Mitochondrial DNA phylogenetic analysis of the genus *Laparocerus* (Coleoptera, Curculionidae, Entiminae). I. The Madeiran clade

ANTONIO MACHADO, MERCEDES LÓPEZ, TERESA ALMEIDA & MARIANO HERNÁNDEZ

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Laparocerus are plant-chewing flightless weevils distributed on oceanic islands in Macaronesia, with a single species in Morocco. The genus has a complicated taxonomic history with several subgenera described. To assist in a taxonomical revision of the group, a molecular study was undertaken. In this first contribution, the species from the Azores and Madeira archipelagos are studied together with representatives of subgenera from the Canary Islands and the single known continental species (46 OTUs). Phylogenetic analyses are based on sequence data from mitochondrial cytochrome oxidase II (COII) and the ribosomal 16S ribosomal RNA (16S rRNA) genes (combined data set 1023 bp), with additional data from the nuclear elongation factor 1α (EF-1α) for some selected species. Maximum likelihood (ML) and Bayesian analyses show that all Madeiran species are monophyletic and form a monophyletic group with the Afro-Canarian samples. Species of the genus *Lichenophagus* appear within the Madeiran and Canarian *Laparocerus* clades, but separated in accordance with their respective island origin. Thus, *Lichenophagus* is here restricted to Madeiran species and proposed as subgenus (*status novo*) of *Laparocerus*. Conversely, the *Laparocerus* subgenus *Drouetius* from the Azores is revealed to be a separate and distant outgroup, in agreement with its morphological distinctiveness, deserving an independent genus status. Internal relationships within the Madeiran clade are discussed and compared with morphologically defined species groups. The Madeiran monotypic subgenus *Cyphoscelis* is not supported by the genetic data and synonymized (nov. syn.) with *Laparocerus*. Subgenera *Laparocerus* and *Atlantis* prove to be polyphyletic. Consequently a restriction to monophyletic and morphologically congruent clades is proposed. A cryptic species vicariant of *Laparocerus morio* was detected and recognized as *L. chaoensis*, *status novo*. Other cases of discrepancy between the genetic results and the traditional taxonomy are discussed in detail in the light of mitochondrial introgression, incomplete lineage sorting or poor taxonomy hypotheses.

Corresponding author: *Antonio Machado*, *c/Chopin 1*, *38208 La Laguna*, *Tenerife*, *Spain*. *E-mail: antonio.machado@telefonica.net*

Mercedes López, *Teresa Almeida and Mariano Hernández*, *Instituto Universitario de Enfermedades Tropicales*, *38071 La Laguna*, *Spain*. *E-mails: merlopez@ull.es*, *tacosalm@ull.es*, *mnhdez@ull.es*

Introduction

Species at present attributed to the genus *Laparocerus* Schönherr, 1834 constitute a heterogeneous group of entimin weevils, endemic to Macaronesia (the Azores, Madeira, Salvages and Canary Islands), with the exception of a single species in West Morocco, on the mainland. They are flightless weevils with free-living edaphic larvae, and most are oliphagous and climb vegetation to feed upon the leaves (Machado 2003). A few species dwell in the leaf-litter, and there are even some which are adapted to the underground environment. The taxonomic history of this genus is complicated, and can be summarized as follows:

The genus *Laparocerus* was established by Schönherr in 1834 to harbour two species described by Boheman in the same work, based on material from 'Lusitania', a patria that was later clarified as being the island of Madeira. *Eremnus tetricus* Boheman, 1834 and *Laparocerus canariensis* Boheman, 1842 from Tenerife (Canary Islands) were added shortly after.

In 1839, Brullé described several *Otiorhynchus* (four species) from the Canaries, and Wollaston, who worked the Madeiran fauna in detail (1854, 1857, 1862), added one more *Laparocerus*, four species of *Omias*, the monotypic genus *Cyphoscelis* Wollaston, 1854, and 18 species of *Atlantis* Wollaston, 1854. All these species were ascribed to the genus *Laparocerus* by Lacordaire (1863), with *Cyphoscelis* and *Atlantis* as sections (subgenera) of it. He also created the group 'Laparocerides' adding several non-Macaronesian genera (*Aomus*, *Elytrodon*, *Merimnetes*, etc.) The related Madeiran genus *Lichenophagus* Wollaston, 1854, which was originally placed in another group, has been recently included in the *Laparocerini* Lacordaire, 1863 (Alonso-Zarazaga & Lyal 1999).

The number of *Laparocerus* and *Lichenophagus* species increased considerably due to further studies by Wollaston (1863, 1864, 1865), Uyttenboogaart (1936, 1937), Uyttenboogaart & Zumpt (1940), Lindberg (1953) and Roudier (1954, 1957, 1958, 1963) among others; the new species being occurring primarily in the Canary Islands. Several additional subgenera were erected: *Amphora* Wollaston, 1864; *Canopus* Wollaston, 1864; *Amyntas* Wollaston, 1865; *Wollastonicerus* Uyttenboogaart, 1937; *Pecoudius* Roudier, 1957 and *Drouetius* Méquignon, 1942. The latter was established for a single known species from the Azores: *Laparocerus azoricus* Drouet, 1859. In the *World Catalogue of Families and Genera of Curculionoidea*, Alonso-Zarazaga & Lyal (1999) fixed and replaced some of these subgeneric names which revealed to be invalid: *Amyntas* Wollaston (1865) (= *Canopus* Wollaston, 1865), *Guanchotrox* Alonso-Zarazaga & Lyal, 1999 (= *Amphora* Wollaston, 1865), and *Machadotrox* Alonso-Zarazaga & Lyal, 1999 (= *Wollastonicerus* Uyttenboogaart, 1937). According to the main catalogues (Winkler 1932; Lona 1938; Lindberg & Lindberg 1958; Lundblad 1958; Kocher 1961; Borges 1990; Machado & Oromí 2000) some 114 taxa (species and subspecies) have been attributed to the genus *Laparocerus*, including *Cyclobarus susicus* Escalera, 1914 from Morocco that was transferred to *Laparocerus* by Ruter (1945).

A revision of the type material of all species assigned to *Laparocerus* and described prior to 2006 yielded 105 valid and 24 invalid species names, but neither the status of subspecies, nor that of subgenera was revised (Machado 2006). Recently, five additional species from Tenerife were described (Machado 2007). The systematic of the group remains complicated and obscure, particularly the boundaries of the several ascribed subgenera. In Table 1, a snapshot of the current status of *Laparocerus* is presented.

A faunal study and taxonomic revision of the genus *Laparocerus* was initiated in 1999. It includes a molecular analysis to better support the systematic decisions and to help infer the phylogeny of the species. The field survey is rendering many new taxa, almost doubling the initial number of OTUs to be analysed. Consequently, and due to financial constraints, only one specimen and two loci per taxa were planned for molecular analysis. Mitochondrial DNA markers (fragments of cytochrome oxidase subunit II (COII) and of ribosomal 16S subunit) were selected as they are standard markers used in many phylogenetic studies (Caterino *et al*. 2000). In addition, a nuclear marker (a fragment of the nuclear elongation factor 1α (EF-1α) gene) was analysed for selected taxa in order to elucidate deeper relationships or checking controversial results.

Table 1 Number of valid taxa (species and subspecies) presently assigned to the genera *Laparocerus* and *Lichenophagus*.

Genus/subgenus	No. of taxa	Distribution
Genus ***Laparocerus*** Schönherr, 1834	Σ 110	
Amyntas Wollaston, 1865	9	Canaries
Atlantis Wollaston, 1854	18	Madeira
Cyphoscelis Wollaston, 1854	1	Madeira
Drouetius Méquignon, 1942	1	Azores
Guanchotrox Alonso-Zarazaga & Lyal, 1999	1	Canaries
Laparocerus Schönherr, 1834	56	6 Madeira, 1 Salvages, 48 Canaries, 1 Morocco
Machadotrox Alonso-Zarazaga & Lyal, 1999	23	Canaries
Pecoudius Roudier, 1957	1	Canaries
Genus ***Lichenophagus*** Wollaston, 1854	Σ 14	
Fernandezius Roudier, 1957	9	Canaries
Lichenophagus Wollaston, 1854	2	Madeira
Mateuius Roudier,1957	3	Canaries

The general study — field prospecting, species description, etc. — is being developed in parallel with the molecular analysis, and mutually supporting each other. First in a series of papers, we present the genetic results for Madeiran species. Our main objective here is to elucidate the genetic relationships among Madeiran *Laparocerus* and to clarify their relationships with the *Laparocerus* lineages occurring on the other Macaronesian archipelagos. The results for the Canarian–Moroccan species will follow in future contributions.

Materials and methods

Sampling and outgroup selection

More than 20 000 specimens of *Laparocerus* have been collected. At least two specimens of each presumably different species were directly placed and killed in ethanol. In many cases this was repeated for different localities. Voucher card-mounted dry specimens and specimens preserved in ethanol from the same locality and date, share the same collection number. Table 2 provides details of specimens' locality data. All specimens were collected by A. Machado, except for *L. azoricus* (*leg.* P. Borges).

The 21 species included in the present analyses (see Table 2) are all those known from the Madeiran Archipelago with the exceptions of *Laparocerus lanatus* (Wollaston, 1854)

Table 2 Specimens used in the phylogenetic analysis.

Species	Sequence	Collection code	Archipelago Island, Locality
Genus *Laparocerus*			
L. (Amyntas) tibialis (Woll. 1864)	CRE	#866	C: Tenerife, Teno Bajo
L. (Atlantis) abditus Roudier, 1963	CR	#267	M: Madeira, Rabaçal
L. (Atlantis) abditus Roudier, 1963	C	#3410	M: Madeira, Porto Novo
L. (Atlantis) aenescens (Woll. 1854)	CR	#4004	M: Madeira, Ribeiro Frío, Levada
L. (Atlantis) angustulus (Woll. 1857)	CR	#4008	M: Madeira, Pico do Ariero
L. (Atlantis) calcatrix (Woll. 1854)	CR	#3397	M: Madeira, Montado dos Peçegueiros
L. (Atlantis) colasi Roudier, 1958	CRE	#841	M: Madeira, Ribeira de Porto Novo
L. (Atlantis) excelsus (Woll. 1854)	CRE	#292	M: Madeira, Ponta do Tristão
L. (Atlantis) inconstans (Woll. 1854)	CR	#852	M: Porto Santo, Calheta
L. (Atlantis) instabilis (Woll. 1854)	CRE	#846	M: Porto Santo, Capela da Graça
L. (Atlantis) lamellipes (Woll. 1854)	CRE	#248	M: Madeira, Balçoes
L. (Atlantis) lamellipes (Woll. 1854)	CR	#4016	M: Madeira, El Folhadal
L. (Atlantis) lauripotens (Woll. 1854)	CR	#3393	M: Madeira, Ribeira Brava
L. (Atlantis) lauripotens (Woll. 1854)	CR	#245	M: Madeira, Santana
L. (Atlantis) lauripotens (Woll. 1854)	C	#3404	M: Madeira, Curral das Freiras
L. (Atlantis) mendax (Woll. 1854)	CR	#3382	M: Madeira, Prainha
L. (Atlantis) mendax (Woll. 1854)	CR	#3415	M: Porto Santo, Campo de Baixo
L. (Atlantis) mendax (Woll. 1854)	CR	#3418a	M: Porto Santo, Playa de Ponta da Calheta
L. (Atlantis) mendax (Woll. 1854)	CR	#3418b	M: Porto Santo, Playa de Ponta da Calheta
L. (Atlantis) mendax (Woll. 1854)	CR	#855	M: Porto Santo, Calhau Serra de Fora
L. (Atlantis) sp. 1	CR	#4003	M: Madeira, Ribeiro Frío, Levada
L. (Atlantis) noctivagans (Woll. 1854)	CR	#266	M: Madeira, Rabaçal
L. (Atlantis) noctivagans (Woll. 1854)	CR	#287	M: Madeira, Ribeira da Tristão
L. (Atlantis) noctivagans (Woll. 1854)	C	#302	M: Madeira, Fanal
L. (Atlantis) schaumii (Woll. 1854)	CR	#3413	M: Porto Santo, Pico do Castelho
L. (Atlantis) ventrosus (Woll. 1854)	CRE	#3422	M: Madeira, Achada Grande
L. (Atlantis) vespertinus (Woll. 1854)	C	#259	M: Madeira, Pico Ruivo
L. (Atlantis) vespertinus (Woll. 1854)	C	#259b	M: Madeira, Pico Ruivo
L. (Atlantis) vespertinus (Woll. 1854)	C	#259c	M: Madeira, Pico Ruivo
L. (Atlantis) vespertinus (Woll. 1854)	CRE	#259d	M: Madeira, Pico Ruivo
L. (Atlantis) waterhousei (Woll. 1854)	CRE	#4020	M: Madeira, Rabaçal
L. (Cyphoscelis) distortus (Woll. 1854)	CR	#4021	M: Madeira, Levada a Caldeirão Verde
L. (Cyphoscelis) distortus (Woll. 1854)	CRE	#304	M: Madeira, Encumeada: El Folhadal
L. (Drouetius) azoricus Drouet, 1859	CR	#308	A: Sâo Jorge, Gruta da Presa do Leao
L. (Drouetius) azoricus Drouet, 1859	C	#309	A: Pico, Enrique Maciel
L. (Drouetius) n. sp.	CR	#3989	A: Terceira, Sierra Sta. Barbara, Lomba
L. (Guanchotrox) canariensis Boh. 1842	CRE	#1886a	C: Tenerife, El Portillo
L. (Laparocerus) clavatus Woll. 1854	CR	#840	M: Madeira, Ribeiro Frio
L. (Laparocerus) sp. 2	CR	#3406	M: Madeira, i. Eira do Serrado
L. (Laparocerus) lindbergi Roudier 1963	CRE	#4147	M: Madeira, Paul da Serra, Campo Grande
L. (Laparocerus) morio morio Boh. 1834	CRE	#4013a	M: Madeira, Encumeada
L. (Laparocerus) morio morio Boh. 1834	CR	#4013b	M: Madeira, Encumeada
L. (Laparocerus) morio morio Boh. 1834	C	#4013c	M: Madeira, Encumeada
L. (Laparocerus) morio morio Boh. 1834	C	#258	M: Madeira, Redondo
L. (Laparocerus) morio morio Boh. 1834	CR	#252	M: Madeira, Punta de Sâo Lourenço
L. (Laparocerus) morio morio Boh. 1834	CR	#305	M: Porto Santo, Pico do Castelo
L. (Laparocerus) morio morio Boh. 1834	CR	#277	M: Deserta Grande, Chão da Doca
L. (Laparocerus) rasus Woll. 1864	CRE	#859	C: Lanzarote, Ermita de las Nieves
L. (Laparocerus) susicus (Esc. 1914)	CR	#4103	Morocco: Agadir, La Fortalessa
L. (Laparocerus) undulatus Woll. 1862	CRE	#3398	M: Madeira, Barranco do Inferno
L. (Machadotrox) excavatus Woll. 1863	CRE	#2135	C: Tenerife, Anaga: Chinobre
L. (Pecoudius) eliasenae (Uytt., 1929)	CRE	#4164	C: Gran Canaria,Valsendero: Cazadores
Genus *Lichenophagus*			
L. (Fernandezius) tesserula Woll. 1864	CRE	#4169	C: La Palma, Punta de Juan Adalid
L. (Lichenophagus) fritillus Woll. 1854	CRE	#4518	M: Porto Santo, Pico de Ana Ferreira
Outgroups			
Otiorhynchus cribicollis Gyll. 1834	CR	#10008	M: Madeira, Curral das Freiras
Otiorhynchus vaucheri Peyer. 1927	CR	#10016	Morocco: Agadir, Imouchá
Rhyncogonus excavatus Van Dyke 1937	CRE	#10019	French Polynesia: Rurutu, Plateau Tenuani

Sequences: C, COII; R, 16S rRNA; E, EFα1. Archipelagos: A, Azores; M, Madeira, and C, Canaries.

which is probably extinct, *Laparocerus navicularis* (Wollaston, 1854), *Laparocerus morio chaoensis* Uyttenboogaart, 1940 and *Laparocerus morio cevadae* Roudier, 1961 that were not recently collected in the field. A few undescribed species were included. To test the monophyly of the Madeiran species, several representatives of the other known subgenera of *Laparocerus* from the Azores and the Canaries were also included (nine species).

Only one specimen for each taxon was analysed, except when morphological differences among specimens from different localities were noticed. Moreover, in a few selected species several individuals from the same locality were analysed to gather an idea of genetic variability within populations.

The Macaronesian genus *Lichenophagus* was initially chosen as outgroup, but a preliminary test revealed it to be nested within the ingroup and paraphyletic. The other genera listed as *Laparocerini* in Alonso-Zarazaga & Lyal (1999) are located in remote areas: Iran–Syria–Saudi Arabia (*Aomus*), Eritrea (*Asmarotrox*), Ethiopia (*Aphyonotus*), Zaire (*Straticus*), India (*Cyrtozemia*) or Australia (*Merimnetes*, *Neomerimnetes*). Unfortunately, we were not able to obtain specimens in ethanol from any of these species. *Otiorhynchus cribicollis* Gyllenhall, 1834 (specimen from Madeira) and *Otiorhynchus vaucheri* Peyerimhoff, 1927 (from Morocco) where also tested as outgroups, but disregarded as they seem to be too distant and introduce topological errors, presumably due to long-branch attraction effect (Anderson & Swofford 2004). Finally, *Rhyncogonus excavatus* Van Dyke, 1937 from the island of Rurutu, in the French Polynesia was selected and used as formal outgroup.

DNA isolation, amplification and sequencing

Genomic DNA was extracted as follows. The specimens were punctured in the abdomen and incubated overnight at 56 °C in 500 µL lysis buffer (100 mM sucrose, 30 mM Tris, 700 mM NaCl, 10 mM EDTA, 0.5% SDS and 80 µg proteinase K). Proteins were removed by phenol–chloroform, chloroform extractions and the DNA precipitated in one volume of isopropanol. The DNA was washed in 70% ethanol, resuspended in 50 µL TE (10 mM Tris, 1 mM EDTA, pH = 8) and stored at –20 °C until use. Mitochondrial COII and 16S ribosomal RNA (16S rRNA) fragments (598 and 425 bp, respectively) were amplified using primers TL-J-3037 and TK-N-3785 (Simon *et al.* 1994; Gómez-Zurita *et al.* 2000), and 16SM (designed by us) and 16SBr (Simon *et al.* 1994), respectively (see Table 3).

The additional nuclear fragment (611 bp) of the EF-1α was amplified using primers EFA754 and EFS149 (Normark *et al.* 1999), the latter with some modifications. The EF-1α sequence for *R. excavatus* was provided by Elin Claridge, University of Berkeley. Details of the sequence data obtained for each specimen are given in Table 2.

Table 3 Sequence of primers used.

TL-J-3037	5′-TAATATGGCAGATTAGTGCATTGGA-3′
TK-N-3785	5′-GAGACCATTACTTGCTTTCAGTCATCT-3′
16SBr	5′-CCGGTCTGAACTCAGATCATGT-3′
16SM	5′-CCAATGAAGTTTTAAATGGCCGC-3′
EFA754	5′-CCACCAATTTTGTAGACATC-3′
EFS149T	5′-AAGGAGGCTCARGAAATGGG-3′

In order to minimize laboratory errors (mislabelling, contamination, etc.), sequencing was always repeated for taxa strangely placed according to traditional morphology; eventually, a second specimen from the same locality was used to check discordant data.

The PCR amplifications were performed on a Biorad® MyCycler thermocycler in a total volume of 50 µL containing 5 µL of 10× buffer (Ecogen, Barcelona, Spain), 150 µM of each dNTP, 0.2 µM of each primer, 0.6 U EcoTaq DNA polymerase (Ecogen) and 40–60 ng of DNA template. Each reaction started with 2 min at 94 °C, then the amplification was carried out for 45 cycles of denaturation at 94 °C for 10 s, annealing at 54, 56 or 50 °C (for COII, 16S rRNA and EF-1α fragments, respectively) for 20 s, and extension at 72 °C for 30 s. A final extension step at 72 °C for 5 min was performed. PCR products were purified using the wizard purification system (ProMEGA, Madison, WI). Both strands were cycle-sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and sequenced in an ABI3770 automated sequencer. PCR profile of sequencing was 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C.

Data analysis

DNA sequences were viewed, edited and assembled using MEGA v3.1 (Kumar *et al.* 2004). Once edited, multiple alignments were performed using CLUSTALW (Thompson *et al.* 1994). The plausibility of the alignment of COII sequences were verified at the amino acid level. Alignment of 16S rRNA sequences was straightforward and it was necessary to include only six gaps. These positions were considered as missing data for all analyses.

Since heterogeneity in base composition is known to affect phylogenetic inference (Lockhart *et al.* 1994) variation among sequences in base pair composition for both fragments was tested using a χ^2 analysis, as implemented in PAUP* (v4.0b10; Swofford 2002).

We used the partition homogeneity test (Farris *et al.* 1994) as implemented in PAUP*4.0b10 (Swofford 2002) (500 replicates) to examine whether there was evidence for different phylogenetic signals between COII and 16S rRNA. No significant differences were found between markers ($P = 0.19$). Thus, we performed the phylogenetic analysis for a composite sequence of 1023 bp.

Phylogenetic relationships were reconstructed using maximum likelihood (ML) and Bayesian inference (BI). For the ML analysis, nucleotide substitution model parameters were determined using MODELTEST v3.7 (Posada & Crandall 1998), while for the BI analysis MRMODELTEST 2.2 (Nylander 2004) was used. Based on arguments presented by Posada & Buckley (2004), we used Akaike Information Criterion (AIC) (Akaike 1973, 1974; Sakamoto *et al*. 1986) to select best-fit models. The ML tree was reconstructed using Treefinder (Jobb, G. 2005 Treefinder version of May 2006, Munich, Germany <http://www.treefinder.de>) under the general time-reversible (GTR + I + Γ) model of nucleotide substitution, with 1000 bootstrap replicates.

BI analysis was conducted with MRBAYES v3.1.2 (Ronquist & Huelsenbeck 2003), using the previously determined model of nucleotide evolution (GTR + I + Γ). Parameters were treated as unknown variables with equal *a priori* probability and subsequently estimated by the programme during the analysis. Starting trees were randomly chosen. Four Monte Carlo Markov chains were run for 6 000 000 generations, trees being sampled every 100 generations for a total of 60 000 trees in the initial sample. Variations in ML scores were examined graphically with the TRACER application (v1.3; Rambaut & Drummond 2003) and stationarity determined as having occurred before the 500 000th generation. However, the first 1 000 000 generations were discarded, thereby ensuring that stationarity was reached. Accordingly, the first 10 000 trees were discarded (as burn-in), and the following 50 000 trees were used to estimate topology and tree parameters. The percentage of times a node occurred within those 50 000 trees was interpreted as the posterior probability of the node. The BI final tree was edited with MRENT v1.2 programme (Zuccon & Zuccon 2006).

The –log likelihood value of the ML and BI trees (GTR + I + Γ) were compared with those of the same trees constructed under molecular clock assumptions. The results showed highly significant differences between the likelihoods of the trees.

In addition, another Bayesian analysis was conducted with the combined nuclear (EF-1α) and mitochondrial data for 19 selected individuals (see Table 2). The partition homogeneity test did not show significant differences among mitochondrial and nuclear genes ($P = 0.84$). Using AIC, MRMODELTEST selected the same GTR + I + Γ model as the most appropriate for the nuclear data set and thus used for the complete data set (1634 bp).

All DNA sequences have been deposited in the GenBank with accession numbers from EF583315 to EF583371 for the COII, from EF583390 to EF583437 for the 16S rRNA, and from EF583372 to EF583389 for the EF-1α, following the species order as listed in Table 2. The EF-1α sequence of *R. excavatus* provided by E. Claridge has accession number EF577111.

Results

DNA sequence variation

For the COII, 263 out of 598 positions (44%) were variable, 88% of the variable sites were parsimony informative. For the 16S rRNA fragment, 134 out of 425 positions (32%) were variables and of these, 82% were parsimony-informative. AT richness across all sites was similar (74%) for both genes, and as is typical in insect mtDNA coding genes, third positions of COII showed low G composition, 1.9%.

A χ^2 test comparing the nucleotide composition across taxa could not reject a null hypothesis of homogeneity for both fragments ($\chi^2 = 31.6$ and 20.32 for COII and 16S rRNA, respectively; d.f. = 132 and $P = 1.0$ for both cases).

Pairwise genetic divergence among species within Madeira showed a maximum of 0.137 corrected evolutive distance (GTR + I + Γ model) between *L. calcatrix* and *L. waterhousei*, a value that is much smaller than the divergence between *L. calcatrix* and *Laparocerus* (*Drouetius*) *azoricus* (0.383) and hereforth considered to be an outgroup.

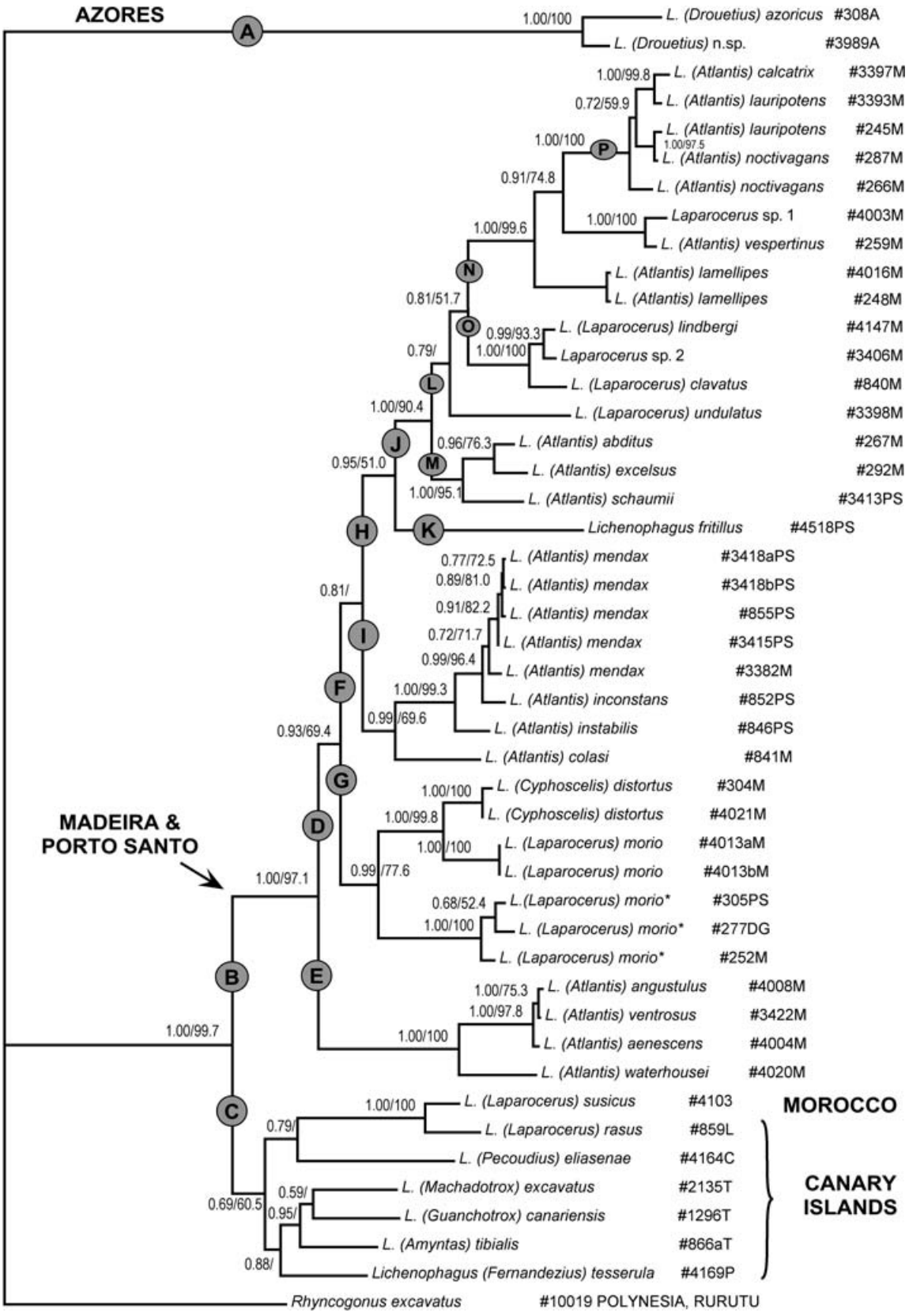
Two specimens of *L. mendax*, three of *L. morio* and four of *L. vespertinus* were sequenced for COII in order to get a rough idea of variation within the same locality. Pairwise distances were 0.002, 0.000 and 0.002, respectively. Divergence increase considerably when comparing interpopulation variation (different localities within the same island): 0.007 for *L. morio* (Madeira, excluded Sao Lourenço), 0.012 for *L. vespertinus*, 0.007 for *L. distortus*, 0.002 for *L. lamellipes*, 0.012 for *L. noctivagans*, 0.029 for *L. lauripotens*, 0.025 for *L. abditus*, and 0.016 for *L. mendax* (only Porto Santo).

Phylogenetic trees

Trees obtained by ML and BI shared the same topology and all main branches were well supported by both methods. Moreover, separate models for data partitions under the Bayesian framework did not change the topology. In Fig. 1, we present the Bayesian tree summarizing posterior Bayesian probabilities (PBP) and ML bootstrap support values.

All species from Madeira and Porto Santo formed a strongly supported monophyletic group (clade B) (PBP = 1.00), directly related to the clade C, which includes all species representatives from the Canary Islands and *L. susicus*, the only known species from Africa (its low PBP = 0.69 increases to 0.99 when adding the EF-1α fragment to the data set; see Fig. 2). The combined Afro-Canarian and Madeiran clade (B + C), is supported by PBP = 1.00 and clearly differentiated from the two *Laparocerus* (subgenus *Drouetius*) from the Azores (clade A), which reveals it to be even more distant than the Polynesian outgroup chosen.

The Madeiran clade includes two subclades: clade E (PBP = 1.00) clustering species of small *Laparocerus* (*Atlantis*) that were originally ascribed by Wollaston to the genus *Omias*, and clade D (PBP = 0.93) with the rest of the species.


AZORES
MADEIRA & PORTO SANTO
MOROCCO
CANARY ISLANDS
A
B
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K
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M
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O
P
1.00/100
L. (Drouetius) azoricus #308A
L. (Drouetius) n.sp. #3989A
1.00/99.8
L. (Atlantis) calcatrix #3397M
0.72/59.9
L. (Atlantis) lauripotens #3393M
L. (Atlantis) lauripotens #245M
1.00/97.5
L. (Atlantis) noctivagans #287M
1.00/100
0.91/74.8
L. (Atlantis) noctivagans #266M
1.00/100
Laparocerus sp. 1 #4003M
1.00/99.6
L. (Atlantis) vespertinus #259M
L. (Atlantis) lamellipes #4016M
0.81/51.7
L. (Atlantis) lamellipes #248M
0.99/93.3
L. (Laparocerus) lindbergi #4147M
0.79/
1.00/100
Laparocerus sp. 2 #3406M
L. (Laparocerus) clavatus #840M
1.00/90.4
L. (Laparocerus) undulatus #3398M
0.96/76.3
L. (Atlantis) abditus #267M
0.95/51.0
L. (Atlantis) excelsus #292M
1.00/95.1
L. (Atlantis) schaumii #3413PS
Lichenophagus fritillus #4518PS
0.77/72.5
L. (Atlantis) mendax #3418aPS
0.89/81.0
L. (Atlantis) mendax #3418bPS
0.81/
0.91/82.2
L. (Atlantis) mendax #855PS
0.72/71.7
L. (Atlantis) mendax #3415PS
0.99/96.4
L. (Atlantis) mendax #3382M
1.00/99.3
L. (Atlantis) inconstans #852PS
0.99/69.6
L. (Atlantis) instabilis #846PS
0.93/69.4
L. (Atlantis) colasi #841M
1.00/100
L. (Cyphoscelis) distortus #304M
1.00/99.8
L. (Cyphoscelis) distortus #4021M
1.00/100
L. (Laparocerus) morio #4013aM
0.99/77.6
L. (Laparocerus) morio #4013bM
1.00/97.1
0.68/52.4
L.(Laparocerus) morio* #305PS
1.00/100
L. (Laparocerus) morio* #277DG
L. (Laparocerus) morio* #252M
1.00/75.3
L. (Atlantis) angustulus #4008M
1.00/97.8
L. (Atlantis) ventrosus #3422M
1.00/99.7
1.00/100
L. (Atlantis) aenescens #4004M
L. (Atlantis) waterhousei #4020M
1.00/100
L. (Laparocerus) susicus #4103
0.79/
L. (Laparocerus) rasus #859L
L. (Pecoudius) eliasenae #4164C
0.59/
L. (Machadotrox) excavatus #2135T
0.69/60.5
0.95/
L. (Guanchotrox) canariensis #1296T
L. (Amyntas) tibialis #866aT
0.88/
Lichenophagus (Fernandezius) tesserula #4169P
Rhyncogonus excavatus #10019 POLYNESIA, RURUTU

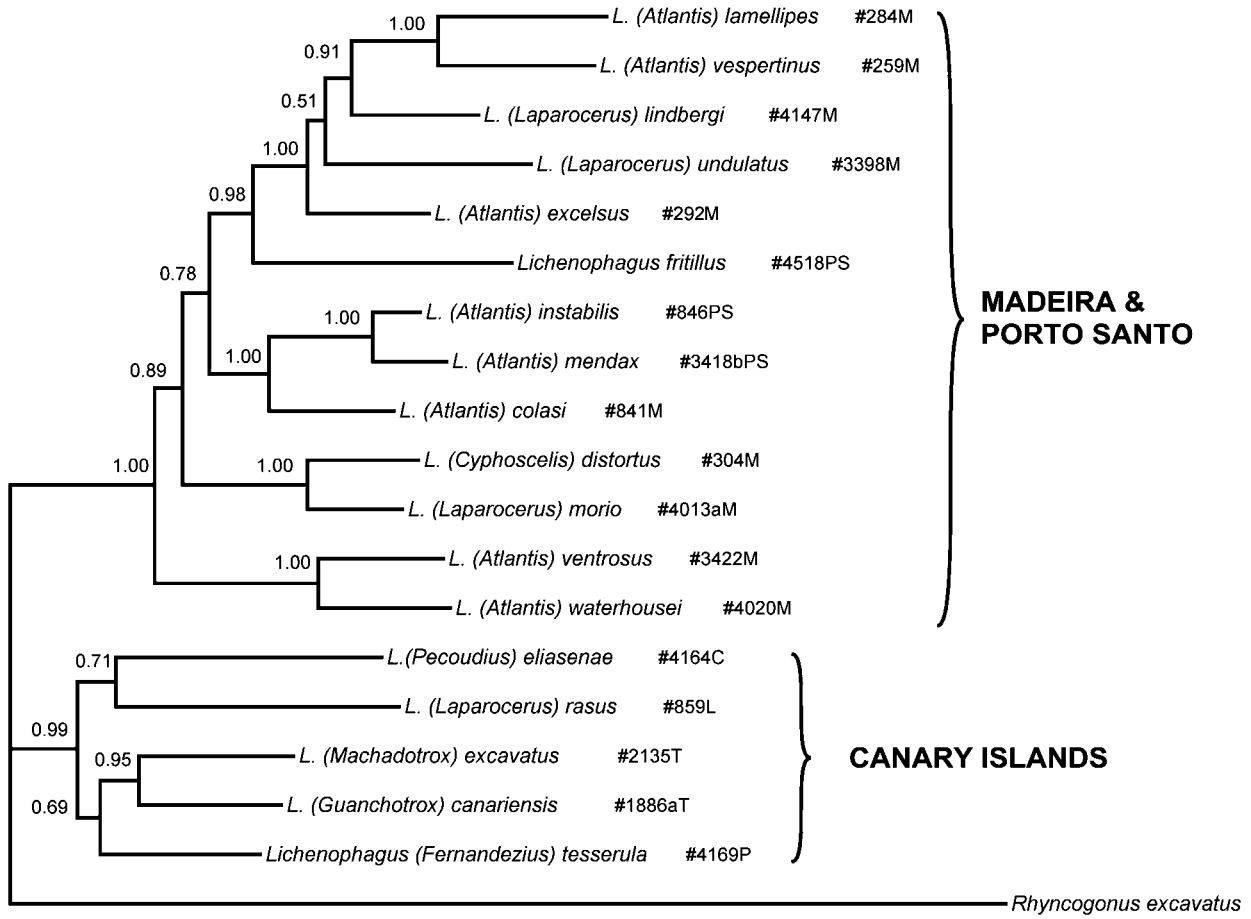

Fig. 2 Bayesian 50% majority rule consensus tree for COII, 16SrNA and EF-1α showing posterior probabilities. Voucher numbers are followed by the initials: C, Gran Canaria; L, Lanzarote; M, Madeira proper; P, La Palma; PS, Porto Santo; and T, Tenerife.

Subgenus *Cyphoscelis* and *L.* (*s.str.*) *morio* cluster together (clade G, PBP = 0.99) and the Madeiran *Lichenophagus*, some *Laparocerus* (*s.str.*), and the rest of subgenus *Atlantis* make up another clade. This latter subclade (clade F) is less well supported (PBP = 0.81) in this tree, but its posterior probability value rises up to 0.96 when considering only the 16S rRNA gene, which is likely more informative for deeper nodes than the COII gene.

In clade G (PBP = 0.99), *L. morio* splits into two branches, one with specimens from Porto Santo, Desertas and Ponta de Sao Lourenço (#252, eastern peninsula of Madeira), and the other with specimens of the interior of Madeira, to which the monotypic subgenus *Cyphoscelis* (with *L. distortus*) is clustered, despite the strong morphological differences among both species.

Clade I (PBP = 0.99) groups most of the *Atlantis* species that were termed by Wollaston (1854) as 'aberrant' for not showing the characteristic sexual dimorphism of the tibiae, as well as *L. colasi* which was described after Wollaston's work (1854) and also lacks sexual dimorphism in the tibiae. The 'aberrant' group is based mainly in Porto Santo. Conversely, clade H (PBP = 0.95) comprises the single *Lichenophagus fritillus* from Porto Santo (clade K) and the species in clade J (PBP = 100) which is taxonomically more complex.

Clade J comprises species from the island of Madeira proper (except *L. schaumii* which also inhabits Porto Santo) clustered in three groups: the rest of 'aberrant Atlantides' (subclade M), the 'true Atlantides' (subclade N) and the group (subclade O) formed by *Laparocerus* sp. 2 and *L. clavatus* plus *L. lindbergi* which, according to the capitate funicle of

Fig. 1 Bayesian 50% majority rule concensus tree for COII and 16S rRNA showing BI posterior probability/ML boostrap values (above 50%). Clades are labelled with letters in the line previous to their nodes. Voucher numbers are followed by the initials: A, Azores; C, Gran Canaria; DG, Deserta Grande; L, Lanzarote; M, Madeira proper; P, La Palma; PS, Porto Santo; and T, Tenerife.

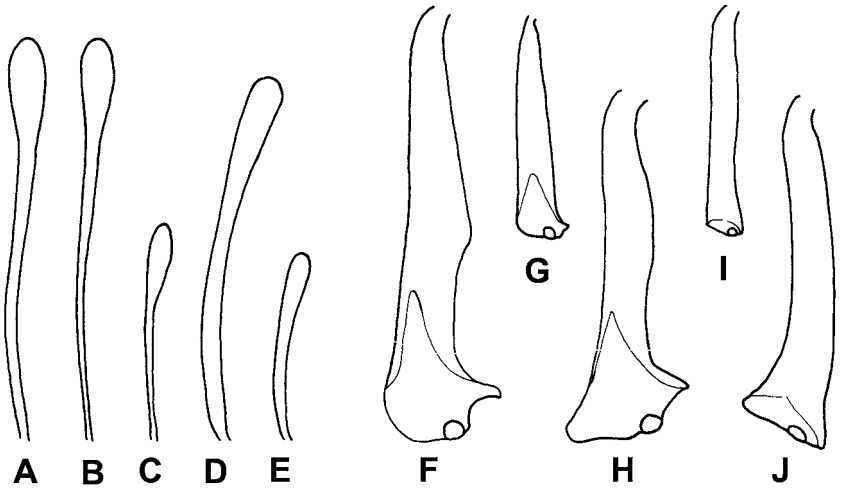

Fig. 3 A–J. Scape of antennae in —A. *Laparocerus morio*, —B. *L. undulatus*, —C. *L. lindbergi*, —D. *L. mendax*, and —E. *L. abditus*. Internal face of metatibiae in —F. *L. undulatus*, —G. *L. lindbergi*, —H. *L. noctivagans*, —I. *L. abditus*, and —J. *L. mendax*.

the antennae (Fig. 3C), have been placed under subgenus *Laparocerus*. These three groups are individually strongly supported, each with PBP = 1.0, but the relationships among them remain unresolved (PBP = 0.79) apparently due to *L. undulatus*, which does not join any of the groups.

Within clade P there are some discrepancies between the molecular phylogenetic relationships and the morphologically defined species: specimens of *L. lauripotens* and of *L. noctivagans* do not cluster conspecifically.

The Afro-Canarian clade (C) is represented by just one representative of each of the known subgenera, plus one of the species attributed to *Laparocerus s.str*. (*L. rasus*), and *L. (Laparocerus) susicus*, from Morocco. Thus phylogenetic relationships among these groups cannot be accurately inferred. However, it is important to highlight that *Lichenophagus tesserula* from the Canary Islands is embedded within the Canarian lineage and thus not closely related to *Lichenophagus* from Porto Santo.

Discussion and conclusions

The phylogenetic tree obtained with molecular data is rather coherent with the present species arrangement based on morphology, but differs in several important points that have implications for the taxonomic concept of the genus *Laparocerus* and its higher systematics.

Drouetius

Subgenus *Drouetius* (clade A) from the Azores is clearly and very distantly separate from the *Laparocerus* of Madeira and the Canaries, deserving to be promoted to genus status. This is consistent with morphological data (Machado, manuscript in preparation).

Lichenophagus

Lichenophagus fritillus from Porto Santo (Madeira), and *Lichenophagus tesserula* from La Palma (Canaries), despite their external morphological similarity, appear separated in the Madeiran and Canarian clades in accordance with their respective island origins. Analysis of the EF-1α gene (Fig. 2) also supports this relationship, arguing against mtDNA introgression as an explanation. Thus, the genus *Lichenophagus* as presently interpreted is paraphyletic and should be split and ascribed as two subgroups of *Laparocerus*. This is an outstanding case of convergent evolution; the peculiar morphology of these insects being probably a response to their ground dwelling habits: feeding in the leaf-litter or on rock-cliff lichens. We propose here a new status for *Lichenophagus* as subgenus of *Laparocerus* and its restriction to *Lichenophagus fritillus* (Porto Santo) and *L. acuminatus* (Deserta Grande). Consequently, the Madeiran and Afro-Canarian monophyletic clade (B + C) would become the new concept of the genus *Laparocerus*, to be redefined.

Laparocerus *and* Cyphoscelis

The monotypic subgenus *Cyphoscelis* — originally described as genus — based on *L. distortus*, seems to be taxonomically irrelevant as it is paraphyletic with respect to the *L. morio* species complex (clade G). Analyses of the nuclear EF-1α gene gave identical results, therefore we reject the hypothesis of a mitochondrial introgression. In relation to *L. morio*, the body of *L. distortus* is abnormally flattened and boat-shaped. Such a disruptive but mainly allometric differentiation is likely related to another habitat shift. *Laparocerus distortus* dwells in the leaf-litter, where it feeds mainly on dead leaves of *Euphorbia mellifera*, while *L. morio* is a nocturnal plant-chewing species that climbs on the vegetation as is normal in *Laparocerus* (Machado 2003). We propose here the synonymy of *Cyphoscelis* Wollaston, 1854 with *Laparocerus* Schönherr, 1834.

According to the trees obtained the subgenus *Laparocerus* with type species *L. morio* (clade G) is clearly polyphyletic as

Table 4 Pairwise distances of mtDNA COII in species of subgenus *Laparocerus*. (DG, Deserta Grande; M, Madeira; M, Ponta de Sâo Lourenço; PS, Porto Santo).

		*Laparocerus morio**			*Laparocerus morio*			*Laparocerus distortus*		
		#252cM′	#252M′	#277DG	#305PS	#258M	#4013aM	#4013bM	#4013cM	#4021M
*morio**	#252M′	0.005								
*morio**	#277DG	0.029	0.031							
*morio**	#305PS	0.031	0.033	0.026						
morio	#258M	0.118	0.116	0.118	0.105					
morio	#4013aM	0.112	0.120	0.122	0.110	0.007				
morio	#4013bM	0.112	0.120	0.122	0.110	0.007	0.000			
morio	#4013cM	0.112	0.120	0.122	0.110	0.007	0.000	0.000		
distortus	#4021M	0.105	0.103	0.102	0.094	0.056	0.060	0.060	0.060	
distortus	#304M	0.105	0.103	0.106	0.094	0.060	0.064	0.064	0.064	0.007

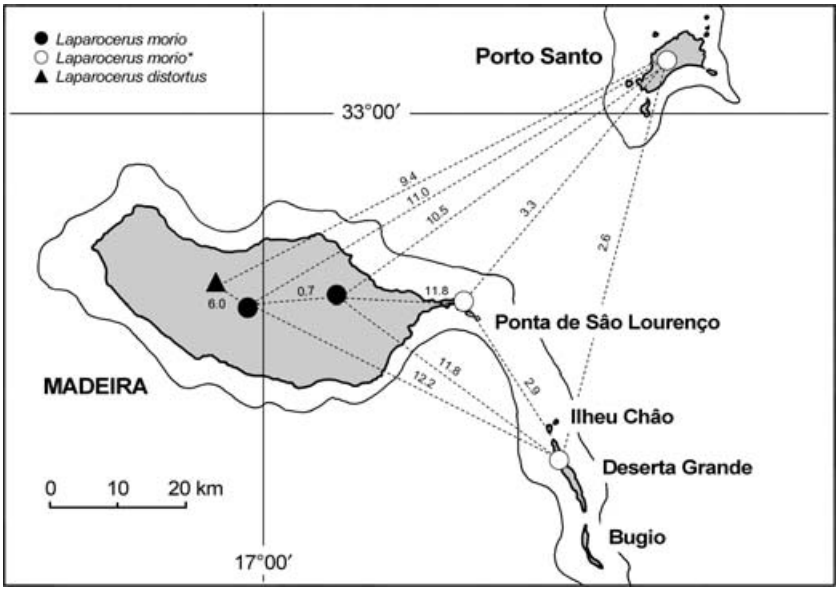

Fig. 4 Distribution of subgenus *Laparocerus* in the archipelago of Madeira with values of COII *p*-distance between species and populations, expressed in percentage.

presently interpreted, with a few species placed in clade L and many more in the Afro-Canarian clade C. We propose the restriction of the subgenus *Laparocerus* to clade G, with *L. morio*, *L. distortus* and a possible cryptic species related to *L. morio* (denoted with * for discussion purposes).

Laparocerus morio and *L. morio** can hardly be distinguished morphologically, but their COII *p*-distance ranges from 0.110 to 0.122 (see Table 4), more than 10 times higher than between normal *L. morio* from two localities within Madeira (0.007), and even higher than that between *L. morio* and *L. distortus* (*p*-distance 0.056–0.060), a clearly distinctive species. *Laparocerus morio* seems to be distributed in Madeira proper with the exception of the extreme eastern peninsula of Ponta de Sâo Lourenço, where it is replaced by populations of *L. morio** (see Fig. 4). This latter population (#252M) is most closely related to specimens from Porto Santo (*p*-distance 0.031–0.033) and from Deserta Grande (*p*-distance 0.029–0.031), a link that is not surprising. It is known from other such groups (e.g. land snails, Cook *et al.* 1990) that the Ponta de Sâo Lourenço has a greater faunal affinity with its extending arc of islets of the Desertas and with Porto Santo, than with the rest of Madeira. In fact, the possibility of the Ponta de Sao Lourenço having been a separate islet that recently fused with Madeira along the valley of Machico is currently being investigated (Susana Prada, pers. com.).

Taking into account the vicariance between *L. morio* and *L. morio**, the extremely high genetic distance between them, and the closer relation of *L. morio* with *L. distortus*, we feel inclined to recognize *L. morio** as a cryptic species. The population of the Ilheu do Desembarcadouro, in the extreme

of Ponta de Sâo Lourenço has been already described as *Laparocerus morio cevadae* Roudier, 1961, and that of the ilheu de Châo (one of the Desertas) as *L. morio chaoensis* Uyttenboogaart, 1940 based on subtle differences in the aedeagus and heal of male metatibiae. Unfortunately, we were not able to obtain fresh material from either of these populations to undertake a DNA analysis, but we assume that they would cluster in coherence with their geographical neighbours of Ponta de Sâo Lourenço and Deserta Grande, respectively. A careful analysis of microcharacters and endophallic structures is needed to attempt characterize this possible cryptic species, which should take the name of *Laparocerus* (*Laparocerus*) *chaoensis* Uyttenboogaart, 1940, according to the principle of priority.

Atlantis

The subgenus *Atlantis* is also polyphyletic according to the molecular data. Species are distributed in clade E, clade I and clade L. Those of clade E are the small *Laparocerus* that Wollaston originally described under genus *Omias*, and later transferred to *Atlantis;* they surely deserve an independent subgenus status. Clade I clusters the bulk of 'aberrant' *Atlantis* in Wollaston's concept, because they do not show the sexual differences in the tibiae typical of the 'true' *Atlantis (*type species *L. lamellipes*). These species are mainly from Porto Santo, with *L. mendax* in the Ponta de Sâo Lourenço (probably a susbspecies), and *L. colasi* in Madeira proper, which is vicariant of *L. navicularis* from Porto Santo (not included in the molecular analysis). The problem arises with two species of 'aberrant' *Atlantis* based on Madeira proper (*L. abditus* and *L. excelsus*) and *L. schaumi* (Sâo Lourenço and Porto Santo) which cluster in clade M having the 'true' *Atlantis*' as sister clade L, within clade J. Their genetic relationship with the other 'aberrant' *Atlantis* is further reduced by the lineage of *Lichenophagus* (clade K) being interposed between them. However, the structure of the short internal sac of the aedeagus of these three species (A. Machado, unpublished data) is the same as that of the other 'aberrant' *Atlantis* (Fig. 5A,B), in agreement with the intuition of Wollaston who grouped them together based on external characters. All other *Atlantis* within clade L — including *L. lindbergi*, *L. clavatus*, *L. undulatus* and *Laparocerus* sp. 2, which are surely not subgenus *Laparocerus* (see discussion above) — share the same and clearly distinct very long internal sac (Fig. 5C), with the seminal duct inserted laterally through a side pouch, instead of apically. This case represents a strong incongruence between internal morphological and molecular data and demands a careful re-examination of morphological characters and their comprehensive evaluation with molecular results. For now, we propose to restrict the concept of subgenus *Atlantis* to species contained in clade L and accept that the capitate or clavate shape of the funicle is a character subject to homologies.

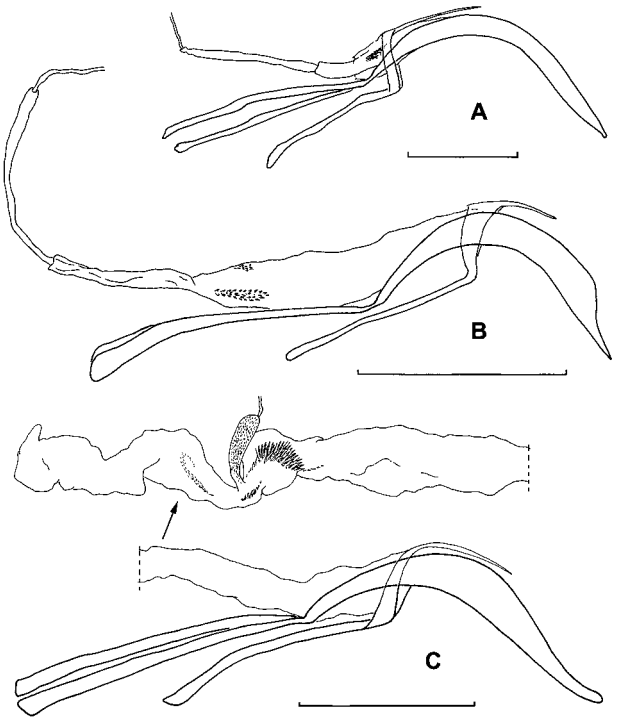

Fig. 5 A–C. Aedeagus —A. *Laparocerus abditus*, —B. *L. mendax*, and —C. *L. calcatrix*. Scale = 1 mm.

Clade L is phenetically well supported, even if the dilatations of male metatibiae in species of subclade O are not as impressive as in subclade N (Fig. 3G). From the genetic point of view, its PBP reduces from 0.99 to 0.79 when *L. undulatus* is included in the data set. This species stands alone within clade L despite its phenetically closer relationship with the species of subclade O. A case of mitochondrial introgression does not seem plausible as it maintains the same position when adding the nuclear marker. In the Canarian *Laparocerus* tree (unpublished data) there are other species showing similar behaviour in deep nodes, and we find no better explanation for such 'misplaced' species (from the morphological viewpoint) than possible homoplasy or a hybrid origin for them.

The inconsistencies shown in subclade P occur at more shallow levels and can be related either to mitochondrial introgressions, incomplete lineage sorting or poor taxonomy (Maddison & Knowles 2006). The complex of *noctivagans–lauripotens–calcatrix* has been controversial since Wollaston described these species (plus *L. australis*, synonymized thereafter) and deserves a thorough taxonomical revision, including material from all of the range of these species. According to Wollaston (1857), *L. noctivagans* dwells in the forest and higher parts of the islands, while *L. lauripotens* would be limited to the lower lands of the leeside. At least this pattern is inconsistent with our sampling results. Nonetheless,

the high intraspecific divergence of COII gene observed when comparing specimens of *L. noctivagans* (*p*-distance 0.000–0.037) and *L. lauripotens* (0.005–0.042) from different localities, can be interpreted as a 'distance effect' (Slatkin 1993). One would expect such an effect in less vagile species like *Laparocerus* and on old and high volcanic islands — like the Canaries or Madeira — which tend to harbour isolated populations occurring in deep valleys or remote regions (Zimmerman 1948; Peck 2006). In this kind of scenario, a recent species derived in parapatry from a given population may still share with it some particular haplotypes and not those of the other units of the meta-population, favouring incomplete lineage sorting for these markers (Funk & Omland 2003). For clarifying hypothesis of incomplete lineage sorting or mitochondrial introgression we would need a much larger set of samples of individuals, localities, and genes.

Colonization

Laparocerus sp. from Madeira constitute a monophyletic clade (B) separate from the *Laparocerus* representatives from the Canary Islands and Morocco (clade C), a fact that would favour the idea of independent colonization processes for each archipelago.

A very rough estimate of the age of the Madeiran *Laparocerus* clade can be obtained by applying the average of 0.0115 substitution rate per site per million years (Brower 1994) to the maximum corrected evolutive distance found within the clade (*L. calcatrix–L. waterhousei* = 0.13756). It gives an estimated age of 6.11 My. Maximum K/Ar datings for Madeira and Porto Santo are 4.45 and 14.3 My, respectively (Geldmacher *et al*. 2000). The hypothesis of a single colonization event for the group seems plausible. However, we confirmed that evolution in the Madeiran *Laparocerus* clade follows a variable nucleotide substitution rate model better than a constant rate model. Assuming that local rates are plausible, nonparametric rate smoothing clock methods should be applied to better estimate divergence times, but we do not have the minimum of two objective calibration points required to use this method (Sanderson 1997).

The age of a given island -representing a maximum divergence time for endemics — is commonly used as one of the calibration points (e.g. Contreras-Díaz *et al*. 2007). Such approach could tautologically support a single colonization event, which is not necessarily the case in a complex group like *Laparocerus*. The deepest branches in the phylogram may well have developed in the continent prior to colonization of the islands. Perhaps, *Laparocerus* was a large and diversified group inhabiting the former Thetyan evergreen forest of North Africa before climate change fostered extinction (Barbero *et al*. 1981). Before total extinction happened, a set of several *Laparocerus* lineages could have reached the islands, where they survived and afterwards radiated. Both hypothesis of colonization of the archipelagos (single lineage vs. multiple lineages) deserve to be tested using geological independent events for calibration. We hope to find such calibration points when studying the Canary Islands clade.

The Canarian clade

Regarding the Canary Islands, the few representative species included in this study do not allow much discussion. The impression that the Canarian *Laparocerus* is a monophyletic group (clade C) is clearly supported when adding the EF-1α fragment to the data set. The only known species from the African mainland, *L. susicus*, falls within this clade, in coherence with the geographical proximity of both territories. We probably face a back-colonization event. As stated in the introduction, a detailed molecular phylogeny of the Canarian clade will be presented in future contributions.

Acknowledgements

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