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Karyotype and Allozyme Characterization of the Iberian *Leptynia attenuata* Species Complex (Insecta Phasmatodea)

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ABSTRACT—Karyological and allozymatic characterizations of recently collected samples of the Iberian *Leptynia attenuata* complex support the occurrence of three genetically differentiated groups, for which parallel morphological observations evidenced only partially diagnostic characters. The groups are: the Portuguese population of Fóia (Serra de Monchique), referred to as the nominal taxon, *L. attenuata*; the Spanish populations of the Sistema Central, referred to as *L. montana*; the populations of the Toledo district, referred to as *L. caprai*. These taxa seem to represent a case of incipient speciation, with chromosomal and genetic differentiation ahead of the morphological one. Chromosome repatternings, affecting autosomes as well as sex chromosomes, appear to go together with the evolutionary events.

INTRODUCTION

Phasmatodea is an order of land insects best known for their mimetic ability (stick or leaf-insects), uncommon reproductive modes and repeated specific hybridization. In Southern Europe, three stick-insect genera are found: *Bacillus* and *Clonopsis*, both ascribed to the family Bacillidae, and *Leptynia*, belonging to the family Heteronemiidae.

The genus *Bacillus* has been deeply investigated by means of morphological, karyological, allozymatic and molecular studies (satellite DNA); these approaches allowed a satisfactory understanding of phyletic relationships and evolution within the genus. This is now known to include the bisexuals *B. rossius* and *B. grandii*, the unisexual *B. atticus* and their related diploid and triploid non-Mendelian hybrids (*B. rossius-grandii*, *B. whitei*, *B. lynceorum*). On the whole, *Bacillus* hybrids represent a good example of reticulate evolution; their reproductive biology has proved of particular interest since, in addition to parthenogenesis, they make use of hybridogenesis and androgenesis (Scali *et al*., 1995; Mantovani *et al*., 1997).

Morphological and karyological investigations on *Clonopsis* revealed the existence of a very widespread unisexual triploid taxon, *C. gallica*, and of the apparently unrelated North-African bisexual species *C. algerica* and *C. maroccana* (Bullini and Bianchi, 1971; Nascetti and Bullini, 1983; Bullini and Nascetti, 1987). These still await deeper analyses.

As a part of a molecular project aimed to characterize European stick-insects, we started the study of the Iberian genus *Leptynia*, formerly known to comprise two well-differentiated species: *L. hispanica* Bolivar, 1878 and *L. attenuata* Pantel, 1890. Within *L. hispanica*, both bisexual (2n=37/38; XO/XX) and parthenogenetic (3n=57, XXX; 4n=76, XXXX) populations have been found, so that the species is now referred to as a complex (Nascetti *et al*., 1983; Bianchi, 1992). Also for *L. attenuata*, karyological data had evidenced two bisexual taxa, characterized by distinct cytotypes (2n=36, XY/XX and 37/38, XO/XX) from Serra de Monchique (Portugal) and El Escorial (Spanish Sistema Central), respectively. A tetraploid unisexual population (4n=76) from Zarzalejo (Sistema Central) was also reported. It was suggested that they could represent different species (Bianchi, 1992).

Sampling campaigns focused on the *L. attenuata* complex were carried out in 1995 and 1996 on the Spanish Sistema Central mountains, the Toledo district hills and the Portuguese Serra de Monchique. SEM analyses on these samples evidenced some trends of morphological differentiation of male body (cerci and last tergite) and of egg (polar mound and operculum features) (Scali, 1996). On these features (which were known to be supported by the findings here presented) their specific splitting was proposed by Scali (1996): the populations of the Spanish Sistema Central and of the Toledo district have been given the names of *L. montana* and *L. caprai*, respectively, while the Portuguese sample was maintained as the nominal species, *L. attenuata sensu stricto*.

In the present paper, we give a preliminary karyological characterization and a detailed allozyme analysis of the collected samples in order to support their systematic rank and outline the likely evolutionary events occurred in the *L. attenuata* complex.

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MATERIALS AND METHODS

Eleven populations, representative of the three species, were sampled: *L. caprai* from Urda (URD), Puerto de Los Majales (PLM) and Los Yebenes (LYE); *L. montana* from Ventorillo (VEN), Pinosol (PIN), El Escorial (ESC), Zarzalejo (ZAR), Puerto Cruz Verde (PCV), Las Navas del Marques (LNM) and Puerto Las Pilas (PLP); *L. attenuata* from the Portuguese locality of Fóia, Serra de Monchique (MON) (Fig. 1). Pertinent information on analyzed samples are reported in Table 1.

In the field, animals were feeding mainly on *Cystus* spp., *Sarothamnus scoparius*, *Ulex* spp. and *Dorycnium suffruticosum*; in the lab they were maintained on the same foodplants, at room temperature and at a natural photoperiod.

GIEMSA stained karyotypes were obtained from chromosome preparations of male and female gonadal tissues, according to Marescalchi and Scali (1990). The same specimens, with additional ones from the same collecting sites, were also analyzed electrophoretically.

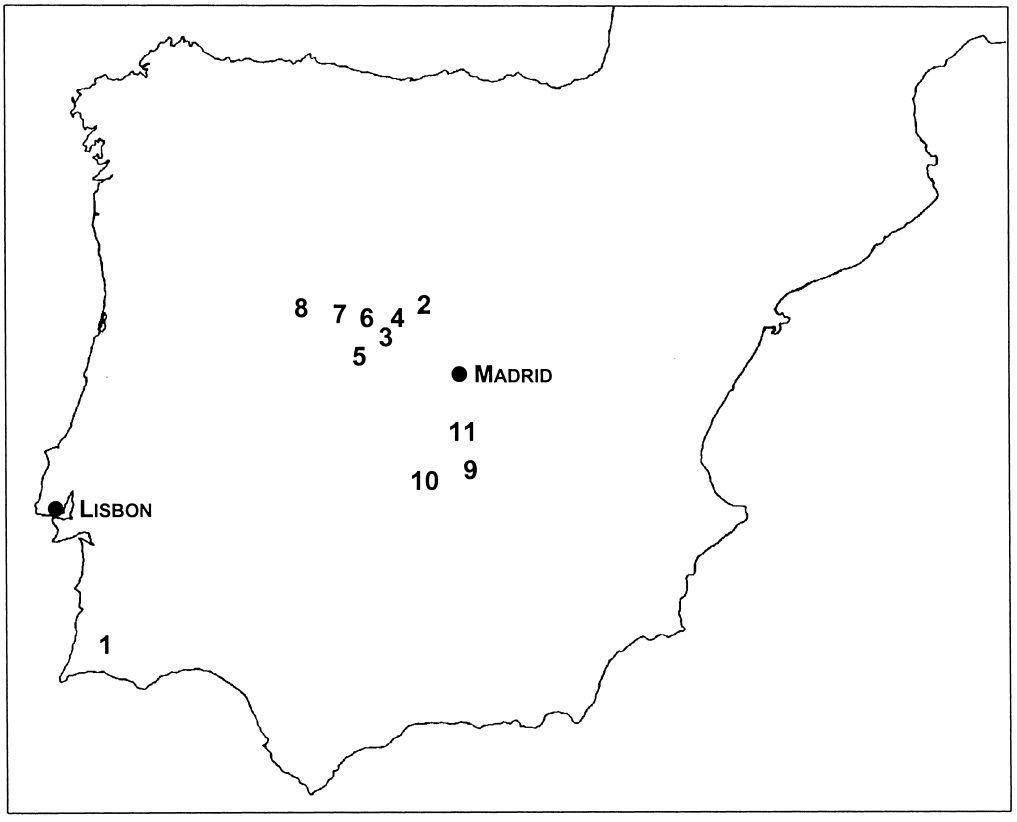

Fig. 1. A map showing sampling sites of *L. attenuata* from Fóia, Serra de Monchique (**1**), of *L. montana* from Sistema Central mountains [Ventorillo (**2**), Pinosol (**3**), El Escorial (**4**), Zarzalejo (**5**), Puerto Cruz Verde (**6**), Las Navas del Marques (**7**) and Puerto Las Pilas (**8**)] and of *L. caprai* from Toledo district [Urda (**9**), Puerto de Los Majales (**10**) and Los Yebenes (**11**)].

Table 1. Collecting sites and size of analyzed samples of *Leptynia*.

species	populations	Karyological analysis		Allozyme analysis
		N specimens	karyotype structure	N specimens
L. caprai	Urda (URD)	6♂, 5♀	2n=39/40 (XO,XX)	8♂, 9♀
	Puerto de Los Majales (PLM)	3♂, 4♀	2n=39/40 (XO,XX)	13♂, 11♀
	Los Yebenes (LYE)	4♂, 3♀	2n=39/40 (XO,XX)	14♂, 11♀
L. montana	Ventorillo (VEN)	3♂, 6♀	2n=37/38 (XO,XX)	11♂, 9♀
	Pinosol (PIN)	12♂, 9♀	2n=37/38 (XO,XX)	14♂, 12♀
	El Escorial (ESC)	3♂, 4♀	2n=37/38 (XO,XX)	3♂, 4♀
	Zarzalejo (ZAR)	3♂, 8♀	2n=37/38 (XO,XX)	3♂, 11♀
	Puerto Cruz Verde (PCV)	5♂, 4♀	2n=37/38 (XO,XX)	11♂, 12♀
	Las Navas del Marques (LNM)	9♂, 9♀	2n=37/38 (XO,XX)	9♂, 12♀
	Puerto Las Pilas (PLP)	2♂, 5♀	2n=37/38 (XO,XX)	7♂, 17♀
L. attenuata	Fóia, Serra de Monchique (MON)	8♂, 9♀	2n=36/36 (XY/XX)	11♂, 9♀

Electrophoretic analyses of leg muscle homogenates were carried out on cellulose acetate membranes, as reported in Mantovani and Scali (1991).

The following enzymes were surveyed: 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), glucose 6-phosphate dehydrogenase (G6PDH, E.C.: 1.1.1.49), glyceraldehyde 3-phosphate dehydrogenase (G3PDH, E.C.: 1.2.1.12), aspartate amino transferase (AAT, E.C.: 2.6.1.1), hexokinase (HK, E.C.: 2.7.1.1), 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). For MDH, IDH, AAT and HK two enzyme systems were identified, so that a total number of 18 loci were analyzed.

At each locus, the commonest allele was scored as 100, while other alleles were reported on the basis of their relative mobility, adding or subtracting from the 100 value a corresponding number of millimetres 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. Hardy-Weinberg disequilibria were tested with the Exact test (Ex; Elston and Forthofer, 1977) and with *f* values (Weir and Cokerham, 1984). Wright's *F*-statistics (Weir and Cockerham, 1984) were calculated for each polymorphic locus. Averages of *F*-statistics and the corresponding standard deviations were obtained using the Jackknife estimator (Weir, 1990). Their significance was evaluated through the permutation procedure (10,000 steps) (Goudet, 1995). Exact test and *f* estimator were carried out using Genepop software (Raymond and Rousset, 1995). Values of Wright's *F*-statistics were obtained using FSTAT software (Goudet, 1995). Genetic distance matrices were estimated according to Nei (1972) and dendrograms were obtained following the UPGMA (Sneath and Sokal, 1973) and the Neighbor-joining method (Saitou and Nei, 1987). In each dendrogram, confidence limits of the clusters were evaluated through the bootstrap analysis (Felsenstein, 1985). Values of genetic distance, dendrograms and bootstraps were actually obtained by the PHYLIP computing programs (3.5 Macintosh executable version, © 1986–1993 by Joseph Felsenstein).

RESULTS

Karyological analysis

Female metaphases from the *L. attenuata* complex revealed the existence of three related but well differentiated karyotypes (Table 1). The first one, observed in the 3 reported populations of *L. caprai*, showed a diploid set of 2n=40, with 3 pairs of small metacentrics (9, 15 and 20) and 17 sharply heterobrachial chromosomes (Fig. 2A). The second karyotype, found in the 7 populations of *L. montana*, evidenced a 2n=38 chromosome complement. Its constitution chiefly differed by the presence of a couple of large metacentric chromosomes (pair 1), absent from the first karyotype (Fig. 2B). Finally, the third karyotype, with 2n=36, was observed in the southern Portuguese *L. attenuata* sample; it appeared even more differentiated by having four large metacentrics (pairs 1 and 2) (Fig. 2C).

In addition, male mitoses and meioses revealed that *L. caprai* and *L. montana* populations possessed an XO male sex determining mechanism, with a clearly heterobrachial X chromosome (Fig. 2A and B), whereas, the *L. attenuata* sample undoubtedly presented an XY sex bivalent with a very large metacentric X chromosome and a much smaller acrocentric Y (Fig. 2C).

Finally, no tetraploid specimens were recorded from neither the newly collected samples from Zarzalejo (*L. montana* range) nor elsewhere.

Allozyme analysis

Out of the 18 scored loci, only *Hk*-1 and *Hk*-2 were monomorphic for the same allele in all populations. At the remaining 16 polymorphic loci, the number of alleles per locus ranged from two (*Mdh*-2) to ten (*6Pgdh*) (Table 2). It was apparent that the *L. caprai* samples (Urda, P. Los Majales and Los Yebenes) shared a similar allelic structure, while differed from all other populations, either by showing alternative alleles (see *Mdh*-2, *Adk* and *Fh* loci) or having as their most common ones, those rarest in the remaining populations (see α*Gpdh*, *6Pgdh*, *Aat*-2). On the other hand, the Portuguese sample of *L. attenuata* showed private alleles at several loci, but no fixed alternative ones. Most loci were at the Hardy-Weinberg equilibrium, main exceptions being those at *Idh*-1, *Idh*-2 and *6Pgdh* loci, for which several samples showed heterozygote deficiencies (Table 2).

As predictable from allelic data, quite different levels of genetic differentiation emerged between samples (Table 3). Interpopulation distances within the *L. caprai* and *L. montana* samples were low, ranging from 0.042 to 0.164 in the former and from 0.019 to 0.078 in the latter. On the contrary, comparisons among the three karyotypic groups evidenced a markedly higher level of differentiation: in pairwise comparisons, *L. caprai* populations turned out to be highly divergent from all other samples (D=0.577 to 1.088), whereas *L. montana* vs. *L. attenuata* was much less divergent (D=0.383–0.450), although still showing decidedly higher values than those within species. The relationships suggested by genetic distances were well featured also in UPGMA and Neighbor-Joining dendrograms, which depicted a similar clustering with very high bootstrap values (Fig. 3 A and B).

When the whole set of data was considered, Wright's *F*-statistics revealed a very high level of genotypic heterogeneity, with *Fit* significant values over all loci. The interpopulation heterogeneity appeared to be the main component of the total variance, *Fst* being highly significant over all loci, except at the *Ald* locus. By contrast, the intrapopulation component (*Fis*) showed a generally lower significance, although *Idh*-1, *Idh*-2, *6pgdh*, *Gox*, *G6pdh*, *Aat*-1, *Pgm* and *Ald* did show some degree of heterogeneity (Table 4).

F values separately calculated for *L. montana* and *L. caprai* populations showed that significant genotypic heterogeneities were rarer, evidencing a more homogeneous structure throughout each sampling area; however, some heterogeneity at both intra- and interpopulation level was still found at some loci (Table 4).

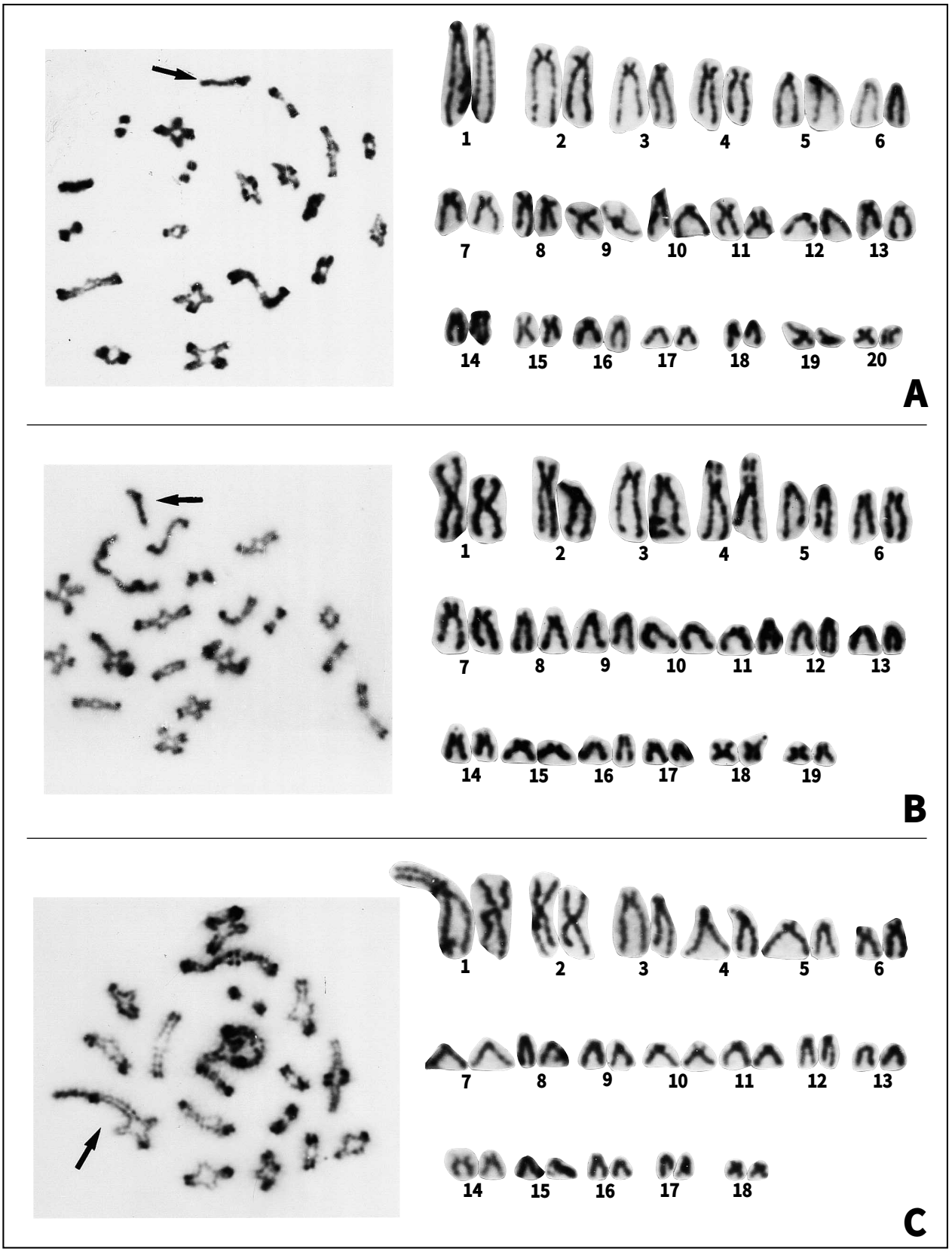

Fig. 2. Male meioses and female karyotypes of the *Leptynia caprai* (2n=39/40, XO/XX) of Urda (Toledo district hills, Spain) (**A**), of the *Leptynia montana* (2n=37/38, XO/XX) of Pinosol (Sistema Central mountains, Spain) (**B**) and of the *Leptynia attenuata* (2n=36/36, XY/XX) of Fóia (Serra de Monchique, Portugal) (**C**). Arrow indicates sex chromosomes.

Table 2. Allelic frequencies at 16 polymorphic loci in 11 *Leptynia* samples.

		URD	PLM	LYE	VEN	PIN	ESC	ZAR	PCV	LNM	PLP	MON
α*Gpdh*	96	–	–	–	–	–	–	–	–	–	–	0.031
	100	0.389	0.109	0.029	0.778	1	1	1	1	1	1	0.969
	102	–	–	–	0.111	–	–	–	–	–	–	–
	106	0.611	0.891	0.971	0.111	–	–	–	–	–	–	–
n.		9	23	17	9	21	7	2	18	17	15	16
Ex												
f		–0.111	–0.100		0.448							
Mdh–1	94	0.059	–	0.980	–	–	–	–	–	–	–	–
	100	0.941	1	–	1	0.885	0.917	0.714	0.957	1	0.977	1
	106	–	–	–	–	0.115	0.083	0.286	0.043	–	0.023	–
	109	–	–	0.020	–	–	–	–	–	–	–	–
n.		17	25	25	20	26	6	14	23	20	22	18
Ex												
f		–0.032				0.265		–0.13	–0.023			
Mdh–2	98	1	1	1	–	–	–	–	–	–	–	–
	100	–	–	–	1	1	1	1	1	1	1	1
n.		17	25	25	20	22	6	13	23	20	22	18
Idh–1	90	–	–	–	–	–	–	–	–	–	–	0.053
	92	1	0.920	0.813	0.250	0.562	0.214	0.364	0.348	0.053	0.211	0.500
	96	–	0.080	0.167	0.056	0.063	–	0.182	0.109	–	–	0.132
	98	–	–	–	–	–	–	–	–	–	–	0.053
	100	–	–	0.021	0.694	0.375	0.786	0.454	0.543	0.947	0.789	0.262
n.		17	25	24	18	24	7	11	23	19	19	19
Ex					d	d		c				c
f			–0.043	–0.048	0.41	0.247	0.625	0.459	0.261	–0.029	0.077	0.226
Idh–2	100	nd	nd	nd	0.941	1	0.929	1	1	1	0.967	0.833
	104	nd	nd	nd	–	–	0.071	–	–	–	–	–
	110	nd	nd	nd	0.059	–	–	–	–	–	0.033	0.167
n.					17	21	7	9	23	20	15	12
Ex					d						d	b
f					1						1	1
6*Pgdh*	92	–	–	–	–	–	–	–	–	–	–	0.107
	95	0.031	0.020	–	–	–	–	–	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	0.071
	98	–	–	–	–	0.024	0.100	–	–	–	–	0.500
	100	0.625	0.160	0.935	–	0.071	–	–	–	0.405	0.025	0.322
	102	0.031	0.020	0.065	0.029	0.167	–	0.417	0.619	–	0.325	–
	105	–	0.040	–	–	–	–	–	–	–	–	–
	106	–	–	–	0.971	0.333	0.700	0.458	0.238	0.547	0.475	–
	108	0.313	0.760	–	–	0.048	–	–	–	–	0.125	–
	111	–	–	–	–	0.357	0.200	0.125	0.143	0.048	0.05	–
n.		16	25	23	17	21	5	12	21	21	20	14
Ex		c	d			b			d		a	d
f		0.167	0.309	–0.048		0.3	–0.2	0.072	0.403	0.043	–0.129	0.463
Gox	98	–	–	–	–	–	–	–	0.059	–	–	–
	100	0.572	0.804	1	0.808	0.778	0.500	0.541	0.647	0.775	0.562	1
	101	–	–	–	–	–	–	0.167	–	–	–	–
	102	0.107	0.022	–	–	–	–	–	–	–	–	–
	104	0.321	0.174	–	–	–	–	–	–	–	–	–
	105	–	–	–	0.154	–	–	–	0.059	–	0.031	–
	107	–	–	–	0.038	0.139	0.500	0.292	0.176	0.225	0.188	–
	111	–	–	–	–	0.083	–	–	0.059	–	0.219	–
n.		14	23	25	19	18	5	12	17	20	16	19
Ex								c				
f		0.268	0.078		–0.154	0.124	–0.091	0.337	0.584	0.022	0.3	

Table 2. (continued)

		URD	PLM	LYE	VEN	PIN	ESC	ZAR	PCV	LNM	PLP	MON
G6pdh	86	–	0.056	–	–	–	–	–	–	–	–	–
	88	–	0.083	–	–	–	–	–	–	–	–	–
	90	–	–	–	–	–	–	–	–	–	–	0.233
	92	–	–	–	–	–	–	–	–	–	–	0.033
	97	–	–	–	–	–	–	–	–	–	–	0.067
	98	–	–	–	0.059	–	–	–	–	–	–	–
	100	0.750	0.861	1	0.941	0.980	1	1	0.875	0.952	0.875	0.667
	103	0.200	–	–	–	0.020	–	–	0.125	0.048	0.125	–
	109	0.050	–	–	–	–	–	–	–	–	–	–
n.		10	18	18	17	25	6	10	20	21	20	15
Ex		b	d									
f		0.769	0.354		–0.032				0.656	–0.026	–0.118	0.358
G3pdh	96	0.029	–	–	–	–	–	–	–	–	–	–
	100	0.971	1	1	0.342	0.174	0.333	0.636	0.348	0.238	0.475	1
	110	–	–	–	0.658	0.826	0.667	0.364	0.652	0.762	0.525	–
n.		17	25	24	9	23	6	11	23	21	20	19
Ex												
f					0.437	–0.189	0.333	0.636	–0.128	–0.29	–0.077	
Aat–1	86	–	–	0.082	–	–	–	–	–	–	–	0.056
	92	0.893	0.860	0.813	–	–	–	–	–	–	–	0.388
	96	0.036	0.020	0.042	–	–	–	–	–	–	–	0.056
	98	0.071	0.120	0.063	–	–	–	–	–	–	–	0.500
	100	–	–	–	1	1	1	0.955	0.947	1	1	–
	105	–	–	–	–	–	–	0.045	0.053	–	–	–
n.		14	25	24	20	25	7	11	19	18	20	18
Ex			d									d
f		0.307	0.366	0.130					–0.029			0.369
Aat–2	91	0.250	–	0.188	–	–	–	–	–	–	–	–
	95	0.625	0.979	0.208	–	–	–	–	0.024	0.026	–	0.235
	100	–	–	–	1	1	1	1	0.976	0.869	1	0.735
	104	0.125	0.021	0.604	–	–	–	–	–	–	–	–
	107	–	–	–	–	–	–	–	–	0.105	–	–
	111	–	–	–	–	–	–	–	–	–	–	0.029
n.		12	24	24	19	23	7	7	21	19	17	17
Ex												
f		0.257		–0.027						–0.098		0.009
Adk	100	–	–	–	0.921	1	1	1	1	0.912	1	–
	105	–	–	–	0.079	–	–	–	–	0.088	–	0.969
	109	1	1	1	–	–	–	–	–	–	–	–
	111	–	–	–	–	–	–	–	–	–	–	0.031
n.		10	20	8	19	24	7	13	17	17	17	16
Ex												
f					–0.059					0.652		
Pgm	89	–	0.056	–	–	–	–	–	–	–	–	–
	93	0.400	0.556	0.523	–	–	–	–	–	–	–	–
	95	0.067	0.278	–	–	–	–	–	–	–	–	–
	96	–	–	–	0.219	–	–	–	–	–	–	0.971
	99	0.033	0.028	0.250	–	–	–	–	–	–	–	–
	100	–	–	–	0.750	0.636	1	0.909	0.750	1	0.658	0.029
	102	–	–	–	0.031	0.341	–	0.091	0.250	–	0.342	–
	105	0.500	0.082	0.227	–	0.023	–	–	–	–	–	–
n.		15	18	22	16	22	5	11	22	17	19	17
Ex			d					d				
f		0.152	0.470	0.059	–0.094	0.262		1.000	0.174		–0.261	
Ald	92	–	–	0.045	–	–	–	–	–	–	–	–
	98	0.067	–	–	–	–	–	–	–	–	–	–

Table 2. (continued)

		URD	PLM	LYE	VEN	PIN	ESC	ZAR	PCV	LNM	PLP	MON
(*Ald*)	100	0.933	1	0.955	1	0.98	1	1	0.957	1	0.950	1
	104	–	–	–	–	0.02	–	–	–	–	0.050	–
	130	–	–	–	–	–	–	–	0.043	–	–	–
n.		15	24	22	20	25	7	12	23	21	20	19
Ex		d							d			
f		1.000		–0.024					1		–0.027	
Fh	91	1	1	1	–	–	–	–	–	–	–	–
	96	–	–	–	0.053	0.021	–	–	–	–	–	0.056
	100	–	–	–	0.684	0.896	1	0.950	0.848	0.395	0.775	0.583
	107	–	–	–	0.263	0.083	–	0.050	0.152	0.579	0.225	0.361
	111	–	–	–	–	–	–	–	–	0.026	–	–
n.		10	24	21	19	24	6	10	23	19	19	18
Ex					d							
f					0.182	–0.075			0.179	–0.008	0.022	0.078
Gpi	90	0.941	1	0.840	–	–	–	–	–	–	–	–
	94	–	–	–	–	–	–	–	–	–	–	0.975
	96	0.059	0.160	–	0.100	–	0.393	0.391	0.079	0.136	–	
	98	–	–	–	–	0.060	–	–	–	0.079	–	0.025
	100	–	–	–	1	0.840	1	0.607	0.609	0.842	0.864	–
n.		17	25	25	20	25	6	14	23	19	22	20
Ex												
f		–0.032		0.127		0.165		0.286	–0.257	–0.108	–0.135	

n. number of analyzed specimens
Ex: Exact test levels of significance a P<0.001, b 0.001<P<0.01, c 0.01<P<0.02, d 0.02<P<0.05
f: Fis in Weir and Cockerham (1984) notation. Population acronyms as in Table 1.

Table 3. Nei's genetic distances between the 11 analyzed *Leptynia* samples

	URD	PLM	LYE	VEN	PIN	ESC	ZAR	PCV	LNM	PLP	MON
URD	–	0.042	0.122	0.838	0.796	0.903	0.793	0.800	0.839	0.816	0.577
PLM		–	0.164	0.848	0.838	0.944	0.838	0.846	0.901	0.855	0.615
LYE			–	0.982	0.956	1.088	0.921	0.978	0.986	1.014	0.726
VEN				–	0.045	0.029	0.061	0.058	0.035	0.031	0.389
PIN					–	0.037	0.041	0.024	0.062	0.029	0.398
ESC						–	0.037	0.047	0.043	0.027	0.450
ZAR							–	0.021	0.078	0.031	0.383
PCV								–	0.063	0.019	0.386
LNM									–	0.040	0.411
PLP										–	0.391
MON											–

Population acronyms as in Table 1.

DISCUSSION

Karyological features corroborate a specific ranking of the three taxa described as *L. caprai*, *L. montana* and *L. attenuata sensu stricto*. Karyological analyses, although preliminary, support the existence of three well defined diploid karyotypes: the first (2n=39/40, XO/XX) found on the Toledo district hills (*L. caprai*), the second (2n=37/38, XO/XX) on the Sistema Central mountains (*L. montana*) and the third (2n=36, XY/XX) on the Portuguese Serra de Monchique (*L. attenuata*). Our investigations did not record the occurrence of all-female tetraploid populations.

The specific ranking of the three groups of populations is also suggested by allozyme data: Nei's D values and phenetic dendrograms (UPGMA and Neighbor-joining) evidence that we are dealing with three genetically differentiated entities. Actually, genetic distances here recorded are of the specific level of differentiation known for stick-insects (0.3 to 1.6) (Bullini and Nascetti, 1987; Scali *et al*., 1995). Also *F*-statistics support a high interpopulation genotypic heterogeneity; on the other hand, the heterogeneity level decreases significantly when *L. montana* and *L. caprai* populations are analyzed separately, suggesting once more that we are dealing with diverged gene pools, otherwise rather homogeneous within themselves.

However, chromosome, allozyme and morphological dif-

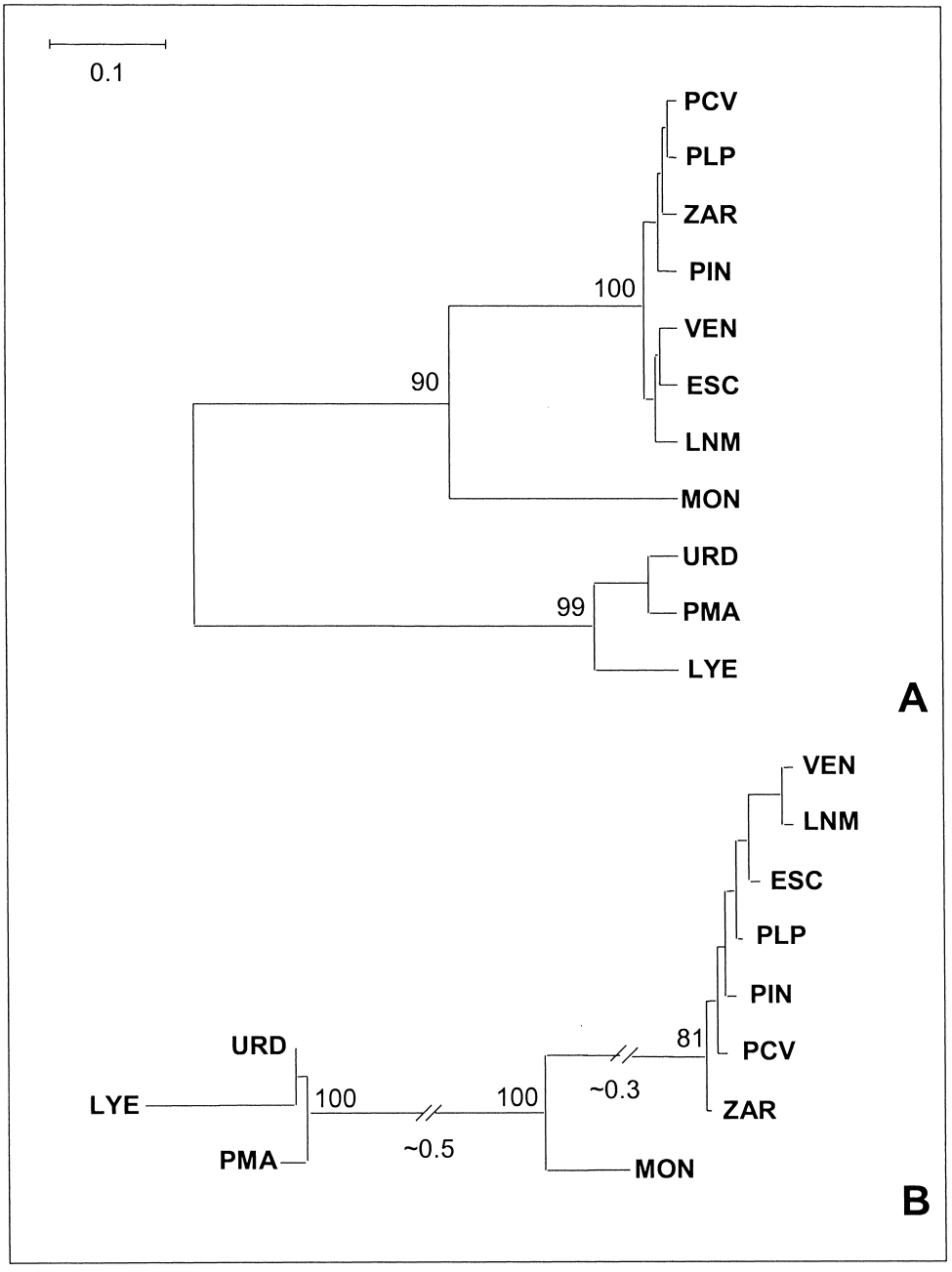

Fig. 3. UPGMA (**A**) and Neighbor-joining (**B**) dendrograms based on Nei's genetic distances obtained between pairs of analyzed *Leptynia* samples. Population acronyms as in Table 1.

ferentiations seem differently coupled in the *L. attenuata* complex as compared to *Bacillus* species: in the latter group a low degree of genetic (see f.i. *B. grandii–B. atticus* distance value of 0.35) and karyotypic differentiation is backed by several fully diagnostic morphological features (reviews in: Bullini and Nascetti, 1990; Scali *et al.*, 1995), whereas in the former species-complex a higher level of genetic and chromosomal divergence goes together with incompletely diagnostic morphological characters (Scali, 1996).

Present findings on *Leptynia* can be believed to depict a case of incipient morphological speciation, with chromosomal and genetic features ahead of the morphological ones in the process. Chromosome repatternings, affecting autosomes as well as sex chromosomes, could have entrained the evolutionary events.

In more detail, the chromosomal rearrangements presumably involved are: *i*, translocations, leading to the appearance of new large metacentric pairs, together with a corresponding decrease of small pairs; *ii*, X-autosome translocations giving rise to X-Y male sex formula, which in stick-insects is at variance with the most generally occurring X-O type.

In this perspective, if a karyotype trend towards lower

Table 4. Wright's *F*-statistics at the polymorphic loci for all (F^{all}), *L.caprai* (F^{caprai}) and *L. montana* ($F^{montana}$) samples.

Locus	Fit^{all}	Fst^{all}	Fis^{all}	Fit^{caprai}	Fst^{caprai}	Fit^{caprai}	$Fit^{montana}$	$Fst^{montana}$	$Fis^{montana}$
α*Gpdh*	0.801[a] (0.817±0.085)	0.793[a] (0.814±0.091)	0.038 (0.028±0.150)	0.098 (0.061±0.207)	0.182[b] (0.155±0.200)	−0.102 (−0.109±0.020)	0.506[a] (0.935±0.435)	0.127[c] (0.230±0.110)	0.435 (0.804±0.373)
Mdh-1	0.093[d] (0.114±0.115)	0.043[b] (0.043±0.016)	0.052 (0.074±0.122)	−0.003 (−0.011±0.013)	0.022 (0.023±0.029)	−0.025 (−0.034±0.022)	0.103 (0.129±0.139)	0.035[d] (0.030±0.022)	0.071 (0.103±0.154)
Idh-1	0.514[a] (0.513±0.083)	0.366[a] (0.366±0.120)	0.234[a] (0.235±0.048)	0.031 (0.042±0.063)	0.072[b] (0.085±0.062)	−0.045 (−0.047±0.005)	0.382[a] (0.383±0.058)	0.126[a] (0.133±0.087)	0.293[a] (0.290±0.049)
Idh-2	0.965[a] (1.012±0.175)	0.703[a] (1.086±0.624)	0.883[a] (0.917±0.144)				0.798[a] (0.854±0.254)	0.002 (−0.005±0.019)	0.798[a] (0.858±0.258)
6Pgdh	0.477[a] (0.483±0.100)	0.362[a] (0.368±0.109)	0.180[a] (0.181±0.071)	0.612[a] (0.666±0.171)	0.516[a] (0.573±0.199)	0.198[d] (0.205±0.073)	0.320[a] (0.327±0.125)	0.216[a] (0.216±0.090)	0.133 (0.138±0.103)
Gox	0.319[a] (0.319±0.070)	0.132[a] (0.127±0.045)	0.216[b] (0.222±0.079)	0.332[b] (0.323±0.222)	0.195[a] (0.164±0.205)	0.171 (0.174±0.104)	0.262[a] (0.267±0.083)	0.038[d] (0.034±0.024)	0.233[a] (0.243±0.101)
G6pdh	0.364[a] (0.373±0.118)	0.080[a] (0.086±0.045)	0.309[a] (0.313±0.118)	0.590[a] (0.581±0.118)	0.091[c] (0.053±0.111)	0.549[b] (0.535±0.236)	0.118 (0.104±0.178)	0.011 (0.014±0.026)	0.108 (0.096±0.201)
G3pdh	0.513[a] (0.509±0.114)	0.490[a] (0.491±0.110)	0.045 (0.039±0.127)	0.003 (0.003±0.004)	0.010 (0.016±0.008)	−0.007 (−0.014±0.004)	0.109 (0.102±0.132)	0.064[b] (0.063±0.049)	0.048 (0.042±0.127)
Aat-1	0.810[a] (0.805±0.042)	0.740[a] (0.733±0.061)	0.266[b] (0.277±0.076)	0.241[c] (0.230±0.098)	−0.007 (−0.004±0.007)	0.247[c] (0.233±0.101)	−0.006 (−0.013±0.011)	0.017 (0.012±0.012)	−0.023 (−0.026±0.008)
Aat-2	0.695[a] (0.709±0.129)	0.685[a] (0.701±0.131)	0.029 (0.023±0.063)	0.497[a] (0.594±0.176)	0.465 (0.623±0.227)	0.060 (0.012±0.138)	−0.007 (−0.009±0.007)	0.063[b] (0.115±0.067)	−0.075 (−0.130±0.075)
Adk	0.947[a] (0.952±0.038)	0.928[a] (0.937±0.050)	0.258 (0.280±0.359)				0.323[b] (0.315±0.437)	0.032 (0.021±0.022)	0.301 (0.304±0.463)
Pgm	0.561[a] (0.565±0.096)	0.481[a] (0.484±0.095)	0.156[c] (0.155±0.089)	0.294[a] (0.290±0.160)	0.096[a] (0.091±0.055)	0.219 (0.212±0.134)	0.201[b] (0.188±0.126)	0.113[a] (0.103±0.056)	0.099 (0.095±0.139)
Ald	0.438[a] (0.445±0.281)	−0.001 (−0.001±0.009)	0.439[b] (0.447±0.285)	0.496[b] (0.494±0.576)	0.012 (0.001±0.026)	0.490 (0.492±0.592)	0.392[c] (0.418±0.456)	−0.009 (−0.011±0.018)	0.398[d] (0.432±0.465)
Fh	0.673[a] (0.684±0.118)	0.649[a] (0.661±0.127)	0.068 (0.068±0.039)				0.228[b] (0.249±0.133)	0.172[a] (0.200±0.149)	0.067 (0.067±0.050)
Pgi	0.699[a] (0.708±0.108)	0.700[a] (0.709±0.100)	−0.003 (−0.009±0.101)	0.166[d] (0.247±0.137)	0.081[b] (0.101±0.072)	0.093 (0.156±0.101)	0.112 (0.099±0.104)	0.133[a] (0.134±0.059)	−0.025 (−0.037±0.127)

F levels of significance as in Table 1.

numbers is accepted, the most ancient taxon of the group could be *L. caprai* (2n=40). The opposite trend towards chromosome increase with a reversion from X-Y to X-O sex-formula was never observed in stick-insects. On the contrary, the trend here suggested would parallel those demonstrated in *Didymuria* stick-insect complex (Craddock, 1970; 1975) and in *Ramulus* (Scali *et al.*, 1990). Such a phylogenetic hypothesis seems to be also supported by the ongoing mt-DNA analysis on Cytochrome-Oxidase subunit II gene: as a matter of fact, in all rooted trees *L. caprai* appears to be basal to other species (unpublished data).

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