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Molecular phylogeny and phylogeography of the Cuban cave-fishes of the genus *Lucifuga*: Evidence for cryptic allopatric diversity

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ABSTRACT

Underground environments are increasingly recognized as reservoirs of faunal diversity. Extreme environmental conditions and limited dispersal ability of underground organisms have been acknowledged as important factors promoting divergence between species and conspecific populations. However, in many instances, there is no correlation between genetic divergence and morphological differentiation. *Lucifuga* Poey is a stygobiotic fish genus that lives in Cuban and Bahamian caves. In Cuba, it offers a unique opportunity to study the influence of habitat fragmentation on the genetic divergence of stygobiotic species and populations. The genus includes four species and one morphological variant that have contrasting geographical distributions. In this study, we first performed a molecular phylogenetic analysis of the *Lucifuga* Cuban species using mitochondrial and nuclear markers. The mitochondrial phylogeny revealed three deeply divergent clades that were supported by nuclear and morphological characters. Within two of these main clades, we identified five lineages that are candidate cryptic species and a taxonomical synonymy between *Lucifuga subterranea* and *Lucifuga teresinarum*. Secondly, phylogeographic analysis using a fragment of the cytochrome *b* gene was performed for *Lucifuga dentata*, the most widely distributed species. We found strong geographical organization of the haplotype clades at different geographic scales that can be explained by episodes of dispersal and population expansion followed by population fragmentation and restricted gene flow. At a larger temporal scale, these processes could also explain the diversification and the distribution of the different species.

1. Introduction

The fragmented structure of groundwater aquifers, historical hydrogeological processes, and the reduced dispersal ability of organisms within these aquifers have produced a rich underground fauna characterized, in most cases, by highly structured populations and species with very restricted distributions (Lefébure et al., 2006; Culver and Pipan, 2009; Zakšek et al., 2009). Notably, phenotypes previously thought to occupy vast regions are, in fact, complexes of species that are geographically restricted. Recently, Culver et al. (2009) suggested that a trade off between vicariant and dispersal process may have acted to produce such patterns. Extreme environments like underground ecosystems may be particularly prone to producing cryptic speciation (Lefébure et al., 2006). Limited dispersal ability leading to strong isolation together with the severe environmental extremes, in terms of physical and ecological characteristics, may lead to diverging populations and ultimately to speciation without appreciable phenotypic changes. Stabilizing selection, reducing or eliminating morphological divergence, associated with the effects of genetic drift on small and isolated populations may thus be a mechanism producing cryptic diversity (Bickford et al., 2007).

Molecular data and the availability of new analysis methods have facilitated the discovery of cryptic diversity (DeSalle et al., 2005) and there are many examples from different groups of stygobiont invertebrates (Leys et al., 2003; Buhay and Crandall, 2005; Lefébure et al., 2006, 2007; Lejeusne and Chevaldonné, 2006; Finston et al., 2007; Page et al., 2008; Bradford et al., 2009; Guzik et al., 2009; Murphy et al., 2009; Trontelj et al., 2009; Zakšek et al., 2009). Although much has been done on the taxonomy, ecology and biology of subterranean fishes (Romero, 2001), few studies

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have been dedicated to analysing the structure of genetic diversity within species and the evolutionary relationships among species (Avise and Selander, 1972; Mitchell et al., 1977; Wilkens, 1988; Pérez and Moodie, 1993; Espinasa and Borowsky, 2000, 2001; Borowsky and Mertz, 2001; Borowsky and Vidthayanon, 2001; Dowling et al., 2002; Strecker et al., 2003; Wilkens and Strecker, 2003; Xiao et al., 2005; Colli et al., 2009). Most of these studies have been on *Astyanax mexicanus*, where strongly genetically structured populations have been identified (e.g. Strecker et al., 2003). One interesting case is the relatively poorly studied group of groundwater-dwelling fishes known as *Lucifuga* Poey that inhabit caves and sinkholes of Cuba and the Bahamas. This genus is one of the most diverse stygobiotic fishes described thus far (Romero, 2001). The distribution of the *Lucifuga* species in Cuba is highly patchy and is mostly restricted to coastal fringes and the exposed karstic plains of the west part of the island that contain phreatic, relatively stable, waters. The karstic rocks, representing about the 60% of the dry land, are not a homogeneous structure and different regions are recognized, depending on the composition of different types of karst and non-karstic rocks (Nuñez-Jiménez, 1967).

The *Lucifuga* are marine in origin and belong to the order Ophidiiformes (Nielsen et al., 1999). At present, seven species are recognized: four of them identified from Cuban fresh and brackishwater caves and sinkholes, *Lucifuga subterranea* (Poey, 1858), *Lucifuga dentata* (Poey, 1858), *Lucifuga simile* (Nalbant, 1981) and *Lucifuga teresinarum* (Díaz, 1988); two from brackish water sinkholes in the Bahamian Islands *Lucifuga speleotes* (Cohen and Robins, 1970) and *Lucifuga lucayana* (Møller et al., 2006) and one, *Lucifuga inopinata* (Cohen and McCosker, 1998), has been described from offshore Galapagos Islands, whose designation as *Lucifuga* remains doubtful (Cohen, personal communication). In addition, a variety of *L. dentata*, *L. dentata* var. *holguinensis*, was originally described from a single locality at the north eastern part of Cuba (Díaz et al., 1987a), however morphologically similar fishes have been also found at other distant localities in the middle south and western parts of the Cuban island (García-Debrás et al., 1999).

Díaz (1988) and Møller et al. (2006) identified a number of characters (e.g. caudal finray numbers, vertebrae number, and eye diameter) that distinguish Bahamian and Cuban species. However, the mosaic distribution of morphological characters within species makes *Lucifuga* taxonomy difficult, and also calls into question the taxonomical usefulness of some of these morphological characters (Nalbant, 1981; Díaz, 1988; Møller et al., 2006).

The only attempt to reconstruct the evolutionary pathways of *Lucifuga* spp. was conducted by Vergara (1980). He analysed 25 morphological characters from the three species described at that time (i.e. *L. dentata*, *L. speleotes* and *L. subterranea*), and two species of the genus *Calamopteryx* as outgroup. Vergara determined plesiomorphic and apomorphic character states and proposed that the Bahamas and Cuba harbour two different evolutionary lineages. He concluded that *L. speleotes* had a higher number of plesiomorphic character states that occurred early during *Lucifuga* evolution whereas *L. subterranea* is the most "adapted" species to underground environments (e.g. smallest eye-balls, lack of palatine teeth, smallest size, lower number of finrays, and a slender body shape). This view remained mostly unchanged until recently, when Møller et al. (2006) suggested that some aspects of *Lucifuga* taxonomy should be reconsidered. These authors proposed that the two subgenera *Lucifuga* and *Stygicola* are invalid, which are defined respectively, by the absence and presence of palatine teeth, and suggested that the lack of palatine teeth in *L. subterranea* and *L. lucayana* is homoplasious.

Given the observed trends from other stygobiotic organisms that are unable to disperse over long distances and that have putatively small effective population sizes, we hypothesize that *Lucifuga* is more diverse than recognized at present. One would expect: (a) relatively deep molecular divergences between morphological recognized species; (b) the occurrence of cryptic diversity in geographically restricted lineages; and (c) deep mitochondrial DNA differentiation between geographically distributed populations characterized by long internal branches connecting geographically restricted haplotypes. In this context, we examined molecular and morphological patterns of *Lucifuga* in Cuba. To maximize our chances of detecting most of the variation within *Lucifuga*, we sampled widely throughout its distribution in Cuba. Using mitochondrial and nuclear DNA sequences from several different *loci*, we first evaluated the phylogenetic relationships between the known species. We also investigated the phylogeographic patterns within the single most widely distributed species in order to identify possible mechanisms that may have operated historically to shape the present patterns of haplotype distribution. We also re-examined morphological variation in the light of the resulting inferred phylogenetic relationships.

2. Materials and methods

2.1. Samples and localities

We sampled *Lucifuga* throughout its distribution in Cuba, from the most eastern locality (Gibara, Holguín province) to Guanahacabibes at the West. Here we report for first time the occurrence of the genus *Lucifuga* outside the mainland in the Cuban archipelago. Five individuals, captured at a new locality, an unnamed crevice at Coco Key, were included in the analysis (Fig. 1, Table 1). Individuals of two of the nominal species (*L. simile* and *L. teresinarum*) were sampled at type localities (Table 1). The fishes were collected by scuba diving using hand nets and transported to the laboratory where they were identified following Nielsen et al. (1999) and original descriptions (Poey, 1858; Cohen and Robins, 1970; Nalbant, 1981; Díaz, 1988; Møller et al., 2006). Type specimens were available for *L. teresinarum* Díaz, 1988 (Holotype, MFP 2015 (reassigned as MFP 18.000385), male, and Paratype, MFP 2016 (reassigned as MFP 18.000386), male, Felipe Poey Museum, Faculty of Biology, University of Havana) and *L. dentata* var. *holguinensis* (no type designation, MFP 18.000420).

Small sections of muscle (~50 mg) or fin clips were dissected from fresh samples and preserved in 95% ethanol. Whole fish were preserved in buffered formalin or 99% ethanol and later transferred to 70% ethanol. For localities where access was difficult or where no fish were found, older tissue samples that had been either frozen or preserved in ethanol were used (Table 1). Each fish was identified by a voucher with species name, and date of collection and locality, and stored in the Felipe Poey Museum (MFP) collection at the University of Havana (Table 1).

2.2. Molecular methods

For old tissue samples, DNA was extracted using the DNAeasy tissue kit (QIAGEN) following supplier guidelines. Fresh tissue, fin or muscle, was digested with proteinase K (100 μg/mL) in 200 μL lysis buffer (100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, and 50 mM DTT) at 50 °C with slow constant shaking, followed by a phenol: chloroform extraction and purification using a Phase Lock Gel™ (Eppendorf).

The polymerase chain reaction (PCR) was used to amplify a total of 1982 bp (aligned positions) of the mitochondrial DNA (mtDNA) genome, including the complete sequence for the ATP8 and ND4L genes, and partial sequences of cytochrome *b* (*cytb*), $tRNA^{Arg}$, $tRNA^{Lys}$, ATP6, and ND2. We also amplified partial sequence of the visual pigment rhodopsin gene (*Rhod*) (373 bp) and the first intron of a homeobox-containing gene (*evx*1) (564 bp). For a selected

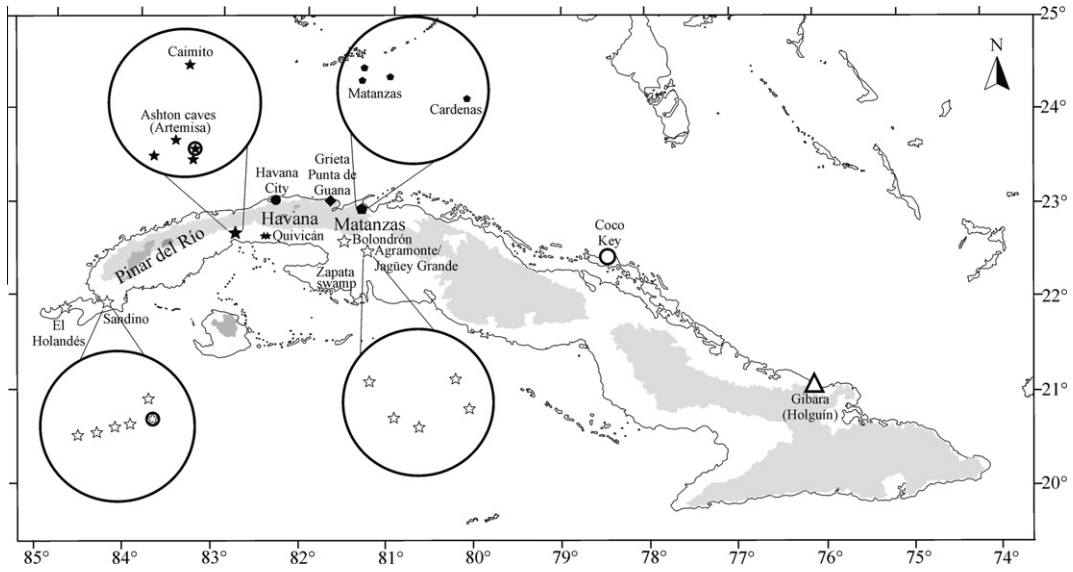

Fig. 1. Sampling localities for *Lucifuga* spp. used in the present study. Regions shaded in grey are mountain ranges (differences in altitude are not indicated). Black star: sampling sites common to *L. dentata* and *L. subterranea*. Black star within a circle: sampling sites common to *L. dentata*, *L. subterranea*, and *L. teresinarum*. White star: *L. dentata* sampling sites. Black pentagon: *L. dentata* (*Lucifuga* sp. 1) sampling sites. Black diamond: *L. simile* sampling sites. Black circle: *L. dentata* (*Lucifuga* sp. 2) sampling sites. White star within a circle: sampling site common to *L. dentata* and *Lucifuga* sp. 3. White circle: *Lucifuga* sp. 4 sampling site. White triangle: *L. dentata* var. *holguinensis* sampling site.

subsample, sequences of two other nuclear genes [calmodulin intron 4 (*CAM*-4) and S7 ribosomal protein intron 1(*S7*-1)] were obtained (Table 1).

Total genomic DNA, 5–100 ng, was used as template in a 50 μL PCR reaction with two units of GoTaq DNA polymerase (Promega), 0.2 μM of each primer, 0.2 μM of dNTPs, and 1.5 mM $MgCl_2$. The primer sequences and the amplification conditions can be obtained by request to the authors (Supplementary material 1).

PCR products were purified using the QIAquick® PCR purification kit (QIAGEN) and cycle-sequenced in both directions using the Big Dye terminator sequencing kit (Applied Biosystems). The fragments were resolved with an ABI 3100 automated sequencer (Applied Biosystems). For *evx*1 and for poorly amplified fragments, PCR products were cloned using the pGEM-T Easy Vector System (Promega). Plasmids were purified using Wizard *Plus* Minipreps DNA Purification System (Promega). Two to three clones were sequenced per sample using T7 and SP6 primers. Sequences were deposited in the EMBL database and accession numbers are provided in Table 1.

2.3. Sequence analysis and phylogeny reconstruction

Sequences were edited using Bioedit Sequence Alignment Editor v5.0.9 (Hall, 1999), and inspected by eye against the original chromatogram. Most alignments were straightforward and did not required further handling, but when necessary multiple sequence alignments were performed with Clustal W (Thompson et al., 1994) as included in MEGA version 4 (Tamura et al., 2007). Secondary structure of the partial tRNA gene sequence ($tRNA^{Lys}$) was inferred to identify homologous sequence positions. The separate gene segments were concatenated in a single alignment using MEGA version 4 (Tamura et al., 2007).

Selection of the outgroups was based on a number of considerations. First, there are relatively limited sequences from this order of fishes in sequence data banks making difficult to test a priori the best candidates as outgroups. Second, as the group lacks strong phylogenetic analysis, the hypothetical sister taxon of *Lucifuga* is unknown at present. We therefore used homologous mtDNA sequences from four Ophidiiformes [*Bassozetus zenkevitchi* (accession number NC004374.1), *Cataetix rubrirostris* (accession number AP004407.1), *Diplacanthopoma brachysoma* (accession number AP004408.1) and *Lamprogrammus niger* (accession number AP004410.1)] reported by Miya et al. (2003). Two species *C. rubrirostris* and *D. brachysoma* are both of the same subfamily (Bythitinae) of *Lucifuga*. In addition we generated new sequence from a fifth species *Stygnobrotula latebricola*, also from the subfamily Bythitinae.

The model of nucleotide substitution best fitting the data was selected using the Bayesian information criterion (BIC; Schwarz, 1978) as implemented in jModelTest version 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008).

We used neighbour joining (NJ), maximum likelihood (ML), and Bayesian methods to generate phylogenetic trees. NJ trees were constructed using MEGA version 4 (Tamura et al., 2007). PhyML 2.4.4 (Guindon and Gascuel, 2003) and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) were used to infer the ML and Bayesian consensus trees respectively. A priori selected model parameters (see above) such as nucleotide substitution matrix and gamma-distributed rate variation across sites were used for tree inference. Stationary base frequencies and substitution rates were optimized during tree inference. The Bayesian analyses consisted of two independent runs using four Metropolis coupled Monte Carlo Markov chains for 1.3×10^6 generations and sampling trees every 100 generations. The first 20% of sampled trees was discarded as burn-in.

For NJ and ML trees, the robustness of the nodes was assessed using the bootstrap method with 1000 and 100 replicates, respectively. Support for the Bayesian analysis was obtained from posterior probabilities of the nodes in the consensus tree.

2.4. Population aggregation analysis of the nuclear sequence variation

Here we proceeded essentially as described by Davis and Nixon (1992). Variable nucleotide site positions were first analyzed among individuals from a single locality assuming that they belonged to the same species, and those populations sharing the same character states were considered conspecific. Groups of populations showing different nucleotide diagnostic profiles were then considered to belong to different evolutionary lineages. We did not establish cutoff levels for polymorphic characters as suggested by

Table 1
Specimens used in the study, identification and accession numbers of the analyzed data sets.

Species	New taxonomical partitions	Sample identification	Voucher (MFP18)[a]	Collection locality (cave, municipality)	mtDNA[b]	*cytb*[c]	Nuclear genes				
							Rhod	*evx*1	*CAM*-4	*S7*-1	Iso[d]
Lucifuga dentata	*Lucifuga dentata*	Ld1Emi	000340	Emilio, Artemisa	FR717320 FR717870 FR718820	FR716705	FR750229 FR750231	FR734114			X
		Ld3Lec	000345	La Lechuza, Artemisa	FR717321 FR717870 FR718823	FR716707	FR750229	FR734125			X
		Ld20Sit	NV	El Sitio, Artemisa[g]	FR717320 FR717870 FR718820	FR716705	FR750229 FR750231	FR734115 FR734116			X
		Ld21Ban	000343	Baño2, Artemisa	FR717320 FR717870 FR718821	FR716700	FR750229 FR750231	FR734110			X
		Ld27Emi	000340	Emilio, Artemisa	FR717320 FR717870 FR718822	FR716702	FR750229	FR734110			X
		Ld33Fel	000320	Felipe, Sandino	FR717318 FR717870 FR718821	FR716690	FR750229	FR734108			X
		Ld46Jag	000318	El Jagüey, Sandino	FR717318 FR717870 FR718821	FR716688	FR750229	FR734108			
		Ld50Gri	000316	El Grillo, Sandino	FR717319 FR717870 FR718821	FR716694	FR750229				
		Ld58Par	000328	Paredones, Caimito	FR717320 FR717871 FR718821	FR716699	FR750229	FR734113	FR750387	FR750343	
		Ld64P	000354	Perico Sanchez, Jagüey Grande	FR717321 FR717872 FR718824	FR716710	FR750229				
		Ld73Ch	000348	Chicharrones, Bolondrón	FR717323 FR717870 FR718826	FR716717	FR750229				
		Ld79Ju	000333	Juanelo Piedra, Quivicán	FR717320 FR717871 FR718821	FR716699	FR750229				
		Ld87Car	000362	La Carreta, Agramonte	FR717321 FR717873 FR718824	FR716711	FR750229 FR750230	FR734108	FR750388 FR750389	FR750343	
		Ld91Rat	000364	Ratonera, Agramonte	FR717322 FR717874 FR718825	FR716709	FR750229	FR775425			
	Lucifuga sp. 1	Lnsp1_003E[f]	000389	El Estadio, Cardenas	FR717324 FR717875 FR718827	FR716722	FR750229 FR750232				
		Lnsp1_004E[f]	000390	El Estadio, Cardenas	FR717325 FR717875 FR718827	FR716723	FR750229 FR750243	FR734122			
		Lnsp1_005E[f]	000391	El Estadio, Cardenas	FR717325 FR717876 FR718827	FR716725	FR750232 FR750243	FR734121			
		Lnsp1_015S[f]	000415	Saturno, Matanzas[h]	FR717327	FR716727	FR750229	FR734120			

(*continued on next page*)

Table 1 (*continued*)

Species	New taxonomical partitions	Sample identification	Voucher (MFP18)[a]	Collection locality (cave, municipality)	mtDNA[b]	*cytb*[c]	Nuclear genes				
							Rhod	*evx*1	*CAM*-4	*S7*-1	Iso[d]
					FR717878 FR718828						
		Lnsp1_025E[f]	000392	El Estadio, Cardenas	FR717325 FR717875 FR718827	FR716724	FR750229 FR750233	FR734124			
		Lnsp1_026E[f]	000412	El Estadio, Cardenas	FR717325 FR717877 FR718827	FR716723					
		Lnsp1_028S[f]	000416	Saturno, Matanzas[h]	FR717327 FR717878 FR718827	FR716728		FR734123			
		Lnsp1_36Ag	000396	del Agua, Matanzas	FR717326 FR717878 FR718829	FR716726	FR750229	FR734119	FR750387	FR750344	X
		Lnsp1_53E[f]	NV	El Estadio, Cardenas	FR717325 FR717875 FR718827	FR716723					
	Lucifuga sp. 2	Lnsp2_012[f]	000397	Cave 1, Habana del Este, Havana City	FR717328 FR717879 FR718830	FR716729	FR750236 FR750237	FR734125			
		Lnsp2_013[f]	000398	Cave 1, Habana del Este, Havana City	FR717328 FR717880 FR718831	FR716729	FR750236 FR750238				
Lucifuga simile	*Lucifuga simile*	Lsim011GPG[e,f]	000408	Grieta Punta de Guana, Matanzas	FR717329 FR717881 FR718832	FR716730	FR750234	FR734124	FR750387	FR750345	
		Lsim014GPG[e,f]	000419	Grieta Punta de Guana, Matanzas	FR717329 FR717882 FR718832	FR716730	FR750234 FR750235	FR734126			
Lucifuga subterranea	*Lucifuga subterranea*	Ls10Ban	NV	Baño2, Artemisa	FR717330 FR717883 FR718834	FR716731	FR750239 FR750240	FR734127			X
		Ls12Emi	000378	Emilio, Artemisa	FR717330 FR717883 FR718833	FR716731	FR750239 FR750241	FR734133		FR750346	X
		Ls30Sit	000383	El Sitio, Artemisa[g]	FR717330 FR717883 FR718834	FR716731	FR750239	FR734127			X
		Ls37Lec	000200	La Lechuza, Artemisa	FR717330 FR717883 FR718834	FR716732	FR750239	FR734129 FR734127	FR750390	FR750346	
		Ls59Par	000199	Paredones, Caimito	FR717331 FR717883 FR718835	FR716732	FR750239	FR734127		FR750346	
		Ls83Ju	000372	Juanelo Piedra, Quivicán	FR717330 FR717883 FR718834	FR716732	FR750239	FR734127			
		Ls84Ju	000373	Juanelo Piedra, Quivicán	FR717330 FR717883	FR716732	FR750239	FR775426			

					FR718834						
		Ls85Ju	000374	Juanelo Piedra, Quivicán	FR717330 FR717883 FR718834	FR716732	FR750239	FR734127			
		Ls86Ju	000198	Juanelo Piedra, Quivicán	FR717330 FR717883 FR718834	FR716732	FR750239	FR734130 FR734127	FR750390 FR750391	FR750347	
		Ls94Ban	000381	Baño2, Artemisa	FR717330 FR717883 FR718834	FR716732	FR750239	FR734134			
		Ls95Ban	000382	Baño2, Artemisa	FR717332 FR717883 FR718834	FR716733	FR750239	FR734131 FR734127		FR750346	
		Ls110Emi	000417	Emilio, Artemisa		FR716732				FR750346	
Lucifuga teresinarum		Lt38Ban[e]	000387	Baño2, Artemisa	FR717330 FR717883 FR718836	FR716732	FR750239	FR734127 FR734132	FR750390	FR750346	
		Lt39Ban[e]	000388	Baño2, Artemisa	FR717332 FR717883 FR718834	FR716733	FR750239	FR734127	FR750390	FR750346	
Lucifuga dentata var. holguinensis	*Lucifuga holguinensis*	Ldh016Taz[f]	399	Tanque Azul, Gibara	FR717333	FR716734	FR750243	FR734137	FR750394	NA	
	Lucifuga sp. 3	Lnsp102PAz	000201	Pozo Azul, Sandino	FR717884 FR717334 FR717885 FR718837	FR716735	FR750242	FR734138	FR750392 FR750393	FR750348	
	Lucifuga sp. 4	Lnsp111CC	000402	Coco Key, Ciego de Avila[i]	FR717335 FR717886 FR718838	FR716736	FR750243		FR750394		
		Lnsp112CC	000401	Coco Key, Ciego de Avila[i]	FR717335 FR717886 FR718838	FR716736	FR750243	FR734139	FR750394		
		Lnsp113CC	000403	Coco Key, Ciego de Avila[i]	FR717335FR717886 FR718838	FR716736	FR750243		FR750394	FR750349	
		Lnsp114CC	000404	Coco Key, Ciego de Avila[i]		FR716736	FR750243		FR750394	FR750350	
		Lnsp115CC	000405	Coco Key, Ciego de Avila[i]		FR716736	FR750243		FR750394	FR750351	FR750352
Out group											
Stygnobrotula latebricola		*Stygnobrotula*	000418	Offshore, North Havana	FR799324 FR839339	FR820784					

NA: unsuccessful amplification.
NV: no voucher number available.
[a] Acronym of the Felipe Poey Museum, Faculty of Biology, University of Havana, Cuba.
[b] Includes the accession numbers of the tRNA-Lys (partial)+atp8+atp6 (partial), nd2 (partial), tRNA-Arg (partial)+nd4L gene domains arranged in the same order.
[c] Intraspecific analyses in *L. dentata*, *Lucifuga* sp. 1 and *L. subterranea*. A NCPA analysis was conducted for the species *L. dentata*. Ascension numbers for supplementary samples included in the study are in the text.
[d] Sample analyzed with isozymes by Hernández (2005), together with several other *L. dentata* ($n = 18$) and *L. subterranea* ($n = 8$) specimens. Note that the exemplar *Lucifuga* sp. 1 (Lnsp1_36Ag) was misidentified as *L. dentata* var. *holguinensis* in the cited work.
[e] Specimens captured at type localities.
[f] Specimens conserved in ethanol or frozen for several years.
[g] Formerly "Los Pérez" cave.
[h] Formerly "Cepero" cave.
[i] Unnamed hole at Coco Key, it represents the first report for the occurrence of the genus *Lucifuga* out of the mainland in the Cuban archipelago.

Wiens and Servedio (2000) because small sample sizes hampered handling frequency data.

2.5. Intraspecific phylogeography

We used the *cytb* sequences (810 bp) to construct haplotype networks in those cases (i.e. *L. dentata*, *Lucifuga* sp. 1, and *L. subterranea*) for which more than one haplotype was identified and where individuals from several localities were available. For the species *L. dentata* and *L. subterranea* the data set was expanded to include more localities as well as additional samples per locality (*L. dentata* accession numbers: FR716685–FR716687, FR716689, FR716691–FR716693, FR716695–FR716706, FR716708–FR716721; *L. subterranea* accession numbers: FR716731–FR716732).

Parsimony networks were obtained using the statistical parsimony method implemented in TCS v 1.21 (Clement et al., 2000). A nested clade phylogeographic analysis (NCPA) (Templeton et al., 1987; Templeton and Sing, 1993; Templeton, 2009) was carried out to uncover major patterns and processes underlying haplotype distribution in *L. dentata*. The program GEODIS v 2.6 (Posada et al., 2000) was used with 10,000 random permutations to calculate the significance of associations between genetic distances and geographic distribution of haplotypes. The inference of historical processes explaining the current patterns of haplotype distribution was obtained using the most recent (April 28th, 2009) inference key provided on the GEODIS webpage (http://darwin.uvigo.es/software/geodis.html).

2.6. Hypothesis testing of phenotypic character evolution

We examined the phylogenetic distribution of four discrete phenotypic traits (caudal–dorsal–anal fin relationships; degree of pigmentation of the eyes; presence/absence of palatine teeth; and number of caudal fin rays) in all individuals. We checked for character stability by analysing, when possible, additional individuals from the fish collection of the Felipe Poey Museum. In total we examined 94 *L. dentata*, two *L. dentata* var. *holguinensis*, 22 *L. simile*, 41 *L. subterranea*, four *L. teresinarum* (including type and paratype), six *Lucifuga* sp. 1, two *Lucifuga* sp. 2, and two *Lucifuga* sp. 3 individuals. For the outgroup, all characters were set as unknown. The program BayesTrait (Pagel and Meade, 2006) was used to estimate character state evolution. To reconstruct ancestral states and obtain their associated likelihood values, a sample of 17,500 trees generated by MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) were used. The estimates were obtained using the reversible-jump MCMC and the BayesMultiState methods (Pagel et al., 2004). The likelihood values of the alternative hypotheses were compared using the Bayes factor criteria according to the expression LR = [log(harmonic mean of the better model) − log(harmonic mean of the worst model)] × 2. Significant association of a tested character state at a given node was assumed when differences were higher than two, values higher than five were considered "strong" and those higher than 10 "very strong" (Pagel and Meade, 2006).

3. Results

3.1. Phylogenetic inference using mitochondrial sequences

For the 45 *Lucifuga* individuals and five outgroups (*B. zenkevitchi*, *C. rubrirostris*, *D. brachysoma*, *L. niger* and *S. latebricola*), the concatenated mitochondrial gene sequences totaled 1982 aligned bp [ATP6 (245 bp), ATP8 (158 bp not including the nucleotides overlapping with ATP6), $tRNA^{Arg}$ (9 bp), $tRNA^{Lys}$ (23 bp), *cytb* (810 bp), ND2 (439 bp), ND4L (297 bp)]. For *Lucifuga* var. *holguinensis* (Ldvh016TAz) we were unable to amplify 22% of the aligned sequence (particularly ND2 and ND4L). Similarly, 38.6% of the data was missing for *S. latebricola* (about half of the *cytb* domain and ND2 segment). A total of 547 variable sites, defining 33 haplotypes, were identified among the ingroup sequences (966 variable sites when outgroups were included), 435 were parsimony-informative. There was no difference in length between protein coding sequences, and no stop codons were detected, indicating that the amplified domains were functional. A single indel was observed in the TψC arm of $tRNA^{Lys}$, *L. subterranea* and *L. teresinarum* shared a nucleotide deletion at this site.

For the phylogenetic inference, we first analyzed each protein coding sequence independently using NJ and ML reconstruction methods. NJ and ML trees were inferred using TrN (Tamura and Nei, 1993) or HKY (Hasegawa et al., 1985) evolutionary models, and the gamma shape or invariant correction parameter estimated for each gene. In all cases, the trees showed essentially the same topology, and invariably the same terminal clades were recovered. A partition homogeneity test analysis (Farris et al., 1995) supported these observations (data not shown). The clade support for some internal nodes was different depending on the gene being analysed and the method being used, possibly due to the relative low number of phylogenetically informative sites at each locus. We therefore concatenated the five protein coding and tRNA gene sequences into a single data set. All subsequent analyses were performed using this dataset.

The evolutionary model estimation indicated that TrN model best fitted the data, with corrections for heterogeneity in site substitution rates (Γ, shape parameter $\alpha = 0.958$) and invariants ($I = 0.348$).

The main incongruence among trees inferred from nucleotide sequence was within the outgroup, in particular the position of *S. latebricola* with respect to the other Bythitidae clades: *Lucifuga* and *Cataetyx* + *Diplacanthopoma*. ML and Bayesian inference placed *S. latebricola* as a sister group to *Cataetyx* + *Diplacanthopoma*, while NJ tree implied a sister relationship with *Lucifuga* (results not shown). However, support values for alternative topologies were not strong, probably because of the amount of missing data for *S. latebricola* (Philippe et al., 2004; Wiens, 2006).

Eight clades were identified within *Lucifuga* (Fig. 2). Individuals of the nominal four species (*L. dentata*, *L. simile*, *L. teresinarum* and *L. subterranea*) that had been previously described based on morphological characters (Poey, 1858; Nalbant, 1981; Díaz, 1988), were found in just three of these clades. A highly supported node (bootstrap = 100% and Bayesian posterior probability = 1.0) links the two lineages that includes the species *L. dentata* + *L. simile* and *L. subterranea*, respectively. The two individuals from the fourth species, *L. teresinarum* (Lt39Ban and Lt38Ban) clustered confidently within the *subterranea* lineage and shared haplotypes with *subterranea* individuals. They were identified as *L. teresinarum* on the basis of only one of the two diagnostic characters, the presence of a caudal fin separated from the dorsal and anal fins; *L. subterranean* has a fused caudal fin (Fig. 2). Five other well supported terminal clades were recovered among the *Lucifuga.* Two are composed of individuals with *L. dentata*-like morphology ("*L. dentata*-like"), designated *Lucifuga* sp. 1 and sp. 2. Three are composed of individuals with *L. dentata* var. *holguinensis*-like morphology ("*holguinensis*-like"), *L. dentata* var. *holguinensis*, and two clades designated *Lucifuga* sp. 3 and sp. 4. Higher level relationships indicate that the *L. dentata* + *Lucifuga* sp. 1 clade together with the *L. simile* + *Lucifuga* sp. 2 clade represents a monophyletic assemblage, and that the lineage leading to *L. subterranea* (+*L. teresinarum*) diverged before the diversification of the *L. dentata* and allies clade (Fig. 2). Finally, the *L. dentata* var. *holguinensis* like clade represents a sister clade to the rest of the *Lucifuga* lineages.

The first *L. dentata*-like clade, *L. dentata sensu stricto*, included individuals collected at the localities from southern Matanzas to

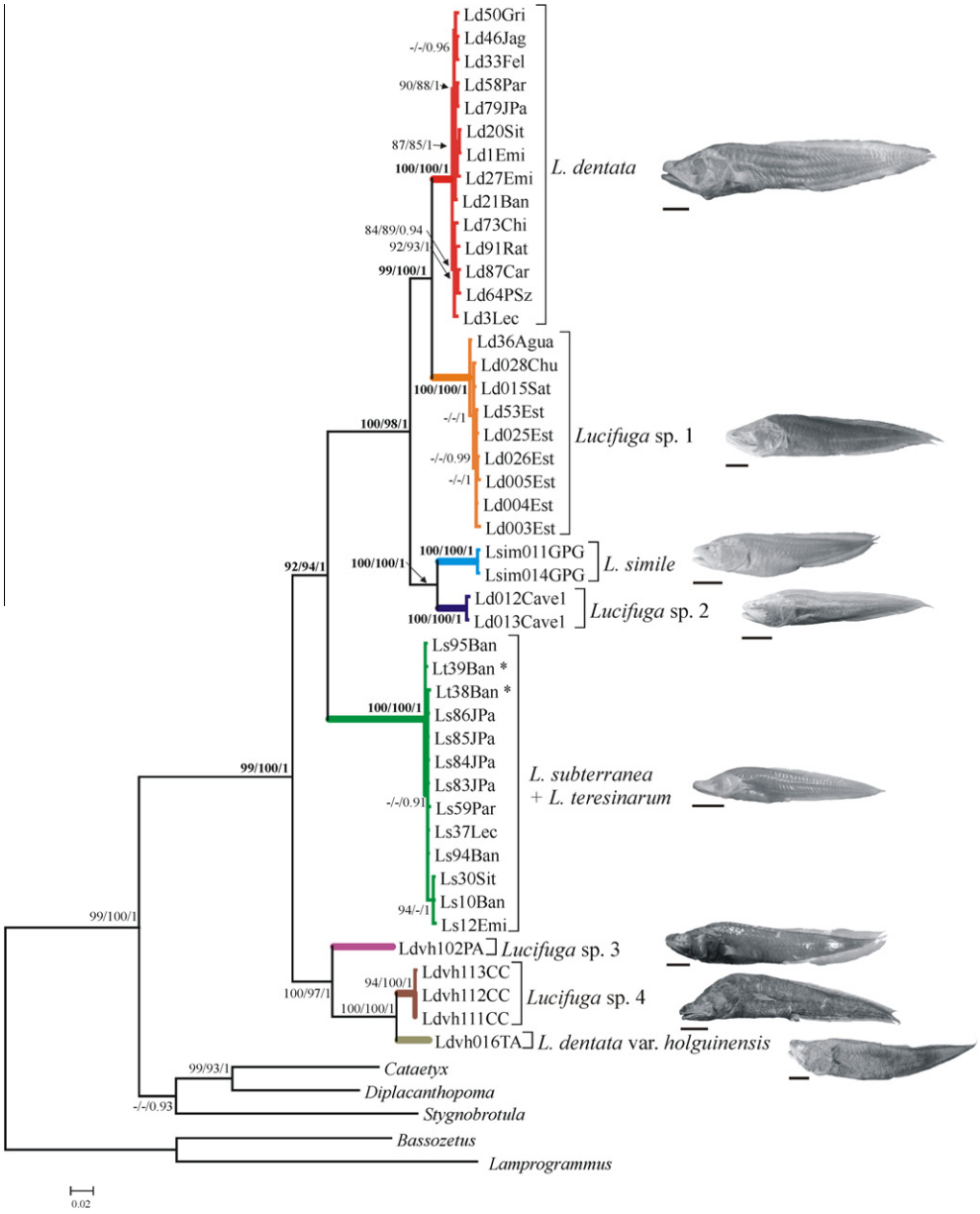

Fig. 2. Phylogenetic tree of the combined mtDNA nucleotide data set (*cytb* + tRNA-Lys + ATP8 + ATP6 + ND2 + tRNA-Arg + ND4L) from *Lucifuga* and outgroups, using NJ, ML and Bayesian inference methods. Bootstrap values higher than 85%, and posterior Bayesian probabilities higher than 90% are shown at nodes in the following order: NJ, ML and Bayesian inference. Asterisks indicate the specimens identified as *L. teresinarum* using morphological characters.

southern Pinar del Río Provinces (see Fig. 1). The second, *Lucifuga* sp. 1 (see Table 1), which fell out as a sister lineage to *L. dentata*, included individuals distributed throughout different localities in the north-eastern Matanzas region (see Fig. 1). The third, *Lucifuga* sp. 2, which fell out as a sister lineage to *L. simile*, contained the two individuals found in just one unnamed cave (Cave 1) near the coast of North Havana City. The *L. dentata* var. *holguinensis*-like clade includes three lineages. The first is a single individual of *L. dentata* var. *holguinensis* from Tanque Azul (Gibara, Holguín) a place relatively close to the first locality where it was reported (Díaz et al., 1987b), the second, *Lucifuga* sp. 4, comprises all individuals (designated as *Lucifuga* sp. 4) sampled at Coco Key locality, which fell out as a sister lineage of *L. dentata* var. *holguinensis*. The third is a single specimen, *Lucifuga* sp. 3, from Pozo Azul (Sandino) at the western part of the island, that fell out as a sister to the other two lineages (Fig. 2, Table 1).

3.2. Character based analysis of nuclear gene variation

The amplification of the nuclear sequence markers was particularly problematic for samples preserved for a long time and for the outgroup (*i.e. S. latebricola*). Nevertheless, we amplified the *Rhod* and *evx* 1 regions for 65 and 63 individuals, respectively, but as we show below the sequence variation was quite low. In an effort to collect more information for nuclear genes, other regions were assayed (*Ald*, *CAM*-4, *CK*, *Ldh*, *Rag*1, *S*7-1, *Tmo*, and *TPI*). However, reliable amplifications were only obtained for *CAM*-4, *Rag*1, and *S*7-1, though the *Rag*1 sequences were invariable between species. These gene sequences were obtained for a reduced subset of samples and clades. An overall low variation was observed at these nuclear gene regions. As shown below, the occurrence of most of the fixed alleles in species with a higher sample number and wider geographic distribution (e.g.: *L. dentata* and *L. subterranea*) justifies

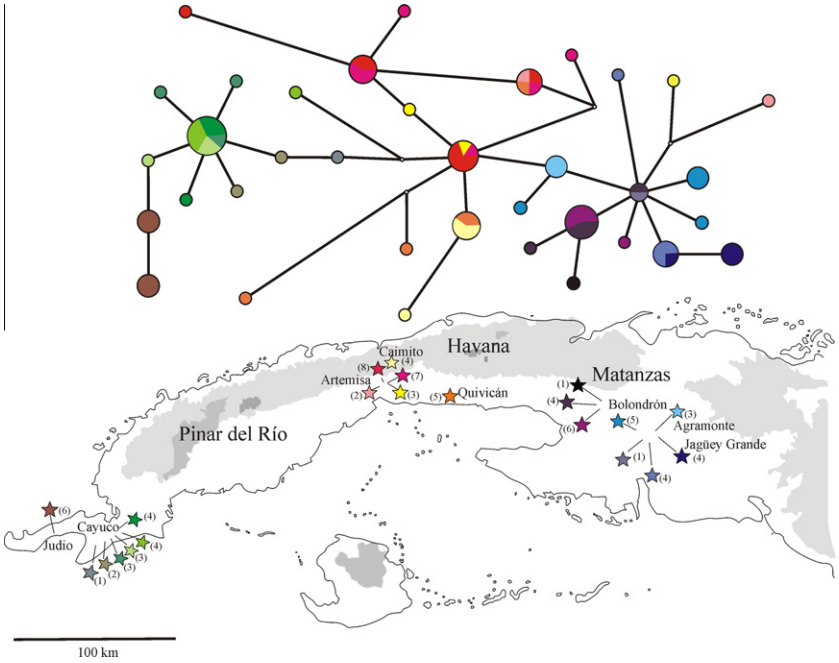

Fig. 3. Statistical parsimony haplotype network reconstructed using the *cytb* sequences from *L. dentata* and the geographic distribution of caves sampled. Caves are indicated as stars with a particular colour code. The colours in circles (haplotypes) indicate the caves where they were found. The length of the connecting branches and the size of the circles are proportional to the number of changes and the number of individuals, respectively. Small empty circles represent inferred haplotypes. Numbers between brackets are the sample sizes for each locality. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the use of PAA in this case. However, as the method is sensitive to sampling errors of individuals and characters (Davis and Nixon, 1992; Sites and Marshall, 2003), the results should be taken with caution because of the limited number of available samples for several taxa.

The *Rhod* gene region (373 pb) had 10 variable sites, six of them were diagnostic for four of the lineages. Conceptual translation of four of these mutations resulted in amino acid substitutions. The *evx*1 intron (565 bp aligned positions) had 15 variable sites, including eight diagnostic sites. The *CAM*-4 gene (441 bp aligned positions) had nine variable sites, and five of them were diagnostic in distinguishing two lineages from the rest. Finally, there were 28 polymorphisms in the *S*7-1 intron sequence, (747 bp aligned positions), and 19 of these diagnostically defined three lineages (Fig. 4).

As observed for the mtDNA, all of the nuclear gene sequences for *L. teresinarum* were identical to that of *L. subterranea* (Fig. 4). This lineage could be distinguished from the others by 12 unique site variations across the four genes, including two amino acid replacement substitutions in the *Rhod* gene (positions 45 and 66). The mtDNA lineages *L. simile* and *Lucifuga* sp. 2 had only one diagnostic site each, in the *Rhod* gene. In *Lucifuga* sp. 2 the observed nucleotide substitution codes for an amino acid replacement. Unfortunately, for these individuals we could not amplify the *CAM*-4 and *S*7-1 regions. *L. dentata* var. *holguinensis* was distinguished by only two nucleotide sites in the *evx*1 intron, information was unavailable for the *S*7-1 region. The *Lucifuga* sp. 3 lineage had the highest number of diagnostic nucleotide sites (13), and one of them was a gap accounting for a several nucleotide repetition in the *evx*1 intron. *Lucifuga* sp. 4 had six diagnostic nucleotide sites, all in the *S*7-1 intron. Unfortunately, no information was available for *L. dentata* var. *holguinensis*, its closest relative as inferred by the mtDNA phylogeny. Finally, *L. dentata* and *Lucifuga* sp. 1 could not be distinguished when all the lineages were analyzed jointly. However, these lineages can be distinguished from each other by two nucleotide sites (positions 36 and 37) in the *S*7-1 intron.

The three major clades identified by the mtDNA data can be distinguished by various diagnostic nuclear nucleotide site positions. We were unable to be definite about whether the variants were diagnostic or not because data is missing for some lineages. Still, the *dentata*-like clade had seven possible diagnostic nucleotide variants (*CAM*-4, positions 185 and 235; *S7*-1, positions 346 and 350; *evx*1, positions 158, 241 and 275), *L. subterranea* (+*L. teresinarum*) clade had at least 13 unique nucleotide variants scattered among all four genes, and in the *holguinensis*-like clade there were probably two in the *S7*-1 (position 373) and *evx*1 (position 146) introns (Fig. 4).

3.3. Intraspecific mtDNA phylogeography

For the three clades with a large enough sample size, i.e. *L. dentata*, *Lucifuga* sp. 1 and *L. subterranea* + *L. teresinarum*, haplotype diversity was examined.

At present, *L. dentata* sensu stricto is the only one species with a wide geographic distribution, whereas all the other studied clades/populations are more or less restricted to particular geographic regions or isolated locations. Although for *Lucifuga* sp. 1 it was not possible to test for associations among haplotypes and their geographic distribution, the distribution of haplotypes per locality indicated that haplotypes are probably not shared between caves (Supplementary material 2). Haplotype and nucleotide diversity were relatively high ($Hd = 0.96 \pm 0.008$; $\pi = 0.0049 \pm 0.00024$), with only one haplotype shared by three individuals out of the nine analyzed. A different pattern was observed in the *L. subterranea* + *L. teresinarum* lineage, where only three haplotypes were detected in a total of 23 individuals from six caves (Supplementary material 2). A single haplotype was shared by all sampled localities, whereas two others were restricted to the region of Artemisa (Baño II, Emilio, La Lechuza, and El Sitio caves). In contrast, there was relative higher haplotypic divergence ($k = 3.99 \pm 0.319$ vs. $k = 1.55 \pm 0.468$ within *Lucifuga* sp. 1) in this lineage, which suggests that there has been a longer period of evolutionary

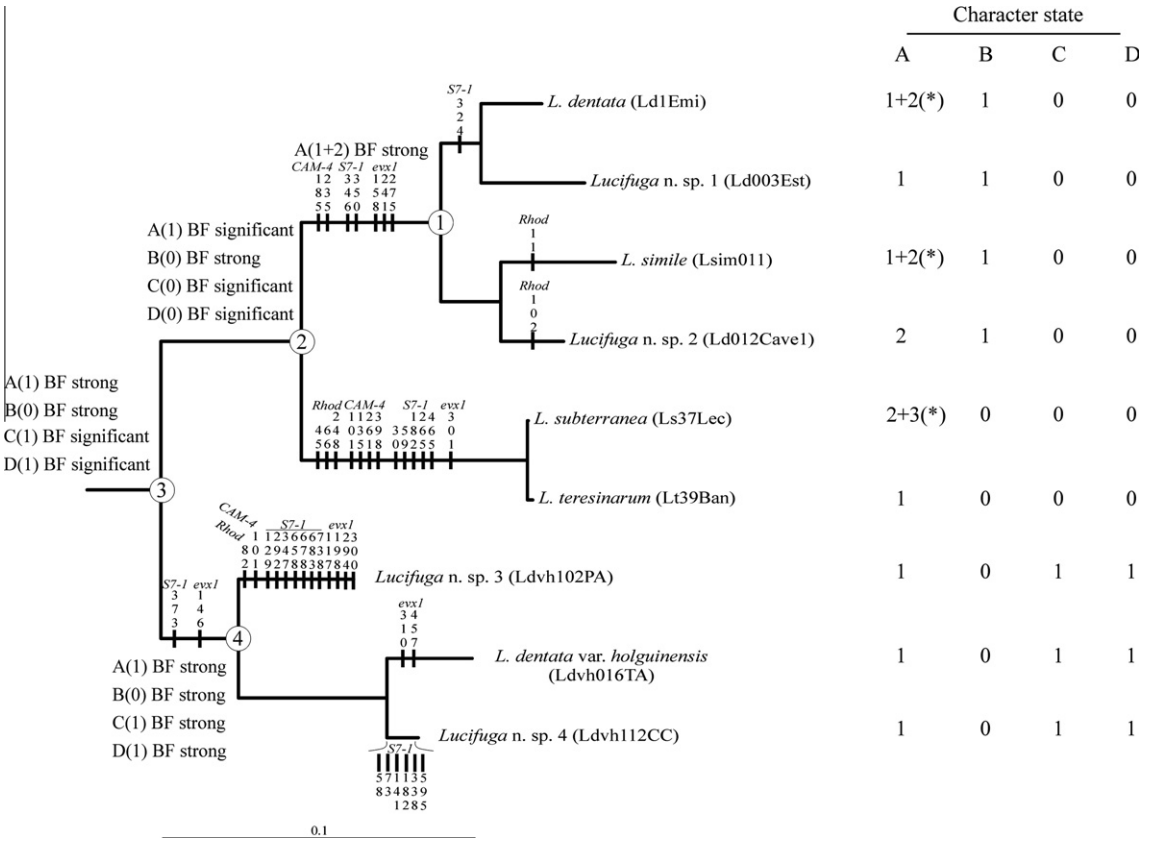

Fig. 4. Analysis of morphological character evolution overlaid on the phylogeny of Cuban *Lucifuga*. The letters and numbers at the right side of the figure represent the characters and their states: (A) relationships between fins (1, not joined; 2, anal–caudal joined; 3, anal–caudal dorsal broadly joined); (B) palatine teeth (0, absent; 1, present); (C) relative eye size (0, 0–0.3% of standard length; 1, 0.54–1.12% of standard length); (D) number of caudal fin rays (0, 8; 1, 10). The asterisk beside the letter A indicates that the frequency of the anal–caudal joined condition is low with respect to the alternative condition.

divergence within *L. subterranea* and probably secondary contact of the two divergent mtDNA lineages in Artemisa. Moreover, it should be noted that *L. teresinarum* had haplotypes LsH1 and LsH3 (Supplementary material 2).

The haplotype network inferred and the nested design for *L. dentata* are shown in Fig. 3 and Supplementary material 2. A total of 36 haplotypes were identified from a total of 80 individuals analyzed. The NCPA showed that significant associations between haplotype clades and their geographical locations were obtained at the highest nesting levels (i.e. 2-step and 3-step clades). For the total cladogram, long-distance colonization [Havana caves (nested level 3-2) to Pinar del Río caves (nested level 3-1)] and past fragmentation [Havana caves vs. Matanzas caves (nested level 3-3)] processes appear to best explain the present haplotype pattern throughout the species range. However, these and other processes were also suggested within very restricted geographic ranges and time periods (Table 2, Supplementary material 2). Namely, the analysis suggests that a recent dispersal event occurred since two highly derived haplotypes, from Matanzas ancestors, were found in two caves (La Lechuza and El Sitio) in the Aston cave system (Artemisa, Havana) (see Fig. 1; Table 1). This event was also inferred with confidence (data not shown) using the coalescent based analysis implemented in the MIGRATE software (Beerli, 1997–2008). Finally, we calculated the number of steps connecting *Lucifuga* sp. 1 *cytb* haplotypes to the *L. dentata cytb* network. A minimum of 31 steps were required, which is outside the 95% confidence level used to infer haplotype connections (data not shown).

Table 2
Evolutionary interpretations of the NCPA *L. dentata cytb* nested contingency results and inferred patterns.

Clade	χ^2	Probability	Inference chain	Inferred pattern
2-1	37.71	0.000	1-2-3-5-15-No	LDC
2-7	64.80	0.000	1-2-3-5-15-No	LDC
3-3	29.00	0.008	1-19-No	AF
Total	147.31	0.000	1-2-11-12-13-14-No	LDC and PF

χ^2 signification based on 10,000 permutations. Inferences were made using the most recent (April 28th, 2009) inference key provided on the GEODIS webpage (http://darwin.uvigo.es/software/geodis.html). AF: allopatric fragmentation; LDC, long distance colonization; PF, past fragmentation.

3.4. Morphological character variation in the light of the phylogeny

Some of the lineages studied had morphological characters previously considered to be restricted to Bahamian species, we therefore re-analyzed four morphological characters that have been previously used in *Lucifuga* taxonomy. In particular, the occurrence of relatively large pigmented eyes and ten caudal fin rays have been used to distinguish the Bahaman species from those in Cuba. *L. dentata*, *L. simile*, *L. subterranea* + *L. teresinarum*, and the newly described lineages (*Lucifuga* sp. 1 and *Lucifuga* sp. 2) all have a dramatic loss of eye pigmentation [0–0.3% of the standard length (SL)] and eight caudal fin rays. However, the *hoguinensis*-like lineages, *L. dentata* var. *holguinensis*, *Lucifuga* sp.3 and 4 showed the same character states observed in Bahamian species: the diameter of the eye pigment ranged from between 0.54% and 1.12% SL, and all have ten caudal fin rays.

The presence/absence of palatine teeth showed a slightly different pattern, all the *L. dentata*-like clades, *L. dentata*, *L. simile*, *Lucifuga*

sp. 1 and 2, have palatine teeth, while those within the *L. subterranea* + *L. teresinarum* and the three *holguinensis*-like clades lack palatine teeth. On the other hand, the caudal–dorsal–anal fin relationships were much more variable. We found that two individuals of the species *L. dentata* (from Perico Sánchez and Chicharrones caves), the two *Lucifuga* sp. 2, and the four *L. subterranea* (from Juanelo Piedra cave) individuals had joined caudal–anal fins and separated dorsal fins. Several individuals of *L. dentata*, one *Lucifuga* sp. 3, and some *Lucifuga* sp. 4 individuals had membranes connecting the three fins basally (included with the individuals with completely separated fins in Fig. 4). These conditions were previously thought to be confined to *L. simile* (Díaz et al., 1987b). Finally, *holguinensis*-like individuals had the caudal fin separated from the dorsal and anal fins.

The result of the analysis using the BayesMultiState option (Pagel et al., 2004) in BayesTrait (Pagel and Meade, 2006) is shown in Fig. 4 and Supplementary material 3. Significant associations between the distribution of character states and the phylogenetic hypothesis were found for all four characters. The analysis of caudal–dorsal–anal fin relationships strongly suggested that for Cuban species the most probable ancestral condition was a separated caudal fin. This state remains the most probable for all the most basal nodes (Fig. 4). Finally, the degree of pigmentation of the eyes and the number of caudal fin rays showed the same phylogenetic distribution. According to the phylogenetic analysis, highly pigmented eyes and ten rays in the caudal fin, as observed in the *holguinensis*-like clade, appear to be the ancestral condition. A reduction on the number of caudal fin rays (eight rays) and a complete or partial loss of eye pigmentation most likely evolved in the common ancestor of *L. dentata* and allies and *L. subterranea* + *L. teresinarum* clade.

4. Discussion

The current study provides the first phylogenetic analysis of the Cuban species of the genus *Lucifuga* using molecular data, and shows that a reappraisal of the taxonomic status and geographic distribution of the evolutionary units of this genus should be undertaken, including the recognition of candidate morphologically cryptic species. Evolutionary lineage distribution and phylogeographic inferences also showed that both dispersal and vicariant processes have occurred in the evolution of this cave-fish genus.

4.1. Phylogeny and phylogeographical patterns

The genus *Lucifuga* is an assemblage with three main clades in Cuba: (i) *L. dentata* and allies; (ii) *L. subterranea* + *L. teresinarum* and (iii) *holguinensis*-like lineage. The first two clades correspond to previously described morphological partitions (Poey, 1858; Vergara, 1980; Nalbant, 1981) but the third one represents a new lineage, which, however, has been previously indicated by COI sequence variation analysis of Cuban freshwater fishes (Lara et al., 2010).

The character based analysis of the nuclear genes supported the main partitions described above, although the number of diagnostic sites per clade was variable. The *L. subterranea* + *L. teresinarum* clade had the highest number of diagnostic sites (13), followed by the *L. dentata*-like clade (7) and the *holguinensis*-like clade (2). However, this result may be highly influenced by the available sample size and the lack of information for some lineages so the results presented here should be taken with caution. Nevertheless, the low sequence variation observed in nuclear genes (i.e. *evx*1 and *Rhod*) for which the number of individuals analyzed was relatively larger (i.e. *L. dentata* and *L. subterranea*) suggests that the general trend is probably similar for other genes. In addition, previous analysis of 28 isozymes and general protein loci revealed complete monomorphy in 24 *L. dentata*, 11 *L. subterranea* and one *Lucifuga* sp. 1 (Lnsp1_36Ag) individuals (see also Table 1). However, 11 loci (*Est*3, 6 and 7; *Mdh*2; *Per*1 and 2; GP 4, 5, 7, 8, 9 and 10; *SOD*) showed clear differentiation of *L. dentata* and *Lucifuga* sp. 1 from *L. subterranea* (Hernández, 2005).

Although well supported, the deepest branches connecting the three major clades are relatively short, strongly suggesting that the most of the internal speciation events happened quite rapidly. This pattern is also apparent when observing the branches connecting sister terminal lineages which show that the splits probably took place relatively simultaneously followed by a rapid sorting of the mtDNA haplotypes. We hypothesize that most of them have occurred during the last few million years when paleogeographic events shaped the present archipelago, and that the most recent splits probably have occurred during a more recent common paleogeographic event. The strong geographical structure within lineages (i.e. *L. dentata*) and among different lineages (species) suggests that the underground aquifers have not been connected for a very long time, if ever. The distribution of most of the populations is peripheral, closely related to coastal margins (see Fig. 1), evidenced by the wide tolerance to salinity in these species. Habitat availability is particularly important in the evolution of this group of stygobiotic fishes whose marine ancestor dwelled in shallow waters of the paleoarchipelago. Thus, Vergara (1981) suggested that *Lucifuga* evolution may have occurred during the late Pliocene–Pleistocene period, when cave and hydrographic systems formed in karstic areas, and recurrent sea level fluctuations may have created conditions suitable for dispersal of these organisms. Indeed, it has been suggested that sea regressions probably represent one of the most significant vicariant events for stygobiotic faunas (Culver et al., 2009). Nonetheless, the relatively recent origin of *Lucifuga* appears challenged by a high level of mtDNA sequence divergence estimated (around 30%) at the deepest nodes of the phylogeny. If Vergara's (1981) hypotheses are correct, it means that this genus experienced an accelerated rate of mtDNA sequence evolution. Conversely, initial diversification of *Lucifuga* may be much older than previously thought.

Intraspecific analyses may shed light on the processes that have moulded the evolution of *Lucifuga*. For the species with a widest distribution (*L. dentata* sensu stricto), mechanisms such as long-range dispersal and range fragmentation were inferred and appear to have produced a pattern of population differentiation highly congruent with spatial distribution. In fact, haplotype distribution shows that in several cases caves have unique mtDNA haplotypes, suggesting very restricted or no gene flow between relatively close geographic localities (Slatkin and Maddison, 1989; Martin and Bermingham, 2000). This was also apparent for *Lucifuga* sp. 1 that has no shared haplotypes among the different caves (Supplementary material 2). This characteristic has been observed for many subterranean species including cave salamanders (Wiens et al., 2003), phalangodid harvestmen (Hedin and Thomas, 2010), amphipods (Lefébure et al., 2006, 2007; Finston et al., 2007), isopods (Verovnik et al., 2005), crayfishes (Buhay and Crandall, 2005), and shrimps (Zakšek et al., 2009). In general, as seen in other stygobiotic fishes [e.g. *Schistura oedipus* (Borowsky and Mertz, 2001), *A. mexicanus* (Strecker et al., 2004), *Sinocyclocheilus* sp. (Xiao et al., 2005)], these findings suggest that under certain circumstances ecologically specialized species can disperse and found new discrete populations, possibly by a very small number of individuals, followed by a rapid drift-induced divergence (Templeton, 1980; Avise, 2000; Bickford et al., 2007). Alternatively, range fragmentation, that is, isolation of cave systems, may have resulted in isolation of populations and genetic differentiation. When extrapolated to the species level, the present distribution of the recognized lineages suggests that dispersal at different geographic scales (e.g. *L. dentata* var.

holguinensis and allies lineages), followed by isolation of available habitats and range fragmentation in other instances (e.g. *L. dentata* and allies lineages) has occurred relatively frequently. In fact, most of the observed terminal lineages have narrow allopatric distributions, closely related to particular karstic patches that have separate geographical histories or are intersected by the orogenesis of mountain ranges that fragmented subterranean aquifers (e.g. the north and south karstic regions of Havana–Matanzas) (see Fig. 1 and also Fig. 115 in Nuñez-Jiménez et al., 1984; Nuevo Atlas Nacional de Cuba, 1989). This phylogeographic pattern has also been observed in other stygobiotic taxa (Buhay and Crandall, 2005; Finston et al., 2007; Lefébure et al., 2007; Zakšek et al., 2007; Page et al., 2008). Although determining the geographic patterns of the speciation process is a difficult task because species ranges certainly change overtime (Losos and Glor, 2003), the evidence from *Lucifuga* suggests that dispersal and allopatric divergence has driven diversification in this genus.

Two species, *L. subterranea* and *Lucifuga* sp. 3 have sympatric distributions with *L. dentata*. The first, *L. subterranea*, has a distribution that completely overlaps with *L. dentata* distribution in the south Havana province while *Lucifuga* sp. 3 is known from a single locality Pozo Azul, Sandino municipality (western Pinar del Río). As none of these three clades have a sister relationship, sympatric speciation seems unlikely to explain present distributions (Avise, 2000). Mechanisms such as range shifts may better explain the occurrence of these divergent evolutionary lineages in sympatry (Avise, 2000; Knowles, 2001). According to the processes inferred from the phylogeographic analysis, it seems likely that *L. dentata* invaded regions previously occupied by other species (i.e. *L. subterranea*) (see Table 2). However, this is less clear in the case of *L. dentata* – *Lucifuga* sp. 3 since the sister clade of the *Lucifuga* sp. 3 taxon is geographically distant (>900 km) (see Fig. 1). Moreover, similar morphotypes to *L. dentata* var. *holguinensis* clade have been found from Zapata swamp karstic system, a much closer locality at the south central region of the island, which suggests that the co-occurrence of *L. dentata* – *Lucifuga* sp. 3 could be due to colonization (see Fig. 1).

4.2. Cryptic diversity and taxonomic revisions

During the last few years candidate cryptic species have been regularly uncovered for several groups of organisms (Bickford et al., 2007) and cave-adapted species appear to be particularly prone to being genetically divergent but morphologically indistinguishable (Lefébure et al., 2006, 2007; Culver and Pipan, 2009; Hedin and Thomas, 2010). Because in an extreme environment (i.e. subterranean environments), organisms are believed to be very constrained in the ways they can adapt, they can be expected to converge in physical characteristics, leading to morphological stasis (Bickford et al., 2007). However, sibling species may evolve, for example via differentiation of mating signals (Bickford et al., 2007). This could be the case for *Lucifuga*, which, in addition to the nominal species (*L. dentata*, *L. simile* and *L. subterranea*), includes five other evolutionary clades. Two of the clades, *Lucifuga* sp. 1 and sp. 2, previously recognized as *L. dentata* (Poey, 2000; García-Debrás and Pérez, 1999) (though *Lucifuga* sp. 2 is actually a sibling lineage to *L. simile*) lack diagnostic morphological characters with respect to its nominal sister species. Similarly, there are few morphological differences within the *holguinensis*-like clade. Mitochondrial DNA sequence divergence was relatively high between sister terminal clades: 4.7–5.7% (3.5–4.2% for the *cytb*); while intraclade divergences (i.e. *L. dentata*, *Lucifuga* sp. 1 and *L. subterranea* for which a larger number of individuals were sequenced) were very low (0–0.4%). However, there were few diagnostic nucleotide changes in the nuclear genes in most cases. The most clearly supported clade was *Lucifuga* sp. 3 that has the largest genetic distance estimates [13.4% (9.5% for *cytb*) and 15.7% (9.6% for *cytb*)] and several distinctive nucleotide site changes with respect to the other two clades (*Lucifuga* sp. 4 and *L. dentata* var. *holguinensis*, respectively) at least for three of the genes (i.e. *CAM*-4, *evx*1 and *Rhod*).

Confirming whether the newly identified clades (i.e. *Lucifuga* sp. 1, *Lucifuga* sp. 2, *L. dentata* var. *holguinensis* and *Lucifuga* sp. 4) represent species is challenging because of the lack of diagnostic differences in nuclear genes and the uncertainty of species delimitation based on only one type of genetic marker (Avise and Ball, 1990; Moritz et al., 1992; Sites and Crandall, 1997; Avise, 2000; Baker et al., 2001; Hudson and Turelli, 2003; Ballard and Whitlock, 2004; DeSalle, 2006). However, the significant population structure and range shift events detected in *L. dentata* suggests that sufficient time has elapsed since the split of this species and *Lucifuga* sp. 1 from a common ancestor. A hierarchical likelihood ratio test (data not shown) did not reject the molecular clock hypothesis, so assuming equal evolutionary rate variation between terminal sister lineages, equivalent evolutionary distances to those observed between *L. dentata* and *Lucifuga* sp. 1 can serve as yardstick to identify real independent evolutionary lineages in this genus.

The species status of *L. teresinarum* was not supported by mtDNA and nuclear genes. It is now apparent that the two morphological characters used to identify *L. teresinarum* (i.e. the relationship between the anal–caudal–dorsal fin and the shape of the male genitalia) are highly variable both within and among species (see Fig. 4). We examined all available males in the different lineages described here and found large variation in hood shapes within each lineage. *Lucifuga simile*, as described by Díaz et al. (1987a), is the only species that has a conical hood shape in all examined male individuals ($n = 14$).

An alternative explanation for the combination of morphological characters observed in *L. teresinarum* is suggested from the sympatric distribution of *L. dentata* and *L. subterranea* in the south Havana region. This suggests that *L. teresinarum* may be a hybrid, a hypothesis which could be tested once diagnostic alleles are available for each parental species (Gow et al., 2006). Although a directional introgression event (*L. subterranea* females and *L. dentata* males), as suggested by the mtDNA results, cannot be ruled out, neither of the two *teresinarum* individuals nor the two *L. subterranea* individuals with a free dorsal fin (Ls84Ju and Ls85Ju from Juanelo Piedra cave) had nuclear alleles diagnostic for *L. dentata*. So, if directional introgression has occurred, this is not apparent from any of the genes sequenced or the evidence has been erased after successive backcrosses with *subterranean* types.

4.3. Morphological character evolution

Several morphological attributes were highly correlated with the three main mtDNA clades. As described previously for *L. dentata* and *L. simile* (Nalbant, 1981; Díaz et al., 1987a) and now also for *Lucifuga* sp. 1 and sp. 2, these clades have palatine teeth, very similar head shapes, but have variable anal–caudal–dorsal relationships (see Fig. 4). The species *L. subterranea* sensu lato is morphologically dissimilar to other *Lucifuga* in that it has a slender body shape, a high frequency of fused anal–caudal–dorsal fins, as well as differences in other morphometric characters (Vergara, 1980). In contrast to the *L. dentata* clade, it lacks palatine teeth (Poey, 1858), a condition shared with *holguinensis*-like clade and one Bahamian species (*L. lucayana*). Finally, the *holguinensis*-like clade has highly pigmented eyes and ten caudal fin rays, in contrast to *L. dentata* and allies + *L. subterranea*, which all have eight caudal finrays and reduced pigment-deficient eyes. A preliminary analysis of the evolution of these morphological characters based on the inferred mtDNA based phylogeny suggests, with moderate statistical support, that the overall character conditions observed in the *L.*

dentata var. *holguinensis* clade was present in the common ancestor of Cuban *Lucifuga*. The analysis also suggests that the acquisition of palatine teeth was a novelty in the *L. dentata* and allies clade and provides further evidence that generic or subgeneric subdivision of the genus (e.g.: *Stygicola* and *Lucifuga*) is unwarranted (Vergara, 1980; Møller et al., 2006). However, phylogenetic relationships between the Cuban and Bahamian species need to be established in order to determine how these discrete morphological features have evolved in *Lucifuga*.

The two Bahamian *Lucifuga* species have been distinguished from Cuban species by several morphological character states (Cohen and Robins, 1970; Vergara, 1980; Møller et al., 2006), however they are also present in *L. dentata* var. *holguinensis*. These findings raise the question of whether the shared morphological similarities between the *holguinensis*-like clade and Bahamian species indicate common ancestry, retention of plesiomorphic character conditions, or merely represent homoplasies.

5. General conclusions

An important conclusion from this study is that the current estimates of species diversity for *Lucifuga* may be underestimated. This was also seen in a broader molecular barcoding survey of freshwater fishes of Cuba (Lara et al., 2010). If we take into account the number of new evolutionary lineages/candidate species identified and the two Bahamian species, the genus becomes probably one of the most species rich cavefish genus described so far (Romero, 2001; Xiao et al., 2005). We also hypothesized that, given the fragmented nature of the distribution of *Lucifuga*, further hidden variation may still be waiting to be discovered.

Recent efforts for protecting vertebrate species in Cuba include the creation of a National Red List of species. This list only includes described species, so the results obtained here make a significant contribution towards the recognition of new evolutionary significant units, as well as supplying information about their distribution. Particularly, the extremely restricted geographic distribution for some of them and the introduction of alien species in subterranean ecosystems implies that conservation plans need to be rapidly implemented to preserve the evolutionary legacy of this peculiar group of cave fishes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.06.015.

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