The *Bag*320 Satellite DNA Family in *Bacillus* Stick Insects (Phasmatodea): Different Rates of Molecular Evolution of Highly Repetitive DNA in Bisexual and Parthenogenetic Taxa

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The *Bag*320 satellite DNA (satDNA) family was studied in seven populations of the stick insects *Bacillus atticus* (parthenogenetic, unisexual) and *Bacillus grandii* (bisexual). It was characterized as widespread in all zymoraces of *B. atticus* and in all subspecies of *B. grandii*. The copy number of this satellite is higher in the bisexual *B. grandii* (15%–20% of the genome) than in the parthenogenetic *B. atticus* (2%–5% of the genome). The nucleotide sequences of 12 *Bag*320 clones from *B. atticus* and 17 from *B. grandii* differed at 13 characteristic positions by fixed nucleotide substitutions. Thus, nucleotide sequences from both species cluster conspecifically in phylogenetic dendrograms. The nucleotide sequences derived from *B. grandii grandii* could be clearly discriminated from those of *B. grandii benazzii* and *B. grandii maretimi* on the basis of 25 variable sites, although all taxa come from Sicily. In contrast, the *Bag*320 sequences from *B. atticus* could not be discriminated accordingly, although they derive from geographically quite distant populations of its three zymoraces (the Italian and Greek *B. atticus atticus,* the Greek and Turkish *B. atticus carius,* and the Cyprian *B. atticus cyprius*). The different rate of evolutionary turnover of the *Bag*320 satDNA in both species can be related to their different modes of reproduction. This indicates that meiosis and chromosome segregation affect processes in satDNA diversification.

Introduction

Stick insects of the holomediterranean genus *Bacillus* have been widely investigated through analyses of morphology, allozyme variability, cytology, and breeding features. These approaches led to the definition of the taxa of this genus and to an understanding of their phylogenetic relationships. There are bisexual species, nonhybrid parthenogens, and unisexual interspecific hybrids. Taxonomically, two species groups can be identified: the first includes only *B. rossius,* whereas the second consists of the bisexual *B. grandii* and the parthenogenetic *B. atticus* (Bullini 1994; Scali et al. 1995). Hybridogenetic strains (*B. rossius-grandii*) and parthenogenetic hybrids (*B. whitei, B. lynceorum*) are, of course, in between (Scali et al. 1995).

Of the three nonhybrid species, the bisexual *B. rossius* (2n = 36, XX female; 35, X0 male) covers most of the western Mediterranean area with eight races mainly defined by allozyme distances (zymoraces). Numerous facultatively parthenogenetic demes are found mainly in the peripheral areas where *B. rossius rossius* and *B. rossius redtenbacheri* subspecies spread (see Scali et al. 1995).

The strictly bisexual *B. grandii* (2n = 34, XX female; 33, X0 male) is differentiated into three formal subspecies which are located in isolated areas of the Sicilian region. *Bacillus grandii grandii* occurs only as a relict population in the southeastern corner of Sicily. The few *B. grandii benazzii* populations range over a very narrow area near Scopello (northwestern Sicily)

and on Levanzo Island (Egadi Archipelago). The only known population of *B. grandii maretimi* is endemic to Marettimo Island (Egadi Archipelago). The three races share the same karyotype, with a common pattern of abundant pericentromeric heterochromatin but slight differences in positions (Marescalchi and Scali 1990). Allozyme analyses of over 20 loci revealed that Nei's *D* between the races ranges from 0.11 to 0.27; scanning electron microscopy of eggs and bodies also supports a clear racial differentiation (Mantovani and Scali 1993*a*; Mantovani, Scali, and Tinti 1991, 1992).

The third species, *B. atticus,* is an all-female parthenogenetic taxon differentiated into three races on the basis of allozyme data and karyotype analysis. *Bacillus atticus atticus* (2n = 34) occurs with only diploid demes in the central Mediterranean basin (Sardinia, Sicily, South Italy, Croatia, and Greece). *Bacillus atticus carius* includes Greek and Turkish triploid demes (3n = 48–51) and a single diploid Turkish population (2n = 34). *Bacillus atticus cyprius* (2n = 32), endemic to Cyprus, is differentiated from the other two zymoraces by several karyological features (Scali and Marescalchi 1987; Marescalchi and Scali 1997). Nei's *D* between pairs of the three races ranges from 0.08 to 0.21 (Mantovani and Scali 1993*b*; Mantovani, Tinti, and Scali 1995).

The low vagility of these apterous insects and their very wide range point to a great antiquity of the species. In interspecific comparisons, *B. grandii* and *B. atticus* share numerous morphological and karyological features; they have a Nei's *D* mean value of 0.35. On the other hand, the high degree of differentiation between *B. grandii* and *B. atticus* versus *B. rossius* is demonstrated by several diagnostic morphological characters, very high values of Nei's *D* (averaging 1.8) and clear differences in number, shape, and structure of their chromosomes (Scali et al. 1995).

Preliminary molecular investigations on highly repetitive satellite DNA (satDNA) of the unisexual *B. at-*

Abbreviation: satDNA, satellite DNA.

Key words: *Bacillus,* concerted evolution, highly repetitive DNA, satellite DNA, stick insects.

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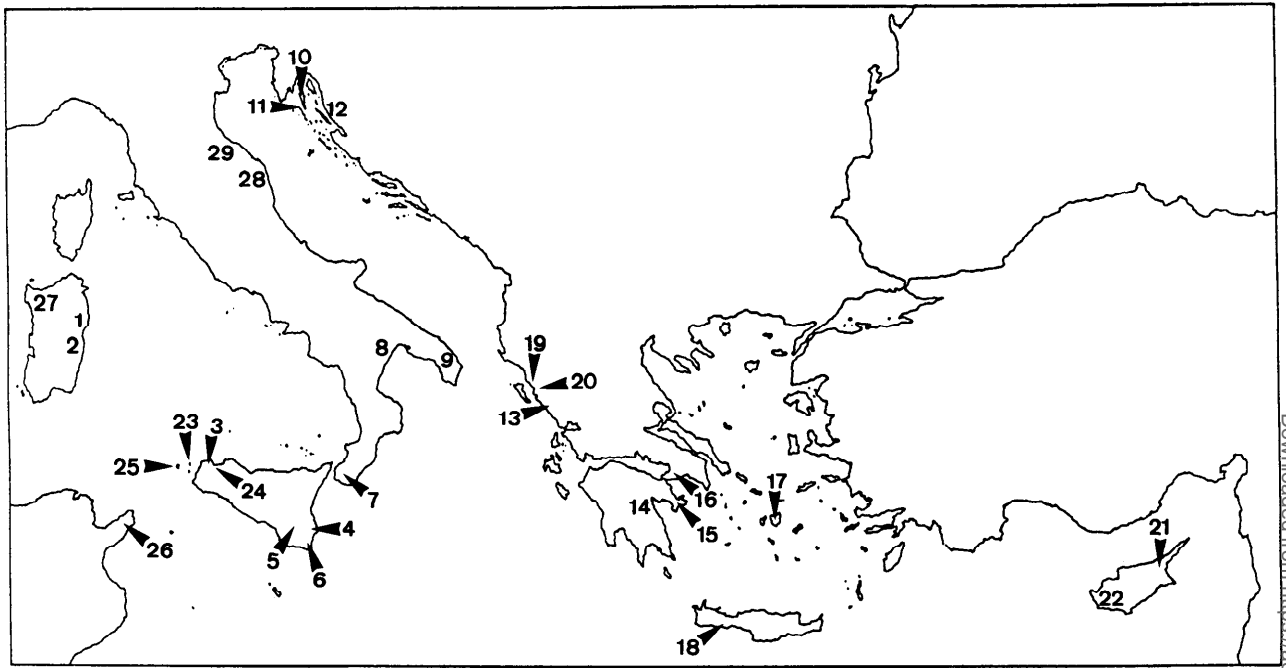
FIG. 1.—Map of the Mediterranean basin showing the collecting sites of the *Bacillus* taxa: *B. atticus atticus* (1–18); *B. atticus carius* (13, 19, 20); *B. atticus cyprius* (21, 22); *B. grandii grandii* (5); *B. grandii benazzii* (23, 24); *B. grandii maretimi* (25); *B. rossius* (24, 26–29).

ticus atticus (Vendicari population) allowed the characterization of the specific *BaB*300 satDNA family. This study also provided evidence of the presence of the *BaB*300 family in the related *B. grandii grandii*. Radioactive in situ hybridization has shown that this family is located at the centromere of a chromosome subset in *B. atticus* (Mantovani et al. 1993), whereas the centromeric regions of all chromosomes are marked in *B. grandii* (unpublished data). However, homologous repeats were not found in the *B. rossius* genome either by blot hybridization or by in situ hybridization analyses (Mantovani et al. 1993; unpublished data). These features indicate that *B. atticus* and *B. grandii* are certainly closely related phylogenetically, although various mechanisms have been suggested to explain the origin and evolution of the all-female *B. atticus* complex (Mantovani and Scali 1993a, 1993b; Scali et al. 1995; Marescalchi and Scali 1997).

Noncoding, highly repeated satDNAs have been successfully used as a tool for taxonomic and phylogenetic investigations within invertebrate groups such as *Caenorhabiditis* (La Volpe 1994), *Artemia* (Badaracco et al. 1987, 1991), *Dolichopoda* (Bachmann, Venanzetti, and Sbordoni 1994, 1996), *Chironomus* (Rovira, Beerman, and Edstrom 1993), *Drosophila* (Bachmann, Raab, and Sperlich 1989, 1990; Bachmann et al. 1992; Bachmann and Sperlich 1993) *Tribolium* (Juan et al. 1993; Plohl et al. 1993), and a few echinoderms (Sainz, Azorin, and Cornudella 1989; Sainz and Cornudella 1990). In the case of *Artemia* species, it has been suggested that the occurrence of two different satDNA families seems to be more related to the mode of reproduction than to the geographic distribution of taxa (Badaracco et al. 1987, 1991).

It is widely agreed that a variety of mechanisms affect the mode of evolution of satDNA (Charlesworth, Sniegowski, and Stephan 1994), resulting in a high level of intraspecific sequence similarity that frequently contrasts a high level of interspecific diversity (Dover and Tautz 1986).

This paper deals with a detailed analysis of the variation of a specific satDNA family which has been found in the bisexual *B. grandii* as well as in the unisexual, closely related taxon *B. atticus*. Comparison reveals the effect of the mode of reproduction on the evolution of satDNA, which, so far, is barely understood. According to the computer simulations of Stephan (1989), it is expected that after a certain period of time population-specific differentiation should occur in sexually reproducing organisms but not in parthenogenetic ones given that meiotic unequal crossing over plays a major role in satDNA evolution. In other words, we could possibly obtain some information to further our understanding of the molecular mechanisms leading to satDNA diversification and, in particular, to evaluate the role that Mendelian reproduction plays in the processes of homogenization and fixation during the evolution of repeated DNA families.

Materials and Methods
Sampling

Individuals belonging to distinct populations of *Bacillus* stick insects were collected during the years 1990–1994 from the following Mediterranean locations (fig. 1): *B. atticus atticus*—Cala Gonone (1), Siniscola (2), San Vito lo Capo (3), Cugni (4), Noto (5), Vendicari (6), Scilla (7), Castellaneta (8), Alimini (9), Cherso (10),

Mali Losinj (11), Borik (12), Parga (13), Argos (14), Epidavro (15) Eleusis (16), Paros (17), Paleochora (18); *B. atticus carius*—Parga (13), Neraida (19), Igoumenitsa (20); *B. atticus cyprius*—Agios Georgios (21), Episkopi (22); *B. grandii grandii*—Noto (5); *B. grandii benazzii*—Levanzo Island (23); Scopello (24); *B. grandii maretimi*—Marettimo Island (25); *B. rossius*—Korbous (26), Sassari (27), Scopello (24), Francavilla (28), Fano (29).

Field-collected specimens were reared in the laboratory in aerated cages on fresh food-plants (bramble or lentisk). Taxonomic identification of specimens was carried out through morphological, allozymic, and karyological analyses. Bodies of field-collected specimens were frozen and stored at −80°C until used for molecular investigations.

Cloning of Satellite DNA

Genomic DNA was prepared from single or a few pooled animals according to the method described by Preiss, Hartley, and Artavanis-Tsakonas (1988). Genomic DNAs of *Bacillus* species were screened for restriction satDNA by endonuclease digestion and subsequent gel electrophoresis. A ladderlike pattern of prominent bands in the background smear indicated the presence of a tandemly repeated satDNA. The following restriction enzymes were applied: *Ava* I, *Bam*HI, *Bcl* I, *Bgl* I, *Cfr*10, *Cla* I, *Dde* I, *Dpn* I, *Dra* I, *Eco*RI, *Eco*RV, *Hae* III, *Hind*II, *Hind*III, *Kpn* I, *Msp* I, *Mva* I, *Nde* II, *Not* I, *Nsi* I, *Pst* I, *Pvu* II, *Rsa* I, *Sac* I, *Sal* I, *Sau*3A, *Sca* I, and *Sma* I. *Alu* I, *Bgl* II, and *Taq* I were already known to produce such a ladderlike pattern in *B. atticus* from an earlier study (Mantovani et al. 1993). SatDNA fragments were extracted from agarose gels using Jetsorb kit (Genomed), ligated to the appropriate plasmid vector, i.e., pGEM 3, pGEM 7, pSP70 (Promega), or pUC19 (BRL), and used to transform *E. coli* DH5α competent cells. Recombinant clones were identified using either the β-galactosidase gene blue-white color system or colony hybridization (Sambrook, Fritsch, and Maniatis 1989).

Standard Molecular Techniques

DNA was transferred to nylon membranes (Boehringer Mannheim) according to the protocol of Southern (1975). Labeling of probe DNA, filter hybridizations, and detection of the hybridization signals were performed as described in the manual "DIG DNA labeling and detection kit–nonradioactive" (Boehringer Mannheim). Plasmid DNA was obtained using the Flexiprep Kit (Pharmacia). Both strands of at least three clones from each population were sequenced by the dideoxychain termination method (Sanger, Micklen, and Coulson 1977) using the Autoread sequencing kit and the A.L.F. automatic sequencer (Pharmacia).

Estimation of Relative Copy Number of Satellite DNA Sequences

Solutions containing defined amounts of denatured genomic DNA and denatured cloned satellite DNA fragments, respectively, were blotted on Hybond N membranes (Amersham) by a "Minifold II" apparatus (Schleicher Schuell). Cloned satDNAs were labeled with ^{32}P dCTP using the Rediprime kit (Amersham) and hybridized to the blotted DNA.

DNA Sequence Analysis

Nucleotide sequences were aligned using the program CLONE (1.0). Genetic distances were calculated according to Kimura's (1980) two-parameter method; constructions and plotting of phylogenetic dendrograms were performed by MEGA (1.0) of Kumar, Tamura, and Nei (1993).

Results

Genomic DNAs of individuals from seven populations of the stick insect species *B. atticus* and *B. grandii* were screened for the presence of tandemly repeated satDNAs by restriction digests and subsequent gel electrophoresis. A typical ladderlike restriction pattern was observed after treatment with *Alu* I, *Bcl* I, *Bgl* II, *Cla* I, *Dra* I, *Eco*RI, *Nsi* I, and *Taq* I in digests from individuals of all analyzed populations. Some stain-intensity differences were noticed in digests of different taxa with the same restriction enzyme. No ladderlike pattern was obtained for genomic DNA from *B. rossius* specimens. Filter hybridization experiments using *BaB*300 clones of Mantovani et al. (1993) supported these observations.

According to the restriction data the approximately 300-bp *Bgl* II fragments of *B. atticus* and *B. grandii grandii*, *Eco*RI fragments of *B. grandii benazzii*, and *Cla* I fragments of *B. grandii maretimi* were regarded as monomers of a tandemly repeated satDNA and chosen for cloning. Twenty-six clones were taken for further analysis: *pAAT/Epi2–4* (*B. atticus atticus*, Epidavro); *pACY/Epk1, 4, 12* (*B. atticus cyprius*, Episkopi); *pACA/Igo2–4* (*B. atticus carius*, Igoumenitsa); *pGG/Not2, 4, 10, 17, 20* (*B. grandii grandii*, Noto); *pGB/Sco1, 2, 4, 7, 8, 10* (*B. grandii benazzii*, Scopello); *pGB/Lev1, 4, 5* (*B. grandii benazzii*, Levanzo Island); *pGM/Mar9, 10, 12* (*B. grandii maretimi*, Marettimo Island). The nucleotide sequences of these clones are given in figure 2. The three sequences *pAAT/Ven1–3* of *B. atticus atticus* (Vendicari population) reported by Mantovani et al. (1993) were also added to the sample. All sequences were deposited in GenBank (accession numbers X74958, X74959, U79495–U79520, and U92526). Figure 2 indicates that all sequences belong to a single satDNA family. The most frequent sequence length is 316 bp, but length variants (ranging from 311 bp in *pAAT/Epi3* to 319 bp in pGB/Sco8) were observed for clones from both species. They result from different numbers of indels of one or two nucleotides. Owing to its presence in all *B. atticus* and *B. grandii* taxa, we more comprehensively termed this satDNA family *Bag*320 instead of *BaB*300, used by Mantovani et al. (1993).

A high degree of sequence similarity was observed among the *Bag*320 clones within each taxon, whereas sequence similarity in pairwise comparisons between taxa was generally lower (table 1). The *Bag*320 sequences

1200   Mantovani et al.

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         BglII                              ClaI  AluI                    BclI
                                            TaqI
              10        20        30        40        50        60        70        80        90
AAT/Ven1  AGATCTGTATGTGTTGATTTATTTCATTTCC*AATCTTATCGATTCAGCTTATTTTGGATATTTTACGTTTATATGATCAAGTAGATAAC
AAT/Ven2  -------G-----------G----A------*-T-------------------------------A-------A-A----------T--
AAT/Ven3  ------------------------------*TT--TA-------------------------------A--A----AT----A-----TT-

AAT/Epi2  ---------*-----A--------------*-T-T-----A----------------------A-------------------------TT-
AAT/Epi3  -----------------------------*-T---A-------------A-----T-T------T--A----T---------CTT-
AAT/Epi4  -------------A---------------*TT-A------------*---C--T--------A-------T----TA----TT-

ACY/Epk1  -----------------------------*-T--------------------G----------A-A----T----C----ATT-
ACY/Epk4  -----------------------------*-T-A-----A------------A-------A-A--AT--------CTT*
ACY/Epk12 ---------------A-------------*-T-AC---T----G------------C--T--------A-------T----AA----TT-

ACA/Igo2  --------------C---------A--A---*T-----------------------TA-A---------A--------TT-
ACA/Igo3  --------------------------*-T---------------A--------------T-A----A-T----AA----TTT-
ACA/Igo4  -------------------------*--------------T-----------TA----A--A----T-----C----TT-

GG/Not2   ----------T---------T----T-----*-T-TG----------A-------T-T-----C-GA--A---------T----AGC-
GG/Not4   -------A--T----------G-----*-T-TG------AG--------T--------A-A-------------TC-
GG/Not10  ----------T---------------T*-T-T----------------A--T-----A-A------------TC-
GG/Not17  ----------T---------------*-G-TG------G----------CT-T-----C--A--C----------T----AGC-
GG/Not20  ----------T-------G-------*-T-TG---A--G--C-------CT-T-----C--A--A---------T--C-GGC-

GB/Sco1   --T-------A---------*-T-T-CG----------AG------T-----C--A--A-----------T---TT-
GB/Sco2   T-T-------A---------*-T-T-C------------T---G--A--A------------TT-
GB/Sco4   T-T-------A---------*-T-T-C-----------TA---C--A--A------------TT-
GB/Sco7   --T-------A---------*-T-T--G----------A-------T-----C--A--A-----------TT-
GB/Sco8   --T------A---G--------A------*-T-T-CG--------A-T-----T-----A--A------------TT-
GB/Sco10  --T-------A---------*-T-T-C-----------T--A--C--A--A----------TT-

GB/Lev1   --T-------A---------*-T-T-CG----------T-----C--A--A----------TT-
GB/Lev4   --T-------A---------*-T-T-CG----------T-----C--A--A------T--*-----TT-
GB/Lev5   --T----A--A-----------AG-T-T-C--------T-----C--A--A----------TT-

GM/Mar9   --T-------A---------*-T-T-C--------G-A-----C-T--------A--A---------G---TT-
GM/Mar10  --T-------A---------*-T---C--------G-G------G--T-----C--A--A---------G-----A----TT-
GM/Mar12  ----------A--C---A------T-----A*-T-T--------------------T-----C--A--A------AT-G----T-TT-
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                                     DraI                    NsiI  DraI            EcoRI         DraI
              100       110       120       130       140       150       160       170       180
AAT/Ven1  ATTTCCA*CTCATTTA*ACTTCTTTCCTATGATTTAAA*TTGTTAT*AATTTATGCATTTAATCTGGTTTATT*CGGTTCTGTT*AAATA
AAT/Ven2  -------*T-------*----------------*G-----*-------A---------A---------*-C-------G*-----
AAT/Ven3  -------*--T----T*-G------*--T-------*G------*----C-----C--C---A----A-A-*----------------
AAT/Epi2  --A-T-T*--T-----*---------------*G-*-----*-----------A----------------*----------*-----
AAT/Epi3  ------*-A----T*----------T--------*G------*----CG-------C---A----A----*----------*-----
AAT/Epi4  ------*--T----T*----------T-------*G-TAAT*----C---------A----A-*----------*------A-*-----

ACY/Epk1  -----G-*--T----C*-G-----A-T-TG-----*G-A----*---GC----AA-C---A----A---*----------*-----
ACY/Epk4  -------*-A----TT-----G----T-------*G----A*----C-------T--A----A---*T----T--T-----
ACY/Epk12 -------*--------*----------A-------G---*-------*----AC----CC-C-A---A---*----------A-----

ACA/Igo2  ------T*--T----*----------------*G-----*----A---------A----T-*-*-T-------*-----
ACA/Igo3  ------*--T-C--T*----------*--T-T-----T-*G-TC---*----C---------A---A-*---A-----*-----
ACA/Igo4  ----T--A--T----T*-GC-----A--T----C---*G------*----C----A--C---A---A-*---------*-----

GG/Not2   ------T*-------C*-T--A----T-------T-*G------*----C--------AA-T--A---*-----A-T-**-----
GG/Not4   ------*-------C*----A-A----T-T----T-*G------*----C--------A-A---A---*-----T-**-----
GG/Not10  ------*-------C*----A-A----T-T----T-*G-A----*----C--------A-A-T--A---*-----T-**-----
GG/Not17  ---G--*-*-------T*----T--C---T----T-*G------*----C--------A-A--------*-----T-**-T----
GG/Not20  ------*-------C*----T------T-------T-*G------*----C--------A-A--A-A---T-C-----T-**-----

GB/Sco1   -A-----*--T----C*-GCC----T--TGC-----T-*G------*----C--------C*A-T-GA---*--T-----T-A*-G-C-
GB/Sco2   -G--T--*-------C*-G-*-----T--TAC-----T-*G------*----C--------CGA-T-GA---*--T-----T-A*-G-C-
GB/Sco4   -G--TT-*T-------C*-G-C----T--TAC-----T-*G------*----C--------C*A-T-GA---*--T-----T-A*-G-C-
GB/Sco7   -A-----*--A----C*-G-C----T--TAC-----T-*G------T----C--------C*A-T-GA---*--T-----T-A*-G-C-
GB/Sco8   -A-----*-------C*TG-C----T--TAC------AG----*--A-C----------A*A-T-GA---*--T-----T-A*-G-C-
GB/Sco10  -G-----*-------C*-G-*----T--TAC-----T-*G------*----C--------C*A-T-GA---*--T-----T-A*-G-C-

GB/Lev1   -A-----*-------C*-GCC----T--TGC-----T-*G------*----C--------C*A-T-GA---*--T-----T-A*-G-C-
GB/Lev4   -A-----*-------C*-G-C----T--TG------T-*G------*-C--C--------C*A-T-GA---*--T-----T-A*-G-C-
GB/Lev5   -G-----*-------C*-G-C----T--TAC-----T-*G------*----C--------C*A*T-GA---*--T-----T-A*-G-C-

GM/Mar9   -------*-------C*-G-C----T--TGC-----T-*G------*----C--------C*A-T-AA-A-*--T-----T-*-TG---
GM/Mar10  -------*-------C*-G-A----T--TGC-----T-*G------*----C--------C*A-T-AA---*--T-----T-*A-G---
GM/Mar12  -------*-------C*-G-*----T--TGC-----T-*G------*T---C--------T-C*A-T-AA---*--T----T-*A-G---
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Fig. 2.—Nucleotide sequences of *Bag*320 satDNA clones aligned with respect to populations. Asterisks indicate gaps (insertions/deletions) introduced to optimize the alignment. The recognition sites discussed in the text are indicated above the sequence. The *Bacillus* taxa are abbreviated as: AAT—*B. atticus atticus* clones; ACA—*B. atticus carius*; ACY—*B. atticus cyprius*; GG—*B. grandii grandii*; GB—*B. grandii benazzii*; GM—*B. grandii maretimi*.

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              190       200       210       220       230       240       250       260       270
AAT/Ven1  ACACTTATTTCATAATAAATGGGTAATTTTTATGGTTTATTATCAATTTTAACTTCGTTTTATTTATATTA*CCCTTTTAGTG*AGAAAC
AAT/Ven2  ---------------------------------T-----G----G----------------T--TC-----CA---T---*---C---G-GT*-AT--T
AAT/Ven3  --------------------G-----AA--T----A--------------------G-----A----------T---*---C----AG-T-----T

AAT/Epi2  ---------------------------T-----G-------------------*------C-C---------T-A-*AA-----*-GT*-----T
AAT/Epi3  ----------------T----------T----AG--C----C---------------TA----------T---A---A---C-GT*------
AAT/Epi4  ----------A--------------T-----G-------T-------------A--T----------T---*---CA--G-GT*-----T

ACY/Epk1  --G-----------G-----AA--T-----G-----G----------A---A---C---------T---*---C---G-G-*----TA
ACY/Epk4  ---------------------T-A-AA--T----T---------AAT----TA----------T---*A--A---T-G-T-----T
ACY/Epk12 --------------------AAA-T----GG-----------C--------TT-C-C------------T-AC*---C---G-G-A-----T

ACA/Igo2  ----------T-------------T----AG----A-------------TA---C--A-------T---*---C---G-GT*-----T
ACA/Igo3  --------------------AA--T-----A--T-CG-----------------C----------T---*---C---G-GT*-----T
ACA/Igo4  -------------------A-T-----G----A------------------C----------TA--*---C---G-GT*-----T

GG/Not2   G-GG-----------------T-----G---C-A----T-G------G*----A----------T---A-A-A----G-GT*-----T
GG/Not4   --------G-T--T-------T--T-----G---CA----ATCG-T---------TA------G-T---A-A-A-C-G-GT*-----T
GG/Not10  G------------T--------T-----G---CA----ATCGTT-------------G-T---A-A-A-C-G-GT*-----T
GG/Not17  ----------A-----------T-----G---C-A----TCG-----------A------T---A-A-A----G-GT*-----T
GG/Not20  --------A-A-----------T-----G---TC-A----TCG-----------A------T---A-A-A----G-GT*-----T

GB/Sco1   --T------------------T-T-----G---C---T---CTT-----------A------C-G-T---A-A-A---T-GT*-----T
GB/Sco2   --------------------T-----A---C------CTT----------A------C-G-T---A-A-A---T-GT*-----T
GB/Sco4   -------------------T-C--G---C-------CT-----------A------C-G-T---A-A-A---T-GT*-----T
GB/Sco7   ----C-----AT---------T-----G---C-------CTT-----------A------C-G-T---A-A-A---T-GT*-----T
GB/Sco8   ---------------------T----AG---A-------CTT----------AA-G---C-G-T---A-A-A---T-GT*------
GB/Sco10  ------------------T-----T-----G--TC-------CTT----A--A---A----T-C-G-T---ATA-A---T-GT*-----T

GB/Lev1   ---------------------T-----G---C-------CTT-----------A------C-G-T---A-A-A---T-GT*-----T
GB/Lev4   ----------G-------------T----AG---C-------CTT-----------G-AA-----C-G-T---A-A-A---T-GT*-----T
GB/Lev5   --------A------A------T-----G---C-------CTT-----------T-----C-G-T---A-A-A-T-GT*-----T

GM/Mar9   ----ACT-------T---------C-----GG--C-------CG-----------A------C--CT---A-A-AAC---GT*-----T
GM/Mar10  --------A------------T-----GG--C-------CGT----------TG-----C---T---A-A-AAC---GT*-----T
GM/Mar12  ------------T-C-------T-----GG--C-----C-GGTT----------A------C-G-T---A-A-A-----GT*-----A
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              280       290       300       310       320     329
AAT/Ven1  CATTTCATTACTATTGTAAAAAGTG**TTTTTGTTTGACGAATTAGTTTTCCGA**CTG
AAT/Ven2  ---------------------C--T--**----------A----------------**-A-
AAT/Ven3  ---------------------C-----**-----------------T---------**---

AAT/Epi2  -----TT-------------C--T--**-----*------------T---------**---
AAT/Epi3  ---------------------G-----**---------------------------**---
AAT/Epi4  A------------------C-----**------------------------------**---

ACY/Epk1  -------------------C-----**AA--*------T-T--------------**---
ACY/Epk4  -----T-------------C-----**----CT--G------A-----------**A--
ACY/Epk12 -------------------C-----**-------A------------------**---

ACA/Igo2  -------------------C-----**------------------------------**---
ACA/Igo3  -------------------C-----**A---------------------------**---
ACA/Igo4  ------G------------C-----**------------------------------**---

GG/Not2   ---C-----T---------C-----**---------AG--T--C--G--------**A--
GG/Not4   ---------T-----C---C-T-C-**-----A---G------C--G--------**A--
GG/Not10  ---------C---------C-----**---------G-----C--G--------**---
GG/Not17  -----A---T---------C-----**---------G-----C--G-------AAG--
GG/Not20  ---------T---------C-----**---------G-----C--G--------**GA-

GB/Sco1   G----T---T-AG------T-----**---------G---T---**A--
GB/Sco2   ---------T--G------CG----**-----------------G--T---**--
GB/Sco4   ---------T--G------CG----**----A-----------C---------T**---
GB/Sco7   T----T---T-AG--A---C-----**---------------G-------**---
GB/Sco8   G--------T-AG------C-----AA-----------------G-------**---
GB/Sco10  -----A---T--G------C-T---**-----------------G-------**---

GB/Lev1   G----T---T-AG------C--T--**---------------AG-------**---
GB/Lev4   G--------T-AG------C-----**---------------------G-----T--**---
GB/Lev5   ---------T--G------CG----**---------------G-------**---

GM/Mar9   T--------T--G------CG----**-----A---G-G-------G------G**--A
GM/Mar10  ---------T---------C-----**-------------------G-------**---
GM/Mar12  -----T---T---------CG----**------G-G---------T--------**A--
```

FIG. 2 *(Continued)*

are A+T rich (74% on average, over all clones). Out of the 329 positions in the alignment, 224 are variable at least once mainly by single-nucleotide substitutions, with transversions being more frequent than transitions (fig. 2). The sites of the eight endonucleases producing the Bag320 restriction satellite pattern were found in the sequences. The *Bgl* II and *Eco*RI sites are modified in most clones derived from *B. grandii benazzii* and *B. grandii maretimi* as well as in all those obtained from *B. atticus* and *B. grandii grandii,* respectively. A *Dra* I

Table 1
Minimum and Maximum Values of Pairwise Sequence Similarity (%) of Bag320 Satellite DNA Monomers Within and Between *Bacillus* Taxa

	B. atticus	B. grandii grandii	B. grandii benazzii	B. grandii maretimi
B. atticus	84.8–89.6	77.7–86.7	76.7–84.2	65.8–73.4
B. grandii grandii		86.3–90.2	79.7–87.0	69.9–76.6
B. grandii benazzii			90.8–96.5	74.1–81.4
B. grandii maretimi				84.8–89.9

site is found at different positions only in sequences of *B. atticus* and *B. grandii grandii*. In both *B. atticus* and *B. grandii,* sequences the most common cleaving sites are those of *Cla* I, *Taq* I, *Alu* I, *Bcl* I, and *Nsi* I (fig. 2).

The *Bag*320 sequences from *B. atticus* and *B. grandii* differ by 12 fixed nucleotide substitutions; *B. atticus* sequences also show a single-nucleotide insertion at position 252 in most clones. No sequence differences could be found that allow discrimination of different populations from the same or from different zymoraces of the obligate parthenogen *B. atticus*. In contrast, 25 race-specific positions were easily recognized among sequences derived from different subspecies of *B. grandii* (i.e., positions 11, 37, 38, 89, 92, 109, 111, 112, 116, 120, 121, 153, 154, 167, 174, 177, 179, 212, 222, 224, 244, 260, 283, 308, 314). At position 260, each of the three *B. grandii* races shows a different fixed nucleotide.

At 17 variable sites, the *B. grandii benazzii* and *B. grandii maretimi* sequences show the same nucleotide as the sole or the most frequent one, and differ from those found in *B. grandii grandii*. At three positions, the *B. grandii grandii* sequences match those of *B. grandii maretimi* while differing from the *B. grandii benazzii* ones. At position 212, *B. grandii grandii* shares the same nucleotide with *B. grandii benazzii*. At positions 111, 283, and 120, the sequences of two subspecies are different (i.e., *B. grandii benazzii* vs. *B. grandii grandii*, *B. grandii grandii* vs. *B. grandii maretimi*, respectively). No characteristic differences were detected among sequences of the two *B. grandii benazzii* populations (Scopello and Levanzo Island).

A neighbor-joining dendrogram (fig. 3) illustrates the similarity among various *Bag*320 sequences. As expected from sequence analysis, the clones of *B. atticus* and *B. grandii* form two separate clades. No race-specific clustering of *B. atticus* clones resulted; on the contrary, the *B. grandii* clones fall into two clades, one consisting of all *B. grandii grandii* sequences, and the other consisting of all clones derived from *B. grandii benazzii* and *B. grandii maretimi*. The clones of the latter races appear to be closely related (fig. 3). Accordingly, the clones *B. grandii benazzii* from Scopello and Levanzo Island do not form distinct clusters. UPGMA and maximum-parsimony dendrograms (not shown) had basically the same topology.

Estimates of relative copy numbers revealed significant differences between *B. atticus* and *B. grandii* (represented by *B. grandii benazzii*). While *Bag*320 repeats contribute approximately 15%–20% to the genome of *B. grandii benazzii*, they make up only 2%–5% of the *B. atticus* genome.

Discussion

The *Bag*320 satDNA family is a characteristic component of the genomes of the stick insect species *B. grandii* and *B. atticus* but was not found in *B. rossius* either by Southern blot or in situ hybridizations, as already noted in earlier studies (Mantovani et al. 1993; unpublished data). The AT-rich *Bag*320 repeats are tandemly arranged and located in the pericentromeric heterochromatin and contribute significantly to the genomes of *B. atticus* (2%–5%) and *B. grandii* (15%–20%). Thus, the molecular characteristics of the *Bag*320 satDNA family are similar to those of other satDNA families found in other invertebrates such as the flour beetles of the genus *Tribolium* (Plohl et al. 1992; Juan et al. 1993; Ugarkovic et al. 1994; Ugarkovic, Podnar, and Plohl 1996), the dipteran *Chironomus pallidivittatus*
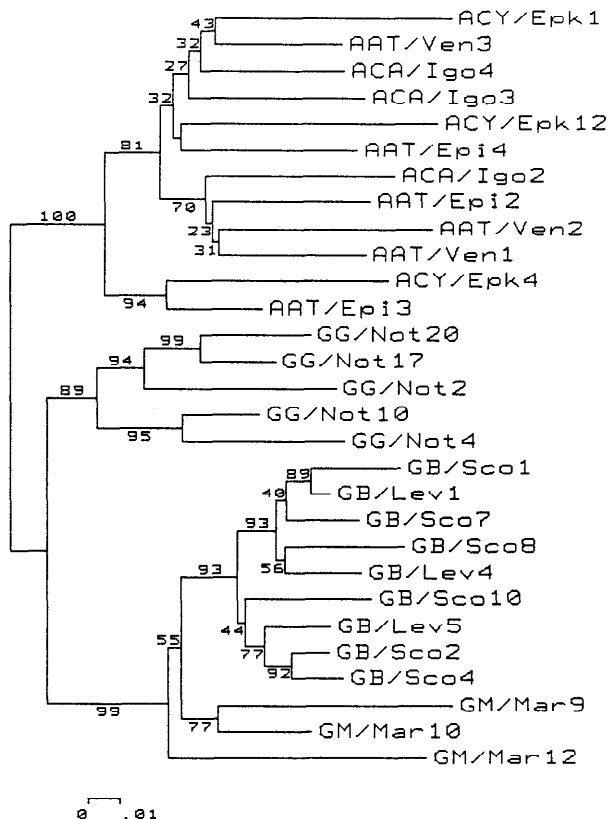

FIG. 3—Neighbor-joining dendrogram of the 29 *Bag*320 sequences. The dendrogram is intended to illustrate the similarity of *Bag*320 satDNA repeats rather than to infer phylogenetic relationships of the species. Numbers above branches indicate bootstrap values for 500 replicates. The scale bar represents a genetic distance of $D = 0.01$, calculated according to the Kimura's (1980) two-parameter method.

(Rovira, Beerman, and Edstrom 1993), and the brine shrimp *Artemia* (Badaracco et al. 1987, 1991), to mention a few.

The *Bag*320 satDNA family is highly conserved in *B. grandii* and *B. atticus*, supporting the close phylogenetic relationships of the two species, established through different experimental approaches (reviewed by Scali et al. 1995).

Interestingly, the *Bag*320 consensus sequences from different races of *B. grandii* are different, while, in spite of the wide range and cytological-allozymic differentiation, those of the three zymoraces of *B. atticus* are not. In phylogenetic dendrograms, a racial clustering of the *Bag*320 repeats of *B. grandii* is evident, especially if *B. grandii grandii* is compared to *B. grandii benazzii* and *B. grandii maretimi*. On the nucleotide sequence level, 25 sites out of 324 (7.7%) are informative for clustering. The differentiation of *Bag*320 repeats in *B. grandii* agrees perfectly with the genic differentiation deduced from morphological and ecological features as well as that deduced from allozyme polymorphisms (Mantovani, Scali, and Tinti 1991, 1992; Mantovani and Scali 1993*b*; Bullini 1994; Scali et al. 1995). However, the *Bag*320 sequences from the two *B. grandii benazzii* populations of Levanzo Island and Scopello could not be discriminated, although the first step toward racial differentiation can be recognized for gene–enzyme systems (Mantovani, Scali, and Tinti 1991; Mantovani and Scali 1993*b*). The assumed period of 15–35 thousand years of geographical isolation for these two populations (Pirazzoli 1987) seems to be too short for fixation of different *Bag*320 variants. This is not surprising when taking into account that the consensus sequences of three specific satDNA families of the Mediterranean cave cricket *Dolichopoda schiavazzii* are still identical in populations geographically isolated for at least 200,000 years (Bachmann, Venanzetti, and Sbordoni 1994, 1996).

To summarize, the evolutionary turnover of *Bag*320 satellite DNA appears to be much slower in the obligate parthenogen *B. atticus* than in the bisexual *B. grandii*. It is hypothesized that this difference is related to the different modes of reproduction of the two species. In other words, the meiotic recombination and the redistribution of chromosomes to the next generation, which typically occur in *B. grandii,* significantly accelerate the evolutionary turnover of *Bag*320 satDNA. This seems to meet the expectation deduced from computer simulations (Stephan 1989), and therefore it would represent the first experimental finding on the issue. Conversely, the parthenogenetic reproduction of *B. atticus* makes each specimen (female) reproductively isolated from all other conspecifics and, through its automictic meiotic process (fusion of the first meiotic division nuclei; Marescalchi, Pijnacker, and Scali 1993), slows down the molecular turnover of satDNA. Thus, in these parthenogenetic females, genomic turnover mechanisms can be active in spreading the highly repeated units on the chromosomes during meiosis, but there is no chromosomal reassortment in the offspring or, consequently, in the population. This view also implies that mitotic recombination such as sister chromatid exchange plays a minor role in satDNA evolution.

In situ hybridization analyses on the mitotic chromosomes of *Bacillus* species revealed that the *Bag*320 satDNA family is localized in the centromeric region on all 34 chromosomes of *B. grandii* and on a large subset of chromosomes (26–30) in the diploid races of *B. atticus* (Mantovani et al. 1993; unpublished data). Thus, on the basis of both cytological and molecular findings, it can be assumed that the *Bag*320 satDNA family was already present in the genome of their common ancestor and differentiated through the fixation of specific mutations after cladogenesis of the *B. atticus* and *B. grandii* lineages. However, since experimentally obtained interspecific hybrids between *B. grandii* and *B. atticus* are fertile (unpublished data), past backcrossing in nature could have horizontally passed the *Bag*320 family from one species (*B. grandii*) to the other (*B. atticus*). Depending on when this horizontal transfer took place, if it did, and also given that *B. atticus* may have been bisexual at that time, such an occurrence could also explain the difference in copy numbers between species. The markedly different copy numbers of *Bag*320 repeats observed for *B. atticus* and *B. grandii* might be related to the mode of reproduction, as sexual reproduction may have facilitated their increase (as well as a higher rate of fixation and homogenization of sequence variants). Alternatively, parthenogenesis may have a bearing on the loss of copies, somehow permitting a satDNA decrease, particularly from some chromosome lineages (pairs 1 and 2; Mantovani et al. 1993).

Bacillus grandii, B. atticus, and *B. rossius* repeatedly hybridized in the past, giving rise to natural interspecific hybrids, which reproduce by cytologically related hemiclonal and clonal mechanisms (Mantovani and Scali 1992; Mantovani, Scali, and Tinti 1992; Tinti and Scali 1995, 1996). In particular, three diploid *rossius/grandii* hybrids (the hybridogenetic strains of *B. rossius-grandii benazzii* and *B. rossius-grandii grandii* and the parthenogenetic *B. whitei*) start their egg maturation processes with the shared steps of an intrameiotic DNA doubling and parental genome segregation. The segregation of unassorted entire *rossius* and *grandii* chromosome haploid sets seems to play a crucial role in balancing the hybrid gametogenetic mechanisms. Actually, in hybridogens, genome segregation allows both the specific degeneration of the sole *grandii* chromosome complement and a normal equational division of the surviving *rossius* haploid set. In the hybrid parthenogens, neither of the segregated parental genomes degenerates, and both are reutilized to rebuild the maternal genetic structure in the clonal progeny (Marescalchi, Pijnacker, and Scali 1991; Tinti and Scali 1992, 1996). It could be speculated that, in this cytological context, *Bag*320 repeats of the pericentromeric heterochromatin of all *B. grandii* chromosomes play an important role in the specific recognition of each parental haploid set.

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