1.77% | 4.87% | New for South Italy |
| *Agonopterix ferocella* (Chrétien, 1910) | 2 | BOLD:ABA0924 | NO | 0.65% | 1.12% | 3.06% | No |
| *Agonopterix heracliana* (Linnaeus, 1758) | 7 | BOLD:ADO3923 | NO | 0.24% | 0.8% | 1.02% | New for South Italy |
| *Agonopterix laterella* ([Denis & Schiffermüller], 1775) | 1 | BOLD:ADN8804 | NO | 0.11% | 0.16% | 1.92% | New for the region |
| *Agonopterix ligusticella* (Chrétien, 1908) | 1 | BOLD:ACF6882 | NO | 0.05% | 0.16% | 1.92% | New for South Italy |
| *Agonopterix liturosa* (Haworth, 1811) | 4 | BOLD:AAE7191 | NO | 0.51% | 1.61% | 3.68% | New for South Italy |
| *Agonopterix nanatella* (Stainton, 1849) | 2 | BOLD:ABA0908 | NO | 0.89% | 1.77% | 2.62% | New for the region |
| *Agonopterix nervosa* (Haworth, 1811) | 9 | BOLD:AAC1846 | NO | 0.21% | 0.8% | 2.8% | No |
| *Agonopterix nodiflorella* (Millière, 1866) | 4 | BOLD:ABV2118 | NO | 0.06% | 0.37% | 3.5% | No |
| *Agonopterix ocellana* (Fabricius, 1775) | 1 | BOLD:AAF7176 | NO | 0.13% | 0.8% | 2.2% | New for South Italy |
| *Agonopterix pallorella* (Zeller, 1839) | 1 | BOLD:ABU5790 | NO | 0.4% | 1.44% | 2.42% | New for the region |
| *Agonopterix pseudoferulae* Buchner & Junnilainen, 2017 | 4 | BOLD:ACW1863 | NO | 0% | 0% | 2.5% | New for the region |
| *Agonopterix purpurea* (Haworth, 1811) | 1 | BOLD:ABY4837 | NO | 0.31% | 0.85% | 1.87% | New for the region |
| *Agonopterix rotundella* (Douglas, 1846) | 1 | BOLD:AAJ6714 | NO | 0.44% | 1.5% | 1.1% | New for the region |
| *Agonopterix scopariella* (Heinemann, 1870) | 21 | BOLD:AAC0206 | NO | 3.92% | 7.33% | 2.2% | No |
| *Agonopterix subpropinquella* (Stainton, 1849) | 2 | BOLD:AER7434 | NO | 0.08% | 0.16% | 1.92% | New for South Italy |
| *Agonopterix yeatiana* (Fabricius, 1781) | 9 | BOLD:AAJ6716 | NO | 0.65% | 1.93% | 3.55% | New for the region |
| *Depressaria* | 1 | BOLD:AFT4983 | YES | N/A | N/A | 2.72% | Unknown |
| *Depressaria albipunctella* ([Denis & Schiffermüller], 1775) | 12 | BOLD:AAF8258 | NO | 0.52% | 1.77% | 3.37% | New for South Italy |
| *Depressaria chaerophylli* Zeller, 1839 | 9 | BOLD:AAF8167 | NO | 0.34% | 0.96% | 4.49% | New for South Italy |
| *Depressaria depressana* (Fabricius, 1775) | 2 | BOLD:AAE7397 | NO | 0.23% | 0.83% | 4.62% | New for South Italy |
| *Depressaria discipunctella* Herrich-Schäffer, [1854] | 1 | BOLD:AAO4681 | NO | 0.38% | 1.12% | 2.62% | New for South Italy |
| *Depressaria douglasella* Stainton, 1849 | 3 | BOLD:ABZ6022 | NO | 0.6% | 1.77% | 1.44% | New for South Italy |
| *Depressaria douglasella* Stainton, 1849 | 1 | BOLD:ABA0668 | NO | 0.25% | 0.8% | 2.84% | New for South Italy |
| *Depressaria floridella* Mann, 1864 | 2 | BOLD:AAO2632 | NO | 0.32% | 1.37% | 2.37% | New for the region |
| *Depressaria incognitella* Hannemann, 1990 | 2 | BOLD:ABZ7952 | NO | 0.2% | 1.28% | 1.44% | New for South Italy |
| *Depressaria marcella* Rebel, 1901 | 1 | BOLD:ABW9330 | NO | 0.19% | 0.37% | 3.74% | New for South Italy |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Depressaria olerella* Zeller, 1854 | 6 | BOLD:AAF8185 | NO | 0.71% | 1.61% | 4.42% | New for South Italy |
| *Depressaria pimpinellae* Zeller, 1839 | 2 | BOLD:AAD6055 | NO | 0.22% | 0.64% | 1.61% | New for South Italy |
| *Depressaria pseudobadiella* Nel, 2011 | 1 | BOLD:ACC4792 | NO | 0.53% | 1.28% | 3.16% | New for Italian mainland |
| *Depressaria radiosquamella* Walsingham, 1898 | 2 | BOLD:ABA1416 | NO | 0.42% | 1.77% | 2.88% | New for South Italy |
| *Depressaria ultimella* Stainton, 1849 | 1 | BOLD:AAL1490 | NO | 0.87% | 1.69% | 2.56% | New for Italian mainland |
| Ethmiinae | | | | | | | |
| *Ethmia bipunctella* (Fabricius, 1775) | 2 | BOLD:AAB2343 | NO | 0.51% | 1.61% | 2.69% | No |
| *Ethmia pusiella* (Linnaeus, 1758) | 2 | BOLD:AAE4537 | NO | 0.16% | 0.64% | 2.73% | No |
| *Ethmia quadrillella* (Goeze, 1783) | 2 | BOLD:AAE7190 | NO | 0.3% | 1.61% | 2.56% | New for the region |
| *Ethmia terminella* Fletcher, 1938 | 2 | BOLD:AAE4514 | NO | 0.53% | 1.84% | 1.6% | New for the region |
| *Ethmia terminella* Fletcher, 1938 | 1 | BOLD:AFT6355 | YES | N/A | N/A | 2.24% | New for the region |
| Hypercalliinae | | | | | | | |
| *Anchinia daphnella* ([Denis & Schiffermüller], 1775) | 2 | BOLD:AAE5098 | NO | 0.51% | 1.01% | 1.18% | No |
| Oditinae | | | | | | | |
| *Odites* | 1 | BOLD:AFS2991 | YES | N/A | N/A | 3.94% | Unknown |
| *Odites kollarella* (Costa, 1832) | 2 | BOLD:ADB7123 | NO | 0.17% | 0.48% | 4.41% | No |
| Peleopodinae | | | | | | | |
| *Carcina quercana* (Fabricius, 1775) | 4 | BOLD:AAB0177 | NO | 0.29% | 2.13% | 6.55% | No |

According to the BOLD identification Engine, our sequences belonged to 47 species, with the BIN BOLD:AAC0206 shared by 4 species, namely *Agonopterix alstroemeriana*, *A. atomella*, *A. carduncelli*, *A. scopariella*. However, the 27 specimens belonging to this BIN were not intermixed but were positioned within intra-BIN clusters corresponding to a given species, each well differentiated by wing morphology (Fig. 1).

In detail, these species are differentiated by 11 (*alstroemeriana*), 5 (*atomella* and *carduncelli*), and 6 (*scopariella*) diagnostic SNPs. On the other hand, two BINs under the name *Depressaria douglasella* were observed at a distance of 2.84% (*p*-dist).

We found five unique BINs, three of which belonging to Depressarinae, one to Ethmiinae and one to Oditinae. The unique BIN BOLD:AFT4983 has a distance of 2.72% from its Nearest Neighbour (NN), which is *Depressaria absynthiella*. Under the genus *Agonopterix,* we found two unique BINs, of which BOLD:AFT9394 is near to *A. thapsiella* (p-dist = 1.12%), and BOLD:AFR9579 near to *A. liturosa* (p-dist = 4.46%). The Ethminae specimen belonging to BIN BOLD:AFT6355 is near to *Ethmia terminella* (p-dist = 2.24%). The unique BIN BOLD:AFS2991, including a specimen we identified as *Odites* sp., has a distance of 3.94% from its unidentified NN. All the specimens belonging to the unique BINs showed a wing pattern very similar to that of their NNs (Fig. 2). The NNs of the latter three unique BINs were also found in this study, while the NNs of the former two BINs were located far from Calabria. The range of the NN of the unique BIN near to *D. absynthiella* is centered in the Alps, while those near *Odites* are even farer, being in Gabon, Africa.

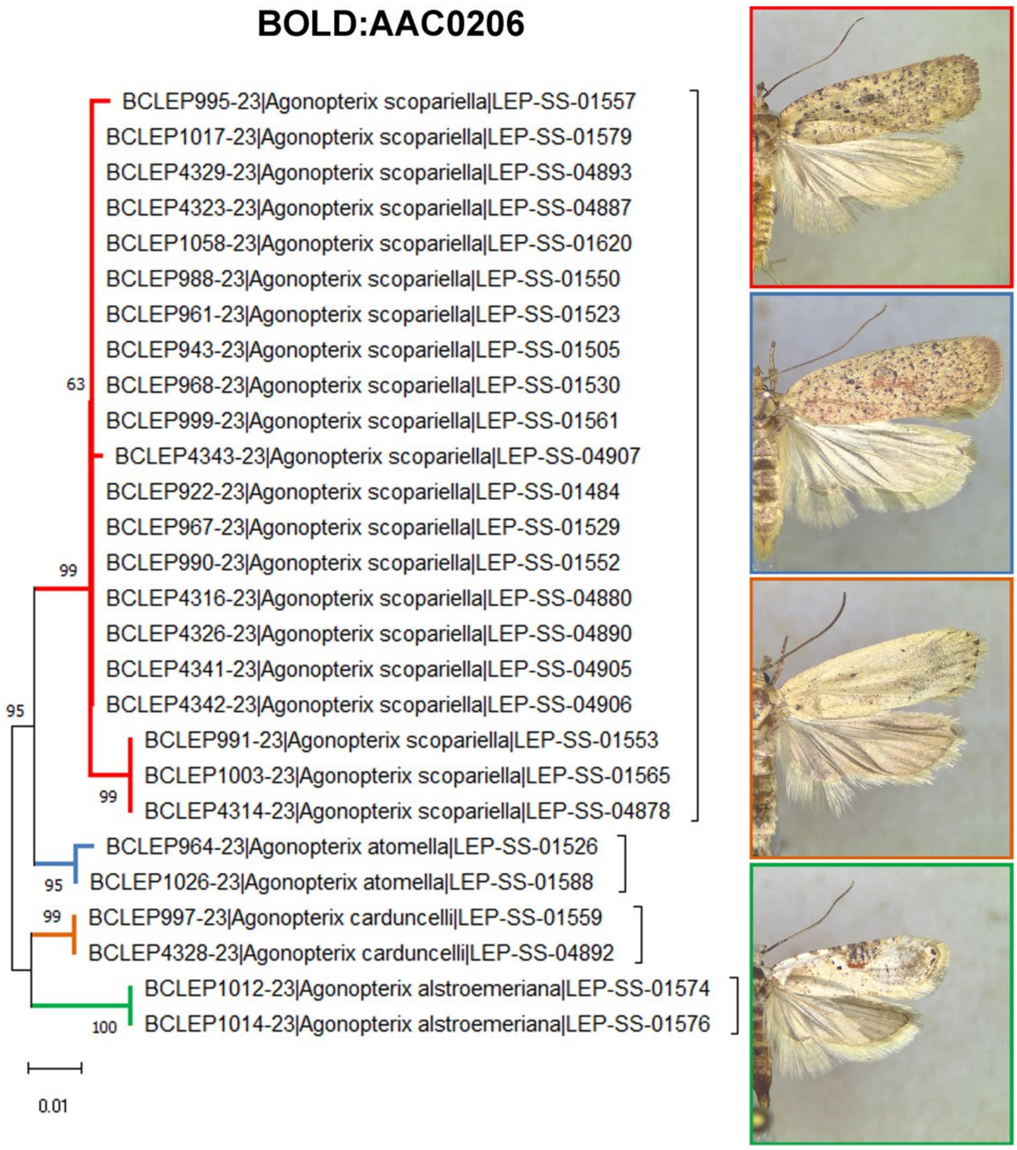


**Fig. 1.** Neighbor-joining tree of sequences belonging to species showing BIN sharing (*Agonopterix alstroemeriana*, *A. atomella*, *A. carduncelli* and *A. scopariella*,). The *p-distance* model was applied using Mega 10.0.5. Node confidence is reported on the lower side.

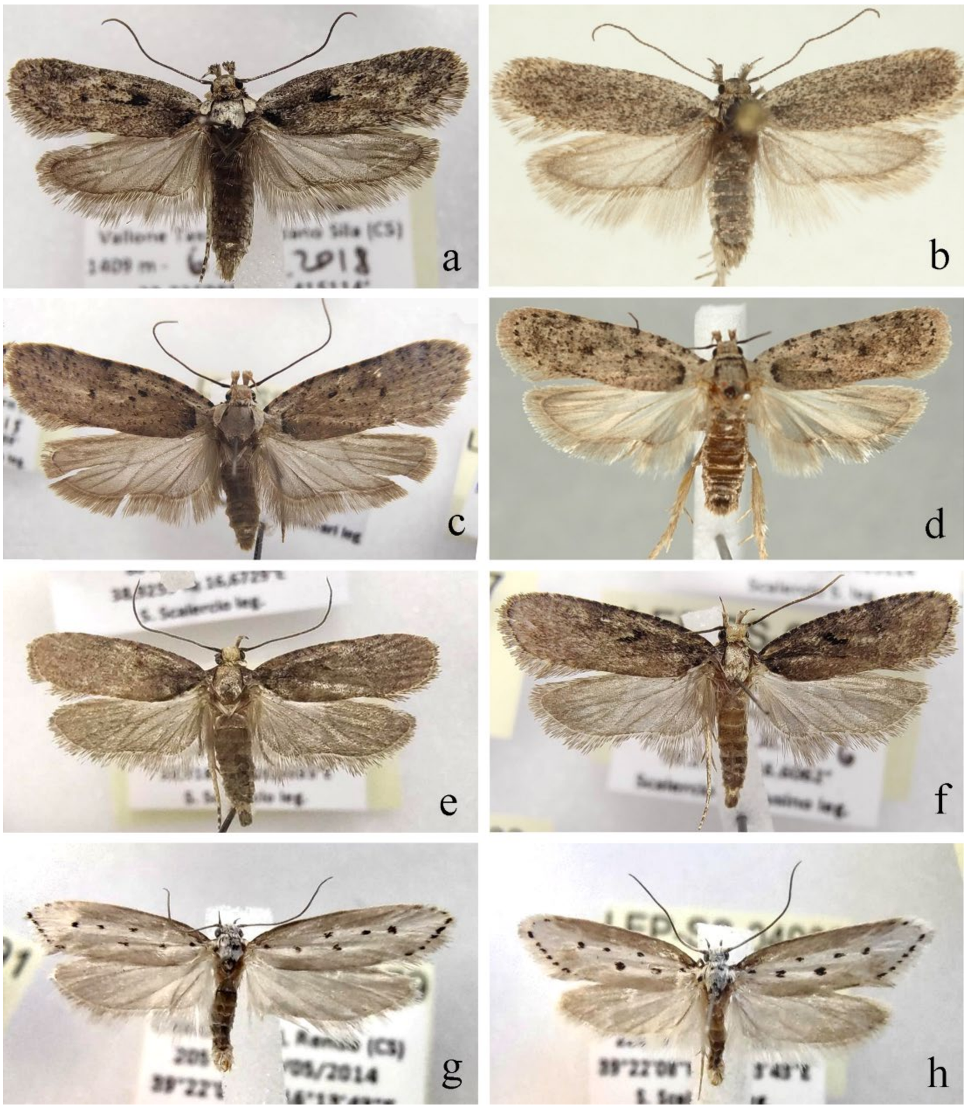

**Fig. 2.** Comparison of specimens belonging to the unique BINs (on the left) with their Nearest Neighbour (on the right) as retrieved from the BOLD system database. a–b: BOLD:AFT4983 (*Depressaria*) vs. BOLD:ADC2233 (*Depressaria absynthiella*); c-d: BOLD:AFT9394 (*Agonopterix*) vs. BOLD:ACE2899 (*Agonopterix thapsiella*); e–f: BOLD:AFR9579 (*Agonopterix*) vs. BOLD:AAE7191 (*Agonopterix liturosa*); g–h: BOLD:AFT6355 (*Ethmia*) vs. BOLD:AAE4514 (*Ethmia terminella*).

**Faunistic results**

167 specimens were assigned to 47 species of which 40 to the subfamily Depressariinae, 1 to Oditinae, 4 to Ethmiinae, 1 to Hypercalliinae, and 1 to Peleopodinae. Among the identified species, *Agonopterix carduncelli*, *Depressaria pseudobadiella* and *D. ultimella* are new for the Italian mainland and 21 more species are new for southern Italy (Table 1). Moreover, 13 species are also new for the fauna of Calabria region, raising the number of Depressariidae known at regional level from 10 species to 47 (Figs. 3–4).

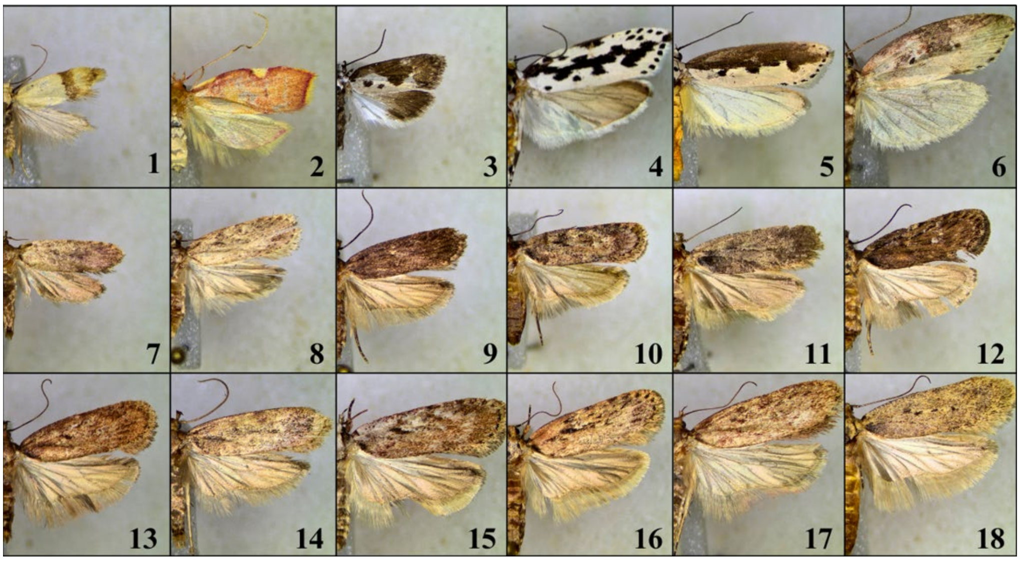


**Fig. 3.** Representatives of the Depressariidae for which there is correspondence between BIN and taxonomy in BOLD. Only one specimen of *Depressaria douglasella* belonging to the BIN BOLD:ABA0668 was figured. *Ethmia terminella* was included in the figure 2. In detail: *Odites kollarella* (1), *Carcina quercana* (2), *Ethmia quadrillella* (3), *Ethmia pusiella* (4), *Ethmia bipunctella* (5), *Anchinia daphnella* (6), *Depressaria marcella* (7), *Depressaria incognitella* (8), *Depressaria ultimella* (9), *Depressaria douglasella* (10), *Depressaria pseudobadiella* (11), *Depressaria radiosquamella* (12), *Depressaria albipunctella* (13), *Depressariae pimpinellae* (14), *Depressaria floridella* (15), *Depressaria chaerophylli* (16), *Depressaria olerella* (17), *Depressaria discipunctella* (18).

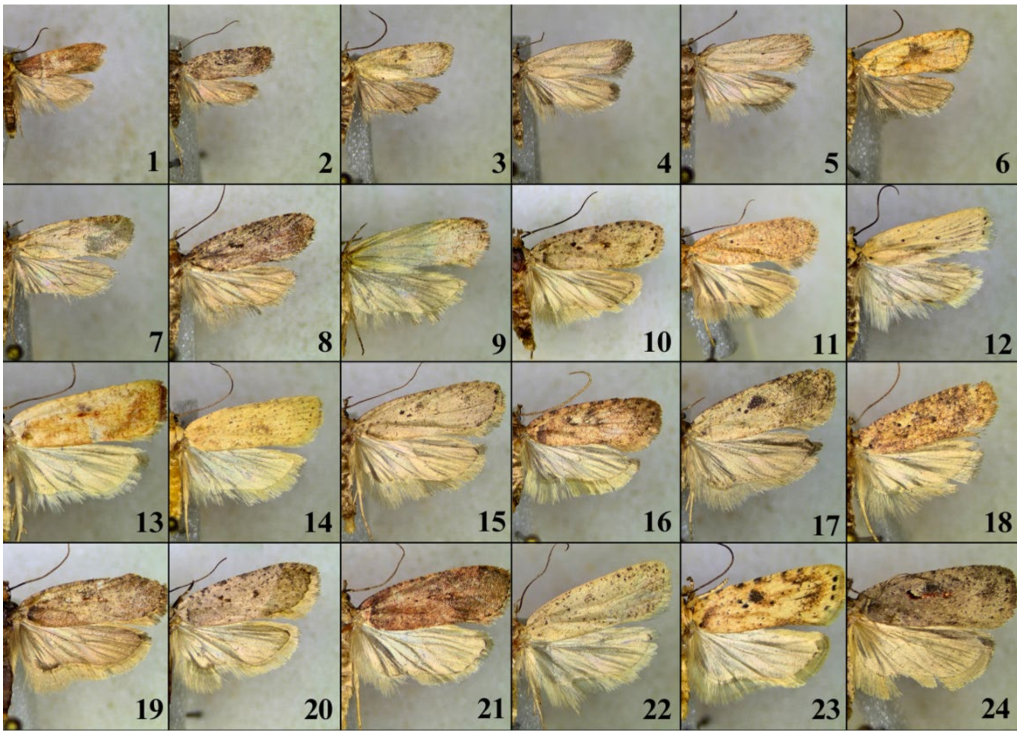


**Fig. 4.** Representatives of the Depressariidae for which there is correspondence between BIN and taxonomy in BOLD. In detail: *Depressaria depressana* (1), *Agonopterix purpurea* (2), *Agonopterix nanatella* (3), *Agonopterix rotundella* (4), *Agonopterix nodiflorella* (5), *Agonopterix doronicella* (6), *Agonopterix ferocella* (7), *Agonopterix liturosa* (8), *Agonopterix ciliella* (9), *Agonopterix chironiella* (10), *Agonopterix ligusticella* (11), *Agonopterix pallorella* (12), *Agonopterix nervosa*

(13), *Agonopterix assimilella* (14), *Agonopterix yeatiana* (15), *Agonopterix heracliana* (16), *Agonopterix subpropinquella* (17), *Agonopterix laterella* (18), *Agonopterix pseudoferulae* (19), *Agonopterix adspersella* (20), *Agonopterix astrantiae* (21), *Agonopterix aspersella* (22), *Agonopterix arenella* (23), *Agonopterix ocellana* (24).

## DISCUSSION

Our results clearly demonstrated that basic taxonomic studies carried out in recent years have significantly improved the performance of automatic analytical tools for species identification. In our study, these tools were able to recover a name for 95% of the Depressariidae we collected in an underexplored region of Southern Europe. Identification rate can be very low in taxonomically understudied insect groups (Kjærandsen 2022), but even in some well-studied orders, such as Lepidoptera, significant gaps still exist. Huemer and Mutanen (2022) emphasized the need of basic taxonomic studies to improve barcode libraries, as they found a relatively low identification success rate for microlepidoptera in a Mediterranean country. Pyralidae, Crambidae, and Tortricidae were among those sufficiently named, with about 80% of correspondence between Linnaean names and BINs. However, only about 50% of Tineidae, an economically important and species-rich family, and about 10% of Autostichidae were named. In their study they barcoded 146 Depressariidae, with all specimens attributed to only nine species, showing a low taxonomic coverage but a high performance of the available library. Taxonomic studies carried out on this family during the DNA barcoding era (Buchner 2015; Buchner et al. 2017; Corley and Buchner 2018; Corley and Ferreira 2019; Corley et al. 2019) ameliorate molecular identifications by strongly reducing misidentifications of voucher specimens and by describing new species, especially in the South European countries. In conclusion, just ten years ago, the success rate of our study should have been lower, not only because of the recent description of new species but also because the taxonomy of voucher specimens was verified through basic taxonomic studies of a large amount of material belonging to well-established species.

### Molecular identification

Despite the high correspondence between BIN and taxonomy observed in this study, there are some exceptions that warrant careful consideration of molecular identification results alone. For example, we found that four species shared a BIN and the same BIN is shared by 48 taxa in total (Huemer and Wieser 2023), becoming BIN-based identification not always affordable. In fact, although a 2% pairwise divergence between BINs is considered the average divergence between species in Lepidoptera (Hebert et al. 2004; Strutzenberger et al. 2011), we found that species sharing the same BIN only diverge by 0.8–1.7%. BIN sharing can occur because of introgression or 'horizontal exchange' of mitochondria (Hausmann et al. 2011). However, BIN sharing among slightly divergent genetic clusters as observed in our study could represent recently separated lineages that have recently speciated or that are still undergoing genetic differentiation (Hausmann et al. 2013). On the other hand, we also found that the same name is attributed to different BINs, leading to an overestimation of the diversity in metabarcoding analyses. However, such cases are typically the focus of taxonomic studies promoted by molecular results, which sometimes lead to the discovery of undescribed cryptic species and, at other times, to the discovery of intraspecific mitochondrial diversity. We found two BINs attributed to *Depressaria douglasella*, but currently, four BINs are listed for this species in BOLD. Further research is required to elucidate the taxonomic significance of these BINs, as they are not contiguous but are separated by clusters composed by other species, such as *Depressaria incognitella* and *Depressaria floridella*. Additionally, *Ethmia terminella* showed two BINs, one of which is unique in BOLD. Despite sympatry and identical wing pattern suggesting the potential for intraspecific variation, further investigations are required, such as the examination of genitalia morphology, to conclusively determine whether it represents a distinct species. The use of nuclear markers can help discriminate between such cases (Scalercio et al. 2021).

The unique BINs we found can be very helpful in improving the Depressariidae DNA barcoding library after *ad hoc* taxonomic studies to ascertain whether they belong to described species that have not barcoded or to cryptic, undescribed species. The role of Southern Italy in general, and Calabria in particular, as a glacial refugium and the complicated geological history of this geographic area (Schmitt et al. 2021), as well as recent discoveries concerning Lepidoptera (Scalercio et al. 2016; Infusino et al. 2018; Govi et al. 2022; Baldizzone 2023; La Cava et al. 2025), suggest that some endemic species may be hidden among these unique BINs. The best candidates are the most divergent genetic lineages, such as the BIN near *Agonopterix liturosa,* which diverges by 4.46% from a BIN found in this study and already present in BOLD, and the BIN belonging to the genus *Odites,* which diverges by 3.94% from a BIN found in Gabon, Africa (Centre for Biodiversity Genomics). However, large intraspecific BIN divergence has been observed (Hausmann et al. 2013; Dapporto et al. 2019), so conclusions should be drawn from integrative taxonomic studies.

**Faunistic insights**

In this study, we were able to increase the regional fauna of Depressariidae by 79%, despite none of the authors being specialist of this taxonomically challenging family, as figures 3 and 4 clearly demonstrate. The most interesting findings, for which the range was significantly enlarged, were the recently described *Agonopterix carduncelli* and *Depressaria pseudobadiella,* and *D. ultimella,* all of which are recorded here for the first time on the Italian mainland.

*Agonopterix carduncelli,* described using morphological and molecular data (Buchner et al. 2017), was known to occur in Portugal, Spain, Greece, Sicily, Sardinia and Morocco (Buchner and Corley 2024). *Depressaria pseudobadiella* whose biology is still poorly studied, was described based to the structure of female genitalia and antennae, which are more distinctly ringed in *D. badiella* (Nel 2011). The species, whose validity was later confirmed by molecular analyses, was known to occur in Portugal, Spain, France, Corsica, and Sardinia (Buchner and Corley 2024). The adults of *Depressaria ultimella* are similar to *Depressaria daucella* (Denis and Schiffermuller 1775), but the larval stages are quite different. It is also similar to *D. halophilella* Chretien, 1908, sharing the same BIN as this species (Buchner et al. 2017). It is commonly widespread in Northern Europe (Buchner and Corley 2024).

## CONCLUSIONS

In this paper, we successfully tested the effectiveness of a DNA barcoding library, improved through basic taxonomic studies, as a tool for investigating the diversity of a little-known taxonomic group within a territory with very scarce knowledge, carried out by a non-specialist team. Biodiversity inventories are increasingly necessary to assess ongoing macroecological dynamics, highlighting trends in species richness trends and changes in species assemblages. However, few taxonomists are available to identify species, a crucial step in understanding functional changes in ecosystems. A significant effort should be made in the coming years by taxonomists to enhance the effectiveness of molecular methods for species identification, overcoming the taxonomic impediment that most ecologists and biologists currently face.

**List of abbreviations**

BIN, Barcode Index Number.

CCDB, Canadian Centre for DNA Barcoding.

BCLEP, Barcoding Calabrian Lepidoptera Project.

BOLD, The Barcode of Life Data System.

COI, Cytochrome *c* Oxidase subunit I.

NN, Nearest Neighbour.

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**Authors' contributions:** SS designed the research, taking the lead in developing the overall research framework and methodology; SLC GZ performed the research, actively engaging in data collection and experimental procedures; SS GZ analyzed the data, processing and interpreting the collected data to draw meaningful conclusions; SS GZ SLC TB wrote the paper, synthesizing the findings into a coherent manuscript.

**Competing interests:** Authors declare no conflict of interest.

**Availability of data and materials:** All data are available in the public repository Barcoding of Life Data System (BOLD), in the dataset DS-DEPREITS (Depressariidae of South Italy). Specimens are preserved in the Research Centre for Forestry and Wood, Rende (ITALY).

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