The Chromosome Complement of the Hybrid *Bacillus whitei* Complex (Insecta Phasmatodea)

I. The paleo- and neo-standard karyotypes

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In Sicily (Southern Italy) five *Bacillus* taxa are found, namely: *B. grandii* ($2n=34$, XX female, 33, XO male), *B. rossius* ($2n=36$, XX female, 35, XO male), the only two bisexuals, the thelytokous *B. atticus* ($2n=34$, XX) and the hybrid taxa *B. whitei* ($2n=35$, XX) and *B. lynceorum* ($3n=52$, XXX), both of relatively recent discovery and endemic to the island (see Scali and Mantovani 1989, for a review).

B. whitei ($2n=35$, XX) is a diploid all-female taxon discovered in Southeastern Sicily with a range widely overlapping that of *B. lynceorum*. The hybrid origin of *B. whitei*—from the cross between *B. g. grandii* and *B. rossius*—has been supported by different fields such as ootaxonomy, karyology, allozyme analysis and DNA cytofluorometry (Nascetti and Bullini 1982, Nascetti *et al.* 1985, Mazzini *et al.* 1987, Scali and Marescalchi 1987a, b, Marescalchi *et al.* 1990).

It has also been shown that *B. whitei* reproduces by thelytokous parthenogenesis (Nascetti and Bullini 1982), which relies on a complex automictic mechanism to maintain both chromosomal and genetical constitution of mothers (Marescalchi *et al.* 1991). However, very recently Scali *et al.* (1991) pointed out the occurrence of hybridogenetic females within *B. whitei* strains syntopic with *B. g. grandii*.

Another hybrid ($2n=35$, XX female; $2n=34$, XO the rare males) between *B. rossius* and *B. g. benazzii*, has been recently found in a northwestern area of Sicily, where it is syntopic with *B. g. benazzii* (Marescalchi and Scali 1989, Scali 1989). Since it shows a hemiclinal reproduction, as indicated by electrophoretic, karyological and hybridological evidence (Mantovani and Scali 1990, 1991, 1992, Mantovani *et al.* 1991a), its systematic status cannot be completely defined (Mantovani and Scali 1992) and it is provisionally indicated as *B. rossius-g. benazzii*, similarly to other hybridogenetic organisms (Shultz 1969, 1989); these hybridogens clearly parallel *B. whitei* hemiclinal strains and obviously originated from independent hybridization events (Mantovani *et al.* 1991b).

Although a karyological account of *B. whitei* has already been given (Bullini *et al.* 1983), we felt it necessary to re-analyze its complement because of heavy inaccuracies in the first description and also because several demes—including hybridogens—with different cytotypes have been found, which, in our opinion, deserve some comments. The cytological study is also completed by C-banding analysis and nucleolus organizer region (NOR) localizations.

Materials and methods

Analyzed field specimens (females) were collected from the sites shown in Fig. 1, namely: 1–Buccheri (8); 2–Canicattini Bagni (68); 3–Carlentini (17); 4–Catania (22); 5–Cava Grande (4); 6–Cugni (17); 7–Noto district (25); 8–Palazzolo Acreide (12); 9–Ponte Manghisi (9); 10–Pedagaggi (11); 11–Piazza Armerina (4); 12–Torre Judica (13); 13–Villasmundo (6); 14–

Fig. 1. Map of Sicily showing *Bacillus whitei* range (inset) and locations of analyzed samples.

A representative number of metaphases was analyzed for Giemsa, C-banding and Ag-NOR techniques. Chromosomes were classified according to the criteria suggested by Levan *et al.* (1964).

Photomicrographs of metaphases were taken on Agfa Ortho (25 ASA) and developed in Neutol.

Measurements for chromosome analysis were taken from enlarged prints of C-metaphases.

Results

Owing to the clearly recognized hybrid constitution of *B. whitei*, it is possible to pair with certainty only those chromosomes which are individually recognizable and comparable in both parental species. This mainly applies to the first six chromosomes of the complement—the fifth and sixth submetacentrics being the X chromosomes—and to a few others, such as the smallest ones and the great majority of NOR bearing chromosomes (Marescalchi and Scali 1990, Manaresi *et al.* 1991).

The “metacentric” standard karyotype

The most widespread karyotype, with $2n=35$, found in specimens from Buccheri, Canicattini Bagni, Carlentini, Cava Grande, Cugni, Ponte Manghisi, Noto, Palazzolo Acreide, Pedagggi, Piazza Armerina and Villa Vela, has been taken as the standard one (Fig. 2).

Characteristic elements of this karyotype—which can be tentatively arranged in pairs—are: two large, slightly heteromorphic, metacentrics (chromosomes 1 and 2), two strongly heteromorphic elements (chromosomes 3 and 4): the larger one, submetacentric, clearly deriving from *B. grandii*; the smaller, almost exactly metacentric, deriving from *B. rossius*: this chromosome characterizes, as we shall see, the paleo-standard karyotype, being replaced in the two others (“acrocentric” and “submetacentric”); two medium-sized, slightly heteromorphic submetacentrics (chromosomes 5 and 6), the sex-chromosomes. A series of elements, whose parentage is uncertain, follows. The series includes the chromosomes which cannot be individually characterized within parental sets and which, therefore, were not arranged in pairs but just according to decreasing size. Among them, however, some can be safely re-

Villa Vela (8). The samples appear to be sufficiently representative of the *B. whitei* range (Fig. 1).

In all populations but three (Catania, Torre Judica, Villasmundo) the majority of specimens, or at least some of them, showed a 35-standard karyotype, here analyzed; the wide variety of the repatterned cytotypes shall be discussed in a separate paper.

Mitotic chromosome sets have been obtained from follicular cells of ovariole tips.

Slides for chromosome observations were prepared using the air-drying technique described by Crozier (1968). Some slides were directly Giemsa stained while others were treated for C-banding or silver impregnation techniques according to the schedule adapted to stick insect tissues by Marescalchi and Scali (1990).

ferred to the parental species, such as the smallest acrocentric of *B. rossius* (chromosome 35, Fig. 2a), and, very often, the satellite-bearing ones.

The C-banding shows that the differences in centromeric heterochromatin among chromosomes are very sharp: they obviously reflect the pattern inherited from the parental species, *B. grandii* being C-heterochromatin rich and *B. rossius* much less so, except for the X chromosome. As a consequence, it has been possible to tell apart the 17 chromosomes of *B. grandii* derivation from the 18 ones of *B. rossius* origin; specifically, the distinction is very easy to make between members of pairs 1 to 3. Within the series of 7–35 chromosomes, the elements rich or poor in C-heterochromatin (i. e. the *B. grandii* and *B. rossius* ones, respectively) do not regularly alternate (Fig. 2b), thus showing that their pairing would not be appropriate.

Fig. 2. *Bacillus whitei* "metacentric" standard karyotype from the sample of Canicattini Bagni; (a) Giemsa and (b) C-banding. Note the metacentric fourth chromosome of paleo-*B. rossius* derivation.

In the standard karyotype some chromosomal clones are recognizable, differing from one another for number, position and size of the satellites and of the corresponding Ag-detected NORs, as summarized in Table 1. We see that, on the whole, there are 8 chromosomes which can bear satellites/NORs in various combinations, namely: 1, 3, 4, 5, 12, 17, 25 and 32, the more frequent locations being on chromosomes number 3 (5 populations), 12 (6 populations), 17 (7 populations), 25 (8 populations) and 32 (9 populations).

Satellites (and NORs) are more often localized on the short arm (chromosomes 1, 3, 4, 5, 12, 25 and 32) than on the long arm (chromosome 17), but in different populations their localization may be reversed (long arms of chromosomes 3 and 5). In the Palazzolo Acreide's population chromosome 3 can alternatively exhibit satellites and NORs on either arm, in the

Table 1. Synopsis of the positively C-banded/NOR chromosomes in the standard karyotype (paleo-*whitei*): Giemsa, C-banding and Ag-NOR from left to right side respectively in each box; only the most clear examples are shown. Figures refer to chromosome position in the karyotype (1-35)

Locality	Chromosome							
	1	3	4	5	12	17	25	32
Buccheri								
Canicattini Bagni								
Carlentini								
Cava Grande								
Cugni								
Ponte Manghisi								
Noto								
Palazzolo Acreide								
Pedagaggi								
Piazza Armerina								
Villa Vela								

same specimen; in some insects from Canicattini Bagni both arms of chromosome 12 are marked (Table 1).

Satellites are always C-positive but show a wide size-range, particularly those on chromosomes 12, 25 and 32 (Table 1).

The "acrocentric" standard karyotype

This karyotype is characterized by an acrocentric fourth chromosome (second heteromorphic pair) and clearly combines the 17 chromosomes of the *B. grandii* haploid set to the corresponding 18 elements of extant *B. rossius*. This karyotype is not common, being found only in some localized demes from the Canicattini Bagni area (Fig. 3a).

Another distinguishing feature of this karyotype is the relative low number of Ag-detected NORs (Fig. 3b) and corresponding satellites which, although varying in size, are always C-heterochromatin positive: these are only found on chromosomes 17, 25 and 32.

Fig. 3. (a) Giemsa and (b) Ag-stained *Bacillus whitei* standard karyotype, with an acrocentric fourth element (neo-*B. whitei*), from a Canicattini Bagni female. Note the satellites (17, 25 and 32) and the corresponding Ag-NOR markings.

The "submetacentric" standard karyotype

In some insects from Cugni, not far from the Canicattini Bagni district, a karyotype slightly differing from both the "metacentric" and the "acrocentric" one is found: its fourth chromosome (second pair) is submetacentric. The same chromosome also shows a C-heterochromatic area at the long arm telomere and a corresponding Ag marking in the same region. Ag-detected NORs are very similar to the acrocentric standard karyotype being localized on chromosomes 25 and 32 (Fig. 4a, b, c).

Discussion

The structural polymorphism shown by the fourth chromosome (metacentric, acrocentric, submetacentric) within an otherwise similar karyotype, deserves detailed comments, because it appears to hold a chief position for tracing *B. whitei* differentiation and evolution. The metacentric condition, found in most demes as either the unique kind or co-existing with another one, is certainly the most widespread. The same metacentric chromosome is found in all

Fig. 4. (a) Giemsa, (b) C-banded and (c) Ag-stained *Bacillus whitei* standard karyotype, with a submetacentric fourth element, from a Cugni female. Note the satellites and the corresponding C-bands (4, 12, 25, 32); are found on chromosomes 4, 25 and 32. Ag-NOR markings.

B. lynceorum populations (Scali and Marescalchi 1987a, b). On the other hand, the alternative acrocentric condition is the only one encountered in extant *B. rossius*. Most interestingly, it can be noticed that, while all "metacentric" demes are known to reproduce by thelytokous parthenogenesis, "acrocentric" specimens from the Canicattini Bagni area reproduce by hybridogenesis (Scali *et al.* 1991, Tinti and Scali 1991).

From all these data, the following scenario of hybridization and microevolutionary events can be suggested: after a first hybridization between *B. grandii* and "metacentric" *B. rossius*—a pale—*B. rossius*, possibly local, which also appears to have contributed to double allotriploid *B. lynceorum*—enough time elapsed for the hybrid to evolve thelytokous parthenogenesis, spread over a considerably large area from the extreme Southeastern corner of Sicily and allow karyological differentiation (Manaresi *et al.* 1992). The first hybridization event, very likely caused the extinction of the *B. rossius* females, owing to the competition of *B. grandii* bisexuals and hybrids. A second hybridization event occurred when "acrocentric", modern *B. rossius* females (neo-*B. rossius*) met bisexual *B. g. grandii* in the same area: the new hybrids show a uniform karyotype, perfectly matching the one of synthetic, lab-obtained specimens, and reproduce by hybridogenesis, renewing their F_1 hybrid constitution at each generation (Scali *et al.* 1991). The phylogenetic relationships of "submetacentric" *B. whitei* are less defined, as these females could derive either from early hybrids with standard karyotype or from the more recent ones, through a pericentric inversion, or, finally, from an independent hybridization event between *B. g. grandii* and a transeunt "subacrocentric" *B. rossius*. Additional evidence on their reproductive biology and cytogenetics is obviously needed to settle the "submetacentric" strains, but, owing to the nowadays absence of *B. g. grandii* from most of Cugni area, we can safely state that they actually reproduce by thelytokous parthenogenesis; at present, however, their capability of hybridogenesis cannot be completely excluded. The low number of Ag-NORs and their chromosome localization would seem to relate the "submetacentric" specimens to the "acrocentric" strains rather than to the "metacentric" ones.

The difference in C-heterochromatin amount observed between the parental species (Marescalchi and Scali 1990, Manaresi *et al.* 1991) makes possible to assign with certainty all chromosomes of the hybrid set to either parental complement and to suggest that the larger homolog of pair 1 is of *B. rossius* derivation, while for the third one (heterochromosomes) the reverse is true. From differences in DNA amount between *B. grandii* and *B. rossius*, it also follows that *B. whitei* has an intermediate genome-size value corresponding to the sum of parental 1C values (Marescalchi *et al.* 1990). From this chromosomal and cytogenetic analysis it also derives that it is not appropriate to define homolog pairs in this hybrid set beyond the third one. It is especially surprising that in the previous description (Bullini *et al.* 1983) the metacentric fourth chromosome (from *B. rossius*), although present, has not been recognized and the same has occurred for the heterochromosomes; finally, a small unrelated acrocentric element has been paired to chromosome 3: all these caused a very misleading pairing of several medium-sized elements.

The C-banding analysis, paralleled by Ag-NOR detection in the same specimens, has also revealed a clear correspondence between C-positive satellites and rRNA synthetic activity, via Ag-stained NORs. A great deal of variation in NOR number and position has been assessed both among and within populations. It is also possible to state that the *B. rossius* set appears much more prone to NOR changes than the *B. grandii* one. It is to be noticed that satellite size-range can be very large for a given localization (see f. i. chromosomes 12 and 32, Table 1): this may suggest possible differences in rDNA duplications. On the whole, from the present study Ag-NORs do not appear to provide reliable long-term cytotaxonomical markers; nevertheless, most of them appear sufficiently stable to allow the conclusion that rDNA cistrons of both parental species are active in the hybrid. Therefore, taking into account

parental and hybrid locations, we may safely suggest that NORs on chromosomes 1, 5, 12, 17 and 25 derive from *B. rossius*, while that on chromosome 32 from *B. g. grandii*; and also that there are two new locations on chromosomes 3 from *B. grandii* and 4 from *B. rossius*. (Marescalchi and Scali 1990, Manaresi *et al.* 1991).

As a final remark we would like to point out that owing to the above shown karyological differentiation, *B. whitei* does not appear to be a homogeneous "historical" group (see Echelle 1990), but rather the outcome of at least two independent hybridization events. Furthermore, the undisputable existence of hemiclonally reproducing females (Scali *et al.* 1991, Tinti and Scali 1991) among self-perpetuating parthenogenetic ones, eventually suggests to formally distinguish the clonal strains (*B. whitei* complex, *sensu stricto*) from the hemiclonal hybridogenetic ones, which could be referred to, following Schultz's indication for *Poeciliopsis* (Schultz 1969, 1989), as *B. rossius-g. grandii*, thus paralleling the Northwestern Sicilian hybridogen *B. rossius-g. benazzii* (Mantovani and Scali 1990, 1991, 1992, Mantovani *et al.* 1991a).

Summary

The thelytokous hybrid *Bacillus whitei* (2n=35, XX female) endemic to Southeastern Sicily, is clearly derived from *B. rossius* × *grandii*, but a variety of cytotypes have been found in these parthenogens. The most widespread, standard karyotype perfectly fits the suggested hybrid derivation except for the fourth metacentric element, certainly deriving from *B. rossius*, in which, however, nowadays invariably shows a corresponding acrocentric chromosome; on the other hand the acrocentric "modern" element has been found in hybridogenetic strains of *B. whitei*, very recently discovered among clonal ones. Linking together reproductive biology and geographical distribution of the "metacentric" and "acrocentric" standard karyotypes, two hybridization events between *B. grandii* and either a "paleo" or "neo" *B. rossius*, respectively, are here suggested.

C-positive satellites and corresponding Ag-NOR, are found on a wide array of chromosomes, mostly reflecting those of both parental species, but also on new locations. The high dynamics of rDNA cistrons, mainly evidenced in the *B. rossius* genome, makes NORs not entirely reliable as long-term cytotaxonomical markers, but rather useful in short-term comparisons.

Key words: Ag-NOR, C-banding, cytotaxonomy, hybridogenesis, parthenogenesis.

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