CARYOLOGIA Vol. 51, n. 3-4: 207-219,1998

Analysis of the karyotypes of four species of the *Leptynia attenuata* complex (Insecta Phasmatodea)

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To Michael White.

SUMMARY - Karyotypes of several bisexual populations of the *Leptynia attenuata* complex were analysed. This complex includes four karyotypically differentiated taxa: *Leptymtl attenuata* s.str., *Leptymtl montana*, *Leptynia caprai* and *Leptynia* sp. The chromosome numbers of the four species are: *2n=36* (♂ XY; ♀ XX) for *Leptynia attenuata*, *2n=38* (♂ X0; ♀ XX) for *Leptynia montana*, *2n=40* (♂ X0; ♀ XX) for *Leptymtl caprai* and *2n=40* (♂ X0; ♀ XX) for *Leptynia* sp. The evolutionary relationships between the four species are discussed

INTRODUCTION

Two species were known in the genus *Leptynia:* L. *hispanica* Bolivar and L. *attenuata* Pantel with similar geographic distribution (Portugal, Central and Northern Spain and France). It was believed that L. *attenuata* reproduced by amphigony with a normal sex ratio, while L. *hispanica* reproduced exclusively by telytokous parthenogenesis. DE SINETY (1901) observed a chromosome number of *2n=36* (35 in the male) in L. *attenuata*, while CAPPE DE BAILLON and DE VICHET (1940) found 56 chromosomes in L. *hispanica* and suggested its triploid constituction.

Researches carried out by means of the electrophoretic techniques for the detection of gene-enzyme systems (NASCETTI *et at.* 1983) have shown - on the basis of the analysis of 20 enzymatic loci - that L. *hispanica* and L. *attenuata* are actually two complexes of species differentiated in their morphology, chromosomes and isozymes.

The L. *hispanica* complex includes bisexual and telytokous taxa; these latter originated by hybridization, because they show fixed heterozygosity in eight enzymatic loci; one of the alleles in heterozygosity is that characteristic of the bisexual taxa (BULLINI and NASCETTI 1987). The L. *attenuata* complex includes several amphigonic taxa. Recently, SCALI (1996) described, on the basis of patterns of enzymatic loci, chromosomal and morphological studies, three species: *Leptynia attenuata* s.str., L. *montana* and L. *caprai.*

We found a fourth karyotypic ally differentiated taxon, temporarily named *Leptym"a* sp.
BIANCHI (1992) examined karyotypes *of Leptynia hispanica* and L. *attenuata* and she pointed out that:
1. in the first complex occur amphigonic taxa with *2n=38,* triploid telytokous taxa with *3n=57* and tetraploid telytokous taxa with *4n=76,*.
2. in the second complex two different cytotypes occur: one, from Monchique (Portugal) with *2n=36* and sex chromosome mechanism XY in the male, the other, from Escorial (Spain) with *2n=38* and sex chromosome mechanism XO in the male.
In that paper, however, there are two mistakes: the first is only a misprint: the same locality, Zarzalejo, is attributed at Spain and at Portugal respectively, in the same table. Zarzalejo, obviously, is in Spain. The second one is heavier: the same unlucky population was described as a parthenogenetic tetraploid taxon of the L. *attenuata* complex, because a switch of signs. Actually, this taxon belongs to the L. *hispanica* complex; in the L. *attenuata* complex are not reported parthenogenetic taxa up to now.
In this paper the karyotypes of *Leptynia attenuata* Pantel, 1890, *Leptynia montana* Scali 1996, *Leptynia caprai* Scali, 1996 and *Leptynia* sp. are described and compared between them; the evolutionary events involved in their speciation are discussed.

MATERIALS AND METHODS

T able 1 shows the list of species and populations collected and examined from 1992 to 1997; their distribution is in Figure 1.
The cytogenetic studies was performed on mitotic metaphases obtained from epithelium of ovarioles for the females and from testicular tissues for the males. The tissues were extracted from the adult animals in insect saline solution, then put in 0.1 % colchicine solution for 90'. After hypotonic treatment (insect saline:distillated water, 1:1, for 20') they were fixed with a 3:1 mixture of methanol and acetic acid for 3-4 hours at room temperature or for 12 hours at *4°C.* Finally, the tissues were put in a drop of 60% acetic acid on a very clean slide which was dried rapidly, moving it, on a hot plate *(40-50"C).* The slides were stained with Giemsa (10%; pH 6.8, for 10 min).
Nomenclature and centromeric index (i) adopted by LEVAN *et at.* (1964) was followed for recognizing chromosome types.

RESULTS

Leptynia attenuata.

The only population of Monchique (Portugal) was examined. The chromosome number is *2n=36* in both sexes because the sex chromosome mecha-

TABLE 1 - List of species and populations examined.

Species	Populations	Geographic origin
Leptynia attenuata s.str.	Monchique	Portugal
Leptynia montana	Arenas	Spain
	Cercedilla	Spain
	Cuevas del Valle	Spain
	Escorial	Spain
	Navarredonda	Spain
	Puerto de Las Pilas	Spain
Leptynia caprai	Cala	Spain
	Cedena	Spain
	El Molinillo	Spain
	Monesterio	Spain
	Puerto de Las Pilas	Spain
	Puerto Ellano	Spain
	Urda	Spain
	Viso del Marques	Spain
Leptynia sp.	Ojen	Spain
	Ubrique	Spain

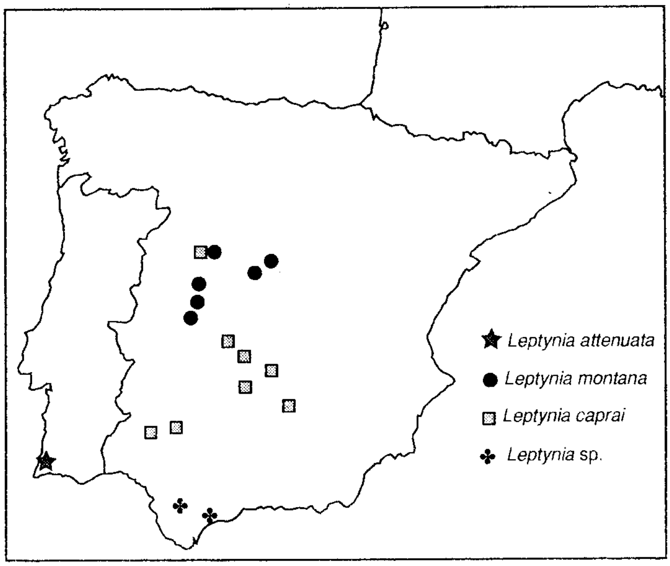

Fig. 1. - Map of Iberian peninsula showing the distribution of the four species of the *Leptynia attenuata* complex.

nism is XY (<3') : XX (2) . The X chromosome is the largest of the all set, metacentric with i=42, while the y chromosome is smaller, submetacentric with i=37 (Fig. 2). The 17 autosomic pairs are:
- four pairs of large size whose one metacentric (2) and three subtelocentric (3,4,5); pair 3 has a satellite on the short arm;
- nine pairs of medium size whose one metacentric (13), the other ones subtelocentric or telocentric;
- four pairs of small size whose two metacentric (16 and 18) and the other ones subtelocentric or telocentric.

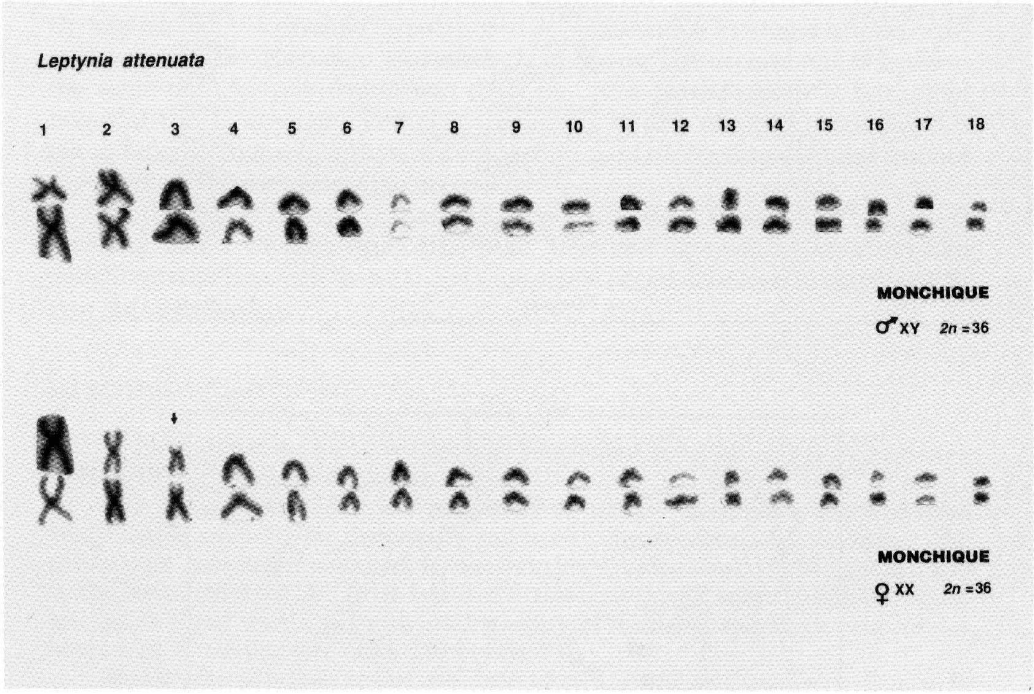

Fig. 2. - *Leptynia attenuata* s.str.: male and female karyotypes, Giemsa staining. The arrow indicates satellites in heterozygosity on pair 3.

Leptynia montana.

Six population of this species have been analysed cytologycally (T able 1) . They have *2n=37* in the male and *2n=38* in the female, with sex chromosome mechanism XO (<3') : XX (2) . The X chromosome is the second in size of the complement, submetacentric with i=35.

The karyotype of Fig. 3 (Cuevas del Valle population) includes:
- five pairs of large size: one metacentric (1), one submetacentric (2, i.e. X chromosome) and three subtelocentric (3,4,5); pair 5 has a satellite on the short arm; this characteristic occurs in all specimens of the six population;
- nine pairs of medium size, subtelocentric (from 6 to 14) ;
- five pairs of small size: two metacentric (16, 19) and three subtelocentric.

Five specimens of Las Pilas population were collected on the same shrub, three belonging - on the basis of karyotypes - to *Leptynia montana* and two to *Leptynia caprai.*

Leptym"a caprai.

In the eight population examined a constant number of 39 chromosomes in the male (40 in the female) was found. The X chromosome is the largest in size, subtelocentric with i=18.
The karyotype (Fig. 4: Puerto Ellano population) consists of:
- five pairs of large size, subtelocentric (from 1 to 5);
- nine pairs of medium size: one metacentric (12), one submetacentric (13), the other subtelocentric (6,7 ,8, 9, 10, 11 and 14);
- six pairs of small size: two metacentric (16, 19) and four subtelocentric (15, 17, 18 and 20).

The presence of satellites on the short arm of pair 17 is constant in all populations.
At Viso del Marques three specimens (two females and a male) were L. *caprai* and two males were *Leptym"a hispanica;* likely, in this locality a population of L. *caprai* and a bisexual population of L. *hispanica* coexist.

Leptynia sp.

Two populations, from Southern Spain, were examined. The chromosome number is 39 in the male and 40 in the female.
The X chromosome is the largest, subtelocentric with i=21 and presents satellites on the short arms. The karyotype (Fig. 5: Ubrique population) consists of:
- five pairs of large size, two metacentric (2, 3) and three subtelocentric (1,4,5);
- nine pairs of medium size, two metacentric (9, 14), the other ones subtelocentric (6,7 ,8, 10, 11, 12, and 13);
- six pairs of small size, three metacentric (16, 17, 18) and three subtelocentric (15,19,20).

Nevertheless the same chromosome number, it is a strong differentiation in chromosome morphology between L. *caprai* and *Leptym"a* sp.

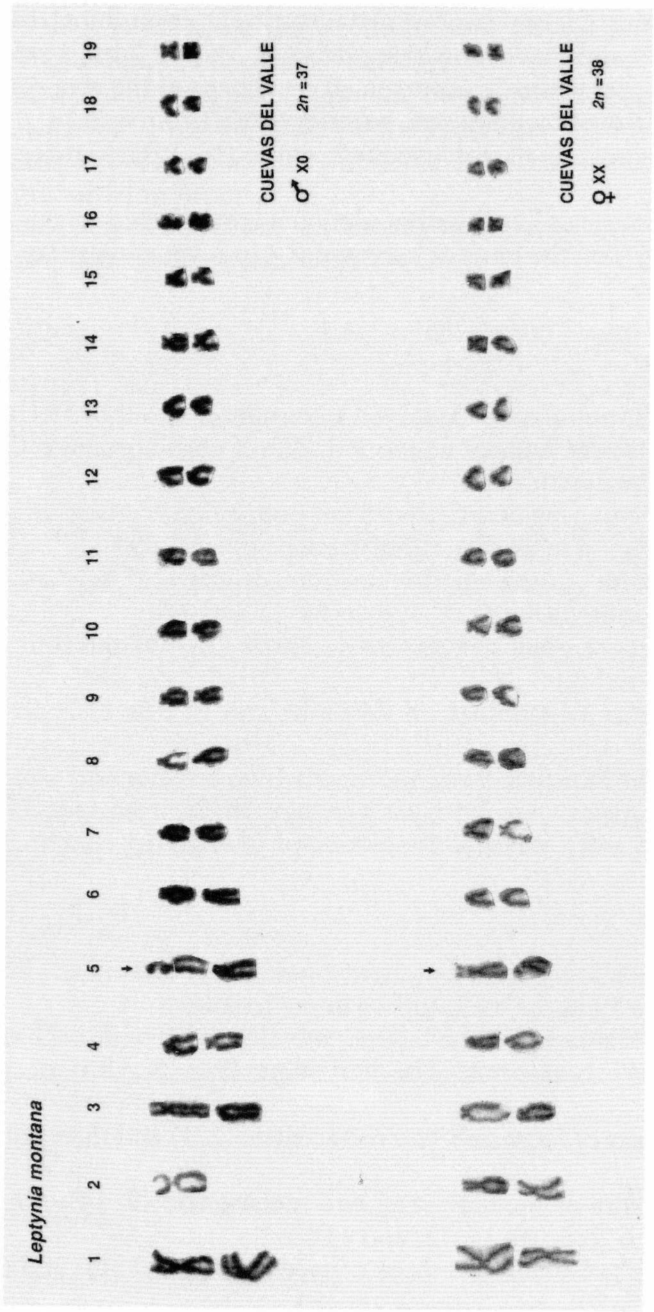

Fig. 3. — *Leptynia montana*: male and female karyotypes, Giemsa staining. The arrow indicates satellites in heterozygosity on pair 5.

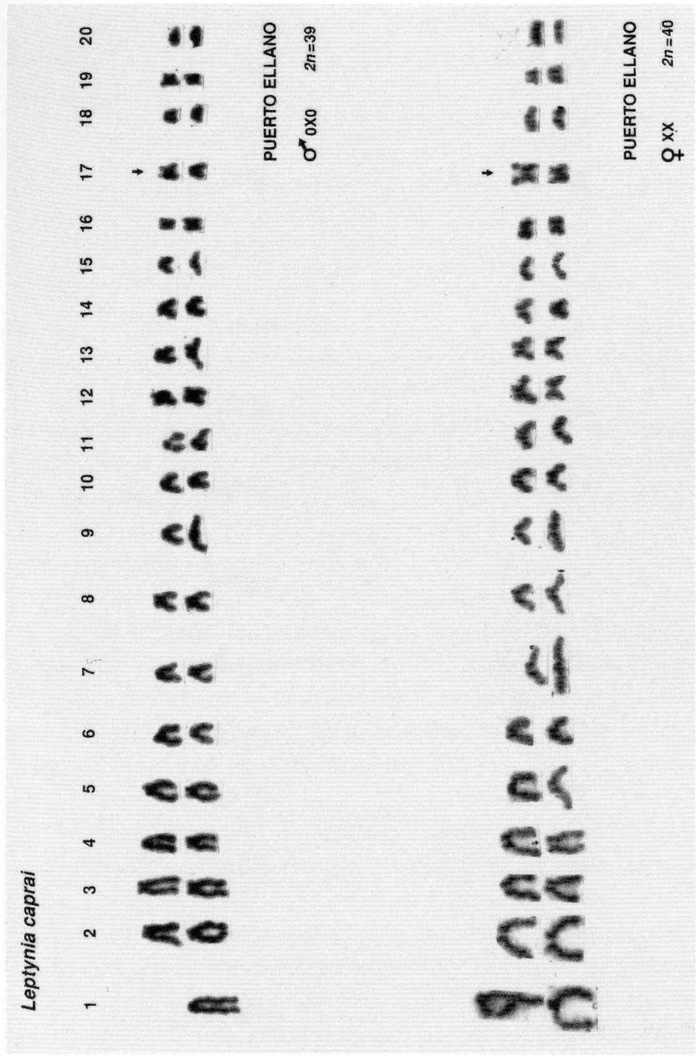

Fig. 4. — *Leptynia caprai*: male and female karyotypes, Giemsa staining. The arrow indicates satellites in heterozygosity on pair 17.

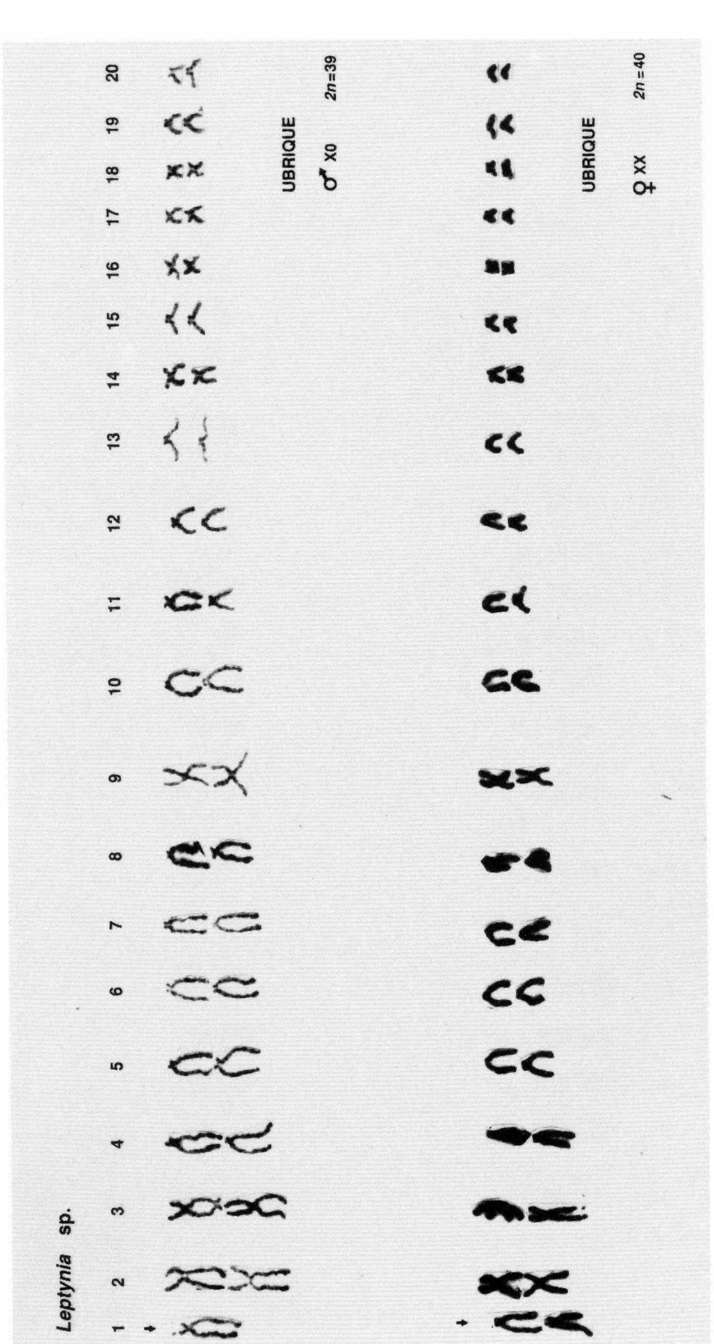

Fig. 5. — *Leptynia* sp.: male and female karyotypes, Giemsa staining. The arrow indicates satellites in heterozygosity on pair 1. The homozygosity is evident only in the female, because pair 1 represent the X chromosome.

DISCUSSION

The karyotypes analysis shows a first interesting point: the remarkable chromosomal differentiation between the four taxa; they differ for chromosome number, chromosome morphology and fundamental number (Fig. 6; T able 3) .
The karyotypical differentiation is furthermore supported by the very different morphology and size of X chromosome in the fourtaxa. These data are summarized in Table 2.

TABLE 2

Species	Chromos. number (2*n*)	Morphology and position of X chromosome
Leptynia attenuata	36	M (i=42) 1st position
Leptynia montana	38	sm (i=35) 2nd position
Leptynia caprai	40	st (i=18) 1st position
Leptynia sp.	40	st (i=21) 1st position

These data are unusual, because normally the X chromosome, in groups of related species, is the same or very similar. Moreover, in *Leptynia attenuata* the sex chromosome mechanism is XY(<3'): XX(2), as result of a X-autosome fusion (neo- XY); indeed the sex chromosome mechanism in the other taxa of the *Leptynia attenuata* complex is Xo (<3') : XX (2) .
It is likely that the ancestral X chromosome should be subtelocentric (as in *Leptynia capraiand* in *Leptynia* sp.). So, according to WHITE (1978) the most probable sequence of events for the neo-XY formation is:
- the subtelocentric X chromosome of L. *caprai* undergoes a fusion to a sub metacentric or a subtelocentric autosome of medium or small size leading thus to a metacentric neo-X chromosome (this is, indeed, the largest one in L. *attenuata);*
- the unfused autosome, confined to the male line, plays the role of the y chromosome and undergoes some structural change, becoming a true y chromosome.
Furthermore, another point is the presence of satellited chromosomes, characteristic for each species, namely: Aps **III** in L. *attenuata,* Aps V in L. *montana,* Aps XVII in L. *caprai* and Aps **I** in *Leptynia* sp. (where A is for acrocentric, p is for the short arm of the chromosome, s is for satellite and the roman number for its position in the karyotype). These satellited chromosomes can be used as "natural morphological markers": in the *Leptynia hispanica* complex also a number of chromosomal markers occur; they are constantly present in the amphigonic diploid populations and in parthenogenetic triploid and tetraploid populations (MELIADO and BIANCHI 1998).

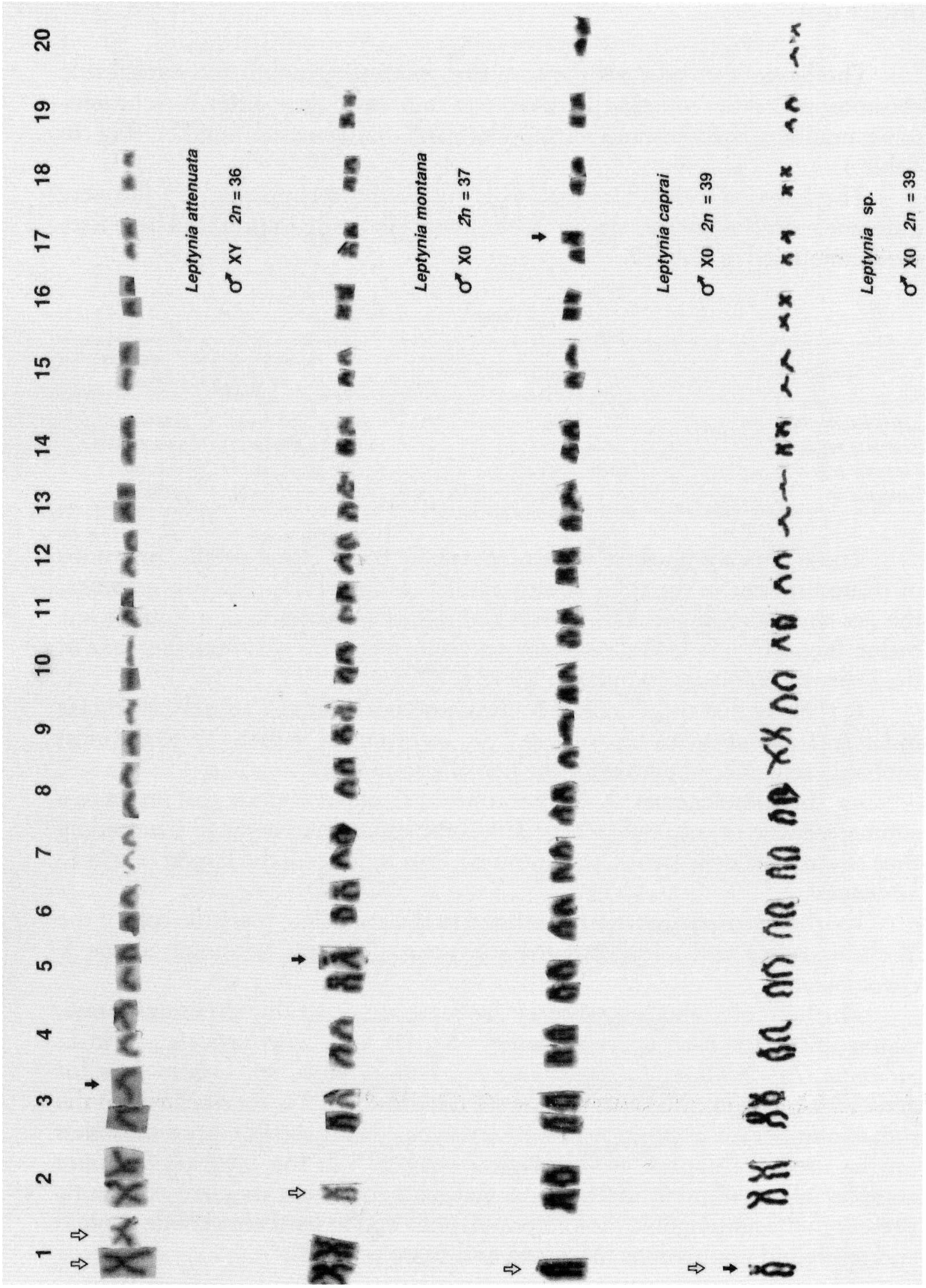

Fig. 6. — Male karyotypes of the four species of the *Leptynia attenuata* complex. Black and white arrows mark satellited chromosomes and X and Y chromosomes respectively.

The suspicion that the taxa of the L. *attenuata* complex are simply "chromosomal races" as in *Didymuria violescens* (CRADDOCK 1970,1974,1975) has been discarded because the laboratory crosses between individuals of different taxa pointed out the existence of post-zygotic reproductive isolating mechanisms of various degrees, from hybrid inviability to hybrid sterility (BIANCHI, in preparation) .

Since the reproductive isolation in the form of post-zygotic RIMs is characteristic of the earlier stages of speciation, it is possible to presume that the taxa of the *Leptynia attenuata* complex are " good species " in the first stage of speciation, or "incipient species".

The available data are yet poor, but we can try to identify some steps of karyotypical evolution in these species. T able 3 is a shema where chromosomal morphology, chromosome numbers, metacentric/acrocentric ratio and fundamental number (n.f.) of the four species are indicated. Then, it is known that in

TABLE 3 - Karyotype morphology of the species of the *Leptynia attenuata* complex examined.

	Leptynia attenuata Monchique $2n=36$		*Leptynia montana* Cuevas del Valle $2n=38$		*Leptynia caprai* P.to Ellano $2n=40$		*Leptynia* sp. Ubrique $2n=40$	
	♂ XY	♀ XX	♂ X0	♀ XX	♂ X0	♀ XX	♂ X0	♀ XX
1	M_{42}	SM_{37}	M	M	A_{18}	0	$A_{ps\ 21}$	0
2	M	A	SM_{35}	0	A	A	M	M
3	A_{ps}	A	A	A	A	A	M	M
4	A	A	A	A	A	A	A	A
5	A	A	A_{ps}	A_{ps}	A	A	A	A
6	A	A	A	A	A	A	A	A
7	A	A	A	A	A	A	A	A
8	A	A	A	A	A	A	A	A
9	A	A	A	A	A	A	M	M
10	A	A	A	A	A	A	A	A
11	A	A	A	A	A	A	A	A
12	A	A	A	A	M	M	A	A
13	M	M	A	A	SM	SM	A	A
14	A	A	A	A	A	A	M	M
15	A	A	A	A	A	A	A	A
16	M	M	M	M	M	M	M	M
17	A	A	A	A	A_{ps}	A	M	M
18	M	M	A	A	A	A	M	M
19	—	—	M	M	M	M	A	A
20	—	—	—	—	A	A	A	A
	5M / 13A		4M / 15A		4M / 16A		7M / 13A	
N.F.	23		23		24		27	

Note. M is for metacentric and submetacentric chromosomes; A is for subtelocentric and telocentric chromosomes; p is for the short arm of the chromosome; q is for the long arm of the chromosome; s is for satellite; N.F. is the fundamental number. The number at the bottom of M or A is the value of the centromeric index.

comparison between the karyotypes of different related species, same chromosome number and different fundamental number indicate the occurrence of pericentric inversions, while different chromosome number and same fundamental number indicate the occurrence of centric fusions or fissions.

Assuming that L. *caprai* is the ancestral species, having the more primitive X chromosome and a higher number of chromosomes, the simplest hypothesis IS:

- from L. *caprai (2n=40;* n.f.=24) should be arose L. *montana (2n=38;* n.f.=23), through a centric fusion and perhaps a pericentric inversion;
- from this latter L. *attenuata (2n=36;* n.f.=23) could be originated, through a centric fusion involving the X chromosome;
- on the other hand, L. *caprai* could originated *Leptynia* sp. *(2n=40;* n.f.=27), through some pericentric inversions (almost three).

This hypothesis seems to be in good agreement with the stasipatric model of chromosomal speciation proposed by WHITE (1978).

SCALI (1996) examined three taxa of the *Leptynia attenuata* complex: the electrophoretic studies on 18 enzymatic loci revealed genetic distance values ranging from 0.28 to 1.09. These sharp values, together with the chromosomal differentiation (for the chromosome number and sex chromosome mechanism in the male), support the formal decription of:

Leptynia attenuata "sensu stricto", corresponding to that described by PANTEL (1890), widespread in Portugal, *Leptynia montana* n.sp., which includes the populations of the Sistema Central mountains and *Leptynia caprai* n.sp. which includes those on the hills south of Toledo.

SEM analyses on bodies of both sexes does not trace clear diagnostic characters for the three groups but only some differentiation trends and the study of egg sculptures gives only two quantitative discriminating characters. So, SCALI (1996) thinks that this is a case of incipient speciation, where genetic and chromosomal differentiation plays a major role in the evolution respect to the morphological one. Therefore, he proposes that, in view of parapatric distribution, karyotypic evolution and gene-f1ow interruption, the three groups reached the species level through a stasipatric mechanism of speciation.

Our data on laboratory crosses (BIANCHI, in preparation) confirm that the four taxa described in this paper are incipient species, presenting post-mating reproductive isolating mechanisms.

All the data obtained in this paper and the deriving inferences seem to be in very good agreement with the SCALI (1996) demonstrations.

Acknowledgements - The Authors are deeply grateful to Prof. Luciano Bullini and to Prof. Giuseppe Nascetti for collecting the materials and to Alessandra Spano for her precious work in the scanner elaboration of the figures.

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Received 10 July 1998; accepted 14 September 1998