SPIXIANA	39	2	273–286	München, Dezember 2016	ISSN 0341–8391

Integrated taxonomy, phylogeography and conservation in the genus *Chelis* Rambur, [1866] in the Iberian Peninsula

(Lepidoptera, Erebidae, Arctiinae)

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Ortiz, A. S., Rubio, R. M., Guerrero, J. J., Garre, M. & Hausmann, A. 2016. Integrated taxonomy, phylogeography and conservation in the genus *Chelis* Rambur, [1866] in the Iberian Peninsula (Lepidoptera, Erebidae, Arctiinae). Spixiana 39 (2): 273–286.

The taxonomy of *Chelis*, a genus distributed within the Palaearctic region, is revised based on morphological and molecular data (DNA barcodes) in the Iberian Peninsula. The neighbour-joining and maximum likelihood trees, combined with adult male genitalia and morphology, support the existence of three species indicating two major lineages, one corresponding to mountainous taxa (*Chelis aragonensis* and *C. cantabrica*), with restricted distribution, and the other represented by *Chelis maculosa*, a taxon with a broad European distribution and a great number of infraspecific taxa. Haplotypic variation is highly concordant with species taxonomy; the variation at a continental scale reveals a significant geographic pattern of haplogroups: *C. arragonensis* is restricted to the mountains of Central Spain and *C. cantabrica* is endemic to the Western Cantabrian Mountains. *C. maculosa* includes several distinct haplotypes with a marked intraspecific genetic divergence (0.79–1.83 %) and *C. simplonica* is endemic to the Alps and presents low interspecific divergence from *C. cantabrica* (1.5 %), requiring further investigation.

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Introduction

Arctiine moths (Erebidae, Arctiinae) are a megadiverse subfamily of Lepidoptera with about 11 000 described species (Scoble 1992). The European fauna is particularly well known with 113 species (Witt et al. 2011), as illustrated by the decrease in the description of new species during the last decades, although such findings are usually confined to the Mediterranean region or to other mountain areas (e. g. Ylla et al. 2010: genus *Setina*, Macià et al. 2013: *Chelis cantabrica*). The genus *Chelis* Rambur, [1866] is considered to include 2–10 species depending on the taxonomic interpretation of the status for a number of taxa (Dubatolov 1988, Karsholt & Razowski 1996, Witt et al. 2011, Karsholt & Nieukerken 2013). Some of these taxa have quite a broad distribution from Europe to the Siberian mountains and steppes, for example with *Chelis maculosa* (Gerning, 1780), *C. caecilia* (Lederer, 1853) and *C. dahurica* (Boisduval, 1834). Other taxa are thought to be restricted to particular areas and mountains from the Eastern Iberian Peninsula (*C. arragonensis* (Staudinger, 1894)), the Cantabrian mountains (*C. cantabrica* Macià et al., 2013) or the Alps (*C. simplonica* (Boisduval, 1840)). *C. maculosa* is an extremely variable species in size,

colouration, wing pattern and size of spots. According to different authors it includes the subspecies *C. m. mannerheimi* (Duponchel, 1836), *C. m. honesta* (Freyer, 1843), *C. m. stertzi* (Schulz, 1902), *C. m. flava* Spuler, 1906, *C. m. radiata* Rebel, 1910, *C. m. rosina* Oberthür, 1911, *C. m. marsicana* Dannehl, 1929, *C. m. centralhispanica* Daniel, 1935 (= *C. m. serratica* Agenjo, 1937), *C. m. arlanzona* Agenjo, 1937, and *C. m. nordiberica* Agenjo, 1937, amongst others. Witt et al. (2011) considered *C. maculosa* to occur in the Central and Southern parts of Europe, from the North Eastern Iberian Peninsula to the Balkans and the Eastern European steppes, but it is absent from the North Western and Northern parts of Europe. The subspecies present in the Iberian Peninsula is *C. maculosa stertzi* (Schulz, 1902); it was described from the Italian Alps although Toulgoët (1985) and Fernández-Vidal (2012) considered it to be an individual form of the nominal subspecies.

C. arragonensis is considered to be endemic to the Iberian Peninsula, from the Pyrenees towards Central Spain. *C. arragonensis* was formerly considered to be a form or a subspecies of *C. maculosa* although it occurs sympatrically with *C. maculosa stertzi* in the Northern half of the Iberian Peninsula. The female genitalia of the two species are very similar but the male genitalia show slight but recognizable differences.

However, Pérez-de Gregorio et al. (2001) only considered the presence of three subspecies in the Iberian Peninsula: *C. m. maculosa* distributed from the Central Pyrenees to the Cantabrian Mountains; *C. m. stertzi* from the Eastern Pyrenees and inner Catalonian Mountains; and *C. m. arragonensis* in the Iberian System, Sierra of Guadarrama and the provinces of Zamora and Burgos. Ylla et al. (2010) considered the presence of only two subspecies, *stertzi* and *arragonensis,* while Fernández-Vidal (2012) and Magro (2013), following the criteria of Witt et al. (2011), displayed the *C. maculosa* and *C. arragonensis* distribution in the North Western half of the Iberian Peninsula. With respect to *C. simplonica*, Fernández-Vidal (2012) considered that the record from Puerto de Pajares could be right (in spite of its low altitude, 1420 m, in comparison with the Alps), but also considered that it could be a mislabelled specimen. Magro (2013) mentioned at least three different overlapping areas for *C. maculosa* and *C. arragonensis* in Central Spain.

In the latest version of the Fauna Europaea (Karsholt & Nieukerken 2013), four taxa have been recognized in Europe, of which only *C. maculosa* is thought to occur in the Iberian Peninsula. Finally, Macià et al. (2013) have described *C. cantabrica*, a species closely related to *C. simplonica*. Therefore, the taxonomic problems concerning the genus *Chelis* in the Iberian Peninsula remain contentious. To solve them, the study of additional characters, rather than solely morphological characteristics, is needed. Although the study of the genitalia provides valuable criteria for species recognition, their preparation is time consuming, limiting the use of this approach for large-scale specimen sorting and examination. The recent integration of morphological and DNA-based approaches has proven to be an effective way to accelerate species discovery and delineation (Lumley & Sperling 2010, Padial & De La Riva 2010, Schlick-Steiner et al. 2010), as well as to assist in detecting previously cryptic species (Hebert et al. 2004, Huemer & Hausmann 2009, Hausmann & Huemer 2011, Mutanen et al. 2012a). Integration of molecular methods with morphological analyses may accelerate biodiversity inventories and corroborate the status of doubtful taxa (Smith et al. 2009). 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 *Chelis* are separated into definite sequence clusters. In this paper we analyse phenotypic characters, COI sequences and geographical distribution of the lineages to assess differences between groups of populations in Iberian Peninsula.

Material and methods

Morphological study

One hundred and fifteen adult specimens of the *Chelis* genus from the Iberian Peninsula and three from the Alps were identified as *Chelis maculosa* (36 specimens), *C. arragonensis* (70), *C. cantabrica* (7) and *C. simplonica* (2). Adult specimens were examined externally in order to evaluate possible differences in their colouration, spot size and wing shape, and were dissected using a standard procedure (Fibiger 1997) with minor modifications. Genital structures were examined using a Zeiss Stemi 2000-C stereomicroscope with a digital camera (Insight Firewire, 18.2. Color Mosaic, Diagnostic Instruments Inc. USA). Final pictures were taken by Canon Digital Camera EOS 550D with an ultra-macro objective. Genital structures were compared with those published by Witt et al. (2011) to get an initial identification. Specimens are deposited in the collection of the Department of Zoology and Physical Anthropology of the Universidad de Murcia (Spain) except *C. simplonica* specimens which are deposited in Tiroler Landesmuseen in Innsbruck (Austria).

Molecular study

Eighteen adult male specimens of *Chelis* 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 Ivanova et al. (2006). Data of sampling localities are indicated in Table 1 and Figure 3. 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 (in progress). Voucher data, images, sequences, and trace files are publicly available on the Barcode of Life Database (BOLD) (Ratnasingham & Hebert 2007).

Sequence divergences for the barcode region were calculated using the Kimura 2 Parameter (K2P) algorithm MEGA6 program (Tamura et al. 2013), including all sites with the pairwise deletion option. Bootstrap values were calculated with 1000 replicates (Kimura 1980), and Neighbour-joining (NJ) and Maximum Likelihood (ML) trees based on distance were constructed with the MEGA6 program.

For the parameter values considered (e.g. sensitivity to codon bias and unequal rates of evolution) the statistical inconsistency of Maximum parsimony (MP) method may occur and was not performed in this study. We selected *Arctia villica* (Linnaeus, 1758), which is systematically related into subtribe Arctiina, as outgroup to root the trees. The number of haplotypes was calculated with DnaSP version 5.10 (Rozas et al. 2003). Data from four species groups were calculated with TaxonDNA version 1.5 (Meier et al. 2006) for subsequent analyses.

Table 1. Sample information for the *Chelis* specimens included in the sequence analysis. *Acronyms for institutional and private collections are as follows: CNCIAN, Canadian National Collection of Insects, Arachnids and Nematodes; RCBD, Research Collection of Bernard Dardenne; RCKZ, Research Collection of Karel Cerny; RCSB, Research Collection of Stoyan Beshkov; TLMF, Tiroler Landesmuseum Ferdinandeum; ZDUM, Zoology Department of University of Murcia.

Taxon	Process ID	Haplotype	Region (Country)	Depository*	COI-5P Seq. Length	GenBank Accession
arragonensis	IBLAO842-12	Hap1	Cuenca (Spain)	ZDUM	643	KT381883
arragonensis	RDNME102-07	Hap1	Spain	CNCIAN	616	
arragonensis	IBLAO555-12	Hap2	Cuenca (Spain)	ZDUM	658	KT381896
arragonensis	IBLAO916-12	Hap2	Teruel (Spain)	ZDUM	646	KT381890
cantabrica	IBLAO788-12	Hap3	Asturias (Spain)	ZDUM	658	KT381888
cantabrica	IBLAO789-12	Hap3	Asturias (Spain)	ZDUM	658	KT381895
cantabrica	IBLAO840-12	Hap3	Asturias (Spain)	ZDUM	658	KT381891
cantabrica	IBLAO841-12	Hap3	Asturias (Spain)	ZDUM	658	KT381886
maculosa	IBLAO919-12	Hap4	León (Spain)	ZDUM	646	KT381885
maculosa	IBLAO920-12	Hap4	León (Spain)	ZDUM	646	KT381892
maculosa	IBLAO922-12	Hap4	León (Spain)	ZDUM	646	KT381894
maculosa	IBLAO923-12	Hap4	León (Spain)	ZDUM	646	KT381898
maculosa	IBLAO926-12	Hap4	León (Spain)	ZDUM	646	KT381881
maculosa	IBLAO927-12	Hap4	León (Spain)	ZDUM	658	KT381897
maculosa	IBLAO921-12	Hap5	León (Spain)	ZDUM	646	KT381887
maculosa	IBLAO924-12	Hap6	León (Spain)	ZDUM	626	KT381893
maculosa	IBLAO556-12	Hap7	Lleida (Spain)	ZDUM	658	KT381882
maculosa	IBLAO843-12	Hap7	Lleida (Spain)	ZDUM	658	KT381889
maculosa	LENOA1182-11	Hap8	Alps (France)	RCBD	658	
maculosa	LENOA1183-11	Hap8	Alps (France)	RCBD	658	
maculosa	IBLAO925-12	Hap9	Cuneo (Italy)	ZDUM	646	KT381884
maculosa	PHLAA351-09	Hap9	Alps (France)	TLMF	658	HM425877
maculosa	PHLAA387-09	Hap9	Cuneo (Italy)	TLMF	658	HM425907
maculosa	PHLAB1155-10	Hap10	Abruzzo (Italy)	TLMF	658	HQ968367
maculosa	PHLSA451-11	Hap11	Pescara (Italy)	TLMF	658	
maculosa	PHLSA582-11	Hap11	Chieti (Italy)	TLMF	658	
maculosa	NMNHL323-10	Hap12	Silistra (Bulgaria)	RCSB	658	HQ966763
maculosa	NMNHL324-10	Hap12	Silistra (Bulgaria)	RCSB	658	HQ966764
maculosa	NMNHL325-10	Hap12	Silistra (Bulgaria)	RCSB	658	HQ966765
maculosa	NMNHL326-10	Hap12	Silistra (Bulgaria)	RCSB	578	HQ966766
maculosa	PHLAF371-11	Hap13	Mavrovo (Macedonia)	TLMF	658	
simplonica	PHLAG180-12	Hap14	Graubünden (Switzerland)	RCKZ	658	

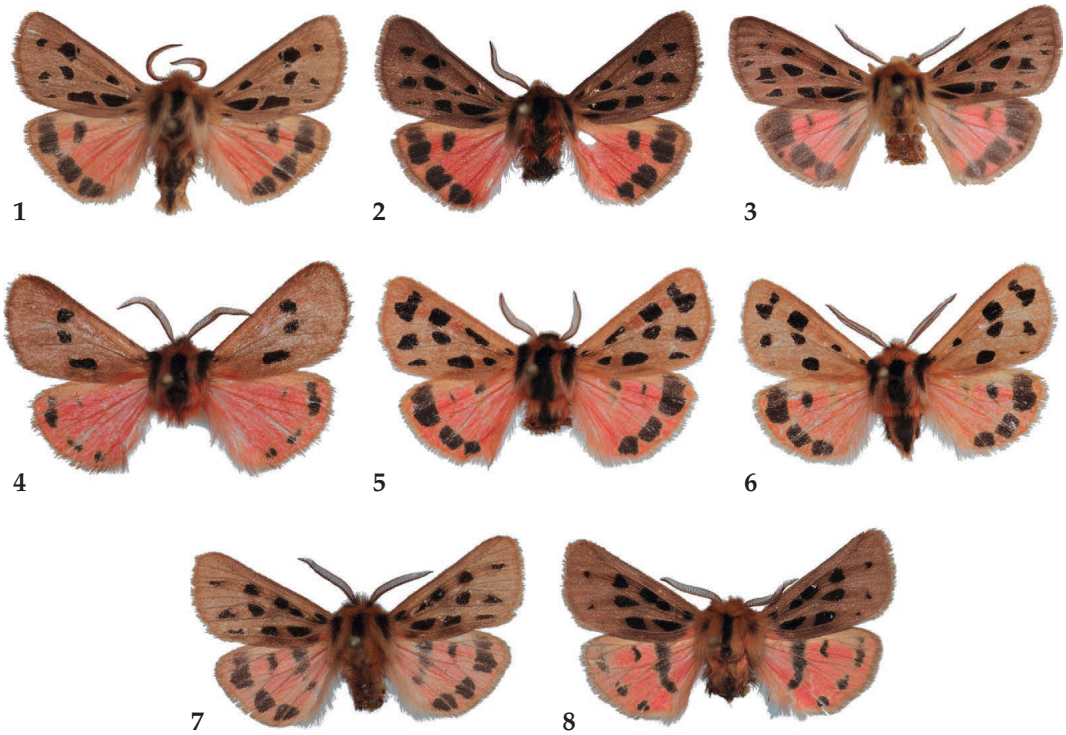

Fig. 1. Adults of *Chelis*. **1–2.** *C. cantabrica* Macià et al., 2013; **3.** *C. simplonica* (Boisduval, 1840); **4–6.** *C. arragonensis* (Staudinger, 1894); **7–8.** *C. maculosa* (Gerning, 1780).

Divergence times between species and between lineages of *Chelis* species were estimated from COI sequences using a Bayesian MCMC speciation method implemented in BEAST version 1.7.5 (Drummond et al. 2012). Substitution models HKY (Hasegawa et al. 1985) used was chosen according to jMODELT-EST version 2.1.3 (Guindon & Gascuel 2003, Darriba et al. 2012). The dataset was analysed under the HKY+I+α substitution model applying a strict molecular clock along the branches. Since it was determined that taxa evolved at equal rates, we applied a strict molecular clock for the estimation of divergence times in BEAST version 1.7.5. Dates for calibration points were the ages obtained applying a molecular clock with substitution faster rate of 2.3 % uncorrected pairwise distance per million years for the entire mitochondrial genome of various arthropod taxa (Brower 1994, Papadopoulou et al. 2010). However, since the reliability of the time estimates will depend on the accuracy and homogeneity of this rate, some caution should be used in the interpretation of these absolute values. The analysis was performed with 10 million generations; chains were sampled every 1000 generations with a burn-in of 1000 generations to ensure convergence. Confidence intervals of divergence times and effective sample size of all parameters were obtained with TRACER version 1.5 (Rambaut et al. 2014). Summary trees were generated using TREEANNOTATOR version 1.7.5. A relaxed molecular clock model combined with a Bayesian speciation Yule process tree prior assumption had the best likelihood for *Chelis* species and was further used in the study.

The *C. simplonica* sequence was provided by Peter Huemer and *C. maculosa* sequences from Europe were obtained from Data Portal of the Barcode of Life Data Systems (BOLD) (Ratnasingham & Hebert 2007), and were combined with our sequences from the Iberian and Italian *Chelis* specimens. Finally, 32 sequences from *Chelis* specimens were used for calculations and tree constructions (Table 1).

Results

Morphological and ecological traits

Chelis is a species-group with high morphological and colour variability: forewings are usually light brown with angular black spots and hindwings are pink with median and marginal maculae (Fig. 1). Superficially, several congeners are very similar and a proper identification requires the preparation of adult male genitalia. *Chelis maculosa* is noticeable because of its variability in size of spots and coloura-

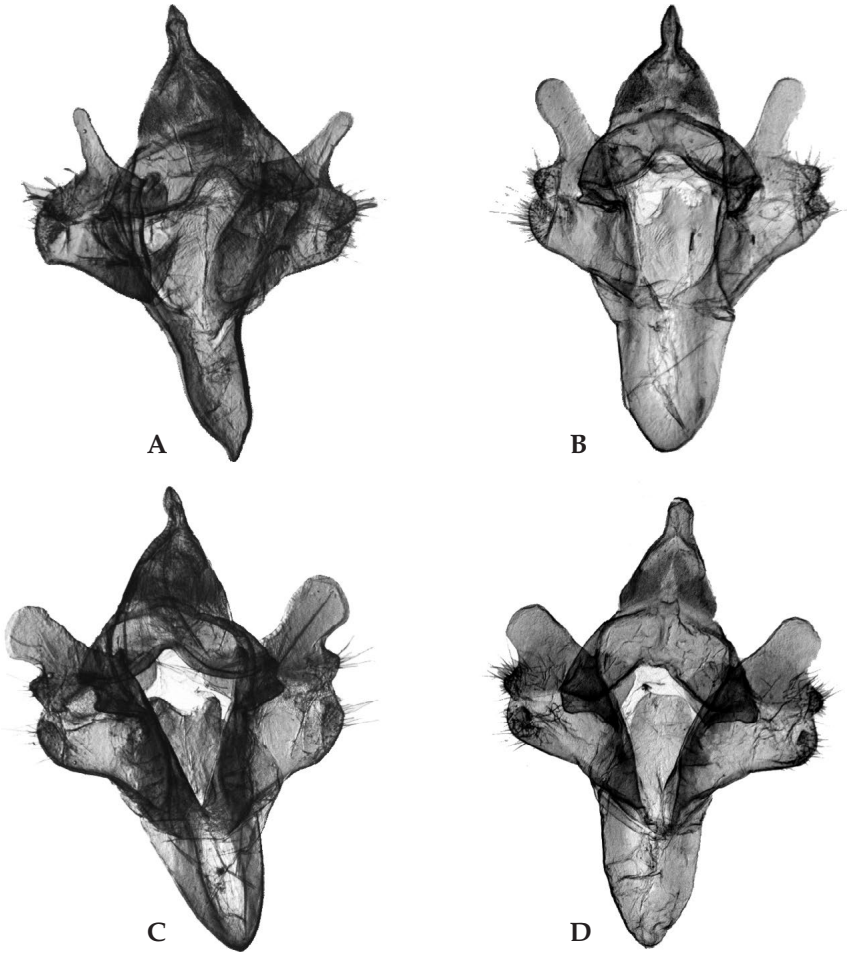

Fig. 2. Male genital structure of *Chelis,* with aedeagus removed. **A.** *C. cantabrica* Macià et al., 2013; **B.** *C. simplonica* (Boisduval, 1840); **C.** *C. arragonensis* (Staudinger, 1894); **D.** *C. maculosa* (Gerning, 1780).

tion (Fig. 1). Wingspan of the male varies from 26 to 35 mm in the subspecies *maculosa* and *stertzi*, and is higher in the subspecies *honesta* (32–38 mm). Forewing is fulvous grey with neat, black and triangular spots including costal margin. Costal vein is darkened. Hindwing is dirty pink coloured with three large, rounded submarginal spots and small spots on the discoidal vein and, at the base, closer to the inner margin. Ventral colouration is dark pink. The species is associated to xerophilous steppes inhabiting many different biotopes from the sea level to ca 2000 m altitudes and flies from May to August as bivoltine with overlapping generations (Witt et al. 2011).

C. arragonensis has often been interpreted as a subspecies of *C. maculosa* (Fig. 1). Wingspan of male varies from 26 to 36 mm (as in *C. maculosa stertzi*). The marginal row of dark spots in the hindwing is reduced or absent, the medial row is enlarged and the basal spots are partly or absent but a high variation is also detected. It is a xero- and thermophilous species inhabiting insolated karstic plateaus and slopes of the Iberian System; it is univoltine and flies from May to August.

C. simplonica is smaller than *C. maculosa* and *C. arragonensis* (wingspan 25–31 mm; Fig. 1). Forewing spots are partly fused into longitudinal stripes and the ground colour is greyish or brown with pinkish reflections. Hindwings are pinkish with a large discal spot and partial fused greyish marginal patches. This species inhabits high montane rocky steppes and dry subalpine meadows in the Alps between 1750–2600 m altitude (Lepidopterologen-Arbeitsgruppe 2000); it is univoltine and flies in July and August. Witt et al. (2011) pointed out that

Fig. 3. Distribution of *Chelis* samples sequenced. ○: *C. maculosa*; ●: *C. simplonica*; ●: *C. arragonensis*; ●: *C. cantabrica*) and distribution ranges of Iberian *Chelis*, according to data original to the present study, Fernández-Vidal (2012), Macià et al. (2013) and Magro (2013).

a sole specimen found in the Cantabrian Mountains may represent a distinct taxon. Recently, Macià et al. (2013) has described a new species *C. cantabrica* related to *C. simplonica*, which is characterized by having a wingspan of 27–30 mm, absence of basal dark spot and pectinate antennae more robust and larger (Fig. 1).

The external morphology for most taxa has been extensively described by Witt et al. (2011) and by Macià et al. (2013) for description of *C. cantabrica*. High external variability can be checked in large specimen series figured by Witt et al. (2011), Fernández-Vidal (2012) and Magro (2013).

The genitalia of the species shows slight differences from broad to narrow digitiform apical valva process and different juxta shapes (Fig. 2). Minor differences in aedeagus morphology were observed, although Macià et al. (2013) pointed out differences in size and spinules density.

Figure 3 illustrates the distribution of Western European *Chelis* taxa sequenced in this study and the distribution of *Chelis* species in the Iberian Peninsula designed according to own data and records from recent bibliographic references in Fernández-Vidal (2012), Macià et al. (2013) and Magro (2013).

Molecular study and phylogeny

The eighteen sampled specimens were sequenced, obtaining more than 625 bp in barcode region (eight of them with 658 bp), and their conspecific sequences were acquired from the databases (BOLD) to analyse taxonomic identification and geographical species grouping. An unpublished *C. simplonica* sequence was provided by Peter Huemer (Tiroler Landesmuseen). In total, 14 different haplotypes were found in the 32 barcode sequences analysed from four lineages of the genus *Chelis* (10 from *C. maculosa*, 2 from *C. arragonensis* and one from both *C. simplonica* and *C. cantabrica*; Table 1). The overall haplotype diversity was 0.929 and the nucleotide diversity 0.022. Nucleotide composition showed a similar A–T bias for the four taxa considered (average A = 30.8 %, T = 38.6 %, C = 16.1 %, G = 14.5 %). No insertions or deletions were found.

Four major groups of haplotypes were found and within them the intraspecific K2P divergence ranges between 0 %–1.83 % (mean 0.84 %; Table 2). These well-supported clades were thereafter treated as four putative species, namely *Chelis simplonica*, *C. cantabrica*, *C. arragonensis* and *C. maculosa*. Divergence

between these four groups varies between 1.5 % and 4.5 % (mean 3.2 %; Table 3). The highest interspecific value was found between *C. maculosa* and the other groups, whereas the lowest one was found between *C. simplonica* and *C. cantabrica* (1.5 %). The total number of nucleotide substitutions between species is high and ranges from 22 to 40 variable sites (Table 3). Neighbour-Joining (NJ) and Maximum Likelihood (ML) trees of COI barcode region recovered the same topology, and all haplotypes were unequivocally assigned to one of the two major clades (Figure 4 shows only NJ tree). ML yielded similar results to those of NJ based on K2P model and the same NJ tree topology was produced under K2P and Tamura 3-parameter (T3P) substitution models (Figs 5A, B). The high bootstrap supports correspond to three clade nodes (Figs 4, 5). This is in concordance with the fast evolving rates of COI that are usually not suitable for the recovery of ancient relationships, but are often useful in resolving more recent splits (Dincă et al. 2011).

The closest relationship between these groups was found between those assigned to *C. cantabrica* and *C. simplonica*. This last taxon was represented by a single sequence from a specimen collected in the Alps. Initially, samples from Somiedo in the Northern Iberian Peninsula were identified as *C. simplonica*, but after the recent description of *C. cantabrica* (Macià et al. 2013) these specimens were re-identified as *C. cantabrica*. Differences in nucleotide composition between these two species was approximately 1 % with 22 nucleotide substitutions (Table 3) and in our trees, the interspecific relationship between *C. simplonica* and *C. cantabrica* are recovered with not enough bootstrap support, suggesting further investigations (Figs 4, 5).

Analyses for the 32-specimen from Table 1 dataset provides support for the monophyly for each lineage of the genus *Chelis* in Europe: the mountainous *Chelis* (clade simplonica-cantabrica-arragonenesis) and the *Chelis maculosa* species groups as strongly supported (Figs 4, 5). Within each of these two main groups, some clades are well supported, whereas some of the relationships in *maculosa* clade are not fully resolved but it can be inferred. The sampling of *C. maculosa* covers all the European species' distribution and this large clade is formed by two haplogroups including Hap4-Hap13 haplotypes. The first haplogroup contained *C. maculosa* samples from northern Iberian Mountains (Hap4, Hap5 and Hap6) nested with populations from Italian Abruzi Mountains (Hap10 and Hap11). The second clade presents wide geographical distribution, including haplotypes from eastern Pyrenees (Hap7), Alps (Hap8, Hap9), Bulgaria (Hap12) and Macedonia (Hap13). These groups are separated by a genetic divergence of 0.89 %. Divergence within *C. maculosa* group ranged from 0 % to 1.83 % (mean 0.45 %) with 23 sequences analysed (Table 2); the maximum value was found between samples from Central Italy and Bulgaria (1.82 %). According to BOLD database, *C. maculosa*'s nearest species is *Chelis dahurica* (Boisduval 1832) from Mongolia, separated by a genetic distance of 2.73 %.

Within the *simplonica-arragonensis* group, COI supports the arragonensis clade as well as the sister relationship between *C. simplonica* and *C. cantabrica*. The four sequences of *C. arragonensis* make up a haplogroup sister to that constructed from sequences of the *C. simplonica-C. cantabrica* clade. In the first clade Hap1-Hap2 falls into *C. arragonenesis* clade, whilst Hap3 and Hap14 fall outside, but into a *simplonica* clade where Hap3 is referred to *C. cantabrica* and Hap14 to *C. simplonica* (Fig. 1). The mountainous clade is composed of two geographically and phylogenetically distinct subclades. The first clade includes a *C. arragonensis* samples from Iberian mountains ranges. The second clade includes two subclades for *C. simplonica* from the Swiss Alps and *C. cantabrica* from Somiedo Valley in Cantabrian Mountains (Iberian Peninsula). The monophyly of genus *Chelis* were well supported by NJ and ML boot-strapping (Figs 4, 5).

In a rough dating attempt (Fig. 6) the age of the basal splits within the genus *Chelis* is estimated to 1.3 Ma. The estimated age for the split between the two

Table 2. Intraspecific mean K2P (Kimura 2-Parameter) divergences and maximum pairwise distances based on the analysis of COI fragments (>500 bp).

	Mean divergence	Maximal distance	Sample size
C. maculosa	0.79	1.83	23
C. arragonensis	0.27	0.30	4
C. simplonica	–	–	1
C. cantabrica	0	0	4
			32

Table 3. Interspecific mean K2P (Kimura 2-Parameter) divergences (mean pairwise distances) based on the analysis of COI fragments (>500 bp). Total number of mutations between brackets.

	C. arragonensis	*C. simplonica*	*C. cantabrica*
C. maculosa	4.5 % (39)	3.6 % (40)	3.7 % (40)
C. arragonensis		2.8 % (32)	2.9 % (32)
C. simplonica			1.5 % (22)

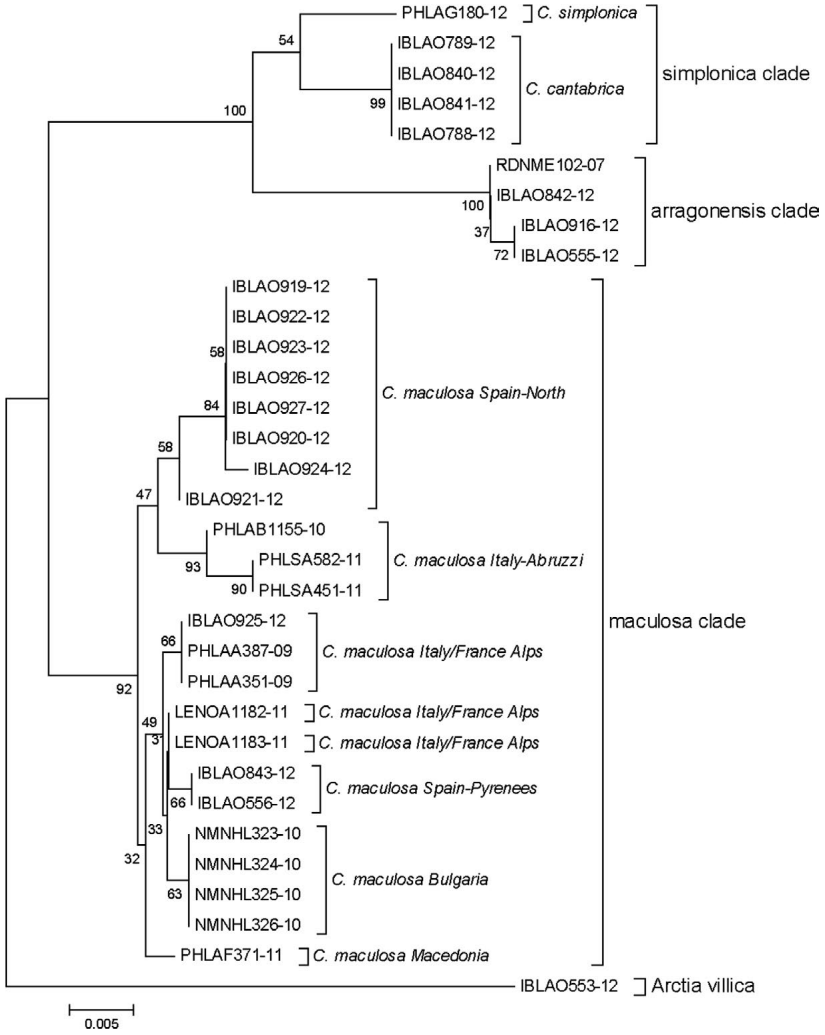

Fig. 4. Neighbour joining tree (K2P) of the *Chelis* genus based on 32 sequences of the mtDNA COI gene (barcode fragment 5', >600 bp), rooted with *Arctia villica* as outgroup. The depth of each branch shows divergence within lineages. Numbers at nodes indicate maximum bootstrap. The scale bar represents 0.005 substitutions/position.

main groups is one Ma and between *C. arragonensis* and *C. simplonica* clade is 0.5–0.6 Ma. In absence of valuably dated arctiine fossiles attributable to genera related to *Chelis*, the calibration of the 'molecular clock' approach had to be based on hypothesized substitution rates which can roughly be estimated at 0.023 per million years for lepidopteran COI sequences (Brower 1994, Papadopoulo et al. 2010).

Discussion

Integration of molecular and morphological data

In different groups of invertebrate taxa, a sequence divergence in the barcode region lower than 2 % often corresponds to intraspecific differences while higher values are typical of interspecific variation (Hausmann et al. 2011). For example, Mutanen et al. (2012b) pointed out that most species of Arctic-Alpine Lepidoptera represented by multiple individuals showed a divergence lower than 2 % for Fennoscandian, Alpine and North American taxa

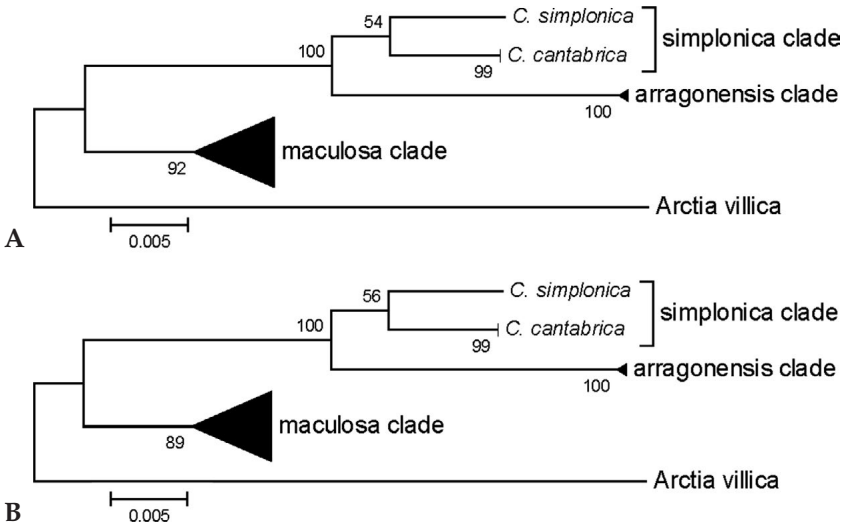

Fig. 5. A. Neighbour-joining tree (K2P) for 33 barcodes COI sequences including *Chelis* species complex, rooted with *Arctia villica* as outgroup. **B.** Neighbour-joining tree (T3P) for 33 barcodes COI sequences including *Chelis* species complex, rooted with *Arctia villica* as outgroup. The depth of each branch shows divergence within lineages. Numbers at nodes indicate maximum bootstrap. The scale bar represents 0.005 substitutions/position.

with regard to intraspecific variation. However, divergence between young sister-species may fall below the 2 % threshold, while unusually variable species may exceed it.

Barcoding data obtained in our study indicate not only the existence of three *Chelis* species in Iberia, but also that there are two well-supported and reciprocally monophyletic groups in Iberian Peninsula. One is made up by sequences of the polymorphic *C. maculosa*, which shows notable sequence variability as expected from its wide geographic distribution across many European localities. The other major clade is split between the Alpine *C. simplonica* plus the Cantabrian *C. cantabrica* samples, and the Iberian endemism *C. arragonensis* (Fig. 4).

C. maculosa presents a high intraspecific variation (mean 0.45 %, maximum 1.82 %) with two main clades diverging by 0.89 %, a value far below the 2 % "threshold" for most interspecific differences, but that clearly separates the North Iberian and Abruzzi samples from other European samples. This suggests that the Pyrenees and the Alps have acted as an effective barrier to gene flow in past geological periods. This genetic variation is related to the wide distributional range of the species from the Northern Iberian Peninsula to Eastern Bulgaria. Populations from the Pyrenees, Provence-Alps-Cote d'Azur and Bulgaria present different haplotypes but show relatively low genetic divergence. Those ones from Macedonia and the Abruzzi are more distinct in both clades (Figure 4). Iberian samples collected in León showed a notable genetic difference of 1.06 % with regard to the sample form the Central Pyrenees (Arán Valley); these two localities are about 720 km distant but there is no evident geographic barrier between them, except for the low altitude of the Western Pyrenees and Basque Country mountains. *C. maculosa* shows a great divergence from congeneric species (from 3.6 % to 4.5 %; Table 3), which suggests a differentiation prior to Pleistocene glaciation events.

Although *C. cantabrica* and *C. simplonica* present some differences, as the presence/absence of basal dark spot and the form of pectinate antennae (Fig. 1), the genetic distance (1.5 %) between Alpine samples of *C. simplonica* and those from *C. cantabrica* is lower than the 2 % threshold, pointed out by Hausmann et al. (2011) for average values between related species. It seems that we are dealing with young taxa recently evolved in isolation, as there is a major geographic gap between these two taxa which precludes gene flow. This hypothesis is consistent with the lack of records of both species in intermediate geographic areas, in spite of the accurate knowledge on the distribution of these taxa.

Both *C. simplonica* and *C. cantabrica* are well separated from *C. arragonensis*, a taxon endemic to the mountains of Central Spain (Table 3; Figs 4, 5). There is a 3.1 % mean pairwise distance between *C. simplonica-cantabrica* group and C. *arragonensis*; this molecular divergence is also reflected in slight but consistent differences in genitalia traits (Fig. 2).

Habitat, biology and conservation of *Chelis* in Iberian Peninsula

Additionally to molecular and morphological characters, the four *Chelis* species discussed above also show differences in ecological characters. *C. maculosa* is bivoltine, with partly overlapping generations and it inhabits xerophilous steppes, hot and dry open biotopes, hilly and montane slopes, karstic slopes and plateaus, from sea level up to 2000 m. *C. maculosa* populations show some plasticity in voltinism similar to many other alpine lepidoptera with reduced generations at higher altitudes (e. g. in the south-western Alps it is univoltine; Huemer pers. com.). The rest of *Chelis* species are univoltine, show a more restricted geographic distribution and inhabit alpine, subalpine and montane rocky steppes, steep slopes, ravines and dry insolated rocky grassland and meadows. The larvae are polyphagous on herbaceous plants, preferring *Galium, Achillea* and *Plantago* species (Witt et al. 2011).

The protection of Macroheterocera, and specially arctiine moths, remains poorly developed in Iberian Peninsula and they are under-represented in threat assessments and biodiversity conservation. In this sense, the Red Data Book of European Butterflies criteria (Van Swaay & Warren 1999) should consider to *C. arragonensis* and *C. cantabrica* as species of conservation concern due to their European endemic status and they must be included into European priorities. These species are a particular European responsibility since they cannot be conserved anywhere else in the world, and such species typically have highly restricted distributions, requiring specific and localized action.

According to Climatic Risk Atlas of European Butterflies (Settele et al. 2008), three different scenarios developed within the ALARM project (see Spangenberg 2007 for further details) were analysed covering a broad range of social, economic, political and geo-biosphere parameters. The moderate (SEDG), intermediate (BAMBU) and maximum change (GRAS) scenarios until 2080 increase the mean expected temperature from 2.4 to 4.1 °C and this indicates a total loss of suitable climatic niche from Cantabrian Mountains for *C. cantabrica* and the other Iberian mountain localities for *C. arragonensis*. Climatic change is a potential threat to several species, notably highly restricted montane endemics in Europe which are closely evolved to specific vulnerable habitats and which have very limited possibility of adapting to change (Dennis 1993).

In this sense, all European endemic species restricted at alpine, subalpine and montane habitats as isolated populations of *C. cantabrica* and *C. arragonensis* must be considered as vulnerable. Settele et al. (2008) recommend that threatened endemic species in Europe should be listed on the Bern Convention and the relevant species listed in any revision of Annexe II of the EC Habitats and Species Directive.

Thus, the classical effect of incorrect taxonomy on conservation efforts is to underestimate the level of biological diversity. The present study illustrates a case of underestimation of biological diversity and that a complete list of species is fundamental requirement of biodiversity studies on evolution, ecology and conservation.

Hypotheses on the evolutionary history of western European *Chelis*

The current distributions of temperate species are the result of multiple range shifts driven by past climatic changes. Cyclic climatic change during the Pleistocene has caused repeated range shifts in most European taxa, profoundly influencing the biodiversity of Europe (for reviews, see Hewitt 1996, 1999, 2000, 2004; Taberlet et al. 1998, Schmitt 2007). The temperate fauna and flora of Europe is generally considered to have survived glaciations in any of three peninsular refugia in the south of the continent, i. e. Iberia, Italy or Balkan.

These refugial regions harbour high biodiversity and endemism due in large part to their long-term environmental stability, which enables the persistence of palaeoendemic taxa (e. g. Jansson 2003, Graham et al. 2006). Isolation among refugial populations promotes genetic and phenotypic differentiation as a result of independent adaptation to local environments and genetic drift, with consequences for reproductive isolation between discrete refugial lineages and the creation of hybrid zones where diverged lineages come into secondary contact (Hewitt 1999).

Assuming a standard sequence divergence rate for Lepidoptera of 0.023 per million years for COI sequences (Brower 1994, Papadopoulo et al. 2010), then the major lineages of *Chelis* may have diversified between 0.65–2 Ma (see Table 3 and Figure 6). The COI gene, however, does not evolve at constant pace over the time scale and neither in different lineages. Assuming standard substitution rates, therefore, leads to very rough and somewhat speculative estimations of the lineage divergences. A comparison of the distribution ranges of the *Chelis maculosa* and the rest of *Chelis* lineages reveal an interesting pattern (Fig. 6). These two complexes are represented by a widespread group of *C. maculosa*, with ten detected haplotypes from Spain to Bulgaria and three species restricted to isolated mountainous areas in Iberian Peninsula and Alps, showing a pattern that it could be considered evidence for similar ecological prefer-

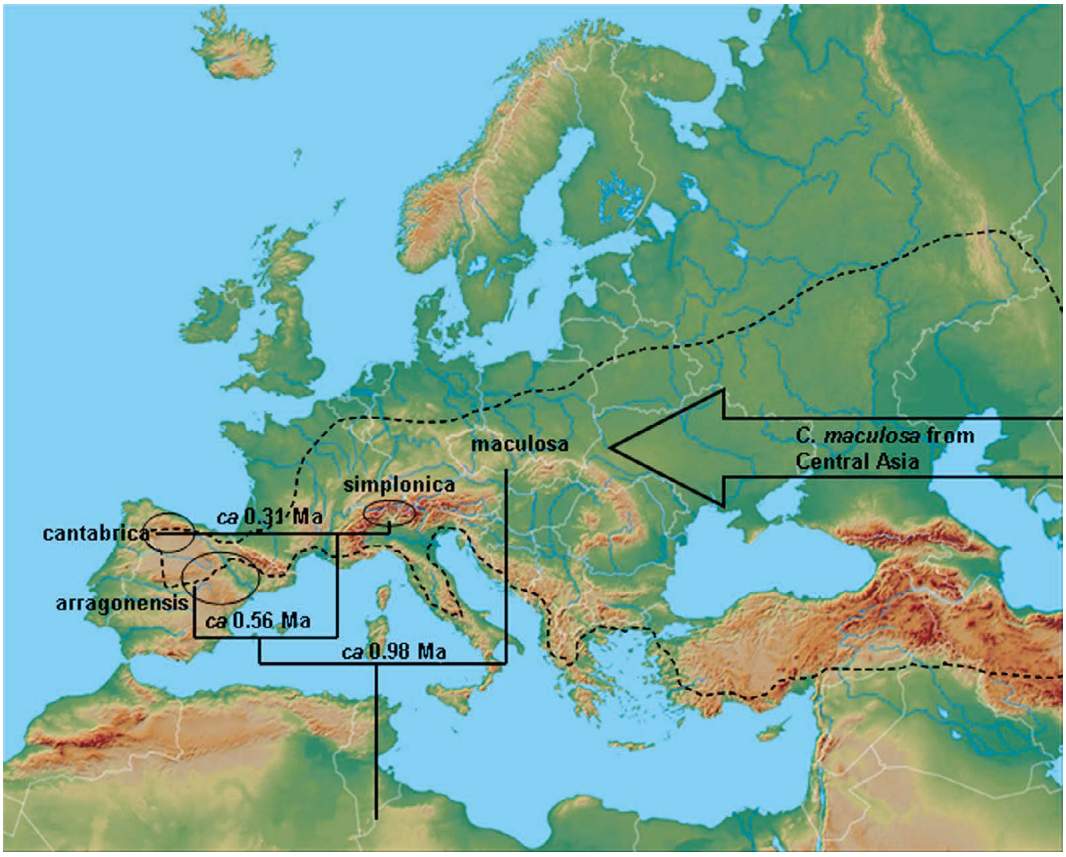

Fig. 6. Biogeographical hypothesis describing the first splits of the genus *Chelis* lineage in Western Europe in the late Pleistocene, and dispersal and isolation in Iberian Peninsula and the Alps, followed by distribution range fragmentation.

ences or parallel histories for these species during the Quaternary. The fact that the three taxa with more restricted distribution *C. simplonica*, *C. cantabrica* and *C. arragonensis*, make up the sister clade to the broadly distributed *C. maculosa*, and suggests that both clades share a recent common ancestor.

Analysis of distribution and phylogeny in the *Chelis maculosa* lineage shows that the phylogeographic history of this complex involved a combination of dispersal and vicariance events with a clear general trend of dispersal from Central Asia, where the group most likely arose to the Western Europe during the interglacial Donau-Günz period (1.4 Ma) (Fig. 6). On a geographical basis, the first lineage split of this ancestor possibly affected isolated populations in the Iberian Peninsula, the Pyrenees and the Alps, giving rise to the *C. simplonica* group, which shows a mean genetic divergence with *C. maculosa* of 3.6 %. This divergence corresponds to a geological time of approximately 1 Ma, thus shortly after the Günz glacial period (1.1 Ma).

After this, *C. maculosa* (or its immediate ancestor) probably survived glaciation effects in temperate refugia at low altitudes (i.e., steppes between ice shields) in the North and in warmer Mediterranean mountains. This phylogeographic pattern has been suggested for other Lepidoptera (e.g. *Agrodiaetus*, *Erebia*, *Melitaea*, *Parnassius*), in which it is postulated a marked role of climatic oscillations during the Pleistocene on population isolation and differentiation (Haubrich & Schmitt 2007, Todisco et al. 2012, Albre et al. 2008, Lenevea et al. 2009, Vila et al. 2010). A second lineage split occurred approximately 0.5–0.6 Ma, thus approximately during the Mindel glacial period (0.6 Ma), in which populations of Central Spain became isolated from others inhabiting the Cantabrian Mountains and the Pyrenees, thus resulting in *C. arragonensis*, a taxon which shows a

2.9 % mean genetic divergence with regard to the *C. simplonica*-*C. cantabrica* group.

The most recent split possibly corresponds to the separation of *C. simplonica* and *C. cantabrica* during the Mindel-Riss interglacial period (0.3 Ma). The alleles of the COI gene in the Cantabrian (n = 3) and Alps (n = 4) mountain lineages show no lineage sorting belong to different haplotype groups (e. g. Hap3 and Hap14; Fig. 6). This apparent absence of lineage sorting (yet with small sample size) and low interspecific variation may be due relatively recent radiation, although it must be studied further with more samples. To date, there are no records for these species in the Pyrenees, which have been well-surveyed for Macroheterocera during the last years, both by ourselves and other lepidopterologists; however, this situation must be corroborated by collecting samples over 2000 m altitude.

The generalized high genetic discrepancy between Iberian lineages and those from the remaining of Europe suggests that Pyrenees represented the main barrier to northward post-glacial expansions. Consequently, Iberian Peninsula retains many endemic lineages evolved by isolation and successively trapped by the Cantabrian and Pyrenees chains and other mountainous systems (Hewitt 1999, 2000).

However, an increasing number of studies indicate that many endemic taxa inhabiting refugial regions are of Pleistocene origin and formed by allopatric fragmentation. In some cases, they are described as distinct species and, in other cases, these taxa are considered to be subspecies or lineages within species. Comparative phylogeographic studies in the Iberian Peninsula have revealed a pattern of divergence in multiple microrefugia, that is largely concordant across taxa and the locations, of which correspond to previously recognized regions of high endemism (for reviews, see Gómez & Lunt 2007). This pattern has become known as 'refugia within refugia'.

The Iberian Peninsula was one of the most important Pleistocene glacial refugia in the European subcontinent (Hewitt 1999, 2001). The high level of endemism in both Iberian plants and animals (Gómez-Campo et al. 1984, Moreno-Sainz et al. 1998; García-Barros et al. 2002) suggests long-term survival, differentiation and speciation, and also a species repository for the northern latitudes of Europe after glaciation events. Iberian Peninsula is geographically isolated on the westernmost point of Europe, and several character favoured survival throughout the Pleistocene according to Gómez & Lunt (2007). First, high physiographic complexity of the Iberian Peninsula, with several large mountain ranges oriented east-west, offers the highest microclimatic scope, allowing survival of populations by altitudinal shifts as general climate changed (Hewitt 1996). Second, Iberian Peninsula enjoyed a wide range of climates, as desert, Mediterranean, Atlantic and Alpine, due to influence of North Atlantic ocean and Mediterranean sea. Besides the fragmented nature of suitable habitats favours the occurrence of multiple glacial refugia, isolated from one another by the harsh climate of the high central Iberian plateau.

The present occurrence of *C. maculosa* in the Iberian Peninsula, showing a sympatric distribution with both *C. arragonensis* and *C. cantabrica*, is thought to be the result of a range expansion of *C. maculosa* since the last glaciation events, thanks to the high ecological plasticity of this species and the finding of many suitable habitats in Iberian Peninsula.

Acknowledgements

We are very grateful to the staff at the Canadian Centre for DNA Barcoding for sequence analysis. Paul D. N. Hebert and many other colleagues of the Barcode of Life project (Biodiversity Institute of Ontario, Guelph, Canada) contributed to the success of this study. The data management and analysis system BOLD was provided by Sujeevan Ratnasingham (BIO, Guelph). We are particularly grateful to Jeremy deWaard, Rodolphe Rougerie and Don Lafontaine for granting access to their projects on BOLD; to Peter Huemer from Tiroler Landesmuseen in Innsbruck (Austria) for loaning specimens and sequences of *C. simplonica* and valuable comments; to Félix González Estébanez who provided *C. maculosa* specimens and to Pablo Valero for the photographic technique. Thanks are also due to Paul Hebert, José Serrano, Carlos Ruiz, Alejandro López and Carmelo Andújar for their comments, suggestions and technical support. John Girdley and Claire Ward also helped with comments and linguistic advice. Special thanks are given to P. N. Somiedo (Asturias), P. N. Serranía of Cuenca and Arán Valley for help with permission for collection and access to field sites. We also thank to various anonymous referees for their comments. We are very grateful for this collegial and kind support. This study has been supported by the project on insect barcoding CGL2009-10906 of the Spanish Ministry of Research and Science. DNA sequencing was supported by Genome Canada (Ontario Genomics Institute) in the framework of the iBOL program, WP 1.9. This work was financed by the Fundación Séneca (project reference 19908/GERM/15) of the Murcia Regional Government.

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