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Molecular Phylogenetics and Evolution 30 (2004) 87–96
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogeny and karyotype evolution of the Iberian *Leptynia attenuata* species complex (Insecta Phasmatodea)

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Received 4 September 2002; revised 29 January 2003

Abstract

An in-depth analysis of the *Leptynia attenuata* species complex has been performed by cytochrome oxidase subunit 2 (*cox2*) gene sequencing as well as karyotype and allozyme analysis. The whole set of data allows to largely resolve the taxonomy of the group and suggests an overall trend of chromosomal repatternings through a progressive reduction of the chromosome number. A previously suggested new species has been also confirmed on a genetic basis. Data are discussed in order to depict a phylogenetic and phylogeographic scenario fitting the observed genetic relationships between the different species of the group. Chromosome rearrangements are proposed as the major speciation driving force within the group and androgenetic reproduction is suggested as a shortcut to overcome the problem of fixing chromosomal rearrangements that are strongly underdominant in heterozygotes.

Keywords: Cytochrome oxidase II; Stick insects; *Leptynia*; Phylogenesis; Biogeography; Androgenesis

1. Introduction

Phasmids are an intriguing group of tropical and subtropical pterygote insects. Although pretty common in most ecosystems, they are scarcely known because of their mimicry (stick and leaf insects) and nocturnal habits. However, a few phasmids have been studied for their uncommon reproductive features and speciation by repeated interspecific hybridization (Bullini and Nascetti, 1990; Pijnacker, 1964, and references therein; Scali et al., 1995).

The holomediterranean genus *Bacillus* proved a good experimental system to study hybridization and unusual reproductive modes, such as parthenogenesis, hybridogenesis, and androgenesis. A large set of research methods have been applied to *Bacillus* taxa (i.e., morphological, karyological, allozyme, and molecular studies), leading to a fairly satisfactory understanding of their phylogenetic relationships and cladogenetic mechanisms (Mantovani et al., 2001, and references therein).

In Europe, two more stick insect genera are present: *Clonopsis*, also belonging to the Bacillidae family, and *Leptynia*, ascribed to the family Heteronemiidae. European *Leptynia* species are only spread in the Iberian Peninsula and Southern France, but some additional *Leptynia* species have been reported from Northern Africa (Bradley and Galil, 1977). However, Northern African species have been only tentatively ascribed to *Leptynia* and a clear taxonomic review of Heteronemiidae taxa is not yet available for that area.

Quite recently, mitochondrial DNA (*cox2*, *rrnL* genes) clearly showed that all European *Bacillus*, *Clonopsis*, and *Leptynia* species pertain to a single monophyletic clade, although ascribed on morphological basis to different families of different suborders, thus evidencing once more that the entire phasmid taxonomy needs a deep revision (Galassi, 2002).

Previous investigations on Iberian *Leptynia* revealed a complex situation that requires a more detailed analysis. Actually, *Leptynia* was formerly known to include two well-differentiated species [*L. hispanica* (Bolivar) and *L. attenuata* Pantel], but from karyological and allozyme analyses it was pretty clear that both had to be considered as species complexes instead (Bianchi, 1992;

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doi:10.1016/S1055-7903(03)00156-8

Bianchi and Meliado, 1998; Passamonti et al., 1999; Scali, 1996). Furthermore, sharply distinct morphological features were found between *L. hispanica* and *L. attenuata* species complexes (i.e., body traits, egg shape and chorionic pattern, genitalia). On the contrary, within *L. attenuata* as well as *L. hispanica*, morphology does not completely help to discriminate the species, since only a few morphological characters show a neat degree of divergence (Scali, 1996, and unpublished data).

Leptynia attenuata comprises only bisexuals, at variance with *Bacillus*, *Clonopsis*, and *L. hispanica*. In *L. attenuata* previous morphological, karyotypic, and allozymic analyses depicted three distinct groups of populations, which were therefore described as different species: *L. attenuata sensu stricto* ($2n = 36$, XY/XX, Portugal), *L. montana* Scali ($2n = 37/38$, XO/XX, Sistema Central Mountains), *L. caprai* Scali ($2n = 39/40$, XO/XX, Montes de Toledo) (Passamonti et al., 1999; Scali, 1996). Moreover a fourth putative species, showing a new and highly divergent karyotype ($2n = 39/40$, XO/XX), has been suggested for Southern Spain populations (Sistema Penibético) (see Bianchi and Meliado, 1998).

The present paper deals with new data on the *L. attenuata* species complex obtained by the cytochrome oxidase subunit II (*cox2*) sequencing. Moreover, new data on karyotype stucture and allozymes have been added to those already published in Passamonti et al. (1999) and here proposed. The overall results allowed us to revise the genetic features of each taxon and to reconsider the phylogeny of the group.

2. Materials and methods

Twenty-four samples, collected over a wide area of the Iberian peninsula, have been utilized for this study. All samples have been characterized for the *cox2* gene (partial sequence), whereas karyologycal and allozyme analyses have been performed on 11 and 9 samples respectively, which were not characterized in Passamonti et al. (1999). These findings have been summed up to get the complete data set to be analyzed in this paper. Pertinent sampling informations are reported in Table 1 and Fig. 1.

2.1. Karyotype analysis

A basic karyotype analysis (GIEMSA stained chromosomes) has been performed on gonad tissues, according to Marescalchi and Scali (1990). Mitoses and meioses have been analyzed in order to define karyotype, male meiotic features and sex formula.

2.2. Mitochondrial DNA analysis

Genomic DNAs of single field collected specimens were prepared according to the method described in Preiss et al. (1988). The *cox2* gene was amplified and sequenced using TL2-J-3034 (5′-AATATGGCAGA TTAGTGCA-3′) and TK-N-3785 (5′-GTTTAAGAG ACCAGTACTTG-3′) insect universal primers (Simon et al., 1994). Partial sequences for the *cox2* gene were obtained for 1–3 individuals from each locality (see Table 1).

Sequences were aligned with the Clustal algorithm of the Sequence Navigator program (ver 1.0.1, Applied Biosystems). Nucleotide alignment corrections were performed taking as a reference the aminoacidic sequences of the partial *cox2* protein, already characterized in 13 insect species belonging to 10 different orders (Liu and Beckenbach, 1992) and in the stick insects of the genus *Bacillus* (Mantovani et al., 2001). The nucleotide sequences of the *cox2* gene from the analyzed *Leptynia* specimens have been entered into GenBank (accession numbers reported in Table 1). Sequences of *Leptynia hispanica* (Bolivar), *Bacillus rossius* Rossi, *B. atticus* Brunner, *B. grandii* Nascetti and Bullini, *Clonopsis gallica* (Charpentier), and *Medaura scabriusculus* (Wood-Mason) were also utilized as outgroups. Codon translations to aminoacids were obtained using the *Drosophila* mitochondrial genetic code, since no inconsistencies were found so far when applied to mitochondrial genes of other insects (Jermiin and Crozier, 1994, and references therein).

Phylogenetic analyses were performed using neighbor joining, maximum parsimony and maximum likelihood approaches, using PAUP (version 4.0, Swofford, 1998). Likelihood scores of each DNA substitution model were calculated using Modeltest software (Posada and Crandall, 1998) and the best scored model (TrN + Γ + I, Tamura and Nei, 1993) was used for maximum likelihood tree reconstructions. According to this, the Tamura–Nei nucleotide distance was utilized for the neighbor joining tree. Support of each dendrogram was obtained using Bootstrap (Felsenstein, 1985). "A posteriori" competing phylogenetic hypotheses (tree topologies) have been also compared using the Shimodaira and Hasegawa test (Goldman et al., 2000; Shimodaira and Hasegawa, 1999) with the SHTest software (v.1.0, available at http://evolve.zoo.ox.ac.uk/software/SHTests).

Moreover, the method described in Takezaki et al. (1995) has been applied both to assess constancy of mutation ratio within the European stick-insects and to obtain a neighbor joining linearized tree, using the Lintree program (Dos executable, available at http://mep.bio.psu.edu). Time calibration obtained for *Bacillus* species (Mantovani et al., 2001), was utilized to estimate divergence time among *Leptynia* clades.

Table 1
Sampling localities, sample identification acronyms and GenBank accession nos. of the investigated *Leptynia* samples

Species	Locality	Allozymes	Cox2	
		No. of samples	Sample	GenBank Accession No.
Ingroups				
L. sp. ($2n = 40/39$, XX/XO)	Sierra de Grazalema, Spain (GRA)	25	GRA1	AF241412
			GRA2	AF241413
			GRA3	AF241414
	Ojen, Spain (OJE)	na	OJE1	AF241415
L. caprai ($2n = 40/39$, XX/XO)	Urda, Spain (URD)	17[a]	URD1	AF241431
	Viso del Marqués, Spain (VIS)	na	VIS1	AF241433
			VIS2	AF241434
	San Lorenzo de Calatrava, Spain (SLC)	6	SLC1	AF241435
			SLC2	AF241436
	Los Yébenes, Spain (LYE)	25[a]	LYE1	AF241437
	Puerto de Los Majales, Spain (PLM)	24[a]	PLM1	AF241438
			PLM2	AF241439
L. montana ($2n = 38/37$, XX/XO)	El Escorial, Spain (ESC)	7[a]	ESC1	AF241416
			ESC2	AF241417
	Puerto Cruz Verde, Spain (PCV)	23[a]	PCV1	AF241418
			PCV2	AF241419
			PCV3	AF241420
	Las Navas del Marqués, Spain (LNM)	21[a]	LNM1	AF241421
			LNM2	AF241422
	Puerto de Las Pilas, Spain (PLP)	24[a]	PLP1	AF241423
			PLP2	AF241424
	Pinosol, Spain (PIN)	26[a]	PIN1	AF241425
			PIN2	AF241426
	Zarzalejo, Spain (ZAR)	14[a]	ZAR1	AF241427
			ZAR2	AF241428
	Ventorillo, Spain (VEN)	20[a]	VEN1	AF241429
			VEN2	AF241430
L. attenuata ($2n = 36$, XX/XY)	São Fiel, Portugal (*locus typicus*) (SFI)	26	SFI1	AF508232
			SFI2	AF508233
			SFI3	AF508234
	Marvão, Portugal (MAR)	8	MAR1	AF241442
	Portalegre, Portugal (POR)	23	POR1	AF241443
	Fundão, Portugal (FUN)	na	FUN1	AF508800
	Peña de Francia, Spain (PDF)	16	PDF1	AF508230
			PDF2	AF508231
	Santibáñez, Spain (SIB)	8	SIB1	AF508235
			SIB2	AF508236
	El Barco de Ávila, Spain (BAV)	9	BAV1	AF508241
			BAV2	AF508242
	Béjar, Spain (BEJ)	23	BEJ1	AF508237
			BEJ2	AF508238
	Guarda, Portugal (GUA)	na	GUA1	AF508239
			GUA2	AF508240
	Monchique, Portugal (MON)	20[a]	MON1	AF241440
			MON2	AF241441
Outgroups				
Leptynia hispanica	Benissa, Spain			AF241444
Bacillus rossius	Capalbio, Italy			AF038206
Bacillus atticus	Cugni, Italy			AF038226
Bacillus grandii	Ponte Manghisi, Italy			AF148301
Clonopsis gallica	Laujaon, Spain			AF096287
Medaura scabriusculus	Bangladesh			AF508243

na, not analyzed.
[a] Samples analyzed in Passamonti et al. (1999).

2.3. Allozyme analysis

New allozyme analysis was here carried out in nine samples (Peña de Francia, Santibáñez, El Barco de Ávila, São Fiel, Béjar, Marvão, Portalegre, San Lorenzo de Calatrava, Grazalema); these data were pooled with those reported in Passamonti et al. (1999). Leg muscle homogenates were run on cellulose acetate membrane (Cellogel), as reported in Passamonti et al. (1999). The following enzymes were analyzed: α-glycerophosphate dehydrogenase (αGPDH, E.C.: 1.1.1.8), malate dehydrogenase (MDH, E.C.: 1.1.1.37), isocitrate dehydrogenase (IDH, E.C.: 1.1.1.42), 6-phosphogluconate dehydrogenase (6PGDH, E.C.: 1.1.1.44), glucose oxidase (GOX, E.C.: 1.1.1.47), glyceraldehyde-3-phosphate dehydrogenase (G3PDH, E.C.: 1.2.1.12), aspartate amino transferase (AAT, E.C.: 2.6.1.1), adenilate kinase (ADK, E.C.: 2.7.4.3), phosphoglucomutase (PGM, E.C.: 2.7.5.1), aldolase (ALD, E.C.: 4.1.2.13), fumarase (FH, E.C.: 4.2.1.2); glucosephosphate isomerase (GPI, E.C.: 5.3.1.9), hexokinase (HK, E.C.: 2.7.1.1). For MDH, IDH, AAT, and HK two enzyme systems were identified, so that a total of 17 allozyme loci were analyzed.

At each locus, the commonest allele was scored as 100, while the others were reported on the basis of their relative mobility, adding to or subtracting from the 100 figure a corresponding number of millimeters for faster or slower alleles, respectively. We assumed a locus to be polymorphic when the frequency of the commonest allele was 0.99 or lower and at least another allele was present at a frequency of 0.01 or higher.

Genetic distance matrices were estimated according to Nei (1972) and dendrograms were obtained following neighbor joining (Saitou and Nei, 1987) and maximum likelihood (as described in Felsenstein, 1981). Confidence limits were evaluated through the bootstrap test (Felsenstein, 1985). Values of genetic distance, dendrograms and bootstraps were obtained by the PHYLIP computing package (3.5 Macintosh executable version, © 1986–1993 by Joseph Felsenstein).

3. Results

3.1. Cox2 gene

Forty-five *Leptynia* specimens from 24 populations have been sequenced (see Table 1 and Fig. 1 for details). Sequencing analysis covered 639 base pairs of the *cox2* gene, coding for 213 aminoacids of the cytochrome oxidase subunit II and corresponding to the gene region sequenced in several insect orders (Liu and Beckenbach, 1992).

Alignment (available from the authors) showed that *Leptynia cox2* sequences have the ATA codon for initiation, as it is in *B. rossius*, *B. grandii*, *B. atticus*, and *C. gallica*. Initiation codon has not been unambiguously sequenced in *M. scabriusculus*. *Cox2* sequences in *L. attenuata* showed 114 polymorphic sites (17.8% of the total). A single codon insertion (386–388) has been ob-

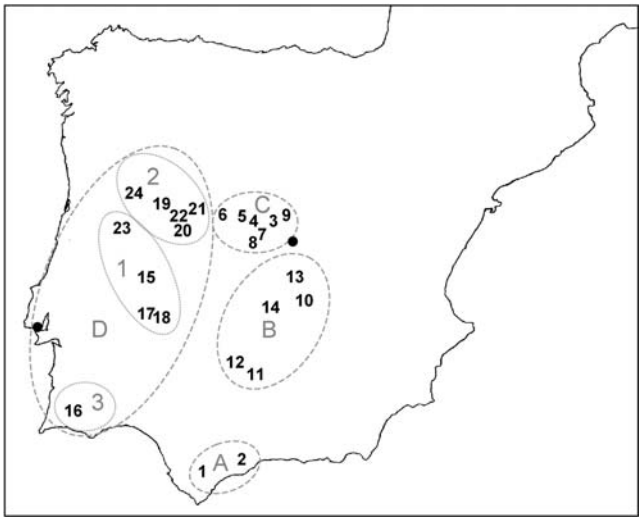

Fig. 1. Geographic distribution of analyzed samples of the *Leptynia attenuata* species complex (*L. species*, A; *L. caprai*, B; *L. montana*, C; *L. attenuata*, D). *L. attenuata* 1, 2, and 3 ranges (see text for details) are also reported. Following localities were sampled: 1—Grazalema (GRA); 2—Ojen (OJE); 3—El Escorial (ESC); 4—Puerto Cruz Verde (PCV); 5—Las Navas del Marqués (LNM); 6—Puerto de Las Pilas (PLP); 7—Pinosol (PIN); 8—Zarzalejo (ZAR); 9—Ventorillo (VEN), 10—Urda (URD); 11—Viso del Marqués (VIS); 12—San Lorenzo de Calatrava (SLC); 13—Los Yébenes (LYE); 14—Puerto de los Majales (PLM); 15—São Fiel (SFI); 16—Monchique (MON); 17—Marvão (MAR); 18—Portalegre (POR); 19—Peña de Francia (PDF); 20—Santibáñez (SIB); 21—El Barco de Ávila (BAV); 22—Béjar (BEJ); 23—Fundão (FUN); 24—Guarda (GUA).

Table 2
Mean distance values within and between species, obtained from *cox2* and allozyme analyses

Comparisons	*Cox2*	Allozymes
	Mean TrN[a] distance	Mean Nei's[b] distance
Intraspecific		
L. sp.	0.014	nc[c]
L. caprai	0.016	0.063
L. montana	0.004	0.043
L. attenuata	0.025	0.184
Interspecific		
L. sp. vs *L. caprai*	0.074	0.579
L. sp. vs *L. montana*	0.078	0.575
L. sp. vs *L. attenuata*	0.073	0.437
L. caprai vs *L. montana*	0.037	0.926
L. caprai vs *L. attenuata*	0.032	0.599
L. montana vs *L. attenuata*	0.033	0.449

[a] Tamura and Nei (1993) nucleotide distance.
[b] Nei (1972) distance.
[c] Not calculated (only one sample has been characterized).

served in *Leptynia* sequences and in *C. gallica*. The insertion is lacking in *Bacillus* and *Medaura*. In the *L. attenuata* species complex a noticeable exception is represented by the sample from Ojen (Sistema Penibético, South Spain), which also lacks the 386–388 insertion.

Mean nucleotide Tamura–Nei pairwise distances ranged from 0.004 to 0.025 in comparisons among populations showing the same karyotype constitution, whereas those between different karyotype groups ranged from 0.032 to 0.078 (Table 2).

Inferred aminoacid sequences showed that the most frequent aminoacids are isoleucine and metionine (both from 9.4 to 10.3%), while the rarest are cysteine (0.9%) and lysine (0.9–1.4%). *Cox2* aminoacid sequence revealed 17 variable sites in *Leptynia* (about 8%); *L.* sp. showed three diagnostic aminoacid substitutions, while the others did not show any diagnostic difference.

3.2. Karyotypes

The 11 new samples showed two distinct cytotypes. Stick insects from São Fiel, Marvão, Portalegre, Peña de Francia, Santibáñez, El Barco de Ávila, Fundão, Béjar, and Guarda invariably showed a $2n = 36$ XX/XY chromosome constitution, so that all have been tentatively referred to as *L. attenuata* in this paper. Two more samples, namely Ojen and Grazalema, showed a markedly different karyotype ($2n = 39/40$, XO/XX) for numerous dibrachial chromosomes, which confirms that reported by Bianchi and Meliado (1998). This karyotype is clearly different from the *L. caprai* one, which conversely showed mostly acrocentric pairs (Passamonti et al., 1999), and likely represents an apomorphy of the taxon (dibrachial chromosomes are likely to derive from pericentric inversion or fusion of acrocentrics).

To summarize, in *L. attenuata* species complex four karyotypes have been detected up to now, namely $2n = 39/40$ XO/XX for *L.* sp., $2n = 39/40$ XO/XX for *L. caprai*, $2n = 37/38$ XO/XX for *L. montana*, $2n = 36$ XY/XX for *L. attenuata*.

3.3. Allozymes

Allozyme data obtained from 17 loci for the new nine populations have been added to those reported by Passamonti et al. (1999). All analyzed loci were polymorphic, the number of detected alleles ranging from 2 (*Hk*-1) to 11 (*Pgm*). A few loci have not been detected in some samples (*αGpdh* and *Adk* in San Lorenzo de Calatrava, and *Idh*-2 in Urda, Puerto de Los Majales, Los Yébenes) (detailed allelic table available from the authors).

Nei's genetic distances showed a high level of genetic divergence between groups showing different karyotypes, while distance values within groups are considerably lower. Detailed mean Nei's distances are reported in Table 2.

3.4. Phylogenetic inferences

Maximum parsimony and maximum likelihood trees based on the *cox2* gene showed comparable clustering patterns (Fig. 2). They clearly indicated that *L.* sp. from Southern Spain (Ojen and Grazalema, Sistema Penibético) has to be considered the most divergent taxon of the *L. attenuata* group. Moreover, *cox2* analysis supports the monophyly of *L. montana*. On the other hand, within *L. caprai* a peculiar situation has been detected for the Viso del Marqués population: it pertains karyologically to *L. caprai* and also the neighbor joining tree supports this position (Fig. 3). However maximum likelihood and maximum parsimony approaches put it apart from the *L. caprai* cluster (Fig. 2).

Furthermore *L. attenuata* never forms a single monophyletic clade, but appears to split into three distinct groups in line with their geographical distribution (see Fig. 1): 1. Marvão, Portalegre, Fundão and São Fiel; 2. Peña de Francia, Santibáñez, Béjar, Guarda and El Barco de Ávila; 3. Monchique. These groups will be therefore referred here to as *L. attenuata* 1, *L. attenuata* 2 *and L. attenuata* 3, respectively. Since *L. attenuata* has been firstly described from São Fiel specimens (see Pantel, 1890), *L. attenuata* 1 has to be considered the *L. attenuata sensu stricto*.

Unfortunately, while the *cox2* gene turned out to be a good marker to separate karyotype groups, it proved far less reliable in reconstructing relationships between *L. attenuata*, *L. caprai*, and *L. montana* (Figs. 2 and 3), since those species showed an even level of divergence.

To better evaluate the phylogenetic scenario, we tested the two major competing hypotheses suggested by the former analyses (i.e., *L. attenuata* monophyly vs. polyphyly and *L. caprai* monophyly vs. paraphyly) using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa, 1999). The analyses showed that likelihood scores of the tested phylogenetic hypotheses are not significantly different, so that neither could be rejected.

The two-cluster test analysis carried out on all analyzed samples showed an overall substitution rate constancy (95% criterion of significance, data not shown), with the only exception of GRA1, GRA2, GRA3, and FUN1 specimens. Removing them from the analysis, we were able to apply the method of Takezaki et al. (1995) to construct a linearized neighbor joining tree to which apply the time calibration obtained for *Bacillus* species (Mantovani et al., 2001) (Fig. 3). According to this approach, we obtain that the major splitting of *L.* sp. would date back as much as 23.31 ± 3.12 million years (Mya). The splitting of the other taxa appear to be more recent and largely overlapping, with *L. caprai* separating

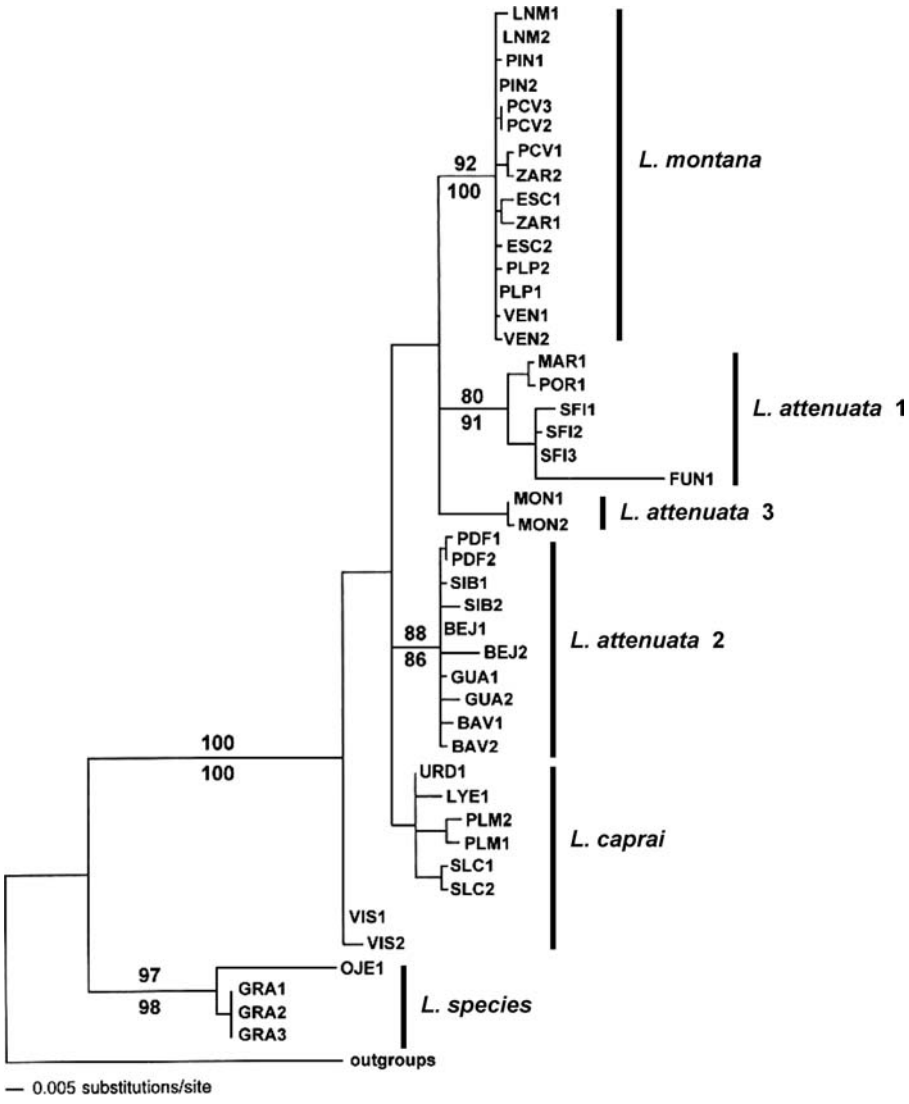

Fig. 2. Schematic maximum likelihood tree based on *cox2* gene, depicting phyletic relationships within the *L. attenuata* species complex. Maximum parsimony tree fully overlap to this. Bootstrap values (100 replicates) obtained by maximum likelihood (above) and maximum parsimony (below) are reported on branches for strongly supported clades only ($\geqslant 60$ bootstrap value). Sample acronyms as in Table 1.

from the remaining taxa 9.01 ± 1.42 Mya before present and the others at 8.22 ± 1.55 Mya (Fig. 3).

Allozyme analysis allowed some additional insights into *Leptynia* phylogenesis: using *L.* sp. as an outgroup (the choice is in line with *cox2* analysis—as illustrated above), we obtained a pattern largely corresponding to that given by *cox2* gene. Monophyly of *L. montana* and *L. caprai* is strongly supported by bootstrap values in both approaches (94 and 100, respectively in maximum likelihood, and 98 for both of them in neighbor joining), even if it has to be noted that *L. caprai* from Viso del Marqués (VIS) could not be analyzed (see *cox* 2 analysis for comparison); on the other hand, as it occurs for the *cox2* gene, relationships between *L. attenuata* 1, 2, and 3 appear to be unresolved. Moreover, allozymes support that *L. caprai* could be basal to *L. montana* and *L. attenuata* (Fig. 4).

4. Discussion

4.1. Taxonomy and phylogenesis of the L. attenuata species complex

In stick insects, karyotype, allozyme, and mitochondrial analyses have proved to be powerful means for phylogenetic inferences (Mantovani et al., 2001). Present data provide new insights on the taxonomy and phylogenesis of the *L. attenuata* species-group.

First, *cox2* analysis clearly demonstrates that the populations from Grazalema and Ojen (Sistema Penibético, Southern Spain) are the most divergent taxon of the group. It appears sound enough to consider it as an undescribed new species of *Leptynia*, as already suggested by Bianchi and Meliado (1998). Conversely, *L. caprai* (with a central distribution in the Iberian

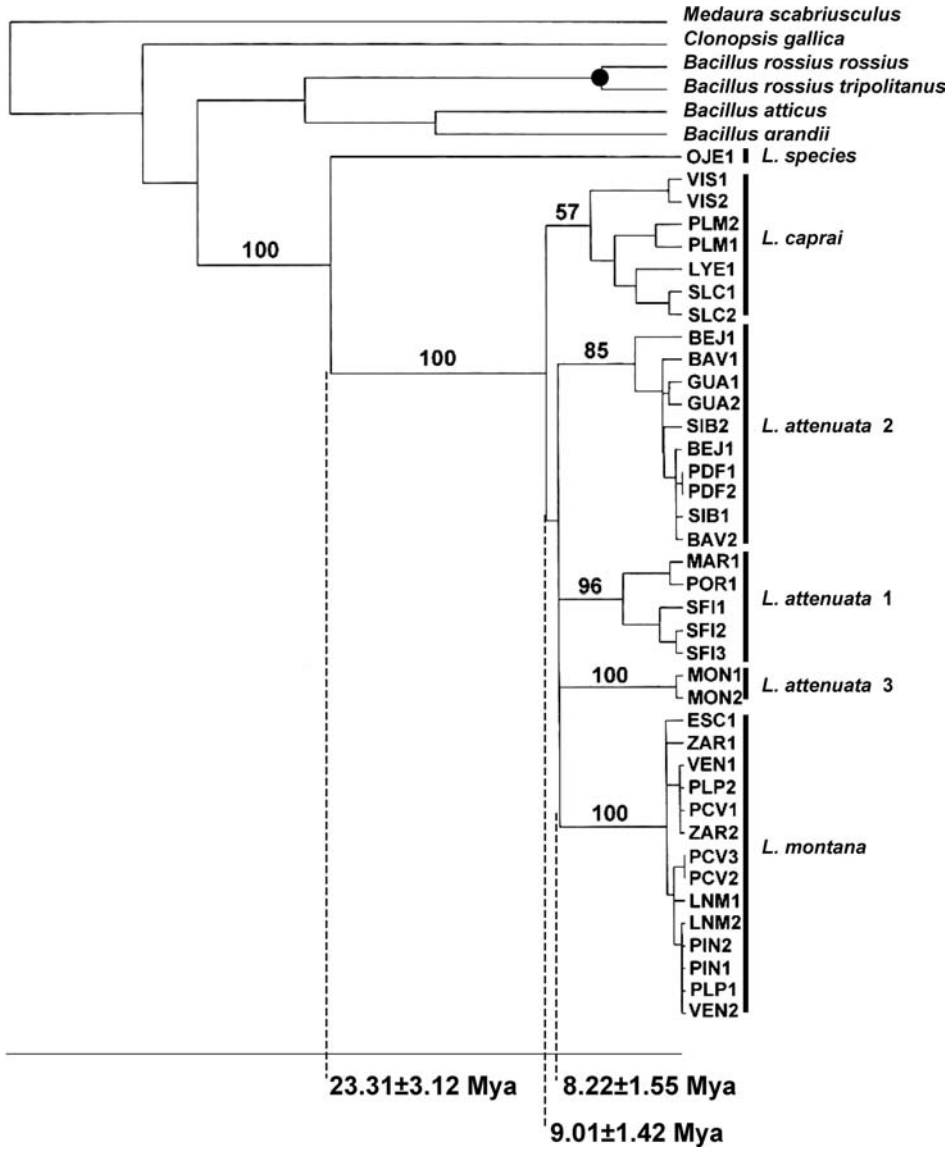

Fig. 3. Linearized neighbor joining tree based on *cox2* gene, obtained according to Takezaki et al. (1995) and putative tempo of cladogenesis within the group, obtained using time calibration of Mantovani et al. (2001). Time calibration start point (as in Mantovani et al., 2001) indicated by a black dot. Bootstrap values (100 replicates) obtained by neighbor joining are reported on branches. Sample acronyms as in Table 1.

Peninsula, see Fig. 1) has a stronger relationship with *L. montana* and *L. attenuata*, and allozymes suggest that this species may be considered basal to those. Moreover, *L. montana*, which is endemic to the mountains of the Sistema Central, is a clearly monophyletic clade, as supported both by allozymes and mitochondrial DNA.

The situation of *L. attenuata* is far more puzzling: data here obtained from allozymes and *cox2* give largely unresolved polytomies, although three groups of populations have been outlined by the *cox2* analysis (*L. attenuata* 1, 2, and 3). At present, it is unclear whether these groups have to be considered as distinct species or not, and additional sequence data are needed. However, it has to be noted that they share the same karyological constitution and, more significantly, the same unusual sex-chromosome formula. Actually, an XX/XY sex determination is very rare in stick insects, as compared to XX/XO one. Incidentally, out of a total of 42 bisexual species of phasmids analyzed in our Lab, none but two (*Gratidia turca* and *L. attenuata*) had revealed such a sex formula (Galassi, 2002; Scali, 1996; Scali et al., 1990). Consequently, this may be considered a good synapomorphy in favor of a monophyletic origin of *L. attenuata*, but we cannot exclude that the XY constitution has independently evolved more than once in *Leptynia.* More in-depth karyotype analyses have been planned to test this point. At any rate, on the whole, allozyme and mtDNA approaches suggest a phylogenetic reconstruction congruent with a trend of chromosomal repatternings, with a progressive reduction of the chromosome number (Passamonti et al., 1999). In this scenario we may speculate that *L.* sp. should be

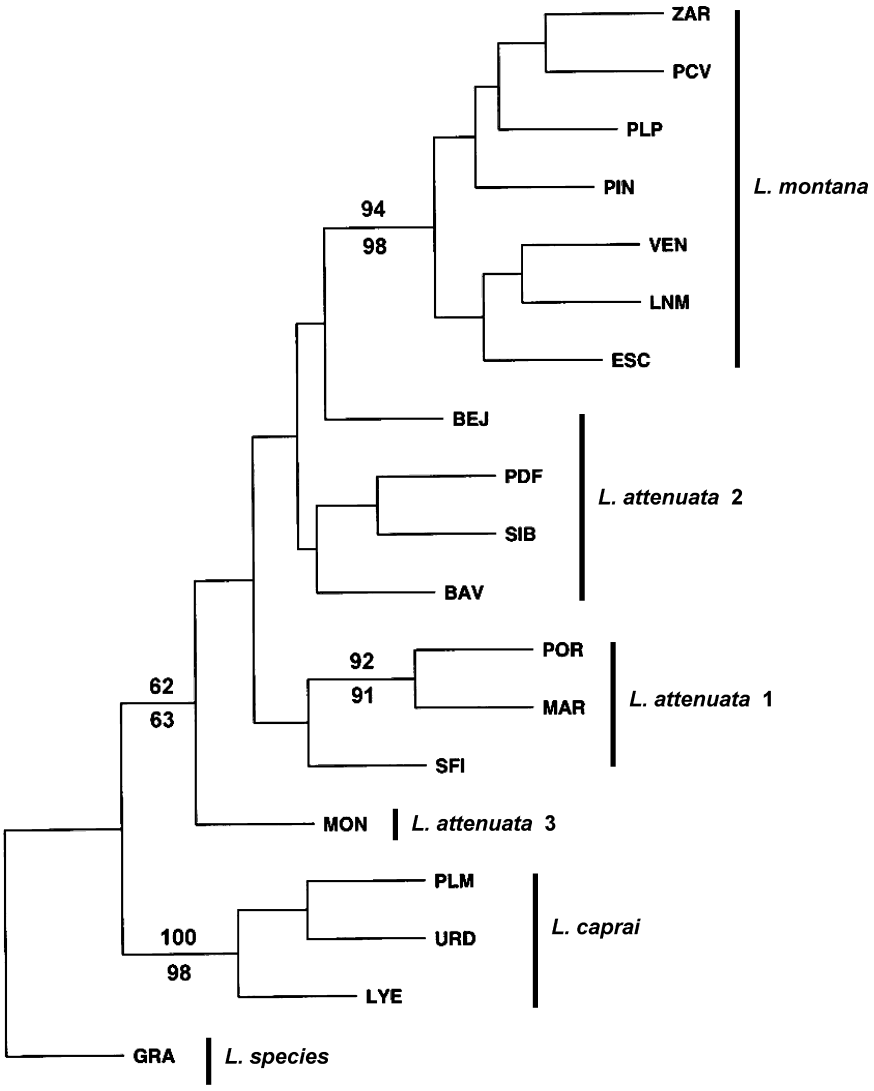

Fig. 4. Schematic maximum likelihood tree based on allozyme data, suggesting phyletic relationships within the *L. attenuata* species complex. Neighbor joining tree fully overlaps to this. Bootstrap values (100 replicates) obtained by maximum likelihood (above) and neighbor joining (below) are reported on branches for strongly supported clades only ($\geqslant$60 bootstrap value). Sample acronyms as in Table 1.

considered as a highly divergent relic species, since Southern Spain is a pretty well known refuge area (Hewitt, 1996). Data from Maximum Likelihood and Maximum Parsimony may suggest that *L. attenuata* and *L. montana* might arise from a subset of the *L. caprai* gene pool, thus depicting a paraphyletic pattern of speciation, although the alternative hypothesis (monophyly of *L. caprai*) cannot be rejected according to the Shimodaira–Hasegawa test and allozyme data. Finally, it is also suggested by the linearized Neighbor Joining tree that this cladogenesis may be likely considered as a species radiation occurring at about nine million years before present.

4.2. Phylogeography

The whole data set on genetics and distribution allows us to propose a phylogeographic pathway on the origin of the *L. attenuata* species-complex.

Leptynia is most likely African in origin. This assumption is mostly based on three points: (i) Heteronemiidae species are common in Africa, but not in Europe; (ii) some North African Heteronemiidae have been tentatively ascribed to *Leptynia* (Bradley and Galil, 1977); (iii) stick insects are mostly tropical and subtropical, and colonized temperate areas secondarily. Moreover, it is worth noting that the distribution of *L.* sp., which apparently is a relic taxon that first separated from the others (see *cox*2), is restricted to the South (Sistema Penibético). This somehow reinforces the idea of an ancestral *Leptynia* taxon spreading from South through the Iberian Peninsula.

Details of those cladogenetic events are partially unresolved by molecular and biochemical data; however, the different methodological approaches gave largely similar results and suggested the idea that, after an ancient major splitting originating *L.* sp., the speciation

process continued later on as a radiation throughout the Iberian peninsula, leading to *L. caprai*, *L. montana* and at least three differentiated *L. attenuata* phylads. This is supported by the large polytomies of maximum likelihood and maximum parsimony *cox* 2 trees (Fig. 2), and by the time calibration procedure, which indicated widely overlapping tempos of speciation for those taxa (Fig. 3).

From our analysis, it is unclear whether climate changes (i.e., glaciations) had a role in the colonization and speciation of the *L. attenuata* group. However, according to our time calibration, Quaternary cold periods—which are largely used in animal phylogeography (Hewitt, 1996, 1999; Taberlet et al., 1998)—cannot be taken into account to explain *Leptynia* distribution and cladogenesis.

4.3. Speciation mechanisms in the L. attenuata species complex

As mentioned above, our comprehensive data set appears to suggest that karyotype rearrangements were the primary driving force for speciation in *Leptynia*. However, we have to mention the possibility that karyotype rearrangements may also occur secondarily after speciation events; this might be the case of the *L. attenuata* 1, 2, and 3 phylads that, while keeping a homogeneous karyotype, might be under incipient speciation.

It has to be noted that the importance of karyotypic changes in speciation has been largely questioned for several reasons, one of the main theoretical difficulties being associated with the fixing of chromosomal rearrangements that are strongly underdominant (i.e., reducing the fitness of the heterozygote) (review in Riesenberg, 2001). Some hypotheses have been provided to bypass such a constraint, but, as far as we know, the possibility that new karyotypes may spread through androgenesis has never been considered. Androgenesis is defined as a reproduction in which diploid offspring carry chromosomes only from the male parent; embryo genome originates from either the doubling or the syngamy of two spermheads (stick-insect eggs are polyspermic), while egg pronucleus does not contribute to the embryo's genome (Mantovani and Scali, 1992). Therefore, if a rearranged heterozygote karyotype is carried by a male, androgenesis could at once provide the opportunity to produce homozygous progeny of both sexes for new chromosomal repatterning. Androgenesis has been found to occur in at least three different kinds of organisms from two kingdoms, thus suggesting that it might be more common than previously thought: this peculiar pattern of reproduction has been firstly demonstrated in *Bacillus* stick insects (Mantovani and Scali, 1992; Tinti and Scali, 1996), but has been proved to occur in several species of the freshwater *Corbicula* clams (Mollusca Bivalvia), which apparently reproduce solely by androgenesis (Byrne et al., 2000; Komaru et al., 1998; Qiu et al., 2001), and in *Cupressus dupreziana* (Saharan cypress tree; Family Cupressaceae), which is the only plant species known to reproduce androgenetically (Pichot et al., 2001).

It is still uncertain whether androgenesis occurs in the *L. attenuata* species complex, as it does in *Bacillus*; however some preliminary crossing experiments suggested that it might play a role in *Leptynia* too (Authors' unpublished data).

In conclusion, our analysis provides insights on taxonomy, phylogenetics, and phylogeography of the *L. attenuata* species complex and gives clues for future research on this fascinating insect group.

Acknowledgments

We gratefully acknowledge Fabrizio Ghiselli, Liliana Milani and Luciana Scali for their precious help in collecting and characterizing *Leptynia* samples, and Miquel A. Arnedo (The Natural History Museum, London) for several suggestions and a critical review of the manuscript. This work has been supported by Italian "Ministero dell'Università e della Ricerca Scientifica" (MIUR) funds.

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