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The Mediterranean-southern African disjunct distribution pattern

Molecular biogeography of Mediterranean and southern African disjunctions as exemplified by pollen beetles of the *Meligethes planiusculus* species-group and related taxa (Coleoptera: Nitidulidae; Meligethinae)

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SUMMARY

We investigated the apparent disjunction expressed in two related assemblages of species of the genus *Meligethes*, i.e. *M. fruticola* and its allies in the Cape region of South Africa and the *M. planiusculus* group in the Mediterranean region. We also inferred possible dynamics in the radiation of the *Meligethes planiusculus* complex within Macaronesia utilizing morphological, molecular and bionomical data, exploring potential historical and palaeoecological scenarios regulated by a molecular clock dating system. We reconstructed phylogenetic relationships of the *M. planiusculus* complex and of related Mediterranean (*M. tristis*), tropical (*M. scotti*) and southern African (*M. chevrolati*, *M. conformis*, and *M. fruticola*) species, using COI mitochondrial gene sequences. Phylogenetic reconstructions support an unambiguous distinction of two major clades grouping European-Mediterranean *M. canariensis*, *M. isoplexidis* and *M. planiusculus* specimens in one clade and the South-African specimens related to *M. fruticola* in another. Molecular markers suggested that the European-Mediterranean taxon *M. tristis* is unambiguously more distantly related to the partly sympatric *M. canariensis*, *M. isoplexidis* and *M. planiusculus*, than to the geographically isolated Southern African taxon, *M. fruticola*. However, morphological data revealed that *M. tristis* is more closely related to *M. planiusculus* and its allies while occupying a position internal to the *M. planiusculus* species group, but external to the *M. planiusculus* complex. Results of divergence estimation analyses suggest a splitting between ancestors of the European-Mediterranean species of the *M. planiusculus* complex and that of the African species *M. fruticola* at ~21-23 MYA. Molecular results also demonstrated that the remaining Afrotropical species are more related to the *M. planiusculus* and *M. fruticola* complexes than to *M. tristis*. This evidence clearly indicates that the Holarctic *M. planiusculus* group represents a paraphyletic assemblage with heterochronic Afrotropical origin. The estimated times of divergence supports evidence from other researchers of an 'Arid Corridor', or of a 'Central or Eastern

High Africa Corridor', which connected several times in the last twenty MY the European-Mediterranean and eastern/southern African areas, and facilitated species migration northwards and southwards. The dynamics of the *Meligethes planiusculus* complex radiation in Macaronesia apparently followed a contradictory biogeographical scenario than the sequence of events recently hypothesized for their host-plants (*Echium*, Boraginaceae).

INTRODUCTION

Most present-day affinities between the Mediterranean flora and fauna and their respective counterparts in the Cape region of South Africa have been analysed via three different hypotheses: 1) the intervening phenomena of community-scale convergent evolution under the pressure of shared macroclimatic parameters (e.g. Cowling et al., 1996, 1997; Cox, 2001; Fryxell, 1967; Gess, 1992; Raven, 1973); 2) the disparity in sampling efforts and knowledge between tropical and subtropical Africa and the Mediterranean and Cape sub-regions (Bowden, 1978; Kirk-Spriggs, 2003) and 3) long-distance dispersal mechanisms (Coleman et al., 2003). A number of well-documented cases illustrating examples of true phylogenetic relationships have been demonstrated in a number of groups involving the presence of relatively recent common ancestors and repeated availability of 'arid corridors', or a 'Central or Eastern High African Corridor' (CEHAC) during the Miocene with subsequent interglacial periods. The CEHAC phenomenon is especially apparent between the arid Horn of Africa and south-western Africa (e.g. Balinsky, 1962; Caujapé-Castells et al., 2002; Coleman et al., 2003; Goldblatt, 1997; Goldblatt & Manning, 2002; Jürgens, 1997; Kirk-Spriggs & McGregor, in press), and have specifically been discussed for both the flora (Bellstedt et al., 2007, Böhle et al., 1996; Hilger & Böhle, 2000; McGuire & Kron, 2005) and insect fauna (Biondi, 1999; Colonnelli, 1984; Louw, 1993; Osella et al., 1998).

Meligethes Stephens, 1830 is a highly specialised anthophagous nitidulid genus that includes some 200 species in the Palaearctic, and more than 300 species in the Afrotropics [1]. Many *Meligethes* species groups have been believed to occur disjunctively in the European-Mediterranean and Cape region of South Africa (e.g. Reitter 1871, 1872a, 1872b). However, a closer examination of most of these purported cases revealed that these instances represent morphological convergence or parallelism (Spornraft and Kirejtshuk, 1993; Kirejtshuk and Audisio, 1995) and do not denote true phylogenetic relatedness. Only a single case remained unresolved, which involved the Mediterranean species *Meligethes planiusculus* (Heer, 1841). The species was cited by Reitter (1872a) from the Cape of Good Hope under the synonym *M. murinus* Erichson, 1845. The species is widespread in the European-Mediterranean region, and belongs to the holarctic *Meligethes planiusculus* species-group, which includes a dozen species with distributions from the Mediterranean and Macaronesian areas to northern Asia, China and western North America (Tab. I).

1) This genus has been very recently separated into several distinct genera, when this paper was in press (Audisio et al., in press).

Two additional species are also included in the *M. planiusculus* species-complex: 1) *M. isoplexidis* Wollaston, 1854, which occurs in the Madeira archipelago and is oligophagous on inflorescences of *Echium* (Boraginaceae); and 2) *Meligethes canariensis* Kirejtshuk, 1997, previously ascribed to *M. isoplexidis* and *M. planiusculus* (Audisio, 1993; Spornraft, 1966; Wollaston, 1865) but now recognised as an endemic species of the western Canary Islands (Kirejtshuk, 1997; Machado and Oromí, 2000). All species in the *M. planiusculus* group are associated at their larval stages with inflorescences of plants in the family Boraginaceae (Audisio, 1993; Audisio et al., 2005; Easton, 1957; Kirejtshuk, 1982, 1992, 1997; Spornraft, 1966, 1967; Williams, 2002; Tab. I). Specifically, *M. planiusculus* complex taxa are oligophagous on species of *Echium* (Boraginaceae).

Spornraft & Kirejtshuk (1993) described a group of new southern African species (*M. fruticola, M. conformis, M. dukei* and *M. univestis*), which were initially placed close to species of the southern African *M. reticulatus* Reitter, 1872 species-group. However, several morphological and bionomical data now indicate that at least *M. fruticola* is more closely related to species of the Holarctic *M. planiusculus* group. This relationship is also confirmed by larval associations with the endemic plant *Lobostemon* (Boraginaceae).

Tab. I - Updated taxonomic, zoogeographic and ecological scenario of the M. planiusculus group (Audisio, 1993, and unpublished data; Spornraft & Kirejtshuk, 1993; Audisio et al., 2005; Williams, 2002; Jelínek & Audisio, 2007). Morphologically distinct complexes are separated by thick lines.

Species	Distribution	Habitat	Alt. range	Larval host-plants (Boraginaceae)	Trophic rank	
M. planiusculus (Heer, 1841)	Europe, Near East, North Africa, Madeira	Xeric meadows, hillsides	0-2000 m	*Echium* spp.	oligophagous	*planiusculus complex*
M. canariensis Kirejtshuk, 1997	Canary Islands	Xeric meadows, maquis, hillsides	0-1200 m	*Echium* spp.	oligophagous	
M. isoplexidis Wollaston, 1854	Madeira	Xeric meadows, maquis, hillsides	0-1600 m	*Echium nervosum* Dryand in Ait., *E. candicans* L.	oligophagous	
M. tristis Sturm, 1845	Europe, Caucasus	Xeric meadows, maquis, hillsides	0-2000 m	*Echium* spp.	oligophagous	*tristis complex*
M. subtristis Easton, 1957	Middle Asia, Afghanistan, Pakistan	Xeric meadows, maquis, hillsides	0-1600 m	*Echium* spp.	oligophagous	
M. bocaki Audisio, Jelínek & Cooter, 2005	China (Yunnan)	Xeric meadows, hillsides	1000-3000 m	unknown	unknown	
M. buduensis Ganglbauer, 1899	SE Europe, Turkey, Caucasus, Middle Asia	Xeric meadows, maquis, hillsides	0-1700 m	*Echium* spp., *Onosma* spp.	oligophagous	*buduensis complex*
M. pectinatus Schilsky, 1894	Turkey, Caucasus	maquis, hillsides	800-2200 m	*Onosma* spp.	oligophagous	
M. saevus J.Le Conte, 1859	western and central North America	Dry open woods, thickets and glades	500-1600 m	*Onosmodium* spp. (*O. molle* complex)	oligophagous	
M. schilskyi Reitter, 1896	Middle Asia, Near East ?, North Africa ?	Xeric meadows, maquis, hillsides, cultivated fields, dry open places	200-2500 m	*Trichodesma* spp.	oligophagous	*schilskyi complex*
M. canadensis Easton, 1955	NW North America	Moist woods, thickets, meadows and streambanks	500-2000 m	*Mertensia paniculata* (Aiton) G.Don	monophagous	*canadensis complex*
M. gurjevae Kirejtshuk, 1984	Mongolia	unknown	1000-2000 m	unknown	unknown	
M. fruticola Spornraft & Kirejtshuk, 1993	South Africa (Western Cape)	Xeric meadows, finbos, hillsides	0-1600 m	*Lobostemon* spp., *Echium* spp. (introduced), *Anchusa* spp.	oligophagous	*fruticola complex*
M. sp. cfr. *fruticola*	South Africa (Western Cape)	Xeric meadows, finbos, hillsides	0-1600 m	*Lobostemon* spp., *Echium* spp. (introduced), *Anchusa* spp.	oligophagous	

Thus, there appears to be an apparent disjunction between *M. fruticola* in the Cape region of South Africa and *M. planiusculus* and its allies (*M. isoplexidis* and *M. canariensis*) in the Mediterranean region. We also note a sister relationship, based on morphological and molecular evidence, between *Echium* (southern Palaearctic) and *Lobostemon* (Cape region), representing the host plants of these *Meligethes* species assemblages (Böhle et al., 1996; Hilger and Böhle, 2000). Furthermore, both plant genera lack close native allies in the rest of the Afrotropics (Böhle et al., 1996). In addition, *M. fruticola* has undergone a recent host-plant shift in some localities in the Western Cape of South Africa from native species of *Lobostemon* to *Echium plantagineum* L. (Audisio unpublished data; De Gaspari, 1995), as well as introduced species of the related genus *Anchusa*. *Echium plantagineum* is an invasive alien weed introduced from Europe to the Southern Hemisphere in the past century (Compton, 1988; Swirepik et al., 1996; Wapshere, 1985). Similar host-plant shifts also have been observed in other apparently host-specific southern African phytophagous beetles associated with Boraginaceae, including the leaf beetle genus *Longitarsus* (Chrysomelidae) (Biondi, 1999).

The distribution of *Meligethes* in Afro-Arabian areas is well-known (e.g. Audisio, 1994; Audisio et al., 1998; Easton, 1954, 1959, 1960; Kirejtshuk, 1980, 1996, 2001, 2003; Kirejtshuk and Audisio, 1995; Kirejtshuk and Easton, 1988; Kirejtshuk and Viklund, 2002; Spornraft and Kirejtshuk, 1993), although the extensive Cape region requires additional sampling effort. However, no species outside the Cape are closely related to members of the *M. planiusculus* group. The single exception is *M. yemenensis* Easton, 1954, and its allies, which are distributed from north-eastern South Africa to the Arabian Peninsula and Dead Sea depression of Israel and Jordan. These species are associated as larvae with *Trichodesma* and related Boraginaceae (Audisio, 1993), but shared morphological characters indicate only a weak relationship to the *M. planiusculus* group.

Our analyses were performed to determine the following: 1) whether the apparently close morphological and bionomical relationships between *M. fruticola* and its allies in South Africa, and *M. planiusculus* and its allies in Mediterranean-Macaronesian areas are due to evolutionary convergence, or if the similarities are indicative of close phylogenetic relatedness; 2) the time of speciation events for the European *M. planiusculus* group and the southern African *M. fruticola*; 3) if genetic relationships, geographic distribution and timing of speciation events support a penetration of African *Meligethes* northwards to the Mediterranean and Palaearctic areas or *vice versa*, and; 4) the time and mode of colonization of the *M. planiusculus* complex in Macaronesia.

MATERIAL AND METHODS

Specimens of all known European-Mediterranean and Macaronesian species of the *M. planiusculus* complex (Tab. II) were studied, as well as the single widespread southern African species *M. fruticola*, and the more distantly related southern African species *M. conformis* Spornraft and Kirejtshuk, 1993, which may belong to the same species-group. *Meligethes tristis* Sturm, 1845, a member of the *M. planiusculus* species-group, also was investigated because it is considered the Palaearctic species most closely related to true examples of the *M. planiusculus* complex, and also is known to be oligophagous as larvae on inflorescences of *Echium* spp. (Audisio, 1993). Unfortunately, specimens of the related middle Asian species *M. subtristis* (Kirejtshuk, 1982) was unavailable for study. Three species (Tab. II) representing different species-groups also were selected to provide additional data for divergence estimations, these include: *M. subglobosus* Reitter, 1875, from the Cape Province, and belonging to the distantly related *M. convexus* species-group (Audisio, 1996); *M. chevrolati* Reitter, 1872, from the Cape Province, and belonging to the *M. amplicollis* species-group (Audisio, 1997; Audisio and De Biase, 2004); and *M. scotti* Easton, 1954, from the southern Arabian Peninsula and eastern Africa, belonging to the *M. scotti* species-group (Easton, 1954, 1959, 1960).

DNA Extraction, PCR and Sequencing Techniques

Specimens were sampled from several localities in Mediterranean Europe, and eastern and southern Africa between February 2000 and February 2007. Live adults were preserved in vials containing ACS grade acetone (Fukatsu, 1999) or 100% ethanol for later analyses. Species identifications were performed using morphological characters (Audisio, 1993). Tab. II lists ID codes for each assayed individual, along with geographical collecting references and EMBL-Bank/GenBank accession numbers.

Total genomic DNA was extracted by standard phenol/chloroform procedure, ethanol precipitation and resuspension in 10mM Tris, 1mM EDTA buffer (TE).

A 637 bp fragment near the 3' terminal end of the mitochondrial cytochrome C oxidase subunit I (COI) was amplified via polymerase chain reaction (PCR) using universal primers C1-J-mod-2183(+)[5'-CAACATTTATTTTGATTCTTTGG-3'] and C1-TL2-N-3014(-) [5'-TCCAATGCACTAATCTGCCATATTA-3'] (Simon et al., 1994, modified). PCR reactions were performed on a MWG® thermal cycler with the following amplification conditions: initial denaturation at 95°C for 5 minutes, followed by 33 cycles of 1 minute each at 94°C, 30 seconds annealing at 55°C, 1 minute extension at 72°C and 7 minute elongation at 72°C. Reactions were performed in a 25µL volume containing $(NH_4)_2SO_4$ 16mM, Tris-

Tab. II - Species and specimens examined for molecular analyses.

Meligethes spp.	Population	
M. canariensis		
CN.1.1	Spain, Canary Islands, La Palma, SW Coast, 10 m, 11.iii.2001, A. Vigna Taglianti leg., on *Echium brevirame* Sprague & Hutch.	AM489798
CN.4.1	Spain, Canary Islands, Tenerife, nr. Adeje, 150 m, 16.iii.2005, P. Audisio & A. De Biase leg., on *Echium bonnetii* Coiney	AM489799
CN.5.1	Spain, Canary Islands, Tenerife, Anaga Region, Las Carboneras, 200-800 m, 17.iii.2005, P. Audisio & A. De Biase leg., on *Echium strictum* L. fil.	AM489800
CN.6.1	Spain, Canary Islands, Tenerife, in the neighbouring of Tamaimo, 500 m, 16.iii.2005, P. Audisio & A. De Biase leg., on *Echium plantagineum* L.	AM489801
CN.7.1	Spain, Canary Islands Tenerife, Anaga Region, Las Carboneras, 200-800 m, 17.iii.2005, P. Audisio & A. De Biase, on *Echium leucophaeum* Webb *ex* Sprague & Hutch.	AM489802
CN.8.1	Spain, Canary Islands, Tenerife, Santiago del Teide, 800 m, 16.iii.2005, P. Audisio & A. De Biase leg., on *Echium aculeatum* Poir.	AM489803
M. chevrolati		
CH.1.1	South Africa, Western Cape, Cape Peninsula nr. Vishoek, 34°09.35'S, 18°25.53'E, m 50, 10.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on *Aspalathus* sp.	AM711601
M. conformis		
FO.1.1	South Africa, Mpumalanga, 16 Km W of Badplaas, 26°02.24'S, 30 24.27'E, 1393 m, 15.ii.2007, P. Audisio & M.Biondi leg., on *Lippia javanica* (Burm.f.) Spreng	AM711602
M. fruticola		
FR.1.1	South Africa, Western Cape, Cape Town, Table Mountain, Tafelberg Road, 33°57.03 S, 18°24.45 E, 300 m, 4.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on *Lobostemon fruticulosus* (L.) Buek	AM489804
FR.2.1	South Africa, Western Cape, Cape Town, Table Mountain, Pipe Track, 270 m, 16.x.2005 33°56.37 S, 18°23.39 E, P. Audisio, M. Biondi & A. De Biase leg., on *Lobostemon fruticulosus* (L.) Buek (in Fynbos)	AM489805
FU.1.1	South Africa, Western Cape, Robinson Pass, 33°52.25 S, 22°01.57 E, 850 m, 10.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on introduced *Echium plantagineum* L. (roadsides)	AM489806
FU.2.1	South Africa, Western Cape, 2 Km N Stellenbosch, 33°52.50 S, 18°50.52 E, 200 m, 5.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on introduced *Echium plantagineum* L. (road edges)	AM489786
FU.3.1	South Africa, Western Cape, 25 Km E Prince Albert, 900 m, 33°16.41 S, 22°15.18 E, 11.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on introduced *Anchusa azurea* Mill. (roadsides)	AM489787
FU.4.2	South Africa, Western Cape, Seweweekspoort, NW Calitzdorp, 33°28.04 S, 21°26.30 E, 560 m, 14.x.2005, P. Audisio, M. Biondi & A. De Biase leg., on introduced *Anchusa azurea* Mill. (roadsides)	AM489788
M. isoplexidis		
IS.1.1	Portugal, Island of Madeira, Caniçal, Radio Farolo do Aeroporto, 24.ii.2000, A. Aguiar leg., on *Echium nervosum* Dryand. in Ait.	AM489789
M. planiusculus		
PL.2.1	Italy, Latium (Rome), Salone, 50 m, 15.iv.2000, P. Audisio leg., on *Echium plantagineum* L.	AM489790
PL.3.1	Portugal, Island of Madeira, Caniçal, Radio Farolo do Aeroporto, 24.ii.2000, A. Aguiar leg., on *Echium nervosum* Dryand. in Ait.	AM489791
PL.4.1	Italy, Latium (Rome), Rome, Tenuta della Cervelletta, 3.iii.2002, M. Mei leg., on *Echium plantagineum* L.	AM489792
PL.5.2	Spain, Andalucia (Malaga), Alhaurin de la Torre, 400 m, 21.iii.2002, P. Audisio leg., on *Echium plantagineum* L.	AM489793
PL.6.1	Greece, Kentriki Makedonia (Seres), between Seres (= Serres) and Lailias, 900-1700 m, 31.v.2005, A. De Biase leg., on *Echium* sp.	AM489794
PL.7.2	Greece, Thessalia (Trikala), Meteora, between Kalambaka e Vlahava, 700-800 m, 29.v.2005, A. De Biase leg., on *Echium* sp.	AM489795
PL.8.1	Greece, Thessalia (Larissa), between Larissa and Trikala (cross to Amigdalea), 100 m, 29.v.2005, A. De Biase leg., on *Echium* sp.	AM489796
PL.9.1	Italy, Latium (Rome), Rome, Centocelle airport, 30 m, 27.v.2005, P. Audisio leg., on *Echium plantagineum* L.	AM489797
M. scotti		
SO.1.1	Kenya, Coast Province near Chakama, 107 m, 24.v.2006, G. Nardi leg., by sweeping on meadows.	AM711600
M. subglobosus		
SG.1.1	South Africa, Western Cape, Cape Town, Table Mountain Pipe Track, 270 m, 16.X.2005, 33°56.37'S, 18°23.39'E, P. Audisio, M. Biondi & A. De Biase leg., Fynbos. AM711599	AM489785
M. tristis		
TS.1.1	Spain, Comunidad Autonoma de Castilla y Leon, S. Domingo de Silos, 41°57.27 N, 3°24.06 W, 1000 m, 30.v.2005, E. Mancini leg., on *Echium* sp.	AM489785

HCl 67mM (pH 8.8 at 25°C), $MgCl_2$ 3mM, Tween-20 0.01%, 1mM of each deoxynucleotide, 0.8pM of each primer, 1.25 units Taq DNA polymerase (BIOLINE®, London, UK). Amplified products were purified by Exo-SAP enzymatic reactions and sequenced with forward primers at BMR genomics S.p.a. (Padua, Italy) employing an Applied Biosystem® 3100 Genetic Analyzer.

Sequences and Phylogenetic Analyses

COI sequences were edited using STADEN® package ver. 2003.1.6 (Bonfield et al., 1999-2002; Staden et al., 2003) and aligned using CLUSTAL X® ver. 1.81 (Thompson et al., 1997). Maximum Parsimony analyses were performed using PAUP*® ver. 4.0b10 (Swofford, 2002) set for a heuristic search with random stepwise addition and TBR algorithms employed. The MaxTrees option was set to 1000 with no autoincrease flag. *Meligethes chevrolati*, *M. scotti*, *M. subglobosus* and *M. tristis*, were alternatively used as outgroups. To test tree topology, a bootstrap analysis with 1000 replications was performed, and a single consensus tree was computed using a 50% majority-rule.

Best fit of molecular evolution model was performed using MODELTEST ver. 3.7 (Posada and Crandall, 1998) and the Akaike information criterion (Akaike, 1974; Posada and Buckley, 2004) to select the substitution model for the Maximum Likelihood (ML) reconstruction, which was accomplished by TREEFINDER® (Jobb, 2007). The inferred ML topology was rooted with *M. subglobosus* as the outgroup, and was successively used to compute corrected pair-wise genetic distances among species on the basis of the estimated substitution model. Topology robustness was tested by bootstrapping 1000 times.

Bayesian analysis was performed under the substitution model selected by MODELTEST® using MrBayes® ver. 3.1.2. These analyses were carried out using random starting trees and runnning 3.5 x 10^6 generations with Markov chains sampled every 1000 generations. To ensure sampling of topologies after chain convergence, the first 1000 trees were discarded as "burn-in". The remaining trees were combined into a 50% majority rule consensus tree that was rooted by setting *M. subglobosus* as the outgroup.

Divergence Time Estimation

The molecular clock was tested by means of a likelihood ratio relative rate test as implemented in R8S® version 1.71 (Sanderson, 2003, 2004). As an exploratory analysis, we performed the test for all nodes of the recovered ML topology, which indicated that the molecular clock hypothesis was not appropriate for estimation of divergence times. Thus, divergence time estimates were calculated by relaxing the molecular clock hypothesis using two methods that incorporate rate

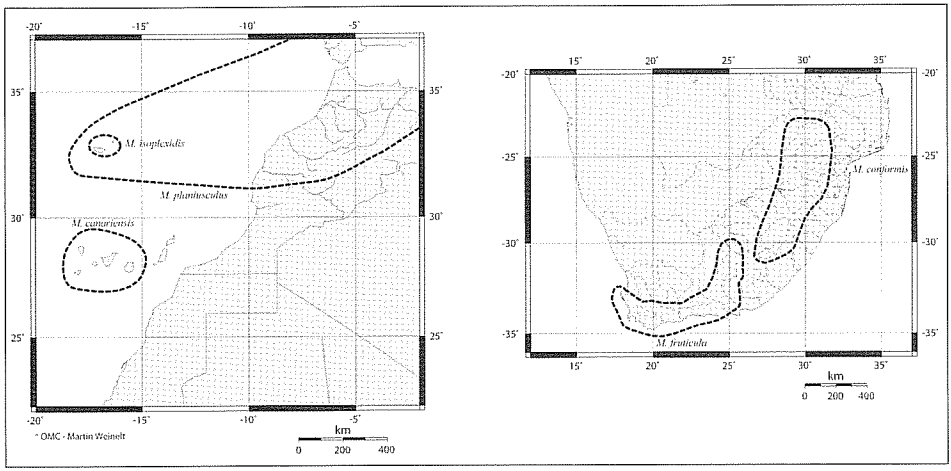

Fig. 1 - Distribution of the *M. planiusculus* complex in Macaronesia (left) and *M. fruticola* and *M. conformis* in South Africa (right).

heterogeneity while estimating divergence times based on rate temporal autocorrelation (Gillespie, 1991).

In the semiparametric Penalized Likelihood (PL) approach (Sanderson, 2002, 2003), rate variation among tree branches is taken into account, using a penalty function, while estimating the divergence times by a Likelihood method. The ML tree topology with branch length estimates was our starting point, and absolute ages of nodes were estimated using optimal smoothing parameter (which accounts for rate change between branches), obtained by the cross-validation procedure in R8S (Sanderson, 2003, 2004), using 80 steps of successive increments of 0.1. A profiling analysis, to summarize node age information, was performed using R8S on topologically identical trees recovered by bootstrapping with TREEFINDER® (Sanderson, 2004).

In the Bayesian analyses, divergence times were estimated following a standard method (e.g. Kishino et al., 2001; Thorne and Kishino, 2002; Thorne et al., 1998). The method is implemented in MULTIDISTRIBUTE® (version 25-Sep-2003, http://statgen.ncsu.edu/thorne/multidivtime.html), which uses a fully probabilistic model to describe evolutionary rate changes over time and over a phylogenetic topology, performing the MCMC procedures to derive posterior probability distribution of rates and times from prior distribution.

Before running the Thorne et al. analysis, we estimated substitution model parameters using version 3.14 of the PAML® package (Yang, 1997), and the inferred parameters were then entered into ESTBRANCHES® (included in MULTIDISTRIBUTE®), to estimate the ML of branch lengths along with the variance-covariance matrix. Successively, the prior distributions of substitution rates and diver-

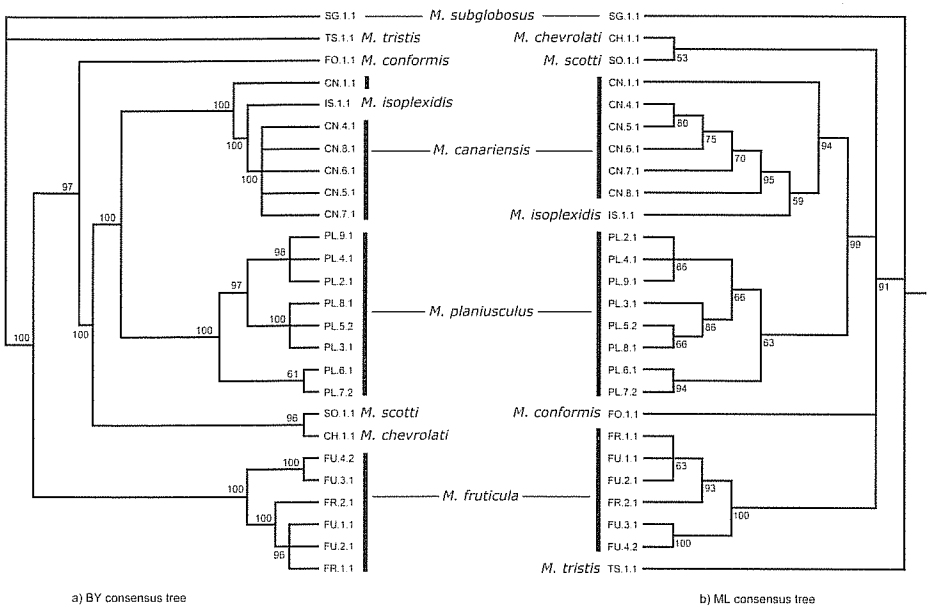

Fig. 2 - Bootstrap consensus trees for (a) Bayesian, and (b) Maximum Likelihood analyses. Refer to Tab. II for acronym definitions.

gence times were approximated using MULTIDIVTIME® (also included in MULTIDISTRIBUTE®). The analysis was performed twice to verify stationarity of prior distribution values. The posterior distributions of substitution rates and divergence times were approximated via MULTIDIVTIME® running in posterior mode. These analyses were then performed three times to verify stationarity of posterior distribution values. The software settings to compute prior and posterior distributions are listed below; each parameter is fully explained in MULTIDIVTIME®. We encourage readers to peruse the software documentation for fully expanded meanings of setting parameters.

Settings used for approximation of prior and posterior distributions:

10000	**numsamps**: How many times should the Markov chain be sampled?
1000	**sampfreq**: How many cycles between samples of the Markov chain?
1000000	**burnin**: How many cycles before the first sample of Markov chain?
38.22	**rttm**: a priori expected number of time units between tip and root
14.28	**rttmsd**: standard deviation of prior for time between tip and root
0.0046880749	**rtrate**: mean of prior distribution for rate at root node
0.0046880749	**rtratesd**: standard deviation of prior for rate at root node
0.03925	**brownmean**: mean of prior for brownian motion constant "nu"
0.03925	**brownsd**: std. deviation of prior for brownian motion constant "nu"

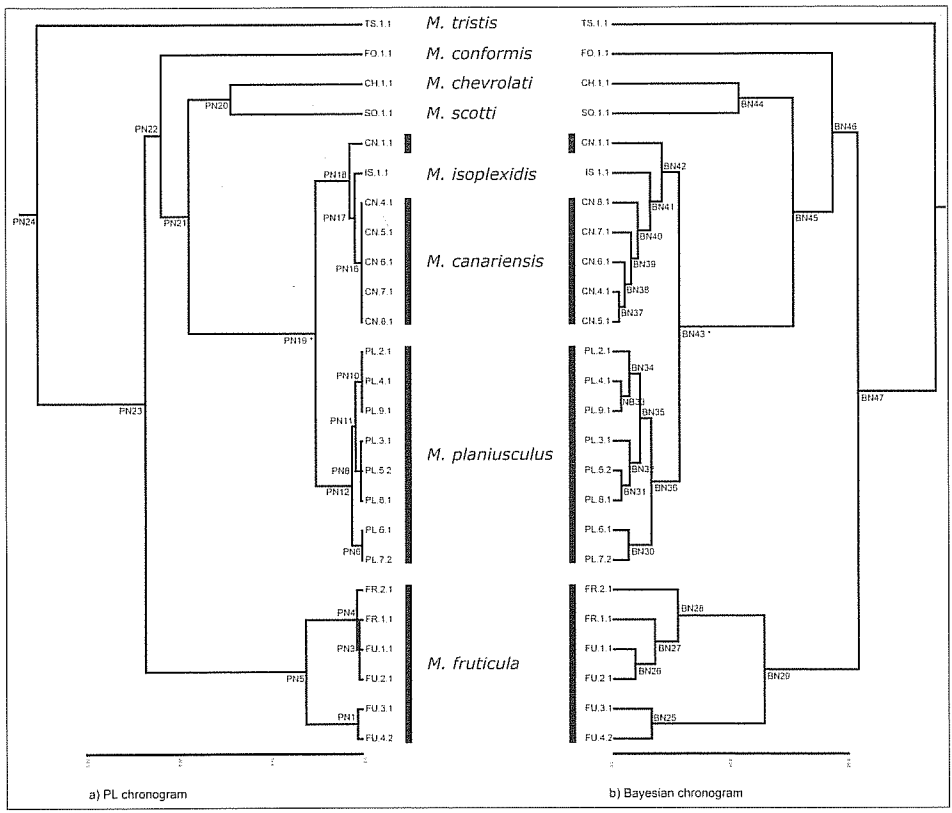

Fig. 3 - Reconstructed chronograms derived from the (a) semiparametric Penalized Likelihood (PL), and (b) Bayesian methods. Average rate inferred by PL = 0.00348 substitutions/site /MY.

The node between *Meligethes canariensis* and *M. planiusculus* (PN19 in the PL analysis and BN43 in the Bayesian analysis, see Fig. 3) was constrained using available geological data for the origin of Madeira Island (5-6 MY; Carvalho and Brandão, 1991). Both analyses discarded the outgroup taxon *M. subglobosus*.

RESULTS

Sequences of 637 bp for COI were obtained for all specimens. We detected 159 (25.9%) variable sites, of which 114 (17.9%) were parsimony informative. Sequences obtained are A+T rich at 71.5% on average, which is similar to previously published data on beetles. Overall comparison of the inferred amino acid sequences showed only low level of variation (6.13% = 13 variable positions on 212 codons), which is likely due to functional constraints of COI.

Mean values of intra- and inter-specific divergences are listed in Tab. III. In taxa 'strictly' assigned to the *M. planiusculus* complex (i.e. *M. canariensis*, *M. iso-*

plexidis and *M. planiusculus*) as well as *M. conformis* and *M. fruticola*, genetic distances range from 0.00-0.169. However, *M. tristis* shows values ranging from 0.183-0.293 (the max value scored without considering the outroup *M. subglobosus*) demonstrating its distinctness from the other species. The highest values of inter-specific distance consistently resulted among comparisons between *M. fruticola* and other taxa. Individuals analyzed for *M. canariensis* share the same haplotype, except CN 1.1, which shows ~1% mean genetic distance when compared to its conspecific. A strict relationship also exists among *M. canariensis* and *M. isoplexidis* as revealed by ~1% average genetic divergence.

The MP consensus trees typically yielded a topology identical to the ML tree, and therefore was not reported here. The MP tree statistics are summarized as follows: TL = 293, CI = 0.6587, HI = 0.3413, RI = 0.8230. ML and Bayesian reconstructions (Fig. 2) support an unambiguous distinction of two major clades grouping European-Mediterranean *M. canariensis, M. isoplexidis* and *M. planiusculus* samples in one clade (bootstrap value for ML = 99 and posterior probabilities for Bayesian analysis = 1), and South African specimens of the *M. fruticola* complex in another (bootstrap value for ML =100, and posterior probabilities for BY = 1). Furthermore, the relatively high value of intra-specific diver-

Tab. III - Genetic distances (GTR+I model) among selected *Meligethes* for the 3' portion of COI. Intraspecific variation values are listed in the first column; nc = not computed.

		M. subglobosus	*M. chevrolati*	*M. scotti*	*M. canariensis*	*M. isoplexidis*	*M. planiusculus*	*M. conformis*	*M. fruticola*
M. subglobosus	nc								
M. chevrolati	nc	0.273 nc							
M. scotti	nc	0.319 nc	0.151 nc						
M. canariensis	0.003 [0.005]	0.264 [0.004]	0.164 [0.004]	0.209 [0.004]					
M. isoplexidis	nc	0.266 nc	0.165 nc	0.211 nc	0.010 [0.000]				
M. planiusculus	0.004 [0.003]	0.249 [0.002]	0.148 [0.002]	0.194 [0.002]	0.040 [0.004]	0.041 [0.002]			
M. conformis	nc	0.214 nc	0.158 nc	0.204 nc	0.149 [0.004]	0.151 nc	0.134 [0.002]		
M. fruticola	0.020 [0.016]	0.209 [0.001]	0.175 [0.001]	0.221 [0.001]	0.166 [0.004]	0.168 [0.001]	0.150 [0.002]	0.115 [0.001]	
M. tristis	nc	0.166 nc	0.247 nc	0.293 nc	0.238 [0.004]	0.240 nc	0.222 [0.002]	0.187 nc	0.183 [0.001]

gence detected for *M. fruticola* (0.020) is reflected in all tree topologies. This pattern indicates a clear split of the *M. fruticola* clade into two subclades: a grouping of FU4.2 and FU3.1 individuals (hereafter referred as *M.* sp. cfr. *fruticola*), and a FU1.1, FU2.1, FR1.1, FR2.1 grouping.

Molecular data suggests that *M. tristis* is more distantly related to the partly sympatric *M. canariensis*, *M. isoplexidis* and *M. planiusculus* species, than the geographically isolated *M. fruticola*. However, morphological data suggests that *M. tristis* is more closely related to *M. planiusculus* and its allies, occupying a position internal to the *M. planiusculus* species-group but external to the *M. planiusculus* complex.

The relative rate tests using a likelihood ratio indicated that the phylogenetic reconstruction did not exhibit a constant substitution rate along its topology. Cross validation procedure yielded several optimal smoothing parameters for running the PL routine to estimate divergence times. The set of values provided outlier elements with low and high scores at the tails of the range. Therefore, we decided to select the smoothing parameter with the lowest value, which was determined to be 16000. Fig 3a illustrates the chronogram with node labels referring to the ages listed in Tab. IV. Node PN19 was constrained on available geological and palaeogeographical dates on the origin of the Madeira archipelago ~5-6 MY (Carvalho and Brandão, 1991). Node PN23 likely mirrors the splitting between ancestors of the European-Mediterranean and African species. Therefore, we tentatively date this splitting to ~23 MY. Results of the Bayesian analysis (Tab. V and Fig. 3b) depict a high degree of congruence with results from the previous analysis, dating the BN47 node at ~21 MY.

DISCUSSION

Meligethes planiusculus, *M. isoplexidis*, *M. canariensis*, *M. fruticola*, *M.* sp. cfr. *fruticola* and *M. tristis* are all associated with Boraginaceae as larvae, whereas *M. conformis*, *M. scotti* and allies are associated as larvae with Verbenaceae, and probably to related families as well (Audisio, unpublished; Easton, 1959, 1960). *Meligethes chevrolati* and allied African species, however, feed on Fabaceae as larvae. Thus, we hypothesize that the purported ancestor of all focal species herein are associated with host-plants belonging to the closely related botanical families that form the Boraginaceae/Rubiaceae/Verbenaceae/Scrophulariaceae clade (Judd et al., 1999); and the ancestral African *Meligethes* group subsequently shifted diets ~27-35 MYA (nodes PN23 and BN47, Fig. 3) to the unrelated Fabaceae (Arnold and De Wet, 1993; Bond and Goldblatt, 1984). Following this dietary shift, *Meligethes* underwent a major radiation paralleling the remarkable species diversity currently showed by Fabaceae in southern Africa (Arnold and De Wet, 1993; Bond and Goldblatt, 1984; Dahlgren,1988).

Tab. IV - Divergence time estimates using PL. Time units in millions of years. For node codes refer to Fig. 3a. Asterisk refers to constrained node based on available palaeogeographical data.

Node	Estimate	Mean	Std dev	Min	Max
PN1	0.50	0.577546	0.475911	0.000000	3.470337
PN2	nc				
PN3	0.35	0.392080	0.268642	0.000000	2.214124
PN4	0.60	0.668111	0.463838	0.000000	3.994961
PN5	6.11	6.459752	3.056114	1.213639	32.528560
PN6	0.00	0.000010	0.000252	0.000000	0.006250
PN7	nc				
PN8	0.13	0.107016	0.125569	0.000000	0.911048
PN9	nc				
PN10	0.00	0.000010	0.000246	0.000000	0.006250
PN11	0.71	0.642744	0.501808	0.041868	3.984481
PN12	1.10	0.926679	0.603338	0.000000	3.531031
PN13	nc				
PN14	nc				
PN15	nc				
PN16	0.00	0.000007	0.000206	0.000000	0.006250
PN17	0.78	0.799370	0.357570	0.000000	3.478319
PN18	1.33	1.375353	0.494565	0.203195	4.520189
PN19 *	5.00	5.000000	0.000000	5.000000	5.000000
PN20	14.18	15.339305	6.197580	4.180595	61.620686
PN21	18.72	20.394328	7.762181	5.803359	88.504920
PN22	21.67	23.502660	8.710152	5.984594	98.136999
PN23	23.42	25.596639	9.704969	6.523323	105.765490
PN24	35.07	38.216156	14.283966	7.095784	170.449485

Overall, morphological, genetic, ecological and biogeographical data demonstrate that the *M. planiusculus* complex and *M. fruticola* are phylogenetically related. However, as shown by all phylogenetic reconstructions, *M. scotti*, and *M. chevrolati* are indicated as sister to the *M. canariensis* and *M. planiusculus* clade, although these two species belong to two different species groups. Furthermore, the European *M. tristis*, despite being a morphologically well recognized member of the *M. planiusculus* group (Tab. I), was shown to be genetically less closely related to the *M. planiusculus* complex than to morphologically more distantly related African species (i.e. *M. chevrolati*, *M. conformis*, *M. scotti*). Therefore, the Holarctic *M. planiusculus* group (Tab. I) likely represents a paraphyletic assemblage despite the observed relationships between the inclusive taxa, which

Tab. V - Estimated posterior distribution times using Bayesian MCMC. Time units in millions of years. For node codes refer to Fig. 3b. Asterisk refers to constrained node based on available palaeogeographical data. The 95% "credibility" intervals refers to intervals estimated by 10000 sorting samples from the Markov chain and and then reporting the 250th and 9750 lowest sampled values for the estimated parameter (time of node).

Node	Mean	SD	95% interval[2]	
BN25	3.16982	2.94722	0.11801	11.12828
BN26	1.80906	1.84729	0.05024	6.93007
BN27	3.45963	2.65466	0.40816	10.33215
BN28	5.38231	3.46835	1.00219	14.19905
BN29	12.64321	5.37157	4.72227	25.11355
BN30	1.25676	1.00738	0.04950	3.81650
BN31	0.65876	0.61975	0.01801	2.30197
BN32	1.36348	0.87819	0.18645	3.53338
BN33	0.66041	0.62203	0.01689	2.37671
BN34	1.35751	0.87934	0.18251	3.48804
BN35	2.23601	1.10341	0.51763	4.69211
BN36	3.17684	1.23821	1.01790	5.46176
BN37	0.48444	0.47402	0.01270	1.76877
BN38	0.97549	0.66277	0.12241	2.60819
BN39	1.50286	0.82655	0.31576	3.44527
BN40	2.08963	0.96418	0.58405	4.23241
BN41	3.10270	1.03539	1.22998	5.05135
BN42	4.09539	1.00362	2.00433	5.66511
BN43*	5.54251	0.28730	5.03479	5.98159
BN44	10.56959	4.23847	4.36123	20.58095
BN45	15.17620	4.94238	8.03774	27.01486
BN46	18.52393	5.78074	10.10691	32.27432
BN47	20.64403	6.44240	11.19807	36.16547
BN48	27.13366	8.44874	14.40281	47.28431

are undoubtedly due to a true common origin based on synapomorphic morphological characters. These affinities were also revealed by recent analyses of novel morphological characters using scanning electron microscopy techniques (Strika and Audisio, unpublished data; Audisio et al., 2009) as well as analyses of nuclear ITS2 molecular markers (Trizzino et al., 2009). These data suggest that the *M. planiusculus* group is cladistically nested in a much wider African assemblage, including members of the moderately related *M. scotti* (including *M. scotti*) and

2) It refers to 95% "credibility" intervals estimated by 10,000 sorting samples from the Markov chain and then reporting the 250th and 9750 lowest sampled values for the estimated parameter (time of node).

M. amplicollis (including *M. chevrolati*) species-groups, as well as members of *M. yemenensis*, *M. reticulatus*, *M. serrator*, and *M. spissus* species groups. Thus, *M. scotti* and *M. chevrolati*, which are currently recognized as members of different species groups, share morphological and molecular characters as well as a common African origin with species of the *M. planiusculus* species group.

Our divergence time estimates (Fig. 3, Tabs. IV, V) suggest that the ancestor of *M. fruticola* and allied African taxa originated ~20-23 MYA, and *M. fruticola* plus *M.* sp. cfr. *fruticola* (the latter likely representing a new undescribed sibling species) subsequently radiated in southern Africa ~6-13 MYA, whereas the *M. planiusculus* complex probably radiated later at ~5-5.5 MYA in the Mediterranean region. A parallel analysis using partial 16S rDNA sequences (Audisio et al., unpublished data; accession numbers: EU716211 to EU716220) yielded an almost identical and congruent divergence time pattern of ~21 MYA) for these focal taxa. Although the recovered estimation of 21 MYA appears to be high for species level divergences, these values fall within ranges found for other large genera combining high morphological and ecological diversification, wide geographical distribution, and ancient origin.

Genetic, biogeographical and ecological data indicate that African ancestors of the focused Mediterranean *Meligethes* species probably specialized in southern Africa on species of Boraginaceae ~17-23 MYA, and subsequently radiated northwards to Mediterranean areas and consequently eastwards towards Middle Asia, China, and western North America (see Tab. I) by way of an established 'arid corridor' connecting southern, eastern, and north-eastern African areas (Axelrod, 1975; Balinsky, 1962; Bellstedt et al., 2007; Biondi, 1999; Böhle et al., 1996; Caujapé-Castells et al., 2001, 2002; Coleman et al., 2003; Goldblatt, 1997; Hilger and Böhle, 2000; Jürgens, 1997; Louw, 1993; McGuire and Kron, 2005; Osella et al., 1998; Werger, 1983; Wilfert et al. 2006; Zinderen Bakker, 1978), or via a CEHAC as proposed by Kirk-Spriggs and McGregor (2009). These specialization/radiation events probably occurred several times in the past 30 MYA (Babe, 2006; Bellstedt et al., 2007), and present Holoartic species, recognized herein as the *M. planiusculus* group (Tab. II), are the result of multiple colonization and speciation events from tropical and subtropical African ancestors[3].

Meligethes planiusculus occurs throughout the European-Mediterranean region and Madeira archipelago, but is absent from the Canary Islands (Fig. 1 left). The biogeographic scenario within the studied species of the *M. planiusculus* complex suggests that colonization of the Canary and Madeira archipelagos, originated from south-western Mediterranean (Iberian) populations of the hypothetical ancestral

3) The classification of the highly speciose genus *Meligethes* is currently the subject of a major generic revision (Trizzino et al., 2009; Audisio et al., in press). These research efforts resulted in the splitting of *Meligethes* into several related genera, wherein some sub-genera are raised to generic rank and some new genera described. One of the new genera comprise the clearly monophyletic African and Holoarctic species assemblage of *Meligethes* s.l. partly discussed and analysed in this paper.

taxon of *M. planiusculus*. Whereas, *M. isoplexidis* originated on the island of Madeira ~0.5-4.5 MYA (nodes PN18-16 and BN42-37, Fig. 3), where it is currently confined. Thus, the Canary archipelago was likely colonized from ancestral Madeiran populations first established in northwestern islands (i.e. La Palma Island), from which *M. canariensis* was subsequently derived. From the morphological point of view, it is easy to confirm that even the genetically isolated *M. canariensis* specimen from La Palma clearly belongs to the true *M. canariensis* despite its nested position within the *M. isoplexidis* cluster. It is then very likely that the La Palma population of *M. canariensis* still retains haplotypes common with *M. isoplexidis* simply due to incomplete lineage sorting, as frequently occurs in recently diverged species. These species, in fact, often can be taxonomically delimited on morphological grounds, despite their present-day evolutionary condition is likely long before the requisite time for reciprocal genetic monophyly to be achieved following speciation. *Meligethes canariensis* exhibits low genetic variation (0.003, Tab. III), suggesting a relatively recent dispersal across the Canary Islands (PN17 = 0.78 MYA; BN41 = 3.10 MYA), probably combined with recent bottleneck events occurred during the Canarian colonization, and followed by its present-day larval association with most of the several endemic species of *Echium* that are present on the islands. The origin of *Echium* in the Canary archipelago dates to ~5 MYA (Böhle et al., 1996). The radiation of *Echium* (including 27 species in Macaronesia) has been hypothesized as occurring from a more ancient Canary center of speciation, to a more recent Madeira area of secondary differentiation, where an endemic species pair with Canary affinities occurs (Böhle et al., 1996). If this radiation recontruction for *Echium* is accurate, the hypothesized beetle/host-plant colonization and radiation should have followed opposite directions and timing, owing to a possible scenario of sequential evolution rather than coevolution (Jermy, 1976, 1984; Ronquist, 1998, 2002; Stireman et al., 2005).

More recently, *M. planiusculus* re-colonized the Madeira archipelago from the Iberian Peninsula, being present in Madeira with populations (PL.3.1) genetically related to Iberian ones (PL.5.2, Figs 2 & 3). This recolonization of Madeira from Portugal or southwest Spain might have been facilitated by volcanic seamounts located between the Macaronesian archipelagos and the continental mainland. The seamounts may have served as 'stepping stones' during recent glaciation events when sea levels were lower (Carine et al., 2004; García-Talavera, 1997).

CONCLUSIONS

Our results indicate that the apparently strong morphological and bionomical affinities of species in the Mediterranean *Meligethes planiusculus* complex and the southern African *M. fruticola* reflect the combined effect of a true common origin and independent evolution on closely related host-plants and habitat types.

The Palaearctic *M. planiusculus* complex and the southern African *M. fruticola* likely originated from a shared common Meligethinae taxon of African descent that was associated as larvae with members of the botanical clade including Boraginaceae, Rubiaceae, Scrophulariaceae and Verbenaceae. The Holarctic *M. planiusculus* group was found to be paraphyletic, and nested in a larger clade that includes other related species-groups from tropical and southern Africa.

Phylogenetic results showed that the ancestor of the *M. planiusculus* complex, *M. fruticola*, and allied African species likely originated ~20-23 MYA; with differentiation of *M. fruticola* in southern Africa ~6-13 MYA (Tab. I), and the *M. planiusculus* complex in Mediterranean areas ~5-5.5 MYA. The extant species of the *M. planiusculus* species-group then radiated ~20 MYA from sub-tropical Africa. The divergence time estimates support the presence of an 'arid corridor' or 'Central or Eastern High Africa Corridor' ~17-20 MYA, which connected the European-Mediterranean and eastern/southern African areas. Penetration of the *M. planiusculus* group (Tab I) into the eastern Palaearctic and western North America likely occurred at the same time. The inferred evolutionary relationships and divergence time estimations of the *M. planiusculus* complex suggest a recent origin from southwestern Mediterranean (Iberian) populations, following the colonization of the island of Madeira ~0.5-4.5 MYA, eventually reaching northern La Palma Island where *M. canariensis* appears as the most recent derivative of the lineage.

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