Flow-cytometric analyses of intraspecific genome size variations in *Bacillus atticus* (Insecta, Phasmatodea)

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Abstract: The stick insect *Bacillus atticus* comprises several populations with different chromosome numbers that are distributed over a large range of the Mediterranean basin. Here we have analyzed the DNA content of nine diploid and three triploid populations by flow-cytometry. The mean genome size of the diploids showed a significant decrease from east to west, ranging from 5.29 ± 0.12 pg for the population from Crete (east) to 4.28 ± 0.10 pg for the population from Sardinia (far west). This longitudinal trend of a decrease in genome size from east to west was also found for the triploid populations (from 6.80 pg for the population in Turkey to 6.08 ± 0.01 pg for the population on the Isle of Rhodes). Differences in DNA content between populations belonging to the same species have been described in animals, but the evolutionary implications of these differences are as yet unclear. What emerges from the present study is a correlation between genome-size variations and geographic distribution. The adaptive nature of genome-size variations in response to environmental changes is discussed, and the class of DNA involved hypothesized.

Key words: C value, flow cytometry, genome size trends, intraspecific DNA variation, DNA classes.

Résumé : Le phasme *Bacillus atticus* comprend plusieurs populations qui montrent un nombre chromosomique variable et qui sont distribuées sur la plupart du bassin méditerranéen. Dans la présente étude, les auteurs ont analysé le contenu en ADN de neuf populations diploïdes et de trois populations triploïdes par cytométrie en flux. La taille moyenne du génome chez les diploïdes diminuait d'est en ouest, allant de 5,29 ± 0,12 pg chez la population de Crète (est) à 4,8 ± 0,10 pg chez la plus occidentale (Sardaigne). Cette tendance longitudinale de diminution de la taille du génome d'est en ouest a aussi été observée chez les populations triploïdes (de 6,80 pg en Turquie jusque 6,08 ± 0,01 pg sur l'Île de Rhodes). Des différences intraspécifiques quant au contenu en ADN ont été décrites chez les animaux mais leurs implications évolutives demeurent nébuleuses. Il ressort de cette étude une corrélation entre les variations de la taille du génome et la distribution géographique. La plasticité adaptative que confèrent des variations du contenu en ADN face à des changements environnementaux ainsi que la classe d'ADN qui est vraisemblablement impliquée sont discutées.

Mots clés : valeur C, cytométrie en flux, tendances quant à la taille du génome, variation intraspécifique du contenu en ADN, classes d'ADN.

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Introduction

The mass of DNA in an unreplicated haploid genome (i.e., a sperm nucleus) is known as the C value (Swift 1950) or genome size (Hinegardner 1976). The genome size is a species-specific character upon which several evolutionary assumptions have been based and that are still a matter of debate (reviews in Cavalier-Smith 1985; John and Miklos 1988). Although it is a common belief that the nuclear DNA content is constant within a species or that any variation would be negligible (Hinegardner 1968; Olmo 1983; Cavalier-Smith 1985), several recent papers have claimed the existence and pointed to the importance of intraspecific variations in both plants (Price at al. 1980; Laurie and Bennet 1985) and animals (Rao and Rai 1987; Gold and Amemiya 1987; Ferrari and Rai 1989; Ragland and Gold 1989; Alvarez-Fuster et al. 1991; Lockwood et al. 1991; Lockwood and Derr 1992; Ruedas et al. 1993), that are presumably caused by gains or losses in various classes of DNA, including satellite DNA.

Within plant-species groups, the geographic, ecological, and taxonomic distributions of DNA content have been shown to be nonrandom; they can evolve in response to stress-related climatic changes through time, and they can be selected (Price 1988). Differences in genome size are mainly the result of changes in C-heterochromatin amounts caused by adaptation to environments (McMurphy and Rayburn

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Table 1. DNA content of somatic nuclei (midgut) in *Bacillus atticus* populations.

Subspecies	Source	No. of specimens examined	No. of cells examined	Estimated 2C DNA value ± SE (pg)	Chromosome number
B. atticus atticus	Italy (Sardinia)	3	40 000	4.28±0.10	34
	Italy (Sicily)	3	40 000	4.31±0.13	34
	Italy (Apulia)	3	40 000	4.79±0.15	34
	Croatia	3	40 000	4.33±0.02	34
	Greece	4	40 000	4.61±0.06	34
	Greece (Crete)	3	40 000	5.29±0.12	34
B. atticus cyprius	Cyprus	3	40 000	4.90±0.14	32
B. atticus carius	Turkey	2	40 000	5.13±0.01	34
	Israel	2	40 000	5.12±0.06	34
	Turkey	3	40 000	6.80[a]	50
	Greece	2	40 000	6.23±0.01	50
	Rhodes	3	40 000	6.08±0.01	49

[a]Standard error (SE) not calculated.

1992). Genome size changes can also be the result of changes in the amount of repetitive DNA (Ceccarelli et al. 1995); typically these DNA sequences have no coding function, so the effect of deletion or addition of their genetic material is primarily nucleotypic. On the whole, the dynamics or patterns of change in genome size at lower hierarchical levels, especially at and around the species level of differentiation, remain poorly understood. Whether or not changes in the genome size of an organism are of an adaptive nature remains an open question.

In animals, there are numerous reports of inter- and intraspecific genome size differences that are due primarily to the amount of highly repeated DNA (Rees et al. 1976; Rao and Rai 1987; Black and Rai 1988); a direct correlation between total DNA content and C-heterochromatic DNA is a general rule for primate species (Manfredi Romanini et al. 1991; Ronchetti et al. 1993). On the contrary, in birds, a larger genome size is associated with increases in single-copy DNA that are larger than the increases found in repetitive DNA (Olmo et al. 1989). In tenebrionids, there are no significant correlations between the genome size and the C-band heterochromatin percentage; the evolutionary variations in their genome size appear therefore to have affected both euchromatin and heterochromatin (Juan and Petitpierre 1989). Accordingly, an evolutionary decrease in genome size from an ancestor with a large genome could even result in a loss of single-copy sequences (Schmidtke and Epplen 1980).

In stick insects, the DNA content per nucleus is known in few species, and only in the genus *Bacillus* has flow-cytometry been used to measure the genome size (Nagl and Schaffner 1981; Marescalchi et al. 1990).

This paper presents data on nuclear DNA content in 12 populations of *B. atticus*. This taxon is a complex of thelytokous obligate parthenogenetic strains and includes both diploids ($2n$ = 32–34, XX) and triploids ($3n$ = 48–51, XXX). They are differentiated into three zymoraces: the diploid *Bacillus atticus atticus*, comprising most of the Greek and all the Croatian and Italian populations; *Bacillus atticus cyprius* ($2n$ = 32, XX), represented by the Cyprian populations; and *Bacillus atticus carius*, embodying diploid and triploid Turkish and triploid Greek populations (Mantovani and Scali 1993; Mantovani et al. 1994).

The relative constancy in chromosome number ($2n$ = 34) and morphology in the majority of populations, the lack of variation in the C-banding pattern (Marescalchi and Scali 1997), and the wide distribution area, make *B. atticus* an interesting species in which to study the extent of intraspecific changes in nuclear DNA amount and its possible evolutionary role.

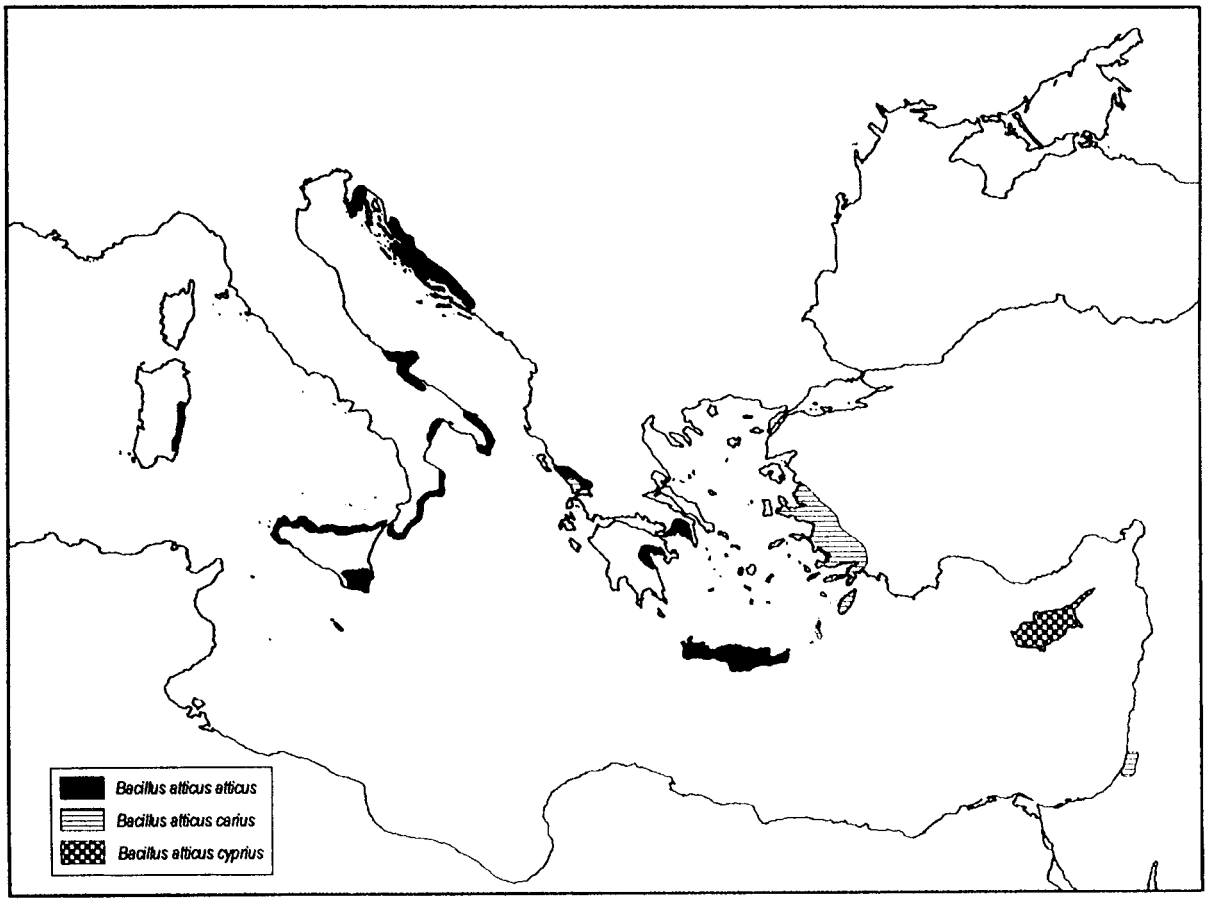
Materials and methods

Genome-size evaluation by flow cytometry was carried out on 12 populations of *B. atticus*. Table I lists the samples studied; subspecies, source, number of analyzed specimens and cells, DNA content in picograms (with relative standard error), and chromosome number are reported for each sample. Zymorace distributions are given in Fig. 1. On the basis of geographic location, some Israeli specimens ($2n$ = 34) are also included in the *B. atticus carius* zymorace, although no gene–enzyme analysis has actually been carried out.

Methods of cell preparation, staining, flow-cytometric analysis, and genome-size evaluation were described in detail by Marescalchi et al. (1990). In short, cells of adult female midintestine were stained by a propidium iodide solution and analyzed with a FACstar flow cytometer (Becton & Dickinson). Mouse (*Mus musculus*) thymocytes, with a reported genome size of 6.80 pg (Manfredi Romanini 1985), were stained simultaneously, and not only served as an internal standard in all cases, but their value was also used to convert relative stick insect DNA values to absolute amounts in picograms. For each insect, a sample of 40 000 cells was measured. The recorded measurements were reprocessed by a Hewlett Packard 217 personal computer, which also provided histograms with 256 frequency classes (channels) on the *x*-axis and the number of cells analyzed on the *y*-axis.

Statistical analyses over all diploid individuals were performed using the SPSS Professional Statistics 6.1 and the SAS Statistical Analysis System. The one-way analysis of variance (ANOVA) with absolute DNA values as the independent variable was carried out to test for heterogeneity among geographic locations. Duncan's multiple range test was applied to establish different groupings, and regression analysis was used to test for correlation between DNA content and longitude.

Fig. 1. Map of the Mediterranean sea basin showing the ranges of *B. atticus* zymoraces.



Results

The mean genome sizes of populations with diploid chromosome numbers ranged from 5.29 ± 0.12 pg for the Cretan sample to 4.28 ± 0.10 pg for the Sardinian sample (Table 1). Within these extreme values, a graded series of DNA contents was observed and Figs. 2–10 visualize the distributions obtained from all samples.

DNA-content peaks of triploids from Turkey always overlapped with the 2C mouse thymocytes, invariably giving a DNA value of 6.80 pg. On the contrary, triploids from the Greek mainland and the Isle of Rhodes showed a decrease in average DNA content, measuring 6.23 ± 0.01 pg and 6.08 ± 0.01 pg, respectively (Figs. 11–13).

The mean DNA content across the 9 strains of diploid *B. atticus* examined was 4.78 ± 0.50 pg. The range of genome-size variation found over all populations was 1.01 pg, which is equal to 21.13% of mean genome size.

The one-way ANOVA of the same diploid specimens evidenced a significant heterogeneity in mean genome size among populations (F = 13.18; df = 8, 17; P <0.0001). The results of Duncan's multiple range test on the same samples are shown in Table 2; DNA averages of the 9 populations form a continuous, overlapping series of groupings.

Significant heterogeneity in mean genome size was also found among different geographic locations: the linear regression analysis of longitude against genome size displayed a significant correlation (r = 0.737; P <0.001) (Fig. 14).

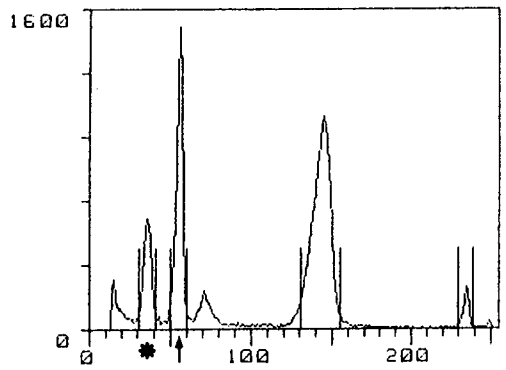
Table 2. Results of Duncan's multiple range test on the distribution of DNA values of diploid *Bacillus atticus* populations.

Source	Mean DNA value* (pg)
Greece (Crete)	5.29*a*
Turkey	5.13*ab*
Israel	5.12*ab*
Cyprus	4.90*bc*
Italy (Apulia)	4.79*bc*
Greece	4.61*cd*
Croatia	4.33*d*
Italy (Sicily)	4.31*d*
Italy (Sardinia)	4.28*d*

*By Duncan's multiple range test, mean DNA values followed by the same letter are not significantly different at P = 0.05.

Discussion

Since all but one of the diploid populations have the same karyotype with $2n$ = 34 chromosomes (Marescalchi and Scali 1997), most or all of the genome-size changes reported above are not due to chromosomal aneuploidy. Therefore, such a significant genome size variation (averaging 21.13%) within diploid *B. atticus* populations was quite an unex-



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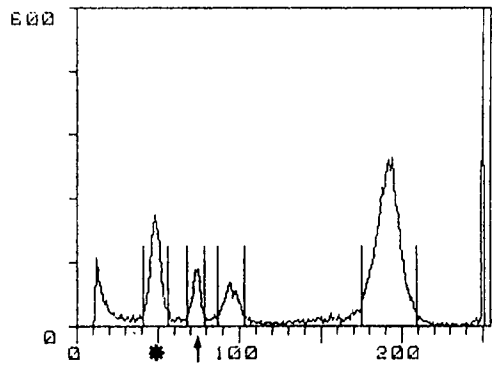

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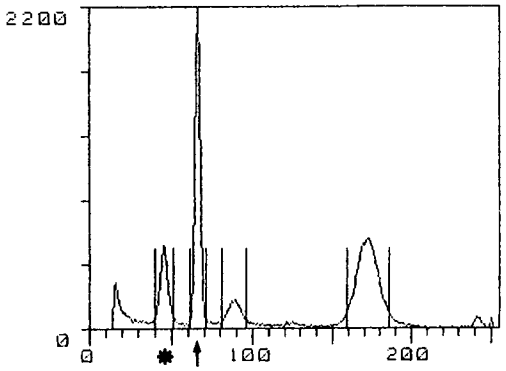

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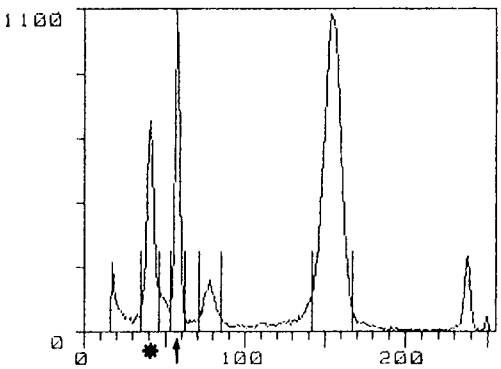

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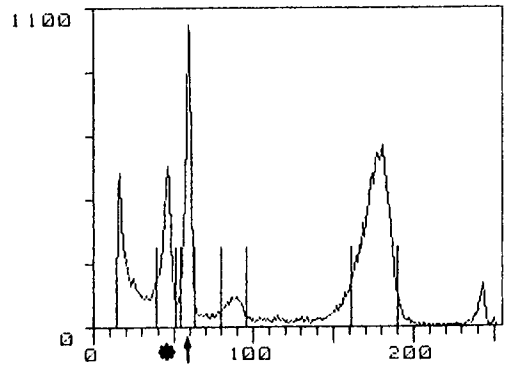

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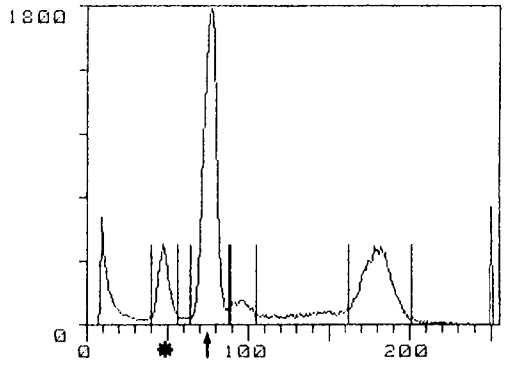

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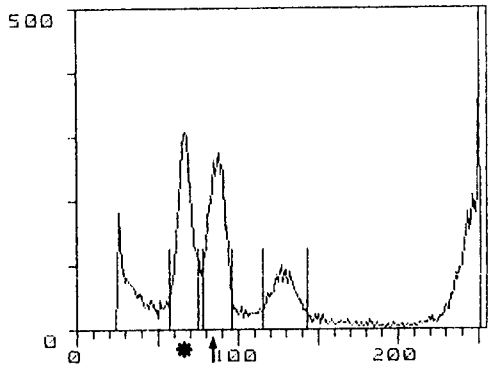

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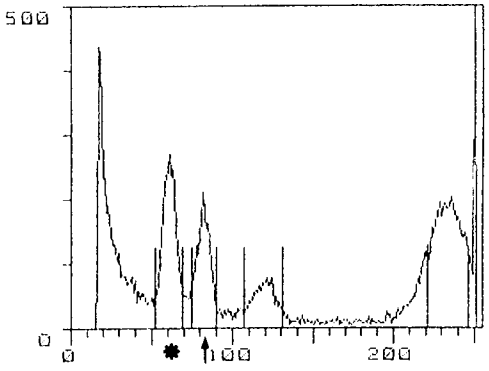
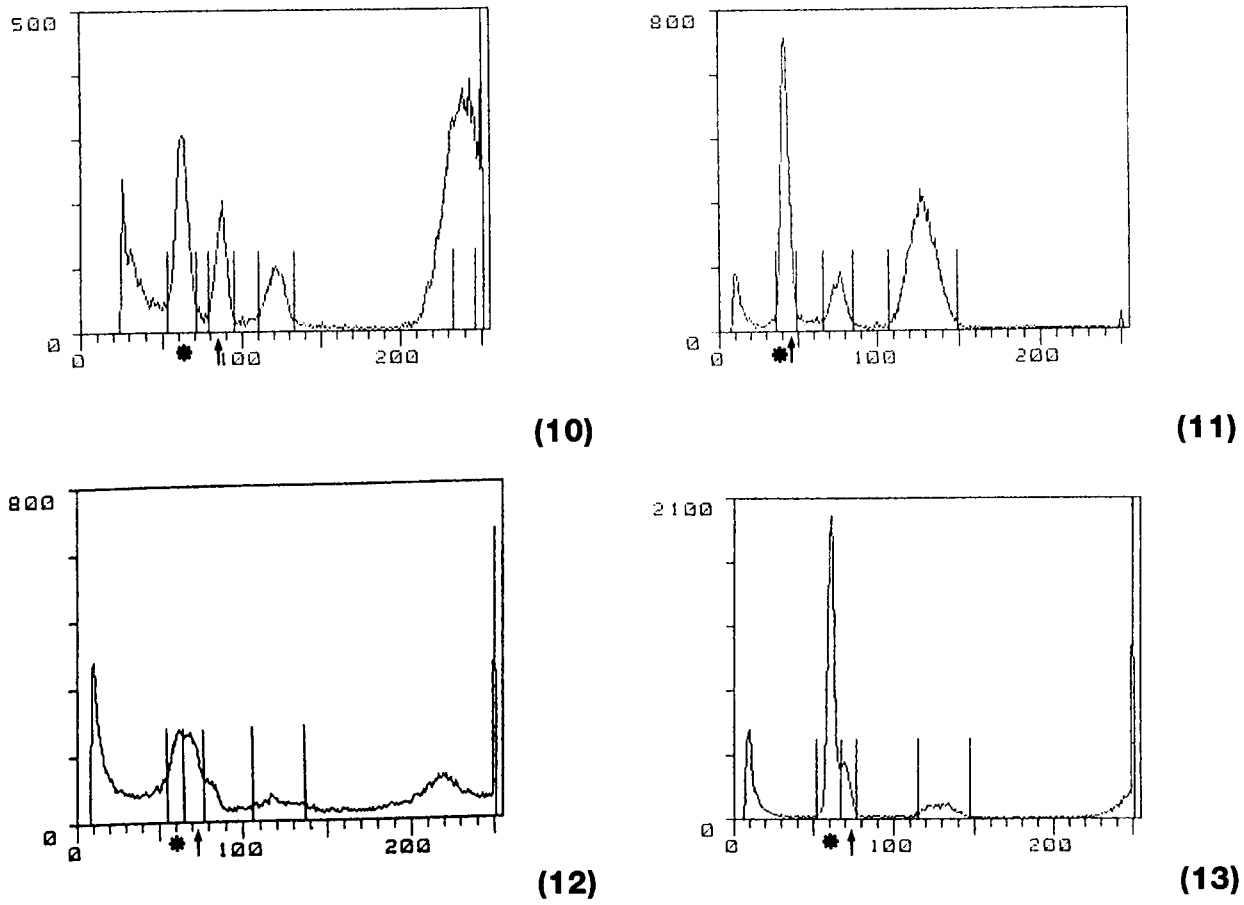


(9)

Figs. 2–9. Histograms showing the distribution of 2C mouse thymocytes (↑) and 2C gut cells (*) in *Bacillus atticus* populations with $2n = 34$. The *x*-axis gives the 256 computer channels corresponding to the class frequency (DNA amount); the *y*-axis gives the number of cells analyzed per channel. In each histogram, the additional barred areas correspond to 4C and 8C *Bacillus* cells. Figures 2–7 refer to *B. atticus atticus* from Sardinia, Sicily, Croatia, Apulia, Greece, and Crete, respectively. Figures 8 and 9 refer to *B. atticus carius* from Turkey and Israel, respectively.

Figs. 10–13. Histograms showing the distribution of 2C mouse thymocytes (↑) and 2C gut cells (*) in *B. atticus cyprius* (Fig. 10) and *B. atticus carius* from Turkey (Fig. 11), the Greek mainland (Fig. 12), and the Isle of Rhodes (Fig. 13).

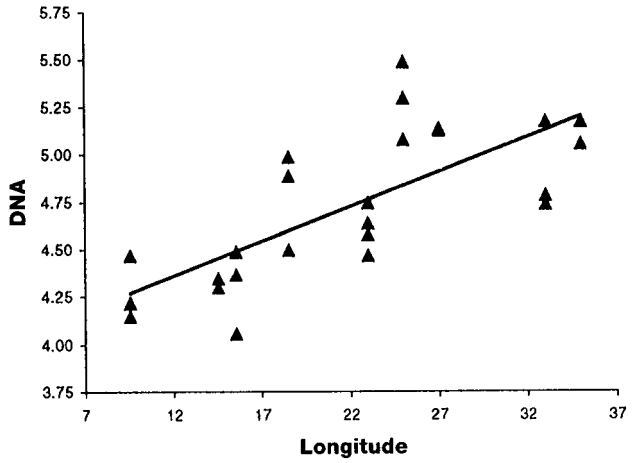


pected finding. The result is even more surprising if we consider the very similar chromosome C-banding pattern of all populations and the overall genetic similarity of these parthenogenetic strains (Mantovani and Scali 1993; Mantovani et al. 1994, 1997; Marescalchi and Scali 1997). In addition, linear regression analysis shows that DNA amounts undergo a significant gradual decrease from the eastern to the western populations. Bier and Muller (1969) measured genome sizes of a variety of insects and found that primitive groups had larger genomes than more recently evolved ones. Our results corroborate the general trend of DNA-content shift from higher to lower values (Marescalchi et al. 1990), and further suggest that the eastern Mediterranean basin, where *B. atticus* is widely distributed, is presumably the center of its origin with the most ancient populations. Accordingly, Italian and Croatian demes, which show the lowest DNA amounts, would be the most recent ones. These DNA variations among geographically distant populations with karyotypically identical chromosomes agree with what has been observed in other insects, such as grasshoppers and *Aedes* mosquitoes (Rees et al. 1978; Rao and Rai 1987).

We would like to emphasize that even the DNA value of *B. atticus cyprius*, in spite of its repatterned karyotype with 32 chromosomes, fits the east–west trend very well. The repatterning of the *B. atticus cyprius* karyotype occurred through extensive translocation events of both the Robertsonian and non-Robertsonian type (Marescalchi 1989), which apparently caused no further DNA loss. Chromosome rearrangements without detectable changes in DNA amount have actually been observed in odonate insects and bats (Burton et al. 1989; Mola and Papeschi 1994).

As far as the size of intraspecific DNA variation is concerned, the 21.13% amount that we observed in conspecific stick insects is higher than the intraspecific values of 13% reported for cyprinids (Gold and Price 1985) or the 15.5% reported for salmonids (Lockwood and Derr 1992). However, intraspecific variation as high as 31.9% has been observed in *Trachemys* turtle species (Lockwood et al. 1991)

Fig. 14. Linear regression of longitude (abscissa) to genome size (ordinate) in diploid *B. atticus* populations. Apparently a positive correlation (r = 0.737, P <0.001) exists between the two parameters.



and as much as 35% has been observed in pocket gophers of the genus *Thomomys* (Sherwood and Patton 1982).

There are two models of variation that can account for the evolutionary differences in genome size. The first one is characterized by a discontinuous variation in genome size, the second one assumes small, continuous, and overlapping changes. When the genome size is affected by many small changes, these are independent in occurrence and their effects are additive (Sokal and Rohlf 1981).

Within *B. atticus*, Duncan's multiple range test revealed that genome-size distribution in 9 distinct populations appears continuous and overlapping, clearly suggesting that differences among these populations accrued by small cumulative changes (Gold and Amemiya 1987; Rao and Rai 1987). Nonetheless, the latter caused, on the whole, a significant intraspecific heterogeneity.

Repetitive DNA sequences form a substantial fraction of the genome of many eukaryotes. This class includes highly repeated satellite DNA, minisatellite and microsatellite sequences (moderately repetitive, tandemly arranged), and transposable elements. Satellite sequences are typically organized in the heterochromatic regions of chromosomes, whereas microsatellite and minisatellite sequences are commonly found in euchromatic regions of the genome. Much of the dispersed moderately repeated DNA of eukaryotes appears to consist of transposable elements, which are nonheterochromatic sequences capable of inserting copies of their own into new genomic locations (Charlesworth et al. 1994).

Repetitive DNA sequences in *B. atticus* were studied by Mantovani et al. (1997): a significant contribution (2–5%) to the genome size is made by the AT-rich satellite Bag320 tandemly arranged sequences located in the pericentromeric heterochromatin. Bag 320 sequences could not be discriminated in geographically isolated and quite distant populations of *B. atticus*, even when belonging to the different zymoraces. If we consider that C-heterochromatin amounts appear to remain constant in all populations, then the fluorometrically detected variations of DNA should be related to nonheterochromatic fractions of the genome. Within plants, it has been reported that geographical clines of decreases in genome size may respond to environmental adaptations (McMurphy and Rayburn 1992); in *B. atticus* however, ecological features do not seem to vary with longitude. In addition, body structures and life history do not appear to be more specialized in western regions of the species' range; therefore, at present, the decrease in nuclear DNA content can be explained neither by obvious adaptations to environment, nor by specialization trends (Hinegardner 1976; McMurphy and Raiburn 1992). Thus, at present, the adaptive value of the *B. atticus* DNA cline remains a completely unanswered question.

Obviously, populations with the highest chromosome number (3*n*) show the largest genome sizes. Even within them we observed a decrease in DNA content triploid specimens of *B. atticus carius* from Turkey show a DNA content of 6.8 pg, those from the Greek mainland one of 6.23 pg, and those from Rhodes one of 6.08 pg. All are much below the expected value, which would be obtained by the addition of a haploset amount to syntopic diploids. A higher chromatin packing ratio in polyploid cells could contribute to the observed decrease in DNA content, and the loss of one chromosome could further explain the DNA decrease recorded for the triploid specimens from Rhodes (Marescalchi 1989), however, a superimposed geographic cline appears to occur as well.

Finally, in view of the report that, in tenebrionid insects, evolutionary changes in genome size appear to have affected both heterochromatin and euchromatin (Juan and Petitpierre 1989), even single-copy DNA cannot be ruled out when considering factors contributing to the shaping of the genome size (Schmidtke and Epplen 1980). We are now beginning a molecular screening of distinct *B. atticus* strains, in order to determine what type of DNA could account for the reported intraspecific variability of genome size in these stick insects, although some kind of repetitive class would appear to be the most likely candidate.

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