Molecular Ecology (2007) **16**, 4256–4268 doi: 10.1111/j.1365-294X.2007.03471.x

The *Leptynia hispanica* species complex (Insecta Phasmida): polyploidy, parthenogenesis, hybridization and more

FABRIZIO GHISELLI, LILIANA MILANI, VALERIO SCALI and MARCO PASSAMONTI
Dipartimento di Biologia Evoluzionistica Sperimentale, Università di Bologna, via Selmi 3, I-40126 Bologna, Italy

Abstract

The *Leptynia hispanica* stick insect species complex includes bisexuals, triploid and tetraploid parthenogenetic populations, suggesting that polyploidy has played a central role in the evolution of this complex. An analysis of karyotype, mitochondrial DNA (*cox2*) and nuclear DNA (*ef1*-α) markers was carried out to clarify phylogenetic relationships and microevolutionary/phylogeographical patterns of the *L. hispanica* complex. Our analyses suggested a subdivision of bisexual populations into four groups, tentatively proposed as incipient species. Moreover, triploids and tetraploids showed two independent origins, the latter being more ancient than the former. From *ef1*-α analysis, triploids showed hybrid constitution, while the hybrid constitution of tetraploids is likely, but more data are needed. We suggest that *L. hispanica* is a case of 'geographical parthenogenesis' with parthenogenetic strains colonizing large peripheral ranges, and bisexuals confined to glacial refuge areas. Moreover, the age, wide distribution and competitive advantage of polyploids over diploids, demonstrate their significance in the evolution of the *L. hispanica* species complex.

Keywords: cytochrome oxidase 2, elongation factor 1-α, hybridization, *Leptynia*, parthenogenesis, polyploidy

Received 8 April 2007; revision received 4 June 2007; accepted 19 June 2007

Introduction

The relative paucity of parthenogenetic animal species is a contentious topic in evolutionary biology, since it interfaces with the adaptive meaning of sexual reproduction and the genetic damage that its disruption might cause (Maynard Smith 1978; Bell 1982; Stearns 1987). However, recent studies showed that unisexuals may perform well, at least over a short-term evolutionary time, but sometimes even over long periods (e.g. bdelloid rotifers). They are nowadays believed far from the genetically uniform, inflexible caricatures often considered in theoretical treatments (Moritz 1993). Moreover, in some well-known cases, parthenogenetic organisms show a higher fitness than their diploid ancestors and have much higher dispersal capability (Hughes 1989). Vandel (1928) proposed the term 'geographical parthenogenesis' since parthenogens are more widespread than their bisexual relatives and manage to live in rough marginal territories such as higher altitudes and latitudes (Peck *et al*. 1998). A strong link exists between geographical parthenogenesis and glaciations: during cold periods, most species suffer a contraction, moving to lower latitudes and altitudes, while during postglacial expansions parthenogens have an advantage over bisexuals in colonizing new habitats.

Parthenogenesis in animals is commonly coupled with polyploidy, although they may occur independently. Polyploidy has been considered pivotal to gene and genome duplication events, which proved to have a central role in evolution (Van de Peer & Meyer 2005). Gene duplication may generate biodiversity by promoting postmating reproductive barriers (Lynch & Conery 2000). Polyploidization is a powerful and rapid speciation mechanism and had a central role in evolutionary processes in some insect taxa (Suomalainen *et al*. 1976). Depending on their origin, polyploids are subdivided into autopolyploids and allopolyploids. The former are the result of several processes within a species during germline maturation, meiosis or fecundation, while allopolyploids usually arise through hybridization between different species, but possibly also between partially cross-fertile progenitors (Gregory

Correspondence: Fabrizio Ghiselli, Fax: +39 0512094286; E-mail: fabrizio.ghiselli@unibo.it

& Mable 2005). Polyploids may have many advantages: the duplication of every gene shelters null alleles from selection by continued functioning of duplicate genes. Also, polyploid genomes can buffer deleterious effects caused by negative dominance (Veitia 2005). Moreover, in allopolyploids, the heterozygosity level at each locus increases dramatically (Bullini & Nascetti 1990). There are also known disadvantages of polyploidy, such as disrupting effects of nuclear and cell enlargement, propensity to produce aneuploid cells and epigenetic instability in gene regulation (Comai 2005). Although polyploidy is common in plants, it is rather exceptional in animals, maybe because hybrids are less common in animals than in plants (Mayr 1963). Muller (1932) first tried to explain the low frequency of polyploids in animals by proposing the disruption of sex determination/differentiation as the main cause. He noticed that dioecious individuals are rare in plants while they are common in animals in which sex is frequently determined by sex chromosomes (see Gregory & Mable 2005 for a wider discussion). In several animals, the heterogametic sex has one degenerate sex chromosome and genetic balance between sexes is ensured by dosage compensation (see Straub & Becker 2007 for a review). Polyploidy does not interfere with chromosomal sex determination *per se*, but it affects the balance normally preserved by dosage compensation. Because animals commonly possess degenerated sex chromosomes while plants do not, polyploidy is rarer among animals than in plants. Accordingly, in animals, polyploidy has fewer disadvantages when associated to parthenogenesis.

Stick insects (Phasmida) are known for their repeated interspecific hybridizations and uncommon reproductive modes; several phasmids are parthenogenetic, either they are hybrids or not. In the Mediterranean area, four stick insect genera are present: *Bacillus* and *Clonopsis* (Bacillidae) and *Leptynia* and *Ramulus* (Heteronemiidae). While the former two genera are distributed in Mediterranean region, *Leptynia* is distributed only in the Iberian Peninsula and Southern France, while *Ramulus* is widespread in Africa and Asia. *Bacillus* hybrids, in addition to parthenogenesis, reproduce through hybridogenesis and androgenesis (Scali *et al*. 1995; Mantovani *et al*. 1997). The genus *Leptynia* was formerly known to comprise two well-differentiated species: *Leptynia hispanica* Bolivar, 1878, and *Leptynia attenuata* Pantel, 1890. Both taxa have now to be considered as species complexes (Bianchi 1992; Scali 1996; Bianchi & Meliado 1998; Passamonti *et al*. 1999). However, within each complex, morphology does not allow clean discrimination, since no morphological character shows a neat divergence (Scali 1996). Within the *L. hispanica* complex, bisexuals, triploid and tetraploid parthenogenetic populations have been found (Nascetti *et al*. 1983; Bianchi 1992), suggesting that polyploidy played a central role in the evolution of the species complex. This complex is therefore a good experimental system for studying these evolutionary and reproductive mechanisms.

This study deals with karyological analysis, cytochrome oxidase subunit 2 (*cox2*) and elongation factor1-α(*ef1*-α) sequencing of *L. hispanica*. The *cox2* mitochondrial gene has been sequenced over a wide variety of taxa and has proved useful for phylogenetic research, especially in Phasmida (Mantovani *et al*. 2001). The *ef1*-α gene is the most popular nuclear protein-coding gene used in phylogenetics. It is a low-copy gene with a relatively low substitution rate, so that its third codon position provides most of the phylogenetic information (Caterino *et al*. 2000). Single-/low-copy genes are becoming increasingly used in phylogenetic studies for several reasons: they are biparentally inherited and, with rare exceptions, they are not subject to concerted evolution (Senchina *et al*. 2003). In addition, these genes have lower homoplasy and provide a large supply of characters (Alvarez & Wendel 2003). The combination of both mitochondrial and nuclear markers is needed to define polyploid taxa ancestors and to clarify the phylogenetic relationships among diploids.

Materials and methods

Leptynia hispanica specimens from 18 populations, collected over a wide area of the Iberian Peninsula and Southern France, were utilized for this study. All samples were characterized for karyotype and the mitochondrial gene cytochrome oxidase subunit 2 (*cox2*, partial sequence); some populations, collected in a following year, were also characterized for the nuclear gene elongation factor 1-α (*ef1*-α, partial sequence). Detailed data are reported in Table 1 and Fig. 1.

A basic karyotype analysis (Giemsa-stained chromosomes) was performed on gonad tissues, according to Marescalchi & Scali (1990). Mitoses and meioses were analysed in order to define karyotype, male meiotic features, sex formula and ploidy.

Total genomic DNA was obtained according to the method described in Preiss *et al*. (1988). Partial sequence of the mitochondrial *cox2* gene was amplified and directly sequenced according to Mantovani *et al*. (2001). The primers were TL2-J-3034 and TK-N-3785 (Simon *et al*. 1994). Sequencing covered 639-bp coding for 213 amino acids of the *cox2* and corresponds to the gene region sequenced in several insect orders (Liu & Beckenbach 1992). Sequences of *Clonopsis gallica*, *Bacillus rossius*, *Bacillus atticus*, *Bacillus grandii*, *Medaura scabriusculus*, *Leptynia attenuata*, *Leptynia montana*, *Leptynia caprai* and *Leptynia* sp., were utilized as outgroups.

Total RNA was obtained using the RNeasy Plus kit (Qiagen), then cDNA was amplified by reverse transcription–polymerase chain reaction (RT–PCR) performed with an oligo(dT) primer using the SuperScript First-Strand

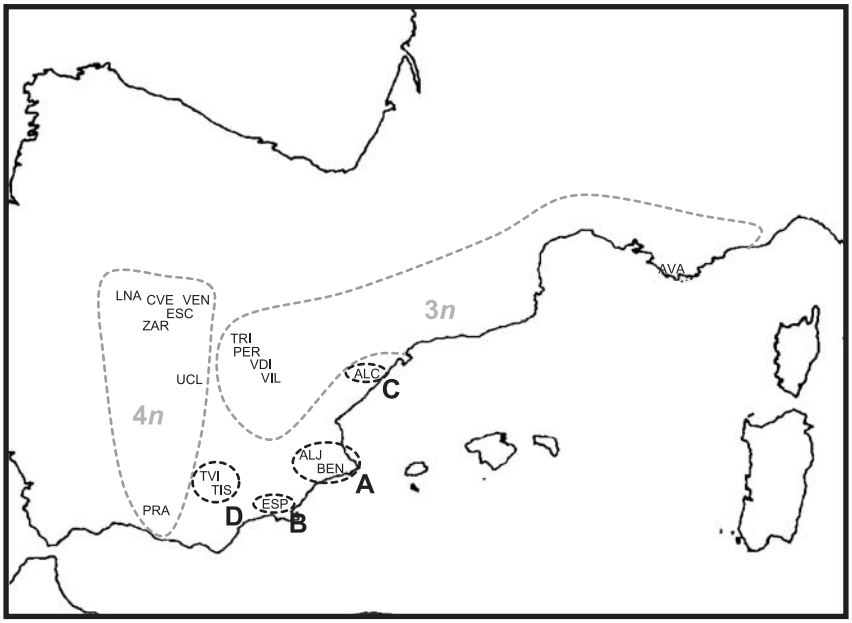

Fig. 1 Geographical distribution of the analysed populations of the *Leptynia hispanica* species complex: BEN, Benissa; ALJ, Alcoy; ESP, Sierra de Espuña; ALC, Alcoceber; TVI, Torre Vinagre, Cazorla; TIS, Puerto del Tiscar; AVA, Abbaje de Valmagne; TRI, Trillo; PER, Peralbeche; VIL, Villalba de Cuenca; VDI, Ventana Diablo; VEN, Ventorillo; ESC, El Escorial; CVE, Puerto Cruz Verde; LNA, Las Nava del Marqués; ZAR, Zarzalejo; UCL, Uclés; PRA, Puerto de La Ragua.

Synthesis System for RT–PCR (Invitrogen). The partial sequence of the elongation factor1-α gene (*ef1*-α) was amplified using a touchdown PCR approach, as follows: 25 cycles with annealing temperature declining from 55 to 45 °C (0.4 °C/cycle), followed by 14 cycles at 45 °C. The primers were M44-1 and rcM52.6 (Cho *et al*. 1995). The amplified products were then cloned using the pGEM-T Easy Vector System Kit (Promega). Recombinant clones were sequenced using the M13 primers. From all the samples, we obtained a library of 81 clones. Sequencing analysis covered 629 bp, coding for 209 amino acids (haplotype GenBank Accession nos in Table 1).

All sequences were aligned with the CLUSTAL algorithm of MEGA 3.1 (Kumar *et al*. 2004). Phylogenetic analyses on the *cox2* gene were performed using neighbour-joining (NJ), minimum-evolution (ME), maximum-parsimony (MP) and maximum-likelihood (ML) approaches, using PAUP (version 4.0, Swofford 2003). Likelihood scores for each DNA substitution model were calculated using MODELTEST (Posada & Crandall 1998) and the best-scored model (GTR + I + Γ) (Rodriguez *et al*. 1990) was used for ML tree reconstructions. Support for each node was obtained using bootstrap (500 replicates) (Felsenstein 1985). Moreover, the method described in Takezaki *et al*. (1995) was applied both to assess constancy of mutation ratio amongst European stick insects and to obtain an NJ linearized tree, using the LINTREE program (Dos executable, available at http://www.bio.psu.edu/people/faculty/nei/lab/software.htm). Time calibration obtained for *Bacillus* species (Mantovani *et al*. 2001) was utilized to estimate divergence time among *Leptynia* clades.

Phylogenetic analyses on *ef1*-α were performed as for the *cox2* gene except for ML, which demanded unavailable computational power. Instead, a Bayesian Analysis was performed using MRBAYES 3.1 (10 000 000 generations; Huelsenbeck & Ronquist 2003). The Tamura–Nei equal frequencies nucleotide distance was utilized for the NJ tree. Templeton's networks (Templeton 1992) were obtained with TCS 1.21 (Clement *et al*. 2000). A gene conversion test was also performed with DNASP (Rozas & Rozas 1999).

Results

Karyotypes

Insects from the bisexual populations of Benissa and Alcoy, Sierra de Espuña, Alcoceber, Tiscar and Torre Vinagre are all $2n = 37/38$, X0/XX. Acrocentric and subacrocentric chromosomes mainly form their karyotype and the largest metacentric chromosomes are the sexual ones (Fig. 2a, b). Parthenogenetic populations are either triploid ($3n = 57$, XXX: Abbaye de Valmagne (Fig. 2c), Sête, Trillo, Peralbeche, Villalba de Cuenca and Ventana Diablo), or tetraploid ($4n = 76$, XXXX: El Escorial (Fig. 2d), Ventorillo, Puerto Cruz Verde, Las Navas del Marquéz, Zarzalejo, Uclés and Puerto de la Ragua).

Cox2 gene

Cox2 alignment showed 60 variable sites out of a total of 639 bp sequenced. Mean p-distance values (p) among bisexuals range from 0.013 to 0.055, showing a good genetic divergence on a geographical basis: we therefore named them *Leptynia hispanica A* (Benissa and Alcoy), *L. hispanica B* (Sierra de Espuña), *L. hispanica C* (Alcoceber) and *L. hispanica D* (Puerto del Tiscar and Torre Vinagre) (not *sensu* Nascetti *et al*. 1983). Triploids show little variation

Table 1 Sampled taxa, localities, acronyms and GenBank Accession nos of the *Leptynia hispanica* species complex. For *cox2* gene, we obtained sequences from 27 *L. hispanica* specimens. For *ef1*-α gene, we obtained 51 haplotypes, derived from the sequencing of 81 clones of the following specimens: 1 haplotype from 1 sample of Benissa (BEN), 5 haplotypes from 2 samples of Serra de Espuña (ESP), 1 haplotype from 1 sample of Alcoceber (ALC), 3 haplotypes from 1 sample of Torre Vinagre (TVI), 18 haplotypes from 2 samples of Ventana Diablo (VDI), 11 haplotypes from 2 samples of Zarzalejo (ZAR), 11 haplotypes from 1 sample of Uclés (UCL) and 1 *Bacillus rossius* haplotype from 1 sample (outgroup)

				GenBank accession	
Taxon	Karyotype	Locality	Acronym	*cox2*	*ef1*-α
L. hispanica A	(2*n* = 37/38, X0/XX)	Benissa (Spain)	BEN	AF241444	EF450635
		Alcoy (Spain)	ALJ	AF241445	
				AF241446	
L. hispanica B	(2*n* = 37/38, X0/XX)	Sierra de Espuña (Spain)	ESP	AF241458	EF450636
				AF241447	EF450637
				AF241448	EF450638
					EF450639
					EF450640
L. hispanica C	(2*n* = 37/38, X0/XX)	Alcoceber (Spain)	ALC	AF241449	EF450641
L. hispanica D	(2*n* = 37/38, X0/XX)	Torre Vinagre (Spain)	TVI	EF507842	EF450642
					EF450643
					EF450644
		Puerto del Tiscar (Spain)	TIS	EF507838	
				EF507839	
				EF507840	
				EF507841	
L. hispanica 3n	(3*n* = 57, XXX)	Abbaje de Valmagne (France)	AVA	AF241450	
				AF241451	
		Trillo (Spain)	TRI	AF241453	
		Peralbeche (Spain)	PER	AF241452	
		Villalba de Cuenca (Spain)	VIL	AF241455	
		Ventana Diablo (Spain)	VDI	AF241454	EF450645
					EF450646
					EF450647
					EF450648
					EF450649
					EF450650
					EF450651
					EF450652
					EF450653
					EF450654
					EF450655
					EF450656
					EF450657
					EF450658
					EF450659
					EF450660
					EF450661
					EF450662
L. hispanica 4n	(4*n* = 76, XXXX)	Ventorillo (Spain)	VEN	AF241463	
				AF241464	
		El Escorial (Spain)	ESC	AF241458	
		Puerto Cruz Verde (Spain)	CVE	AF241456	
				AF241457	
		Las Navas del Marqués (Spain)	LNA	AF241459	
L. hispanica 4n	(4*n* = 76, XXXX)	Las Navas del Marqués (Spain)	LNA	AF241460	
		Zarzalejo (Spain)	ZAR	AF241465	EF450674
					EF450675
					EF450676
					EF450677
					EF450678
					EF450679
					EF450680

Table 1 *Continued*

Taxon	Karyotype	Locality	Acronym	GenBank accession	
				cox2	*ef1-α*
			ZAR		EF450681
					EF450682
					EF450683
					EF450684
		Uclés (Spain)	UCL		EF450663
					EF450664
					EF450665
					EF450666
					EF450667
					EF450668
					EF450669
					EF450670
					EF450671
					EF450672
					EF450673
		Puerto de la Ragua (Spain)	PRA	AF241461	
				AF241462	
Outgroups					
Clonopsis gallica		Laujaon (Spain)		AF096287	
Bacillus rossius		Capalbio (Italy)		AF038206	
Bacillus rossius		Lussino (Italy)			EF450634
Bacillus atticus		Cugni (Italy)		AF038226	
Bacillus grandii		Ponte Manghisi (Italy)		AF148301	
Medaura scabriusculus		Bangladesh		AF508243	
Leptynia attenuata		São Fiel (Portugal)		AF508232	
Leptynia montana		El Escorial (Spain)		AF241416	
Leptynia caprai		Urda (Spain)		AF241431	
Leptynia species		Sierra de Grazalema (Spain)		AF241412	

($p = 0.002$) and are similar to *L. hispanica C* ($p = 0.001$). Moreover, tetraploids show a higher variability than triploids ($p = 0.006$) and they are more similar to *L. hispanica D* ($p = 0.007$). ME, ML and MP trees (not shown) have overlapping topologies and confirm the above-mentioned grouping. Triploids and tetraploids are always split into two genetically well-differentiated groups: all triploids cluster with *L. hispanica C*, while all tetraploids cluster with *L. hispanica D*. This immediately supports the thesis that parthenogenetic taxa are not directly related to each other, and the transition to unisexuality occurred in two distinct times. Two-cluster test analysis carried out on all samples showed an overall substitution rate constancy (95% criterion of significance, data not shown) with the single exception of the two outgroups *Leptynia caprai* and *Leptynia* species which were then removed from the analysis to apply the method proposed by Takezaki *et al*. (1995) and construct a linearized NJ tree (not shown) to which the time calibration utilized for *Bacillus* species was applied (Mantovani *et al*. 2001). Because of LINTREE software limitations, it was not possible to apply the general time reversible (GTR) distance (not implemented in the program), so the most similar distance was chosen, that is TrN distance (Tamura & Nei 1993). The separation of *L. hispanica A* from the ancestral *Leptynia* lineage dated 16.57 million years ago (±2.65), while the splitting of other bisexual taxa (*B*, *C* and *D*) is more recent, about 5 million years (5.00 ± 1.32 million years) ago. *Leptynia hispanica 4n* is the older of the two parthenogenetic taxa and its origin has been dated at 2.86 ± 1.01 million years, in the Pliocene. In contrast, because of the extremely low variability (only one mutation found), it was not possible to obtain a time calibration for *L. hispanica 3n*, even though it is evident that its origin is much more recent, in spite of a wide geographical range, comparable to that of tetraploids. Templeton's analysis (Fig. 3) confirmed that the karyotype and geographical grouping we applied is supported. Moreover, a close relationship between triploids and *L. hispanica C* is evident: most of the triploid sequences are identical to the Alcoceber ones. The same applies to the relationship between tetraploids and *L. hispanica D*, even though both of them are more variable. From Templeton's analysis it is also clear that polyploid taxa are not related.

Ef1-α gene

To simplify analysis, the 81 nucleotide sequences obtained, when identical, were pooled into haplotypes, which were

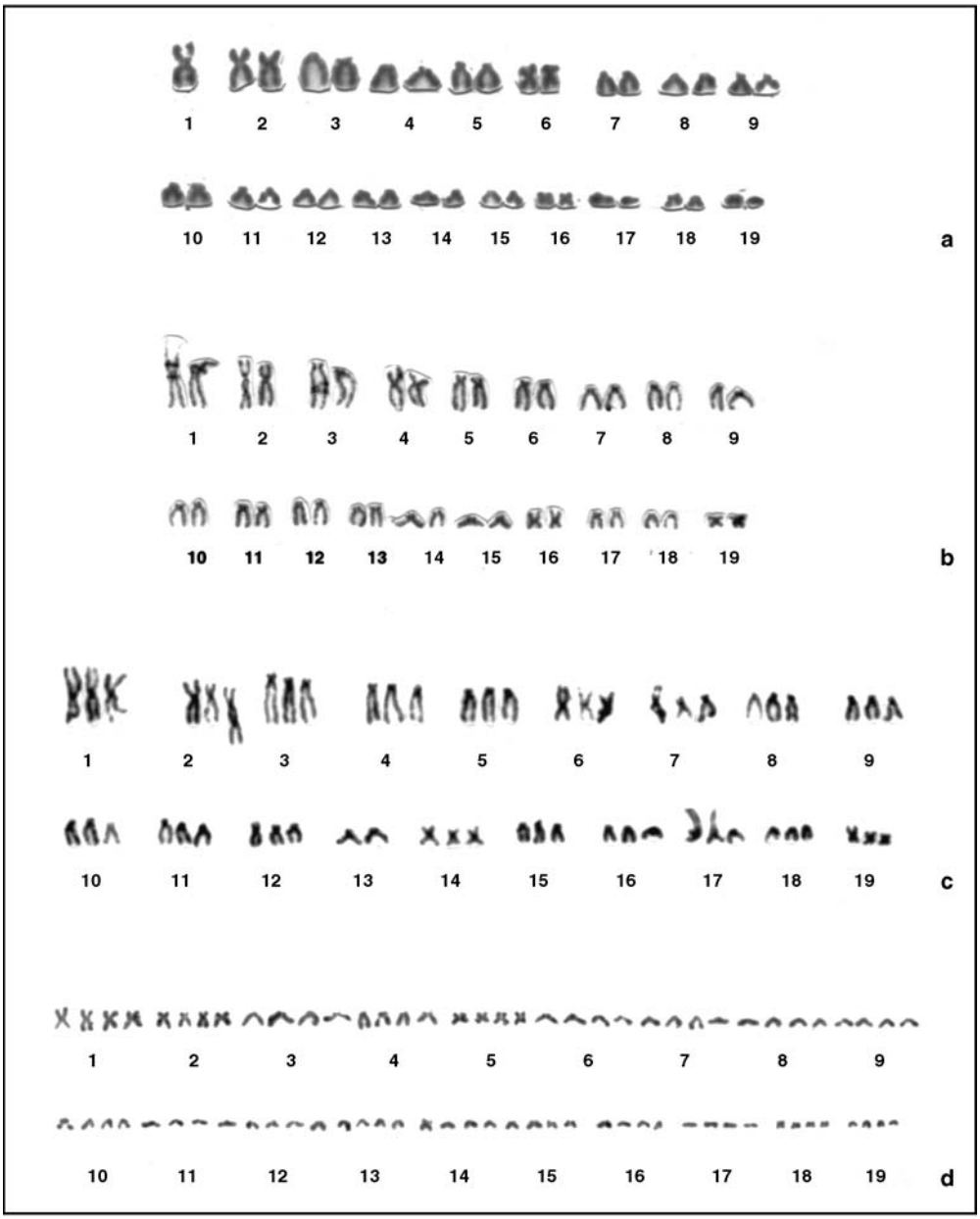

Fig. 2 Karyotypes of *Leptynia hispanica* species complex. (a) Benissa, male ($2n = 37$). (b) Sierra de Espuña, female ($2n = 38$). (c) *L. hispanica* $3n$ from Abbaje de Valmagne ($3n = 56$; note the autosomal translocation from triplet 13 to 2). (d) *L. hispanica* $4n$ from El Escorial ($4n = 76$).

used in all phylogenetic studies; 51 haplotypes were therefore analysed. *Ef1*-α alignment showed 194 variable sites out of a total of 629 bp sequenced. Excluding *L. hispanica A*, this value drops to 67. Mean p-distance values (p) among bisexuals range from 0.019 to 0.265, with *L. hispanica A* being the most differentiated form (0.259–0.265). On the whole, *ef1*-α analysis confirms the subdivision of diploids into four groups, as obtained from *cox2*. Mean p-distance among triploids is 0.009 and it is 0.013 within tetraploids. The maximum number of haplotypes (= different alleles) in each individual was 3 in diploids, 8 in triploids and 11 in tetraploids. This means that the minimum copy number for the haploid genome is 2 for bisexuals, and 3 for polyploids. Analysis using DNASP software (Rozas & Rozas 1999) showed no gene conversion traits in the obtained haplotypes (data not shown). ME, MP and Bayesian trees have comparable clustering patterns. The same applies to Templeton's network, which is shown in more detail (Fig. 4). *Leptynia hispanica A* is the most differentiated form and all

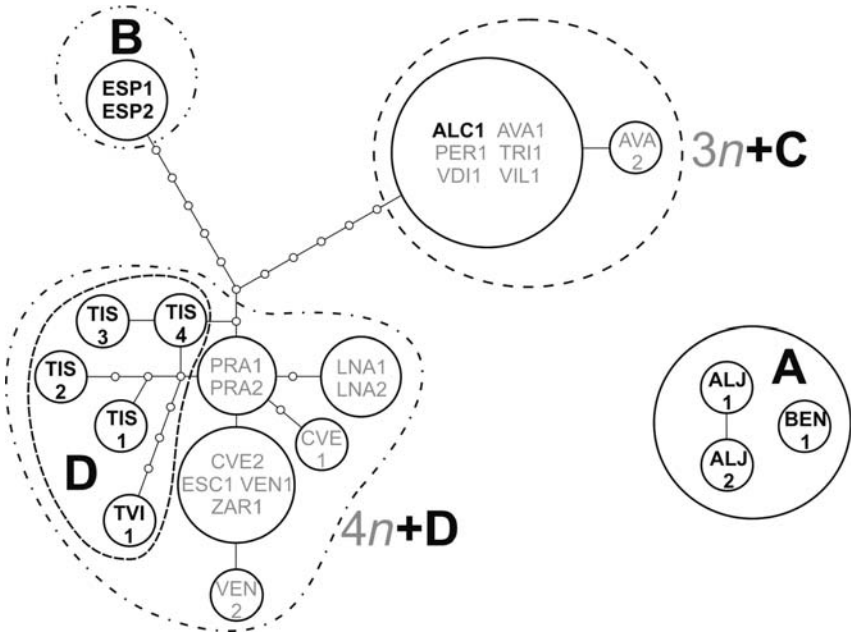

Fig. 3 Templeton network for *Leptynia hispanica cox2* sampled sequences. Sampling locality acronyms as in Table 1. Each dash indicates a single mutation and empty circles indicate intermediate unsampled haplotypes. Diploid haplotypes are in bold. Note the clustering of ALC1 (*L. hispanca* C) in *L. hispanica 3n* clade.

diploid populations are distinct; triploids are subdivided into two different clusters, one strictly related to *L. hispanica C* ($3n_m$), the other not showing any relationship with known diploid taxa ($3n_p$). It is noticeable that this second group of haplotypes seems to be better represented in the triploids genome (see Templeton's network), since after cloning and random sequencing, we found more than twice the number of these clones. Tetraploids' relationships are even more complicated: the most noticeable finding is that all sequences are different from the *L. hispanica D* ones, which, according to *cox2*, is the maternal taxon. Although sequences from *4n* specimens do not form a clear cluster, nevertheless sequences of *L. hispanica D* form a different one supported by high bootstrap values, thus confirming no direct relationship between the two. Moreover, haplotypes from *L. hispanica D* are so differentiated that no significant parsimonious connection was possible using Templeton's network (Fig. 4).

Discussion

Karyotype characterization

In *Leptynia hispanica*, chromosomal differences among diploid karyotypes are low and it is not easy to distinguish the four diploid groups using this analysis (Fig. 2a, b). This situation is in sharp contrast to what was observed in the *Leptynia attenuata* complex, where karyotype rearrangements have been the main force in the speciation process (Passamonti *et al*. 2004). Polyploid parthenogenetic populations can be easily subdivided into two groups: one triploid ($3n = 57$) and the other tetraploid ($4n = 76$). The triploid karyotype suggests a likely hybrid origin, since some triplets are formed by a couple of similar chromosomes and a single slightly different chromosome (see triplets 1, 3, 6, 14, 19; Fig. 2c); also chromosome satellites (triplets 7 and 17) may support this interpretation. The translocation of an autosome from triplet number 13 to triplet number 2 in the triploid population of Abbaye de Valmagne could be consistent with the suppression of meiosis, which allows lighter constraints on chromosome organization. However, at present, we are not able to determine if the parthenogenesis is apomictic or automictic. In contrast to the triploids, the tetraploid quartets are formed by undistinguishable chromosomes (Fig. 2d), so a hybrid structure of tetraploids is not obvious from the karyotype. The finding of an X0 sex determination mechanism in bisexuals is consistent with most stick insects, while only a few showed an XY mechanism (Passamonti *et al*. 2004). Accordingly, we can assume that sex determination in *L. hispanica* is obtained by the X/autosomes ratio. Consequently, in polyploid strains, the male offspring would have had problems in sexual development and therefore risk sterility, so polyploid *L. hispanica* strains entrained parthenogenesis. Although we did not detect any aneuploid karyotype in *L. hispanica* parthenogens, Brock (1991) reported putative gynandromorphs among *L. hispanica* populations in southern France, which are nowadays known to be triploids. Moreover, in several other polyploid stick insects, the lack of one X chromosome determines the production of intersexual individuals, showing a variable degree of sterility (Tinti & Scali 1996).

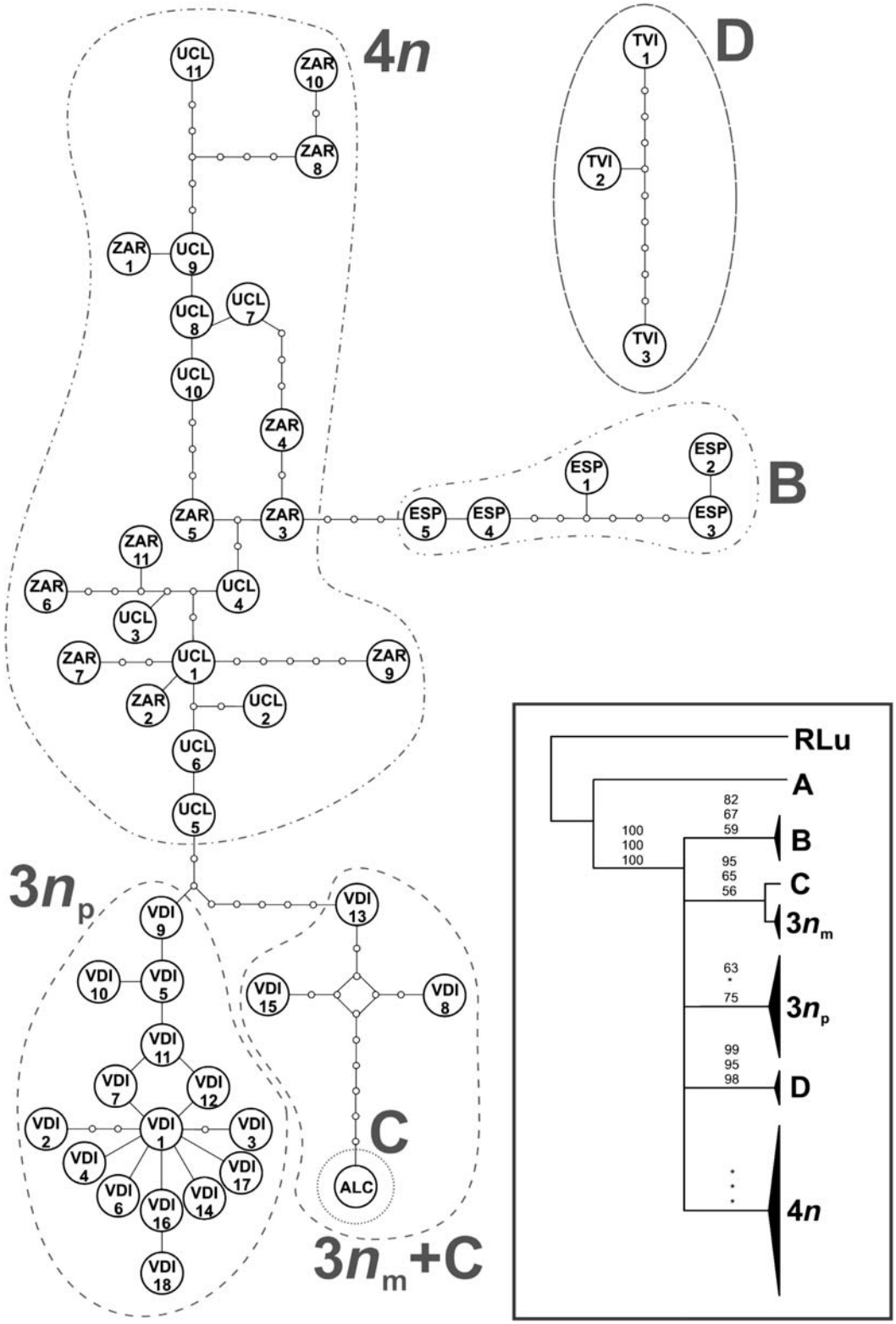

Fig. 4 Templeton network for *Leptynia hispanica ef1-α*. Each dash indicates a single mutation and empty circles indicate intermediate unsampled haplotypes. A schematic tree obtained with Bayesian Likelihood approach (10 000 000 generations) is reported (lower right) for better comparison. Posterior probabilities and bootstrap values obtained from MP and ME (respectively) are shown on clades. Asterisks indicate nonsignificant supporting values. Rlu — *Bacillus rossius* from Mali Losinje (Croatia); A–*L. hispanica A*; B–*L. hispanica B*; C–*L. hispanica C*; D–*L. hispanica D*; $3n_p$ — *L. hispanica 3n*, paternal; $3n_m$ — *L. hispanica 3n*, maternal; 4*n*–*L. hispanica 4n*. Sample acronyms as in Table 1.

Cytochrome oxidase 2 analysis: tracing the maternal ancestors of L. hispanica *polyploids*

Mitochondrial *cox2* gene sequencing and phylogenetic analysis highlighted several aspects of the microevolution in this complex. The occurrence of four diploid bisexuals and two polyploid parthenogenetic groups is strongly supported. In particular, among diploids, *L. hispanica A* is the most differentiated form. The rarity of this form and its narrow range (in two sampling seasons we collected only 10 individuals in Benissa and Alcoy) suggest that we are dealing with a relic taxon. *Leptynia hispanica B*, *C*, *D* show a neat but lower differentiation level, and their sharply disjunctive ranges lead us to hypothesize that they are incipient species. *Leptynia hispanica C* results the population that originated the triploids, since their sequences are almost all identical (Fig. 3). Moreover, the strict relationship between *L. hispanica D* and tetraploids points to an unequivocal kinship. Triploid and tetraploid populations appeared to have distinct temporal and spatial origins. Our time calibration suggests that tetraploids are more ancient than triploids and that *L. hispanica 4n* is quite an old taxon (approximately 2–3 million years). The observed *cox2* variability is greater in tetraploids than in triploids and this might be linked to their different ages. Moreover, multiple hybridization events might have occurred in tetraploids, while the high homogeneity in triploids might be indicative of a single event. However, only further analyses on more variable zones of genome might help.

Elongation factor 1-α: nuclear markers and hybridization

While mtDNA traces the maternal ancestry, the only way to determine whether polyploids are hybrid or not and to find the paternal contribution is to analyse nuclear DNA markers. The *ef1*-α gene is considered one of the best available markers (Cho *et al*. 1995; Jordal 2002; and references therein). Although largely accepted, this needs some further discussion, since our data suggest that *ef1*-α gene in *Leptynia* has multiple copies, as it is also found in *Artemia salina* (Lenstra *et al*. 1986), and in holometabolous insects such as *Apis mellifera* (Danforth & Ji 1998), *Drosophila melanogaster* (Hovemann *et al*. 1988) and Curculionidae (Normark *et al*. 1999; Jordal 2002). The one we report here seems to be the first case in hemimetabolous insects, since analyses on orthopteroid insects did not find more than a single copy (Zhou *et al*. 2002; Broughton & Harrison 2003). Since we found multiple gene copies in *L. hispanica*, a clear understanding of sequence relationships is necessary when sequences from multicopy genes are used for phylogenesis (Sanderson & Doyle 1992; references therein). If sequences are taken as orthologous, when they are paralogous, the relationships can be incorrectly inferred (Bailey *et al*. 2003), so efforts should be made to exclude the possibility of paralogous copies. In this attempt, Danforth & Ji (1998) found that the minimum divergence observed between paralogues in their studies on *Apis mellifera* is >18% (overall sequence divergence). In *L. hispanica,* the overall sequence divergence is 2.3%, a value that appears to us a significant clue in excluding the presence of paralogous copies in our analysis. Also, we have to consider the possibility that we are dealing with pseudogene copies. Pseudogenes have been defined as nonfunctional copies of genomic DNA derived from functional genes (Balakirev & Ayala 2003). Since duplicated genes are redundant at origin, their usual fate is the nonfunctioning of one copy (Lynch & Conery 2000). In addition, the duplicates usually lack promoter and regulatory sequences, so they can no longer produce a functional mRNA (Vanin 1985; Zhang 2003). In this study, we obtained *ef1*-α sequences from mRNA copies, thus excluding nonfunctional pseudogenes from our analysis. In addition, we did not find incomplete mRNAs, frameshift mutations or any other evidence of malfunctioning sequences. There are also a few known cases in which some retroposition-mediated duplicated genes are actually expressed ('processed pseudogenes': Vanin 1985), probably because of their insertion downstream of a promoter sequence. Nevertheless, this is an unlikely event (Vanin 1985); therefore, we can reasonably exclude that this happened in *L. hispanica*. For all the above-mentioned reasons, we feel we are not dealing with wrong phylogenetic signals because of paralogous and/or pseudogene copies in our data set.

Tracing hybrid constitution of L. hispanica *polyploids*

Phylogenetic analysis on *ef1*-α agrees with mtDNA data: the four *L. hispanica* diploid groups are clearly distinct from each other, and the basal position of *L. hispanica A* is confirmed (Fig. 4). For the unisexuals, two different haplotype clusters found within triploids suggest that *L. hispanica 3n* is a hybrid taxon, as found in other parthenogenetic stick insects (Mantovani *et al*. 1999; Morgan-Richards & Trewick 2005). One sequence cluster is, as expected, similar to *L. hispanica C*, confirming mtDNA data and reinforcing the conclusion that it is the maternal taxon of the *3n* hybrid. The other cluster probably represents the paternal contribution to the hybrid, but unfortunately it could not be associated with any known bisexual. The numeric proportion of clones might also suggest that *L. hispanica C* contributed to the triploid hybrid genome with only one haploset, while the other two sets derived from an unknown taxon. This observation is congruent with karyotype morphology (Fig. 2c). We can trace two different scenarios in the origin of the *3n* hybrid *L. hispanica*: a single hybridization event in which a haploid

egg of *L. hispanica C* fused with two sperms of the parental taxon (physiological polyspermy), or a two-step origin, through an intermediate diploid parthenogenetic hybrid which subsequently incorporated a further paternal genome. From our data, we cannot prefer one scenario to the other: probably only further studies on hyper-variable regions of the genome could solve the issue. The origin of tetraploids is more complex because of a higher genetic variability within the group. However, it seems to us that the most interesting result is that no *ef1-α* haplotypes of *L. hispanica D* (which indeed contributed with mitochondria to tetraploids, as demonstrated by mtDNA) have been found. Consequently, tetraploids would still have to be considered hybrids, although the nuclear maternal contribution is nowadays missing. We can hypothesize three different scenarios to explain this unexpected finding. Actually, we could assume that *L. hispanica D* contributed to a hybrid *4n* genome but its alleles were lost because of gene conversion. In this case, tetraploids would have to have an automictic meiotic mechanism, otherwise meiosis suppression would not allow any recombination event. However, using DNASP (Rozas & Rozas 1999), we did not find gene conversion tracts between tetraploids and their putative maternal ancestor (*L. hispanica D*). The second hypothesis is that tetraploids could originate through androgenesis, so they do not keep trace of the maternal nuclear genome, while maintaining maternal mtDNA. Androgenesis is a reproduction in which diploid offspring inherit chromosomes only from the male (Mantovani & Scali 1992). Androgenesis, first demonstrated in *Bacillus* stick insects (Mantovani & Scali 1992; Tinti & Scali 1996; Mantovani *et al*. 2001), has been proved to occur in several species of freshwater *Corbicula* clams (Komaru *et al*. 1998; Byrne *et al*. 2000; Qiu *et al*. 2001), and in the cypress tree *Cupressus dupreziana* (Pichot *et al*. 2001). To explain *L. hispanica* data with androgenesis, we have to assume that only paternal genomes are present in the nucleus. However, even though androgenesis has already been found in Phasmida, direct evidence is needed to ascertain whether it really occurred in *4n L. hispanica*. Finally, we would like to mention that recent studies highlighted some cases of nonequivalent contributions from the two parents to gene expression in allopolyploids and hybrids (Pikaard 2000; Adams *et al*. 2003; Veitia 2005). It was suggested that these changes in transcription could be attributable to a regulatory mismatch between effectors and target genes. During hybridization, there is a merger of two different genomes and the new cellular and nuclear volumes could be suboptimal for the expression of the parental genes. The transcriptional mechanism of the parents could be otherwise similar, while the promoter-activator system could be different. Additionally, a hybrid or allopolyploid could carry a pool of molecules which have different affinities for DNA in a volume that is different from that of the parents (Veitia 2005). Dufresne & Hebert (1994) analysed *Artemia salina* and suggested that hybridization between genetically divergent species leads to maternal genome expulsion or silencing. In fact, the situation appears similar to *L. hispanica*: divergence between *L. hispanica 4n* and *L. hispanica D* might support this hypothesis.

Phylogeography of the L. hispanica *species complex*

Based on gathered data, it is possible to outline a consistent phylogeographical scenario for the *L. hispanica* complex. Most likely, the original range of the complex could have been the territory North of Alicante, where at present it is possible to find *L. hispanica A*, the most relic and differentiated taxon. Nowadays, detectable diploid groups are probably the result of a genetic differentiation due to low dispersal and fragmentation of suitable habitats into isolated multiple glacial refugia, because of the harsh climate of the high central Iberian plateau during glaciations (Gómez & Lunt 2007). Since they seem to be quite old, bisexuals of *L. hispanica* probably went through cyclical climate changes that strongly influenced their phylogeography, as also observed for example in *Deinacrida* (Trewick *et al*. 2000) and *Heteronotia* (Strasburg & Kearney 2005). Parthenogenetic groups show distinct geographical distributions, with ranges of tetraploids and triploids being contiguous to those of their diploid ancestors (*L. hispanica D* and *L. hispanica C*, respectively; Fig. 1). Moreover, *L. hispanica 3n*, whose range extends far to the North, is less variable than the southern *4n*, and this is quite a typical pattern as observed by Hewitt (2001, 2004). In addition, Law & Crespi (2002b) noted that younger asexuals tend to be found further North than older ones, and this fully agrees with what we observed in *L. hispanica*. *L. hispanica* distribution fits the 'geographical parthenogenesis' model with a broad distribution of polyploid populations together with a confined distribution of diploids in small and fragmented areas. As noticed in many other cases (Suomalainen 1978; Mantovani 1998; Sandoval *et al*. 1998; Schön *et al*. 2000; Law & Crespi 2002a; Baxevanis *et al*. 2006), the original range of each diploid group was probably wider. After the rising of polyploid populations, diploids were displaced, perhaps due to the parthenogens' greater fitness. In *L. hispanica*, triploids show a large range, despite their recent origin; their fast expansion, also at the expense of the contiguous tetraploid populations, could well be in progress. In fact, *4n L. hispanica* is a more ancient taxon, so we can hypothesize that it might suffer from some degree of genetic decay. Most likely, triploids were formed after the last ice age and quickly colonized territories previously covered by ice (Pyrenees) or too cold for their ancestors (high central Iberian plateau) which remained in the refuge areas, as happened for several known parthenogenetic insects (Law

& Crespi 2002a; Kearney 2003; Stenberg *et al*. 2003; Strasburg & Kearney 2005; Kearney *et al*. 2006). Post-glacial territories represent a big challenge for colonizing organisms because of the likely occurrence of unknown physical and biotic conditions (Hewitt 1996; Kearney 2005). Hybridization quickly creates new and much higher range of genetic combinations with different fitness (Barton 2001) easily allowing the transition to new adaptive peaks and therefore the colonization of new territories (Lewontin & Birch 1966). Niches and spatial separation reduce competition and the possibility of backcrossing between polyploids and diploid parents, which often produces low-fitness offspring; consequently, separation could be favoured by selection (Martins *et al*. 1998).

Evolutionary perspectives of L. hispanica *hybrids*

In *L. hispanica*, geographical parthenogenesis appears to be strictly related to polyploidy and hybridization. The main genetic effect of hybridization is to enhance heterozygosis. Moreover, the formation of a hybrid lineage from multiple individuals (polyphyly) also has important consequences: polymorphism can be drastically lowered in hybrids descended from a single ancestor, while it can be increased in hybrids descended from multiple events. According to Fisher (1930), polymorphism represents the 'evolutionary hope' of a population, which has lost bisexual reproduction and therefore the possibility of genetic assortment. Furthermore, the polyploid condition enhances genetic versatility by gene duplication. According to the above-mentioned rationale, the high variability within the *L. hispanica 4n* taxon could be the main reason for its evolutionary success and long-lasting existence. For the same reason, the low level of triploids' variability might still not be significantly affecting them, simply because of their relatively recent existence and consequent slight genetic decay. However, the hybrid condition entails some problems: the fixation of a new hybrid strain needs reproductive isolation, otherwise gene flow would stop the cladogenetic process. Moreover, hybrids must overcome the constraints of meiotic pairing of heterospecific chromosomes. Parthenogenesis can overcome both problems. Also polyploidy can lead to reproductive isolation and contribute to egg maturation: therefore both parthenogenesis and polyploidy can be considered as major pathways leading to hybrid balancing and persistence (Kearney 2005). This seems to be what has happened twice in the *L. hispanica* species complex, leading to tetraploid (earlier) and triploid (later) strains. Why this happened so frequently in *L. hispanica*, as well as in other circum-mediterranean stick insects, is a matter of conjecture, but there is some evidence that phasmids have a pre-adaptation to develop parthenogenesis (Marescalchi *et al*. 2002) and usually lack premating isolation mechanisms (Scali *et al*. 2003).

Acknowledgements

We thank Prof Barbara Mantovani, Dr Andrea Luchetti and Dr Andrea Ricci for their suggestions and help. This work was supported by the Italian 'Ministero dell'Università e della Ricerca Scientifica' (MIUR) funds and by the 'Donazione Canziani' bequest.

References

Adams KL, Cronn R, Percifield R, Wendel JF (2003) Genes duplicated by polypolidy show unequal contributions to the transcriptome and organ-specific silencing. *Proceedings of the National Academy of Sciences, USA*, **282**, 4649–4654.

Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, **29**, 417–434.

Bailey CD, Carr TG, Harris SA, Hughes CE (2003) Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. *Molecular Phylogenetics and Evolution*, **29**, 435–455.

Balakirev ES, Ayala FJ (2003) Pseudogenes: are they 'junk' or functional DNA? *Annual Review of Genetics*, **37**, 123–151.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology*, **10**, 551–568.

Baxevanis AD, Kappas I, Abatzopoulos TJ (2006) Molecular phylogenetics and sexuality in the brine shrimp *Artemia*. *Molecular Phylogenetics and Evolution*, **40**, 724–738.

Bell G (1982) *The Masterpiece of Nature: the Evolution and Genetics of Sexuality*. University of California Press, Berkeley, California.

Bianchi AP (1992) Karyological studies of Mediterranean stick-insects belonging to the genera *Clonopsis* and *Leptynia* (Insecta Phasmatodea). *Caryologia*, **45**, 1–19.

Bianchi AP, Meliado P (1998) Analysis of the karyotypes of four species of the *Leptynia attenuata* complex (Insecta Phasmatodea). *Caryologia*, **51**, 207–219.

Brock P (1991) *Stick-Insects of Britain, Europe and the Mediterranean*. Fitzgerald Publishing, London.

Broughton RE, Harrison RG (2003) Nuclear gene genealogies reveal historical, demographic and selective factors associated with speciation in field crickets. *Genetics*, **163**, 1389–1401.

Bullini L, Nascetti G (1990) Speciation by hybridization in phasmids and other insects. *Canadian Journal of Zoology*, **68**, 1747–1760.

Byrne M, Phelps H, Church T, Adair V, Selvakumaraswamy P, Potts J (2000) Reproduction and development of the freshwater clam *Corbicula australis* in southeast Australia. *Hydrobiologia*, **418**, 185–197.

Caterino MS, Cho S, Sperling FAH (2000) The current state of insect molecular systematics: a thriving tower of Babel. *Annual Review of Entomology*, **45**, 1–54.

Cho S, Mitchell A, Regier JC *et al*. (1995) A highly conserved nuclear gene for low level phylogenetics: Elongation Factor-1α recovers morphology- based tree for Heliotine moths. *Molecular Biology and Evolution*, **12**, 650–656.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1659.

Comai L (2005) The advantages and disadvantages of being polyploid. *Nature Reviews Genetics*, **6**, 836–846.

Danforth BN, Ji S (1998) Elongation Factor 1-α occurs as two copies in bees: implications for phylogenetic analysis of *ef1*-α sequences in insects. *Molecular Biology and Evolution*, **15**, 225–235.

Dufresne F, Hebert PDN (1994) Hybridization and origins of polyploidy. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **258**, 141–146.

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.

Fisher RA (1930) *The Genetical Theory of Natural Selection*. Chapter 6. Oxford University Press, Oxford, UK.

Gómez A, Lunt DH (2007) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: *Phylogeography in Southern European Refugia: Evolutionary Perspectives on the Origins and Conservation of European Biodiversity* (eds Weiss S, Ferrand N), pp. 155–188. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Gregory TR, Mable BK (2005) Polyploidy in animals. In: *The Evolution of the Genome* (ed. Gregory TR), chapter 8, pp. 427–517. Elsevier. Academic Press, San Diego, CA.

Hewitt GM (1996) Some genetic consequences of ice ages and their role in divergence and speciation. *Biological Journal of Linnean Society*, **58**, 247–276.

Hewitt GM (2001) Speciation, hybrid zones and phylogeography — or seeing genes in space and time. *Molecular Ecology*, **10**, 537–549.

Hewitt GM (2004) Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **359**, 183–195.

Hovemann B, Richter S, Walldorf U, Cziepluch C (1988) Two genes encode related cytoplasmic Elongation Factors 1-α (*ef1*-α) in *Drosophila melanogaster* with continuous and stage specific expression. *Nucleic Acid Research*, **16**, 3175–3194.

Huelsenbeck JP, Ronquist F (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Hughes RN (1989) *A Functional Biology of Clonal Animals*. Chapter 3. Chapman & Hall, London and New York.

Jordal BH (2002) Elongation Factor-1α resolves the monophyly of the haplodiploid ambrosia beetles Xyleborini (Coleoptera Curculionidae). *Insect Molecular Biology*, **11**, 453–465.

Kearney M (2003) Why sex is so unpopular in the Australian desert? *Trends in Ecology & Evolution*, **18**, 605–607.

Kearney M (2005) Hybridization, glaciation and geographical parthenogenesis. *Trends in Ecology & Evolution*, **20**, 495–502.

Kearney M, Blacket MJ, Strasburg JL, Moritz C (2006) Waves of parthenogenesis in the desert: evidence for the parallel loss of sex in a grasshopper and a gecko from Australia. *Molecular Ecology*, **15**, 1743–1748.

Komaru A, Kawagishi T, Konishi K (1998) Cytological evidences of spontaneous androgenesis in the freshwater clam *Corbicula leana* prime. *Development Genes and Evolution*, **208**, 46–50.

Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Law JH, Crespi BJ (2002a) The evolution of geographic parthenogenesis in *Timema* walking-sticks. *Molecular Ecology*, **11**, 1471–1489.

Law JH, Crespi BJ (2002b) Recent and ancient asexuality in *Timema* walkingsticks. *Evolution*, **56**, 1711–1717.

Lenstra JA, Van Vliet A, Arnberg AC, Van Hemert FJ, Moller W (1986) Genes coding for the Elongation Factor *ef1*-α in *Artemia*. *European Journal of Biochemistry*, **155**, 475–483.

Lewontin RC, Birch LC (1966) Hybridization as a source of variation for adaptation to new environments. *Evolution*, **20**, 315–336.

Liu H, Beckenbach AT (1992) Evolution of the mitochondrial cytochrome oxidase 2 gene among 10 orders of insects. *Molecular Phylogenetics and Evolution*, **1**, 41–52.

Lynch M, Conery JS (2000) The evolutionary fate and consequences of duplicate genes. *Science*, **290**, 1151–1155.

Mantovani B (1998) Satellite sequence turnover in parthenogenetic systems: the apomictic triploid hybrid *Bacillus lynceorum* (Insecta, Phasmatodea). *Molecular Biology and Evolution*, **15**, 1288–1297.

Mantovani B, Scali V (1992) Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-grandii benazzi* (Insecta Phasmatodea). *Evolution*, **46**, 783–796.

Mantovani B, Tinti F, Bachmann L, Scali V (1997) The Bag320 satellite DNA family in *Bacillus* stick insects (Phasmatodea): different rates of molecular evolution of highly repetitive DNA in bisexual and parthenogenic taxa. *Molecular Biology and Evolution*, **14**, 1197–1205.

Mantovani B, Passamonti M, Scali V (1999) Genomic evolution in parental and hybrid taxa of the genus *Bacillus* (Insecta Phasmatodea). *Italian Journal of Zoology*, **66**, 265–272.

Mantovani B, Passamonti M, Scali V (2001) The mitochondrial cytochrome oxidase 2 gene in *Bacillus* stick insects: ancestry of hybrids, androgenesis and phylogenetic relationship. *Molecular Phylogenetics and Evolution*, **19**, 157–163.

Marescalchi O, Scali V (1990) Cytogenetic studies on *Bacillus grandii grandii* and *Bacillus grandii benazzii* (Insecta Phasmatodea): karyotype description, constitutive heterochromatine and nucleolus organizer regions. *Genetica*, **82**, 117–124.

Marescalchi O, Zauli C, Scali V (2002) Centrosome dynamics and inheritance in related sexual and parthenogenetic *Bacillus* (Insecta Phasmatodea). *Molecular Reproduction and Development*, **63**, 89–95.

Martins MJ, Collares-Pereira MJ, Cowx IG, Coelho MM (1998) Diploids vs. triploids of *Rutilus alburnoides*: spatial segregation and morphological differences. *Journal of Fish Biology*, **52**, 817–828.

Maynard Smith J (1978) *The Evolution of Sex*. Cambridge University Press, Cambridge, UK.

Mayr E (1963) *Animal Species and Evolution*. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

Morgan-Richards M, Trewick SA (2005) Hybrid origin of a parthenogenetic genus. *Molecular Ecology*, **14**, 2133–2142.

Moritz C (1993) The origin and evolution of parthenogenesis in the *Heteronotia binoei* complex: synthesis. *Genetica*, **90**, 269–280.

Muller HJ (1932) Some genetic aspects of sex. *American Naturalist*, **66**, 118–138.

Nascetti G, Bianchi Bullini AP, Bullini L (1983) Speciazione per ibridazione nei fasmidi del bacino mediterraneo (Cheleutoptera: Bacillidae). *Atti XIII Congresso Nazionale Italiano Entomologia, Sestriere — Torino*, 475–478.

Normark BB, Jordal BH, Farrell BD (1999) Origin of a haplodiploid beetle lineage. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **266**, 2253–2259.

Passamonti M, Mantovani B, Scali V (1999) Karyotype and allozyme characterization of the Iberian *Leptynia attenuata* species complex (Insecta Phasmatodea). *Zoological Science*, **16**, 675–684.

Passamonti M, Mantovani B, Scali V (2004) Phylogeny and karyotype evolution of the Iberian *Leptynia attenuata* species complex (Insecta Phasmatodea). *Molecular Phylogenetics and Evolution*, **30**, 87–96.

Peck JR, Yearsley JM, Waxman D (1998) Explaining the geographic distribution of sexual and asexual populations. *Nature*, **391**, 889–892.

Pichot C, El Maâtaoui M, Raddi S, Raddi P (2001) Surrogate mother for endangered *Cupressus*. *Nature*, **412**, 39.

Pikaard CS (2000) The epigenetics of nucleolar dominance. *Trends in Genetics*, **16**, 495–500.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Preiss A, Hartley DA, Artavanis-Tsakonas S (1988) Molecular genetics of enhancer of split, a gene required for embryotic neural development in *Drosophila*. *EMBO Journal*, **12**, 3917–3927.

Qiu A, Shi A, Komaru A (2001) Yellow and brown shell color morphs of *Corbicula fluminea* (Bivalvia Corbiculidae) from Sichuan Province, China, are triploids and tetraploids. *Journal of Shellfish Research*, **20**, 323–328.

Rodriguez F, Oliver JF, Marín A, Medina JR (1990) The general stochastic model of nucleotide substitution. *Journal of Theoretical Biology*, **142**, 485–501.

Rozas J, Rozas R (1999) DNASP, version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Sanderson MJ, Doyle JJ (1992) Reconstruction of organismal and gene phylogenies from data on multigene families: concerted evolution, homoplasy, and confidence. *Systematic Biology*, **41**, 4–17.

Sandoval C, Carmean DA, Crespi BJ (1998) Molecular phylogenetics of sexual and parthenogenetic *Timema* walking-sticks. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **265**, 589–595.

Scali V (1996) Descrizione di due specie incipienti di insetti stecco (Phasmatodea) del complesso *Leptynia attenuata* Pantel: *L. montana* n.sp. e *L. caprai* n.sp. *Redia*, **76**, 123–136.

Scali V, Tinti F, Mantovani B, Marescalchi O (1995) Mate recognition and gamete cytology features allow hybrid species production and evolution in *Bacillus* stick-insect. *Italian Journal of Zoology*, **65**, 59–70.

Scali V, Passamonti M, Marescalchi O, Mantovani B (2003) Linkage between sexual and asexual lineages: genome evolution in *Bacillus* stick insects. *Biological Journal of Linnean Society*, **79**, 137–150.

Schön I, Gandolfi A, Di Masso E *et al*. (2000) Persistence of asexuality through mixed reproduction in *Eucypris virens* (Crustacea, Ostracoda). *Heredity*, **84**, 161–169.

Senchina DS, Alvarez I, Cronn RC *et al*. (2003) Rate variation among nuclear genes and the age of polyploidy in *Gossypium*. *Molecular Biology and Evolution*, **20**, 633–643.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of The Entomological Society of America*, **87**, 651–701.

Stearns SC (1987) *The Evolution of Sex and its Consequences*. Birkhauser-Verlag, Basel, Switzerland and Boston, Massachusetts.

Stenberg P, Lundmark M, Knutelski S, Saura A (2003) Evolution of clonality and polyploidy in a weevil system. *Molecular Biology and Evolution*, **20**, 1626–1632.

Strasburg JL, Kearney M (2005) Phylogeography of sexual *Heteronotia binoei* (Gekkonidae) in the Australian arid zone: climatic cycling and repetitive hybridization. *Molecular Ecology*, **14**, 2755–2772.

Straub T, Becker PB (2007) Dosage compensation: the beginning and the end of generalization. *Nature Reviews Genetics*, **8**, 47–57.

Suomalainen E (1978) Two new cases of parthenogenesis in moths. *Nota Lepidopterologica*, **1**, 65–68.

Suomalainen E, Saura A, Lokki J (1976) Evolution of parthenogenetic insects. In: *Evolutionary Biology*, Vol. 9 (eds Hecht M, Steer W, Wallace B), pp. 209–257. Plenum, New York.

Swofford DL (2003) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer & Associates, Sunderland Massachusetts.

Takezaki N, Rzhetsky A, Nei M (1995) Phylogenetic test of the molecular clock and linearized trees. *Molecular Biology and Evolution*, **12**, 823–833.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512–526.

Templeton NS (1992) The polymerase chain reaction. History, methods, and applications. *Diagnostic Molecular Pathology*, **1**, 58–72.

Tinti F, Scali V (1996) Androgenetics and triploids from an interacting parthenogenetic hybrid and its ancestors in stick-insects. *Evolution*, **50**, 1251–1258.

Trewick SA, Wallis GP, Morgan-Richards M (2000) Phylogeographical pattern correlates with Pliocene mountain building in the alpine scree weta (Orthoptera, Anostostomatidae). *Molecular Ecology*, **9**, 657–666.

Van de Peer Y, Meyer A (2005) Large-scale gene and ancient genome duplications. In: *The Evolution of the Genome* (ed. Gregory TR), Chapter 6, pp. 329–368. Elsevier. Academic Press, San Diego, CA.

Vandel A (1928) La parthénogenèse geographique. Contribution α l'étude biologique et cytologique de la parthénogenèse naturelle. *Bulletin Biologique de la France et de la Belgique*, **62**, 164–281.

Vanin EF (1985) Processed pseudogenes: characteristics and evolution. *Annual Review of Genetics*, **19**, 253–272.

Veitia RA (2005) Paralogs in polyploids: one for all and all for one? *Plant Cell*, **17**, 4–11.

Zhang J (2003) Evolution by gene duplication: an update. *Trends in Ecology & Evolution*, **18**, 292–298.

Zhou S, Zhang J, Fam MD, Wyatt GR, Walker VK (2002) Sequences of elongation factors-1α and -1γ and stimulation by juvenile hormone in *Locusta migratoria*. *Insect Biochemistry and Molecular Biology*, **32**, 1567–1576.

The main focus of Authors' research interests are: phylogenetics, biogeography, evolution of stick insects (Insecta Phasmida) and bivalve mollusks; mitochondrial DNA structure and evolution; Doubly Uniparental Inheritance of mtDNA in bivalve mollusks.
