Chromosome Research **11**: 165–178, 2003.

Karyological and genetic variation in Middle Eastern lacertid lizards, *Lacerta laevis* and the *Lacerta kulzeri* complex: a case of chromosomal allopatric speciation

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Received 2 October 2002. Received in revised form and accepted for publication by Michael Schmid 9 December 2002

Key words: chromosomal speciation, cytochrome b, karyotypes, *Lacerta kulzeri*, lacertid lizards, sex chromosomes, 12S rRNA

Abstract

Karyological (standard and C, Ag-NOR and Alu-I banding methods) and mtDNA analyses (cytochrome b and 12S rRNA) were conducted on specimens from eight allopatric populations of the *Lacerta kulzeri* complex. Parallel analyses were performed for comparison on *Lacerta laevis* specimens. Karyological and molecular studies support the morphological and ethological evidence indicating the specific separation between *Lacerta laevis* and *Lacerta kulzeri*.

In the *Lacerta kulzeri* complex, chromosomal analysis substantiated an interpopulation differentiation roughly along a north–south trend, mainly regarding the sex chromosome morphology and heterochromatin. The cytochrome b and 12S rRNA gene analyses showed minor genetic differences that were considerably smaller than those commonly found in genetically isolated populations. The *L. kulzeri* populations from Barouk, Druze and Hermon show a mean genetic distance that, in other saurians, characterises subspecies.

The conditions found in *L. laevis* and *L. kulzeri* are reminiscent of King's model of chromosomal primary allopatry and support the hypothesis that in these lacertid lizards chromosome variations can become fixed before the accumulation of the genetic mutations.

Introduction

Field work in the Middle East (e.g. Bischoff & Schmidtler 1994, in den Bosch 1998) indicated that a number of populations previously ascribed to the common polymorphic wall lizard of the Middle East and Turkey, *Lacerta laevis* Gray, 1838, were in fact members of a discrete species complex. The two distinct taxa, *Lacerta laevis* and the *Lacerta kulzeri* complex, live in separate habitats at

different altitudes. *Lacerta laevis* is distributed from sea level up to around 1500 m over a large interconnecting area and is active most of the year; the lizards in the *Lacerta kulzeri* complex occupy habitats from approximately 1300–2000 m above sea level (a.s.l.) and are active only during the warmer months. Whereas *L. laevis* specimens are generally ground dwellers and are found in comparatively humid areas, the members of the *L. kulzeri* complex usually prefer rocky outcrops in relatively dry zones in higher regions, and, in effect, these assemblages correspond to island populations. Seven groups are distinguished in this complex, each confined to a mountain range in Lebanon, Syria, Israel and Jordan. They have been provisionally called (in den Bosch 1998) *Lacerta kulzeri* Müller & Wettstein, 1932, a lacertid originally described from the cedar wood at 1900 m near Bcharré, Makmel Mts., northern Lebanon (synonymy in in den Bosch 2002). Recent studies based on morphological and ethological characters (in den Bosch & Bischoff 1996, in den Bosch *et al.* 1998, in den Bosch 1998, 1999, Bischoff & Müller 1999, Bischoff & Schmidtler 1999) and hybridization experiments (in den Bosch, unpublished observations) suggested that *L. kulzeri* should be considered a group of forms related to, but definitively distinct from, *L. laevis*. Taxonomic revisions of the *L. kulzeri* group are in progress (Bischoff & Müller 1999, Bischoff & Schmidtler 1999, in den Bosch & Zandee 2001); however, there is no agreement on the status of the several forms.

In this paper, we discuss the results of karyological and mitochondrial gene (12S and cytochrome b) analyses performed in *L. laevis* and in different populations of *L. kulzeri* stressing the short genetic distance as opposed to the remarkable chromosomal differentiation both between *L. laevis* and *L. kulzeri*, and among the different *L. kulzeri* populations.

Materials and methods

The number, sex and origin of the specimens of *Lacerta laevis* and the seven *Lacerta kulzeri* forms studied, are listed in Table 1. All material was collected by the first author, except Ma'alula (Bischoff & Schmidtler), the initial specimens of Petra (Müller) and Dana (Modrý).

Mitochondrial gene analysis

Total DNA was extracted from all *L. kulzeri* specimens according to the Jeffreys & Flavell (1977) method. One gram of tissue was homogenized and incubated in 150 mmol/L NaCl, 100 mmol/L EDTA, pH 8.0, 1% SDS, and 0.1 mg/ml proteinase K. The DNA was then purified twice by chloroform/isoamylalcohol extraction followed by ethanol precipitation, and then PCR-amplified using the universal oligonucleotide primers for

Table 1. Range, origin and number of specimens studied of the *Lacerta kulzeri* complex and *Lacerta laevis*. (Only two *L. kulzeri* forms have yet been properly described; the remaining forms are therefore named after their locality.)

	Range	Origin of specimens studied
***Lacerta kulzeri* forms**		
Barouk	Jabal Barouk: Lebanon	Top of pass across J. Barouk (Lebanon); 1 male, 1 female
Druze	Jabal Druze: southern Syria	J. Druze; 1 male, 1 female
Hermon	Mt. Hermon: Lebanon, Syria, Israel	Mt. Hermon (Israel); 1 male, 2 females
Ma'alula	Anti Lebanon Mts.: Lebanon, Syria	Ma'alula (Syria); 2 males, 1 female
Northern Lebanon	Akkar region: northern Lebanon	Charbiné (Lebanon); 1 male, 1 female
L. k. petraea (Petra + Dana)	Kerak, al Iraq, Petra, Dana: Jordan	Dana (Jordan); 2 males, 1 female; Petra (Jordan); 2 males, 2 females
L. k. kulzeri (Sannin)	Sannin and Makmel Mts.: Lebanon	Above Faraïa, Sannin (Lebanon); 2 males, 1 female
***Lacerta laevis* forms**		
L. l. laevis	Turkey, Syria, Lebanon, Israel, Jordan	Byblos (Lebanon); 1 male, 1 female

cytochrome b and 12S rRNA genes designed by Kocher *et al.* (1989). Amplification conditions were as follows (30 cycles): 94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min. The amplified DNA was directly sequenced on an automated DNA sequencer (ABI PRISM 310, *PE* Biosystems) using a fluorescence dye terminator cycle sequencing kit (*PE* Biosystems). The sequences were aligned with *Lacerta laevis* GeneBank sequences (accession number for 12S AJ238183, for cytochrome b AF080332) used as outgroup. The alignment was performed with the CLUSTAL W programme (Thompson *et al.* 1994) set at default parameters.

The trees were produced using maximum parsimony (MP) with PAUP 4.0 beta 8 version (Swofford 1998). The MP trees were produced following branch and bound search. The search was carried out using equal character weighting, and random stepwise addition with 10 replications, retaining only minimal trees; bootstrap values, indicating robustness of nodes, refer to 1000 replications. The alignment based on 12S rRNA and cytochrome b genes were 390 and 308 nucleotides long, respectively. The alignments can be requested from the authors. The nucleotide sequences have been deposited in GeneBank (accession numbers from AJ519706 to AJ519721).

Karyological analysis

All specimens were injected with a solution of 0.5 mg/ml of colchicine (0.1 ml/10 g body weight). Two hours later, they were exposed to ethyl ether vapour, killed, and the intestine, lungs, spleen and gonads were removed. The organs were incubated for 30 min in 0.7% sodium citrate hypotonic solution, then fixed in methyl alcohol and acetic acid. The chromosomes were obtained by scraping (Odierna *et al.* 1996). In addition to conventional staining with 5% Giemsa solution at pH 7.0, the following banding methods were used: Ag-NOR banding according to Howell & Black (1980); C-banding according to Sumner (1972) using a solution of $Ba(OH)_2$ at 45°C; and Alu-I banding according to Mezzanotte *et al.* (1983).

Results

Mitochondrial gene analysis

The sequences of both mitochondrial genes analysed were identical in all the specimens belonging to the same populations. All the nodes in the MP trees for 12S and cytochrome b genes are supported by bootstrap values higher than 50.

Analysis for 12S rRNA gene (Figure 1) showed one group of *L. kulzeri* specimens which included those from Barouk, Hermon and Druze, and another which included the specimens from Petra and the Sannin Mountains. The individuals from Ma'alula appear to be similar to these two groups, whereas those from Northern Lebanon and Dana occupy a basal position. In the phyletic tree based on

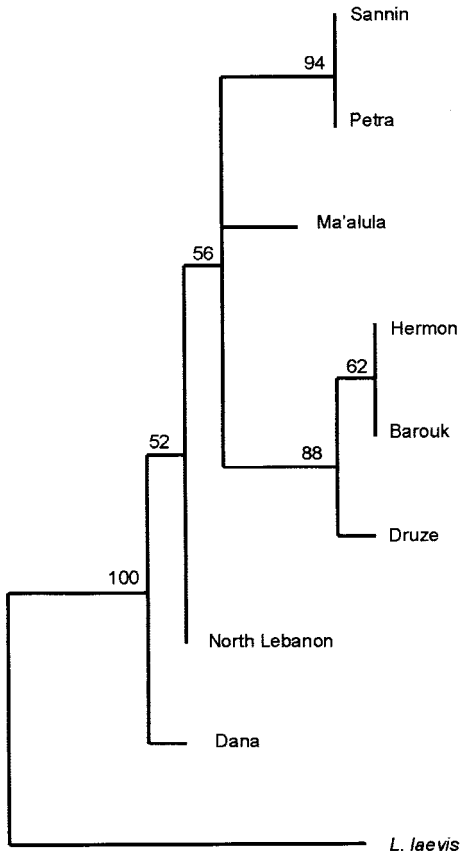

Figure 1. Maximum parsimony tree based on mitochondrial 12S-gene analysis.

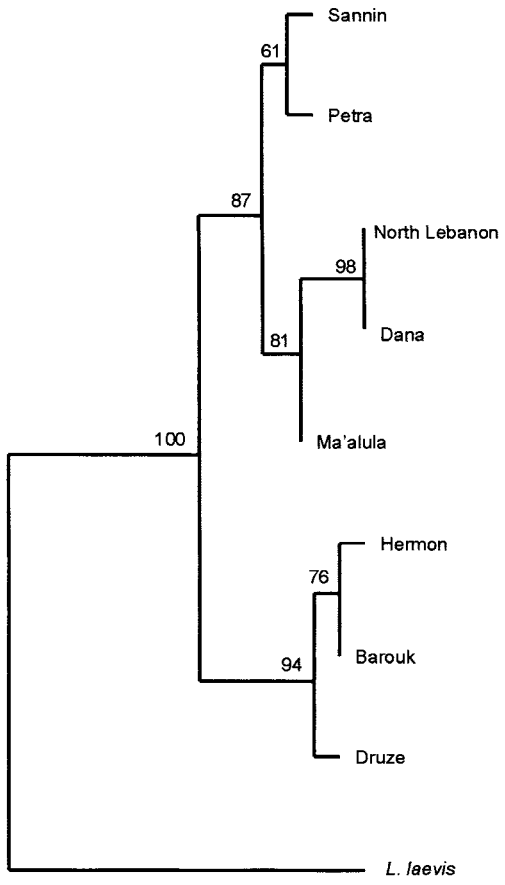

Figure 2. Maximum parsimony tree based on mitochondrial cytochrome b gene analysis.

cytochrome b gene analysis (Figure 2), the position of the individuals from Barouk, Hermon, Druze, Sannin and Petra was similar to that obtained by 12S gene analysis, the remaining forms from Northern Lebanon, Dana and Ma'alula were all grouped in a cluster that was more similar to the set formed by the Petra and Sannin populations.

The genetic distances are reported in Table 2. We normalised the Nei indexes in percentage of the genetic distance between *L. laevis* and *L. kulzeri*, which ranged from 0.038 to 0.048 for the 12S rRNA gene and from 0.130 to 0.140 for the cytochrome b gene.

The genetic distances between the various forms of *L. kulzeri* and between these and *L. laevis* were quite short.

Karyological analysis

Chromosome differences between Lacerta laevis *and the* Lacerta kulzeri *complex*

Both *L. laevis* and the *L. kulzeri* complex possess a karyotype with 36 uniarmed macrochromosomes, two microchromosomes (Figure 3), and ZW sex chromosomes (Figures 4, 5 & 6). However, some differences were detected. A first dissimilarity was observed in the number and localization of the nucleolous organizer region (NORs): in *L. laevis*, a single NOR was observed on the telomeres of the 16th chromosome pair (Figure 3A), whereas *L. kulzeri* displayed an additional NOR on the telomeres of the microchromosomes (Figure 3B). CMA_3 banding confirmed Ag-NOR evidence (data not shown).

A further difference was found in the morphology of the W chromosome, which, in *L. laevis*, was intermediate in size between the microchromosomes and the smallest macrochromosomes (Figure 4A). After C banding, this chromosome appeared completely heterochromatic except for the telomeric region (Figure 5A), whereas, after digestion with the restriction enzyme Alu-I, only this region remained undigested and stainable (Figure 6A). Although W chromosome morphology was different in the various forms of *L. kulzeri* (Figure 4), its heterochromatin was considerably resistant to digestion with Alu-I (Figures 5 & 6).

Differences were also observed in autosome heterochromatin. In the various forms of *L. kulzeri*, all autosomes had C bands at the centromeric tip (Figure 5); in *L. laevis*, instead, such bands were centromeric only in the chromosomes of the 2nd, 10th and 14th pairs, whereas, in all the other chromosomes, they were exclusively pericentromeric (Figure 5 A). In both species, the treatment with the Alu I enzyme yielded bands identical to those obtained with C-banding.

Intraspecific chromosome differences in Lacerta kulzeri

The populations of *L. kulzeri* studied here showed different W chromosome morphology and heterochromatin distribution (Figures 4, 5 & 6): The W chromosome in Northern Lebanon specimens was as large as the chromosomes of the 8th or 9th pair and exhibited Alu-I-resistant centromeric, telomeric and interstitial C bands (Figures 4C, 5C &

Table 2. Genetic distances among the various populations of *L. kulzeri* as percentages of the genetic distance between *L. laevis* and *L. kulzeri* in: (**A**) the 12S rRNA mitochondrial gene and (**B**) the cytochrome b mitochondrial gene. **Bold face**: the same genetic distances expressed directly in Nei index.

	Sannin	Petra	N. Lebanon	Dana	Ma'alula	Hermon	Barouk	Druze	*L. laevis*
A									
Sannin	—	**0.000**	**0.010**	**0.015**	**0.012**	**0.018**	**0.018**	**0.018**	**0.048**
Petra	0	—	**0.010**	**0.015**	**0.012**	**0.018**	**0.018**	**0.018**	**0.048**
N. Lebanon	20	20	—	**0.005**	**0.007**	**0.012**	**0.012**	**0.012**	**0.038**
Dana	30	30	13	—	**0.012**	**0.012**	**0.012**	**0.017**	**0.038**
Ma'alula	25	25	18	31	—	**0.015**	**0.015**	**0.015**	**0.046**
Hermon	37	37	31	31	33	—	**0.000**	**0.005**	**0.046**
Barouk	37	37	31	31	33	0	—	**0.005**	**0.046**
Druze	37	37	31	45	33	11	11	—	**0.046**
L. laevis	100	100	100	100	100	100	100	100	—
B									
Sannin	—	**0.006**	**0.026**	**0.026**	**0.013**	**0.055**	**0.049**	**0.049**	**0.130**
Petra	4.6	—	**0.026**	**0.026**	**0.013**	**0.049**	**0.042**	**0.042**	**0.130**
N. Lebanon	20	20	—	**0.000**	**0.013**	**0.062**	**0.055**	**0.055**	**0.140**
Dana	20	20	0	—	**0.013**	**0.062**	**0.055**	**0.055**	**0.140**
Ma'alula	10	10	9	9	—	**0.055**	**0.049**	**0.049**	**0.133**
Hermon	42	37	44	44	41	—	**0.006**	**0.019**	**0.140**
Barouk	37	32	39	39	37	4.3	—	**0.013**	**0.140**
Druze	37	32	39	39	37	14	9	—	**0.140**
L. laevis	100	100	100	100	100	100	100	100	—

Figure 3. Male Ag-NOR-banded karyotypes of *L. laevis* (**A**); and *L. kulzeri* specimens from Ma'alula (**B**).

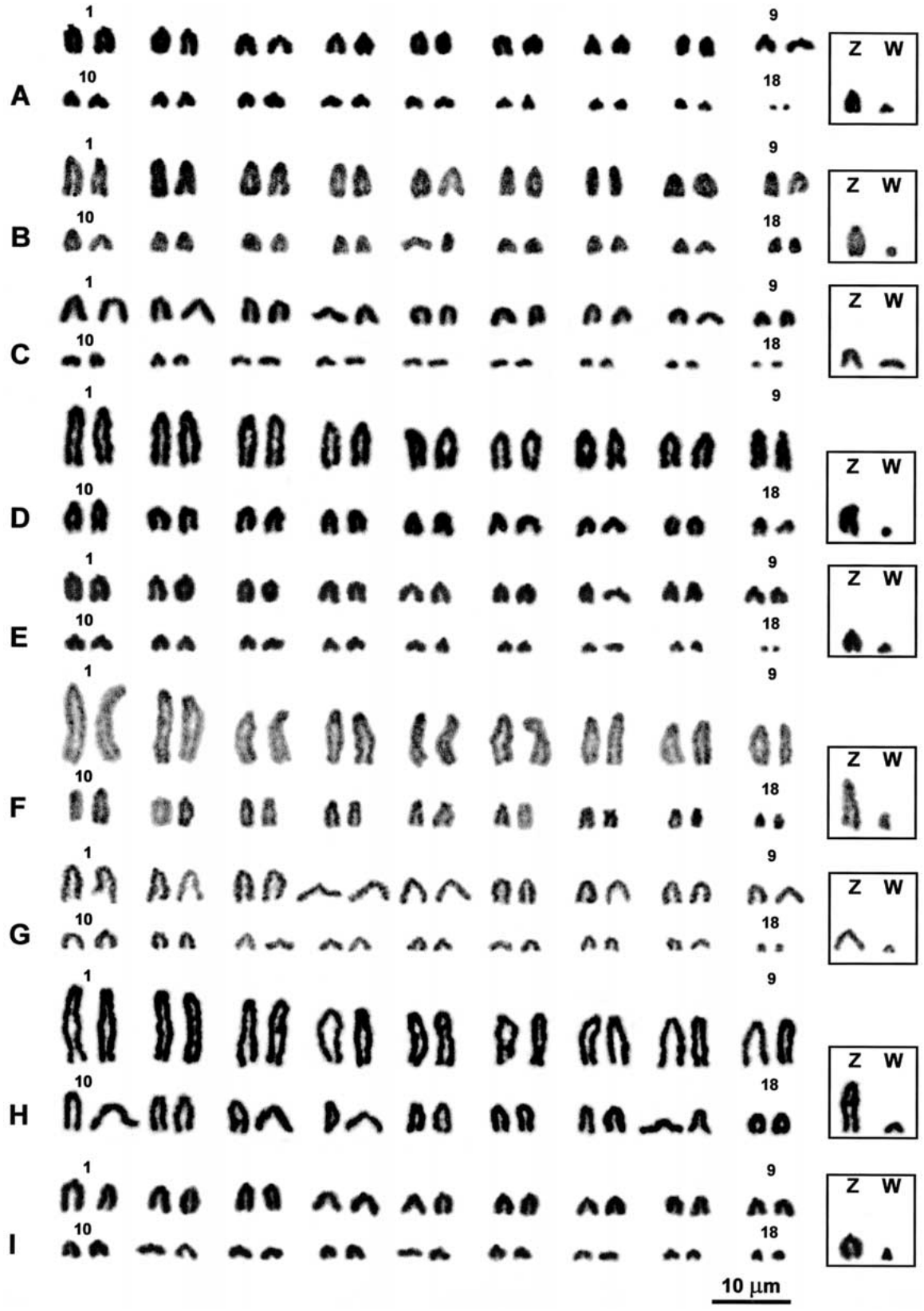

Figure 4. Female Giemsa-stained karyotype of *L. laevis* (**A**) and *L. kulzeri* complex specimens from Barouk (**B**), Northern Lebanon (**C**), Dana (**D**), Druze (**E**), Ma'alula (**F**), Hermon (**G**), Petra (**H**) and Sannin Mountains (**I**).

and 6C). In Druze specimens, the W was as large as the 17th pair chromosomes and completely heterochromatic (Figures 4E, 5E & 6E). In Ma'alula and Sannin specimens, the W had the same size as a microchromosome (Figures 4F & I); in the lizards from Ma'alula, it was completely heterochromatic (Figures 5F & 6F), whereas, in those from Sannin, only the centromeric tip was C banded (Figures 5I & 6I). In Barouk, Dana, Hermon and Petra populations, the W was dot-like (Figures 4B, D, G & H) and completely heterochromatic (Figures 5B, D, G & H). Differences were also found in the heterochromatin content and distribution of the microchromosomes and the smallest macrochromosomes (17th pair). In some populations, both the microchromosomes and the 17th pair macrochromosomes were completely heterochromatic, while, in other populations, they showed only centromeric or telomeric C bands (Figure 5). The heterochromatin of the autosomes was Alu-I resistant in all the *L. kulzeri* forms (Figure 6), except the heterochromatin of the 17th pair chromosomes of Northern Lebanon specimens, which was Alu-I sensitive (Figure 6C). The morphological features and the heterochromatin characteristics of the various populations are summarized in Figure 7.

Discussion

L. laevis–L. kulzeri *diversification*

Our karyological studies confirm the morphological and ethological data indicating that *L. laevis* and *L. kulzeri* are to be considered distinct species (in den Bosch & Bischoff 1996, in den Bosch *et al.* 1998, in den Bosch 1998, 1999, Bischoff & Müller 1999, Bischoff & Schmidtler 1999, Schmidtler & Bischoff 1999, in den Bosch & Zandee 2001). In fact, we found differences in several karyological features such as NOR localization and sex chromosomes morphology, which generally characterize different species of reptiles, particularly lacertid lizards (Odierna *et al.* 1987, 1996, Porter *et al.* 1994, King *et al.* 1982, Pellegrino *et al.* 1999).

However, the divergence of these two species might date back to a relatively recent period because the comparative analysis of mitochondrial genes showed that the genetic distance between them is among the shortest measured in different species of various saurians, lacertids included (Hedges *et al.* 1991, Gonzalez *et al.* 1996, Mayer & Arribas 1996). A calculation based on the mean evolutionary rate measured in various lizards suggests that change per lineage is between 0.61 and 0.70% per million years, in species for which branching events have been confidently dated (Moritz *et al.* 1987, Macey *et al.* 1998, 1999). On this basis, we estimate that *L. laevis* and *L. kulzeri* separated approximately 200 000 years ago, and that the *L. kulzeri* forms displaying higher values of genetic divergence started to split no more than 90 000 years ago. It could well be that Pleistocene climate changes, with periods of major temperature and precipitation fluctuations affecting the area (e.g. Zohary 1973, Wolfart 1987), were indeed a likely initial driving force in the light of the currently different ecological requirements of *L. laevis* and the *L. kulzeri* group.

L. kulzeri *interpopulation variability*

The taxonomic status of the seven forms of *L. kulzeri* is still debated (Bischoff & Müller 1999, Bischoff & Schmidtler 1999, in den Bosch & Zandee 2001). They clearly differ in morphology and courtship behaviour (in den Bosch & Zandee 2001), are reproductively isolated in the field, and in preliminary laboratory crossing experiments have shown severe hybridization problems. Indeed, crossing experiments concerning six *L. kulzeri* forms, in which 7 males and 10 females were involved, never resulted in offspring (in den Bosch, unpublished observations). Mitochondrial gene analysis of the various *L. kulzeri* populations did not provide reliable data to clarify their systematic relationships: the mean genetic distances between populations are very short (Table 2), hardly reaching values that, in other saurians, characterise different subspecies (e.g. Gonzalez *et al.* 1996: mean genetic distance observed between subspecies of *Gallotia* in the 12S rRNA gene 29%; in the cytochrome b gene 35%). Here, this is only the case between the group including the specimens from Barouk, Hermon and Druze and all the other populations. We do observe that the gene trees seem to result in groupings whereas, in the ethological phylogenetic tree, one form of *L. kulzeri* branches off at each bifurcation. The karyological

Figure 5. Female C-banded karyotypes of *L. laevis* (**A**) and *L. kulzeri* complex specimens from Barouk (**B**), Northern Lebanon (**C**), Dana (**D**), Druze (**E**), Ma'alula (**F**), Hermon (**G**), Petra (**H**) and Sannin Mountains (**I**).

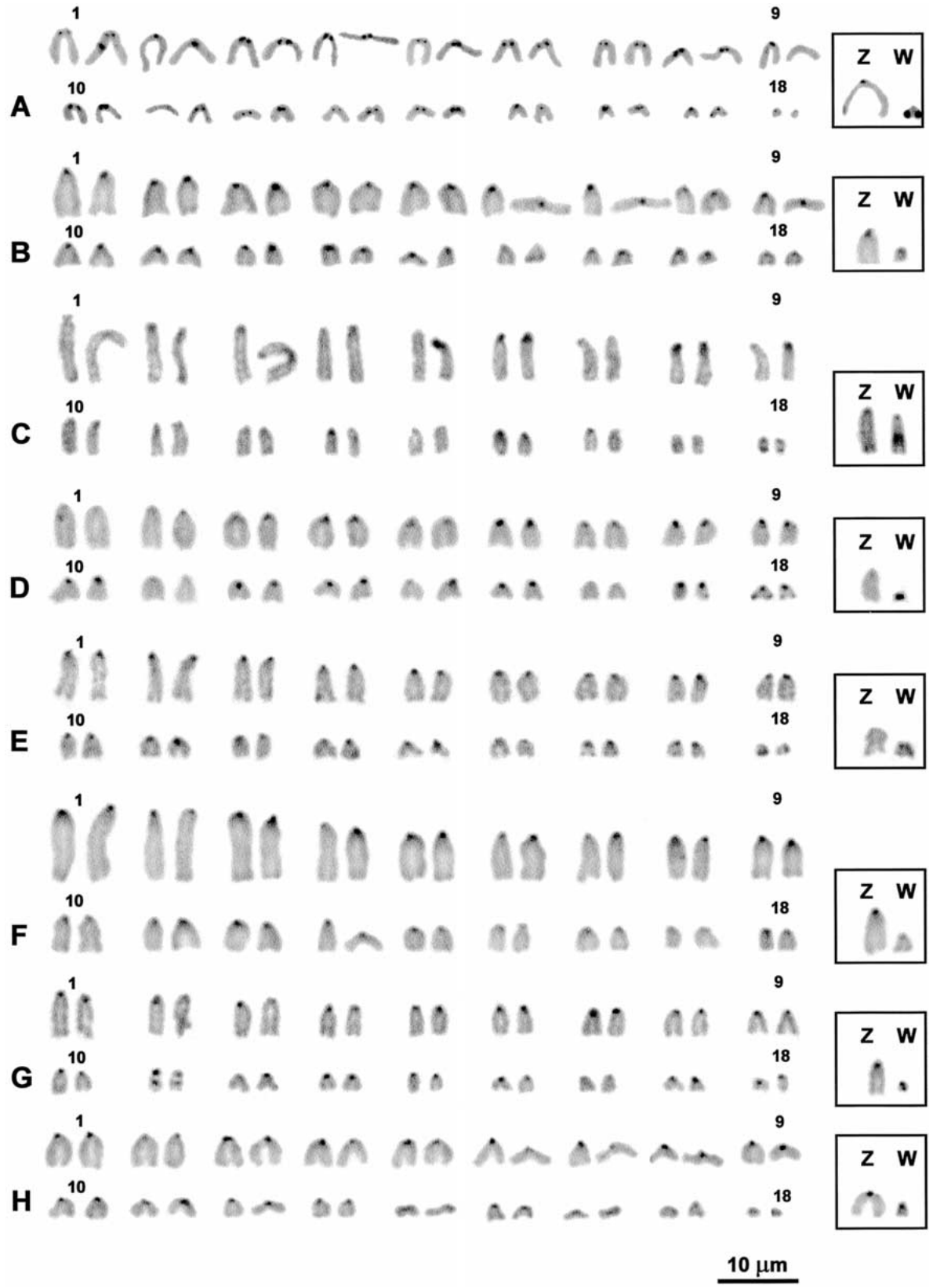

Figure 6. Female Alu-I-digested karyotypes of *L. laevis* (**A**) and *L. kulzeri* complex specimens from Barouk (**B**), Northern Lebanon (**C**), Dana (**D**), Druze (**E**), Ma'alula (**F**), Petra (**G**) and Sannin Mountains (**H**).

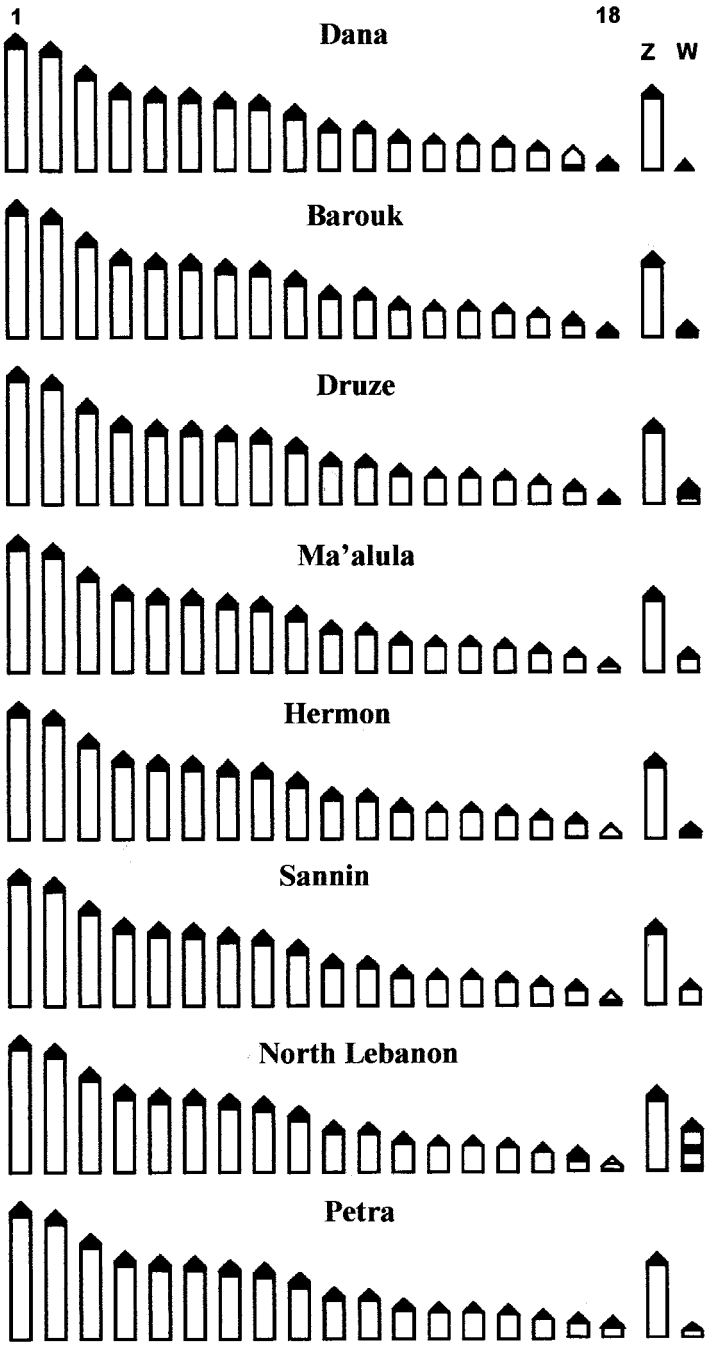

Figure 7. Ideogram summarizing the karyological characters of the various *L. kulzeri* forms.

data are more informative. The different *L.* cf. *kulzeri* populations showed clear differences in W chromosome morphology, in heterochromatin content, and in the amount and composition of the heterochromatin of the microchromosomes and the smallest macrochromosomes. The more primitive form of W chromosome was found in the specimens from Northern Lebanon, which are also

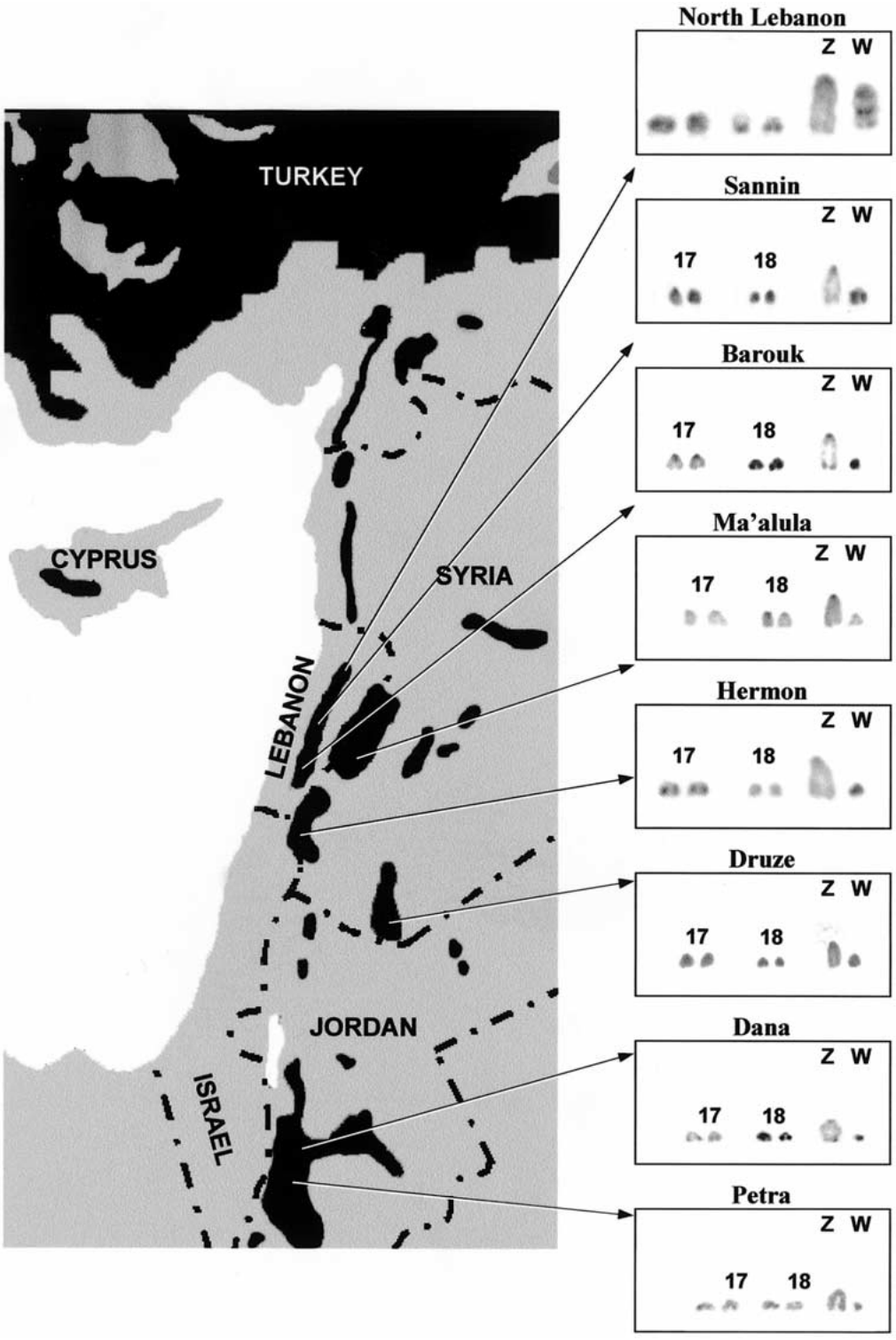

Figure 8. Differences in sex chromosomes, microchromosomes and 17th pair chromosomes found in the different *L. kulzeri* populations.

ethologically the most pleisiomorph (in den Bosch & Zandee 2001). In the other populations, this chromosome seems to have undergone a progressive reduction in length as a result of sequential heterochromatin deletions and modifications. This reduction followed a slightly north-to-south trend (Figure 8), although it cannot be excluded that the deletion processes occurred independently in each population.

Either between *L. laevis* and *L. kulzeri* or among the various populations of the last species complex, obvious morphological, ethological and chromosomal differences are apparent, but, at the same time, their genetic distances are very short, and with regard to the *L.* cf. *kulzeri* populations inadequate to justify the lack of interbreeding.

The greater level of karyological variability in comparison with the genetic one suggests that chromosomal differences became fixed before the various species or populations began to accumulate genetic differences. This situation is reminiscent of King's model of chromosomal primary allopatric speciation, also observed in several saurians (King *et al.* 1982, King 1993). According to this model, multiple or sequential chromosome mutations arise in peripheral, isolated populations of species and become fixed because of intensive inbreeding favoured by modest vagility or by presence of territorial males. The population thus arisen spreads, and, in the case of secondary contact with the parental species and formation of a hybrid zone, genetic introgression is prevented by the adverse effect of chromosome differences on the hybrid's fertility.

L. laevis–L. kulzeri *complex – role of the chromosome change*

According to various authors (e.g. John 1981, King 1993), the chromosome rearrangements can act as a post-mating isolating mechanism only if they prevent or negatively affect the meiotic pairing and segregation of the hybrids. However, not all rearrangements are negatively heterotic (King 1993). In the case of the *L. laevis–L. kulzeri* complex, an important role might have been played by the differences in the sex chromosomes. Indeed, alterations in morphology and/or heterochromatin content of sex chromosomes are known to negatively impact on hybrids' fertility (Hewitt *et al.* 1989, Coyne & Orr 1989, McKee 1991, King 1993), probably due to the recognized involvement in meiotic pairing and segregation of these chromosomes (King 1993, McKee *et al.* 2000). Moreover, Thelma *et al.* (1988) have shown that, in *Nesokia indica*, individuals differing in the amount of sex chromosome heterochromatin were unable to reproduce under laboratory conditions. A situation very similar to one observed by us in *L. kulzeri*. In contrast, it is not easy to evaluate the possible consequence of quantitative and compositional variations of autosome heterochromatin that we have observed between the different *L. kulzeri* forms. Indeed, in several eukaryotes, heterochromatin is known to affect genetic variability (King 1993, Pimpinelli 2000); however, various reports also exist of normal hybrids' fertility between cytotypes possessing gross structural heterozygosity for the amount and localization of heterochromatin (John & King 1983, King 1993).

It has been generally observed that the mitochondrial genes evolve faster than most of the nuclear genes (Wilson *et al.* 1985, Matthee *et al.* 2001). Thus the level of variability which we have observed in the 12S and cytochrome b mitochondrial genes of *L. laevis* and *L. kulzeri* complex can be considered good evidence of the overall genetic variability of these species. Therefore, we can confidently conclude that the results of our research indicate that, in the separation between *L. laevis* and *L. kulzeri* and among the various populations of this last species, the chromosome rearrangements' fixation has preceded the accumulation of the genetic mutations and that some of these karyological differences might have collaborated to reinforce the barrier against hybridization between incipient species according to the King's model of chromosome primary allopatry.

In order to obtain additional information on genetic variability of the *L. laevis* and *L. kulzeri* complex, and to confirm the findings of the mtDNA analyses, we currently study the variation of several nuclear genes.

Acknowledgements

This work was supported by a grant from the P.R.I.N. 99 (Programma di Ricerca di Interesse

Nazionale) of the Ministero dell'Istruzione, dell'-Università e della Ricerca (M.I.U.R.).

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