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Parental species and hybrid descendants of *Bacillus* (Insecta Phasmatodea) show different patterns of highly amplified, colocalized ribosomal and telomeric sequences

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Abstract

We investigated by dual-color fluorescence in situ hybridization (FISH) with 28S ribosomal and (TTAGG)_n telomeric probes all species of the circum-Mediterranean genus *Bacillus* encompassing bisexual and parthenogenetic taxa, namely the three parental species (*B. grandii*, *B. atticus*, *B. rossius*) and the two derived hybrids (*B. whitei*, *B. lynceorum*). Specimens were collected in Italian mainland, Sardinia and Sicily. In all species the presence of colocalized, highly amplified ribosomal and telomeric sequences was demonstrated by the double labelling of the cytological satellites. These satellites varied in size, number and location both among and within species. In *B. grandii* and *B. atticus* a maximum of two FISH-labeled locations were observed, whereas in *B. rossius* and in the two hybrids up to 11 different positions were recorded. Moreover, our investigations showed a significant occurrence of chromosome breakages and rearrangements. The overall meaning of the ribosomal and telomeric sequence colocalization as well as the Nucleolar Organizer Region mobility and activity are discussed in both the ancestors and their hybrid descendants. It is noteworthy that the same trait has been shown in seven additional phasmid species belonging to distantly related genera. This trait could be a shared ancestral character in phasmids.

Keywords: *Stick insects, FISH, parthenogenesis, cytological satellite variation, chromosome repatterning*

Introduction

Stick insects (Insecta Phasmatodea) represent an order of particular interest owing to their reproductive biology: most members show bisexual reproduction, but thelytokous parthenogenesis, hybridogenesis and androgenesis can occur as well. About 15% of the 3,200 species of this order are obligatory parthenogenetic (Velonà et al. 2015), often as a consequence of hybridization between species (Ghiselli et al. 2007; Scali 2009a; Milani et al. 2015).

A peculiar karyotypic feature of stick insects is the variable number and size of cytological satellites, which may vary both among congeneric taxa and within same species (Manaresi et al. 1991, 1992a, 1993; Tinti & Scali 1991; Ghiselli et al. 2007; Scali et al. 2012). The large cytological satellites are usually labeled after the AgNO₃

reaction, thus supporting their active role in the ribosome synthesis (Manaresi et al. 1991, 1992a, 1993; Tinti & Scali 1991; Ghiselli et al. 2007).

Recently in Iberian phasmids, namely *Leptynia montana* Scali (2n = 38/37; XX/XO) and two subspecies of *L. attenuata* Pantel (2n = 36; XX/XY) (Scali 2009b; Scali et al. 2012), we analyzed the chromosomal location and features of nucleolar organizer regions (NOR) by AgNO₃ and dual-color fluorescence in situ hybridization (FISH) of 45S rDNA together with the (TTAGG)_n insect telomeric repeat. In dual-color FISH, the probes always marked the cytological satellites and usually overlapped, demonstrating a colocalization of the two highly amplified repetitive sequences; furthermore FISH and AgNO₃ markings always matched (Scali et al. 2016).

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Five additional phasmid species, either bi- or unisexual, belonging to distantly related genera, were found to share the same cytogenetic feature (Liehr et al. 2017). Based on this connection, in this study, we have investigated the issue in all known species of the circum-Mediterranean genus *Bacillus*, which encompasses both bisexual (*B. grandii*, *B. rossius*) and unisexual diploid (*B. atticus*, *B. whitei*) or triploid (*B. lynceorum*) species. About one myr ago, in the south-eastern Sicily (Iblei Hills), *B. grandii* males and parthenogenetic *B. rossius* females hybridized giving rise to the diploid parthenogen *B. whitei*, which in turn was fertilized by *B. atticus*, which was bisexual at the time (Scali 2009a, 2013), thus producing the triploid trihybrid parthenogen *B. lynceorum*. Hybridogenetic and androgenetic strains were also produced, thus realizing a complex pattern of reproductive and phyletic relationships, described as a case of “reticulate evolution” (Scali 2009a).

The new investigations on all species of the genus *Bacillus* help to shed light on the colocalization of the amplified NOR/telomeric sequences and on their number variability and mobility on the chromosomes of five more species. Furthermore, the analysis of species with markedly different genetic structures provide us with the chance to follow the transmission features of those repeated sequences from parents to diploid and triploid hybrid descendants.

Materials and methods

In this research, all applicable international, national and Cagliari University institutional guidelines for the care and use of animals were followed.

A total of 47 field-collected specimens were analyzed: 4 *B. grandii* males; 15 *B. atticus*; 12 *B. rossius* (3 males, 9 females), 12 *B. whitei*, and 4 *B. lynceorum*. Their collecting sites are reported in the map (Figure 1) and summarized in Table I.

Chromosome preparations

The chromosomal preparations were obtained by manual dissection of the gonads of anesthetized specimens in insect Ringer’s solution, a 10 minutes hypotonic shock in 1% sodium citrate solution, followed by gentle teasing of the Carnoy-fixed tissue fragments in few drops of 60% acetic acid on a laboratory glass and subsequent cell drying on a hot plate (for more detailed information see Scali et al. 2016). Tens of chromosomal plates were obtained from the follicular cells of ovarioles or spermatogonia/spermatocytes;

more than 20 mitotic/meiotic plates per individual were analyzed in depth. Then, a few karyograms of each species were prepared. In each karyotype the chromosomes were arranged according to their decreasing length. In the karyotype of the parental species the usual arrangement in pairs was applied, whereas in the two hybrids (2n and 3n) only the six/nine largest chromosomes that were individually recognizable, could be arranged in pairs/triplets. For the remaining chromosomes, only the size criterion was singly adopted. The unusual chromosomes derived from fissions or fusions have been placed in the position of the corresponding unrepeated chromosomes, in accordance with the standard species karyotype. Whole chromosomes or chromosome fragments could be sometimes traced from their specific centromeric heterochromatin amounts.

Dual-color FISH

Dual-color FISH with ribosomal 28S and telomeric (TTAGG)_n sequences was performed according to Salvadori et al. (2014). The 28S probes were derived from *Nephrops norvegicus*, *B. rossius* and *B. atticus* DNAs by polymerase chain reaction (PCR) amplification, using the universal primers D1F and D1R (Zardoya & Meyer 1996). The amplification of the 28S gene was based on the following cycling parameters: an initial denaturation of 5 minutes at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 47°C in *Bacillus* species or 54°C in *N. norvegicus* for the annealing of primers, and 1 minute at 72°C for the extension of fragments, and then a final extension phase of 5 minutes at 72°C. The PCRs were set up in a 25 µl reaction volume containing 2.5 µl of 10X Buffer (Dream Taq® buffer Thermo scientific), 2.5 µl of 2 mM dNTPs, 1 µl of 25 mM of MgCl₂, 0.2 µl of each 10 mM primers, 0.16 µl of Taq polymerase (Dream Taq® Thermo scientific) and 1 µl of DNA (50–100 ng). The telomeric probe was PCR generated according to Ijdo et al. (2001). The probes have been labeled with biotin-16- deoxyuridine triphosphate (dUTP) or digoxigenin-11- dUTP using a nick translation kit (Roche diagnostics) and detected with avidin conjugated to fluorescein isothiocyanate (Vector Laboratories), or anti- digoxigenin-rhodamine (Sigma-Aldrich), respectively. The chromosomes were counterstained using 4',6-diamidino-2-phenylindole (Sigma Aldrich).

Single FISH always marked the whole large cytological satellites with either green (fluorescein isothiocyanate) or red color (rhodamine), according to the utilized fluorochrome. Dual-color FISH produced

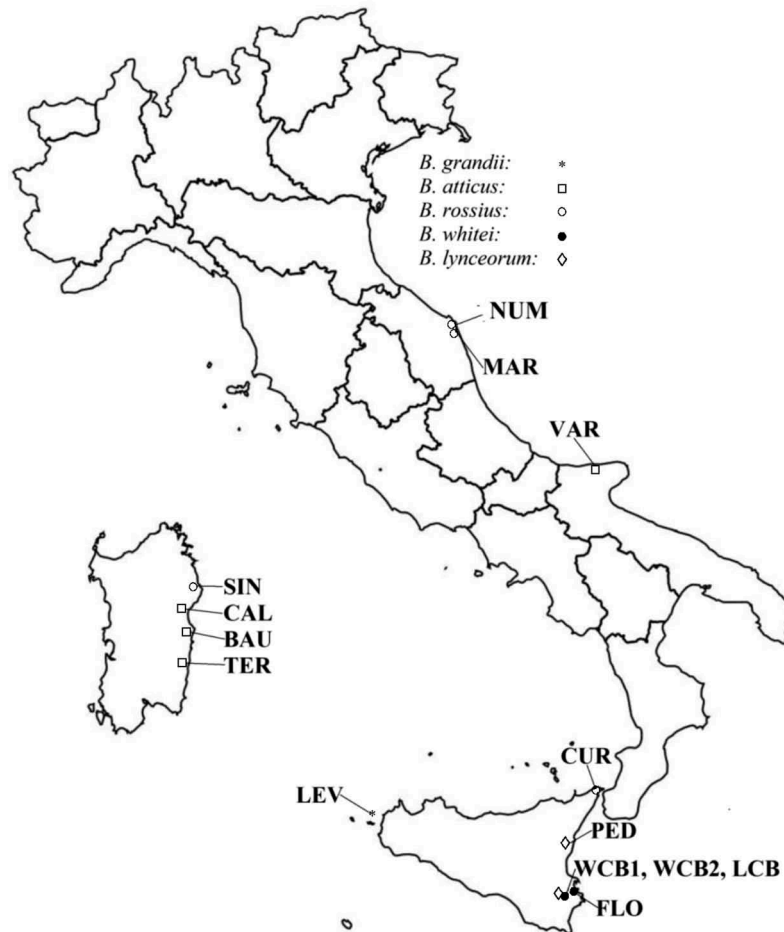


Figure 1. Collecting sites of the investigated *Bacillus* specimens. * *B. grandii*: Levanzo Island (LEV); □ *B. atticus*: Baunei (BAU), Calagonone (CAL), Tertenia (TER), Varano (VAR); ○ *B. rossius*: Curcuraci (CUR), Marcelli (MAR), Numana (NUM), Siniscola (SIN); ● *B. whitei*: Canicattini Bagni (WCB1, WCB2), Florida (FLO); ◇ *B. lynceorum*: Canicattini Bagni (LCB), Pedara (PED).

a yellowish/pink fluorescence on the cytological satellites as a result of the overlapping labelings.

Image processing

The mitotic and meiotic metaphases were observed under a Zeiss Imager M1 fluorescence microscope. The images were captured with a Hamamatsu digital camera C8484 and processed using a karyotyping FISH-dedicated image-analysis system (Cromowin Plus, TESI Imaging).

Results

By dual-color FISH, we analyzed the 28S rDNA and (TTAGG)_n telomeric repeats of five *Bacillus* species characterized by different reproductive modes. Specifically, the species were: i) *Bacillus grandii* (2n = 34/33, XX/X0) strictly bisexual, endemic to Sicilian Islands; ii) *B. rossius* (2n = 36/35,

XX/X0) bisexual – but also with facultative-parthenogenetic populations in Europe – from the Western circum-Mediterranean; iii) *B. atticus* (2n = 34, XX from Italian mainland and Sicily; 2n = 33, XX from Sardinia), presently a thelytokous parthenogen, ranging from eastern to central Mediterranean countries; iv) *B. whitei* (*rossius/grandii*, 2n = 35, XX), and v) *B. lynceorum* (*rossius/grandii/atticus*, 3n = 52, XXX), both thelytokous parthenogens and endemic in southeastern Sicily (Scali 2009a). The karyograms were arranged according to a size criterion. Owing to the varying size of cytological satellites, only the constant body of satellite-bearing chromosomes was considered for their ranking.

Bacillus grandii

The analyzed specimens showed the standard chromosome complement described by Marescalchi and

Table I. Synopsis of the number of specimens and collection sites of the analyzed species, of 28S rDNA markings, and of the chromosome pairs/single chromosome (chr) on which the FISH marks were located.

Species and site of collection	Specimens	28S rDNA signals	28S/tel FISH pattern
<i>Bacillus grandii</i> (LEV)	4	2	13 th pair
<i>B. atticus</i> (BAU, CAL, TER, VAR)	15	2	17 th pair
<i>B. rossius</i> (NUM)	3	6	1 st , 2 nd , 13 th pairs
<i>B. rossius</i> (MAR)	2	6	1 st , 13 th , 17 th pairs
<i>B. rossius</i> (SIN)	4	6	5 th , 13 th , 14 th pairs
<i>B. rossius</i> (CUR)	3	2	5 th pair
<i>B. whitei</i> (WCB1)	2	4	7, 10, 12, 25 chrs
<i>B. whitei</i> (WCB2)	6	4	1, 6, 12, 17 chrs
<i>B. whitei</i> (FLO)	4	5	1, 3, 4, 6, 32 chrs
<i>B. lynceorum</i> (LCB)	3	1	39 chr
<i>B. lynceorum</i> (PED)	1	3	5, 15, 39 chrs

Scali with outstanding pericentromeric blocks (1990). In the dual-color FISH, the telomeric (TTAGG)_n probe, beside ordinary telomeres, marked also the short arm of the 13th pair, which bears the cytological satellites, usually large, but often varying in size. The 28S rDNA probe consistently marked the same chromosomal satellites, thus producing a distinct summed-up yellowish color of the two probes (Figure 2(a), Table I).

Bacillus atticus

The specimens collected in the Italian mainland, showed the standard karyotype with 2n = 34 chromosomes, whereas all the Sardinian ones revealed a 2n = 33 chromosome set as a consequence of a Robertsonian fusion of two medium-sized acrocentrics, possibly the 11th pair, as described by Tinti and Scali (1991) (Figure 2(b)). Most of the chromosome pairs showed pericentromeric heterochromatic blocks, that are similar to but smaller than those of *B. grandii*. In both peninsular and insular samples (15 specimens in total) the large satellites located on the short arm of the 17th pair were always double-marked by 28S and telomeric probes; in several individuals the labeled satellites were observed in a heterozygous condition (see inset of Figure 2(b) and Table I).

Bacillus rossius

All specimens showed the standard karyotype (2n = 36, XX, female; 35, XO, male) (Manaresi et al. 1991). In this species, differently from *B. grandii* and *B. atticus* the chromosomes featured a faint pericentromeric heterochromatin.

After dual-color FISH, the 28S signals showed differences in chromosome location and number (ranging from 1 to 6 signals) in individuals of different collecting sites. Among the 12 investigated specimens, the 13th pair was labeled in individuals from NUM, MAR, and SIN; the 1st pair was marked in individuals from NUM, MAR and the 5th pair was marked in individuals from SIN and CUR; the 2nd, 14th and 17th pairs were labeled in individuals from NUM, SIN and MAR, respectively (Figure 2(c), Table I). In three specimens the labeling of the 13th and 14th pairs showed a different size signal between homologs (heterozygous marking). The 28S labeling was constantly observed in all the sizeable cytological satellites. The telomeric probe, in addition to ordinary telomeres, marked the same satellites labeled by the 28S FISH. However, some instances of amplified telomeric marking without or with notably faint 28S labeling were observed as shown in the long arm of the 1st pair in Figure 2(c): in this chromosome the 28S probe labels a very small region and after dual-color FISH only the telomeric labeling can be seen. Figure 2(d) shows a meiotic metaphase I of a SIN male after dual-color FISH with the 13th bivalent double-labeled.

Bacillus whitei

WCB1 and WCB2 specimens from the Canicattini Bagni area of this thelytokous hybrid parthenogen were collected in two near sites but sharply separated by a large and deep canyon. Both samples showed the standard *B. whitei* karyotype for chromosome number and composition (2n = 35, XX) as expected by the chromosome contribution of the parental species, namely 18 chromosomes from *B. rossius* and 17 from *B. grandii* (Manaresi et al. 1992a). Within the hybrid complement, the chromosomes of the latter species can be positively traced thanks to their larger pericentromeric heterochromatic blocks; thus, in addition to the six largest elements whose origin can be individually recognized, also the species derivation of the decreasing chromosomes that follow in the karyogram, can be inferred; notably, however, no pairs of homologous

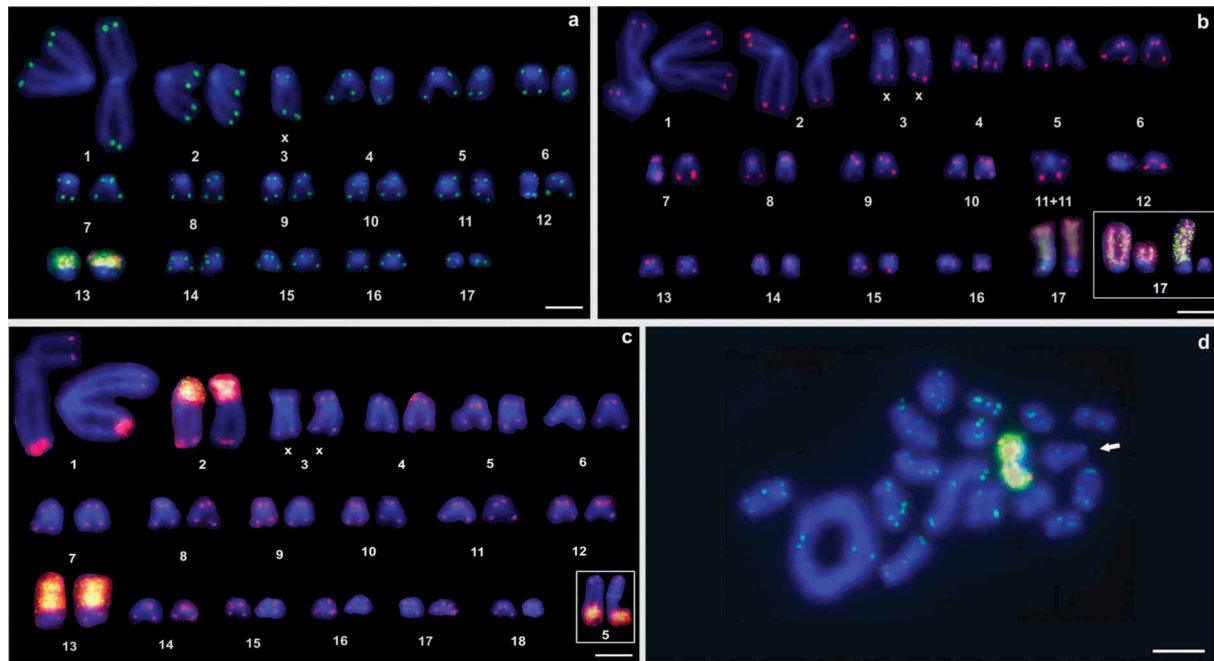


Figure 2. *Bacillus grandii* (LEV) (a), *B. atticus* (TER) (b), *B. rossius* (NUM) (c) karyograms: where labelings overlap in dual-color FISH, the resulting fluorescence gives a yellowish/pink color. (a) 28S (red fluorescence) and telomeric (green fluorescence) probes are overlapped on the 13th pair cytological satellites. (b) 28S (green fluorescence) and telomeric probes (red fluorescence) are overlapped on the 17th pair cytological satellites, which can be heterozygous in different individuals (inset). (c) 28S (green fluorescence) and telomeric (red fluorescence) probes are overlapped on 1st, 2nd and 13th pairs. The 28S signal of 1st pair is extremely weak/missing. The inset shows the only marked 5th pair of a CUR specimen (long arms). (d) Male meiotic metaphase I of a *B. rossius* (SIN) specimen: 28S (red fluorescence) and telomeric (green fluorescence) probes are overlapped on the 13th bivalent; to its right the arrow indicates the X chromosome. Bar = 5 μ m.

chromosomes can be formed (Manaresi et al. 1992a, 1992b) (Figure 3(a,b,c); Table I).

In the WCB1 specimens 28S and telomeric dual color FISH were overlapped on four chromosomes, namely acrocentrics 7, 10, 12 and 25. The labeling of the 25th element could well correspond to one homolog of the satellite-bearing 13th pair derived either from *B. rossius* or *B. grandii*; however, the size of the centromeric heterochromatin would support *B. grandii*. On the other hand, the labeled 7th and 12th chromosomes, corresponding to the 4th and 6th pairs of parental species, respectively, represent two newly acquired NOR locations (Figure 3(a)).

In the WCB2 specimens, a quite different situation was observed. After the dual-color FISH, four large cytological satellites were marked, and the labeled chromosomes were the following: the large metacentric 1 of *B. rossius* derivation, the submetacentric 6 (that is one of the two X chromosomes), the small submetacentric 12, and the acrocentric 17 of *B. grandii* derivation (Figure 3(b)).

The FLO *B. whitei* specimens showed a rearranged karyotype with 36 chromosomes, owing to the fission of one of the largest

metacentrics (chromosome 2 in our ranking) of *B. rossius* derivation.

After dual-color FISH also in these specimens the pattern for the highly enriched ribosomal and telomeric sequences differed from those of WCB1 and WCB2, as five chromosomes showed large co-localized signals, namely, the largest metacentric 1 of *B. grandii* derivation, the submetacentric 3, the metacentric 4, submetacentric 6 (one X chromosome), and one acrocentric, around position 32 (Figure 3(c)). Overall, in just 12 specimens of *B. whitei*, 10 different chromosomes and 12 locations were involved in dual-color FISH labeling. A marked pattern of satellite variations for number and location notably occurred in the *B. whitei* parthenogens.

Bacillus lynceorum

The chromosome set of this all-female parthenogen derives from the contribution of three different parental species, namely the maternal *B. rossius* ($n = 18$) and the two paternal ancestors *B. grandii* ($n = 17$) and *B. atticus* ($n = 17$). Therefore the standard triploid

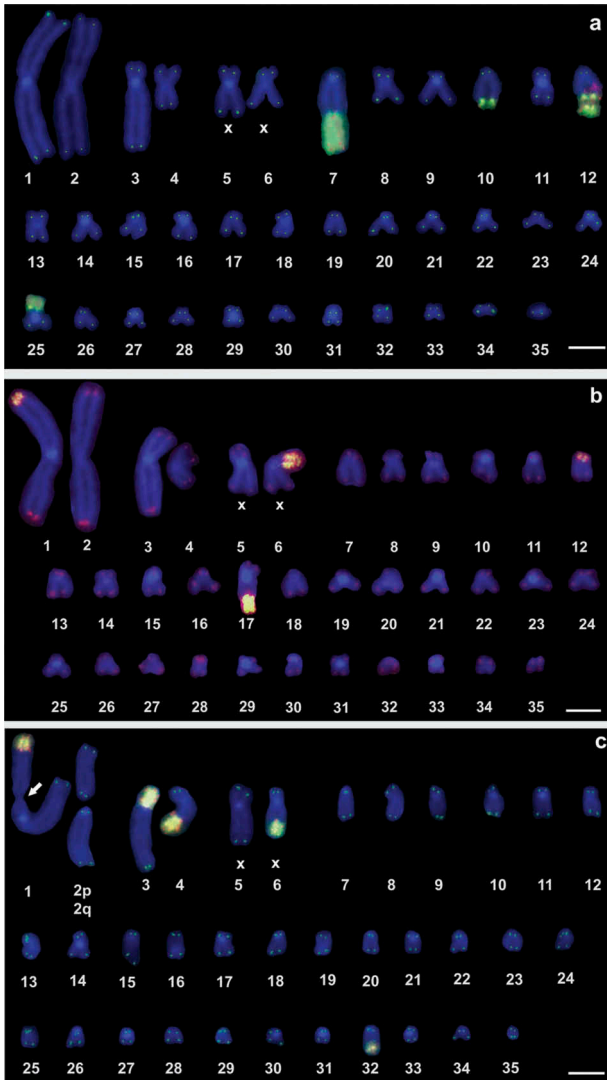


Figure 3. *B. whitei* dual-color FISH karyograms: where labelings overlap, the resulting fluorescence gives a yellowish/pink color. (a) in WCB1 females 28S (red fluorescence) and telomeric (green fluorescence) probes are overlapped on the acrocentrics 7, 10, 12, and 25. (b) in WCB2 females, 28S (green fluorescence) and telomeric (red fluorescence) probes are overlapped on chromosomes 1, 6 (one X chromosome), 12, and 17. (c) The 36 repatterened karyotype of an FLO female with a broken 2nd chromosome (2p and 2q arms). Chromosome 1 features a constricted trait (arrow). 28S (red fluorescence) and telomeric (green fluorescence) probes are overlapped on chromosomes 1, 3, 4, 6 (one X chromosome) and one acrocentric around position 32. Bar = 5 μ m.

karyotype of *B. lynceorum* consists of 52 elements (Manaresi et al. 1993). However, several fissions and translocations had been observed in many populations over the species range, leading to a variety of cytotypes showing a tangled sharing of active NORs (see Manaresi et al. 1993 for an overall picture): none of the specimens here analyzed presented the

standard karyotype, as LCB females contain $3n = 53$ chromosomes and the PED specimen showed a chromosome set with 55 elements.

In the 20 analyzed plates, the three LCB females showed a chromosome set with 53 elements owing to the Robertsonian fission of a large metacentric of *atticus* derivation (1st triplet). Another karyogram feature of these specimens is the pericentric inversion and the partial deletion of the short arm of chromosome 4 (of *B. grandii* origin); furthermore a part of the deleted piece has been apparently translocated onto a small acrocentric, giving origin to an odd medium-sized submetacentric chromosome (Figure 4(a), 29th element). According to the FISH analysis, the LCB specimens possessed just one labeled chromosome, marked by both ribosomal and telomeric probes. It ranks at the 39th position, possibly corresponding to the 13th *B. grandii* pair; this chromosome might have received part of the deleted short arm of the 4th chromosome (Figures 4(a), 5(c); Table I).

The further numeric increase of the karyotype, up to 55, observed in all the 20 analyzed plates of the PED female, is due not only to the Robertsonian fission of one of the large metacentrics of the 1st triplet, which possibly is of *B. rossius* derivation, but also to a deletion of the short arm of the 5th submetacentric chromosome (2nd triplet, of *B. atticus* derivation), which gave rise to two minute chromosomes (Figures 4(b), 5(d)). Only the 52nd small chromosome could be identified as an odd, new chromosome since no telomeric FISH signals were observed at its chromosomal ends; the second chromosome could possibly be the minute 54th element (Figure 4(b)). A very long constriction of one large metacentric of the 1st triplet (of *B. grandii* origin, arrow in Figure 4(b)) was also observed.

After dual-color FISH, three chromosomes of different sizes were marked, namely the abovementioned deleted 5th submetacentric of the 2nd triplet, the long arm of a medium-sized acrocentric of *B. grandii* derivation, at around the 15th position, and the short arm of a small acrocentric, at about the 39th position (Figure 4(b); Table I).

Overall, in spite of the small *B. lynceorum* sample, at least four different chromosomes were marked in dual-color FISH.

Discussion

Highly amplified and colocalized ribosomal/telomeric sequences

FISH results showed that the chromosome complement of all *Bacillus* species embodies a high enrichment of mostly colocalized ribosomal and telomeric

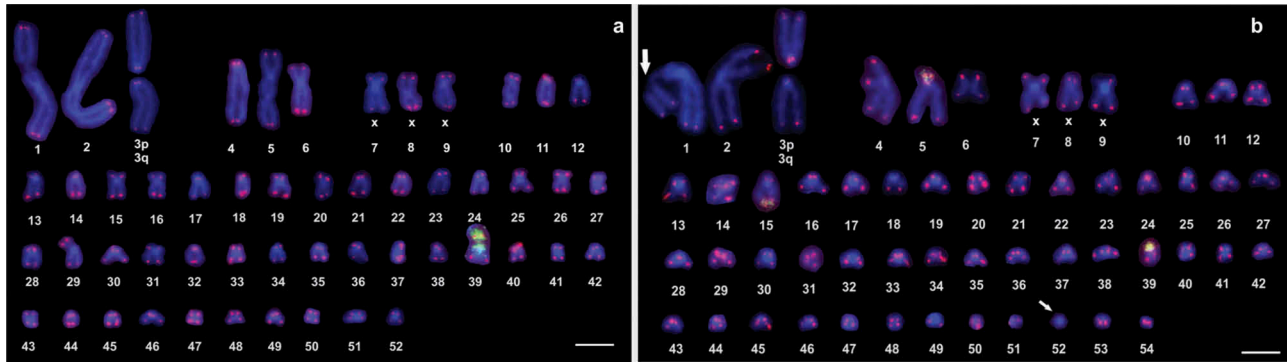


Figure 4. *B. lynceorum* dual-color FISH repatterned karyograms: where labelings overlap the resulting fluorescence gives a yellowish/pink color. (a) In LCB females the 53 chromosomes derive from the fission of one metacentric of the 1st triplet (3p and 3q); furthermore the 4th chromosome shows a pericentric inversion and partial deletion of the short arm: the deleted traits possibly translocated on to the 29th and 39th chromosomes. (b) In the PED female the 55 chromosomes possibly derive from the fission of one metacentric of 1st triplet (3p and 3q) and from the multiple breakage of the short arm of the 5th chromosome originating two minute chromosomes: the 52nd, lacking the telomeric labeling (small arrow) and possibly the 54th. Chromosome 1 features a constricted trait (large arrow). The 28S (green fluorescence) and telomeric (red fluorescence) probes are overlapped in: a) the repatterned 39th chromosome; b) the short arm of the 5th, the long arm of the 15th, and the short arm of the 39th chromosomes. Bar = 5 μ m.

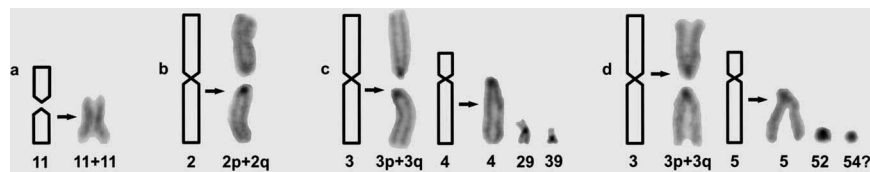


Figure 5. Synopsis of chromosomal rearrangements found in the specimens studied. (a) all the Sardinian *B. atticus* showed the fusion of two acrocentrics, likely the homologs of 11th pair. (b) The *B. whitei* (FLO) specimens showed the fission of one metacentric (chromosome 2). (c) *B. lynceorum* (LCB) specimens showed the fission of one metacentric of the first triplet. Furthermore the 4th chromosome was deeply repatterned by a pericentric inversion and the partial deletion of the short arm: the deleted traits possibly translocated on to the 29th and 39th chromosomes. (d) *B. lynceorum* (PED) showed the fission of one metacentric of the 1st triplet, and a deletion of the short arm of the 5th chromosome, giving rise to two minute chromosomes, the 52nd and possibly the 54th.

sequences, located on sizable cytological satellites. The newly acquired observations fully support the first finding described in *Leptynia* (Scali et al. 2016) and also reported for five additional phasmid species belonging to distantly related genera (Liehr et al. 2017). The shared trait of amplification/colocalization of NOR and telomeric sequences among all investigated phasmid species, strongly suggest that this trait could be an ancestral character. The rare instances of highly repeated telomeric sequences without or with faint ribosomal signals (see Figure 2(c)) could be either due to the low copy number of ribosomal units (that is, below the threshold detectable by FISH), or indicate that in stick insects the expansion of telomeric sequences could sometimes occur without or before ribosomal amplification. Furthermore the finding that, as a result of the overlapping labelings, the fluorescence ranges from yellowish to pink, appears to reflect a varying amplification of telomeric and ribosomal repeats.

The most common telomeric sequence is the hexameric (TTAGGG)_n repeat, which is present

in all vertebrates and considered the ancestral motif of telomeres in animals (Meyne et al. 1990; Fajkus et al. 2005). The pentameric (TTAGG)_n sequence is deemed the ancestral motif of telomeres in arthropods (Sahara et al. 1999; Vitková et al. 2005) while the eptameric one (TTTAGGG)_n in most higher plants (Richards & Ausubel 1988). All these telomeric sequences have been found co-localized with rDNA in a variety of animals such as arthropods (Salvadori et al. 2012) and, among vertebrates, in fishes (Salvadori et al. 1995; Ocalewicz 2013), amphibians (Nanda et al. 2008), reptiles (Srikulnath et al. 2009) and mammals (Zhdanova et al. 2003, 2007; Milioto et al. 2019) as well as in plants (Dvořáčová et al. 2015). The biological meaning of such an association is largely unknown, although the relationship between these two highly dynamic and frequently adjacent genome domains raises several questions that are reviewed and discussed in numerous papers

(Salvadori et al. 1995; Ocalewicz 2013; Dvořáčová et al. 2015; Liehr et al. 2017; Milioto et al. 2019).

Variability of ribosomal sequences and chromosome rearrangements

The occurrence of colocalized ribosomal and telomeric sequences were recorded for all the investigated *Bacillus* species (Table I). The parental *B. grandii* and *B. atticus* maintain a steady pattern of one labeled chromosome pair (13th or 17th, respectively) and, to date, this feature is the most common situation among phasmid species (Marescalchi & Scali 1990, 1993; Scali et al. 2006; Liehr et al. 2017). In *B. rossius*, in addition to the 13th pair, up to four more chromosome pairs show this trait. Similarly, the pattern of numerous and variable NORs and telomeric amplified sites occurs in both *B. whitei* and *B. lynceorum*, that is the hybrids originating from the hybridization of Sicilian *B. rossius* mothers with *B. grandii* and *B. atticus* fathers. Overall, 11 different chromosomes were found to be involved in the NOR localization. Possibly, for the hybrids, in addition to the sharing of the *B. rossius* maternal haploset, the heterospecific chromosome constitution and the peculiar egg-maturation mechanisms further increase the number and variability/mobility of the amplified sites.

The same populations of *B. grandii*, *B. atticus*, *B. whitei* and *B. lynceorum* analyzed by FISH, were previously thoroughly investigated by AgNO₃ reaction; (Marescalchi & Scali 1990; Tinti & Scali 1991; Manaresi et al. 1991, 1992a, 1992b, 1993): the comparison between the AgNOR investigations and the FISH labeling data here obtained shows a full correspondence in number and location, so that no inactive NORs have been detected in *Bacillus*. The same conclusion could be reached when the comparison was made for three *Leptynia* taxa where the same specimens were analyzed by both FISH and the AgNO₃ reaction (Scali et al. 2016). Thus, no inactive ribosomal sequences seem to occur in all investigated phasmids.

The FISH analyses fully support the wide AgNO₃ survey on Sicilian *B. rossius* populations (Manaresi et al. 1991), which revealed that up to six chromosome pairs could bear odd NORs, with a maximum of eight sites per cell. Furthermore, in this facultative parthenogenetic species, the occurrence of multiple sites of amplified NOR/telomere sequences is more frequent in females of unisexual populations than in specimens of bisexual demes. We suggest a link between the

variability for number and location of colocalized NOR/telomeric sequences and the presence of R2 retrotransposons. R2 retrotransposons, presenting a strict sequence specificity for an insertion target-site in the 28S rDNA, have been reported in bisexual and parthenogenetic populations of *B. rossius*, with a very high copy-number (Martoni et al. 2015; Satovic et al. 2016; Bonandin et al. 2017; Scavariello et al. 2017). Moreover, increased chromosome breakages and rearrangements have been observed in all taxa embodying the *B. rossius* genome, particularly in the parthenogenetic hybrids (Figure 5(b,c,d)). In this connection, several NOR features could be passed from *B. whitei* to *B. lynceorum* at the time of *B. atticus* backcrosses to the former parthenogen (Marescalchi & Scali 2001). The LCB and PED females of *B. lynceorum* are fully in line with this issue for fissions, deletions and new chromosome/fragments appearance. We can hypothesize that the additional chromosomes derived from fissions were maintained as independent elements in some instances, whereas they could have been lost in others, possibly owing to their impaired centromeric function and/or telomere erosion (see f.i. the PED chromosome 52 in Figure 4(b)).

Polymorphic cytological satellites and NOR inheritance

The sharp polymorphism of cytological satellites between homologs and among specimens observed in *Bacillus* could be related to the occurrence of unequal crossing over; furthermore, this trait appears to be enhanced in *B. rossius* and its hybrids, where ribosomal genes host R2 transposons, since a striking intraspecific variability has been found in those taxa, particularly in *B. whitei*.

Considering the labeled chromosome pairs of parental species, we could see that all NOR can be passed to hybrid descendants; this condition could perhaps provide the hybrids with an overall higher physiological advantage, as implied by their colonizing ability and parent's displacement (Manaresi et al. 1993; Scali 2009a). Based on the pericentromeric heterochromatin features of the ancestors, the attribution of a given NOR to a specific parental haploset has often been possible and we could observe that the ribosomal sites deriving from two (in *B. whitei*) or three (in *B. lynceorum*) different genomes can be at work in the same individual. By contrast, we could also observe that both hybrids can thrive with a single NOR from anyone ancestral species, as observed in LCB specimens with just one labeled chromosome. Therefore, despite the sharp genetic interspecific differences

within the genus *Bacillus*, such as those occurring between *B. rossius* one side, and *B. grandii*/*B. atticus* the other (Mantovani et al. 2001), the ribosomal activity is not impaired in the hybrids, even if two out of three parental NOR sites are missing (see Table 2 in Manaresi et al. 1993). In this connection we can observe that no plate of the WCB1 specimens showed two marked chromosomes in the 25th/26th karyotype positions (corresponding to the 13th pair of parents) as one could expect from the inheritance of both parental satellites; thus, at least one satellite of the ancestors has been lost in the hybrid. These findings demonstrate that the ribosomal machinery from anyone species can cope with the needs of both parental and hybrid *Bacillus* species. These features of the ribosomal compartment add new information to papers dealing with hybrid species onset and persistence in *Bacillus* (Bullini & Nascetti 1990).

Concluding remarks

What seems peculiar to the so far investigated stick insects is that all specimens of all investigated species, even belonging to very distant genera, show a colocalization of highly amplified NOR and telomere repeats; this trait could be a shared ancestral character in phasmids, although its functional meaning is not clear. FISH unequivocally provides evidence that colocalized ribosomal and telomeric repeats can easily move along the chromosomes, increase in number or all-but-one be lost as observed in the triploid trihybrid *B. lynceorum*. In *Bacillus* the colocalized ribosomal and telomeric repeated sequences seem to represent an hot spot of chromosome rearrangements allowing unequal crossing-overs as highlighted by the sharp polymorphism of cytological satellites between homologs and among specimens. Furthermore in *B. rossius* and its hybrids, where 28S rDNA hosts R2 transposons, this feature appears to be enhanced since a striking intraspecific variability has been witnessed particularly in *B. whitei*.





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