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Cantharidin is conserved across phylogeographic lineages and present in both morphs of Iberian *Berberomeloe* blister beetles (Coleoptera, Meloidae)

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Intra-specific coloration polymorphism coupled with an ancient process of lineage differentiation in *Berberomeloe majalis* (Linnaeus, 1758) offers the opportunity to analyse the temporal scenario in which the correlation between toxicity and coloration might have evolved. Based on phylogenetic and phylogeographic analyses, we identified the timing for the split between red-striped and entirely black morphotypes of *B. majalis*. To evaluate whether coloration patterns and toxicity are related in this species, we quantified the concentration of cantharidin across morphotypes and phylogeographic lineages. Phylogenetic analyses based on *cox1* mitochondrial DNA sequences recovered three major clades where both morphotypes were intermingled, indicating a multiple homoplastic origin for the entirely black coloration. Our analyses showed that cantharidin content did not differ between morphs of *B. majalis*; however, it significantly increased in haemolymph in females kept isolated from males, which reveals the females' ability either to concentrate cantharidin towards haemolymph or to synthesize cantharidin themselves. Lack of monophyly and absence of genetic isolation in both morphotypes favour the hypothesis of a recent homoplastic phenomenon to explain the loss of the striped pattern. Our phylogenetic and phylogeographic analyses show that changes in coloration are recent, suggesting that the ancient pressures that fixed and maintained red-striped colorations are no longer acting today on *B. majalis*. The absence of change in cantharidin content (i.e. entirely black and red-striped specimens are equally poisonous) suggests that the evolution of colour polymorphisms in *B. majalis* is probably decoupled from toxicity.

ADDITIONAL KEYWORDS: meloids – phylogeny – polymorphism – toxicity.

INTRODUCTION

Predation by visual hunters is considered one of the most important selection pressures involved in the evolution of colour patterns of animals (Gamberale-Stille & Tullberg, 1999; Summers & Clough, 2001; Lindstedt *et al.*, 2011). Conspicuous warning colorations are frequently associated with unpalatability or toxicity (Lindstedt *et al.*, 2011), and consequently detectability, memorability, and ease of identification are advantageous cues upon which selection may act (Roper, 1990; Aronsson & Gamberale-Stille, 2008). Although predation might favour intermediate signals (i.e. allowing for signal variation, see Rowe, Lindstrom & Lyytinen, 2004; Darst, Cummings & Cannatella, 2006), toxicity and coloration patterns remain invariant as long as predation pressure is active. However, predation risk may vary significantly through time, likely changing the benefits of warning patterns (Mappes, Marples & Endler, 2005). If selective pressures are reduced or eliminated by a change in the predator community, the system would be free to change, either retaining the toxicity and conspicuous coloration, returning to the ancestral coloration and even losing toxicity, or allowing for colour polymorphisms with toxic and/or non-toxic specimens (Blomberg & Garland, 2002).

Colour polymorphisms in toxic or unpalatable species can also be maintained by alternating selective

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pressures such as changes in substrate which might modify visibility (Endler, 1978; Blanco & Bertellotti, 2002; Bond & Kamil, 2006), or by sexual selection (Eisner & Meinwald, 1995; Ayasse, Paxton & Tengö, 2001). Colour polymorphisms might then be associated with varying degrees of toxicity or unpalatability as a consequence of tandem evolution (Summers & Clough, 2001; Cooper, 2008) or concurrent reciprocal selection (Weldon, 2013; Weldon & Burghardt, 2015). Therefore, the consequences of relaxing selective pressures on warning coloration indicating toxicity would be related to the temporal scenario in which warning colorations were free to change (Harvey *et al.*, 1982; Gamberale-Stille & Guilford, 2004; Skelhorn & Rowe, 2010). Empirical evidence in support of this reasonable theoretical framework is scarce. The analyses of alternative temporal frameworks within a phylogenetic context to detect changes in selection pressures on warning coloration could offer new insights into this issue (Brower, 1996; Summers & Clough, 2001; Beltrán *et al.*, 2002; Mappes *et al.*, 2005). Here, we study colour polymorphisms in toxic blister beetles (Coleoptera: Meloidae) to contribute to such new insights.

Blister beetles of the family Meloidae are capable of synthesizing cantharidin, which is a monoterpene anhydride compound highly toxic to vertebrates and invertebrates (Eisner *et al.*, 1990; Dettner, 1997; Barr *et al.*, 1998). A first obvious function of cantharidin could be to deter predators. The efficiency of cantharidin as a deterrent for predators (Carrel & Eisner, 1974) and its poisoning effects on livestock have been widely reported (Bahme, 1968; Ray *et al.*, 1980; Beasley *et al.*, 1983). In fact, only a few species consume blister beetles (Eisner *et al.*, 1990; Bartram & Boland, 2001; Bravo et al., 2014, 2016). A second function of cantharidin could also be to help blister beetles avoid internal parasites, due to its fungicidal, nematocidal, and bactericidal properties (Carrel & Eisner, 1974; Bravo *et al.*, 2014). Among blister beetles, males usually have more cantharidin than females (Capinera, Gardner & Stermitz, 1985; Blodgett, Carrel & Higgins, 1991; Carrel *et al.*, 1993; Carrel, 1999; Nikbakhtzadeh & Tirgari, 2002; Mebs *et al.*, 2009; Nikbakhtzadeh *et al.*, 2012), and apparently only males are able to synthesize this toxin (Sierra, Woggon & Schmid, 1976; Holz *et al.*, 1994). Therefore, cantharidin content could also be under sexual selection pressures. Female blister beetles would acquire it from males during copulation. Nevertheless, there are some research reports where no final conclusions about the biosynthesis pathway of cantharidin are provided (Yin & Jin, 2010). Thus, new studies focused on sexual differences of cantharidin biosynthesis in blister beetles are necessary. In this study, we test sexual differences in synthesis of cantharidin in blister beetles. Using a multidisciplinary approach, we also infer the historic process which gave rise to colour polymorphism in a species of blister beetle, and analyse the relationship between coloration pattern and toxicity.

Although a correlation between conspicuous coloration and its function as a warning signal to predators has not been tested in Meloidae, many species of blister beetles display bright and conspicuous colorations and occasionally may adopt a frightening attitude (Varley, 1939; Pinto, 1975). One extreme case is represented by giant beetles of the genus *Berberomeloe* Linnaeus in the Iberian Peninsula. The coloration in the widespread species *Berberomeloe majalis* (Linnaeus, 1758) is characterized by the presence of bright blood-red or orange transverse stripes across a solid black swollen abdomen (the length of this beetle may reach up to 9 cm, Fig. 1A, García-París, 1998). A similar conspicuous coloration, including transverse

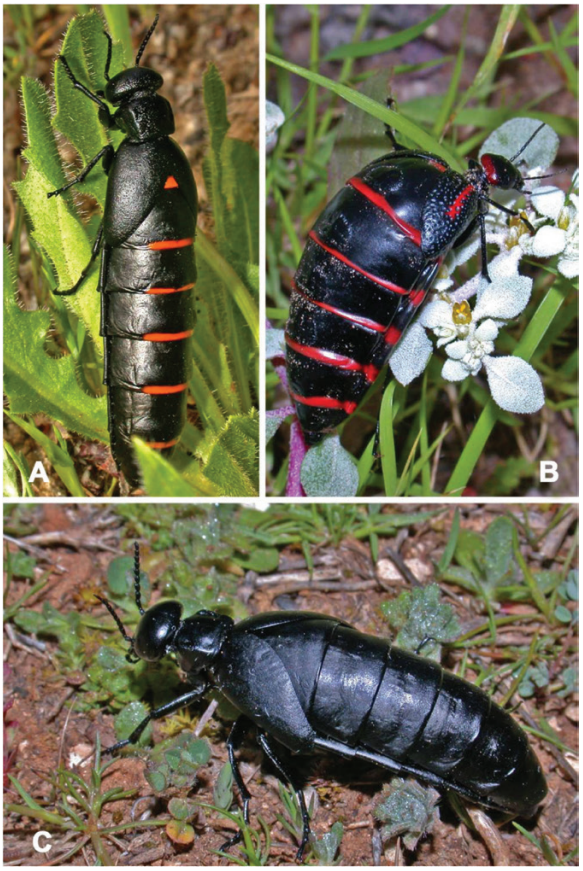

Figure 1. (A) Red-striped morph of *Berberomeloe majalis*, characterized by the presence of bright blood-red or orange transversal stripes across a solid black swollen abdomen; (B) genus *Megetra*, whose coloration has evolved independently of genus *Berberomeloe*; and (C) black morphotype of *B. majalis*, characterized by the entirely black body without any red stripes.

red stripes on a swollen abdomen, has evolved independently in the distantly related North American genus *Megetra* LeConte (Fig. 1B), suggesting that this coloration pattern is subjected to high selective pressure. The three species of the genus *Megetra* show the characteristic striped coloration (Fig. 1B) with no intra-specific variability for this trait (Selander, 1965; Pinto, 1984). However, *B. majalis* varies extensively in colour. Populations of entirely black specimens without any red markings (Fig. 1C) are found scattered over much of the distribution range of *B. majalis*. These populations are often found close to populations consisting of red-striped specimens, but both morphs seem to be spatially segregated and no mixed series have been found in the field (García-París, 1998; personal observation).

A recent hypothesis on the geographic distribution of genetic variability of *B. majalis* in the Iberian Peninsula (Percino-Daniel, Buckley & García-París, 2013) revealed the existence of differentiated mitochondrial lineages within the species. The presence of intra-specific coloration polymorphism coupled with an ancient process of lineage differentiation in *B. majalis* offers an excellent opportunity to analyse the correlation between toxicity and conspicuousness and the temporal scenario in which the relation might have evolved.

We use two approaches to study this issue. First, we evaluate the temporal frame in which conspicuous coloration is lost, by phylogenetic and phylogeographic analyses of Iberian populations of *B. majalis*, with emphasis in (1) identifying the relationship between red-striped and entirely black populations and (2) determining the timing for the split between the morphotypes. Second, we quantify the concentration of cantharidin across sexes, morphotypes, and phylogeographic lineages, with the specific objective of (3) evaluating whether toxicity and conspicuous coloration are related in the genetically diverse *B. majalis*.

METHODS

Sampling

Sampling of *B. majalis* for phylogeographic and phylogenetic analyses was designed to cover a large portion of the species' known distribution range in the Iberian Peninsula (n = 77) and Morocco (n = 5; Table 1; Fig. 2A), in order to capture the mitochondrial diversity of the species in the area. Sampling included specimens of red-striped and entirely black populations (Fig. 2A). Specimens for cantharidin analyses were collected in April–May 2012 in Central Spain, and also included both coloration patterns (29 red-striped and 57 entirely black) (Fig. 2B).

Mitochondrial DNA analyses

Tissue was extracted from the coxae or femora of 63 specimens preserved in ethanol (96° to absolute) stored at –20 °C. Total genomic DNA was extracted using protocols described by Alcobendas *et al.* (2008). Polymerase chain reaction (PCR) was used to amplify 657 base pairs (bp) of the mitochondrial Cytochrome Oxidase I gene (*cox1*), using the primers LCO1490 (Folmer *et al.*, 1994) and COI-H (Machordom *et al.*, 2003). PCR reactions were performed as explained in Percino-Daniel *et al.* (2013). Sequencing was as indicated in Martínez-Solano et al. (2006).

Sequences were compiled using Sequencer v.4.9 to assemble and to edit the sequence contigs. The *cox1* sequences were aligned in Mesquite v.3.04 (Maddison & Maddison, 2015) to correct the final alignments. The data set consisted of 67 specimens from *B. majalis* and 15 additional specimens obtained by Percino-Daniel *et al.* (2013) (GenBank accession numbers: KC853086–KC853103). For the phylogenetic analyses, we included the other known species of the genus, *Berberomeloe insignis* (Charpentier, 1818), as a related outgroup.

Cantharidin content analyses

We analyzed cantharidin content in haemolymph and in the dried body. Individuals were subjected to two extractions of haemolymph to test whether cantharidin content increased or decreased in the second extraction compared to the first. In this way we aimed to explore differences between sexes in the capability of cantharidin synthesis. We also measured the cantharidin content in the dry body to analyse the relationship between cantharidin content and phylogeny.

To analyse the sexual differences in cantharidin synthesis, cantharidin content was examined in haemolymph (n = 96 haemolymphs of 72 individuals, Table S1). Defensive behaviour of *B. majalis* consists of thanatosis and evident autohaemorrhea (Bologna, 1989, 1991), which may take place even without direct contact with the suspected aggressor. Seventy-two specimens of *B. majalis* were kept indoors in plastic boxes (15 × 10 × 5 cm, W × L × H, one adult per box). Cages were cleaned and fresh leaves of *Vicia sativa* and flowers of Asteraceae were provided on a daily basis. Beetles were sexed by observing their external sexually dimorphic characters (last visible abdominal segment). Individuals were weighed every other day to control for any dehydration, and beetles which lost more than 10% of their weight were discarded from all analyses (n = 9). We took advantage of the *B. majalis* bleeding reflex to obtain 0.0001–0.4 mL of haemolymph. Haemolymph droplets expelled through body segment joints, either along legs or around neck, were collected in 2 mL tubes while gently handling specimens using

Table 1. Localities, number and species information, and GenBank accession numbers for the sequences and specimens used in the molecular study.

Taxon	Locality (country: province: town)	Voucher field number	GenBank accession number
B. majalis	Spain: Albacete: El Bonillo	MAB140	KC853090
B. majalis	Spain: Albacete: El Bonillo	MAB169	KC853089
B. majalis	Spain: Almería: El Puntal, Uleila	MAB185	KC853091
B. majalis	Spain: Almería: El Puntal, Uleila	MAB197	KC853092
B. majalis	Spain: Burgos: Monasterio de Rodilla	MAB116	KC853094
B. majalis	Spain: Burgos: Monasterio de Rodilla	MAB123	KC853093
B. majalis	Spain: Cáceres: 3 km S Navas del Madroño	MAB174	KC853095
B. majalis	Spain: Cáceres: 3 km S Navas del Madroño	MAB175	KC853096
B. majalis	Spain: Cádiz: 3 km S Alcalá de los Gazules	MAB107	KC853097
B. majalis	Spain: Ciudad Real: 2 km N Carrión de Calatrava	MAB103	KX495702
B. majalis	Spain: Ciudad Real: 2 km N Carrión de Calatrava	MAB105	KX495703
B. majalis	Spain: Ciudad Real: 6 km S Malagón	MAB156	KX495729
B. majalis	Spain: Ciudad Real: Argamasilla de Calatrava	MAB138	KX495719
B. majalis	Spain: Ciudad Real: Piedrabuena	MAB500	KC853098
B. majalis	Spain: Ciudad Real: Piedrabuena	MAB501	KC853099
B. majalis	Spain: Cuenca: Fuentes	MAB110	KX495706
B. majalis	Spain: Cuenca: Villar de Cañas	MAB163	KX495732
B. majalis	Spain: Cuenca: Villarejo de Fuentes	MAB139	KX495720
B. majalis	Spain: Granada: Santa Cruz del Comercio	MAB115	KX495708
B. majalis	Spain: Guadalajara: 5 km ENE Illana	MAB168	KX495735
B. majalis	Spain: Guadalajara: 5 km NE Illana	MAB126	KX495712
B. majalis	Spain: Guadalajara: 5 km O Cogolludo	MAB142	KX495721
B. majalis	Spain: Guadalajara: 5 km O Cogolludo	MAB147	KX495724
B. majalis	Spain: Guadalajara: Uceda	MAB109	KX495705
B. majalis	Spain: Guadalajara: Uceda	MAB118	KX495709
B. majalis	Spain: Guadalajara: Uceda	MAB134	KX495717
B. majalis	Spain: Guadalajara: Uceda	MAB135	KX495718
B. majalis	Spain: Guadalajara: Uceda	MAB150	KX495726
B. majalis	Spain: Guadalajara: Uceda	MAB167	KX495734
B. majalis	Morocco: Oumallal: Oum er Rbia (Middle Atlas)	MAB176	KX495739
B. majalis	Morocco: Oumallal: Oum er Rbia (Middle Atlas)	MAB177	KX495740
B. majalis	Morocco: Oumallal: Oum er Rbia (Middle Atlas)	MAB178	KX495741
B. majalis	Morocco: Oumallal: Oum er Rbia (Middle Atlas)	MAB179	KX495742
B. majalis	Morocco: Oumallal: Oum er Rbia (Middle Atlas)	MAB180	KX495743
B. majalis	Spain: Jaen: 10 km N Ubeda	MAB161	KX495731
B. majalis	Spain: Jaén: 10 km N Ubeda	MAB119	KX495710
B. majalis	Spain: Jaén: 10 km N Ubeda	MAB127	KX495713
B. majalis	Spain: Jaén: Orcera	MAB153	KX495727
B. majalis	Spain: Madrid: 1 km NE Meco	MAB203	KX495753
B. majalis	Spain: Madrid: 1 km SE Villanueva de la Torre	MAB202	KX495752
B. majalis	Spain: Madrid: 3 km E Villaconejos	MAB157	KX495730
B. majalis	Spain: Madrid: 4 km S Colmenar de Oreja	MAB146	KC853100
B. majalis	Spain: Madrid: 4 km S Colmenar de Oreja	MAB159	KC853101
B. majalis	Spain: Madrid: 5 km NE Molino de la Aldehuela	MAB133	KX495716
B. majalis	Spain: Madrid: 5 km NE Molino de la Aldehuela	MAB172	KX495738
B. majalis	Spain: Madrid: 5 km S Villaconejos	MAB164	KX495733
B. majalis	Spain: Madrid: Alcalá de Henares	MAB186	KX495745
B. majalis	Spain: Madrid: Alcalá de Henares	MAB198	KX495751
B. majalis	Spain: Madrid: Brea de Tajo	MAB191	KX495750
B. majalis	Spain: Madrid: Canencia	MAB187	KX495746
B. majalis	Spain: Madrid: Colmenar de Oreja ★	Ber85	KX495697

Table 1. *Continued*

Taxon	Locality (country: province: town)	Voucher field number	GenBank accession number
B. majalis	Spain: Madrid: Colmenar de Oreja ★	Ber90	KX495698
B. majalis	Spain: Madrid: Colmenar de Oreja ★	Ber91	KX495699
B. majalis	Spain: Madrid: Colmenar de Oreja ★	Ber92	KX495700
B. majalis	Spain: Madrid: Daganzo de Arriba ★	Ber136	KX495696
B. majalis	Spain: Madrid: Daganzo de Arriba ★	Ber113	KX495689
B. majalis	Spain: Madrid: Montejo de la Sierra	MAB183	KX495744
B. majalis	Spain: Madrid: Montejo de la Sierra	MAB189	KX495748
B. majalis	Spain: Madrid: Montejo de la Sierra	MAB190	KX495749
B. majalis	Spain: Madrid: Rivas de Jarama	MAB100	KX495701
B. majalis	Spain: Madrid: Talamanca ★	Ber131	KX495693
B. majalis	Spain: Madrid: Talamanca ★	Ber133	KX495694
B. majalis	Spain: Madrid: Talamanca ★	Ber135	KX495695
B. majalis	Spain: Madrid: Valdepiélagos ★	Ber117	KX495690
B. majalis	Spain: Madrid: Valdepiélagos ★	Ber118	KX495691
B. majalis	Spain: Madrid: Valdepiélagos ★	Ber127	KX495692
B. majalis	Spain: Segovia: Navafría	MAB143	KX495722
B. majalis	Spain: Segovia: Prádena	MAB129	KX495715
B. majalis	Spain: Toledo: 2 km NE El Emperador	MAB120	KX495711
B. majalis	Spain: Toledo: 2 km NE El Emperador	MAB122	KC853102
B. majalis	Spain: Toledo: 2 km NE El Emperador	MAB173	KC853103
B. majalis	Spain: Toledo: 2 km O Urda	MAB170	KX495736
B. majalis	Spain: Toledo: 2 km O Urda	MAB171	KX495737
B. majalis	Spain: Toledo: 6 km N El Emperador	MAB148	KX495725
B. majalis	Spain: Toledo: Zarza de Tajo ★	Ber101	KX495686
B. majalis	Spain: Toledo: Zarza de Tajo ★	Ber103	KX495687
B. majalis	Spain: Toledo: Zarza de Tajo ★	Ber104	KX495688
B. majalis	Spain: Valencia: Camporrobles	MAB112	KX495707
B. majalis	Spain: Valladolid: San Miguel del Arroyo	MAB106	KX495704
B. majalis	Spain: Valladolid: San Miguel del Arroyo	MAB128	KX495714
B. majalis	Spain: Valladolid: San Miguel del Arroyo	MAB144	KX495723
B. majalis	Spain: Valladolid: San Miguel del Arroyo	MAB154	KX495728

Note: All specimens were collected by the authors from 2001 to 2012. Stars indicate populations and specimens selected for cantharidin content analyses.

nitrile gloves. Haemolymph extraction was performed twice. The first extraction was after 2–4 days in captivity (n = 72) and the second extraction (n = 24) was after 8–10 days (Table S1). A haemolymph sample (0.4–10.2 g) was diluted with hexane (0.5 mL) and homogenized with dichloromethane (0.5 mL) in glass tubes with Teflon caps. Then, the samples were sonicated (Elma Transonic 700) for 3 min.

To analyse the relationships between cantharidin content and phylogeny, 58 specimens were used to estimate cantharidin content per gram of dry weight. The sample consisted of individuals subjected to non-haemolymph extraction (n = 14), one haemolymph extraction (n = 28), and two haemolymph extractions (n = 16, Table S1). Individuals were frozen and then lyophilized. Body dry weight was determined after 48 h of freeze drying in a VIRTIS 25 LE-53 GENESIS SQ. Lyophilized individuals (0.057–0.725 g) were transferred to a Teflon digestion reactor with hydrochloric acid (3 mL) and were heated to 120 °C for 3 h. Once the reactor was cooled its content was added to chloroform (5 mL), followed by vortex mixing for 15 s. Once phases were separated, the lower layer of chloroform was collected with a Pasteur pipette. The procedure was repeated twice for each individual to ensure complete extraction.

Samples of haemolymph and lyophilized individuals were placed in 2 mL vials for analysis by gas chromatography coupled to mass spectrometry (GC–MS). The GC–MS system consisted of a Thermo Finnigan Trace GC 2000 coupled with a Trace MS mass selective detector. The chromatographic conditions were controlled using the Xcalibur software v. 1.2 (Thermo Finnigan, San Jose, CA). The GC column was a SLB-5 ms (30 m × 0.32 mm, 0.25 µm, Supelco Analytical, Bellefonte, PA). The flow rate of helium was 0.8 mL/

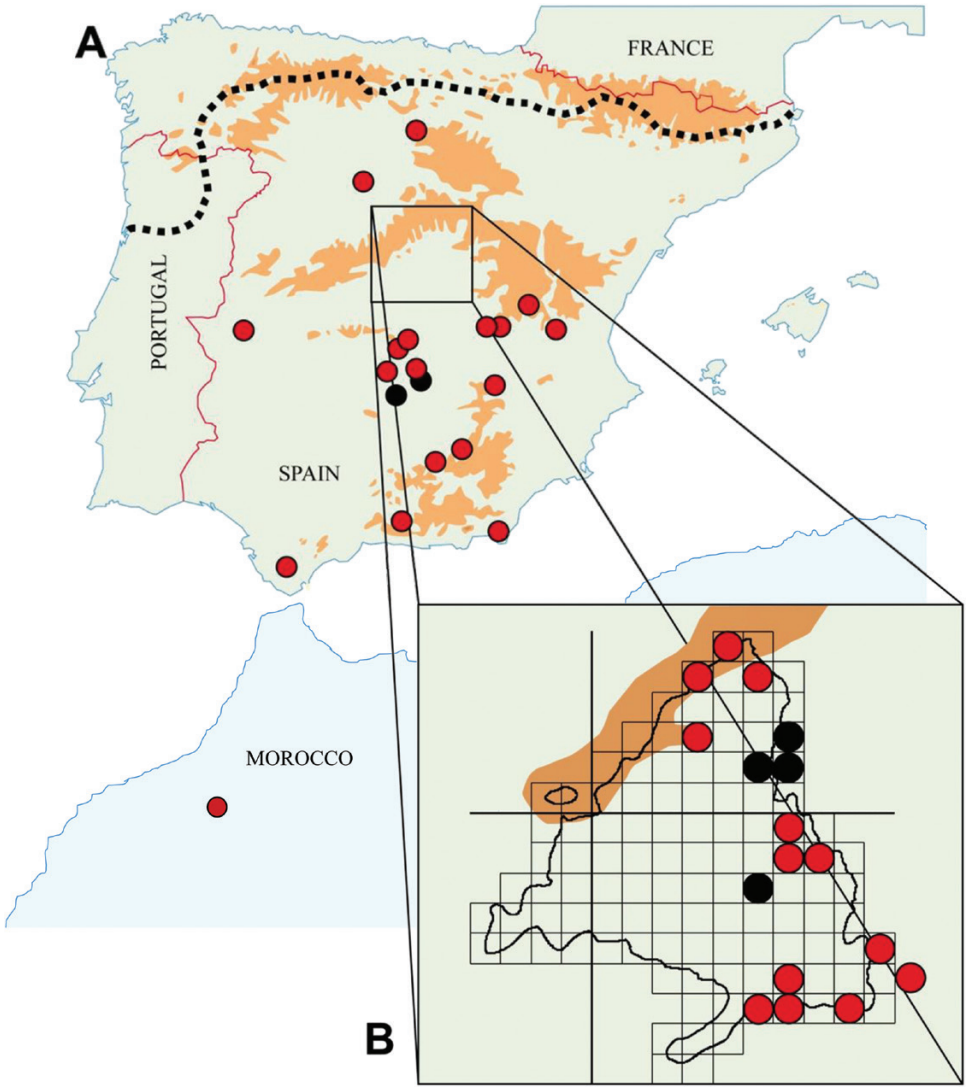

Figure 2. Study area showing the sampling localities of red-striped (red dots) and entirely black populations (black dots) of *Berberomeloe majalis*. (A) Specimens for phylogeographic and phylogenetic analyses. (B) Specimens for cantharidin content analyses. Dotted line represents the northern limit of the species distribution.

min. The injection volume was 1 µL in splitless mode for 2 min. Injector conditions were 250 °C in constant flow mode. The column oven had an initial temperature of 50 °C for 2 min. The subsequent temperature was programmed at a heating rate of 10 °C/min to 310 °C. The final temperature was held isothermally for 5 min. Total run time was 30 min. Cantharidin detection was performed by selected ion monitoring, registering m/e 128 which is the majority ion of cantharidin's mass spectra. Cantharidin identification was performed by comparison with mass spectra available in NIST MS search 2.0 library. Confirmation and quantification was achieved with the retention time and calibration curves (range: 0.015–48 µg/mL, slope: 476.401, r^2 = 0.999) obtained from the injection of cantharidin standard purchased from Sigma–Aldrich (St. Louis, MO).

Statistical analyses

Phylogenetic analyses were carried out under Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks. ML analyses were estimated with RAxML (Stamatakis, Hoover & Rougemont, 2008) using 1000 bootstrap replicates to assess node support. BI analyses were performed using MrBayes 3.2.2 (Ronquist *et al.*, 2012). We ran different analyses with the partition scheme suggested in PartitionFinder (Lanfear et al., 2012, 2014) with partitions cox1_PoS1 1–655/3, cox1_PoS2 12–656/3, and cox1_PoS3 3–657/3).

We explored the substitution model space with the option lset nst=mixed rates=invgamma. To estimate the posterior probabilities of all the parameters, we ran four Markov Chains Monte Carlo (MCMC) for 100×10^6 generations sampling every 10 000 generations, in two independent runs (for further details see Percino-Daniel *et al.*, 2013). To generate the final phylogenetic hypothesis, we discarded 25% of the obtained trees as burn-in, and generated an annotated 50% Majority Rule consensus tree in MrBayes.

By using a Bayesian-coalescence approach implemented in BEAST 1.8.2 (Drummond *et al.*, 2012), we estimated divergence times between lineages within *B. majalis*. Given the absence of fossil records for the species, we calibrated the molecular clock using the substitution rates for *cox1* estimated in Papadopoulou, Anastasiou & Vogler (2010). We ran the analyses for 100×10^6 sampling every 10 000 generations, under a lognormal relaxed molecular clock, a HKY + I + G substitution model calculated in JModelTest v.2 (Darriba *et al.*, 2012) and under a Birth-Death Incomplete Sampling speciation tree prior (Stadler, 2009). To encompass the pairwise differentiation range of 2.3–3.54% (Papadopoulou *et al.*, 2010), we specified as a prior the substitution rates for *cox1*: lognormal distribution in real space with initial value = 0.015 Log(Mean) = 0.015 Log(SD) = 0.2. We examined the trace plots and Effective Sample Size values in Tracer v.1.5. to evaluate the convergence of the MCMC. The results were summarized and annotated in a maximum clade credibility tree generated in TreeAnnotator (Drummond *et al.*, 2012), previously removing the first 25% as burn-in. Haplotype networks for *cox1* marker to Iberian Peninsula populations were constructed with HaploViewer, which converts a BI tree into a haplotype genealogy (available at http://www.cibiv.at/~%20greg/haploviewer).

Ancestral character states were reconstructed in RASP (Yu *et al.*, 2015), with the aim of analyzing the ancestral state of red-striped coloration within *B. majalis*. We performed BBM (Bayesian Binary MCMC) analyses implemented in RASP v.3.2 (Ali *et al.*, 2012; Yu *et al.*, 2015). As an input we introduced the consensus tree from BEAST with branch lengths in units of time and we assigned A for red-striped specimens and B for entirely black specimens. We ran ten MCMC chains simultaneously for 5×10^4 generations, sampling every 100 steps and discarding the first 100 samples as burn-in.

We identified variables that significantly affected the cantharidin content in haemolymph using general linear mixed-effects models (LMM, McCullagh & Nelder, 1989). In the model we included cantharidin content in haemolymph (μg/g) as a dependent variable, and sex (male, female), morphotype (red-striped and entirely black) locality, and extraction (a single extraction, two sequential extractions) as independent variables. The individual was fitted as a random factor in the models. Following Zuur, Ieno & Smith (2007), we compared beyond optimal models (the most complex models, with all factors and their plausible interactions) with different random error structure using the restricted ML estimation procedure. Once the random structure was defined, we determined the fixed effects structure using ML. Model averaging was performed because more than one model had ΔAICc < 2 (Table S2). The variance explained was estimated by calculating the marginal R^2 (R^2_m: variance explained by the fixed factors) and conditional R^2 (R^2_c: variance explained by both the fixed and random factors, Nakagawa & Schielzeth, 2013).

Differences in cantharidin content of body dry weight (mg/g d. w.) was analyzed with a general linear model (GLM); the variables sex (male, female), morphotype (with red stripes, entirely black), and number of extractions (0, 1 or 2 extractions) were defined as independent variables. The dependent variable was log-transformed to better conform to assumptions of normality and homogeneity of variance. Effect of locality on cantharidin content of dry weight body was analyzed with a non-parametric Kruskal–Wallis test. The sample was split by sex and morphotype, so differences in cantharidin content in body dry weight between localities were analyzed within same sex and same morphotype.

We also performed Generalized Estimating Equations (GEE) to analyse the statistical relationship between cantharidin content and morphotype (red-striped and entirely black) taking into account their phylogenetic relationship (Paradis & Claude, 2002). We performed these analyses using the function 'compar.gee' in R package 'ape' (Paradis, Claude & Strimmer, 2004). GEE models are suitable for data with discrete response variables through the specification of binomial error structure. The response variable was morphotype (entirely black, red-striped) and the mean cantharidin content in haemolymph per population (n = 6) was defined as independent variable. A simplified distance-based phylogenetic analysis was calculated using Neighbor Joining algorithms with PAUP v 4.0a146 (Swofford, 2002). The resulting topology was edited and exported to Newick format in FigTree v.1.4.0.

We verified the normal distribution of LMM and GLM model residuals visually by checking normal probability plots and with Shapiro–Wilk test. We verified the homogeneity of variances and goodness of fit by plotting residuals vs. fitted values. These analyses were carried out in *R* version 2.15.0. Simple statistics are reported in the main text as mean ± SD.

RESULTS

CANTHARIDIN CONTENT RESULTS

Model-averaging of cantharidin content in haemolymph included sex and number of extractions as significant

factors (Table 2). Cantharidin content in haemolymph did not depend on morphotype and locality ($P > 0.05$; Table 2). Cantharidin content in haemolymph was significantly higher in males (80.9 ± 106.5 µg/g) than in females (20.0 ± 41.5 µg/g; Table 2 and Fig. 3). Cantharidin content in haemolymph was also significantly higher in the second than in the first extraction in both sexes (first extraction: 33.76 ± 53.11 µg/g; second extraction: 135.36 + 143.49 µg/g; Fig. 3). Finally, the content of cantharidin in dried bodies was also significantly higher in males (males: 64.22 ± 51.28 mg/g d. w; females: 9.10 ± 12.64 mg/g d. w; $F_{1,48} = 49.9$, $P < 0.001$), with no significant effects on morphotype ($F_{1,48} = 0.681$, $P = 0.413$) and number of extractions ($F_{2,48} = 0.863$, $P = 0.428$). Locality had no significant effect on cantharidin content in dry weight body either in entirely black populations (females: $\chi^2 = 0.10$, $P = 0.752$, males: $\chi^2 = 0.61$, $P = 0.736$) or in red-striped populations (females: $\chi^2 = 0.72$, $P = 0.157$, males: $\chi^2 = 0.41$, $P = 0.522$). The GEE model showed that cantharidin content in dry weight body did not depend on morphotype ($t = -0.24$, $P = 0.848$).

Phylogenetic results

Sequences of 657 bp of the Cytochrome Oxidase I mitochondrial gene (*cox1*) were obtained for 63 specimens of Iberian *B. majalis*. The phylogenetic analysis set under ML (bootstrap support, BS) and BI (posterior probabilities, PP) methods yielded a topology that reflects three major clades (Fig. 4). A poorly supported Clade A included samples from the red-striped populations of various localities in central Spain, together with entirely black specimens from Ciudad Real. Clade B included only red-striped specimens and showed two subclades, the first from western and southern Spain (PP = 1; BS = 86), and the second from Almería in southern Spain and Oumallal in the Middle Atlas. Clade C was the only strongly supported monophyletic clade (PP = 1; BS = 99), split into two subclades. The first subclade (PP = 1; BS = 91) grouped entirely black specimens from Guadalajara (Uceda, Valdepiélagos) and Madrid (Talamanca), together with red-striped individuals from Madrid (Meco, Daganzo de Arriba), all of them in central Spain (Fig. 2, inset). The second subclade grouped mainly red-striped specimens from northern Spain (PP = 0.89; BS = 95), together with a black specimen from Madrid (Rivas de Jarama). Red-striped and entirely black morphotypes were mixed across the phylogeny within clades A and C. Groupings reflect geographic structure rather than coloration patterns (see the following text on network analyses).

The haplotype network, which includes the 49 *cox1* haplotypes obtained, shows that the mtDNA variability in *B. majalis* is well structured geographically (Fig. 5A). No clear pattern of segregation was observed among entirely black and red-striped specimens (Fig. 5B). In fact, entirely black specimens from Guadalajara (Uceda) and one red-striped specimen from Madrid (Daganzo de Arriba) shared the same haplotype (Fig. 5B). Time to the most recent common ancestors (TMRCA) for the known *Berberomeloe* species was placed about the end of the Miocene (Fig. 6).

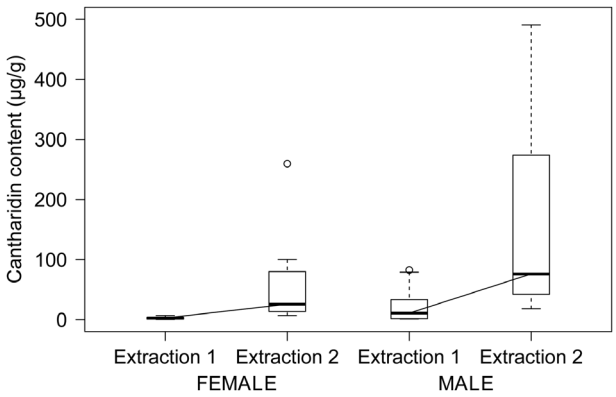

Figure 3. Sexual differences in cantharidin content in haemolymph (µg/g) of *B. majalis* individuals subjected to two haemolymph extractions.

Table 2. Model-averaging for cantharidin content in haemolymph of *Berberomeloe majalis* as a function of sex, morphotypes, locality, and number of cantharidin extraction.

Fixed effects	**Estimate ± SE**	***z*-value**	***P*-value**
Intercept	–0.03 ± 0.40	0.07	0.947
N extractions	0.99 ± 0.15	6.39	<0.001
Sex (male)	0.71 ± 0.22	3.28	0.001
Morphotype	–0.07 ± 0.61	0.11	0.912
Locality (Colmenar de Oreja)	–0.70 ± 0.38	1.84	0.066
Locality (Talamanca de Jarama)	–0.31 ± 0.17	1.73	0.083
Locality (Valdehornos)	–0.35 ± 0.39	0.88	0.379
Locality (Zarza de Tajo)	0.11 ± 0.38	0.29	0.773
Sex × *N* extractions	–0.29 ± 0.23	1.16	0.246

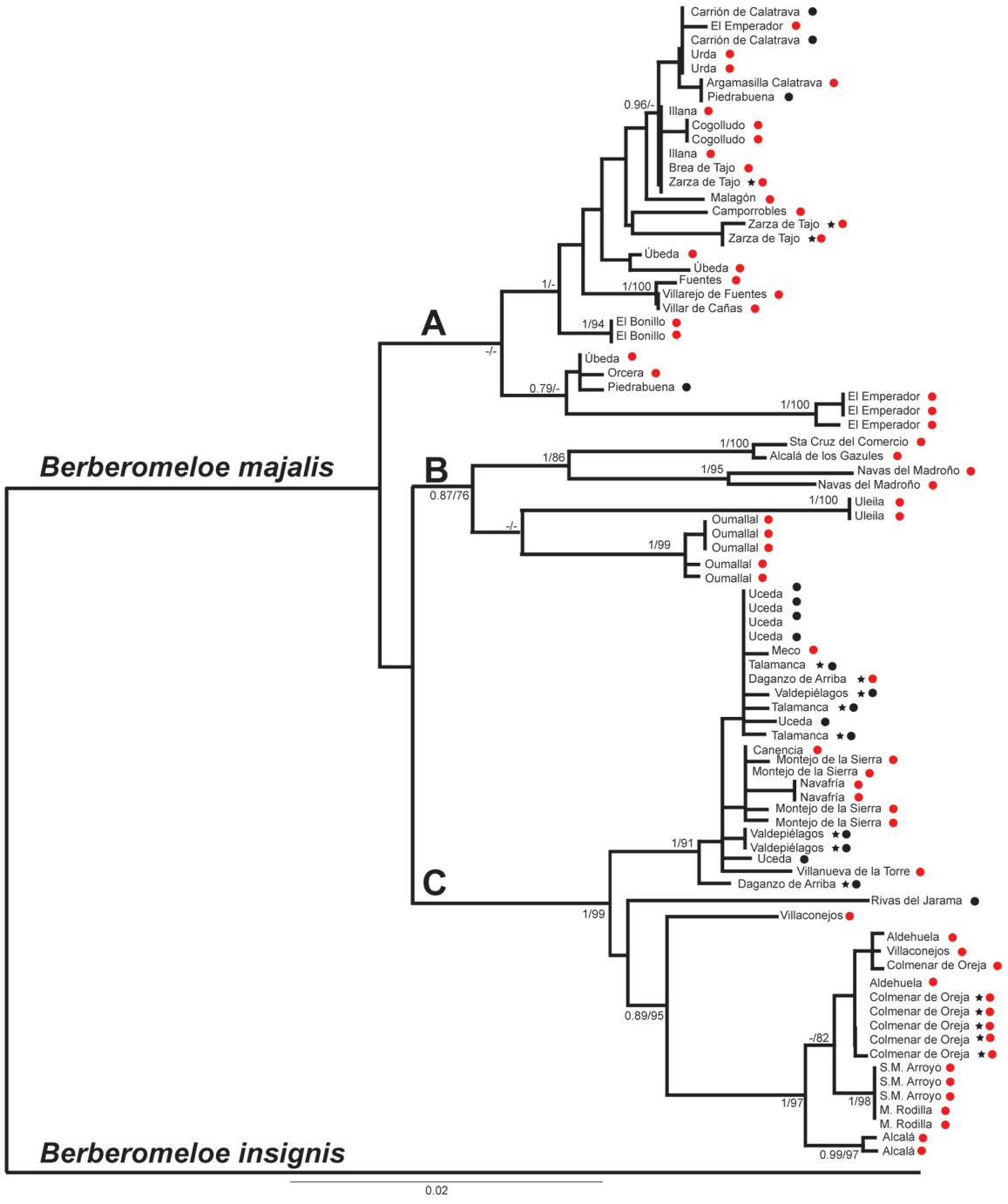

Figure 4. Phylogenetic relationships among Iberian populations of *Berberomeloe majalis*. Bayesian phylogenetic tree based on 657 bp of *cox1* mtDNA sequences. Support for each node is represented by the posterior probabilities (PP) resulting from the Bayesian Inference analysis and the bootstrap support values (BS) obtained for the Maximum Likelihood tree (PP/BS). The dots on the right side correspond to the two colour morphs: black dots represent entirely black specimens and red dots represent red-striped specimens. Stars indicate populations and specimens selected for cantharidin content analyses.

Differentiation of the main clades within *B. majalis* occurred during the Pliocene–Pleistocene transition 2.94 Mya (95% HPD: 1.31–5.15). TMRCA for entirely black specimens occurred about 1.13 Mya (95% HPD: 0.46–2.1) and the rate of diversification increased at the Upper Pleistocene about 0.8–0.5 Mya.

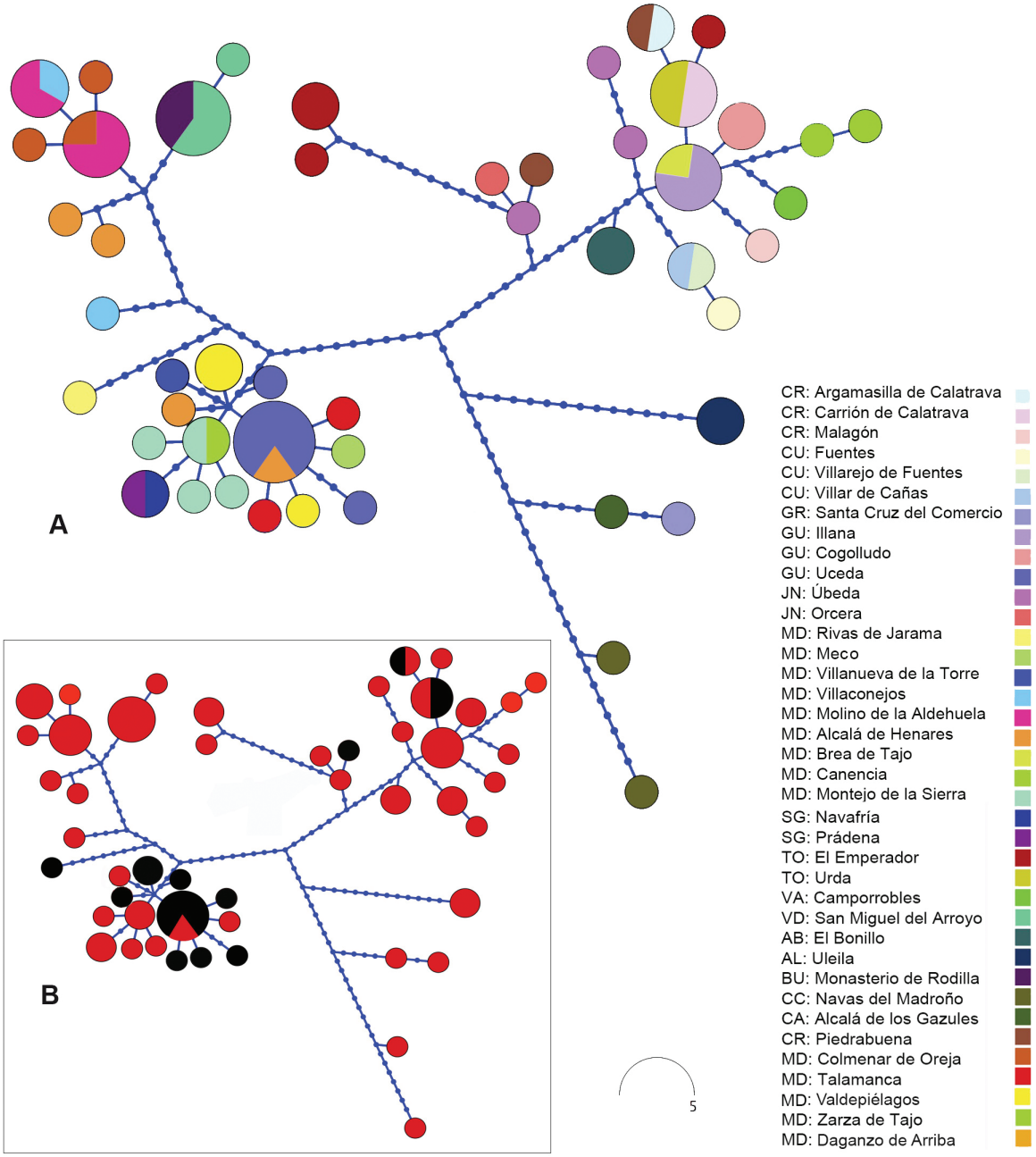

Figure 5. Mitochondrial *cox1* haplotype network of *Berberomeloe majalis* lineages within the Iberian Peninsula (49 haplotypes). The size of the circles (haplotypes) is proportional to the number of individuals presenting each haplotype. (A) The different colors assign haplotypes to the geographical location reported in Table 1. (B) Black and red lineages from the haplotype network correspond to the two colour morphs (red-striped vs. entirely black specimens).

The ancestral character reconstruction showed that red-striped coloration is ancestral for *B. majalis*. The oldest node representing the most recent common ancestor of black specimens is more recent than that representing red-striped coloration, which appears as an ancestral character in all clades (Fig. S1).

DISCUSSION

Our results revealed that the cantharidin content in *B. majalis* is influenced by sex and haemolymph extraction, but does not differ between morphotypes, nor across phylogeographic lineages. Cantharidin content was always higher in males than in females,

Figure 6. Temporal estimates (95% high probability intervals – HPD) depicted in the phylogenetic tree generated in a Bayesian-coalescence framework for *Berberomeloe majalis*. Blue bars at nodes indicate the 95% highest posterior density (HPD) intervals and units of *X*-axis reflect Mya. Asterisks correspond to posterior probabilities values over 0.95.

suggesting that the amount of this toxic compound could be under sexual selection rather than under predator deterrence pressures. On the other hand, the cantharidin content in haemolymph increased significantly in the second extraction in both sexes. Previous studies suggest that cantharidin biosynthesis occurs during early stages of ontogenetic development, and that females are unable to synthesize this toxin, obtaining it from males during copulation (Sierra *et al.*, 1976; Holz *et al.*, 1994). However, our results showed an increase in cantharidin content in haemolymph in females, which could only be explained by their ability to either concentrate cantharidin in haemolymph or synthesize cantharidin themselves. Our results support this latter possibility, that is that females are also able to synthesize cantharidin. First, the cantharidin content measured as dry weight per beetle was higher than in haemolymph, which suggests that beetles may accumulate cantharidin somewhere in their bodies, and that they are able to mobilize this toxin towards haemolymph. Second, if females received a finite amount

of cantharidin from males during copulation, the cantharidin content of females would show a clear decrease between successive haemolymph extractions. However, extraction factor did not significantly influence the cantharidin content in dry weight of beetle. Our data support the suggestions from previous authors, that is females may be able to synthesize this toxin independently of the males' nuptial transfer (Nikbakhtzadeh *et al.*, 2012), as well as the earlier proposed mechanisms of cantharidin synthesis in blister beetles (Yin & Jin, 2010).

The occurrence of red over black patterns has appeared multiple times along the phylogeny of meloid beetles (Pinto & Bologna, 1999; Bologna & Pinto, 2002), but striped patterns over exposed heavy abdominal segments are less common. Species of the genus *Megetra* in North American deserts and *B. majalis* in the western Palearctic share both heavy exposed abdomens and conspicuous red stripes over them (Fig. 1). Phylogenetic analyses of Meloidae did not show a close relationship between *Megetra* and *Berberomeloe*. In fact, the two clades in which these genera are included are not sister groups and belong to different tribes (Bologna *et al.*, 2008). Evolution of the striped pattern is thus homoplastic, probably as a consequence of convergence in a system subjected to strong selective pressures (Wu *et al.*, 2010).

Phylogenetic analyses reveal that changes in coloration are recent, and indicate that that red-striped coloration is ancestral for *B. majalis*. TMRCA for clades including black specimens dated back to the Pleistocene. Red-striped and entirely black populations of *B. majalis* occur together in three main clades, two of them separated for at least 2.6 My (over the Pliocene–Pleistocene limit). Most populations within these lineages showed the typical red-striped conspicuous coloration pattern, while populations composed of entirely black specimens are scattered all over the geographic range of the species. Red-striped patterns occur through all lineages, including those relating directly to the basal split within the species (Fig. 4). This shows that red-striped coloration is ancestral for *B. majalis*, that is it evolved in the ancestor of all current lineages within the species (Fig. S1). The lack of monophyly of entirely black populations indicates a multiple homoplastic origin for the entirely black coloration, which emerged independently on multiple occasions. Percentage of *cox1* sequence divergence across entirely black and red-striped neighbouring populations is similar to the divergence found within red-striped populations, suggesting that a possible genetic isolation among them is the result of recent demographic or isolating events. Lack of monophyly and absence of genetic isolation support the hypothesis of a recent loss of the striped pattern across the entire geographic range of the species.

Cantharidin content does not differ significantly between red-striped and entirely black populations, or across phylogeographic lineages in *B. majalis*. These results point to a decoupling of coloration and toxicity, consistent with the widespread presence of cantharidin in Meloidae, independent of the conspicuousness of their coloration. However, predators avoid unprofitable preys if they are markedly visible (Roper & Redston, 1987; Prudic, Skemp & Papaj, 2007). Entirely black coloration is frequent among large non-poisonous Mediterranean beetles that share habitat and activity with *Berberomeloe*, that is tenebrionoid beetles of the genera *Pimelia* and *Akis*, which are frequent prey of diurnal and nocturnal predators, respectively (Hódar, Campos & Rosales, 1996; Tigar & Osborne, 2000; Del Bove & Isotti, 2001; Sánchez-Piñero, 2007; Bravo *et al.*, 2016), suggesting that black coloration and large size do not deter predators. The existence of toxic populations composed of entirely black specimens within the red-striped lineages suggests that the selective pressures that maintained the red-striped coloration were either not related to visual predation, or alternatively, selective pressures became relaxed in recent times, when entirely black populations appeared. Although a few predators tolerate cantharidin toxicity (Eisner *et al.*, 1990; Bartram & Boland, 2001; Bravo et al., 2014, 2016), reports on blister beetle predation are very scarce (see Bologna, 1991).

In sum, the phylogenetic analyses show that changes in coloration of *B. majalis* are recent, and therefore that the ancient pressures that fixed and maintained red-striped colorations in this species are no longer acting today. However, these changes in coloration are not correlated with changes in cantharidin content (i.e. entirely black specimens and red-striped are equally poisonous), suggesting that the evolution of colour polymorphisms in *B. majalis* is decoupled from toxicity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Sample sizes of haemolymph samples and lyophilized individuals according to sex and morphotypes (entirely black, red-striped) with non-haemolymph extraction (extraction 0), in the first extraction (2–4 days after capture) and the second extraction (8–10 days after capture).

Table S2. Candidate linear mixed-models (LMMs) for cantharidin content in haemolymph of *B. majalis* as a function of sex, morphotypes, and number of cantharidin extraction. Model selection was based on the Akaike's Information Criterion corrected for small sample sizes (AICc). Delta (ΔAICc) and weight values (wAICc) for each AICc are also shown. Marginal R^2 (R^2_m; proportion of variance explained by the fixed factors alone) and conditional R^2 (R^2_c; proportion of variance explained by both the fixed and random factors) were computed for each model using methods described by Nakagawa & Schielzeth (2013).

Figure S1. Ancestral character reconstruction with temporal estimates on the Bayesian-coalescence tree (BEAST). The red colour corresponds to red-stripped morphotypes, black colour to entirely black morphotypes, and blue for both morphotypes. The ancestral character reconstruction showed the red-striped coloration is ancestral for *B. majalis*. Clades A, B, and C are enlarged to allow visual inspection.

SUPPORTING INFORMATION

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Test	Extraction	Entirely black		Red-stripes		Total
		Female	Male	Female	Male	
Haemolymph	1	23	27	11	11	72
	2	5	12	4	3	24
Body	0	4	3	4	3	14
	1	7	9	5	7	28
	2	3	7	4	2	16

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Candidate models		df	AICc	ΔAICc	wAICc	R^2_m	R^2_c
1)	N extractions + Sex + N extractions * Sex	5	208.3	0.0	0.42	0.51	0.67
2)	N extractions + Sex + Morphotype + N extractions * Sex + Sex * Morphotype + N extractions * Morphotype	14	208.7	0.4	0.34	0.46	0.69
3)	N extractions	6	209.4	1.2	0.23	0.45	0.68
4)	Sex	10	217.4	9.1	0.00	0.49	0.69
5)	N extractions + Morphotype + N extractions * Morphotype	4	238.8	30.5	0.00	0.25	0.25

Figure S1. Ancestral character reconstruction with temporal estimates on the Bayesian–coalescence tree (BEAST). Red colour corresponds to red-stripped morphotypes, black to entirely black morphotypes and blue for both morphotypes. The ancestral character reconstruction showed that red-striped coloration is ancestral for *B.majalis*. Clades A, B and C are enlarge to allow visual inspection.