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Messinian Salinity Crisis and Quaternary glacial events shaped genetic diversification in Siculo-Maghrebian blister beetles (Coleoptera: Meloidae)

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Three non-phoretic blister-beetle species (*Mylabris schreibersi*, *Actenodia distincta* and *Cabalia segetum*) were investigated to trace their Siculo-Maghrebian biogeographic origins and to understand how Tertiary and Quaternary paleogeographic events (e.g. the 'Messinian Salinity Crisis' and the cyclic Quaternary glacio-eustatic marine oscillations) shaped their phylogeographic patterns. Data from *CAD* and *RpP01* nuclear genes highlighted a clear lineage sorting between Sicilian and Maghrebian populations of *M. schreibersi*, explainable by a vicariance event likely occurred after the 'Messinian Salinity Crisis'. A less marked – but still noticeable – genetic distinction among Sicilian and Maghrebian populations of *A. distincta* and *C. segetum* was observed, which could be more referable to moderately old (during Pliocene or late Quaternary) dispersal events. In addition, the low genetic variability and lack of population structure found in Sicily for the three blister-beetle species suggest some drastic reduction in size of Sicilian populations during Pleistocene glacial phases. In general, our results on Meloidae beetles are consistent with published literature, indicating that vicariance or dispersal scenarios can be both plausible explanations of the Siculo-Maghrebian distribution currently observed in a number of animal and plant species.

ADDITIONAL KEYWORDS: biogeography – dispersal – mediterranean – phylogeography – vicariance.

INTRODUCTION

Meloidae is a family of Coleoptera Tenebrionoidea including some 130 genera and nearly 3000 species. Blister beetles are of particular interest because of their distinctive biology, which includes hypermetamorphic development, parasitoid larval habits, defensive attributes and diverse courtship behaviour (Bologna & Di Giulio, 2011) as well as for their importance to applied science (pharmacology, veterinary and agricultural problems: see Bologna (1991) for a review of these aspects). An extensive review on meloid systematics, bionomics and biogeography (Bologna, 1991) as well as more recent phylogenetic studies (Bologna & Pinto, 2001; Bologna *et al.*, 2008b) based on morphological (adults and larvae), behavioural (sexual and cleaning behaviour) and molecular characters have been carried out to investigate the evolutionary history of this family.

Species of this family are primarily distributed in temperate steppes and xeric regions, but also in subtropical/tropical savannas and in other open habitats (Bologna, 1991). Bologna & Pinto (2002) formerly described the high diversity of this family in the Old World, and newly discovered taxa were subsequently described (Bologna, 2003, 2009; Turco & Bologna, 2008). Out of the 80 Old World genera (but other genera endemic to southern Africa are under description, Bologna, Amore & Pitzalis, In press), 53 occur in the Palaearctic, 43 in the Afrotropical (with 19 genera common in both regions) and 22 in the Oriental

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regions. Thirty endemic genera (56.6%) belong to the Palaearctic Region, but the world's greatest diversity of meloid genera likely occurs in the Saharan-Mediterranean subregion. This zone, transitional with the Afrotropical one, includes 39 of the 53 genera recorded from the Palaearctic: 17 are Saharan-Mediterranean endemics and represent 32.1% of the Region's diversity (Bologna & Pinto, 2002).

Blister beetles from the Saharan–Mediterranean subregion, as well as several other animals and plants, show diverse patterns of geographic distribution, which have been shaped by Cainozoic palaeogeographic events of the West Mediterranean area. The causes of such distributions formerly interpreted as originated in the paleo-continent of Tyrrhenis (e.g. Jeannel, 1942; Furon, 1959) have to be traced back to tectonic events occurred in the Late Oligocene and Early Miocene (e.g. Rosenbaum, Lister & Duboz, 2002), during the end of the Miocenic period named 'Messinian Salinity Crisis' (e.g. Krijgsman *et al.*, 1999) and the cyclic Quaternary glacio-eustatic sea-level oscillations (e.g. Imbrie *et al.*, 1993; Gibbard & van Kolfschoten, 2004). In particular, the Siculo-Maghrebian distribution (La Greca, 1957; corresponding to the N African chorotype with extension in Sicily: Vigna Taglianti *et al.*, 1999), involves about 5% of the Sicilian Fauna (http://www.faunaitalia.it/ckmap/) and Flora (Pignatti, 2002). It is an intriguing biogeographic pattern, but only investigated in some amphibians (e.g. Stöck *et al.*, 2008, 2016), reptiles (e.g. Giovannotti *et al.*, 2007; Stöck *et al.*, 2016), mammals (e.g. Dubey *et al.*, 2008) and Asteraceae plants (e.g. Hilpold *et al.*, 2011; Troia, Raimondo & Geraci, 2012), and only rarely in insects (e.g. Habel, Dieker & Shmitt, 2009; Habel *et al.*, 2011) and other invertebrates (e.g. Giusti & Manganelli, 1984; Altaba, 1998; Guiller *et al.*, 2001; Sanmartín, 2003; Pfenninger *et al.*, 2010). Actually, many of these studies concern species with more extended distribution in West Mediterranean. Moreover, some of the examined species show great vagility or passive dispersion (e.g. Habel *et al.*, 2011).

The Meloidae family includes six species having a strict Siculo-Maghrebian distribution [*Cabalia segetum* (Fabricius, 1792); *Mylabris schreibersi* Reiche, 1865; *Mylabris impressa* Chevrolat, 1837; *Actenodia distincta* (Chevrolat, 1837), *Meloe luctuosus* Brandt & Erichson, 1832; *Zonitis bellieri* Reiche, 1860], while few other West Mediterranean species are distributed not only in Maghreb and Sicily but also in the Iberian Peninsula, southern France, Sardinia and Corsica [e.g. *Meloe murinus* Brandt & Erichson, 1832; *Zonitis fernancastroi* Pardo Alcaide, 1950, *Leptopalpus rostratus* (Fabricius, 1792)]. While species of the genera *Meloe*, *Zonitis* and *Leptopalpus* have high dispersion capacity, due to phoretic larvae dispersed by bees (hosts or occasional vectors), species of the genera *Cabalia*, *Mylabris* and *Actenodia* have non-phoretic larvae and adults with scarce flying capacity, which greatly reduces their dispersion ability especially across sea barriers (Bologna & Marangoni, 1990; Bologna, 1991; Bologna & Pinto, 2002).

The most prominent Caenozoic palaeogeographic event, which affected the Mediterranean, was certainly the 'Messinian Salinity Crisis'. As well demonstrated (Krijgsman *et al.*, 1999, 2002; Roveri *et al.*, 2014), the closure during the Messinian period (Miocene) of both the Riffian corridor (6.0 Myr) and the Betic passage (6.3 Myr) produced a dramatic desiccation of the whole Mediterranean basin, which ended 5.33 Myr after the opening of the Gibraltar Strait. During this period, the new terrestrial connections made available between European and North African lands and the subsequent extension of Mediterranean, xeric and temperate ecosystems (Fauquette *et al.*, 2006) promoted dispersion of a number of plant and animal organisms. Afterwards, no other terrestrial connections occurred among northern and southern Mediterranean shores, also during marine regression, due to the repeated Quaternary glacial events (Rohling *et al.*, 1998; Lambeck *et al.*, 2004). However, these cold periods dramatically conditioned the World biota and, particularly, the European ones (e.g. Hewitt, 1996, 1999, 2000, 2004, 2011). During Pleistocene glaciations, Sicily was connected to the Italian Peninsula, but not to Tunisia, being the minimal width of the Sicilian channel about 40–50 km (Hilpold *et al.*, 2011).

The marine barriers and the lack of land connectivity between north-western and south-western Mediterranean territories since the opening of the Gibraltar strait stimulate to explore micro- and macro-evolutionary processes occurred in animal populations currently distributed on both sides of the sea. The origin of these populations could be inferred by phylogeographic analyses aimed at evaluating the genetic divergence among them.

In our study, three Siculo-Maghrebian non-phoretic meloid species (*C. segetum*, *M. schreibersi* and *A. distincta*) were selected in order to trace and compare evolutionary histories and phylogeographic patterns, likely shaped by paleogeographic events occurred across the Sicilian channel. The aims of this study are to (1) assess levels of genetic divergence between Sicilian and Maghrebian populations within the three selected blister-beetle species; (2) assess the genetic structure within Maghreb and Sicily and relate it to both Tertiary and Quaternary palaeogeographic events; and (3) compare the phylogeographic patterns observed in these species and interpret them in the light of the Siculo-Maghrebian biogeographic pattern.

MATERIAL AND METHODS

TARGET SPECIES AND SAMPLE COLLECTION

Mylabris schreibersi (tribe Mylabrini) belongs to a Palaearctic genus and is distributed from the West and North Morocco to North Tunisia and widespread all over Sicily (with two ancient records in Southern Calabria, which have never been confirmed). The first instar larvae are parasite of eggs of Orthoptera Acridoidea; adults feed primarily on Asteraceae and Dipsacaceae and are active between April and August from sea level to *c*. 2000 m in Morocco and 1700 m a.s.l. in Sicily (Bologna, 1991).

Actenodia distincta (tribe Mylabrini) belongs to an Afrotropical-Saharan genus and is distributed in the whole part of non-desert Morocco, through North Algeria to North Tunisia, and in Sicily (with old records in Andalusia, but not confirmed). The larval stage has a poorly known biology, even if its morphology excludes phoretic habits. Larvae are putative parasite of Orthoptera Acridoidea; adults feed primarily on Asteraceae, Convolvulaceae and other plant families and are active between April and August from the sea level to *c*. 2000 m in Morocco and 1700 m a.s.l. in Sicily (Bologna, 1991).

Cabalia segetum (tribe Lyttini) is the only Mediterranean element of the genus, which has an Afrotropical-Saharan distribution. *C. segetum* is distributed along the coastal areas of Algeria, Tunisia and Libya (Cyrenaica) (with doubtful records from North Morocco) and widespread all over Sicily. The larval stage has an unknown biology: its morphology excludes phoretic habits and suggests parasitism on Hymenoptera Aculeata, as in almost all Lyttini. Adults feed primarily on Convolvulaceae and Malvaceae and are mostly active between May and July, from sea level to *c*. 1300 m a.s.l. (Bologna, 1991).

Samples of *M. schreibersi*, *C. segetum* and *A. distincta* were collected in the field by hand and net-sampling (2004–2016) in Sicily, Tunisia and Morocco (Fig. 1; Table 1). Collected specimens were preserved in ethanol 95%, stored at 4 °C and afterwards morphologically identified by dichotomous keys (Kaszab, 1948; Bologna, Di Giulio & Pitzalis, 2008a; Pan & Bologna, 2014).

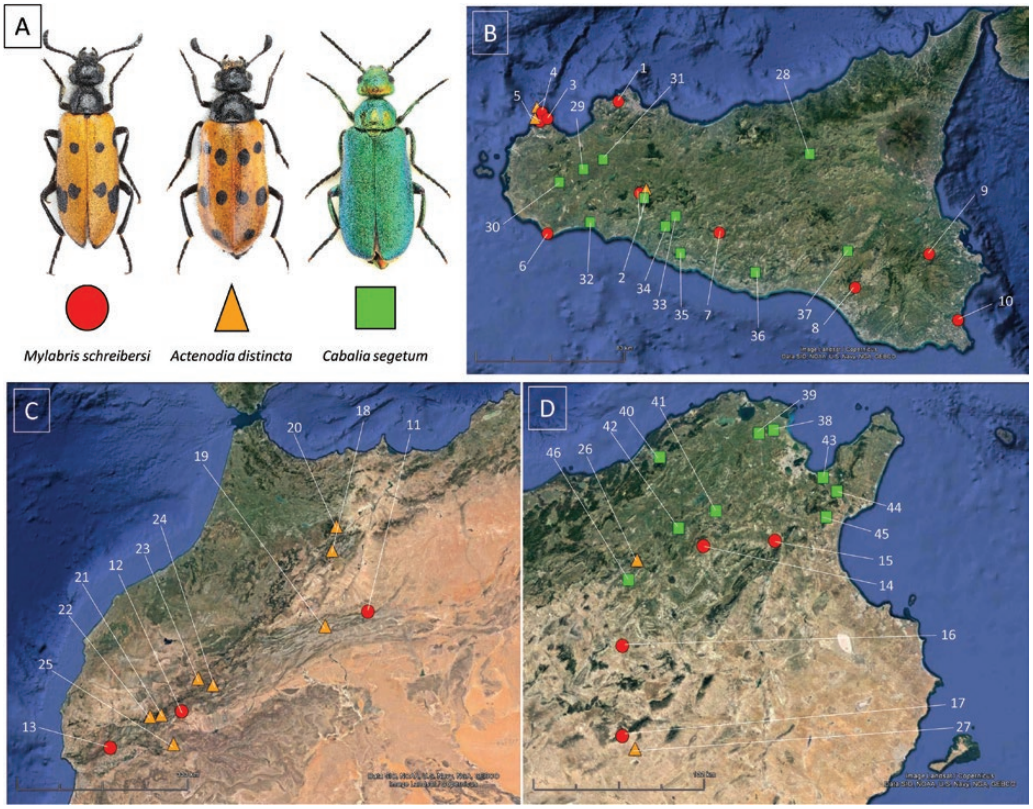

Figure 1. Meloidae species and sampling sites. (A) The three analysed species and their associated symbols; (B) sampling sites in Sicily; (C) sampling sites in Morocco; (D) sampling sites in Tunisia. In the maps, each population is identified with a number corresponding to those listed in Table 1.

Table 1. Localities, coordinates, sampling dates and number of analysed individuals for both gene markers

Mylabris schreibersi

Localities and collectors			Coordinates		Dates	*CAD*	*Rpp01*
Sicily	1.	Capaci (Palermo) M. Romano	38.1577	13.2404	02/06/2013	7	6
	2.	Palazzo Adriano (Palermo) M. Bologna, E. Mancini, A. Riccieri	37.6956	13.3174	10/06/2015	4	5
	3.	Torre Impiso (Trapani) M. Bologna	38.1355	12.7861	28/05/2010	1	1
	4.	San Vito lo Capo (Trapani) M. Romano	38.1671	12.7518	15/06/2013	3	3
	5.	Castelluzzo (Trapani) M. Bologna, J.D. Pinto	38.1258	12.7299	28/05/2010	9	8
	6.	Granitola (Trapani) M. Bologna	37.5704	12.6609	08/05/2016	3	0
	7.	Milena (Caltanissetta) M. Bologna, E. Mancini, A. Riccieri	37.4475	13.7300	09/06/2015	4	5
	8.	Acate (Ragusa) M. Bologna, E. Mancini, A. Riccieri	37.0552	14.5012	08/06/2015	1	1
	9.	Sortino (Siracusa) M. Bologna, E. Mancini, A. Riccieri	37.1546	14.9805	08/06/2015	4	4
	10.	Vendicari (Siracusa) M. Bologna	36.8054	15.0884	17/05/2013	1	1
Morocco	11.	Talsint (Figuig) D. Salvi	32.6364	3.6409	05/06/2014	6	2
	12.	Tizi-n-tichka (Marrakech) D. Salvi	31.3083	-7.3685	05/05/2008	3	2
	13.	Missirat (Taroudant) D. Salvi	30.7718	-8.8549	31/05/2011	2	1
Tunisia	14.	Gaafour (Gov. Siliana) M. Bologna, E. Mancini	36.3484	9.3682	14/05/2015	4	7
	15.	El Fahs (Gov. Zaghouan) M. Bologna, E. Mancini	36.2980	9.9785	13/05/2015	1	1
	16.	Kalaat Khasba (Gov. El Kef) C. Settanni, S. Carloni, F. Turco	35.7320	8.5735	29/04/2004	1	1
	17.	Darnayah (Gov. Kasserine) C. Settanni, S. Carloni, F. Turco	35.1143	8.4979	29/04/2004	1	1
Total						55	49

Actenodia distincta

Localities			Coordinates		Dates	*CAD*	*Rpp01*
Sicily	2.	Palazzo Adriano (Palermo) M. Bologna. E. Mancini. A. Riccieri	37.6956	13.3174	10/06/2015	2	1
	4.	San Vito lo Capo (Trapani) M. Romano	38.1671	12.7518	02/05/2013	4	4
	5.	Castelluzzo (Trapani) M. Bologna. J.D. Pinto	38.1258	12.7299	28/05/2010 - 10/04/2011 - 16/04/2011	4	4
Morocco	18.	R 504 (Sefrou) D. Salvi	33.6404	-4.2007	06/05/2010	3	3
	19.	Rich. road P21 (Midelt) A. Di Giulio. F. Turco	32.4444	-4.4872	08/08/2006	1	1
	20.	TazaJebel Tazzeka National Park (Taza) A. Di Giulio. E. Maurizi & P. Hlaváč.	34.0266	-4.0806	12/05/2009	2	2
	21.	Oukaimeden. road P2028 (Al Haouz) A. Di Giulio. E. Maurizi & P. Hlaváč	31.2379	-7.8172	08/05/2009 - 16/05/2012 - 12/05/2013	8	6
	22.	Asni. road S501 (Al Haouz) A. Di Giulio. F. Turco	31.2235	-8.0294	05/05/2006	3	3
	23.	R 208 (El Kelaa Des Sraghna) L. Cerny	31.7802	-7.0245	03/05/2012	0	2
	24.	Aït Blal. R302 (Azilal) L. Cerny	31.6653	-6.7358	02/05/2012	1	0
	25.	(Ouarzazate) D. Salvi	30.7439	-7.6096	01/06/2014	0	1

Table 1. *Continued*

Tunisia	26.	Le Kef (Gov. Le Kef) M. Bologna. E. Mancini	36.2784	8.7921	15/05/2015	1	0
	27.	Thelepte (Gov. Kasserine) F. Turco. C. Settanni. S. Carloni	35.0134	8.5807	29/04/2004	1	1
Total						30	28

Cabalia segetum

Localities			Coordinates		Dates	*CAD*	*Rpp01*
Sicily	28.	Sperlinga (Enna) M. Bologna. E. Mancini. A. Riccieri	37.7497	14.3614	11/06/2015	5	5
	2.	Palazzo Adriano (Palermo) M. Bologna. E. Mancini. A. Riccieri	37.6956	13.3174	10/06/2015	6	6
	29.	Pantani di Anguillara (Trapani) M. Bologna	37.8579	12.9488	07/05/2016	2	2
	30.	Salemi (Trapani) M. Bologna	37.8190	12.7989	08/05/2010	4	5
	31.	Camporeale (Trapani) M. Bologna	37.8980	13.0949	07/05/2016	4	3
	32.	Menfi (Agrigento) M. Bologna. J.D. Pinto	37.5909	12.9398	14/04/2011	4	4
	33.	Alessandria della Rocca (Agrigento) M. Bologna. C. Marangoni	37.5514	13.4798	26/05/2010	1	1
	34.	Cianciana (Agrigento) M. Bologna. J.D. Pinto	37.5218	13.4061	14/04/2011	2	4
	35.	Raffadali (Agrigento) M. Bologna. E. Mancini. A. Riccieri	37.3758	13.4675	09/06/2015	6	6
	36.	Campobello di Licata (Agrigento) M. Bologna. E. Mancini. A. Riccieri	37.2070	13.9055	09/06/2015	6	6
	37.	Caltagirone (Caltagirone) M. Bologna A. Di Giulio	37.2380	14.4923	20/05/2013	5	5
Tunisia	38.	Utique Ruins (Gov. Bizerte) G. Sabatinelli	37.0581	10.0632	27/04/2013	2	2
	39.	Utique (Gov. Bizerte) M. Bologna. E. Mancini	37.0387	9.9526	17/05/2015	5	4
	40.	Nefza (Gov. Bejà) M. Bologna. E. Mancini	36.9579	9.0905	16/05/2015	6	6
	41.	Testour (Gov. Bejà) G. Sabatinelli	36.5625	9.5094	2013	2	2
	42.	Téboursouk (Gov. Bejà) M. Bologna. E. Mancini	36.4754	9.1698	15/05/2015	4	4
	43.	Hammam Lif (Gov. Nabeul) M. Bologna. E. Mancini	36.6951	10.4357	18/05/2015	3	2
	44.	Grombalia (Gov. Nabeul) M. Bologna. E. Mancini	36.5738	10.5349	17/05/2015	5	5
	45.	Sidi (Gov. Nabeul) M. Bologna. E. Mancini	36.4141	10.4098	13/05/2015	8	5
	46.	Le Kef (Gov. Le Kef) M. Bologna. E. Mancini	36.1770	8.6850	15/05/2015	1	1
Total						81	78

Each population is identified by a locality number, as reported in Figure 1.

Molecular methods

Genomic DNA was extracted from the cephalic portion of 191 specimens (*C. segetum* = 89; *M. schreibersi* = 67; *A. distincta* = 35) with a 'Salting Out' protocol (Sunnucks & Hales, 1996). PCR amplifications were performed on selected portions of two nuclear DNA (nucDNA) genes, known to be variable at the species rank in beetles (Gómez-Zurita *et al.*, 2004; Wild & Maddison, 2008): *CAD* (a portion of exon encoding the carbamoylphosphate synthetase enzyme) and *RpP01* (a portion of intron 1 of *RpP0* gene encoding the 60S acid ribosomal protein P0). For PCR amplifications, the following primer pairs were used: *CAD* = CD439F/CD688R (Wild & Maddison, 2008); CD439F/CAD_int2_R (5′-AATTATCATAAGCATCACGTAC-3′); *RpP01* = P0-5p (Gómez-Zurita *et al.*, 2004)/Rpp0mylR (5′-GCTGYAAACAAAATACGRACAAT-3′).

PCR were conducted in a volume of 25 µL containing 3 µL of 10× buffer, 3 µL of $MgCl_2$ (50 mM), 0.5 µL of dNTPs (10 mM), 0.2 µL of Taq DNA polymerase (5 U/µL; BIOTAQ Bioline) and 0.5 µL of each primer (25 mM), 16.8 µL of H_2O MilliQ and 1 µL of DNA template. Amplifications were carried out using a BIO-RAD MyCycler. A touchdown PCR protocol was used to amplify the *CAD* marker with the following thermal cycling conditions: 94 °C for 3.5 min, followed by 20 cycles of 94 °C, 30 s, annealing temperatures step-downs every cycle of 0.4 °C (from 58 to 50 °C), 35 s, 72 °C, 2.5 min and additional 20 cycles of 94 °C, 30 s, 55 °C, 35 s, 72 °C, 2.5 min. Amplification of *RpP01* was carried out with the following thermal cycling conditions: 94 °C, 3 min, followed by 35 cycles of 94 °C, 30 s, 52–54 °C, 45 s, 72 °C, 1 min. Agarose (1%) gel run stained with 1 µL of SimplySafe (EurX) allowed at determining the quality and quantity of amplified products to be sent to Macrogen Europe (the Netherlands) for sequencing. All sequences were deposited in GenBank (accession number: MF426271 -MF426912).

Polymorphism and phylogeographic analyses

Editing of chromatograms and alignment of genotype sequences were performed using the Staden Package ver. 2003.1.6 (Staden, Beal & Bonfield, 1998). Alleles were phased using the PHASE algorithm (Stephens, Smith & Donnelly, 2001) as implemented in DNAsp v5.10 (Librado & Rozas, 2009). For each species/marker, phased sequences were assigned to three groups corresponding to the geographic origins of sampled individuals, that is 'Sicily', 'Morocco' and 'Tunisia' (the two latter groups were also merged together in the 'Maghreb' group). Genetic polymorphism parameters and Tajima's *D* (Tajima, 1989) and Fu and Li's (Fu, 1997) neutrality tests (based on the total number of segregating sites) were carried out using DNAsp. Pairwise *p*-distances among and within geographic groups were calculated with MEGA7 (Kumar, Stecher & Tamura, 2016), whereas F_{ST} were estimated using Arlequin v3.5.2.2 (Excoffier & Lischer, 2010). Statistical significance of F_{ST} values was tested in Arlequin v3.5.2.2 through permutation tests (number of permutations set to 100, $P < 0.05$). The analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) was performed with Arlequin, and *F*-statistic was used to estimate the proportion of genetic variability among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). For this purpose, individuals were included in two main groups ('Sicily' and 'Maghreb') according to their geographic origin. Networks, representing genealogical relationships among haplotypes, were inferred by applying the median-joining algorithm (Bandelt, Forster & Röhl, 1999) in NETWORK v4.6 (http://www.fluxus-engineering.com).

RESULTS

Genetic polymorphism and haplotype networks

A total of 166 *CAD* and 155 *RpP01* unphased sequences were obtained for the three analysed meloid species (Table 1). Partial *CAD* coding sequences of 753 bp (=251 amino acidic positions, a.a.), 627 bp (=209 a.a.) and 756 bp (=252 a.a.) were obtained for *M. schreibersi*, *A. distincta* and *C. segetum*, respectively. Non-coding fragments of 198 bp, 183 bp and 180 bp for *RpP01* were obtained for *M. schreibersi*, *A. distincta* and *C. segetum*, respectively. Heterozygous positions were detected along alignments of both *CAD* [*M. schreibersi* *n* = 44 (5.8%), *A. distincta* *n* = 37 (5.9%), *C. segetum* *n* = 11 (1.4%)] and *RpP01* [*M. schreibersi* *n* = 11 (5.5%), *A. distincta* *n* = 16 (8.7%), *C. segetum* *n* = 7 (3.8%)]. For all species, most heterozygous sites were found to be exclusive of Maghrebian individuals at both *CAD* (*M. schreibersi* 82%, *A. distincta* 81%, *C. segetum* 91%) and *RpP01* (*M. schreibersi* 100%, *A. distincta* 87%, *C. segetum* 71%).

Genetic polymorphism parameters computed for *CAD* and *RpP01* in all three species are reported in Table 2. Overall, *CAD* showed higher values of haplotype diversity than *RpP01* in all species (and, particularly, in *M. schreibersi* and *C. segetum*). For both markers, a greater nucleotide diversity was observed in *M. schreibersi* and *A. distincta* than in *C. segetum* as well as a higher genetic polymorphism in Maghrebian populations than in Sicilian ones.

Non-significant departures from neutrality were evidenced in both markers for the analysed species, except for an excess of rare *RpP01* variants in the overall *M. schreibersi* data set (i.e. negative *F** and *D**)

Table 2. *CAD* and *RpP01* polymorphisms and neutrality tests for the three species

Geographic origin of samples		2*N*	*S*	*H*	Hd (%)	π (%)	ϑ_w (%)	*D*	*F**	*D**
Mylabris schreibersi										
CAD (753 bp)	Overall	110	46	39	91.6	1.1	1.2	−0.14	−0.28	−0.29
	Sicily	74	8	11	82	0.2	0.2	−0.35	−0.34	−0.26
	Tunisia	14	30	13	98.9	1.5	1.2	0.87	0.56	0.34
	Morocco	22	28	15	94	1.3	1	1.12	0.74	0.42
	Maghreb	36	37	28	97.6	1.5	1.2	0.89	0.38	0.04
RpP01 (198 bp)	Overall	98	14	11	49.9	0.6	1.4	−1.49	**−2.90**	**−2.97**
	Sicily	68	0	1	0	0	0	n.a.	n.a.	n.a.
	Tunisia	20	8	7	68.9	0.7	1.1	−1.30	−1.19	−0.92
	Morocco	10	8	5	86.7	1.3	1.4	−0.38	−0.43	−0.39
	Maghreb	30	13	10	76.1	0.9	1.6	−1.44	−2.17	−2.03
Actenodia distincta										
CAD (627 bp)	Overall	60	39	39	94.7	1.2	1.3	−0.44	0.06	0.35
	Sicily	20	7	3	53.2	0.5	0.3	1.82	**1.67**	1.30
	Tunisia	4	8	3	83.3	0.6	0.7	−0.82	−0.81	−0.82
	Morocco	36	31	33	99.5	1.1	1.2	−0.29	0.01	0.16
	Maghreb	40	34	36	99.5	1.1	1.3	−0.43	0.27	0.03
RpP01 (183 bp)	Overall	56	25	20	87	2	3	−1.03	−0.06	0.50
	Sicily	18	2	2	11.1	0.1	0.3	−1.51	−2.13	−1.99
	Tunisia	2	0	1	0	0	0	/	/	/
	Morocco	36	23	19	95.7	2.7	3	−0.37	0.26	0.51
	Maghreb	38	25	20	96	2.7	3.2	−0.56	0.60	0.24
Cabalia segetum										
CAD (756 bp)	Overall	162	13	25	69	0.4	0.3	0.54	−0.58	−1.09
	Sicily	90	1	2	2.2	0	0	−1.04	−2.00	−2.00
	Tunisia	72	12	23	94.5	0.5	0.3	1.54	0.41	−0.29
RpP01 (180 bp)	Overall	156	7	9	39.5	0.3	0.7	−1.23	−1.76	−1.62
	Sicily	94	2	3	4.2	0	0.2	−1.39	−2.77	−2.80
	Tunisia	62	5	7	70.7	0.5	0.6	−0.16	0.00	0.08

2*N*, number of alleles; *S*, number of segregating sites; *H*, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; ϑ_w, Watterson Esitmator; *D*, Tajima's D; *F** and *D**, Fu and Li test; n.a., not applicable. Statistically significant results are indicated in bold ($P < 0.05$).

and of *CAD* intermediate-frequency variants (i.e. positive *F**) in Sicilian populations of *A. distincta*.

Networks showing phylogenetic relationships among *CAD* and *RpP01* haplotypes (coloured according to their geographic origin) are depicted are depicted in Fig. 2, and with further details on haplotype-specific geographic origins in Fig. 3. A clear separation between Sicilian and Maghrebian haplo-groups was evidenced in *M. schreibersi* by both *CAD* and *RpP01* (with a minimum number of mutational steps = 6 and 1, respectively). Such a separation was far less marked in both nuclear genes of *C. segetum* and *A. distincta* in which, however, the absence of shared *CAD* haplotypes among Sicilian and Maghrebian individuals was observed. In contrast with results obtained for *M. schreibersi*, *RpP01* Sicilian-specific haplotypes were shared with some Maghrebian individuals (Fig. 2) in both *C. segetum* and *A. distincta*.

Gene flow and population structure

In general, F_{ST} values were relatively higher when comparing Sicily with Maghreb/Morocco/Tunisia (Sicily vs. Maghreb: *M. schreibersi*: *CAD* = 0.65, *RpP01* = 0.74; *A. distincta*: *CAD* = 0.37, *RpP01* = 0.13; Sicily vs. Morocco: *M. schreibersi*: *CAD* = 0.72, *RpP01* = 0.84; *A. distincta*: *CAD* = 0.40; *RpP01* = 0.15; Sicily vs. Tunisia: *M. schreibersi*: *CAD* = 0.76, *RpP01* = 0.85; *A. distincta*: *CAD* = 0.58; *RpP01* = 0.90; *C. segetum*: *CAD* = 0.56, *RpP01* = 0.39), than in comparisons between Morocco and Tunisia (*M. schreibersi*: *CAD* = 0.12, *RpP01* = 0.08; *A. distincta*: *CAD* = 0.23; *RpP01* = 0.23). All values were statistically significant ($P < 0.05$).

Overall, results of AMOVA (Table 3) confirmed the presence of phylogeographic structure in our data. Both nuclear markers indicated a strong subdivision ($F_{CT} > 0.6$) between Maghrebian and Sicilian

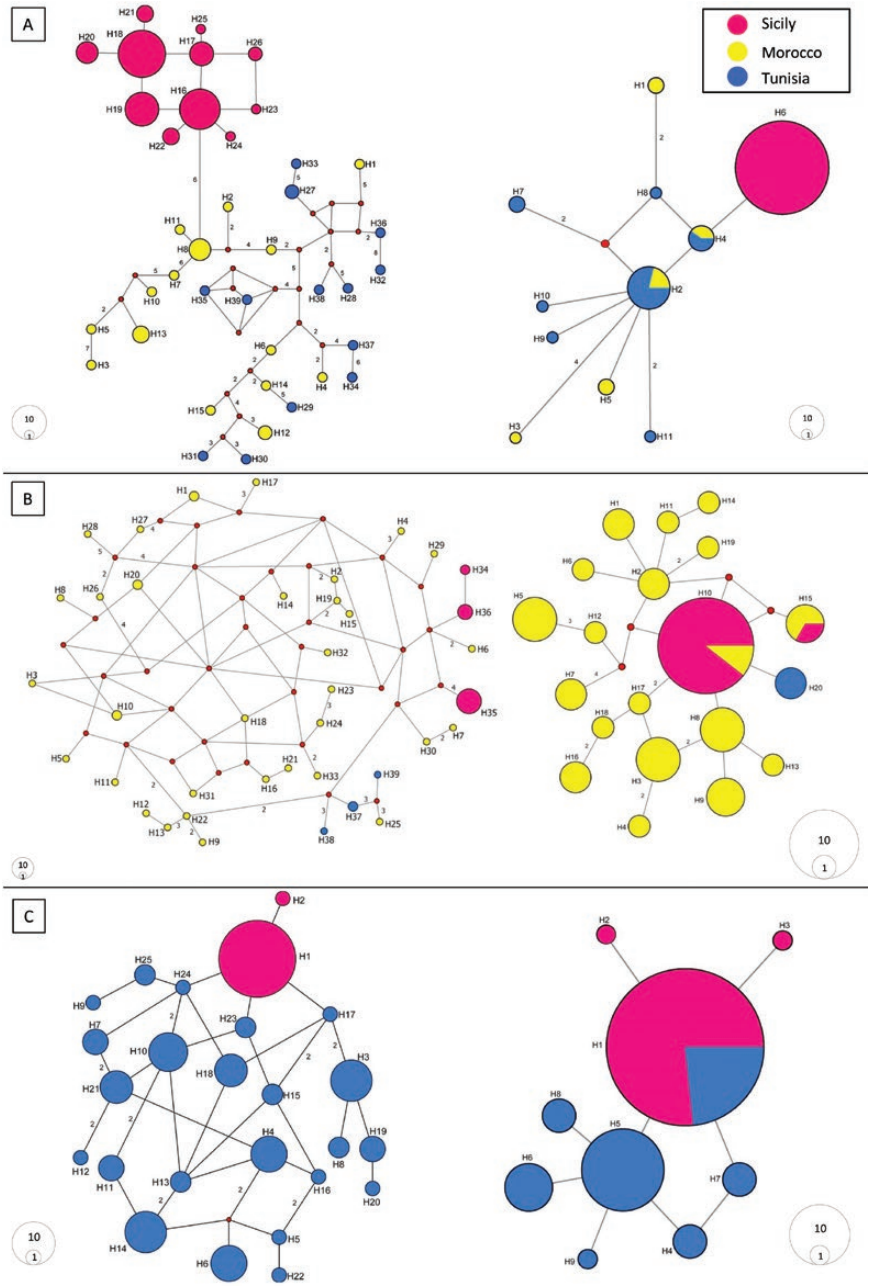

Figure 2. Haplotype networks showing phylogenetic relationships among *CAD* (left) and *RpP01* (right) haplotypes in the three Meloidae species: (A) *Mylabris schreibersi*, (B) *Actenodia distincta*, (C) *Cabalia segetum*. Colours assigned to the three main geographic areas are reported in the legend (top right). Number of mutations separating haplotypes is reported above network connections (not scaled), except for haplotypes separated by one mutational step. Not sampled intermediate haplotypes are indicated by red dots.

populations in *M. schreibersi*, with most of molecular variation distributed between the two groups (*CAD* = 63.9%; *RpP01* = 72.9%). The averaged genetic *p*-distance between Sicily and Maghreb in *M. schreibersi* was 2% (±0.4) at *CAD* and 0.7% (±0.4) at *RpP01*. In addition, *CAD* data (but not *RpP01*) suggested a relatively slighter, but significant, differentiation among *M. schreibersi* populations within groups (F_{SC} = 0.19): in general, the mean genetic pairwise distance among populations in Sicily [*CAD* = 0.2% (±0.1); *RpP01* = 0.0% (±0.0)] was lower than among those in Maghreb [*CAD* = 1.5% (±0.3); *RpP01* = 0.8% (±0.3)] (see also Table 2). *CAD* data also suggested segregation between Maghrebian and Sicilian populations in

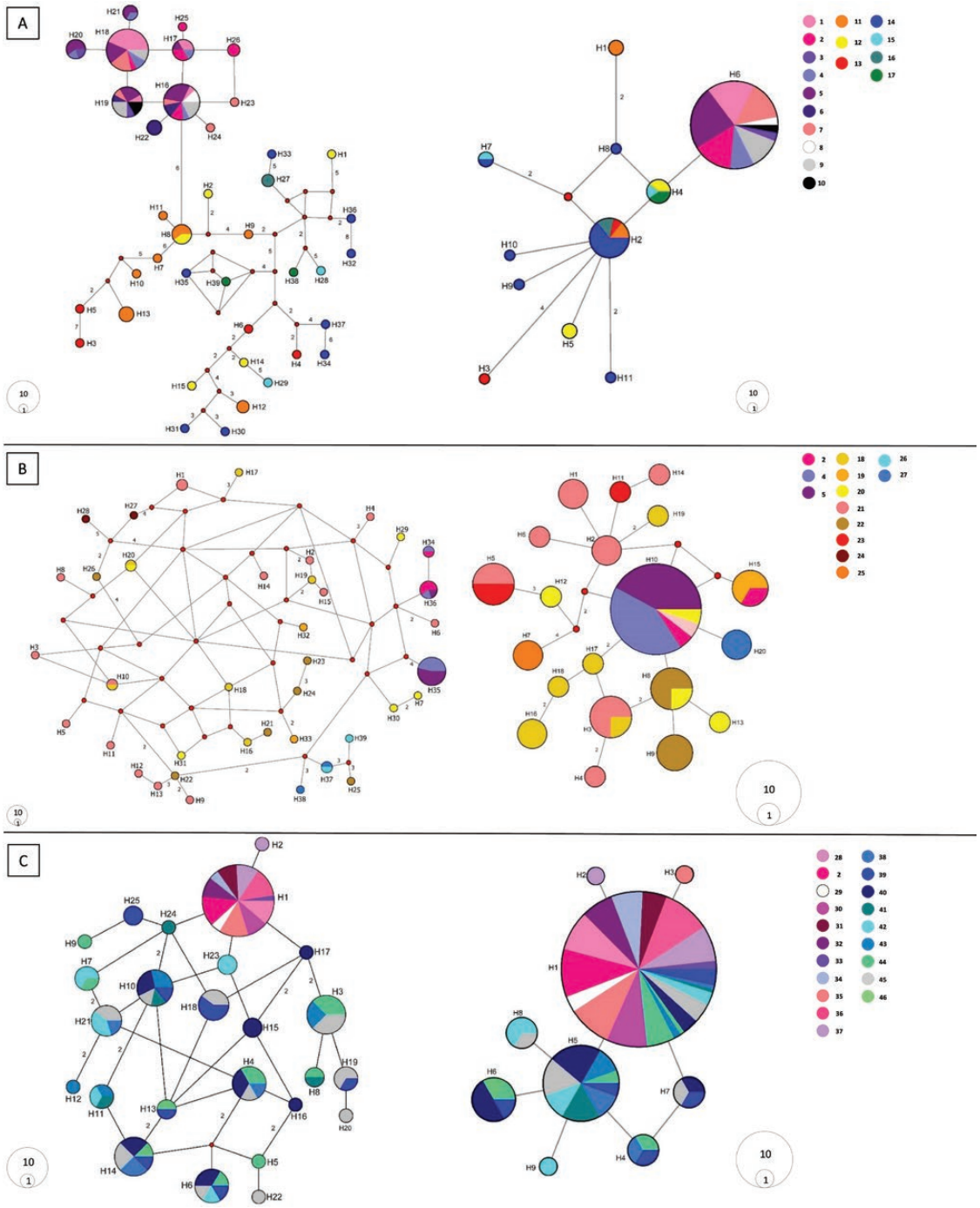

Figure 3. Haplotype networks showing phylogenetic relationships among *CAD* (left) and *RpP01* (right) haplotypes in the three Meloidae species: (A) *Mylabris schreibersi*, (B) *Actenodia distincta*, (C) *Cabalia segetum*. Colours assigned to each population are reported in the legends (top right) and are identified by a number corresponding to localities listed in Table 1. Number of mutations separating haplotypes is reported above network connections (not scaled), except for haplotypes separated by one mutational step. Not sampled intermediate haplotypes are indicated by red dots.

C. segetum (F_{CT} = 0.55) (although the distribution of molecular variation was comparable with that scored within populations, i.e. absence of structure), with 0.5% (±0.2) of mean genetic distance between Sicily and Tunisia. *RpP01* genetic variance was mostly distributed within *C. segetum* populations (60.89%),

Table 3. Results of AMOVA for the three species for gene markers *CAD* (1) and *RpP01* (2)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*F*-statistics
Mylabris schreibersi					
(1) Among groups	1	207.687	4.17 Va	63.89	F_{CT} = **0.64**
Among populations within groups	15	70.216	0.45 Vb	6.97	F_{SC} = **0.19**
Within populations	93	176.752	1.90 Vc	29.14	F_{ST} = **0.71**
(2) Among groups	1	33.468	0.78 Va	72.95	F_{CT} = **0.73**
Among populations within groups	14	8.552	0.07 Vb	6.38	F_{SC} = 0.23
Within populations	82	18.214	0.22 Vc	20.67	F_{ST} = **0.80**
Actenodia distincta					
(1) Among groups	1	48.233	1.51 Va	33.34	F_{CT} = **0.33**
Among populations within groups	9	51.804	0.70 Vb	15.51	F_{SC} = **0.23**
Within populations	49	113.896	2.32 Vc	51.14	F_{ST} = **0.49**
(2) Among groups	1	8.145	0.04 Va	2.35	F_{CT} = 0.02
Among populations within groups	9	44.367	0.82 Vb	41.87	F_{SC} = **0.43**
Within populations	45	49.417	1.10 Vc	55.77	F_{ST} = **0.44**
Cabalia segetum					
(1) Among groups	1	87.05	1.07 Va	55.43	F_{CT} = **0.55**
Among populations within groups	18	20.588	0.04 Vb	2.10	F_{SC} = 0.05
Within populations	142	116.721	0.82 Vc	42.47	F_{ST} = **0.57**
(2) Among groups	1	10.52	0.13 Va	39.56	F_{CT} = **0.40**
Among populations within groups	18	3.573	–0.00157 Vb	–0.45	F_{SC} = –0.007
Within populations	137	28.85	0.21 Vc	60.89	F_{ST} = **0.40**

Statistically significant results are indicated in bold ($P < 0.01$).

indicating the lack of a significant genetic distance between the two main geographic groups [Sicily vs. Maghreb *p*-distance = 0.4% (±0.3)]. In *A. distincta*, most of the molecular variation at both *CAD* and *RpP01* was distributed within populations (51.4%, 55.7%) and only 33.3% of variance was distributed between the two main geographic groups. However, in *A. distincta*, Sicily vs. Maghreb genetic subdivision [*p*-distance = 1.4% (±0.3)] was statistically supported by *CAD* (F_{CT} = 0.33) (but not by *RpP01*).

DISCUSSION

Our choice of blister-beetle species with a limited dispersion capacity and strong biological constraints was intentioned at investigating how the paleogeographic events of both Tertiary and Quaternary shaped their genetic structure. More in general, our findings can provide hints about when (Messinian vs. post-Messinian periods) and how (vicariance vs. dispersal) the distribution in both Sicily and Maghreb originated, in the frame of the theoretical discussion on the Siculo-Maghrebian pattern, which could represent a generalized model of vicariance, distinct events of dispersal or both (e.g. Croizat, 1964; Nelson & Platnick, 1981).

Genetic data from both nuclear markers pointed out a clear lineage sorting between Sicilian and Maghrebian populations in *M. schreibersi*. The high genetic divergence between these lineages within *M. schreibersi* (e.g. 2.0% in *CAD*) and the lack of shared haplotypes is likely the result of a long-term isolation of populations in the two geographic regions. The level of genetic divergence suggests that they may represent two different taxonomic entities. However, no significant morphological differences were observed in this species (Pan & Bologna, 2014), except for a gradual distinction in the mesosternal structure between Sicilian and Tunisian individuals and between Tunisian and Moroccan ones. Consequently, without additional data, no conclusion

can be drawn about the possible existence of cryptic species within *M. schreibersi*. According to such unexpected, large genetic divergence suggested by nuclear markers, the time of separation among Sicilian and Maghrebian populations could be dated back to the period after the Messinian Salinity Crisis (5.3 Myr). A similar pattern of old separation was observed in mammals, in particular within *Crocidura* shrews (Dubey *et al.*, 2008).

Although less pronounced, a signal of genetic distinction among populations from Sicilian and Maghrebian regions was also detectable on both markers in *A. distincta* and *C. segetum*. The observed genetic divergence supports a scenario of a more recent isolation of Sicilian populations of *A. distincta* and *C. segetum* than in *M. schreibersi*, probably occurred during the late Pliocene or early Quaternary. Small differences in the elytra pattern of Sicilian populations of *A. distincta*, described as var. *sicula* Baudi, 1878, could be the result of isolation of Sicilian populations, also because this phenotype rarely occurs in Maghreb. On the contrary, no morphological differences were identified in *C. segetum* (Bologna, 1991; Bologna *et al.*, 2008a). Long-distance dispersal events could be hypothesized to explain the presence of *A. distincta* and *C. segetum* in Sicily and their reduced degree of genetic divergence. This hypothesis falls in line with a possible southern Mediterranean origin of both species since they both belong to Afrotropical-Mediterranean genera and are clearly related to other Saharian or South-Mediterranean species (Kaszab, 1948; Bologna *et al.*, 2008a). In addition, no land connections were demonstrated to emerge during Glacial Maximum peaks, but only a reduction in the width of the Sicilian channel. A 'stepping-stone' scenario cannot be ruled out; however, if the lack of records of both species in Pantelleria Island might be addressed to a more recent volcanic activity, which almost completely erased its biota, the formerly reported presence of *A. distincta* in Lampedusa and *C. segetum* in Malta (Bologna, 1991, 1995) has never been confirmed by recent surveys. Long-distance dispersals from Maghreb to Sicily were hypothesized by floating vegetation rafts in species of the lizard genus *Chalcides* (Giovannotti *et al.*, 2007; Mezzasalma *et al.*, 2016) as well as in toads of the *Bufo viridis* complex (Stöck *et al.*, 2008), whose Sicilian populations were eventually described as belonging to a different species. However, floating rafts of large masses of undifferentiated debris should not have constituted a meaningful way of dispersal for blister-beetle species, which have a very short adult lifespan and a long and hypermetabolic larval phase totally dependent on the host presence. Alternatively, the wind could be considered as a plausible mean of dispersal for small animals such as beetles.

Genetic data revealed low variability and a lack of population structure in Sicily as opposed to the high polymorphism observed in Maghreb for all three blister-beetle species. Such a pattern (also observed in *Chalcides chalcides*; Giovannotti *et al.*, 2007) might be explained by a different effect of Pleistocene glaciations in Sicily as compared to the Maghreb. During glaciations, the habitat of these Mediterranean species was probably greatly reduced in Sicily so that small isolated populations may have survived mainly in southern and western Sicily, with subsequent depletion of most of their genetic diversity (e.g. Suc, 1984; Hewitt, 1996, 2004; Incarbona *et al.*, 2010). On the other hand, the effect of the glaciations on Mediterranean species was likely less severe in North Africa, with a wide extent of Mediterranean habitat shifted southwards providing a wide glacial refugium for Maghrebian populations of all three analysed species. This scenario may explain the maintenance of polymorphism and gene flow between Maghrebian populations as already suggested in other taxa (e.g. Husemann *et al.*, 2013).

CONCLUSIONS

Levels of genetic divergence between Sicilian and Maghrebian populations observed in the three blister-beetle species suggest different origins of their present Siculo-Maghrebian distribution. *Mylabris schreibersi* distribution can be explained by an old vicariance event, due to the post-Messinian opening of the Sicilian channel, whereas *C. segetum* and *A. distincta* distributions by a moderately old dispersal event (probably during a late Quaternary marine regressions). The exact mean of trans-marine dispersal cannot be defined, especially because of the strong constraints of the biological larval requirement of both *C. segetum* and *A. distincta*, even if the reduction of the marine distance and the existence of dominant southern winds (sirocco) could support stochastic events, as known in other insects (dragonflies, butterflies, grasshoppers). This multiple biogeographic scenario in Meloidae is well consistent with the published literature on both animals and plants, denying the existence of a generalized zoogeographic model.

A multitaxa approach, including several examples of West Mediterranean distributions, such as the Siculo-maghrebian, the Ibero-Maghrebian and the Sardo-Maghrebian distributions, would help to elucidate palaeoecological constraints in dispersal routes and generalized events of vicariance, which led to those biogeographic patterns involving biotas of the two facing lands on West Mediterranean.

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