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Eucosma callei sp. nov.—a new species of Olethreutinae from South-eastern Iberian Peninsula (Lepidoptera, Tortricidae)

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Abstract

Eucosma callei **sp. nov.** is described and illustrated from the Iberian Peninsula. It differs from its Iberian congener, *Eucosma gonzalezalvarezi* Agenjo, 1970, in external appearance and genitalia. The 5' barcode fragments of the mitochondrial gene COI (the DNA barcode) are presented and confirms the description of this new species.

Key words: Integrative taxonomy, new species, DNA barcode

Introduction

The family Tortricidae is represented by 11,000 species worldwide (Gilligan *et al.* 2024); the subfamily Olethreutinae Walsingham, 1895, includes nearly one-half of that total. The genus *Eucosma* Hübner is the largest Holarctic genus with more than 346 named species and subspecies (Gilligan *et al.* 2024). However, *Eucosma* has been challenging to circumscribe because type specimens of type species are lacking (see review by Gilligan & Wright 2013b and Gilligan *et al.* 2014). In Europe, 47 species of *Eucosma* were recorded by Razowski (2003), and the Lepiforum.de list has recently updated that number to 60 (Lepiforum 2023).

The use of DNA barcode as a tool for species diagnosis in the animal kingdom has been suggested as the best option to overcome the gap between available taxonomic information and the need for an effective and reliable species identification, especially in Lepidoptera (Hebert *et al.* 2003). DNA barcoding offers a rapid and cost-effective alternative strategy for the identification of described species and the discovery of new ones (Hebert *et al*. 2003; Savolainen *et al*. 2005; Mitchell 2008). Integrating molecular methods with morphological species identification can accelerate biodiversity inventories and elucidating the status of doubtful species.

During recent studies on Tortricidae in the Iberian Peninsula, the authors discovered a species of *Eucosma* that exhibit some morphological differences from *E. gonzalezalvarezi* Agenjo, 1970, its apparent closest relative. The new species collected from Spain differs from other *Eucosma* species by morphological and molecular characters and is described here as *Eucosma callei*, **sp. nov**. The aim of the present paper is to describe this new species comparing it with its putative sister species, *Eucosma gonzalezalvarezi*, and additionally report new records for *Eucosma gonzalezalvarezi* in the Iberian Peninsula.

Material and Methods

Morphological study

This study was based on the morphological study of twenty-two adult specimens collected from different localities in the southeast of the Iberian Peninsula (Fig. 1; map was created using www.simplemappr.net.). The specimens were

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examined externally to evaluate possible differences in their colouration and wing pattern based on the taxonomic traits provided in Agenjo (1970) and Razowski (2003), and were dissected using standard procedures (Yela 1992; Fibiger 1997) with minor modifications. Images of the adults (Figs. 2A, B) were captured with a Nikon D70 digital camera and were z-stacked using Zerene software. Morphology of the male genital structures (Fig. 3A, B) were studied using a Zeiss Stemi 508 stereomicroscope with a Zeiss Axiocam ICc5 digital camera and were compared with those published by Agenjo (1970) and Razowski (2003). The abbreviation "g. prep." refers to the number of a genitalia preparation. Specimens are deposited in the Research Collection of Biología Animal (RCBAUM) in the Department of Zoology and Physical Anthropology of the University of Murcia (Spain) and in the Museo Nacional de Ciencias Naturales (MNCNM) (Madrid, Spain).

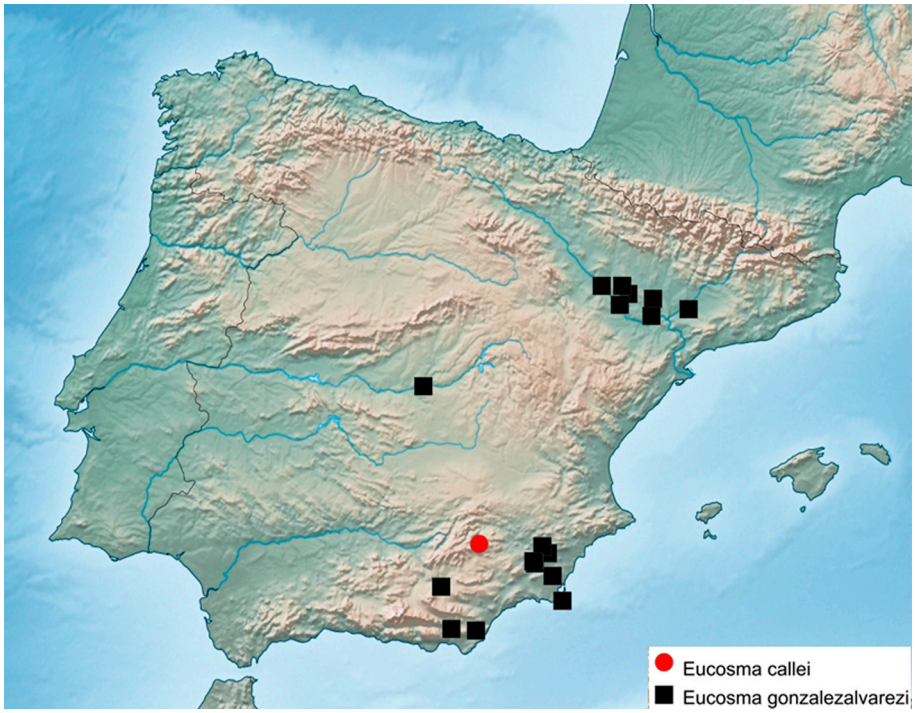

FIGURE 1. Distribution of *Eucosma callei* **sp. nov.** and *Eucosma gonzalezalvarezi* (Agenjo, 1970) including samples collected and bibliographic references. Note that each point may represent more than one specimen. The map was created using www.simplemappr.net.

Material studied of *Eucosma gonzalezalvarezi*: Spain; province of Murcia, Murcia, Hacienda Riquelme, lat: 37.896348, long: -0.968830, 190 m, 1 ♂, 10 Oct 2023, leg. J. Girdley & D. Grundy; 1 ♂, 22 Oct 2023, leg. J. Girdley; 1 ♂, 23 Oct 2023, leg. J. Girdley; Fortuna, Humedal del Ajauque y Rambla Salada, lat: 38.176022, long: -1.100857, 150 m, 1 ♂, 1 ♀, g. prep. 1406MG, 8 Oct 2020, leg. M. Garre; 1 ♂, g. prep. 1407MG, 26 Oct 2020, leg. M. Garre; 1 ♂, 10 Oct 2022, leg. M. Garre; Molina de Segura, Sierra de la Pila, La Poza Amarga, lat: 38.253410, long: -1.207176, 720 m, 1 ♂, g. prep. 2097MG, 9 Oct 2023, leg. M. Garre; Las Torres de Cotillas, La Pilica, lat: 38.020329, long:-1.285840, 150 m, 4 ♂♂, 1 ♀, g. prep. 2098MG, 19 Oct 2022, leg. M. Garre; 1 ♂, g. prep. 1813MG, 25 Oct 2022, leg. M. Garre; Campos del Río, Embalse de los Rodeos, lat: 38.044581, long:-1.312778, 150 m, 2 ♂♂, g. prep. 1973MG, 17 Oct 2023, leg. M. Garre; Cartagena, Calblanque, lat: 37.600857, long: -0.751967, 15 m, 1 ♀, 15 Oct 2020, leg. J. Girdley.

Molecular study

Nine adult specimens of *Eucosma* c.f. *gonzalezalvarezi* were processed and sequenced at the Canadian Centre for DNA Barcoding (CCDB, Guelph) to obtain DNA barcodes (Table 1) using the standard high-throughput protocol described by deWaard *et al.* (2008) which can be accessed at www.dnabarcoding.ca/pa/ge/research/protocols. Ultimately, the analysis (i.e., sequence alignment, calculations of similarity, and tree construction) included nine

Eucosma c.f. *gonzalezalvareci* from Spain, all of which had sequence data more than 500 bp (Table 1). Voucher data, GPS coordinates, images, sequences, Genbank Accession and trace files are available through the public data set (dx.doi.org/10.5883/DS-EUCOSIB) in BOLD (BOLD: www.boldsystem.org) (Ratnasingham & Hebert 2007). Sequences were compared to the reference library of Lepidoptera barcodes using the BOLD-ID identification engine.

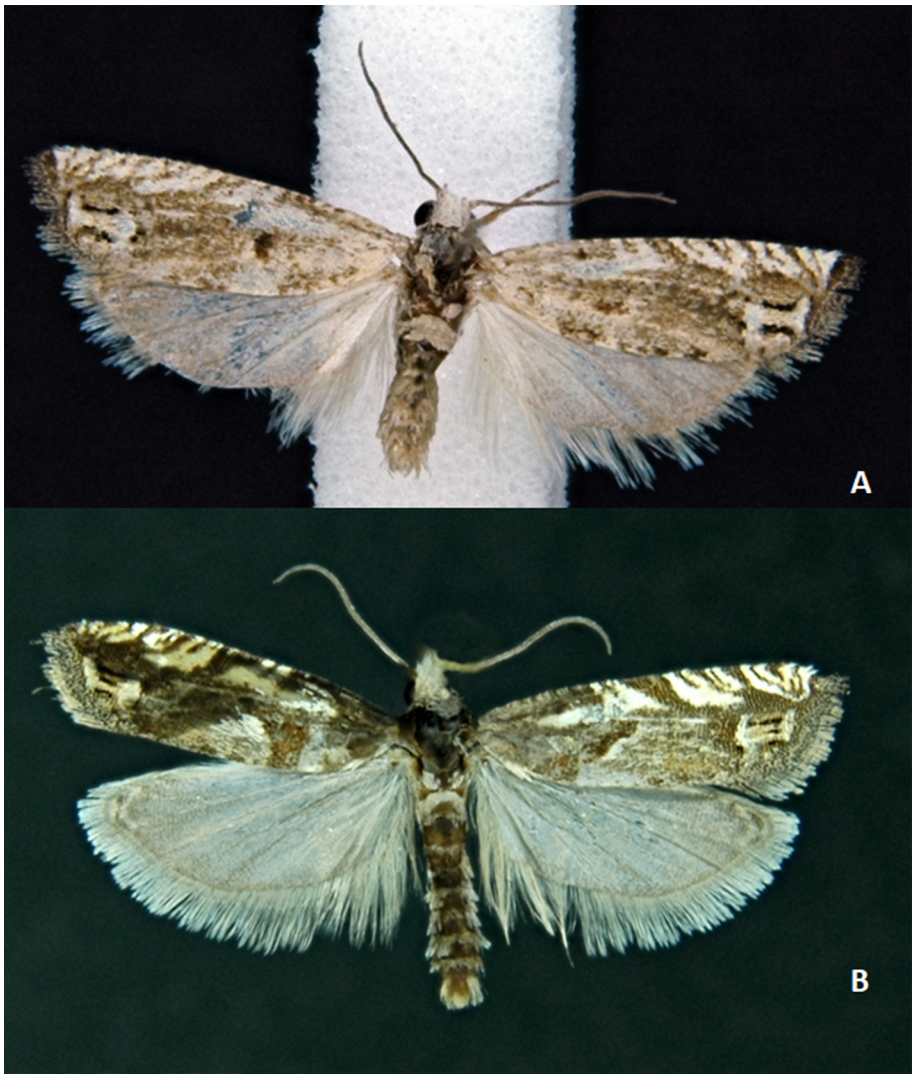

FIGURE 2. Adult specimens. A. Holotype *Eucosma callei* **sp. nov.** ♂; B. *Eucosma gonzalezalvarezi* Agenjo, 1970 ♂. Photograpy by Rubio and Ortiz.

Sequence divergences for the barcode region were calculated using the Kimura 2-parameter (K2P) model (Kimura 1980) and interspecific genetic distances were calculated using the analytical tools of BOLD. All new and public species sequences were downloaded and aligned with the CLUSTAL algorithm of the MEGA6 software (Tamura *et al.* 2013). Bootstrap values were calculated with 1000 replicates, and initial Neighbor-joining (NJ) and Maximum Likelihood (ML) trees based on distance were constructed with the MEGA6 software. We selected the following taxonomically related Eucosmini species (Gilligan *et al.* 2014) as outgroups to root the tree: *Eucosma aemulana* (Schläger), *E. albidulana* (Herrich-Schäffer), *E. balatonana* (Osthelder), *E. conterminana* (Guenée), *E. lacteana* (Treitschke), *E. rubescana* (Constant) and *E. tripoliana* (Barrett), for congeneric comparison and, *Epiblema costipunctana* (Haworth), *Pelochrista infidana* (Hübner) and *Phaneta pauperana* (Duponchel), which are taxonomically related into subfamily Olethreutinae, Eucosmini (Gilligan *et al.* 2014), as outgroups to root the tree. In order to assess the COI divergences between the taxa, we included all sites with the pairwise deletion option. All trees presented the same topology therefore, only the ML tree is presented (Fig. 4).

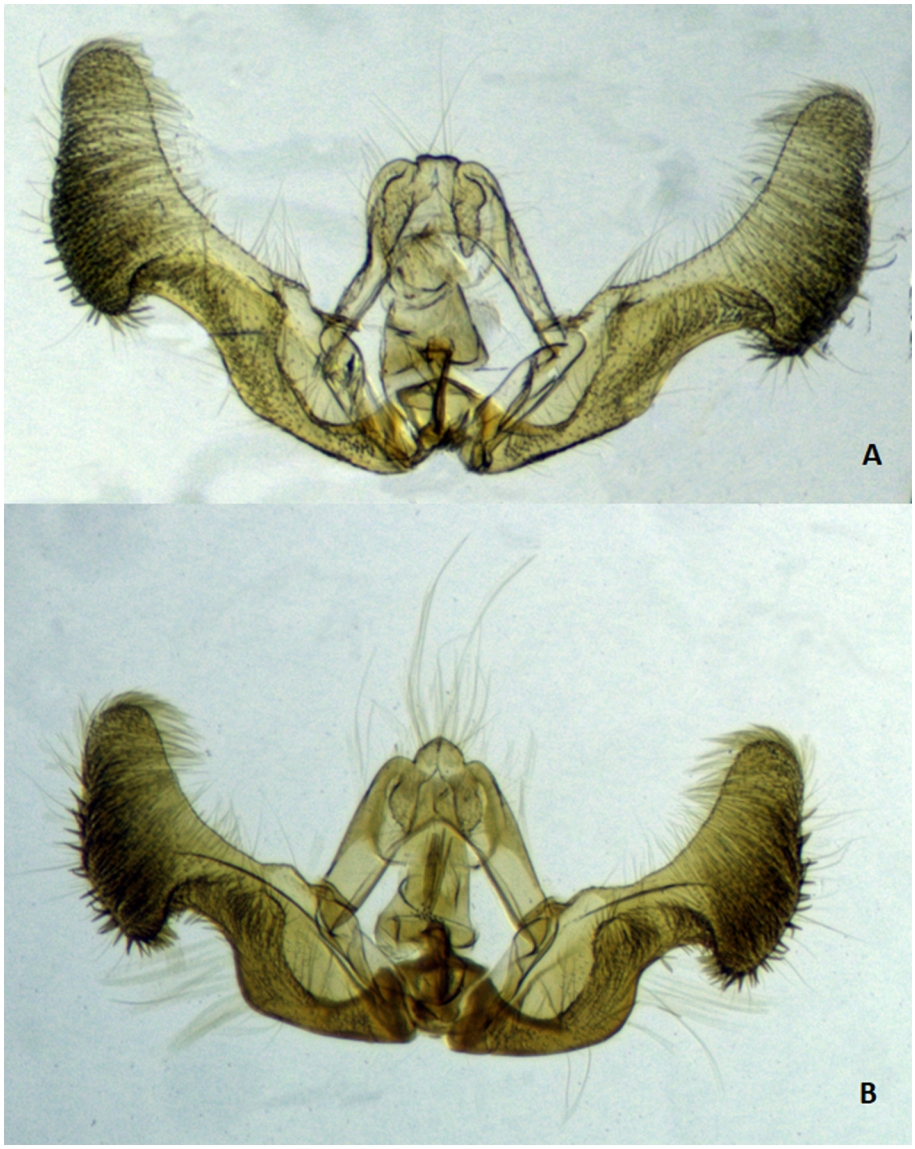

FIGURE 3. Male genitalia. A. *Eucosma callei* **sp. nov.**, Genitalia slide JG-412; B. *Eucosma gonzalezalvarezi* Agenjo, 1970, Genitalia slide MG-1407. Photograp by Rubio and Ortiz.

Taxonomy

Eucosma callei Girdley, Garre, Rubio, & Ortiz, sp. nov.

Barcode Index Number. BOLD: AEO5630

Type material. ***Holotype*, *male*.** Spain; province of Murcia, Moratalla, Fuente de los Almeces, lat: 38.150384, long: -2.226821, 1,087 m, 20 Sep 2020, g. prep. JG-412, IBLAO2392-22, leg. J. Girdley, RCBAUM collection.

***Paratypes*.** 4 ♂♂ with same data as holotype, g. prep. 2099MG, g. prep. 2100MG, g. prep. 2101MG, g. prep. 2102MG, leg. J. Girdley, RCBAUM and MNCNM collections.

Diagnosis. Externally the new species is similar to *Eucosma gonzalezalvarezi* but differs in the lighter ground colour of the forewing that attenuates the contrast with the costal strigulae and the ill-defined markings (Figs 2A, B). The male genitalia of *E. callei* differ from those of *E. gonzalezalvarezi* in the position of ventral edge of sacculus, the angle of sacculus, the length of neck of valve, the depth of the ventral incision of valva and the size of uncus (Figs 3A, B). Finally, these two and related species are separated by DNA barcode sequences, except *E. aemulana* and *E. tripoliana* which have identical barcodes (Fig. 4).

TABLE 1. Taxon names, BOLD accession numbers for the specimens used in distance estimations (Process ID), Barcode Index Number (BIN) and locality information with exact site, province (Prov.) and GenBank accession number.

Taxon	Process ID	BIN	Exact Site	Prov.	GenBank Acc. Nr.
E. callei	IBLAO2255-21	AEO4373	Fuente de Los Almeces-Moratalla	Murcia	PQ388225
E. callei	IBLAO2256-21	AEO4373	Fuente de Los Almeces-Moratalla	Murcia	PQ388228
E. callei	IBLAO2390-22	AEO4373	Fuente de Los Almeces-Moratalla	Murcia	PQ388231
E. callei	IBLAO2391-22	AEO4373	Fuente de Los Almeces-Moratalla	Murcia	PQ388232
E. callei	IBLAO2392-22	AEO4373	Fuente de Los Almeces-Moratalla	Murcia	PQ388226
E. gonzalezalvarezi	IBLAO2254-21	AEO5630	Aparcamiento Calblanque-Cartagena	Murcia	PQ388224
E. gonzalezalvarezi	IBLAO2719-22	AEO5630	La Pilica-Las Torres de Cotillas	Murcia	PQ388226
E. gonzalezalvarezi	IBLAO2720-22	AEO5630	La Pilica-Las Torres de Cotillas	Murcia	PQ388229
E. gonzalezalvarezi	IBLAO2721-22	AEO5630	La Pilica-Las Torres de Cotillas	Murcia	PQ388230
E. gonzalezalvarezi	IBLAO2288-21	Fail barcode	Humedal del Ajauque y Rambla Salada-Fortuna	Murcia	-

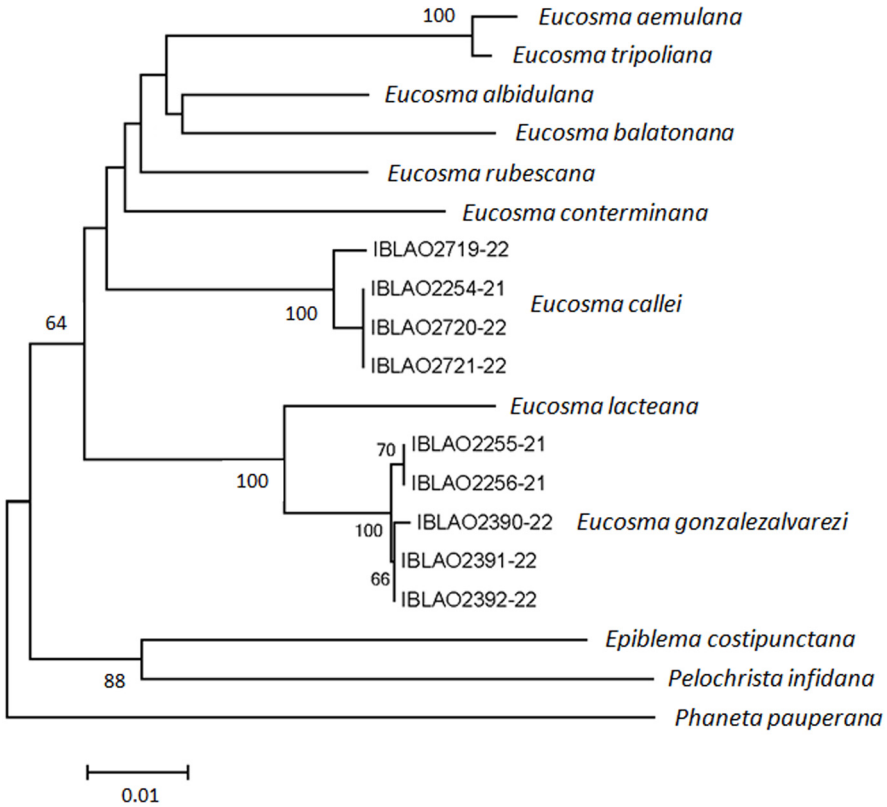

FIGURE 4. Maximum Likelihood tree of *Eucosma* species, obtained from 70 nucleotide COI sequences. The depth of each branch shows divergence within lineages. Bootstrap values are provided at major nodes. The scale bar represents 0.01 genetic difference.

Description. Male. Adult (Fig 2A). Head: frons whitish; vertex whitish; antenna filiform with scape and pedicellus whitish; labial palpus whitish somewhat ochreous on sides, porrect. Thorax: patagium pale brown; mesonotum ochreous; tegula ochreous; metanotum grey; thorax ventrally whitish; forewing length 5-6 mm (mean 5.5 mm, n= 5), costal fold 0.3 mm length of forewing; upperside ground colour creamy with brown admixture; nine pairs of distinct white costal strigulae, the one closest to median fascia is continued in a white striae; speculum white with two inner black elongate dots and refractive lines; three punctiform black spots anterior to speculum;

basal blotch pale, dark brown distally and dorsally; subtornal spot small, dark brown; median fascia extending over costal edge of speculum towards the termen; cilia grey with dark brown admixture in apical area; underside dark grey on the costal region with strigulae repeating those of upperside and ochreous on the rest. Variation: without observed variation in forewing pattern and colouration. Hindwing upperside pale ochre, whiter basally, cilia whitish; underside concolourous with hindwing upperside. Abdomen grey.

Male genitalia based on 5 preparations (Fig. 3A). Uncus reduced, rounded apically, slightly hairy. Socii drooping, thick and convex on the external side. Tegumen simple, large, straight-edged and tapering distally. Gnathos and anal cone slightly sclerotized. Basal part of valva relatively narrow. Sacculus slightly convex ventrally with weakly rounded caudal angle; neck of valva slender; ventral incision of valva distinct, but rather shallow. Cucullus large, with rounded dorsal angle and distinct ventral lobe, densely setous. Phallus short, tapering terminally, which contains a clump of deciduous cornuti.

Female. Unknown.

Distribution. *Eucosma callei* **sp. nov.** appears to be endemic to the southeastern part of the Iberian Peninsula, where it is only known from one locality in northwest Murcia Region.

Biology. The adult is active in September for one short generation annually. The early stages are unknown. Specimens were collected in a mountainous area at 1,087 m altitude of the Upper Mesomediterranean bioclimatic belt. This locality is characterized by forests of oaks (*Quercus* spp.) and pines (*Pinus* spp.) with some hackberries (*Celtis* spp.) and walnut trees (*Juglans* spp.) in moist soils. A great diversity of bushes and shrubs thrive in the forest clearing. In contrast, *Eucosma gonzalezalvarezi* inhabits xerothermophilic habitats such as sand dunes, salt marshes, wasteland and scrubland at altitudes below 800 m. It is especially abundant in anthropogenic areas with disturbed soils, like old abandoned cropland in which there are nitrophilous plant communities dominated by *Artemisia barrelieri* Besser and *A. campestris* L. subsp. *glutinosa* (Besser) Batt., both potential host plants of this species, as is *Artemisia herba-alba* Asso after Agenjo (1970) (Fig. 5).

FIGURE 5. Habitat in the Fuente de los Almeces, Moratalla Mountains, Murcia. Photograph by J. Girdley.

Etymology. The name of the species is dedicated to Dr. José Amador de la Calle Pascual, an eminent Spanish lepidopterologist who published his doctoral thesis on the Iberian Noctuidae in the 1980s and who has been a mentor to many other lepidopterologists.

Molecular characterization. The COI sequences form a BIN BOLD:AEO4373 (n=5; Table 1; sequence length 658 bp), with 0.36% maximum intraspecific variation (mean 0.17%). Based on COI divergence, the new species is isolated from *E. gonzalezalvarezi* (n=4) by a genetic mean distance of 6.2% (BOLD:AEO5630; Mean of 0.44 and

Maximum distance of 0.97%) (Table 1). The new species differs from the seven other *Eucosma* species compared by average of 6.25% (max.: 7.9%; min.: 3.2%) (Table 2) and belongs to a phylogenetically isolated lineage which is well supported by morphology and genetic data.

TABLE 2. Interspecific mean K2P (Kimura 2-Parameter) divergences (mean pairwise distances) based on the analysis of COI fragments (>500 bp) among *Eucosma callei* and other *Eucosma* species (TRI: *E. tripoliana*; ALB: *E. albidulana*; BAL: *E. balatonana*; RUB: *E. rubescana*; CON: *E. conterminana*; GON: *E. gonzalezalvarezi*; LAC: *E. lacteana*).

	TRI	ALB	BAL	RUB	CON	GON	CAL	LAC
E. aemulana	0.6	6.0	6.5	5.8	7.0	6.4	7.9	8.8
E. tripoliana		6.0	6.2	5.8	6.7	6.1	7.8	8.7
E. albidulana			5.6	4.8	6.2	6.0	6.5	7.1
E. balatonana				6.1	6.7	6.5	7.1	8.1
E. rubescana					5.5	5.5	3.4	7.8
E. conterminana						5.9	3.4	8.1
E. gonzalezalvarezi							6.2	7.4
E. callei								3.2

Molecular analysis

In the COI molecular dataset, five specimens of *E. callei* **sp. nov.** and four specimens of *E. gonzalezalvarezi* were sequenced to analyse taxonomic identity and geographical species grouping, obtaining up to 658 bp for the barcode region.

The molecular results indicate the presence of two species groups: the clade including *E. gonzalezalvarezi* and its sister species *E. lacteana*, which differ from each other by 7.4% (K2P distance), and the larger group containing *E. callei* and all other *Eucosma* species in the dataset (Table 2, Fig. 4). Neighbour-Joining (NJ) and Maximum Likelihood (ML) trees of the COI barcode region recovered the same topology, and all haplotypes could be unequivocally assigned to one of the clades (Fig. 4). A phylogenetic hypothesis with ML as an optimality criterion was generated using MEGA software and the topology obtained was chosen as the basis for our discussion with branch support values (Fig. 4). The monophyly of the genus *Eucosma* was recovered by all methods, and all *Eucosma* species examined were clustered together and separated from *Epiblema costipunctana*, *Pelochrista infidana* and *Phaneta pauperana*, the outgroups used to root the tree (Fig. 4).

Discussion

Eucosma gonzalezalvarezi is an Iberian endemic known from the type locality in El Regajal (Madrid) (Agenjo 1970) and a few localities in Huesca, Zaragoza and Lérida in the northeast (Sauter 1999; King 2003; Sumpich 2011; Ylla *et al.* 2015; Redondo *et al*. 2020) and Almería and Granada in the southeast (Sumpich 2011; Ylla & Macià 2010) (Fig. 1).

External features, morphology and COI divergence of twenty-three specimens tentatively identified as *E. gonzalezalvarezi* from the southeast of the Iberian Peninsula were studied and compared with other *Eucosma* species.

The results of the DNA barcode analysis indicate that a new species, *Eucosma callei* **sp. nov.**, is rather isolated from other congeneric species studied, as well as from *Epiblema costipunctana*, *Pelochrista infidana* and *Phaneta pauperana* (Fig. 4). Interspecific mean distances of the new species to other *Eucosma* species ranged from 3.2% to *E. lacteana* to 7.9% to *E. aemulana* (Table 2). In addition, *E. callei* differs from other *Eucosma* species by 3.4% or more (Table 2). According to Hausmann *et al.* (2011), sequence divergence in the barcode region of 2% or more are typical of interspecific variation and recognized as distinct MOTUs, while lower values often correspond to intraspecific differences.

With regards to the external features, the wing pattern of *E. callei* is most similar to that of *E. gonzalezalvarezi*, and particularly to those of *E. tripoliana* and *E. aemulana*. Male genitalia of *E. tripoliana* and *E. aemulana* are very

similar to that of *E. gonzalezalvarezi* and all three are rather different to that of *E. callei*. The main differences are: ventral edge of sacculus is slightly convex with weakly rounded caudal angle in *E. callei*, while it is distinct and straight in the other three species; neck of valve slender, nearly as long as sacculus in *E. callei*, while it is shorter than sacculus in the other three species; ventral incision of valve in *E. callei* is shallower than in the other three species; cucullus with long dorsal portion in *E.callei*, shorter in the other three species; and size of uncus, more prominent in *E. callei* and smaller in the other three species. Although barcodes of *E. callei* differ from those of *E. aemulana* and *E. tripoliana* by mean genetic distance of 7.9% and 7.8%, respectively, *E. aemulana* and *E. tripoliana* interestingly share the same BIN (BOLD:AAE3780; n=21) and they also share a similar distributional range since *E. aemulana* is a transpalaearctic species occurring in the Iberian Peninsula while *E. tripoliana* occurs mainly north of the Pyrenees (Razowski 2003). The taxonomic status of these two species should be investigated in detail, especially considering Swatschek (1958) treated them as synonyms based on their early stages. Distinguishing *Eucosma callei* by external morphology is at first glance very difficult, owing to its similarity with other species of the genus. However, *Eucosma callei* differs by external morphology, male genitalia, barcodes, and its known distribution, which is above 1,000 m of altitude above sea level.

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Environmental Authorities in Región de Murcia and Junta de Andalucía provided permission for collection and access to field sites. We also thank various anonymous referees for their comments. This study was supported by the projects Fauna Ibérica XII—Lepidoptera: Noctuoidea I (PGC2018-095851-B-C63) and Fauna Ibérica: Noctuoidea II (PID2023-149039NB-I00) of the Spanish Ministry of Research and Science.

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