



DNA barcoding of the genus *Apaidia* Hampson, 1900 for species delimitation from Western Mediterranean fauna (Lepidoptera, Erebidae)

A. S. ORTIZ^{1*}, R. M. RUBIO¹, J. J. GUERRERO¹, M. GARRE¹ & J. L. YELA^{2*}

¹Department of Zoology and Physical Anthropology, University of Murcia, Campus de Espinardo; E-30100 Murcia, Spain

✉ aortiz@um.es; <https://orcid.org/0000-0002-3877-6096>

✉ rmrubio@um.es; <https://orcid.org/0000-0002-0109-7874>

✉ juanjogf@um.es; <https://orcid.org/0000-0002-9645-5266>

✉ manuel.garre@fripozozo.com; <https://orcid.org/0000-0002-5846-8621>

²Grupo DITEG, Área de Zoología, Facultad de Ciencias Ambientales, Universidad de Castilla-La Mancha, Avda. Carlos III, s.n.; Campus Real Fábrica de Armas, E-45071 Toledo, Spain

✉ joseluis.yela@uclm.es; <https://orcid.org/0000-0003-1371-8495>

*Corresponding authors: ✉ aortiz@um.es; ✉ joseluis.yela@uclm.es

Abstract

The genus *Apaidia* Hampson, 1900 is a relict Western Mediterranean genus in the South-western part of Europe and the North-western areas of the Mediterranean Africa comprising so far three species, *Apaidia rufeola* (Rambur, 1832), *Apaidia mesogona* (Godart, [1824]) and *Apaidia barbarica* Legrand, 1939. According to the examined material, COI mitochondrial DNA sequences and adult morphology integration supports the existence of three main lineages of *Apaidia* with sequence divergence rates of approximately 4.5%, which are within the range reported for other well-defined insect species. In addition, we recovered three different BINS, suggesting the presence of different species with unique and specific identifier for *A. mesogona* (AEC6797), *A. rufeola* (AEI9539), and the Iberian-Balearic *A. barbarica* (AEI9540). This study contributes to a better understanding of the taxonomy of the genus *Apaidia* and challenges future revision of this genus in Northern Africa, as well as the presence of the *Apaidia* species in Western Mediterranean islands and populations located in Italy.

Key words: DNA barcoding, COI, *Apaidia*, species delimitation, Western Mediterranean

Introduction

The European Arctiinae (Erebidae) fauna is particularly well known, with 113 species (Witt & Ronkay 2011). The genus *Apaidia* Hampson, 1900 is a small Western Mediterranean genus restricted in the South-western part of Europe and the North-western areas of Mediterranean Africa comprising so far three species, *Apaidia rufeola* (Rambur, 1832), *Apaidia mesogona* (Godart, [1824]) and *Apaidia barbarica* Legrand, 1939 (e.g. Witt & Ronkay 2011). The species are small and fragile moths (wingspan between 14–25 mm) with slender body and relatively broad and apically finely pointed forewings. The ground colour of the forewing varies from ochreous-grey to dark brown with a few dark dots or a more or less continuous, diffuse stripe. Their external appearance resembles mostly certain genera of the subtribe Nudarina, but the genitalia features indicate their close relationship with species in the subtribe Lithosiina (Witt & Ronkay 2011).

The male clasping apparatus has several common features with that of *Eilema* s.l. while the configuration of the vesica is more or less globular, with small frontal diverticula covered by fine denticles. The female genitalia are characterised by the tubular, sclerotised and anteriorly cristate-ribbed ductus bursae and the bilobate bursae with large, elliptical-ovoid appendix bursae and smaller, discoidal-globular corpus bursae; both parts are weakly membranous (Witt & Ronkay 2011).

Apaidia mesogona is a species with high individual variability in the forewing colouration and the intensity of the dark forewing stripe but without geographic variation in the European and Moroccan populations. According

to Witt & Ronkay (2011), *Apaidia mesogona* differs from *A. rufeola* in its smaller size, having shorter wings and in general darker colouration, especially remarkable in the hindwings. Interestingly, the females of *A. mesogona* are smaller in wingspan than the males while the situation is just the opposite in *A. rufeola*. The dark forewing stripe is also differently marked in the two species, which is thinner but sharper in *A. mesogona*, often consisting of blurred spots, forming sometimes a continuous line. In *A. rufeola*, the stripe is more scattered and broader, usually forming a wide and straight fascia at the place of the antemedial line, and some other patches are present in and under the cell and at the base of the wing.

Apaidia rufeola can be distinguished from *A. mesogona* by its paler colouration, especially the hindwing, which is remarkably paler, ochreous-grey. Their forewing markings are more diffuse, consisting of a larger number of dark grey patches including most often a few of such spots in the basal area of the wing. In addition, the ciliation of the male antenna is longer than in *A. mesogona*.

Apaidia barbarica was originally described as an Algerian subspecies of *A. rufeola*, supported by external features which suggested that *barbarica* was conspecific with *A. rufeola*, while the genitalia suggested that *barbarica* could be considered a subspecies of *A. mesogona* (Witt & Ronkay 2011). *A. barbarica* was raised to species status by Durante & Panzera (1998) when they described the Sicilian population as subspecies *messapia*, while Witt & Ronkay (2011) synonymized the subspecies *messapia* with *barbarica*. Gastón *et al.* (2013) considered the Iberian putative *A. barbarica* as a species and compared the male and female genitalia of the three mentioned species. *A. barbarica* differs from *A. mesogona* by its larger size, paler ochreous-grey forewings, and much paler, whitish-grey or ochreous-grey hindwings; the forewing pattern is more diffuse and the basal patch is usually present. *A. barbarica* is externally very similar to *A. rufeola* but somewhat larger in size and the forewing markings are less diffuse. The male genitalia of Algerian *A. barbarica* show closer resemblance to those of *A. mesogona*.

The male and female genitalia of the three species were illustrated by Witt & Ronkay (2011) and Gastón *et al.* (2013) and are very similar, despite the conspicuous external differences between the adults. The male genitalia presents a short and broad clasper -actually, a saccular extension- in the Iberian-Balearic *A. barbarica*, conspicuously longer and thinner in *A. mesogona* and short and slightly slenderer in *A. rufeola*. Vinculum of *A. barbarica* is bifurcated, with a slightly variable notch and with short and thick lateral ends; in *A. mesogona* it is also bifurcated, with a wide horseshoe-shaped notch and with thin and long lateral ends; *A. rufeola* shows no bifurcation at vinculum. Cucullus is rounded in *A. barbarica* and in *A. rufeola* but pointed in *A. mesogona*. The female genitalia present a very wide ductus bursae in *A. barbarica*, moderately wide in *A. rufeola*, and narrow and sclerotized in *A. mesogona*. The corpus bursae, including the appendix bursae, of *A. barbarica* is smaller than that of *A. mesogona* and *A. rufeola* (see Witt & Ronkay 2011 and Gastón *et al.* 2013).

The present investigation was prompted by results obtained during an effort to barcode all Macroheteroceran Lepidoptera species in the Iberian Peninsula, which revealed that specimens of the genus *Apaidia* were separated into definite sequence clusters. In this paper we analyze COI sequences and geographical distribution of the lineages to assess differences between Western Mediterranean *Apaidia* species to further clarify their taxonomic status and relationships.

Material and Methods

Morphological study

The present study was based on the morphological study of 25 specimens collected from different localities in the Iberian Peninsula and Corsica (Table 1). These adult specimens were examined externally to evaluate possible differences in their colouration, spot size and wing shape based on the taxonomic key provided by Witt & Ronkay (2011) and Gastón *et al.* (2013), and were dissected using a standard procedure (Fibiger 1997, with minor modifications). Adult images were originally published in Gastón *et al.* (2013) and are published here again under author permission (Fig. 1). Genital structures were examined using a Zeiss Stemi 508 stereomicroscope with a Zeiss Axiocam ICc5 digital camera and were compared with those published by Witt & Ronkay (2011) and Gastón *et al.* (2013). Specimens were deposited in the Research Collection of Biología Animal in the Department of Zoology and Physical Anthropology of the University of Murcia (Spain) and in the Research Collection of J. L. Yela (Toledo, Spain).

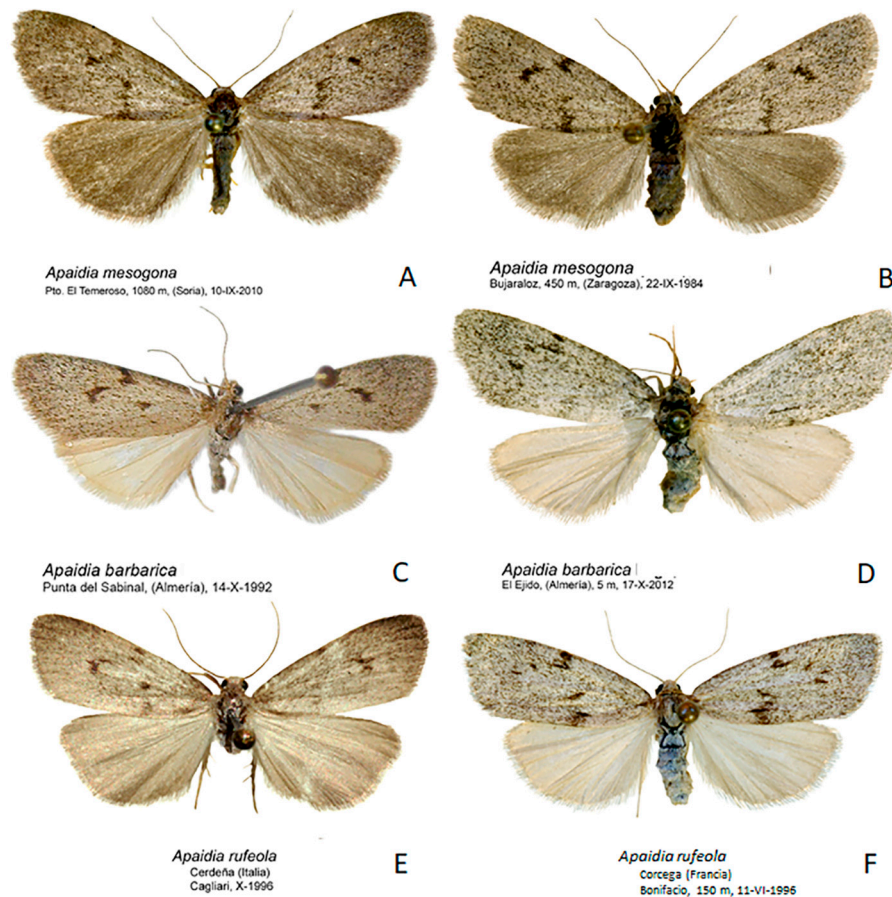


FIGURE 1. A, B. Male and female specimens of *Apaidia mesogona*; C, D. Male and female specimens of *Apaidia barbarica*; E, F. Male and female specimens of *Apaidia rufeola* (Photographies by J. Gastón).

Molecular study

Fifteen adult specimens of *A. mesogona* and *A. barbarica* were processed and sequenced at the Canadian Centre for DNA Barcoding (CCDB, Guelph) to obtain DNA barcodes using the standard high-throughput protocol described by deWaard *et al.* (2008), which can be accessed at www.dnabarcoding.ca/page/research/protocols. Data of sampling localities are indicated in Table 1 and Figure 2. DNA extracts are currently stored at the Canadian Centre for DNA Barcoding and sequences were deposited in GenBank according to the iBOL data release policy. Voucher data, GPS coordinates, images, sequences, trace files, and Genbank acc. nr are publicly available through the public data set (<https://doi.org/10.5883/DS-APAIDIA>) in the Barcode of Life Database (BOLD) (Ratnasingham & Hebert 2007). The *A. rufeola* sequences were provided by Rodolphe Rougerie and were combined with our sequences from the Iberian *Apaidia* specimens. Finally, twenty sequences from *Apaidia* specimens were used for calculations and tree constructions (Table 1).

Sequence divergences for the barcode region were calculated using the Kimura 2-parameter (K2P) model (Kimura 1980) and the degrees of interspecific genetic variation were calculated using the analytical tools of BOLD. All the new and public species sequences were downloaded and aligned with the K2P algorithm of the MEGA6 software (Tamura *et al.* 2013). Boot-strap values were calculated with 1000 replicates (Kimura 1980), and initial Neighbor-joining (NJ) and Maximum Likelihood (ML) trees based on distance were constructed with the MEGA6 software. We selected *Mitochondria miniata* (Forster, 1771) and *Paidia rica* (Freyer, 1858) from the subtribe Nudariina and *Pelosia muscerda* (Hufnagel, 1766) and *Lithosia quadra* (Linnaeus, 1758) from the subtribe Lithosiina, which are systematically related in the tribe Lithosiini, as outgroups to root the tree. In order to assess the COI divergences, we included all sites with the pairwise deletion option.

Results

Molecular analysis

In the dataset composed of 24 sequences, 20 specimens of the genus *Apaidia* were sequenced to analyse taxonomic identity and geographical species grouping, obtaining more than 654 bp for the barcode region (13 of them with 658 bp). The four additional sequences correspond to the outgroups.

Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees of the COI barcode region recovered the same topology and were unequivocally assigned to one of the three major clades considered as putative species: *A. mesogona*, *A. rufeola* and Iberian-Balearic *A. barbarica* (Table 1). 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. 3). The monophyly of the *Apaidia* species was recovered by all those methods and was corroborated with the inclusion of *Miltochrista miniata* (Process-ID IBLAO512-12 in BOLD; <http://v4.boldsystems.org/>) and *Paidia rica* (IBLAO972-14) from the subtribe Nudariina and *Pelosia muscerda* (IBLAO1809-21) and *Lithosia quadra* (IBLAO537-12) from the subtribe Lithosiina as outgroups to root the tree.



FIGURE 2. Distribution of *Apaidia* samples studied (red squares: *A. mesogona*; green triangles: *A. rufeola*; black circles: *A. barbarica*) Note that each point may represent more than one specimen. The map was created using www.simplemapp.net.

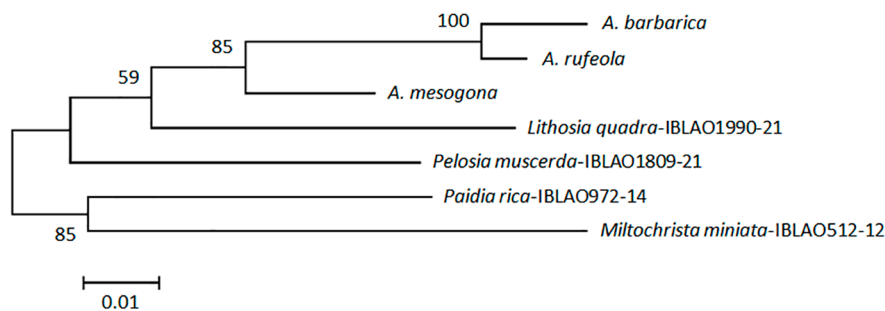


FIGURE 3. Neighbour-Joining tree (K2P; constructed with MEGA6; COI 5' > 600 bp) including 20 sequences of selected *Apaidia* and compared to Lithosiina species *Pelosia muscerda* and *Lithosia quadra*, and Nudariina species *Miltochrista miniata* and *Paidia rica* as outgroups. The depth of each branch shows divergence between lineages. Bootstrap values > 50% are provided at major nodes. The scale bar represents 0.01 genetic difference.

Within these three species, the intraspecific K2P mean divergence ranges between 0 %–0.24% (mean 0.08 % with 0.8% maximal distance in *A. mesogona* and no variation within Iberian-Balearic *A. barbarica*; n=4) (Table 2). Divergence between these three groups varies between 2.0% and 6.1% (mean 4.5%; Table 3). The highest interspecific value was found between *A. mesogona* and *A. barbarica* (6.1%) and between *A. mesogona* and *A. rufeola* (5.4%), whereas the lowest one was found between Iberian-Balearic *A. barbarica* and *A. rufeola* (2.0%) (Table 3). The total number of nucleotide substitutions between species is 44 variable sites.

TABLE 1. Taxon names, BINs, BOLD and GenBank accession numbers for the specimens used in distance estimations (Process ID) and locality information (Country, Region, Exact site, decimal coordinates and collectors).

Species	Process ID	BIN	GenBank	Country	Region	Exact Site	Lat	Lon	Collectors
<i>A. barbarica</i>	IBLAO1833-21	AEI9540	OP721274	Spain	Almeria	El Ejido	36.70	-2.67	F. Morente
<i>A. barbarica</i>	IBLAO1395-20	AEI9540	OP721262	Spain	Almeria	El Ejido	36.69	-2.71	J.J. Guerrero
<i>A. barbarica</i>	IBLAO1885-21	AEI9540	OP721272	Spain	Almeria	El Ejido	36.69	-2.70	F. Morente
<i>A. mesogona</i>	IBLAO1718-20	AEC6797	OP721261	Spain	Cadiz	Los Barrios	36.28	-5.58	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1499-20	AEC6797	OP721273	Spain	Cadiz	Los Barrios	36.33	-5.57	J.M. Gaona
<i>A. mesogona</i>	IBLAO2455-22	AEC6797	OP721257	Spain	Cadiz	Punta Paloma-Tarifa	36.06	-5.71	T. Ranki
<i>A. mesogona</i>	IBLAO2454-22	AEC6797	OP721263	Spain	Cadiz	Punta Paloma-Tarifa	36.06	-5.71	T. Ranki
<i>A. mesogona</i>	Unprocessed			Spain	Huelva	Almonte	37.02	-6.58	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1700-20	AEC6797	OP721259	Spain	Huesca	Villanueva de Sigena	41.70	0.03	J.J. Guerrero
<i>A. mesogona</i>	IBLAO910-12	AEC6797	OP721269	Spain	Albacete	Nerpio	38.15	-2.35	J.J. Guerrero
<i>A. mesogona</i>	IBLAO911-12	AEC6797	OP721256	Spain	Albacete	Nerpio	38.15	-2.35	J.J. Guerrero
<i>A. mesogona</i>	Unprocessed			Spain	Cuenca	Villalba de la Sierra	40.25	-2.05	J.J. Guerrero
<i>A. mesogona</i>	Unprocessed			Spain	Cuenca	Villalba de la Sierra	40.25	-2.05	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1709-20	AEC6797	OP721266	Spain	Gerona	Pau	42.29	3.11	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1711-20	AEC6797	OP721267	Spain	Gerona	Aiguamolls de l'Emporda	42.21	3.09	J.J. Guerrero
<i>A. mesogona</i>	Unprocessed			Spain	Murcia	Sierra de Carrascoy	37.86	-1.30	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1613-20	AEC6797	OP721268	Spain	Murcia	Sierra Espuna	37.86	-1.51	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1614-20	AEC6797	OP721258	Spain	Murcia	Sierra Espuna	37.86	-1.51	J.J. Guerrero
<i>A. mesogona</i>	IBLAO1416-20	AEC6797	OP721264	Spain	Murcia	Yecla	38.67	-1.05	J.J. Guerrero
<i>A. rufeola</i>	LPRCL431-20			France	Corsica	Santo-Pietro-di-Tenda	42.67	9.21	J. Piolain
<i>A. rufeola</i>	LPRCL524-20	AEI9539	OP721265	France	Corsica	Santo-Pietro-di-Tenda	42.67	9.21	J. Piolain
<i>A. rufeola</i>	LPRCL474-20	AEI9539	OP721271	France	Corsica	Santo-Pietro-di-Tenda	42.67	9.21	J. Piolain
<i>A. rufeola</i>	LPRCL472-20	AEI9539	OP721260	France	Corsica	Santo-Pietro-di-Tenda	42.67	9.21	J. Piolain
<i>A. rufeola</i>	LPRCI1494-21	AEI9539	OP721270	France	Corsica	Ventiseri	41.93	9.39	A. Jailloux
<i>A. rufeola</i>	LPRCI1495-21	AEI9539	OP721255	France	Corsica	Ventiseri	41.93	9.39	A. Jailloux

TABLE 2. Intraspecific mean K2P (Kimura 2-Parameter) divergences and maximum pairwise distances based on the analysis of COI fragments (> 600 bp).

	Mean divergence	Maximal distance	Sample size
<i>Apaidea mesogona</i>	0.24	0.80	10
<i>Apaidea rufeola</i>	0.05	0.16	6
<i>Apaidea barbarica</i>	0	0	4

TABLE 3. Interspecific mean K2P (Kimura 2-Parameter) divergences (mean pairwise distances) based on the analysis of COI fragments (> 600 bp).

	<i>A. barbarica</i>	<i>A. rufeola</i>	<i>A. mesogona</i>	<i>L. quadra</i>	<i>P. muscerda</i>	<i>P. rica</i>	<i>M. miniata</i>
<i>A. barbarica</i>		2.0	6.1	8.8	10.6	10.6	10.9
<i>A. rufeola</i>	98.012		5.4	7.8	9.8	9.9	10.4
<i>A. mesogona</i>	93.921	94.648		7.0	7.6	9.0	10.6
<i>L. quadra</i>	91.185	92.202	93.009		8.5	10.1	11.6
<i>P. muscerda</i>	89.362	90.214	92.401	91.489		9.3	11.1
<i>P. rica</i>	89.362	90.061	91.033	89.818	90.729		10.5
<i>M. miniata</i>	89.058	89.602	89.362	88.450	88.906	89.514	

The BIN System is an online framework that clusters barcode sequences algorithmically and is recalculated from time to time as the number of sequences of each species increases. All the COI sequences from the three species were uploaded and examined into the Barcode of Life Data System (BOLD), resulting in three BINs: AEC6797 for *A. mesogona*, AEI9539 for *A. rufeola* and AEI9540 for Iberian-Balearic *A. barbarica*.

Discussion

The three yet described species of the genus *Apaidia* are present in the western part of the Mediterranean area. As said, all of them show small size, slender body, and forewing varying from ochreous-grey to dark brown with relatively broad and apically finely pointed forewings (Witt & Ronkay 2011). Gaston *et al.* (2013) argued that the genital morphology of the three species is different enough in both sexes, with sufficient interspecific variability for the definition of their specific status.

Available molecular data also support the existence of three main lineages of *Apaidia* with sequence divergence rates of approximately 4.5%, which are within the range reported for other well-defined insect species. Sequence divergence rates between *A. mesogona* and *A. barbarica* (~6.1%) are within the range reported for the COI mitochondrial gene in other congeneric insect species, supporting their genetic distinctiveness (Hebert *et al.* 2003, 2004). The *A. rufeola* lineage from Corsica shows a great divergence from congeneric *A. mesogona* (5.4%) and Iberian-Balearic *A. barbarica* (2.0%) species. Iberian *A. barbarica* samples collected in Almería showed a mean genetic difference of 2% with regard to the *A. rufeola* samples from Corsica. These two species present morphological differences and the genetic distance is the 2% threshold, pointed out by Hausmann *et al.* (2011) for average values for interspecific variation. No material of north African (topotypic) *A. barbarica* was available for study.

Comparison of the three *Apaidia* species with other species of the Nudariina and Lithosiina subtribes indicates that their sequences show a closer relationship with the Lithosiina species, as well as occurs with their genitalia features, as was pointed out in Witt & Ronkay (2011) (Figure 3).

Barcode Index Number (BIN) system is a persistent registry for animal MOTUs recognized through sequence variation in the barcode region. The BIN pipeline analyzes new sequence data for the barcode region as they are uploaded to BOLD and BIN metadata are dynamic because key elements of specimen records on BOLD, especially taxonomic assignments, are frequently revised by data providers and because of the high flow of new records (Ratnasingham & Hebert 2013). In our analyses, BOLD calculated three different BINS, suggesting the presence of different species with a unique and specific identifier for each one: AEC6797 for *A. mesogona*, AEI9539 for *A. rufeola* and AEI9540 for Iberian-Balearic *A. barbarica*.

The major lineage of *Apaidia* are mainly present from Morocco to Tunisia and may have diversified in three complexes represented by a widespread group of ancient *A. mesogona* from Morocco to mainland France and the Balearic Islands, Corsica and Sardinia and two species as *A. rufeola*, distributed from Algeria and Tunisia to areas in Italy and the Tyrrhenian islands (Corsica, Sardinia and Sicily), and *A. barbarica*, restricted to Algeria, areas in eastern Italy, Eivissa, Formentera and a small population in Punta Entinas (Almería, South-eastern Spain) (Gastón *et al.* 2013; Maciá *et al.* 2014), showing a pattern that suggests similar ecological preferences or parallel histories for these species during the Quaternary, a phylogeographic hypothesis that would be worth testing in future studies.

Integrating the evidence from COI mitochondrial DNA sequences and adult morphology, we conclude that the *Apaidia* specimens collected in Punta Entinas (Almería, Spain) are genetically different from specimens from Corsica (*A. rufeola*) and, at the current state of knowledge, they must be considered as *Apaidia barbarica*. Barcodes of these specimens would have to be compared with a sample from North African *A. barbarica* to know if they correspond to a migrant colony or a relict population, or even a different species; actually, the male genitalia of the Iberian-Balearic *A. barbarica* (e.g., Gastón *et al.* 2013; Maciá *et al.* 2014), show some differences with those of the Algerian, topotypic *A. barbarica* as illustrated in Witt & Ronkay (2011). Therefore, it could still not be ruled out that the Iberian-Balearic and the Algerian-Sicilian-South Italian populations referred here as *A. barbarica* represent different specific entities.

In light of this, despite the well-supported species discrimination by both morphology and barcodes, several faunistic questions remain unresolved. This is partly due to the fact that the distribution and sympatric occurrence of *Apaidia* species in Northern Africa, as well as the presence of the *Apaidia* species in Western Mediterranean islands and the *A. barbarica* and *A. rufeola* located populations in Italy, are not fully understood. The future expansion of molecular data sets including data from North African and West Mediterranean isolated *Apaidia* populations will be crucial for the resolution of the different questions that are currently unresolved, such as intraspecific variability and whether subsequent taxa can be delimited by means of integrated taxonomy.

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