Mitochondrial DNA phylogeography and population history of *Meladema* diving beetles on the Atlantic Islands and in the Mediterranean basin (Coleoptera, Dytiscidae)

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Abstract

The phylogeny and population history of *Meladema* diving beetles (Coleoptera, Dytiscidae) were examined using mitochondrial DNA sequence from 16S ribosomal RNA and cytochrome oxidase I genes in 51 individuals from 22 populations of the three extant species (*M. imbricata* endemic to the western Canary Islands, *M. lanio* endemic to Madeira and *M. coriacea* widespread in the Western Mediterranean and on the western Canaries), using a combination of phylogenetic and nested clade analyses. Four main lineages are observed within *Meladema*, representing the three recognized species plus Corsican populations of *M. coriacea*. Phylogenetic analyses demonstrate the sister relationship of the two Atlantic Island taxa, and suggest the possible paraphyly of *M. coriacea*. A molecular clock approach reveals that speciation within the genus occurred in the Early Pleistocene, indicating that the Atlantic Island endemics are not Tertiary relict taxa as had been proposed previously. Our results point to past population bottlenecks in all four lineages, with recent (Late-Middle Pleistocene) range expansion in non-Corsican *M. coriacea* and *M. imbricata*. Within the Canary Islands, *M. imbricata* seems to have independently colonized La Gomera and La Palma from Tenerife (although a colonization of La Palma from La Gomera cannot be discarded), and *M. coriacea* has independently colonized Tenerife and Gran Canaria from separate mainland lineages. In the Mediterranean basin this species apparently colonized Corsica on a single occasion, relatively early in its evolutionary history (Early Pleistocene), and has colonized Mallorca recently on multiple occasions. On the only island where *M. coriacea* and *M. imbricata* are broadly sympatric (Tenerife), we report evidence of bidirectional hybridization between the two species.

Keywords: Canary Islands, colonization, conservation, hybridization, nested clade analysis, speciation

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Introduction

Many current paradigms in evolutionary biology and biogeography have arisen from the study of oceanic island radiations. While most evolutionary research on island flora and fauna has focused on Pacific island groups, a growing body of information is emerging on the islands of the North Atlantic, particularly within the biogeographical region of Macaronesia (Juan *et al*. 2000). Macaronesia includes the Canary Islands, Madeira, the Azores, the

Selvages and the Cape Verdes, and is characterized by the presence of what has been assumed to be relict floral assemblages with affinities to the Tertiary floras of Europe (Médail & Quézel 1999; Nakamura *et al*. 2000). New molecular work, however, suggests a more recent origin for some of the endemic taxa (e.g. Helfgott *et al*. 2000; Fuertes-Aguilar *et al*. 2002).

Among the islands of Macaronesia, the Canaries are particularly rich in endemic radiations of invertebrates, many of which lack obvious mainland sister groups and have been interpreted traditionally as Tertiary relicts (e.g. Machado 1987). However, phylogeographical analysis of

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several beetle radiations (Juan *et al.* 1995; Emerson *et al.* 1999, 2000a, 2000b; Rees *et al.* 2001; see Juan *et al.* 2000 for review) suggests much more recent speciation dates, mainly within the Pleistocene. Most studies to date have investigated clades which are endemic to the archipelago, and whose mainland sister group was not identified with certainty. Here we report on the mitochondrial DNA (mtDNA) phylogeography of a clade of diving beetles which is present on both the Canarian and Madeiran archipelagos, and occurs widely within the western Mediterranean basin. The colonization history of this group in Macaronesia is apparently more complex than that of other invertebrates considered to date, and the presence of members of the clade outside the region allows the Atlantic Island lineages to be viewed in a wider context than has been possible in most previous studies.

Meladema Castelnau (Coleoptera: Dytiscidae) is a small genus of diving beetles endemic to the western Palaearctic region (Franciscolo 1979). Members of the genus occur exclusively in permanent running waters, typically occupying deep pools in streams at intermediate altitudes. There are three currently recognized species: *M. imbricata* (Wollaston), endemic to the western Canary Islands of Tenerife, La Gomera and La Palma, where it is severely endangered and restricted to a total of four permanent high altitude streams; *M. lanio* (Fabricius), endemic to the island of Madeira, where it is still relatively abundant; and

M. coriacea Castelnau, found throughout the western Mediterranean basin in southern Europe and North Africa, and also on the western Canary Islands of Gran Canaria, Tenerife and La Gomera (Franciscolo 1979; Machado 1987; Balke *et al.* 1990). The genus therefore contains two narrow-range endemic species, restricted to Macaronesia, and a single widespread taxon, which occurs both within Macaronesia and elsewhere.

We present a mtDNA analysis of these beetles for an investigation of the colonization history and speciation of island populations, and provide evidence for a surprisingly complex pattern of diversification that includes multiple invasions of the Atlantic islands, exchange between islands, and hybridization.

Materials and methods

Taxon sampling and DNA sequencing

All three known species of *Meladema* were included in the study (Table 1, Fig. 1). All known populations of *M. imbricata* on the Canary Islands (Machado 1987; Balke *et al.* 1990) were sampled (16 specimens from Tenerife, La Gomera and La Palma). Three specimens from two populations of *M. lanio* were sampled from Madeira. On the Canaries *M. coriacea* occurs on three islands, of which two were sampled (Tenerife and Gran Canaria), no
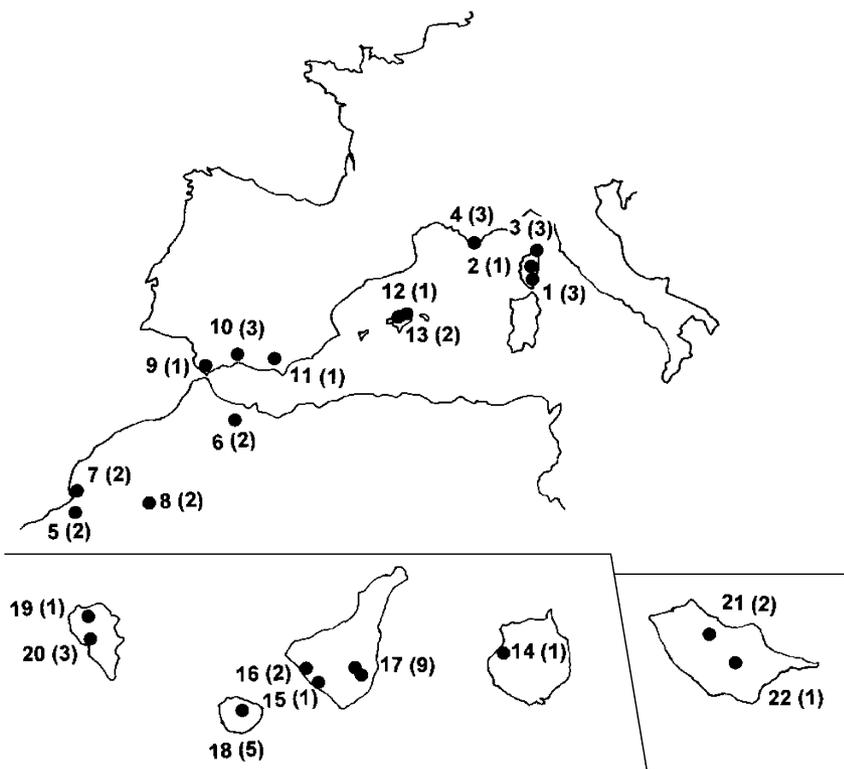

Fig. 1 Location of the studied populations of *Meladema*, with the number of specimens in parentheses. Each locality is identified with the same number given in Table 1.

Table 1 List of the specimens used in the study, with the collection data and haplotype number for the genes COI and 16S rRNA

No.	Species	Locality	Site	Latitude	Longitude	Date	Collector	COI	16S
1.1	coriacea	Corsica	Porto-Vecchio: l'Ospedale	41 35 00 N	9 16 00 E	19.9. 1999	IR & A. Cieslak	1	1
1.2	coriacea	Corsica	Porto-Vecchio: l'Ospedale	Id.	Id.	19.9. 1999	IR & A. Cieslak	1	1
1.3	coriacea	Corsica	Porto-Vecchio: l'Ospedale	Id.	Id.	19.9. 1999	IR & A. Cieslak	1	—
2.4	coriacea	Corsica	Ghisoni: rd. to Campannella	42 06 00 N	9 12 00 E	19.9. 1999	IR & A. Cieslak	2	1
3.5	coriacea	Corsica	Cap Corse: Bertolacce	42 57 00 N	9 25 00 E	21.9. 1999	IR & A. Cieslak	3	2
3.6	coriacea	Corsica	Cap Corse: Bertolacce	Id.	Id.	21.9. 1999	IR & A. Cieslak	4	2
3.7	coriacea	Corsica	Cap Corse: Bertolacce	Id.	Id.	21.9. 1999	IR & A. Cieslak	5	1
4.8	coriacea	France	Var, La Londe-les-Maures: rallon de Valcros	43 08 00 N	6 14 00 E	5.10. 1999	P. Ponel	6	3
4.9	coriacea	France	Var, La Londe-les-Maures: rallon de Valcros	Id.	Id.	5.10. 1999	P. Ponel	6	3
4.10	coriacea	France	Var, La Londe-les-Maures: rallon de Valcros	Id.	Id.	5.10. 1999	P. Ponel	7	3
5.11	coriacea	Morocco	Anti Atlas, Oued Massa: Assif Oumarhuz	29 40 00 N	8 58 00 W	21.7. 1997	IR	8	3
5.12	coriacea	Morocco	Anti Atlas, Oued Massa: Assif Oumarhuz	Id.	Id.	21.7. 1997	IR	9	3
6.13	coriacea	Morocco	Moyen Atlas, Taza: Tazzeka N.P.	34 16 00 N	4 01 00 W	15.7. 1997	IR	10	3
6.14	coriacea	Morocco	Moyen Atlas, Taza: Tazzeka N.P.	Id.	Id.	15.7. 1997	IR	11	3
7.15	coriacea	Morocco	Immouzer-des-Ida-Outanane, Assif Tanit	30 39 46 N	9 21 13 W	21.4. 2001	IR & A. Cieslak	12	3
7.16	coriacea	Morocco	Immouzer-des-Ida-Outanane, Assif Tanit	Id.	Id.	21.4. 2001	IR & A. Cieslak	13	3
8.17	coriacea	Morocco	Tachokchte, Assif Siroua	30 47 51 N	7 31 35 W	19.4. 2001	IR & A. Cieslak	13	3
8.18	coriacea	Morocco	Tachokchte, Assif Siroua	Id.	Id.	19.4. 2001	IR & A. Cieslak	14	3
9.19	coriacea	Spain	Cádiz: Fancinas	36 09 00 N	5 41 00 W	26.7. 1998	IR	13	3
10.20	coriacea	Spain	Córdoba, Baena: Arroyo de las Beatas	36 37 00 N	4 20 00 W	26.9. 1999	M. Baena	14	4
10.21	coriacea	Spain	Córdoba, Baena: Arroyo de las Beatas	Id.	Id.	26.9. 1999	M. Baena	14	4
10.22	coriacea	Spain	Córdoba, Baena: Arroyo de las Beatas	Id.	Id.	26.9. 1999	M. Baena	15	3
11.23	coriacea	Spain	Murcia, river Mula: Fte. Caputa	38 02 00 N	1 29 00 W	19.9. 1999	A.Millan	16	3
12.24	coriacea	Mallorca	Els Casals: Te. Son March	39 54 00 N	3 00 00 E	12.11. 2000	IR & A.Cieslak	13	3
13.25	coriacea	Mallorca	Mortixet: tributary te. Son March	39 54 00 N	2 55 00 E	12.11. 2000	IR & A.Cieslak	17	7
13.26	coriacea	Mallorca	Mortixet: tributary te. Son March	Id.	Id.	12.11. 2000	IR & A.Cieslak	18	3
14.27	coriacea	Gran Canaria	Bco. Guguy grande	28 06 00 N	15 45 00 W	14.4. 2001	IR & A.Cieslak	19	3
15.28	coriacea	Tenerife	Bco. Del Infierno, 900 m	28 15 00 N	16 45 00 W	12.1. 2000	DTB	20	3
16.29	coriacea	Tenerife	Bco. De Masca	28 30 00 N	16 45 00 W	14.1. 2000	DTB	20	3
16.30	coriacea	Tenerife	Bco. De Masca	Id.	Id.	14.1. 2000	DTB	20	3
17.31	imbricata	Tenerife	Barranco del Río	28 30 00 N	16 30 00 W	4. 1998	DTB	20	3
17.32	imbricata	Tenerife	Barranco del Río	Id.	Id.	4. 1998	DTB	20	—
17.33	imbricata	Tenerife	Barranco del Río	Id.	Id.	4. 1998	DTB	27	—
17.34	imbricata	Tenerife	Barranco del Río	Id.	Id.	4. 1998	DTB	28	5
17.35	imbricata	Tenerife	Barranco del Río, 1600 m	Id.	Id.	13.1. 2000	DTB	20	3
17.36	imbricata	Tenerife	Barranco del Río, 1600 m	Id.	Id.	13.1. 2000	DTB	26	8*
17.37	imbricata	Tenerife	Barranco del Río, 1600 m	Id.	Id.	13.1. 2000	DTB	28	5
17.38	coriacea	Tenerife	Barranco del Río, 600 m	Id.	Id.	13.1. 2000	DTB	21	5
17.39	coriacea	Tenerife	Barranco del Río, 600 m	Id.	Id.	13.1. 2000	DTB	21	5
18.40	imbricata	Gomera	El Cedro	28 00 00 N	15 30 00 W	4. 1998	DTB	22	5
18.41	imbricata	Gomera	El Cedro	Id.	Id.	4. 1998	DTB	22	5

Table 1 *Continued*

No.	Species	Locality	Site	Latitude	Longitude	Date	Collector	COI	16S
18.42	imbricata	Gomera	El Cedro	Id.	Id.	4.1998	DTB	22	5
18.43	imbricata	Gomera	El Cedro	Id.	Id.	15.1.2000	DTB	22	5
18.44	imbricata	Gomera	El Cedro	Id.	Id.	15.1.2000	DTB	23	5
19.45	imbricata	La Palma	Barranco del Río above Santa Cruz	28 30 00 N	17 50 00 W	4.1998	DTB	24	5
20.46	imbricata	La Palma	Barranco del Hoyo Verde	28 15 00 N	17 50 00 W	4.1998	DTB	25	5
20.47	imbricata	La Palma	Barranco del Hoyo Verde	Id.	Id.	4.1998	DTB	25	5
20.48	imbricata	La Palma	Barranco del Hoyo Verde	Id.	Id.	4.1998	DTB	25	—
21.49	lanio	Madeira	Ribera dos Cedros			6.1998	L.C. Kelly	29	6
21.50	lanio	Madeira	Levada das Faias			6.1998	L.C. Kelly	30	6
22.51	lanio	Madeira	Levada das Faias			6.1998	L.C. Kelly	31	6

*Only the first part of the fragment (511 bp).
Haplotypes of *M. lanio* were not used in the nested clade analysis.

specimens being found on La Gomera, despite an intensive search. A representative selection of populations of *M. coriacea* was also sampled on the mainland on both shores of the western Mediterranean, from southwestern Morocco to southern France, and on the Mediterranean islands of Mallorca and Corsica (Table 1, Fig. 1). There are some records of *M. coriacea* further east in the Mediterranean region up to Turkey (Guignot 1933; Franciscolo 1979), although it seems to be exceedingly rare in this area and no specimens could be obtained. Outgroups for phylogenetic analyses included two other Western Palaearctic genera of Colymbetini, *Rhantus* and *Colymbetes* (the tribe to which *Meladema* is grouped), plus two genera of Agabini (*Agabus* and *Ilybius*), all within the subfamily Colymbetinae (Nilsson & Roughley 1997). Some of these outgroup sequences were obtained from Ribera *et al.* (2001a, 2001b).

Specimens were collected in absolute ethanol, and muscular tissue used for DNA isolation via a phenol–chloroform extraction as described in Vogler *et al.* (1993). Sequences of 16S rRNA were amplified as a single fragment of *c.* 800 base pairs (bp), using primers 16Sa (5′ ATGTTTTTGTTAAACAGGCG) for the 5′ end of the gene and 16SAlf1 (5′ GCATCACAAAAAGGCTGAGG) for the 3′ end. A single fragment of *c.* 800 bp of COI (from the middle of region E3 to the 3′ end, Lunt *et al.* 1996) was amplified using primers 'Jerry' (5′ CAACATTTA-TTTTGATTTTTTGG) and 'Pat' (5′ TCCAATGCACT-AATCTGCCATATTA) (Simon *et al.* 1994). The 16S haplotype of the specimen with COI27 could not be obtained, and for haplotype 16S8 only the first 511 bp could be sequenced (Table 1). All sequences generated in this study have been deposited in GenBank (accession nos AF428187–AF428237).

The following PCR cycling conditions were used for DNA amplification: 1–2 min at 95 °C, 30 s at 94 °C, 30 s at 47–50 °C (depending on the melting temperatures of the primer pair used), 1–2 min at 72 °C (repeated for 35–40 cycles) and 10 min at 72 °C. Amplification products were purified using a GeneClean II kit (Bio 101, Inc.). Automated DNA sequencing reagents were supplied by Perkin Elmer Applied BioSystems Ltd (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit). Sequencing reactions were purified by ethanol precipitation and were electrophoresed on an ABI3700 sequencer. Sequencing errors/ambiguities were edited using the SEQUENCHER 3.0 software package (Gene Codes Corporation).

Phylogenetic analysis

Sequences for COI were of identical length, and 16S rRNA sequences differed in length only minimally and could be aligned by indels at six nucleotides positions. There was no length variation in the ingroup. Alignment was performed

manually by attempting to maximize sequence similarities. Phylogenetic analysis was performed with PAUP4.0b6 (Swofford 1999), using parsimony procedures for tree reconstruction, and using gaps as a fifth character. Coding gaps as a missing character did not affect tree topologies. Constraint trees for determining Bremer support values (Bremer 1994) and partitioned Bremer support values were generated with TREEROT (Sorenson 1996).

Maximum likelihood searches were conducted with a reduced data set, including all outgroups plus the most basal haplotype of each of the four main clades found in the parsimony analysis (see below). The optimal evolutionary model was estimated using MODELTEST 3.04 (Posada & Crandall 1998). To estimate node ages we fitted ML branch lengths assuming a molecular clock using the optimal model as estimated with MODELTEST, and compared the likelihood to that obtained assuming no clock (Felsenstein 1981).

Rate of diversification

To study the rate of diversification of lineages within *Meladema* we used the log-lineage through time approach (Harvey *et al.* 1994; Nee *et al.* 1995). Using the ultrametric cladogram obtained for estimating node ages (see above), we plotted the logarithm of the cumulative number of lineages against the molecular distance of each node from the root. Under a constant birth–death model, the expectation is a straight line of slope b–d (b = speciation rate, d = extinction rate), with an upturn in the number of lineages towards the present with slope b. If there is a change in the rate of diversification, or a high level of background extinction, the plot should show a pronounced upturn towards the present (Nee *et al.* 1995; Barraclough & Nee 2001). The upturn as a result of extinction is expected to start at around 1/(b−d) time units before the present, whereas a change in diversification rate could occur at any time (Barraclough & Nee 2001). It is thus possible to refute extinction only when the time of the change in diversification rate significantly differs from 1/(b−d) time units. On the contrary, incomplete sampling is expected to lead to an apparent decrease in diversification rate over time, because recently formed lineages will tend to be underrepresented in the sample.

To calibrate branch lengths we applied an approximate early divergence rate of 2% per million years (Myr) for arthropod mitochondrial DNA (Brower 1994), which corresponds to a base rate (per branch) of 0.01 substitutions/site/Myr. In Ribera *et al.* (2001b) it is shown that for species of Colymbetinae (genus *Agabus*) the 2% per Myr estimation for the combined COI plus 16S rRNA genes is equivalent to the much lower estimate of Gómez-Zurita *et al.* (2000) in *Timarcha* leaf beetles based on the 16S rRNA gene alone (0.76% divergence per Myr).

Nested clade analysis

Minimum spanning networks for nested clade analysis were computed using TCSv1.06 (Clement *et al.* 2000), which estimates gene genealogies from DNA sequences following the method described in Templeton *et al.* (1992). Networks were constructed initially with the cut-off limit of a number of steps (single mutational events) corresponding to a probability of 95%, although for the COI gene the number of steps was subsequently increased to allow the integration of the two networks within *M. coriacea*. The haplotypes were grouped manually in increasingly inclusive clades (or nestings) following the algorithm described in Templeton *et al.* (1987) and Templeton & Sing (1993), up to the final level of nesting comprising the entire network.

The relationship between the geographical distribution of haplotypes and the parsimony networks was tested using the method of random permutations proposed by Templeton & Sing (1993) as implemented in GEODIS 2.0 (Posada *et al.* 2000), with 10 000 permutations. According to this method, clades with genetic and/or geographical variation within a nested category are tested against their geographical locality, with the null hypothesis of no geographical association. Results were interpreted with the inference key provided on the GEODIS web page, which is modified from Templeton *et al.* (1995) and Templeton (1998).

Results

Parsimony analysis

Eight different 16S rRNA haplotypes of 832 bp were found among the ingroup specimens studied (Table 1, see Table 2 for distances). A branch and bound search coding gaps as a fifth character resulted in a single tree with the haplotypes 16S1 and 16S2 (Corsican *Meladema coriacea*) basal and the haplotypes of the remaining *M. coriacea* sister to *M. imbricata* and *M. lanio* (not shown). All sequences of the partial COI gene were 802 bp in length (see Tables 1 and 2 for the list of haplotypes and distances). A heuristic search of 1000 Tree-Bisection-Reconnection (TBR) replicates resulted in five equally parsimonious trees with an unresolved polytomy of four main clades: *M. lanio*, *M. imbricata*, the Corsican populations of *M. coriacea* and the remaining *M. coriacea*.

The partition homogeneity test was not significant (*P* = 0.38, 100 replicated heuristic searches of 100 TBR replicas each), indicating overall character congruence among the two genes. The strict consensus of the 12 most parsimonious trees obtained in the combined analysis placed the Corsican *M. coriacea* as sister to an unresolved polytomy of three monophyletic groups: the remaining *M. coriacea*, *M. imbricata* and *M. lanio* (Figs 2 and 3). Within *M. imbricata*,

Table 2 Length of the aligned sequences of 16S rRNA and COI, with character variability and tree statistics

Partition	Size	Inf.	Min. *P*	Max. *P*	No. trees	Length	CI
16S	838	133 (10)	0.001 (1–2)	0.10 (6–8)/0.13	1	324	0.72
COI	802	185 (56)	0.001 (26,27–28)	0.04 (25–31)/0.16	5	604	0.57
COI + 16S	1640	318 (66)	0.0006	0.03/0.15	12	938	0.60

Size, size of the (aligned) data matrix; Inf., parsimony informative characters (in brackets, ingroup only); Min. P, minimum uncorrected distances (in brackets, corresponding haplotypes); Max. P, maximum uncorrected distances within the ingroup before the slash (in brackets, corresponding haplotypes), and with outgroups after the slash; No. trees, number of most parsimonious trees; Length, length of the most parsimonious trees; CI, consistency index.

Fig. 2 Phylogenetic hypothesis for *Meladema* haplotypes based on a combined maximum parsimony analysis of the 16S rRNA and COI genes (strict consensus of the two reweighted trees). Numbers above the branches are partitioned Bremer support values (16S/COI) (node 25, with a zero global Bremer support, was not recovered in the strict consensus of the 12 shortest trees). Numbers below the branches are bootstrap proportions (only shown if > 50%). Numbers at the branch points are node identifiers. See Table 1 for details of the haplotypes.

the haplotypes of the islands of La Palma and La Gomera were reciprocally monophyletic, while the ones from Tenerife were paraphyletic and basal (Figs 2 and 3). Within the remaining *M. coriacea* the main lineage split separated Oriental (SE France, SE Spain and NE Morocco) from Occidental populations (Tenerife, Gran Canaria, SW Morocco,

S Spain, with the inclusion of Mallorca). Within each of these main groups there was no apparent geographical structure. The Mallorcan haplotypes (COI17–16S7 and COI18–16S3) were paraphyletic with respect to the Moroccan haplotype COI12–16S3. The haplotype found in the specimen from Gran Canaria (COI19–16S3) was sister to that
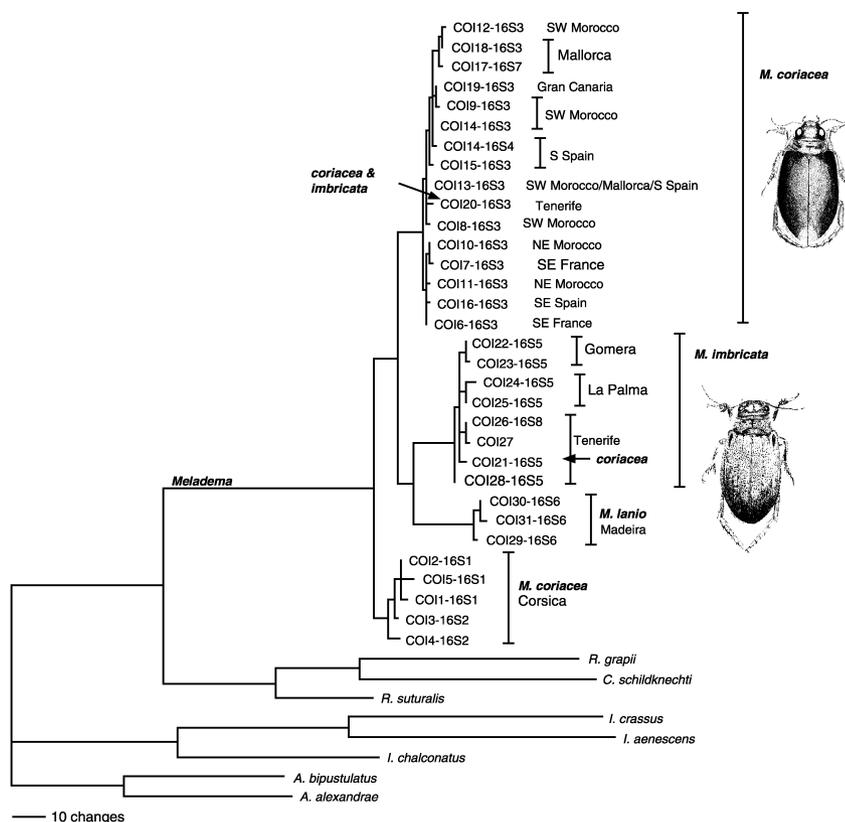
Fig. 3 Phylogram of one of the 12 most parsimonious trees obtained in the combined maximum parsimony analysis of the 16S rRNA and COI genes, with the origin of the specimens and the morphological species. Note the distinct morphology of the two coexisting species *M. coriacea* and *M. imbricata* (*M. lanio* is externally very similar to *M. imbricata*).

found in specimens from SW Morocco (COI9–16S3), and the haplotype from Tenerife (COI20–16S3) was placed in an unresolved basal polytomy (Figs 2 and 3).

After a single round of reweighting according to the CI, the new search resulted in two equally parsimonious trees of CI = 0.8. The polytomy among three of the main lineages was resolved by the placement of *M. lanio* as sister to *M. imbricata*, and both sister to the remaining *M. coriacea* (Fig. 2). The protein sequence of the COI gene provided further support for the sister relationship between *M. imbricata* and *M. lanio*.

For the calculation of the partitioned Bremer support and the estimation of branch lengths a tree with the same topology as the reweighted tree was chosen from among the 12 most parsimonious unweighted trees. Most of the nodes had high bootstrap and Bremer support (Fig. 2), in particular those corresponding to the genus *Meladema* (node 16) and the four main clades within the genus (*M. lanio*, node 24; *M. imbricata*, node 22; Corsican *M. coriacea*, node 9; and the remaining *M. coriacea*, node 26), all with bootstrap values over 90%.

Two specimens morphologically identical to other *M. coriacea* from the island of Tenerife (Barranco del Río at 600 m) had a unique mitochondrial haplotype (COI21–16S5) which was most closely related to other haplotypes of *M. imbricata* from the same island (Figs 2 and 3). In the

same area, but at higher altitude (1600 m), three specimens morphologically identical to co-occurring *M. imbricata* exhibited the COI20–16S3 haplotype, which is otherwise restricted to *M. coriacea* and more widespread (Table 1, Figs 1 and 2). The high altitude stretches of the Barranco del Río are the only known locality for *M. imbricata* on Tenerife (Machado 1987), and this stream is the only one where two species of the genus can be found within the same drainage basin, although *M. imbricata* is restricted to the upper, and *M. coriacea* the lower part of the stream.

Maximum likelihood analysis

A maximum likelihood analysis was performed to assess the relationships of the four main lineages of *Meladema* using a reduced data set including all outgroups and the most basal haplotype of each main lineage (COI28–16S5, COI29–16S6, COI4–16S2, COI6–16S3). The optimal substitution model of the reduced data set was identical to that for the whole data, as estimated with MODELTEST (base frequency estimated, unequal rates with a gamma shape parameter of 1.22). A heuristic search of 100 random addition replicates resulted in a single tree (not shown) of –lnL 6090.6, which differed with respect to the maximum parsimony tree in the rooting of the ingroup. The haplotype of the continental *M. coriacea* (COI6–16S3) was basal, and paraphyletic with

Table 3 Comparison of ML values for different roots of the ingroup when enforcing a molecular clock for the combined (16S + COI) analysis

	Root				
	(M. coriacea) + (M. imb + lanio)	M. coriacea Corsica	M. coriacea continental	M. imbricata	M. lanio
−LnL (no clock)	2802.23	2802.23	2802.23	2802.23	2802.23
−LnL (clock)	2825.46	2825.92	2825.92	2825.46	2824.92
χ^2	46.46	47.38	47.38	46.46	45.38
P	0.03	0.02	0.02	0.03	0.04

−LnL(clock), maximum likelihood enforcing a molecular clock; χ^2, chi-square value of the ML ratio (30 degrees of freedom); P, probability associated to the χ^2 (see text for the substitutions models).

respect to that of the Corsican *M. coriacea* (COI4–16S2). *Meladema imbricata* (COI28–16S5) and *M. lanio* (COI29–16S6) were sisters and derived within *M. coriacea*, in agreement with the reweighted maximum parsimony tree. An exploratory search (six replicas) with the full data set obtained the same result (− lnL 6466.8). Maximum likelihood searches of the ingroup only for both genes confirmed the sister relationship between *M. lanio* and *M. imbricata*, to the exclusion of the two lineages of *M. coriacea.*

Rate variation

We estimated an ultrametric tree for the ingroup using the preferred ML model for the combined analysis and enforcing a molecular clock, using a tree in which *M. lanio* plus *M. imbricata* were sister to *M. coriacea* (Table 3, Fig. 4). Different rootings resulted in very similar trees, with the estimated length of all branches within each of the four main lineages almost identical. The only differences were in the relative length of the basal branches defining the relationships among the four main clades, which were always very short.

The test for departure from a molecular clock model was significant for the combined analyses (P = 0.03). Using different rootings of the tree did not change this significance (Table 3), but due to the small amount of total variation, and the distortion of the branch lengths introduced by alternative methods (such as the nonparametric rate smoothing of Sanderson 1997; see Sanderson 2002), we opted for enforcing a molecular clock using the optimum estimated ML model.

The shortest of the branches between lineages is the one leading to the Corsican *M. coriacea* (0.0091 substitutions/site), while the longest of the within-lineage branches is more than three times shorter (0.0028, also within Corsican *M. coriacea*). On average, branches between lineages are an order of magnitude longer than those within. These differences are also reflected in the raw data: the average P distance within lineages is 0.003 (n = 153 ± SD 1.55×10^{-6}), while the average distance between lineages is 0.019 (n = 200 ± SD 1.43×10^{-5}).
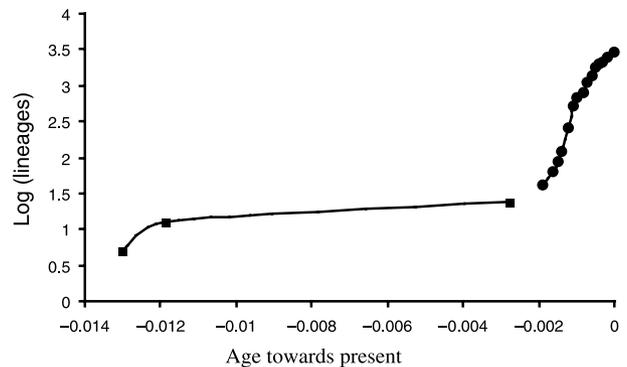

Fig. 5 Lineages through time plot for *Meladema*. The y-axis is the log number of lineages. The x-axis represents the relative age towards the present, based on the branch lengths in the ultrametric tree in Fig. 4. The basal polytomy with the three species was resolved arbitrarily at half the length of the branch leading to *M. coriacea* (i.e. 0.0011). Squares, main four lineages; circles, diversification within main lineages.

To assess the possible role of extinction in shaping the tree we use the log-lineages though time approach. The resulting plot (Fig. 5) clearly reflects the change in rate variation between and within the main clades. There is an obvious change in slope when crossing the within–clade boundary, with a sudden increase in the number of lineages, with again a slight decrease towards the present. The estimated slopes are significantly different (as measured with 95% confidence intervals of the regression parameters). The estimated 1/b−d = 0.02, while the inflexion point is at 0.003 units before present, an order of magnitude smaller. Hence, the increased slope towards the present cannot be interpreted as high background extinction (see Methods).

Geographical nested clade analysis

All 16S rRNA haplotypes were joined in a single network with 95% probability, with a maximum distance of three mutational steps between haplotypes. The unrooted
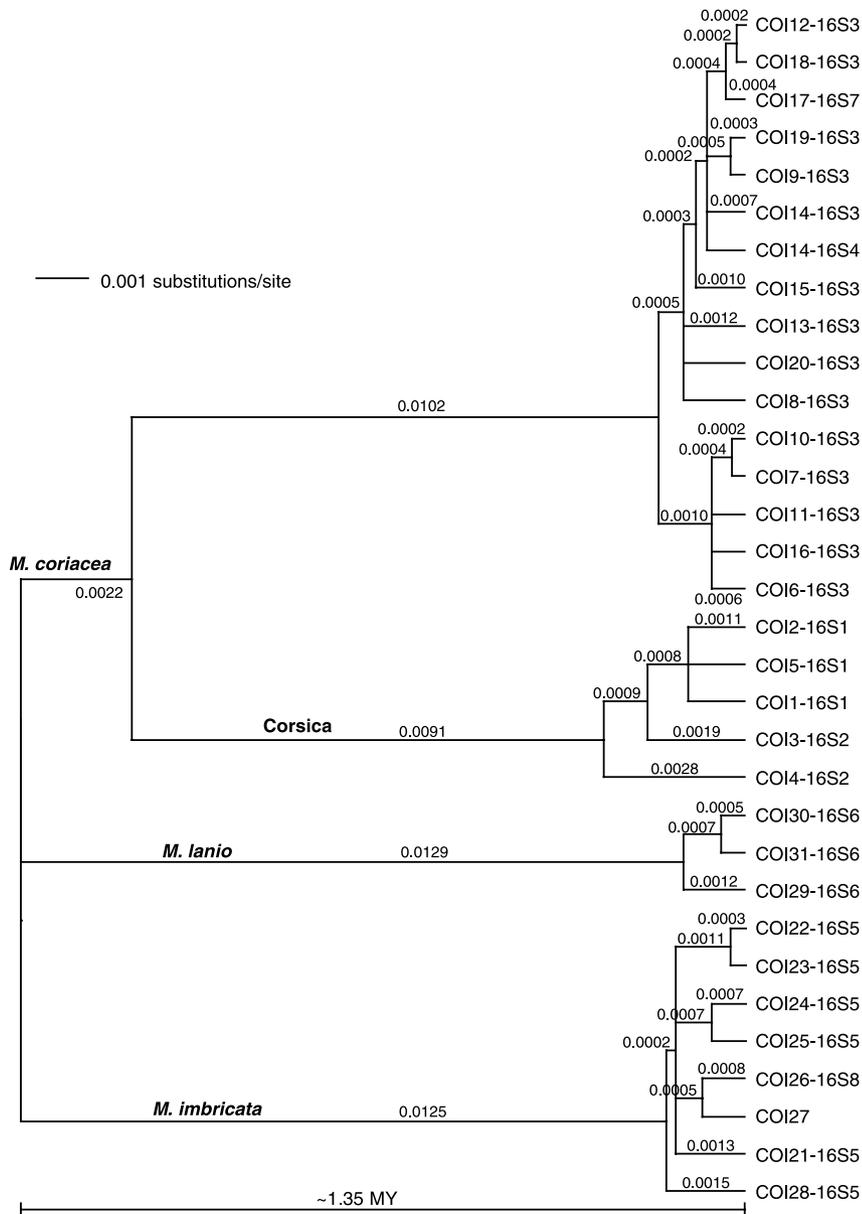
Fig. 4 Ultrametric tree for *Meladema*, obtained with maximum likelihood (optimal model) enforcing a molecular clock and the monophyly of *M. coriacea*. Numbers on branches are their estimated length. The absolute time scale corresponds to an estimated rate of 0.01 substitution/site/Myr/branch, equivalent to the standard divergence rate of 2% per My.

network reflected the topology of the maximum parsimony tree, with *M. lanio* linked to Corsican *M. coriacea* (Fig. 6a). The whole network was included in three two-step clades, and the central haplotype was estimated to be 16S3, the most widespread of the non-Corsican *M. coriacea*, in agreement with the topology of the ML analysis with outgroups included.

In the geographical association analysis, one one-step clade showed a significant association, comprised of haplotypes exclusive to two of the three specimens from Cordoba (Spain), 16S4, Table 4 and Fig. 6. According to the inference chain this geographical arrangement can be interpreted as restricted gene flow with isolation by distance. Among the two-step clades, only that containing the haplotypes from

Corsica and Madeira was significant (2.3, Table 4) and interpreted as the result of long-distance colonization. The total cladogram also had significant geographical structure, indicating contiguous range expansion (Table 4).

For the COI data set, the 95% threshold for linking the haplotypes in the nested clade (12 steps) produced four independent networks, representing the four main clades. If the number of steps was increased to 13, the network of the Corsican *M. coriacea* was united to that of the remaining *M. coriacea*. The network for *M. lanio* (not shown) could not be statistically analysed, as there was not enough variation within clades. The whole network of *M. imbricata* was included in two two-step clades, and that of *M. coriacea* in two four-step clades (Fig. 6b). The central haplotype for *M.*
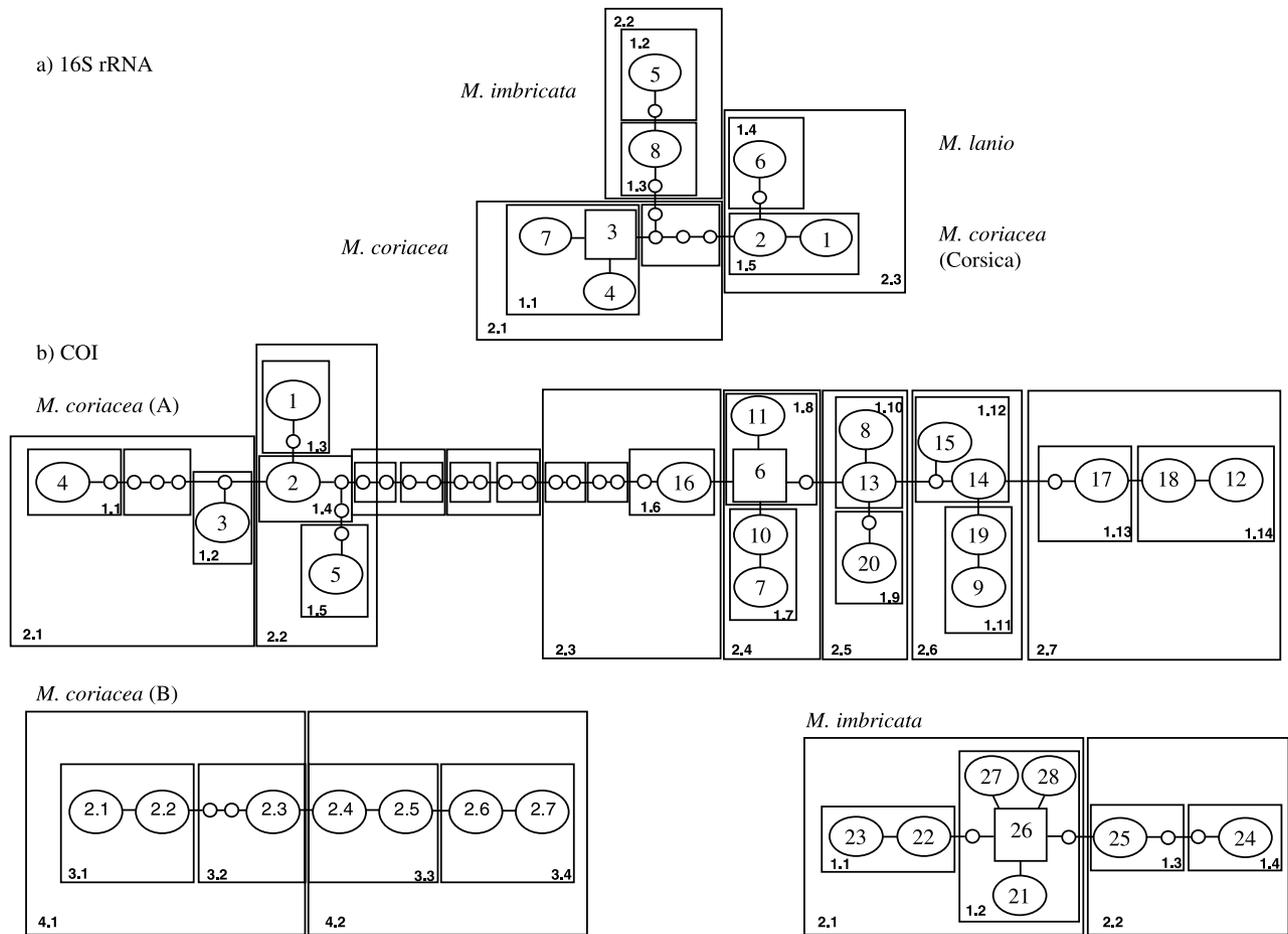
Fig. 6 Unrooted haplotype networks for the genes 16S rRNA (a) and COI (b), with the associated nested clade design. The haplotype network of 16S rRNA is the estimated 95% probability cladogram obtained with tcsv1.06 (see Methods). The network for COI is one step longer than the 95% estimation, to allow the inclusion of all specimens of *M. coriacea* in the same network (see Results). Haplotype numbers refer to Table 1; each connection represents one mutational step. Intermediate missing haplotypes represented by empty circles. Boxes represent clades of increasing number of steps, numbers inside boxes represent the number of steps (first) and the serial order of the clade (second). For clarity, three- and four-step clades of *M. coriacea* are represented in a separate network (*M. coriacea* B).

Table 4 Clades with significant geographical structure (*P* < 0.05), with their interpretation according to Templeton *et al.* (1995) (updated in the GEODIS web page) (see Fig. 5 for the composition of the clades, and Table 1 for the localities of the haplotypes). t.c., total cladogram. Numbers refer to the consecutive steps in the inference chain, with the final interpretation (see the GeoDis web page)

Network	Clade	Chain inference
16S	1.1	2-3-4-NO restricted gene flow with isolation by distance (16S4)
	2.3	2–11 YES range expansion — 12–13 YES long distance colonization (16S6 from 16S2)
	t.c.	2–11 YES range expansion — 12 NO contiguous range expansion
COI *imbricata*	2.1	2–11 YES: range expansion — 12–13 YES long distance colonization (COI22 and COI23 from COI26)
	t.c.	2–11 YES range expansion — 12 NO contiguous range expansion (2.2) (marginally significant, p 0.6 for 2.1: 13 YES: long distance colonization)
COI *coriacea*	2.5	2-3-4-9 NO past fragmentation (COI20)
	t.c.	2-3-5-15 NO past fragmentation (COI1-COI5, COI20)

imbricata was estimated to be COI26 (from Tenerife), and that for *M. coriacea* COI6 (from SE France, Table 1 and Fig. 3).

The geographical correlation for clade 2.1 in *M. imbricata* was significant and interpreted as a long-distance colonization of La Gomera by haplotypes from Tenerife. The structure of the entire network was also significant, indicating contiguous range expansion for clade 2.2 (of La Palma haplotypes from Tenerife) or, with a marginal significance ($P = 0.6$), long-distance colonization of La Gomera from Tenerife (Table 4). In *M. coriacea*, the two-step clade 2.5 had a significant structure, interpreted as past fragmentation of the haplotype from Tenerife (COI20), the southwestern Anti Atlas (COI8, the locality closest to the Canaries) and the Haute Atlas (COI13) (Table 4). This interpretation is supported by the fact that the Tenerife haplotype is separated from the rest by an additional mutational step (Fig. 6). For the total cladogram, the interpretation is again past fragmentation, involving both the haplotypes from Tenerife (COI20) and Corsica (COI1–COI5).

Discussion

Phylogeny of the species and populations of Meladema

Our phylogenetic analysis revealed four divergent highly supported clades, but with poorly defined relationships among them. Three of these clades represent the three recognized species of the genus, but the separation of the Corsican *Meladema coriacea* was entirely unexpected, as they are morphologically identical to other populations of the species. The populations from Corsica are genetically as divergent from the remaining *M. coriacea* as from any of the other species of the genus. The level of detected genetic variation within this Corsican clade is similar, if not larger than that seen within other lineages. This level of genetic divergence is particularly surprising when compared to the remaining *M. coriacea*, which show lower levels of divergence, despite their much wider distribution from the Canary Islands and southern Morocco to SE France, and being represented by a larger number of individuals in our analyses (25 vs. 7). Three individuals sampled in a single locality, a small residual stream pool on Cap Corse, had three different haplotypes, with the highest within-clade sequence divergence overall.

There is no reason to attribute this pattern to a sampling artefact. As shown in the lineage-through-time plot, the decrease in the slope towards the present is not pronounced, suggesting that the sampling within the main lineages is appropriate. All extant species of the genus and all known populations of *M. imbricata* were sampled, together with two populations of *M. lanio* from different areas of the single island on which this species is found. The sampling of western populations of *M. coriacea* — the ones likely to be related to the Atlantic Island endemics — is also comprehensive, and only eastern Mediterranean populations of the species are missing. However, in the light of the unexpected level of divergence of Corsican beetles, it may be that other populations of *M. coriacea* (including those of Sardinia and mainland Italy) would also prove to be highly divergent genetically. The change in diversification rate from between to within clades cannot be attributed to random extinction of lineages, as shown by the position of the inflexion point in the lineages-through-time plot.

Our mtDNA data suggest that Corsican *Meladema* have been isolated from other populations for considerable time, and that this isolation may predate the divergence of the Atlantic Island lineages. Several synapomorphies would diagnose the Corsican population as a distinct phylogenetic species (Cracraft 1983). Although it may be desirable that this fact is reflected in the taxonomic status of this population, formal changes to the taxonomy of the group should await further sampling of the eastern populations. The question of species status also needs to be seen in the light of the lack of morphological differences, and in particular the lack of any detectable differences in male genitalia, which presumably establish mating incompatibility at early stages of divergence in Coleoptera (Eberhard 1985). Such differences clearly exist between each of the two Atlantic Island taxa and *M. coriacea* (Machado 1987), and presumably restrict interbreeding, although the molecular analysis attests to some hybridization in zones of sympatry between *M. imbricata* and *M. coriacea* (see below). The complete allopatry of the *M. coriacea* populations in Corsica and elsewhere, and the lack of evidence of divergence in specific mate recognition systems (*sensu* Patterson 1985), makes it impossible to assess their species status on the basis of criteria of reproductive isolation (Mayr 1963).

It is, however, interesting that the Atlantic Island species have diverged morphologically and ecologically during a time window that is equivalent to the separation of the Corsican populations, which did not acquire obvious morphological differences. This observation may point to the role of ecological factors in allopatric speciation (Schluter 1998, 2000, 2001), because the high-altitude streams occupied by endemic *Meladema* species on the subtropical Atlantic Islands differ in flow regime and climate from those in which members of the genus occur elsewhere. The two Atlantic Island taxa appear as sister groups in almost all our analyses, indicating that they arose from a common ancestor, apparently derived from mainland populations of *Meladema* in the Early Pleistocene.

The main weakness of our phylogenetic analysis is the difficulty to establish the basal ingroup node. Based on the combined parsimony analysis, and in agreement with the COI gene alone, the most basal lineage would be the Corsican *M. coriacea*, although with low bootstrap (45%) and Bremer support values. According to ML the basal clade

would be the non-Corsican *M. coriacea*, in agreement with the estimated central haplotype in the cladistic unrooted network (for both 16S rRNA and COI), and the parsimony analysis of 16S rRNA. In all cases, the most probable scenario is a sister relationship between the endemic island species, *M. lanio* and *M. imbricata*, with the paraphyly of *M. coriacea*. However, this conclusion is clearly dependent on the rooting of the ingroup clade, which is hampered by the great divergence of the closest relatives of *Meladema*. Hence the insertion of the root node, and any conclusions about the most basal node within *Meladema*, remain questionable. In all analyses performed on the ingroup lineages only, the sister relationship of *M. imbricata* and *M. lanio* was well supported, to the exclusion of the two *M. coriacea* clades, and hence the morphological similarity of the latter could be best explained as a plesiomorphic feature common to both of them.

Phylogeography and rate variation

According to our results, the split among the four main lineages within *Meladema* would have taken place in close temporal succession. There is a general lack of data to calibrate molecular clocks in beetles, but accepting the standard rate of variation of *c*. 2% per Myr (Brower 1994) for the combined 16S rRNA and COI genes (equivalent to the estimated rate of Gómez-Zurita *et al*. 2000 for the gene 16S rRNA alone, see Ribera *et al*. 2001b), this split would have taken place between 1.35 and 1.15 Myr (Fig. 4). This places diversification within the genus, and the origin of the Atlantic Island endemics, well within the Early Pleistocene. Their association with the laurel forest on the islands (Machado 1987; Balke & Hendrich 1989; Balke *et al*. 1990) is therefore secondary, as these species do not appear to be late Tertiary relics. In fact, *M. imbricata* is not restricted to *laurisilva*, as suggested by Balke *et al*. (1990), but instead occurs in high altitude permanent streams in either laurel or pine woodlands (DTB, unpublished). Similar conclusions apply to the Corsican populations, which would not be the product of a deep vicariant separation from the continent. The last land connection between Corsica and the mainland was at the end of the Messinian salinity crisis 5.33 Myr ago (Krijgsman *et al*. 1999), and the formation of the Tyrrhenian islands dates to approximately 12–15 My (see Steinfartz *et al*. 2000 and references therein). Both dates are well beyond the origin of the Corsican lineage, even allowing for great uncertainty with our clock estimation. A recent origin of the Atlantic Island endemics is in keeping with recent molecular work on terrestrial radiations on the Canaries (e.g. Juan *et al*. 2000), all of which point to relatively recent speciation events.

With a standard estimate, the maximal divergences within each of the four major lineages range from 270 000 (Corsican *M. coriacea*) to 120 000 years (*M. lanio*). The rel-

atively long branches leading to the main lineages have to be interpreted as a recent coalescence of the extant haplotypes, due probably to marked population bottlenecks in each lineage. This is not unexpected in the case of the three island endemic lineages (*M. imbricata*, *M. lanio* and Corsican *M. coriacea*), where bottleneck events may have accompanied long-distance colonization. It is more surprising, however, for the populations of *M. coriacea* from the western Mediterranean, including Mallorca and the Canaries.

Although the coalescence within the four major lineages dates to approximately the same time, their diversification cannot be attributed readily to a single common environmental or geological trigger, as the uncertainty in our clock estimation is much larger than many of the cycles of climatic change affecting the western Mediterranean area (Tzedakis *et al*. 1997; Charles 1998). What seems clear is that at some point the non-Corsican *M. coriacea* experienced a rapid range expansion, with a split among oriental and occidental lineages, but with little geographical structure within each of these clades, as seen in the nested clade analysis. Only the haplotype of the populations from Tenerife shows a significant association with distance, suggesting an early colonization of the island. The sampled population in Gran Canaria is of an independent, more recent origin, and clearly related to some haplotypes in south Morocco and southern Spain. The island of Mallorca was also recently and repeatedly colonized, with two of the three haplotypes sampled here paraphyletic with respect to a haplotype from Morocco, and a third haplotype in common with Morocco. The similarity in the level of genetic variation within each of the four lineages (both when estimated in an ultrametric tree or when considering the raw sequences), despite the large difference in the number of individuals sampled and the distributional areas of the species, also points to a similar recent expansion of *M. coriacea*.

Within the Corsican *M. coriacea*, the deep divergences in such a small area may be related to the mountainous geography of the island (with a maximum altitude over 2000 m). This would have provided ample opportunities for repeated cycles of isolation in small streams in valley bottoms in cold periods with the subsequent mixing of populations when conditions ameliorated (Hewitt 1996, 2000). Within *M. imbricata*, the most divergent haplotypes are present on Tenerife, with four haplotypes in the same population in Barranco del Río (one of them corresponding to the specimens morphologically identical to *M. coriacea*). These haplotypes are paraphyletic with respect to the monophyletic populations of La Gomera and La Palma, and their split can be dated to approximately 135 000 years BP according to our calibration. The haplotypes of Gomera and La Palma are unrelated, as established in the nested clade analysis, and hence represent lineages independently derived from Tenerife (although the significance of the

geographical association with La Palma was only marginal, and thus not conclusive on this point). The colonization sequence does therefore proceed from the oldest to younger islands, but this cannot be related to their sequence of geological origination, as the evolutionary origin and population divergence of *M. imbricata* is much more recent than the emergence of both Tenerife and La Gomera (*c*. 11.6 and 10 Mya, respectively, Juan *et al*. 2000).

Conservation issues and hybridization

The species of *Meladema* are the largest freshwater animals native to the Macaronesian islands, and the largest endemic Coleoptera. They are restricted to the upper reaches of larger permanent streams, in well-preserved *laurisilva/Pinus* forest, and as such are considered to be highly vulnerable and potentially under threat on all islands on which they occur. The situation is particularly serious on the Canary Islands, where anthropogenic water abstraction and diversion has resulted in a 10-fold decrease in the number of permanent stream systems since the mid-20th century (Malmqvist *et al*. 1995; Rodríguez Brito 1995). *Meladema imbricata* is possibly the most severely endangered aquatic invertebrate within the European Union, being restricted to a single stream on each of Tenerife and La Gomera, and two localities on La Palma. Although it is still possible that the species persists elsewhere on the latter, there are no further streams suitable for this species remaining on Tenerife or La Gomera. On Tenerife, *M. imbricata* occurs exclusively in the upper reaches of Barranco del Río where *M. coriacea* is abundant in the lower portions. Although both species have never been found to coexist, the fact that three of seven individuals of *M. imbricata* from this locality exhibit a *M. coriacea* haplotype (COI20–16S3) is indication of genetic introgression between the two taxa. This can only be interpreted as hybridization, despite the lack of morphological intermediates between these two distinct species (Fig. 2), and not as ancestral polymorphism, as the COI haplotype is derived within *M. coriacea*, and the same as other specimens of *M. coriacea* from Tenerife (including Barranco del Río). Conversely, some specimens of *M. coriacea* carried mtDNA haplotypes from within the *M. imbricata* clade. Wirtz (1999) noted that, as a result of female choice, most animal hybridization is unidirectional between females of a rare species and males of a more common one. Our results show that hybridization in *Meladema* has been bidirectional, but this can still be related to the local relative abundance of each species. Bidirectional hybridization can occur if females of one species colonize an area dominated by the other, accepting heterospecific males if given no choice, and the resulting hybrid females backcross with males from their parental population. *Meladema imbricata* individuals may be washed downstream to areas occupied by *M. coriacea*, particularly during periods of high water flow, whereas extreme droughts in low elevation streams may favour upstream movements of *M. coriacea*.

This scenario would suggest that *M. imbricata* is doubly threatened on Tenerife, because of its geographical restriction to a single locality and the risk of continued genetic introgression from *M. coriacea*. The high genetic diversity of *M. imbricata*, the lack of morphological intermediates and the apparent long presence of *M. coriacea* on Tenerife suggest, however, that current hybridization between these two species is limited and the two taxa remain distinct, probably because of their habitat differentiation. However, with continued water extractions from lowland streams the two species may come into contact more frequently, and hybridization may reach a point where it threatens the existence of *M. imbricata* as a distinct taxon (Wolf *et al*. 2001).

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