

Revision of the stick insect genus *Leptynia*: description of new taxa, speciation mechanism and phylogeography

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Abstract

Leptynia specimens were analyzed by karyotype analysis, mitochondrial gene sequencing and SEM of bodies and eggs. Here we describe a new species, *Leptynia annaepaulae*, and three subspecies of *L. attenuata* Pantel (*L. attenuata attenuata*, *L. attenuata iberica*, *L. attenuata algarvica*). The phylogeny of the genus *Leptynia* is congruent with a karyotype trend toward a reduction of chromosome number and the shift from the shared XX/X0 sex chromosome formula to the unusual XX/XY one. Chromosome repatterning appears to occur ahead of genetic differentiation, following a chromosome model of cladogenesis. Chromosome and genetic differentiation, in turn, appears to precede morphological distinction, thus realizing a condition of incipient species for most of the *Leptynia* taxa. Actually, morphological analyses revealed that, only rarely clear cut differences exist among and between taxa, while, more often, just trends in the differentiating traits occur, since the investigated characters generally suffer from some overlapping: In this study, only the 10th:9th ratio value and the subanal vomer appear to be diagnostic for *L. annaepaulae* against all other *Leptynia* taxa. As a consequence, the subanal vomer as well as cercus tooth features with egg chorion traits are not sharply diagnostic for the remaining co-generic taxa; however, comparisons are quite helpful in reducing uncertainties. A likely phylogeographic scenario for the genus supports that *Leptynia* ancestors spread from Northern Africa into Southern Spain where an ancestral taxon originated *L. annaepaulae* (2n = 40/39, XX/X0, with 2 large dibrachial pairs). Later on, a northbound colonization, should have originated *L. caprai* (2n = 40/39, XX/X0, all acrocentrics), from which *L. montana* (2n = 38, XX/X0) and *L. attenuata* (2n = 36, XY/XX) originated; supporting instances of chromosome repatterning have been actually observed. In this connection we like to stress that, particularly in stick insects, androgenesis has been a preferential pathway to quickly make homozygous those odd chromosome rearrangements likely responsible for low fitness in the heterozygotes.

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Introduction

According to the traditionally accepted systematics, Iberian representatives of Phasmida belong to either the family Bacillidae (*Bacillus*, *Clonopsis*) or Diapheromeridae (*Leptynia*, *Pijnackeria*), actually assigned to the different suborders of Areolatae, the former, and Anareolatae, the latter, on the basis of the presence/absence of the tiny tibial *area apicalis*, respectively (see Otte and Brock, 2005; Bradler, 2009). A splitting of the former genus *Leptynia* Bolivar into *Leptynia* and *Pijnackeria* has been implemented of recent (Scali, 2009a); *Leptynia sensu novo* mainly spreads into Portugal, Western and Central Spain - where it partially overlaps with the other genus -, whereas *Pijnackeria* ranges from Central/Eastern Spain up to Southern France (Brock, 1993). The splitting has been based on multidisciplinary approach findings, obtained from karyological, genetical and morphological investigations. The same study also allowed to settle the confused systematic status of the originally reported *L. hispanica* (Bolivar, 1878) and *Leptynia attenuata* (Pantel, 1890) species by definitively indicating *Pijnackeria hispanica* as type species of the new genus.

Distinct body traits differentiating specimens of the two genera appeared to be the presence/absence of the subanal vomer in the males and the abdomen tip features, including cercus morphology, in the females. In more detail, while in *Leptynia* males a well developed subanal vomer is always present, it is lacking in

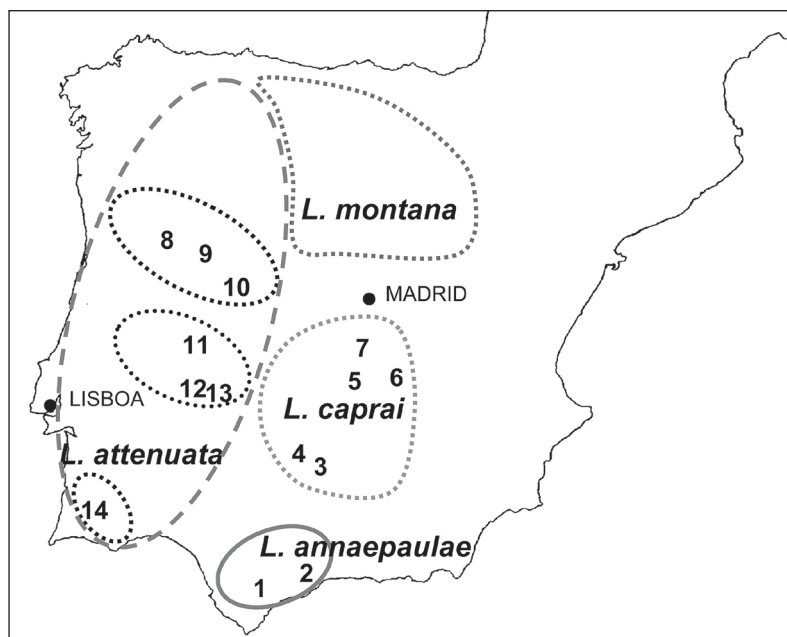


Fig. 1. Map showing the ranges of *Leptynia* species and subspecies. *L. annaepaulae*: 1) Grazalema (GRA), 2) Ojen (OJE). *L. caprai*: 3) Viso del Marqués (VIS), 4) San Lorenzo de Calatrava (SLC), 5) Puerto de los Majales (PLM), 6) Urda (URD), 7) Los Yébenes (LYE). *L. attenuata iberica*: 8) Guarda (GUA), 9) Peña de Francia (PDF), 10) Santibañez (SIB). *L. attenuata attenuata*: 11) São Fiel (SFI), 12) Marvão (MAR), 13) Portalegre (POR). *L. attenuata algarvica*: 14) Monchique (MON). All acronyms are those reported in Mr Bayes tree of Fig. 2.

Pijnackeria males; at the same time, the slightly tapered and soft *terminalia* of *Leptynia* females, with back-directed protruding cerci, are sharply contrasting with the pointed, compressed and stiff last abdomen tergite, almost concealing the short, often downward directed, cerci of *Pijnackeria* females (Scali, 2009a). Also gross morphology of the egg, as well as its SEM chorionic pattern, proved sharply diagnostic: the dark grey egg capsule of *Leptynia* is remarkably thinner and smoother than that of *Pijnackeria* egg, which is sand coloured, thicker and all covered by an outstanding ribbon network. Furthermore, while all *Pijnackeria* females loosely anchor their eggs to the substrate (soil, stones), *Leptynia* eggs are firmly glued to the food-plant twigs.

In addition, the two genera could be well characterized through mitochondrial gene analyses, since they appeared to be neatly separated (Scali, 2009a).

Both genera encompass a few specific taxa. *Leptynia* species are bisexual diploids with karyotypes clearly differing for both chromosome number (40 to 36) and structure, including the sex chromosomes: the common XX/X0 formula, for female and male respectively, is realized in the numerically highest sets ($2n = 40/39$ and $38/37$), while the unusual, clearly derived, XX/XY in the $2n = 36$ taxa. The karyotypic differences appear to be due to Robertsonian translocations (mainly fusions and inversions), which have been envisaged as a driving force of their speciation, coupled

Table 1. Collecting sites (with acronyms), sample size and chromosome number (with sex formula) of new *Leptynia* taxa utilized for karyology and SEM.

Collecting sites	Sample size	Taxon	Chromosome number
São Fiel (SFI), Portugal	16	<i>L. attenuata attenuata</i> Pantel, 1890	$2n = 36$ (XX/XY)
Marvão (MAR), Portugal	8	<i>L. attenuata attenuata</i> Pantel, 1890	$2n = 36$ (XX/XY)
Portalegre (POR), Portugal	13	<i>L. attenuata attenuata</i> Pantel, 1890	$2n = 36$ (XX/XY)
Guarda (GUA), Portugal	5	<i>L. attenuata iberica</i> ssp.n.	$2n = 36$ (XX/XY)
Peña de Francia (PDF), Spain	8	<i>L. attenuata iberica</i> ssp.n.	$2n = 36$ (XX/XY)
Santibañez de la S. (SIB), Spain	28	<i>L. attenuata iberica</i> ssp.n.	$2n = 36$ (XX/XY)
Serra de Monchique (MON), Portugal	10	<i>L. attenuata algarvica</i> ssp.n.	$2n = 36$ (XX/XY)
Sierra de Grazalema (GRA), Spain	31	<i>L. annaepaulae</i> sp.n.	$2n = 40/39$ (XX/X0)
Ojen (OJE), Spain	2	<i>L. annaepaulae</i> n.sp.	$2n = 40/39$ (XX/X0)

with a weak morphological differentiation (Scali, 1996; Passamonti *et al.*, 1999, 2004).

At variance, *Pijnackeria* encompasses both bisexual and parthenogenetic polyploidy taxa; furthermore, the karyotypes of the bisexuals have kept a numerically invariant haploid set ($n = 19$), also very similar in structure, including sex chromosomes, to always give a $2n = 38/37$, XX/X0 chromosome complement. The polyploid parthenogens, with $3n = 57$, XXX, and $4n = 76$, XXXX, apparently originated through the addition of one or two such haploid sets of 19 chromosomes, respectively. Molecular analyses have even demonstrated that those parthenogens are hybrids derived from different maternal ancestors and independent routes (Ghiselli *et al.*, 2007).

The *Leptynia* taxa so far described are:

- *Leptynia attenuata* Pantel, 1890 ($2n = 36$, XX/XY), first reported from the Portuguese Castelo Branco region (Pantel, 1890). Since Pantel did not indicate the species holotype, a lectotype has been designated from his original collection housed at the Natural History Museum of Paris (Scali, 1996). Later, *Leptynia attenuata* had been first reported from the Algarve region in South Portugal, and initially indicated as *L. attenuata* tout court on the basis of cercus morphology (Scali, 1996). Afterwards, additional *L. attenuata* was recovered from the Castelo Branco region and further populations were also discovered from the Guarda and Peña de Francia areas (Passamonti *et al.*, 2004) (see map, Fig. 1);
- *L. montana* Scali, 1996 ($2n = 37/38$, X0/XX) spreads on the Sistema Central mountains, north-west of Madrid;
- *L. caprai* Scali, 1996 ($2n = 39/40$), found in the hilly area south of Toledo down to the Calatrava region.

A preliminary series of bidirectional crosses suggested that among the species a considerable degree of reproductive isolation occurs with low hatching rates (from 6 to 36 %) and sterile hybrid adults (unpublished data).

Moreover, within *L. attenuata* three geographically distinct clades could be recognized by the *cox2* gene analysis (*L. attenuata* 1-3) (Passamonti *et al.*, 2004). Although the phylogenetic inference failed to retrieve *L. attenuata* as a clade, the same chromosome number and unusual sex determination ($2n = 36$; XX/XY) of those clades, coupled with a low reproductive isolation and a limited morphological differentiation (see below and Appendix), suggest that they should pertain to the same species. As a consequence, *L. attenuata* 1-3 should be better described as subspecies. The subspe-

cies here defined as groups of populations with an allopatric distribution (Fig. 1) and a partial gene-flow reduction are:

- *L. attenuata attenuata*, the one located in the range of species' *patria typica* (São Fiel; Pantel, 1980);
- *L. attenuata iberica*, that spreading from the Portuguese region of Guarda (Serra da Estrêla) to the Spanish areas of Villablino and Peña de Francia;
- *L. attenuata algarvica*, spreading on the southern Portuguese Serra de Monchique (Algarve region).

Furthermore, among *Leptynia* samples an unnamed, sharply differentiated, basal taxon was additionally found to occur on the Sierra de Grazalema (Southern Spain); this will be here formally described as a new species, named *Leptynia annaepaulae*.

Material and methods

Specimens derive from a series of collecting campaigns between 2004 and 2010; derivations and composition of pertinent samples are listed in Table 1 and shown in the map (Fig. 1).

Karyotypes were obtained from mitotic plates prepared from either testes or ovariole tips after 30min fixation in Carnoy solution, teasing in 45% acetic acid, drying on a hot plate (60°C) and Giemsa staining. Slides were observed in either Leica or Zeiss photomicroscopes. From 10 to 50 metaphasic plates per taxon were counted, from which karyotypes were derived; about 90% mitoses gave a constant and coherent chromosome number. In each karyotype chromosomes are arranged according their decreasing size, but also centromeric index (C.I., Levan *et al.*, 1964), and, particularly for the small elements, the amount and shape of pericentromeric heterochromatin were taken into account. We must however admit that not always a completely sound assignment could be achieved; in this case the overall appearance of the chromosome arms guided the disposition in pairs.

The *cox2* partial sequences of 45 *Leptynia* specimens (GenBank Accession Numbers: AF241412/AF241431; AF241433/AF241443; AF508800; AF508230/AF508242) were aligned with the Clustal algorithm of MEGA 3.1 (Kumar *et al.*, 2004) and analyzed by Mr Bayes 3.1 (5,000,000 generations; Huelsenbeck *et al.*, 2003), using the General Time Reversible (GTR + I + G) substitution model (Lanave *et al.*, 1984; Tavaré, 1986; Rodríguez *et al.*, 1990), that showed the best score with Modeltest (Posada and Crandall, 1998). *Pijnackeria hispanica* was utilized as

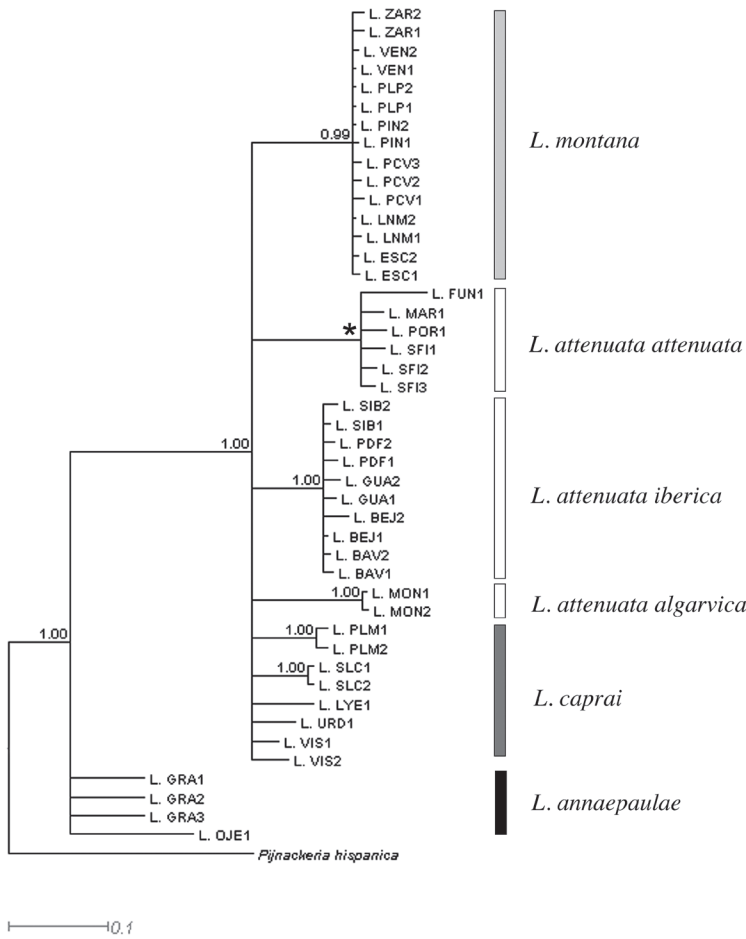


Fig. 2. Bayesian phylogenetic tree. The *cox2* partial sequences of 45 *Leptynia* specimens were analyzed by Mr Bayes 3.1 (5,000,000 generations; Huelsenbeck *et al.*, 2003). A clear splitting between *L. annaepaulae* and the other *Leptynia* samples (pp = 1.00) is apparent. The remaining *Leptynia* are joined in a vast unresolved polytomy, in which some well defined clades can be recognized: *L. montana* (pp = 0.99), *L. attenuata algarvica* (pp = 1.00), and *L. attenuata iberica* (pp = 1.00). For detailed observations see the text. *Pijnackeria hispanica* was utilized as outgroup.

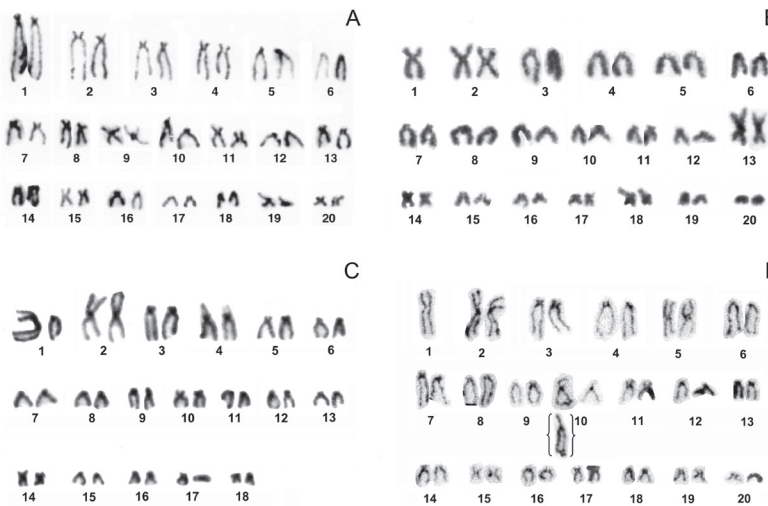


Fig. 3. A) Karyotype of *L. caprai* female: $2n = 40, XX/X0$. Note the acrocentric/subacrocentric structure of all large pairs, including the X chromosomes (the largest). B) Karyotype of *L. annaepaulae* male: $2n = 40, XX/X0$. Note the submetacentric X, the 2nd meta-centric pair and the 13th and 18th pairs with satellites. C) Karyotype of *L. attenuata attenuata* male, $2n = 36, XX/XY$, with a large submetacentric X, and a small acrocentric Y (1st pair). D) Karyotype of *L. caprai* found at Viso del Marqués: an intermediate situation between *L. caprai* and *L. annaepaulae* can be appreciated; furthermore a re-patterned chromosome (# 10) can be observed. In brackets is reported the same re-patterned chromosome taken from another plate to support its maintenance in germ cells.

outgroup (GenBank Accession Numbers: AF241444, see Fig. 2).

Scanning Electron Microscope (SEM) preparations were obtained after at least three days fixation of bodies or eggs in 70% and a complete dehydration in a graded series of ethanol solutions. An ultrasonication of 30s to eliminate, or at least significantly reduce, surface debris on specimens was then applied, which were then air dried and attached to specimen-holders with a suitable adhesive tape. Afterwards, bodies and eggs were coated with gold in a Bio-Rad SC 502 sputter-coater evaporator and observed in a Hitachi 2400 or in a Jeol JSM 5200 scanning electron microscope. The adopted terminology for body and egg descriptors is the one commonly used for stick insects (see Bradley and Galil, 1977; Scali and Mazzini, 1981). The terminology for body and egg parameters has been derived from both Clark (1976) and Milani *et al.* (2009). Egg and body size measures were either taken directly under a dissecting microscope or automatically evaluated by the SEM apparatus.

Both SEM and light microscope images were recorded through an electronic apparatus and then processed for publishing.

Results

Quite clearly, mitochondrial DNA genes had demonstrated that all European species of *Bacillus* and *Clonopsis* (Areolatae) and *Leptynia* (Anareolatae) pertain to a same monophyletic clade (Galassi, 2002); therefore the commonly accepted suborder splitting into Areolatae (represented *Bacillus* and *Clonopsis*) and Anareolatae (now represented by *Leptynia* and *Pijnackeria*) must be questioned; in this context the occurrence of the tiny *area apicalis* among the Phasmida (= Phasmatodea) should be considered a homoplasious (or convergent) character state, although it has been also envisaged as a homologous plesiomorphic trait (Bradler, 2009). At any rate, while further evaluation is pending, we can keep the attribution of *Leptynia* to the family Diapheromeridae, subfamily Pachymorphinae, tribe Gratidiini (Otte and Brock, 2005), since, after all, all morphological and structural body traits of *Leptynia* are coherent with the general features of those systematic groupings (see Appendix).

The karyotypes neatly separate all species, but are not diagnostic among the three *L. attenuata* subspecies (Fig. 3). It must be noted, however, that while to the same number reported for *L. attenuata* subspecies

practically indistinguishable karyotypes correspond, in *L. caprai* and *L. annaepaulae* to the same score of 39/40 chromosomes consistently different karyotypes occur (Fig. 3). In this connection we want to mention that stable karyotypes have been always obtained from all analyzed specimens within each species; with one exception, however, since the karyotype of Viso del Marques specimens (Calatrava Region, southern part of *L. caprai* range) was different from the other *L. caprai* populations, and surprisingly more similar, although not identical, to that of *L. annaepaulae* by having a submetacentric X chromosome - the largest in the karyotype - and also possessing the second large metacentric pair just like *L. annaepaulae*, both lacking from *L. caprai*, whose sex chromosomes and large pairs are all acrocentrics or subacrocentrics (Fig. 3). This finding is to be confronted with the observation that the linearized NJ *cox2*-based tree (see Passamonti *et al.*, 2004) put these specimens within the *L. caprai* cluster. The karyotype of *L. attenuata attenuata* is characterized by a large submetacentric X, and a small acrocentric Y (1st pair). Additional outstanding pairs are the metacentric 2nd, and the acrocentric 4th with satellites. This karyotype, with only minor differences, is also found in *L. attenuata iberica* and *L. attenuata algarvica*.

The Bayesian tree based on *cox2* mitochondrial gene (Fig. 2) showed a clear splitting between *L. annaepaulae* and all other *Leptynia* samples (pp = 1.00). The remaining *Leptynia* are joined by a vast unresolved polytomy: *L. attenuata* is not retrieved as a clade, possibly due to the low variability of the *cox2* gene, but also *L. caprai* was not recovered as a single clade; the issue should be further investigated. However some groups can be recognized: *L. montana* (pp = 0.99), *L. attenuata algarvica* (pp = 1.00), and *L. attenuata iberica* (pp = 1.00); on the other hand, since the *L. attenuata attenuata* clade was supported in previous analyses through Maximum Parsimony and Maximum Likelihood, based on the very same sequences (see Passamonti *et al.*, 2004), we think we are allowed to maintain this node (here marked with an asterisk). Moreover, Fig. 4 highlights the substitutions observed in the same sequenced haplotypes of *cox2* gene: all *Leptynia* taxa, with the noteworthy exception of *L. caprai*, show diagnostic mutations; on the other hand, *L. caprai* shares several mutations with the other taxa.

On the basis of karyotype features and genetic differentiation, a corresponding morphological analysis has been carried out, by investigating most of the commonly recorded parameters of bodies and eggs (see Scali, 1996; Scali and Milani, 2009) (Table 2).

		1111111111	1222222222	2222333333	3333334444	4444445555	5555555555	556666
		1223366689	0011345667	8012234456	7789013356	7789990123	3357790112	2233445556
		5290636911	5815813234	7702511704	6984356973	8940365132	5864731692	5947340584
<i>L. montana</i>	LNM1	AAGATCTTTC	ACTATTCTCA	TCACCCCTC	TTGAACTTT	AGTCATTAC	TCCTACAT	ATCACATT
	LNM2	..A.....C...
	PCV1	..A..T...C...
	PCV2	..A..T...C...
	PCV3	..A.....C...
	PIN1	..A.....C...
	PIN2	..A.....C...
	PLP1	..A.....C...
	PLP2	..A.....C...
	ZAR1	..A..A...C...	..A..C...
	ZAR2	..A.....C...
	VEN1	..A.....C...
	VEN2	..A.....C...
	ESC1	..A.....C...
	ESC2	..A.....C...
<i>L. attenuata attenuata</i>	FUN1	..A.....T	..T.....T	..TT.....	..C.C..CC..	..C.T..CTA	..C.TT...T	..T..A...T
	MAR1	..A.....T	..T.....T	..T.....	..C.....	..TG..C...	..C.T..CC.A	..C.T...T.C
	POR1	..A.....T	..T.....T	..T.....	..C.....	..TG..C...	..C.T..CC.A	..C.T...GT.C
	SFI1	..A.....T	..T.....T	..TT.....	..T.....	..C.T...C...	..C.T...C.A	..C.T...T.C
	SFI2	..A.....T	..T.....T	..TC.....	..T.....	..C.C...C...	..C.T...C.A	..C.T...T.C
	SFI3	..A.....T	..T.....T	..TC.....	..T.....	..C.T...C...	..C.T...C.A	..C.T...T.C
<i>L. attenuata iberica</i>	SIB1	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GT...
	SIB2	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G..A....	..T..GT...
	PDF1	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GTG...
	PDF2	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GTG...
	GUA1	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CCC...	..G.....	..T..GT...
	GUA2	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CCC...	..G.....	..T..GT...
	BEJ1	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GT...
	BEJ2	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GT...
	BAV1	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GT...
	BAV2	..A.....T	..T..C....	..T..T...T	..T..T...T	..T..CC...	..G.....	..T..GT...
<i>L. attenuata algarvica</i>	MON1	..A.....T	..T.....	..T.....	..T.....	..C.CGTTTC	..A.....	..T.....
	MON2	..A.....T	..T.....	..T.....	..T.....	..C.CGTTTC	..A.....	..T.....
<i>L. caprai</i>	PLM1	..A.....T	..T..A...	..TT..T...T	..TG..C...TT..T...	..C..C..T
	PLM2	..A.....T	..T..AC...	..TT..T...T	..TG..C...TT..T...	..C..C..A
	SLC1	..CT...T	..GT...CC	..TT..T...T	..CG..C...TT..T...	..T..C..T
	SLC2	..CT...T	..GT...CC	..TT..T...T	..CG..C...TT..T...	..T..C..T
	LYE1	..A.....T	..T..ACT...	..TT..T...T	..CG..C...TT..T...	..T..C..T
	URD1	..A.....T	..T..CC...	..TT..T...T	..CG..C...TT..T...	..T..C..T
	VIS1	..A.....T	..TA...C..G	..TT..T...T	..TG..CC..CATT..T...	..T..TC..T
	VIS2	..A.....T	..TA...C..G	..TT..T...T	..TG..CC..CATT..T...	..T..TC..T
<i>L. annaepaulae</i>	OJE1	TTA..A..CCCT	TATC..ATCT	CA..TTT..TCT	AT..T...T	TT...A	ATTT...T
	GRA1	TTA..A..CCCT	TAT..ATCT	CA..TTT..T.T	CAT..T...T	TT...A	AT...T
	GRA2	TTA..A..CCCT	TAT..ATCT	CA..TTT..T.T	CAT..T...T	TT...A	AT...T
	GRA3	TTA..A..CCCT	TAT..ATCT	CA..TTT..T.T	CAT..T...T	TT...A	AT...T

Fig. 4. Substitutions in the sequenced *cox2* partial gene of *Leptynia*. Private mutations marked in grey. Sample acronyms as in Fig. 2. All taxa, excluding *L. caprai*, show diagnostic mutations. *L. caprai* shares several mutations with the other taxa.

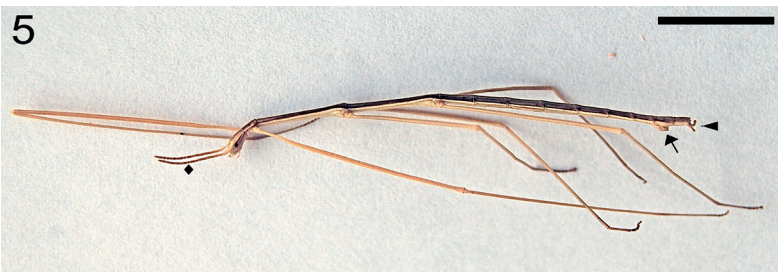


Fig. 5. Holotype (male) of *Leptynia annaepaulae* sp. n. Note the widespread brown pigmentation of the tergites, the sharp white line above the pleura and the canella colour of sternites. It is also possible to just see the relatively long antennae (lozenge), particularly if compared with those of the female (Fig. 6; see also Fig. 7), the profile of the subgenital operculum (arrow) and the terminal claspers (arrowhead). Bar = 1 cm.



Fig. 6. Paratype (female) of *Leptynia annaepaulae* sp. n. Note the pink nuance of the whole body colour, the bright white lateral line, the short antennae (lozenge; see also Fig. 7) and the straight projecting cerci (arrowhead). Bar = 1 cm.

Table 2. Chromosome numbers and main body and egg parameter ranges of *Leptynia* taxa. Lengths are given in mm and angles in degrees. Body measures do not include antennae and cerci. The number of measured specimens are given in brackets: for *L. caprai*, *L. montana* and *L. attenuata algarvica* it could also include, when appropriate, those cumulatively reported in Scali (1996), amounting to about 40 for each taxon; abdom. terg. = abdominal tergite.

Character	Sex	<i>L. attenuata attenuata</i>	<i>L. attenuata iberica</i>	<i>L. attenuata algarvica</i>	<i>L. montana</i>	<i>L. caprai</i>	<i>L. annaepaulae</i>
Chromosomes	M	36	36	36	37	39	39
	F	36	36	36	38	40	40
Body length	M	42.5-47.5 (5)	39.5-44.0 (9)	34.0-40.0 (6)	37.0-43.0 (4)	39.0-44.0 (13)	42.0-43.0 (3)
	F	49.5-57.0 (3)	49.2-54.4 (9)	46.0-50.0 (4)	44.0-57.0 (5)	44.0-55.0 (7)	48.0-56.0 (7)
Antennal articles (N°)	M	15-16 (10)	15-18 (17)	14-18 (12)	14-18 (8)	14-18 (13)	16-17 (6)
	F	15-16 (4)	14-16 (18)	13-16 (8)	13-16 (10)	13-16 (7)	16-17 (14)
Length of antennae	M	5.2-6.5 (10)	5.0-6.0 (17)	5.7-6.7 (6)	5.0-6.0 (8)	4.5-7.0 (13)	6.0-7.0 (6)
	F	3.8-4.0 (4)	4.0-5.0 (18)	3.1 (2)	3.5-4.5 (8)	3.4-4.5 (5)	3.5-4.2 (14)
Mesonotum length	M	8.0-9.5 (5)	7.0-8.5 (8)	6.3-7.5 (4)	6.8-7.5 (4)	7.0-9.0 (6)	8.3-8.5 (3)
	F	9.0-10.5 (3)	8.5-10.1 (8)	8.0 (1)	8.0-9.0 (4)	8.5-9.5 (3)	9.0-10.2 (7)
Metanotum length	M	8.0-9.5 (5)	7.5-8.7 (8)	6.6-7.8 (4)	7.0-9.0 (4)	8.3-9.5 (5)	8.4-8.5 (3)
	F	10.0-10.5 (3)	9.0-10.6 (8)	8.5 (1)	8.0-9.0 (4)	9.0-10.0 (3)	9.0-10.2 (7)
Length of median femur	M	12.0-13.5 (9)	10.5-13.0 (20)	10.0-10.2 (3)	10.0-12.0 (4)	13.5-14.0 (9)	12.5-15.5 (5)
	F	11.8-15.0 (6)	10.0-13.0 (21)	8.0-10.0 (2)	9.8-12.5 (6)	12.0-13.0 (5)	12.5-18.0 (8)
Length of hind femur	M	15.5-18.0 (9)	14.5-16.5 (19)	12.6-14.0 (5)	14.0-16.0 (4)	15.5-17.0 (5)	12.5-17.0 (5)
	F	15.8-19.0 (6)	12.0-17.0 (21)	12.0 (2)	12.0-16.0 (4)	13.5-17.0 (6)	14.0-18.0 (8)
Hind femur reach	M	7½ (9)	7½ -8½ (19)	6½-7½ (5)	7½ (4)	7½ (5)	6½-7¾ (5)
	F	7¼ (6)	6½-7 (21)	5½ (2)	4-5 (4)	4-5 (6)	6½-7 (8)
Ratio 10 th :9 th abdom. terg.	M	0.72-0.85 (10)	0.68-0.75 (8)	0.72-0.88 (6)	0.77-0.96 (4)	0.77-0.94 (8)	0.60 (3)
	F	1.20-1.80 (7)	1.45-1.80 (8)	1.66-2.00 (4)	1.40-2.10 (4)	1.60-1.80 (3)	1.30-2.0 (8)
Egg length		3.5-4.5 (7)	3.5-4.8 (6)	4.1-4.8 (8)	3.7-4.0 (8)	5.7-7.2 (6)	5.5-5.9 (8)
Egg width:length ratio		0.24-0.32 (6)	0.22-0.26 (10)	0.22-0.23 (5)	0.25-0.28 (8)	0.13-0.19 (8)	0.17-0.19 (8)
Egg operculum angle		-5/+10 (6)	+20/+30 (5)	+15/+20 (5)	+10/+18 (8)	+27/+33 (6)	+30 (2)

Also the new *Leptynia* taxa are small phasmids - around 40mm the males and 50mm the females - with the same slender body appearance as that observed in co-generic species; the common sexual dimorphism is always apparent, with males much thinner than females (Figs 5-6).

From an overview of the reported metric and meristic traits it can be seen that in the males the 10th:9th ratio value appears to be fully diagnostic for *L. annaepaulae*, whereas no other sharp distinguishing trait exists to separate a given taxon from all others; rather, only morphological or dimensional trends could be observed for the analyzed traits, since the recorded features or values always suffer some overlapping (Table 2). However, additional, mainly qualitative characters - such as the subanal vomer features and cercus tooth shape/size - although rather tricky, allowed a reasonably safe diagnosis of a few *Leptynia* taxa (Table 3 and Appendix).

L. attenuata algarvica males are the smallest, while those of the other two *L. attenuata* subspecies largely overlap each other and with the remaining species. *L.*

attenuata algarvica females too tend to be small, but their size range amply overlaps or even lays within those of the remaining taxa. The article number of antennae is the same in males and females of all species; however, their length is consistently higher in males than females, thus confirming the well known sexual allomorphy for this character in stick insects (Fig. 7); the very low values of antennal lengths recorded for *L. attenuata algarvica* females could be linked to their small body size, but also the reduced sample could be responsible for this finding.

One additional trait often utilized to characterize related orthopteroid taxa, independently from absolute specimens' size, is the hind femur reach when positioned parallel to the abdomen. In tested males of the already described species, the hind leg femur invariably attained about the half of the 7th abdominal segment, thus being of no diagnostic value. The same parameter gave similar results for *L. attenuata* and *L. annaepaulae* males; the only finding at variance has been obtained from *L. attenuata iberica*: out of six

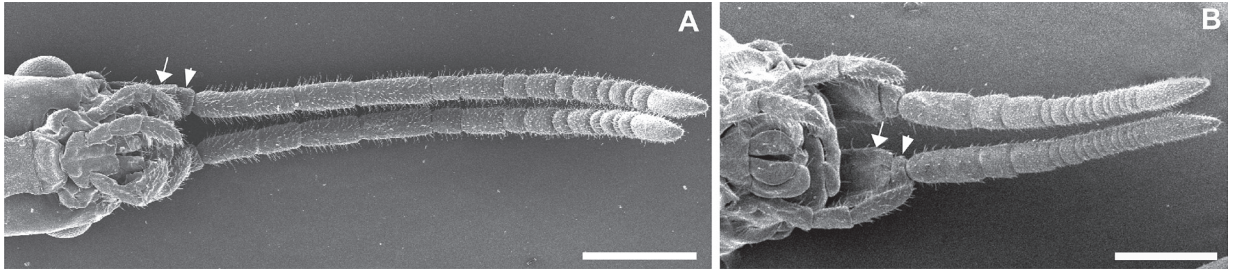


Fig. 7. A) Antennae of a *Leptynia* male, with 17 articles: all taxa share the same antennal structure and have a similar length range with higher values than females (sex allomorphy). Bar represents 1 mm. B) Antennae of a *Leptynia* female, with 16 articles. The structural antennal plan is the same as that of males, but the single articles are shorter. Incomplete or additional segmentations can modify the basic article number in both sexes. Legend: arrow - scapus; arrowhead - pedicellus. Bar = 0.8 mm.

measured males two had proportionately longer legs, since their femur reached almost half of the 8th abdominal segment. A slightly more significant result has been obtained from females: the hind femur reach has been observed to increase from *L. attenuata algarvica* (half of the 5th segment) to *L. attenuata iberica* (from 5th to 6th/7th segment joint) and, further, to *L. attenuata attenuata* (from the base of 7th segment to half of it). In *L. annaepaulae* females the hind femur constantly reaches from half of 6th up to 7th segment articulation.

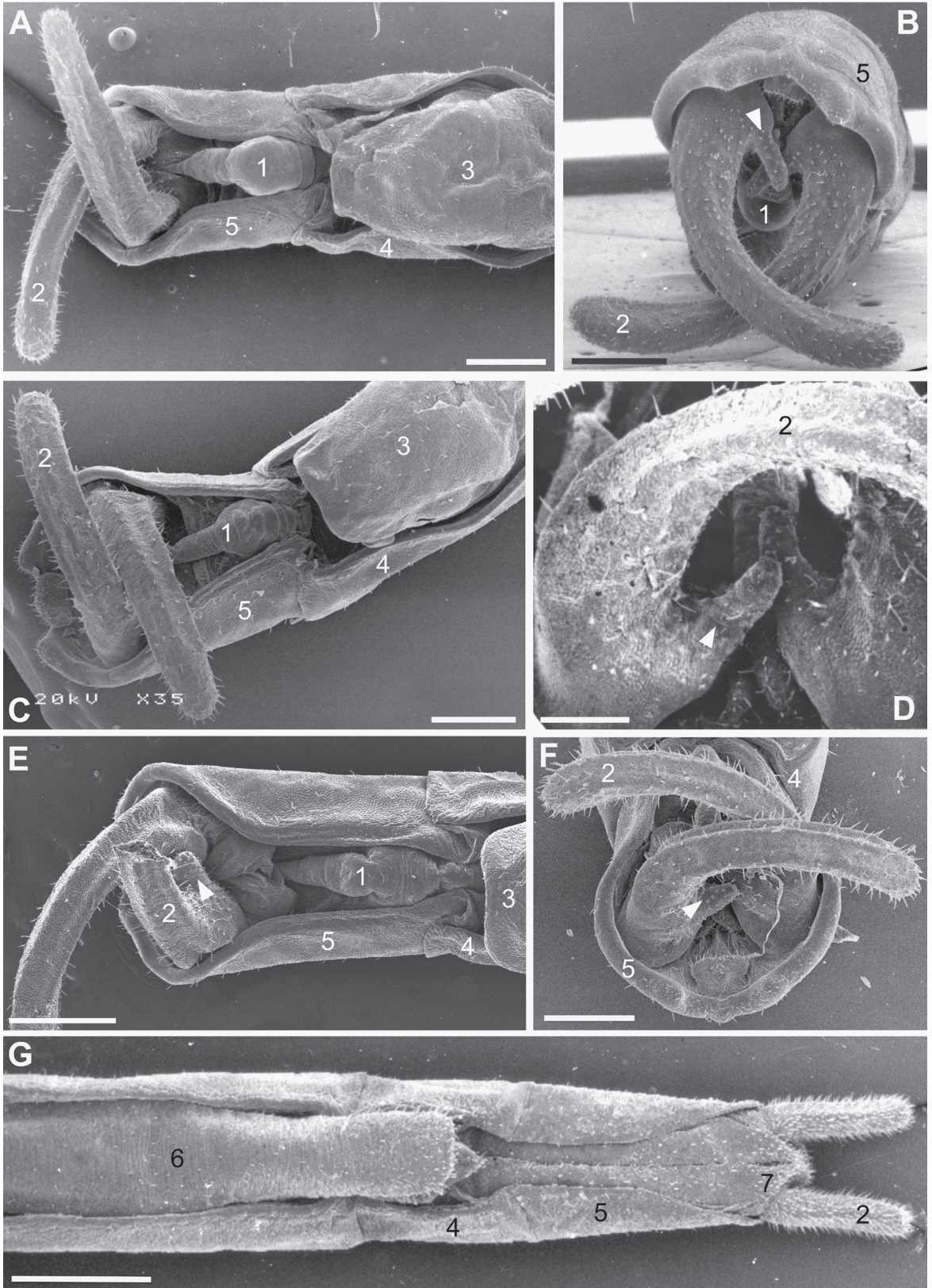
As already pointed out, the presence of a subanal vomer (about 1mm long) is a major diagnostic character of *Leptynia* males, when compared to those of the

related *Pijnackeria* genus, which lack it (Scali, 2009a, b). In addition, its shape has now turned out to realize different patterns among *Leptynia* species and even subspecies. As a matter of fact, the proximal part the vomer base is swollen, almost spherical, in most specimens of *L. attenuata attenuata* and *L. attenuata algarvica*; moderately swollen and only slightly wider in *L. attenuata iberica*, flat and decidedly wider than its following segment in *L. annaepaulae*, where it also shows a lobate structure (Fig. 8A, C, E). Furthermore, in many *L. attenuata* males the vomer may appear furrowed on the base and/or the distal, narrower part of it (Fig. 8C, E). The same observations revealed that even for the already known *L. attenuata* and *L. caprai*

Table 3. Comparisons between subanal vomer and cercus traits in *Leptynia* males (see Fig. 8).

Taxon	Vomer base features	Cercus tooth features
<i>L. a. attenuata</i> <i>L. a. iberica</i>	swollen and wider than the vomer stem close to it moderately swollen and slightly wider than the vomer stem	slender and of medium length (Fig. 8B) of intermediate thickness (wide base) and short (Fig. 8F)
<i>L. a. algarvica</i> <i>L. montana</i>	swollen and wider than the vomer stem moderately swollen and narrow, almost of the same thickness of the vomer stem	thick, long and stout (Fig. 8D) thick and short (Scali, 1996)
<i>L. caprai</i> <i>L. annaepaulae</i>	not swollen, narrow as the vomer stem flat, very wide with lateral lobes	very thick (wide base) and very short (Scali, 1996) slender and short (Fig. 8E)

Fig. 8. Sample of male and female terminalia of *Leptynia*, showing some significant structures. A-B) *L. attenuata attenuata* male: the swollen smooth subanal vomer base (1) its furrowed distal segment and the cercus (2) with a slender tooth (arrowhead); the tapered operculum (3). C-D) *L. attenuata algarvica* male: the vomer swollen base, the stout tooth (arrowhead) and the truncated operculum (3). E) *L. annaepaulae* male: the flat and lobed appearance of the vomer base (1); the short, pointed tooth (arrowhead); the posterior operculum border (3). F) *L. attenuata iberica* male: the low conical tooth (arrowhead), with a wide base. G) Female terminalia of *L. attenuata attenuata* showing the 1st ovipositor valve (6) the tapered, soft 10th sternite (7) and straight cerci (2). Legend: 1 - vomer; 2 - cercus; 3 - male operculum; 4 - 9th abdominal tergite; 5 - 10th abdominal tergite; 6 - ovipositor valve (8th abdominal sternum); 7 - 10th sternite; arrowhead - cercus tooth. Scale bars: A, B, C, E, F = 0.5 mm; D = 0.2 mm; G = 1 mm.



differences can be perceived, so that a complete taxon-specific series could be obtained (Table 3).

Male cerci are well developed and in the adults assume a typical conformation of claspers; a peculiar trait of *Leptynia* claspers is the occurrence of a projecting tooth of variable size and shape, placed near the base of each cercus. When such teeth were compared among

the first described species (*L. caprai*, *L. montana* and *L. attenuata*), species-specific patterns could be appreciated: it appeared very low with a wide base in *L. caprai*, longer and thick in *L. montana*, longest and stout in *L. attenuata*, so that tooth features appeared fully diagnostic (Scali, 1996). The new taxa as well show differing cercus tooth traits: slender, short and pointed in *L. annaepaulae*; slightly longer, thin with a blunt apex and obliquely inserted in *L. attenuata attenuata*; low, conical with a wider base in *L. attenuata iberica*; long and more robust in *L. attenuata algarvica* (Fig. 8B, D-F).

Therefore, the clear cut differences observed among the first described species have become rather subtle and careful comparisons are now needed to make a sound assessment of each taxon.

A partially diagnostic condition is found when egg size and chorion features are compared. All *Leptynia* species lay thin, elongated eggs, but *L. caprai* and *L. annaepaulae* appear to have comparably longer eggs than others (Table 2); accordingly *L. montana* and *L. attenuata* lay shorter eggs, amply overlapping each other for size range. The higher length values of *L. caprai* and *L. annaepaulae* are mainly due to their shared traits of developing both a raised polar mound and a dome-shaped operculum, while in the shortest eggs a shallow polar mound and a flat operculum are realized (Fig. 9). All this is mirrored in the width to length ratios: owing to the almost invariant egg width values in all taxa, the lowest ratio figures are found for *L. caprai* and *L. annaepaulae*, while for the remaining clearly higher values are obtained (Table 2). At the same time it can be seen that *L. annaepaulae* eggs, together with *L. caprai*, show the highest values of

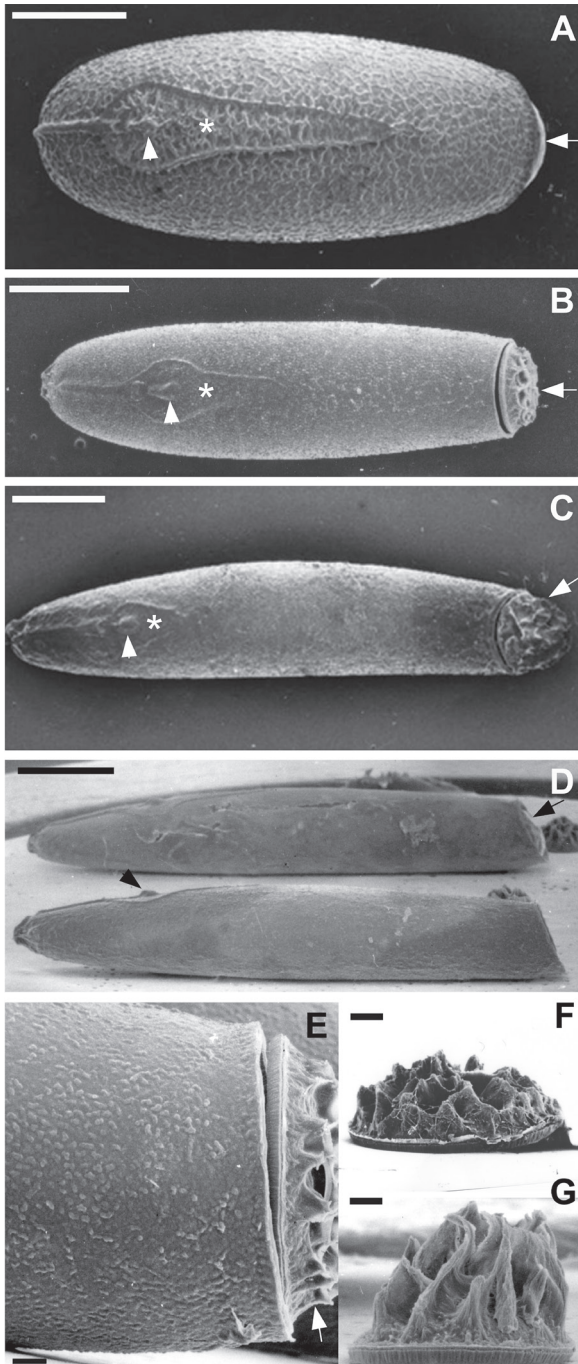


Fig. 9. Eggs of Iberian phasmids. A) Dorsal view of egg capsule of *Pijnackeria*, showing its outstanding ribbon pattern, the micropylar area (asterisk), micropylar cup (arrowhead) and opercular opening (arrow). B) Dorsal view of the slender egg of *L. attenuata attenuata* with much lower ribbon-net development and a flat operculum (arrow). C) Dorsal view of the very elongated capsule of *L. caprai*, showing, the micropylar area (asterisk), the raised operculum (arrow) and the developed polar mound at the opposite pole. D) Side view of *L. annaepaulae* eggs with the positive angle of the opercular opening (right) and the raised polar mound (left). E) anterior part of *L. attenuata attenuata* egg with small pin heads and an almost perpendicular operculum. F) The shallow operculum of *L. attenuata algarvica*. G) The dome-shaped one realized in *L. caprai* and *L. annaepaulae*. Scale bars: 1 mm in A-D and 0.1 mm in E-G. Legend: arrow, operculum; arrowhead, micropylar cup; asterisk, micropylar plate.

positive opercular angle; the value is intermediate in *L. montana* and variable within *L. attenuata*, where in the nominal subspecies it can be neutral or even become negative (Fig. 9B-E). On the whole, the series of egg data sets heavily blurs the picture resulting from the early description of *Leptynia* species, where the opercular angles appeared to constitute fully diagnostic traits (Scali, 1996). The fine chorionic pattern on both egg capsule and the lance-tip shaped micropylar area is similar in the new taxa and is made by a carpet of irregularly shaped pin-heads (about 2 μ m in diameter), with a superimposed irregular pattern of sparse ribbons. These attain a maximum development and density on the roundish operculum and the polar mound, particularly in *L. annaepaulae*, where they may take the appearance of projecting cristae as it also occurs in *L. caprai* (Fig. 9).

Detailed descriptions of the new taxa and their comparative diagnosis, are given in the Appendix (Tables 4-5).

Discussion

This paper provides the formal description of *Leptynia* species and subspecies embodied into the recently re-defined genus (Scali, 2009a) and also makes a comprehensive comparison among them by analyzing their taxonomical features.

Leptynia species' distinction chiefly resides on karyological findings and it is supported by parallel molecular data obtained from the mitochondrial *cox2* gene.

It has been suggested that the *Leptynia* speciation driving force has been the karyotypic differentiation entraining a certain amount of reproductive isolation and a subsequent genetic differentiation (Passamonti *et al.*, 2004; Ghiselli *et al.*, 2007). On the whole, these observations appear to support that the chromosomal changes occur ahead of genetic differentiation of taxa and these, in turn, precede their morphological characterization. As a matter of fact, nowadays the morphological characterization process rather provides an intricate mosaic of partially diagnostic traits: owing to these new findings even the cercus tooth and egg features, which appeared to be sharply diagnostic traits (Scali, 1996), actually realize a series of characters with faint boundaries, if the whole range of taxa has to be considered; at present, they just give hints to the right classification. As far as our records now tell, only the 10th:9th tergite ratio and the vomer traits of *L. an-*

naepaulae are unambiguously identifying the species against all other *Leptynia*. On the whole, *Leptynia* taxa seem to realize a condition of incipient morphological differentiation, whereas karyotype and genetic differentiations have been already built. It is in this kind of incomplete morphological information that multidisciplinary approaches to taxon definition appear of paramount utility.

The comprehensive phylogenetic relationships suggested by chromosomal and molecular data sets are congruent with the karyotype trend toward a numerical reduction of chromosomes and, at the same time, support the view that *L. annaepaulae* can be considered the outcome of an older cladogenetic event, while *L. caprai*, *L. montana* and *L. attenuata* could derive from the same genetic pool on a more recent time. From the *cox2* mutation pattern and phylogenetic reconstruction, *L. caprai* might be deemed as representative of the ancient gene pool from which *L. montana* and *L. attenuata* arose. Moreover, *L. caprai* should be considered as an assembly of populations sharing the same chromosome number, but different karyotype morphologies. This finding is supported by the observation that the Viso del Marquès specimens are karyologically similar to *L. annaepaulae* although genetically close to *L. caprai*; in addition the occurrence of clearly translocated chromosomes is supporting their inferred important role in *Leptynia* cladogenesis. In our opinion Viso del Marquès could be an incipient separate species, but, for the limited set of observations due to very small sample available, we prefer not to formally define the issue.

If we now integrate these assumptions with the actual distribution of taxa, we may suggest the following phylogeographic scenario for the genus: it is unanimously accepted that stick insect are tropical and subtropical in origin; therefore *Leptynia* ancestors should be originated in Africa, where also other *Leptynia* have been provisionally recorded (Bradley and Galil, 1977), and spread into Southern Spain through the Gibraltar Straits. In the Iberian Peninsula an ancestral taxon, with a numerically high karyotype (around 40 chromosomes) should have originated *L. annaepaulae*, restricted to the southern range of *Leptynia*. Later on, a northern colonization, paralleled by Robertsonian translocations and inversions, could have originated *L. caprai*, from which *L. montana* and *L. attenuata* arose. While molecular data sets clearly support *L. montana*, this is not clearly so for *L. attenuata*, notwithstanding that the shared rare XY male sex-formula appears to constitute a good

synapomorphy. For this reason, we cautiously take *L. attenuata* as a unique species, and the three groups as subspecies.

Karyotype repatterning, repeatedly invoked above as a triggering cladogenetic event, has been much questioned mainly in view of the theoretical difficulties in the fixation of chromosome rearrangements, responsible for a low fitness in the heterozygotes. In addition to the various hypotheses put forwards to overcome such limitation (see Riesenberger, 2001), we would like to recall here the one envisaging androgenesis as a preferential pathway to quickly making homozygous odd chromosome rearrangements, particularly in stick insects (Passamonti et al., 2004). As a matter of fact, the all-paternal nuclear inheritance of androgens allows the embryo to immediately duplicate the heterozygous chromosome novelties, as it has been first witnessed in *Bacillus* and, of recent, in *Clonopsis* stick insects (Mantovani and Scali, 1992; Tinti and Scali, 1996; Milani et al., 2009, 2010; Scali, 2009b; Scali and Milani, 2009). Androgenesis is also shared by the whole fresh-water bivalve genus *Corbicula* (Komaru et al., 1998; Byrne et al., 2000; Qiu et al., 2001) and even by the Saharan cypress *Cupressus dupreziana* (Pichot et al., 2001). Actually, the peculiar cytological features of androgenesis appear to easily overcome the major genetic and evolutionary constraints believed to be entrained by the heterozygous condition of freshly repatterned karyotypes.

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Appendix

Systematics of the genus Leptynia and descriptions of new taxa

All taxa of the genus have a slender body, typical of most stick insects. They are also characterized by the absence of wings, have only compound eyes without *ocelli* and show a prognathous head with a well developed *gula*. The comprehensive cladistic phylogenetic analysis of Phasmatodea by Bradler (2009) fully supports their monophyletic status, but also deeply upsets the traditionally accepted definition and composition of taxa as defined by Bradley and Galil (1977), because most of the groupings turned out to be polyphyletic. However, since the Bradler's proposal to transfer most Families to a new single Family 'Neophasmatidae' is controversial (see Brock, 2011; Zompro, 2004), we here maintain the traditional taxonomy of Phasmatodea (Bradley and Galil, 1977), and the formal systematic of *Leptynia* is summarized accordingly.

Systematic account

Family Diapheromeridae Kirby, 1904
 Subfamily Pachymorphinae Brunner, 1893
 Tribe Gratiidiini Gunther, 1953
 Genus *Leptynia* Pantel, 1890

The family Diapheromeridae embodies anareolate stick insects, namely lacking of *area apicalis* (see Introduction), with distinctly segmented antennae. Insects of the subfamily Pachymorphinae have articulated antennae shorter than fore femora, as well as smooth ventral edges of hind femora. To tribe Gratiidiini belong stick insects with the anal segment truncate or slightly emarginated in the males, while variable in the females. Their 2nd abdominal segment is at least 2x longer than wide. Middle and hind femora are unarmed (or rarely spinulose); cerci may be elongated but never lobed. Their distribution is very wide being found in Africa, Madagascar, Central Asia, China and Southern Europe. The genus *Leptynia* is an Iberian genus characterized by male antennae with a larger than long *scapus*. From the originally described genus, the Spanish taxa whose males lack the subanal vomer and females show a compressed abdomen tip (see Introduction) have been separated and put in the new genus *Pijnackeria* (Scali, 2009a). On the other hand, the reshaped *Leptynia* encompasses only diploid sexual species, with a well developed subanal

vomer in the males and a soft, tapered, abdominal tip in the females.

Updated definition of specific and infraspecific taxa

Leptynia attenuata Pantel, 1890, subdivided into 3 subspecies, namely: *Leptynia attenuata attenuata* subsp. n., Pantel, 1890; *Leptynia attenuata iberica* subsp. n., Scali, Milani, Passamonti, 2012; *Leptynia attenuata algarvica* subsp. n., Scali, Milani, Passamonti, 2012.

Leptynia caprai Scali, 1996;

Leptynia montana Scali, 1996;

Leptynia annaepaulae sp. n., Scali, Milani, Passamonti, 2012.

All holotypes and the paratype have been deposited in the Museo dell'Evoluzione of Bologna University.

Body colouration of *L. attenuata* subspecies and of *L. annaepaulae* is much the same as that of the already known co-generic species (*L. montana* and *L. caprai*), for both colour shadows and pattern (Figs 5-6). Males are painted by brown stripes on the head vertex and genae; a median patch is normally found on the pronotum and deep brown stripes run along the thoracic and abdominal tergites, paralleled by a sharp white line above the pleura. Beside the brown coloured areas a light canella ground colour is found; the ground canella colour of males assumes greenish nuances on both dorsal and ventral areas, so that the lighter areas give the males an overall brown and light-green shade. Females are uniformly canella or bright-green all over the body, but genae and pleurae show a brilliant white stripe along both thoracic and abdominal segments (Fig. 6).

On the mesonotum, metanotum and last abdominal tergites of *L. attenuata* specimens of both sexes a faint median carina is generally present, although the thoracic carina may fade out in *L. attenuata iberica* and *L. attenuata algarvica*; *L. annaepaulae* lacks median carina in males; only the 10th abdominal tergite may show it. The rim of the anal tergite of males is variably incised and appears to follow subspecific trends: the notching is generally shallow in *L. annaepaulae* and *L. attenuata* subspecies, excepting *L. attenuata iberica*, whose 10th tergite incision is somewhat deeper; the shallow type of emargination closely reminds that of *L. montana*, whereas the second one, that of *L. caprai*. In females, beyond the round, often lightly notched

posterior rim of the 10th tergite, the supra-anal valve can be perceived.

Excepting legs and cerci, males and females of *L. annaepaulae* and *L. attenuata* subspecies are uniformly covered by very minute hemispheres (about 1 μ m). Males are glabrous excepting for cerci and last abdominal tergites, while females show diffuse short hairs, which become denser and black on the abdomen tip (Fig. 8G).

A peculiar trait shared by *Leptynia* females is a series of regularly spaced teeth (likely sensorial organs) along the lateral border of the meso- and meta-notum, just above the pleura, on each body side. This thoracic crenation had not been reported in the already described *Leptynia* taxa.

Males of *L. annaepaulae* and all *L. attenuata* subspecies show muticous fore legs, whereas the mid and hind pairs may develop few (1-5) very small teeth; in females all leg pairs may similarly show few small teeth; this condition also occurs in the already described species of the genus. The shape and size of the subgenital operculum do not reveal taxon specific characters, always being dome shaped with a roundish, lightly undulated posterior rim just approaching the 9th/10th sternite articulation (Fig. 8A, C, E).

All *Leptynia* females share a very similar general morphology, particularly for their short antennae and leg segments. Also female *terminalia*, are very uniform throughout the genus, since, in addition to the gently tapered and soft tip, with straight protruding cerci, are also invariably characterized by the inferior ovipositor valve with a narrowing distal third, only approaching the 9th/10th sternite articulation, and allowing the 1st and 2nd valve pairs to be seen (Fig. 8G). At any rate, all these features sharply differ from those of the *Pijnackeria* female *terminalia*, that are characterized by a paddle-shaped subgenital valve tip, ending beyond the 9th/10th sternite boundary and completely concealing the other ovipositor valves. Also, in *Pijnackeria* females cerci are almost completely hidden by the pointed abdomen tip (Scali, 2009a).

Leptynia attenuata attenuata, Pantel 1890, new rank

The subspecies embodies specimens collected primarily in the *patria typica* of São Fiel (Castelo Branco, Portugal) as indicated by Pantel (1890) in his seminal paper, as well as samples collected in nearby locations (see map, Fig. 1; Table 1), shown to cluster together by molecular analyses (Passamonti *et al.*, 2004). Its definition resides on the genetic distances and the likely phy-

letic relationships, based on the mitochondrial *cox2* gene (Fig. 2) and allozyme data, respectively. Its karyotype obviously fits in *L. attenuata* for number, general structure and sex-chromosome formula ($2n = 36, XX/XY$; Fig. 3C); its fine structural differentiation from the karyotype of the two new subspecies is not a sound one.

From size ranges of both males and females it can be seen that *L. attenuata attenuata* specimens are among the largest of the species.

Male *terminalia* present two traits worth mentioning: the swollen base of the subanal vomer and its common peculiar narrowings subdividing it into three-four parts; however, these traits are not subspecific diagnostic features being also shared by the other two:

- An apparently sounder characterizing trait is given by the small tooth projecting from the base of the cercus arm: the tooth (about 0.3 mm long) is here slender, almost cylindrical with a blunt apex (Fig. 8B).

- No peculiar features have been observed in the females; however, the subspecies appears to be characterized by the lowest egg operculum angle recorded among *Leptynia* taxa (Table 2), its egg capsule being cut almost perpendicularly by the opercular opening (Fig. 9E).

- Owing to the above described morphological traits, we choose a male as reference specimen from Portalegre, whose main parameters are recorded in Table 4. From the direct inspection of the reference male we can see that its left foreleg is missing and that its general colouration is deep brown. The compound, hemispherical eyes are uniformly tinged by a deep brown pigment; beyond the eye the brown pigment draws two stripes on each side of the head, the higher being larger. From the head vertex a narrow median brown line starts to continue also on the thoracic and abdominal tergites. On each side the median line is encircled by a brown patch on the pronotum and paralleled by a thick brown stripe along the thoracic and abdominal tergites. The whole ventral side and the dorsal areas free from the brown pigment, are of a light canella colour.

- As in most *L. attenuata* specimens a subtle but neat median carina is noticeable on the meso- and meta-notum; a similar carina is also found on the anal, 10th, abdominal tergite, which, as mentioned above, even shows a wide, shallow incision (Fig. 8B). The ventral side of the male *terminalia* bears the most characteristic traits of the subspecies, since the subanal vomer possesses a characteristic swollen base and the cerci a typical slender tooth of appropriate size for the subspecies (Fig. 8A-B).

Derivatio nominis. The indication of the subspecies directly derives from the nomenclature rules, because its populations just occupy the area from which the species *L. attenuata* was first described by Pantel (1890).

Leptynia attenuata iberica subsp. n.

The specimens of this taxon come from both the northern Portuguese areas of Guarda (Serra da Estrê-la) and the Spanish locations on the Sierra de Peña de Francia (see map, Fig. 1; Table 1). Its definition resides on the genetic distances and the likely phyletic relationships based on the mitochondrial *cox2* gene and allozyme data, respectively (see also Passamonti *et al.*, 2004). Its karyotype too fits in the *L. attenuata* for number, structure and sex-chromosome formula ($2n = 36$, XX/XY) (Passamonti *et al.*, 1999); its structural differentiation is too weak to be diagnostic.

Both male and female size is, on average, just smaller than those of *L. attenuata attenuata*, but larger than that of *L. attenuata algarvica*. Its morphological characterization is widely incomplete being actually limited to the shape of the subanal vomer base and cercus tooth.

From the direct inspection of the holotype and making reference to Table 4, we can observe that its colouration is mainly brown on the tergites and cinnamon on the sternites, while the eye has a blackish tinge, deeper in the posterior half. The colour distribution pattern is the one shared by most *L. attenuata* specimens, but the two retroocular stripes on the genae run very close each other and the two dorsal stripes are so wide to fuse on the median line over the whole body length; as usual, each brown stripe is externally accompanied by a white narrow line. As already pointed out for the other *Leptynia* taxa, the meso- and meta-notum bear a faint median carina, which is also found on the anal tergite.

Derivatio nominis. The subspecies is up to now the only one found to spread in both Portuguese (Guarda area) and Spanish (Peña de Francia and, further north, west of Villablino) locations in the central Iberian peninsula; this geographical feature has suggested us the denomination.

Leptynia attenuata algarvica subsp. n.

The third subspecies has been found in an isolated narrow area of Southern Portugal (Algarve region) subdivided into small populations on the Serra de Mon-

chique, on the rare patches of surviving wild bush vegetation. This subspecies had been referred as the nominal species *Leptynia attenuata* (Scali, 1996) before the re-discovery of *Leptynia* samples in the *patria typica* of São Fiel (Castelo Branco). Later on the southern Monchique populations turned out to be genetically well differentiated from the nominal ones, and therefore had to be treated as a separate taxon. A general characterizing trait of the latter is the very small size of males with comparative short hind femurs, although with relatively long antennae; correspondingly also the females are the smallest (Tables 2-3). The vomer shape with its swollen base and the long stout cercus tooth also appear characterizing traits (Fig. 8C-D). Furthermore, a limited series of crosses (3) of this subspecies with the nominal one, gave a suggestion of a low fecundity (21% egg hatching), with even a hint of sex-ratio unbalance (7 males, no females) (unpublished data).

From the male holotype inspection it can be seen that the brown colouration in addition to the lateral parts of the tergites also marks the median line along the whole body; on the thorax the usually separate and parallel running stripes are so wide to actually fuse each other on the median line. In this subspecies some males may present an uninterrupted brown median line on the sternites of the whole body, as it occurs in the holotype, while in others only short brown segments can be observed.

Derivatio nominis. Owing to its very limited range, apparently restricted to the Algarve region, we thought that this geographic feature would be adequate for the taxon.

Leptynia annaepaulae sp. n. (Figs 5-6)

The new species has been localized in the Ronda district (Sierra de Grazalema), where it colonizes small patches of shrubs and grasses (*Genista*, *Cystus*, *Rubus*). This is a genetically and chromosomally well differentiated taxon (Figs 2-4), long waiting its formal description (Bianchi, 1992; Bianchi and Meliado, 1998; Passamonti *et al.*, 2004).

While males are very much alike those of the remaining taxa for pigmentation shadows and pattern (Fig. 5), females show a peculiar light-pink nuance (Fig. 6). A morphological distinguishing trait is the very short 10th, anal tergite of males which exhibits the lowest ratio value versus the 9th one (Table 2). On the other hand, the 10th abdominal segment of females does not appear to be shorter than that of the co-generic species, since the same range of ratio values have

been obtained. Additional male traits of systematic value, although tiny ones, are the flat, wide vomer base (Fig. 8E) followed by a decidedly narrower subsequent trait, and the short, acute cercus tooth: these traits, as far as we can judge, tell apart *L. annaepaulae* males from those of the remaining known taxa. In addition to the pink shadow, no additional morphological distinctions have been observed in females; even ootaxonomical characters have turned out not fully diagnostic, although rather well defined: *L. annaepaulae* lays long eggs with a high, positive opercular angle and ensuing low width:length ratios, but these same traits are also shared by *L. caprai* (Table 2). Therefore this species too appears to be a mix of more or less complete diagnostic features.

Holotype and paratype description. The body size of the male holotype is on the small side of the species range and has an overall striped appearance due to the sharply brown dorsal areas contrasting with the lateral white narrow lines and the cinnamon colour of the ventral body side. The brown colouration follows the generic pattern, with the two dorsal stripes just wider than usual to almost fuse each other on the median line on both the thorax and the abdomen. Eyes are light brown and almost pigment-less in their anterior half. The left hind femur presents a minute thorn, whereas all other leg segments are smooth. The sub-genital operculum has standard shape and size; its posterior rim is gently notched. The subanal vomer clearly shows the specific flat base, decidedly wider than the caudal part of it. Also the branching cercus tooth, covered with black hairs, has a fairly typical short and pointed appearance. The notching of the last tergite - the only one provided with a faint median carena - is shallow and narrow, affecting just one third of the tergite border.

The female paratype is average for body size and, at present, appears of a uniform light canella colour, since its native pink shadow has faded out in ethanol (compare to Fig. 6); limited light-brown areas can be perceived on the genae, pronotum and 2nd abdominal

tergite. The serrated series of lateral thoracic teeth is conspicuous; all legs are muticous. We here like to remind the main *terminalia* features of females, such as the ovipositor ventral valve tip - covered with black hairs - just shorter than the 1st and 2nd valve pairs, the soft last tergite uncovering the supra-anal valve and the straight protruding cerci (refer to Fig. 8G). Both holotype and paratype are housed in the Museo dell'Evoluzione of Bologna University, with collection numbers Gra096 and Gra092, respectively.

Derivatio nominis. The species is dedicated to the distinguished Italian student of circum-Mediterranean stick-insects Anna Paola Bianchi, of Rome University La Sapienza, who first pointed out the occurrence of this southern Spanish *Leptynia* taxon with the same chromosome number as *L. caprai*, but with a clearly differing karyotype structure for some large pairs of the set (Bianchi, 1992).

We now try summarizing the more or less complete diagnoses made possible by the here analyzed characters (Table 5). It can be seen that: in the males the ratio of abdominal tergite 10th vs the 9th is fully diagnostic for *L. annaepaulae* as compared to all species and subspecies, while in *L. attenuata* it is diagnostic against the other species but not between *L. attenuata* subspecies. Among the latter, *L. attenuata algarvica* appears to be the most characterized, since its length of metanotum and median or hind femur are diagnostic vs most of the other taxa (Table 5). On the other hand, the reach of the female hind femur is fully diagnostic for *L. attenuata algarvica* and almost completely so when each other taxon is considered. Egg parameters are very similar in *L. annaepaulae* and *L. caprai*, but fully diagnostic when the two are compared to all other taxa.

On the whole, it could be concluded that taking into account the whole series of the here reported qualitative and quantitative traits (in addition to karyotype) a reasonably safe identification of *Leptynia* taxa can be reached.

Table 4. Holotype and paratype parameters and characterizing features of *Leptynia attenuata* subspecies and *L. annaepaulae* sp. n. Body length (in mm) does not include antennae and cerci. * = reference male of our collection; h = holotype, male; p = paratype, male for *L. attenuata attenuata*, female for *L. annaepaulae*; R = right; L = left. In the ‘hind femur reach’ column the ordinal refer to abdominal tergites.

Taxon	Body length	Antennal articles	Hind femur reach	Vomer base	Cercus tooth features	Body colour
<i>L. a. attenuata</i> (*)	43.5	R15-15L	7 th + 1/2	swollen	slender, medium	brown/light canella
<i>L. a. iberica</i> (h)	44.0	R16-16L	8 th + 1/3	intermediate	wide base, short	brown/light canella
<i>L. a. algarvica</i> (h)	40.0	R16-16L	6 th + 1/2	swollen	stout, long	brown/light canella
<i>L. annaepaulae</i> (h)	42.0	R16-16L	7 th + 3/4	flat	slender, pointed, short	dark brown/light canella
<i>L. annaepaulae</i> (p)	56.0	R15-15L	6 th + 1/2	-	-	light pink/dark canella

Table 5. Diagnostic value of analyzed metric traits of *Leptynia*. The bold character indicates the compared taxon. + = diagnostic trait; o = not diagnostic trait.

Taxon		Chromosome structure		Body length		Mesonotum length		Metanotum length		Ratio 10 th :9 th abdominal terg.		Med. femur length		Hind femur length		Hind femur reach		Egg length		Egg width:length ratio	
		M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F				
<i>L. annaepaulae</i>	<i>L. caprai</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	o	+	o	o	o	
	<i>L. montana</i>	+	+	o	o	+	o	o	o	+	o	o	o	o	o	o	+	+	+	+	
	<i>L. a. attenuata</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	o	+	+	+	+	
	<i>L. a. iberica</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	o	o	o	+	+	
	<i>L. a. algarvica</i>	+	+	+	o	+	+	+	+	+	o	+	+	+	+	o	+	+	+	+	
<i>L. caprai</i>	<i>L. annaepaulae</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	+	+	o	o	o	
	<i>L. montana</i>	+	+	o	o	o	o	o	o	o	o	+	o	o	o	o	o	+	+	+	
	<i>L. a. attenuata</i>	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	
	<i>L. a. iberica</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	o	o	+	+	+	
	<i>L. a. algarvica</i>	+	+	o	o	o	+	+	+	o	o	+	o	+	+	+	+	+	+	+	
<i>L. montana</i>	<i>L. annaepaulae</i>	+	+	o	o	+	o	o	o	+	o	+	o	o	o	+	+	+	+	+	
	<i>L. caprai</i>	+	+	o	o	o	o	o	o	o	o	+	o	o	o	o	o	+	+	+	
	<i>L. a. attenuata</i>	+	+	o	o	+	o	o	+	o	o	o	o	o	o	o	+	+	o	o	
	<i>L. a. iberica</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	o	+	+	o	o	
	<i>L. a. algarvica</i>	+	+	o	o	o	o	o	o	o	o	+	o	o	o	o	+	+	+	+	
<i>L. a. attenuata</i>	<i>L. annaepaulae</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	+	+	+	+	+	
	<i>L. caprai</i>	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	+	
	<i>L. montana</i>	+	+	o	o	+	+	o	+	o	o	o	o	o	o	o	+	+	o	o	
	<i>L. a. iberica</i>	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	o	o	
	<i>L. a. algarvica</i>	o	o	+	o	+	+	+	+	o	o	+	+	+	+	o	+	+	o	o	
<i>L. a. iberica</i>	<i>L. annaepaulae</i>	+	+	o	o	o	o	o	o	+	o	o	o	o	o	+	o	+	+	+	
	<i>L. caprai</i>	+	+	o	o	o	o	o	o	+	o	+	o	o	o	o	+	+	+	+	
	<i>L. montana</i>	+	+	o	o	o	o	o	o	+	o	+	o	o	o	o	+	+	o	o	
	<i>L. a. attenuata</i>	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	
	<i>L. a. algarvica</i>	o	o	o	o	o	+	o	+	o	o	+	o	o	o	o	+	+	o	o	
<i>L. a. algarvica</i>	<i>L. annaepaulae</i>	+	+	+	o	+	+	+	+	+	o	+	+	+	+	o	+	+	+	+	
	<i>L. caprai</i>	+	+	o	o	o	+	+	+	o	o	+	+	+	+	o	+	+	+	+	
	<i>L. montana</i>	+	+	o	o	o	o	o	o	o	o	o	o	o	o	o	+	+	+	+	
	<i>L. a. attenuata</i>	o	o	+	o	+	+	+	+	o	o	+	+	+	+	o	+	+	o	o	
	<i>L. a. iberica</i>	o	o	o	o	o	+	o	+	o	o	+	o	o	o	o	+	+	o	o	