Salmon: A New Autosomal Mutation Demonstrating Incomplete Dominance in the Boa Snake Boa constrictor

R. N. Ihle, G. W. Schuett, and K. A. Hughes

An unusual and attractive pigmentation pattern mutation termed “salmon” has been identified in the United States in several captive colonies of the common neotropical boa snake boa constrictor [Boa constrictor (Boidae)]. Boa constrictors expressing the Sa pigmentation pattern appear to be restricted to regions of Panama. Animals with the Sa phenotype exhibit a sharp decrease in melanophore pigments (e.g., melanin) and an increase of xanthophore pigments (e.g., pteridines and carotenoids) throughout the body, including ventral and caudal regions. Moreover, the dorsal saddles (blotches) and lateral diamond patterns are greatly reduced or absent. Our study was initiated using a female B. constrictor born in captivity and expressed the Sa pigmentation pattern. Results from breeding experiments indicated an inheritance mode of autosomal incomplete dominance for the Sa and Wt alleles.

Snakes are a remarkably diverse group of reptiles in terms of variation in pigmentation pattern (Bechtel 1995). Nonetheless, few studies on the inheritance (e.g., Zweifel 1981) and development of pigmentation patterns (e.g., Murray and Myerscough 1991) have been conducted. The majority of research on pigmentation patterns in snakes has been performed by either zoo personnel or private breeders [reviewed by Bechtel (1995)].

The mode of inheritance of melanism (an all-black pigmentation pattern) in eastern garter snakes (Thamnophis sirtalis) from populations of the western Lake Erie region (Blanchard and Blanchard 1941) and albinism (amelanism) in the red rat snake (Elaphe g. guttata) (Bechtel 1995; Bechtel and Bechtel 1962) and San Diego gopher snake (Pituophis melanoleucus annectens) (reviewed in Bechtel 1995) were the first to be studied. In the above cases, and in nearly all subsequent ones (Bechtel 1995), pigmentation pattern mutations have been found to behave as a simple two-allele Mendelian trait, with the mutant allele recessive to the wild-type (Wt) allele. Two notable exceptions are the California kingsnake (Lampropeltis getulacaliforniae) and reticulated python (Python reticulatus). In L. g. californiae, the pigmentation pattern termed “striping” (common in several populations) is dominant to the typical pattern termed “banding” (Klauber 1944; Zweifel 1981). A newly described pattern mutation termed “tiger” in P. reticulatus involves tendencies for the Wt body pattern (blotched) to be replaced by elongated blotches in heterozygotes and by longitudinal stripes in homozygotes, a condition termed “super tiger” [reviewed in Barker and Barker (1997); see pp. 49, 53, and 64 for color plates of Wt, tiger, and super tiger, respectively]. Several breeders of P. reticulatus have reported that the tiger mutation is autosomal and shows incomplete dominance or codominance (Barker and Barker 1997).

In this article we report a new and unusual pigmentation pattern mutation, termed “hypomelanistic” or “salmon,” in the boa snake Boa constrictor (Kluge 1991; Henderson et al. 1995; de Vosjoli et al. 1998:38–39). A female boa constrictor with the salmon (Sa) mutation was acquired by one of us (R.N.I.) as a juvenile and used in breeding experiments to determine the inheritance mode of this trait. When compared to animals expressing the Wt pigmentation pattern (Figure 1A), the Sa mutation (Figure 1B) is characterized by a sharp decrease in the expression of melanophore pigments (e.g., melanin) and a sharp increase in the expression of xanthophore pigments (e.g., pteridines and carotenoids) throughout the body, including ventral and caudal regions. Moreover, the dorsal saddles (blotches) and lateral diamond patterns are greatly reduced or absent.

Materials and Methods

The Sa mutant female described above was crossed to a Wt male (obtained from a commercial source) and produced F1 progeny in 1994, 1995, and 1996 (Table 1). In 1996 and 1997, crosses between the above F1 progeny were performed to determine the mode of inheritance of the Sa mutation. In the F1 crosses that involved only Sa individuals (i.e., Sa × Sa), another novel phenotype occurred, which appeared to be an amplified version of the Sa phenotype. This new morph showed extreme loss of melanism and increased salmon (sometimes orange) coloration throughout the body, including the eye (iris) and tongue. Furthermore, the dorsal blotches were further reduced in size and the lateral pattern was frequently absent (Figure 1C,D). This phenotype will be referred to as “super salmon” (Ss) in this article. Also, from 1996 to 1999, several other F1 crosses (dom × sire) were performed and include Wt × Wt, Sa × Al (albino mutant; Kahl strain, and is autosomal recessive inheritance), Wt × Ss (performed by G. Hobbs, private breeder), Sa × Wt, and Sa × Sa.

All animals in our facility were housed individually in commercial Neodesha plastic cages with a sliding glass front (122 cm × 81 cm × 46 cm). A commercial heat pad (Mylar) was positioned beneath one end of each cage to provide a basking site (35°C). Water was provided in ceramic bowls and available ad libitum. Domestic rodents (rats) were offered weekly. All experimental matings were performed from...
Tests with one degree of freedom were performed using Yates’ correction for continuity. Only one phenotypic class is expected in the progeny, thus no association tests could be performed. Note that the dorsal blotches show extreme fading and size reduction.

Cross Table 1. Test of the incomplete dominance model

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Shown are crosses producing F1 and F2 progeny, and crosses of F1 salmon (Sa) females to an albino (Al) male.

* Tests with one degree of freedom were performed using Yates’ correction for continuity.

Only one phenotypic class is expected in the progeny, thus no association tests could be performed. Note that the progeny phenotypes match those expected under the incomplete dominance model.

Figure 1. Comparison of (A) Wt, (B) Sa, and (C,D) Ss Boa constrictor progeny produced in this study (Table 1). Note in (D) that the iris is nearly orange (versus gray and tan in Wt), the tongue is pale (versus black in Wt), and the dorsal blotches show extreme fading and size reduction.

Results and Discussion

We present in Table 1 the results of parental crosses and crosses of their F1 progeny, along with results of chi-square tests (Zar 1996) of the fit to a model of incomplete dominance. Chi-square values $<=0.05$ were scored to deviate significantly from the expected model. The parental crosses involving the Sa female and Wt male were successful in three consecutive years (1994–1996), and both Wt and Sa F1 progeny were produced in each of the three litters (Table 1). Ten different pairs of F1 crosses from our parental stock were scored to deviate significantly from the expected ratios. The two Sa progeny from our parental stock were crossed (Sa $\times$ Wt, Sa $\times$ Sa, and Sa $\times$ Ss), yielding Wt, Sa, and Ss F2 progeny (Table 1).

Moreover, we performed three additional crosses involving F1 animals. One cross, Wt $\times$ Wt, yielded 100% Wt progeny, and two other crosses, Wt $\times$ Sa (performed in another facility, G. Hobbs; permission was granted to publish the data) and Ss $\times$ Wt, yielded 100% Sa progeny (Table 1).

To test for allelism, two additional crosses were performed using two F1 Sa females and a single, unrelated albino (Al) male (Kahl strain). The Kahl strain Al trait is autosomal recessive (de Vosjoli et al. 1998:37). Both of the Al $\times$ Sa crosses yielded expected ratios of Wt and Sa progeny (Table 1).

In summary, a total of 17 crosses were performed, producing 381 healthy progeny (145 Wt, 185 Sa, and 51 Ss). The sex ratio was close to one in all litters, with no evidence of sex-limited expression of the Sa trait.

Preliminary inspection of the results suggested an autosomal model of incomplete dominance for the inheritance of the Sa trait. The data were fit to this model by conducting chi-square tests (Zar 1996). For all tests having a single degree of freedom we used Yates’ correction for continuity (Zar 1996:464).

The chi-square tests did not reveal significant deviation from the expected ratios under the model of incomplete dominance (Table 1). The first two litters of the parental cross (F1, and F2) were marginally nonsignificant (both at $P = .07$). However, litter F3 of the parental cross showed a strong fit to the model ($P = .90$). We attribute the tendency for deviation from the model in the first two litters to errors in scoring offspring. It is possible that we scored some of the Sa progeny as Wt because in some situations Sa neonates are difficult to distinguish from Wt until after several ecdyses. Unfortunately at least 10 of the Wt animals from the first two litters were not retained; thus we cannot re-score those individuals. In all but two cases, subsequent litters produced ratios close to expected values. The two Sa $\times$ Al crosses demonstrated no interaction between the Sa mutation and the recessive allele for the Al mutation. These results are consistent with the traits being determined by alleles at different loci.

We therefore propose that the Sa mutation in B. constrictor is autosomal and shows incomplete dominance, with the alleles behaving as a pair and segregating in predicted Mendelian fashion (e.g., Russell 1992). Individuals with a single copy of the Sa allele (heterozygotes) deviate from Wt in appearance (Figure 1B), and those with two copies of the Sa allele (Ss, homozygotes) deviate substantially more, in some cases bearing little resemblance to the Wt
pigmentation pattern of the species (Figure 1C,D; de Vosjoli et al. 1998). There is, however, a range of phenotypes exhibited by snakes possessing Sa and Ss alleles. Primarily affected are the size and pattern of dorsal and lateral body blotches, and the degree to which melanin is expressed in scales. Undoubtedly pigmentation pattern in Sa and Ss individuals is influenced by modifiers and/or the environment. Future work includes performing crosses between individuals possessing both the salmon (Sa and Ss) and albinoism (AI) trait, and those that are homozygotes, Ss × Ss.

It is known that the Sa trait we report in captive B. constrictor occurs in certain populations from Panama (Porras 1999; Hardy D Sr, personal communication). According to Porras (1999, personal communication), B. constrictor from Isla Taboaguilla (Bahia de Panama) show the Sa trait (p. 60), and some individuals bear a strong resemblance to the Sa phenotype (pp. 51–52). D. Hardy, Sr. (personal communication) collected a juvenile female B. constrictor from the region of Gamboa, Panama, that showed the Sa condition. Because we have limited information on the Sa trait in B. constrictor, the extent of its geographic distribution remains to be established. Nonetheless, at present we are aware of no other localities than those provided above (Porras L, personal communication), and thus tentatively conclude that the Sa mutation has a limited geographic distribution in B. constrictor. Of importance, these data provide information that will be of utility to systematists and taxonomists working on this species. Based on our findings, populations of B. constrictor expressing the Sa or Ss pigmentation pattern should not be considered as distinct geographic races or other type of evolutionary unit. Rather it is our opinion that these populations show polymorphism with respect to pigmentation pattern.

From 1030 W. Pecos Ave., Mesa, Arizona (Ibue), and Department of Life Sciences, Arizona State University West, P.O. Box 7100, Phoenix, AZ 85069-7100. G. Hobbs kindly supplied data (Wt × Ss cross) and granted permission for its inclusion in this article. L. Clarke and K. Hanley photographed the animals. K. Dixon made helpful suggestions in the initial analysis. D. Hardy Sr. and L. Porras provided information on the occurrence of the salmon trait in B. constrictor from certain Panamanian populations. Address correspondence to G. W. Schuett at the above address or e-mail: gschuett@asu.edu.

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References


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Physical Mapping of rRNA Genes in Medicago sativa and M. glomerata by Fluorescent in situ Hybridization

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Fluorescent in situ hybridization (FISH) was applied to diploid and tetraploid subspecies of alfalfa (Medicago sativa L.) to investigate the distribution of rRNA genes and to utilize the sites of 18S-5.8S-25S rDNA and 5S rDNA sequences as markers for studying the genome evolution within the species. Medicago glomerata Balb., the species considered to be the ancestor of alfalfa, was included in this study in order to obtain more information on the phylogenetics of alfalfa. Simultaneous in situ hybridization was performed with the probes pTa71 and pXVI labeled with digoxigenin and biotin, respectively. In the diploid taxa, M. glomerata, M. sativa ssp. coerulea Schmalh and ssp. falcata Arcangelii, the 18S-5.8S-25S rDNA sequences were mapped to two sites corresponding to the secondary constrictions of the nucleolar chromosome pair, while 5S rDNA appeared to be distributed in two pairs of sites. Chromosomes carrying 5S loci could be distinguished on the basis of their morphological characteristics. The number of rDNA sites detected in the tetraploid M. sativa ssp. falcata and ssp. sa- tiva (L.) L. & L. were twice the number found in the respective diploid ssp. falcata and ssp. coerulea. The results of this study show that the distribution of ribosomal genes was maintained during the evolutionary steps from the primitive diploid to the cultivated alfalfa. Modifications of the number of rRNA loci were not observed. The importance of in situ hybridization for improving karyotype analysis in M. sativa L. is discussed.

Medicago sativa L., the most studied spe- cies of the genus Medicago, includes forms with different morphological traits and chromosome numbers. Diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) taxa with violet flowers and coiled legumes are classified as M. sativa ssp. coerulea (Less. ex Ledeb.) Schmalh and M. sativa ssp. sa- tiva (L.) L. & L., respectively. Cytogenetic characteristics have revealed that these two forms are very closely related species (Gillies 1970; Ho and Kash A 1972; Lesins 1957; Stanford 1959). Meiotic studies on micro- and macrosporogenesis have dem- onstrated that ssp. sativa originated by sexual polyploidization following the production of 2n gametes in ssp. coerulea (McCoy and Bingham 1988; Pfeiffer and Bingham 1983). The original area of distribu- tion of the primitive diploids probably included present-day Anatolia, Armenia, Iran, and the areas around the Caspian Sea. It was in these localities that the tet- raploids appeared, which through domestica- tion gave rise to the cultivated form of alfalfa. At present alfalfa is considered the most important forage legume in both Eu- rope and North America (Michaud et al. 1988).

M. sativa ssp. falcata (L.) Arcangelii differs from the previous taxa in that it bears yellow flowers and produces straight pods. It has both diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) forms, both of which are widely distributed over eastern Europe. The tetraploids occur with greater frequency than the diploids, which are considered evolutionary relics. De- spite the morphological differences, many cytological characteristics have demon- strated a close genetic relatedness be-
between ssp. falcata and ssp. sativa (Cleveland and Stanford 1959; Lesins 1957). At the same ploidy level they intercross easily and produce viable hybrids with normal meiotic behavior (Ho and Kasha 1972). Furthermore, pachytene studies have demonstrated that their chromosomes are almost identical (Gillies 1970). Recently RFLP maps have shown that there is a perfect collinearity between the genomes of the two species (Tavoletti et al. 1996). It is well documented that they share a common ancestry, most probably identifiable with M. glomerata Balb., a diploid species with yellow flowers and coiled pods (Gillies 1971; Lesins and Lesins 1964). Differentiation of ssp. coerulescens and ssp. falcata has been proposed as the consequence of the spatial isolation of the ancestral populations of M. glomerata. Under different selection pressures they developed morphological traits, such as violet flowers and straight pods, which are characteristic of ssp. coerulescens and ssp. falcata, respectively (Quiros and Bauchan 1988).

Cytogenetic studies have been carried out to understand the phylogenetics of alfalfa and to analyze the genetic similarity with the related taxa (Gillies 1971). However, the mitotic chromosomes have a limited usefulness because they are small and very similar in size and are generally considered extremely difficult for detailed cytogenetic studies. These limitations have been partially overcome in recent years by the application of the C-banding technique, which was used to characterize the somatic chromosomes of M. sativa CADL (cultivated alfalfa at the diploid level) (Masoud et al. 1991) and tetraploid alfalfa (Falistocco et al. 1995). Recently Bauchan and Hossain (1997) applied it to M. sativa ssp. coerulescens and ssp. falcata. The C-banding pattern revealed a larger amount of constitutive heterochromatin in the chromosomes of tetraploid ssp. sativa and ssp. coerulescens than in those of ssp. falcata, which exhibited bands only at the centromeric regions.

Molecular cytogenetic techniques are currently being applied to study genomic organization and determine the evolutionary process. DNA:DNA fluorescent in situ hybridization (FISH) used in conjunction with fluorochrome staining has turned out to be an extremely valuable method for studying species with small or uniform chromosomes (Maluszynska and Heslop-Harrison 1993; Schmidt et al. 1994). It is possible to analyze in detail the chromosome structure and to detect the genomic changes that occurred during the evolution of the species by means of physical mapping of repetitive genes or other sequences directly on metaphase chromosomes (Cuadrado and Jouve 1997; Fomi- naya et al. 1994; Pedersen et al. 1995). Physical mapping of the chromosomes also provides useful information for breeding programs (Heslop-Harrison and Schwarzacher 1996).

Identification of the sites of the ribosomal genes (rDNA) is generally the first step toward the physical mapping of chromosomes. Such genes constitute the most important multigene families in the eukaryotic genome. In plants they are organized in separated clusters: 18S-5.8S-25S and 5S genes, usually located in different chromosomes (Lapitan 1992). The 18S-5.8S-25S ribosomal genes are present in many hundreds of repeated units arranged in tandem arrays corresponding to the nucleolar organizer regions (NORs) (Lapitan 1992). Genes coding for the 5S ribosomal RNA have a similar organization, however, their sites along the chromosome are not revealed by any morphological characteristics.

FISH to chromosomes of diploid subspecies of M. sativa and tetraploid alfalfa has been utilized for determining the sites of the ribosomal 18S-5.8S-25S gene cluster (Calderini et al. 1996), however, loci of 5S genes have never been identified. This study was undertaken to determine the complete physical mapping of rRNA genes in diploid ssp. coerulescens, ssp. falcata, tetraploid ssp. falcata and ssp. sativa, and to use the ribosomal gene sites as cytogenetic markers for studying the genome evolution within the species. Investigations were also conducted on M. glomerata Balb., the species related to M. sativa and considered its ancestor, to obtain more information on the evolution of the ribosomal gene sites.

Simultaneous in situ hybridization was

![Figure 1. Fluorescent in situ hybridization of rDNA probes, pTa71 (green) and pXVI (red), to mitotic chromosomes of M. glomerata (a–c), M. sativa ssp. coerulescens (d–f), and M. sativa ssp. falcata (g–i). (a) Metaphase of M. glomerata showing DAPI staining, (b) sites of 18S-5.8S-25S rDNA, and (c) sites of 5S rDNA. (d) Metaphase of ssp. coerulescens showing DAPI staining, (e) sites of 18S-5.8S-25S rDNA, and (f) sites of 5S rDNA. (g) Metaphase of ssp. falcata showing DAPI staining, (h) sites of 18S-5.8S-25S rDNA, and (i) sites of 5S rDNA. Large and small arrows indicate 18S-5.8S-25S sites and 5S sites, respectively. The bar represents 5 μm.](image-url)
performed with probes pTa71 and pXVI labeled differently to determine the localization of both 18S-5.8S-25S and 5S rDNA sequences in the same experiment.

Materials and Methods

Plant Material

The materials used for this study consisted of the accessions 2956 and 3401 of *M. sativa* ssp. *coerulea* from the Institute of Plant Breeding of Perugia (Italy), *M. sativa* ssp. *falcata* 2x WISFAL-1 and 4x WISFAL-1 (Bingham 1990), kindly provided by Professor E. T. Bingham, University of Wisconsin, Madison, *M. sativa* ssp. *sativa* cv. Turrena, a synthetic variety from the Institute of Plant Breeding of Perugia, and the accessions PI 516906, PI 577567, and PI 577568 of *M. glomerata* from the Western Regional Plant Introduction Station, Pullman, Washington.

Chromosome Preparation and FISH

Seeds of each accession were germinated in petri dishes at room temperature. Actively growing root tips were excised when they were about 1 cm in length, pretreated in a saturated aqueous solution of a-bromonaphthalene for 4 h, and then fixed in ethanol:acetic acid (3:1) overnight.

Chromosome preparation was carried out in the following way. Fixed root tips were washed in enzyme buffer (10 mM citric acid/sodium citrate pH 4.6) for 30 min then placed on poly-L-lysine-coated slides with 1–2 drops of enzyme solution (4% cellulase Onozuka R10 and 1% pectolyase Sigma in distilled water) for 2 h at 37°C. For each slide, three root tips were used. After removing the enzyme with distilled water and eliminating excessive water, 1–2 drops of ethanol:acetic acid (3:1) were added. Root tips were broken with a thin needle to give a fine emulsion and spread on the slide. Preparations were air dried. FISH was accomplished by pretreating slides with 100 μg/ml of RNase A in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) for 1 h at 37°C and washed three times in 2×SSC. After incubation with 80 U/ml of pepsin (Sigma) in 10 mM HCl for 15 min at 37°C, the chromosome preparations were stabilized by immersion at room temperature for 10 min in freshly depolymerized 4% (w/v) paraformaldehyde in water, washed in 2×SSC, dehydrated in a graded ethanol series, and air dried. Heterologous probes were used for in situ hybridization. Clone pTa71 contains a 9 kb EcoRI fragment of *Triticum aestivum* L. consisting of the 18S-5.8S-25S rRNA genes and nontranscribed spacer sequences (Gerlach and Bedbrook 1979). Clone pXVI contains the complete gene of 5S rRNA and the spacer region of *Beta vulgaris* L. (Schmidt et al. 1994). Clone pTa71 was labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation, while pXVI was labeled with biotin-11-dUTP (Sigma) using the polymerase chain reaction (PCR).

The hybridization solution consisting of 150 ng/μl of DNA probe, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% (w/v) SDS (sodium dodecyl sulfate), and 300 ng/μl sheared salmon sperm DNA, was incubated for 10 min at 70°C and chilled on ice. Forty microliters of hybridization mixture were applied to each chromosome preparation and covered with a plastic coverslip. The hybridization mixture and the chromosomes were denaturated together at 70°C in a modified thermocycler for 5 min, then the temperature was gradually decreased to 37°C. The hybridization was carried out overnight at 37°C. The one-day in situ hybridization was also performed; in this case the hybridization time was reduced to 3 h. Since both methods gave good results, they were used without distinction. After hybridization the coverslips were floated off in 2×SSC at 42°C, then the slides were washed for 10 min each in 20% formamide (v/v) in 0.1×SSC at 42°C, 2×SSC at 42°C, and 2×SSC at room temperature. Detection of the probes labeled with digoxigenin and biotin was carried out simultaneously. The slides were transferred to detection buffer (4×SSC/0.1% Tween 20) for 5 min, treated with 5% (w/v) bovine serum albumin (BSA) in detection buffer for 5 min, and incubated in 20 μg/ml of sheep antidigoxigenin antibody conjugated with FITC (Boehringer Mannheim) and 5 μg/ml of streptavidin conjugated with Cy3 in detection buffer containing 5% (w/v) BSA for 1 h at 37°C. After incubation the slides were washed in detection buffer three times for 8 min each at 37°C. The slides were counterstained with 2 μg/ml of DAPI (4’,6-diamidino-2-phenylindole) and then mounted in antifade solution Vectashield (Vector Laboratories). Slides were examined with a Microphot Nikon epifluorescence microscope.

![Figure 2](image1.png)  
**Figure 2.** Fluorescent in situ hybridization of rDNA probes, pTa71 (green) and pXVI (red), to mitotic chromosomes of tetraploid *M. sativa* ssp. *falcata* (a–c) and *M. sativa* ssp. *sativa* (d–f). (a) Metaphase of ssp. *falcata* showing DAPI staining, (b) sites of 18S-5.8S-25S rDNA, and (c) sites of 5S rDNA. (b) Metaphase of ssp. *sativa* showing DAPI staining, (e) sites of 18S-5.8S-25S rDNA, and (f) sites of 5S rDNA. Large and small arrows indicate 18S-5.8S-25S sites and 5S sites, respectively. The bar represents 5 μm.

![Figure 3](image2.png)  
**Figure 3.** Homologous chromosome pairs carrying 5S sites. A representative example of the physical location of 5S rDNA genes in the diploid taxa; the chromosomes are of ssp. *falcata*. 

258 The Journal of Heredity 2000:91(3)
Results
Simultaneous in situ hybridization with probe pTa71 labeled with digoxigenin and probe pXVI labeled with biotin allowed the sites of the ribosomal genes 18S-5.8S-25S and 5S to be identified in the same metaphase plates. Figures 1 and 2 show metaphase chromosomes from the root tips of diploid and tetraploid taxa, respectively, after FISH with the two probes and counterstaining with DAPI.

Localization of 18S-5.8S-25S and 5S rRNA Genes in M. glomerata, M. sativa ssp. coerulea, and M. sativa ssp. falcata
After in situ hybridization with probe pTa71 labeled with digoxigenin detected with antibody conjugated with FITC and clone pXVI labeled with biotin detected with streptavidin Cy3 the two clusters of 18S-5.8S-25S and 5S rDNA genes were mapped to the chromosomes of diploid M. glomerata, ssp. coerulea and ssp. falcata (Figure 1). Two hybridization sites of probe pTa71 were observed in all the accessions of the three taxa. The green fluorescent signals showed that loci of 18S-5.8S-25S sequences are localized in the satellited chromosome pair in correspondence to the regions of the nucleolar organizers (Figure 1b,e,h). No other sites were detected. The two hybrid sites were clearly detected because signals were large and of high intensity. The size of the mapped region did not show detectable variation among accessions or taxa when chromosomes with an equal degree of contraction were compared.

Two pairs of 5S rDNA sites were identified in each diploid taxa (Figure 1c,f,i). The strength of the signals allowed the number of the hybrid sites to be ascertained with accuracy. In some metaphases the signals appeared as two bright red spots corresponding to the loci of the 5S genes on each of the chromatids. The chromosome pairs carrying the 5S genes could be identified in each taxa by selecting metaphases with less contracted chromosomes; they could be distinguished from each other by means of their different lengths: the largest pair showed the hybridization signal in the short arm, while in the smaller pair it was in the long arm. In both cases signals appeared quite near the centromeres (Figure 3). In each of the diploid taxa the clusters of 5S and 18S-5.8S-25S ribosomal genes are located on different chromosomes.

Localization of 18S-5.8S-25S and 5S rRNA Genes in M. sativa ssp. falcata (4x) and M. sativa ssp. sativa
In situ hybridization carried out in the tetraploid ssp. falcata and ssp. sativa revealed two pairs of 18S-5.8S-25S sites. Probe pTa71 mapped to the secondary constrictions of the four nucleolar chromosomes (Figure 2b,e). No signals were detected outside these regions. Differences in the strength of hybridization signals of the four sites were not observed either in ssp. falcata or ssp. sativa.

Loci of 5S rDNA genes were found in four pairs of chromosomes (Figure 2c,1). The identification of the hybrid sites was difficult, however, good preparations without overlapping chromosomes or background allowed the number of sites to be accurately determined. Simultaneous utilization of probe pTa71 and pXVI showed that in the tetraploid complements 5S and 18S-5.8S-25S genes are also localized on different chromosomes.

Discussion
Investigations of rRNA genes are of great importance to phylogenetic studies because their number may change substantially during evolution. Variations may affect the number of loci within the genome and the copy number of the repeat units at single loci (Flavell 1989). Analysis of the number and distribution of the sites of rDNA sequences provides important information for understanding the genome organization of related species and for evaluating genomic modifications that occurred during the evolution of the species. Identification of ribosomal gene sites on plant chromosomes by FISH has been achieved in many species including Hordeum vulgare L. (Leitch and Heslop-Harrison 1992; Pedersen and Linde-Laursen 1994), Beta vulgaris L. (Schmidt et al. 1994), Festuca arundinacea Schreb. (Thomas et al. 1997), Brassica ssp. (Maluszynska and Heslop-Harrison 1993), and Populus species (Prado et al. 1996). This research offers the first report of the physical mapping of the 18S-5.8S-25S and 5S rRNA genes in diploid and tetraploid subspecies of M. sativa and in its putative ancestor M. glomerata. FISH to chromosomes of these forms enabled the sites of 18S-5.8S-25S and 5S rDNA sequences to be examined at different evolutionary phases, that is, from the early diploid to the cultivated tetraploid form.

The sites of 18S-5.8S-25S rDNA in the diploid taxa correspond to the secondary constriction of the satellited chromosomes, while those of 5S rDNA are present in two pairs of chromosomes. Such results indicate that the distribution of ribosomal gene sites detected in the putative ancestral genome of M. glomerata was conserved during the evolution of the diploid ssp. coerulea and ssp. falcata. Moreover, the morphology of the chromosomes with the 5S loci and the position of this cluster of ribosomal genes seems to suggest that structural rearrangements involving the two chromosome pairs did not occur in the diploid taxa.

The number of rDNA sites detected in tetraploid ssp. falcata and ssp. sativa were twice the number found in the respective diploid forms. This situation demonstrates that in M. sativa, the number of ribosomal gene sites did not change during evolution. Consistent variations of the copy number at single loci also appear unlikely since the fluorescence intensity did not show significant differences among the 18S-5.8S-25S sites and the 5S sites.

This study provides further information about the genome structure and phylogenetic relationships of M. sativa and shows that FISH is also a valuable method for improving the karyotyping analysis in this species. The physical mapping of the 5S rDNA genes, for example, may be used as a marker for identification of the two chromosomes. This is of particular interest for investigating chromosomes in ssp. falcata in which C- and N-banding techniques are not useful for karyological analysis (BauChan and Hossain 1997, 1998). Since the distinction of the two chromosomes carrying the 5S genes relies on morphological characteristics that cannot always be unequivocally ascertained, in situ hybridization experiments can be carried out using different types of sequences as probes. By means of physical location of 5S ribosomal genes and other sequences it will be possible to identify these chromosomes in diploid and tetraploid metaphases with certainty and to verify if possible structural rearrangements occurred after evolution.

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Karyotype Analysis, Banding, and Fluorescent in situ Hybridization in the Scarab Beetle Gymnopleurus sturmi McLeay (Coleoptera Scarabaeoidea: Scarabaeidae)

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Conventional staining, differential banding, and in situ hybridization with both ribosomal and telomeric probes to mitotic chromosomes of Gymnopleurus sturmi (Scarabaeoidea: Scarabaeidae) are described. The karyotype is distinguished by a pericentric inversion polymorphism in chromosome 3, which is either acrocentric or subteloentric. Silver staining (Ag-NOR) and chromomycin A3 (CMA3), failed to study the detection of nucleolar organizer regions (NORs), due to the extensive silver and CMA3 stainability of all GC-rich heterochromatin. Fluorescent in situ hybridization (FISH) using a Paracentrurus lividus (Echinodermata) rDNA probe mapped the ribosomal RNA genes (rDNA). FISH with the all-human telomeric sequences (TTAGGG), revealed a lack of homology between the telomeric probe and the telomeres of G. sturmi. This suggests that the telomeric hesanucleotide (TTAGGG), is not so conserved within eukaryotes as it has been hypothesized.

The cosmopolitan superfamily Scarabaeoidea (Coleoptera) is an ancient group whose origins can be traced back at least to the late Mesozoic (more than 200 million years ago) and comprises about 33,000 different species. Details regarding the morphology, body size, habitat, feeding behavior, and reproductive biology of these interesting organisms have been compiled by many authors [see Halffter and Matthews (1966), Morón (1984), Hanaski and Cambefort (1991), and the authors quoted by them] who have pointed out the impressive diversity among species and groups. On the contrary, data available about the chromosomes of Scarabaeoidea are very scanty, comprising the haploid and/or diploid numbers of about 200 species and the karyotype description of some of them (Salamanna 1965, 1972; Vidal et al. 1977; Yadav et al. 1979, 1989).

Conventional banding techniques have been attempted on chromosomes of four Scarabaeoidea species—the Dynastinae Enema pan (F.) (Vidal and Giacomozzi 1979), the Geotrupidae Thorectes intermedius (Costa) (Colomba et al. 1995), and the Scarabaeidae Bubas bison L. and Glyphodorus sterquilinus (Westwood) (Colomba et al. 1996). Recently, for the first time in Scarabaeoidea, fluorescent in situ hybridization (FISH) has been used to map rDNA encoding for “large” rRNA molecules in the geotrupid Thorectes intermedius (Vitturi et al. 1999).

The genus Gymnopleurus Illiger, 1803 (Scarabaeidae: Scarabaeinae), whose distribution is Palearctic, Afrotropical, and Oriental (Halffter and Edmonds 1972), comprises about 50 species (Balthasar 1963), but only three of them have been partly investigated from a karyologic point of view. Dasgupta (1963) reported the haploid number n = 10 for G. koenigii.
Figure 1. (a) Giemsa-stained male karyotype of *G. sturmi* showing pair 3 with the homozygous condition for the subtelocentric type (3a3a). (b) C-banded female karyotype showing pair 3 with the heterozygous condition for the subtelocentric and the acrocentric type (3a3b). (c) Diagramatic representation of the average karyotype.

and Lahiri (1972) established the diploid number 2n = 18 for *G. sinuatus*, and Kacker (1976) found 2n = 20 for *G. cyaneus*.

This study on *Gymnopleurus sturmi* chromosomes by means of Giemsa staining, a number of banding techniques, and FISH is the first advanced cytologic analysis of the subtribe Gymnopleurina, whose phylogeny is still controversial (cfr. Cambefort 1991; Zunino M, unpublished data).

**Materials and Methods**

A total of 120 sexually mature specimens (64 male and 56 females) of *G. sturmi* McLeay (Scarabaeoidea: Scarabaeidae), collected in the field at Bellolampo (Palermito, northwest Sicily, Italy) from May 1996 to September 1998 were used for this study. All animals, identified following the guidelines laid down by Baraud (1992), were reared in breeding boxes according to Zunino and Monteresino (1994). Due to the scantiness of cells actively dividing, only a few specimens (9 males and 2 females) provided high-quality metaphase spreads that could be utilized successfully in this study.

Chromosome preparations were obtained, using the air-drying method (Vitturi et al. 1996), either from male gonads or the intestinal epithelium of individuals of both sexes after in vivo colchicine treatment. Conventional Giemsa staining followed current methods (Vitturi et al. 1996). C-banding was performed according to Sumner (1972). Silver staining was carried out following the one-step method described by Howell and Black (1980). Staining with the fluorescent dyes CMA3 (GC specific) and DAPI (AT specific) was performed according to Schmid et al. (1983). Chromosomes were classified according to the criteria proposed by Levan et al. (1964).

FISH was performed on fixed metaphases of six males using a *Paracentrotus lividus* (Echinodermata) 4.3 kb rDNA probe (prR14) consisting of sequences from the 3’ end of the 18S to the 3’ end of the 26S rDNA, provided by R. Barbieri (University of Palermo). FISH was also carried out on chromosomes of two males using the all-human telomeric repeat (TTAGGG)4 (P4097-DG.5, Oncor).

Nick translation labeling with digoxigenin and hybridization experiments were performed according to manufacturers’ instructions (Boehringer Mannheim and Oncor). Slides were mounted in an antifade solution containing propidium iodide (5 μg/ml) and viewed under a Leica I3 filter set (BP 450–490; LP 515). Photographs were taken using a Leica microscope on Kodak Agfaortho 25 ASA and Kodak Ekta-color 1000 ASA films.

**Results**

Counts of 10 Giemsa-stained metaphases per specimen gave a diploid number of 2n = 20. We arranged the chromosomes, according to their dimension and centromere position, into nine pairs of autosomes and one sex chromosome pair that was homomorphic (XX) in the females and heteromorphic (XY) in the males. Autosomal pairs range from 7.68 to 1.78 μm, and comprise two metacentric (pairs 1 and 4), five subtelocentric (pairs 2 and 6–9), and one acrocentric (pair 5), pair 3 is heteromorphic, consisting either of two subtelocentric chromosomes or one subtelocentric and one acrocentric. These chromosomes are equal in length and the subtelocentric type is designated as 3a while the acrocentric type is designated as 3b. The X is a small subtelocentric of about 1.5 μm and the Y is a metacentric microchromosome (Figure 1a,b). A haploid average karyogram was obtained from 10 high-quality spreads (Figure 1c).

Application of C-banding revealed the occurrence of large heterochromatic blocks, corresponding to the centromeric and pericentromeric regions of chromosomes 1, 2, 3a, and 4. In the subtelocentric pairs 6–9 and in the X, C-positive material was spread over the paracentromeric area of the long arm and on the whole short arm. Chromosomes 5 and 3b showed a centromeric C band. No C-positive reac-
tion was observed in the Y, probably due to its minute dimension (Figure 1b).

All heterochromatic material was stained by silver (Figure 2A) and fluoresced brightly after CMA, treatment (Figure 2B). Chromosomes were homogeneously DAPI stained (Figure 2C).

dNA FISH performed on 40 spermatogonial metaphases of five males allowed localization of the major ribosomal clusters in the heterochromatic regions of a maximum of three chromosome regions besides the ribosomal clusters.

Two kinds of interindividual chromosome polymorphism have been observed in the G. sturmi population under study. The first type deals with the occurrence of different numbers of rDNA copies, revealed by FISH signals differing in number and size. This kind of polymorphism, however, is a very common finding in animals (see Schmid 1982; Suzuki et al. 1990).

Another type of interindividual chromosome polymorphism consists of two different configurations of chromosome 3, including the homozygous condition for the subtelocentric chromosome 3a3a and the heterozygous for the subtelocentric 3a and the acrocentric 3b. The lack of the homozygous condition for the acrocentric chromosome 3b3b is likely due to the limited number of specimens analyzed. Since 3a3a and 3a3b configurations were found both in males and females, it is clear that this chromosome rearrangement is inherited but not linked to the sex determination mechanism. Furthermore, on the basis of a general karyoevolutionary trend toward an increase in karyotype symmetry (Stebbins 1950), we suggest that in G. sturmi, a transformation from the original acrocentric into the neotelocentric occurred through a pericentric inversion.

Pericentric inversion polymorphism has been described in mammals (Davis et al. 1986; Hale 1986), birds (Ansari and Kaul 1979; Christidis 1986), reptiles (Moritz 1984), Amphibia (Schmid et al. 1995), and some fish species (Turner et al. 1985; Vit et al. 1993), whereas, as far as we know, within invertebrates, it has been reported for Orthoptera [see Cabrero and Camacho (1982), and authors quoted by them] and for different populations of the genus Drosophila (Norry et al. 1997; Paik et al. 1998; Singh 1998).

After CMA, staining, G. sturmi chromosomes selectively fluoresce in correspondence to all heterochromatic material. This strongly indicates that heterochromatic DNA is compartmentalized in GC base pairs. The lack of complementarity, revealed after treatment with the AT-specific fluorochrome DAPI, is an unusual reaction whose explanation remains unclear.

In most fish (Mayr et al. 1986; Phillips and Hartley 1988) and amphibians (Schmid et al. 1995; Schmid and Gutenbach 1988), CMA individualizes both active and inactive NORs as a consequence of the AT-rich GC content of the rDNA. In G. sturmi, although NORs are found to be enriched in GC base pairs, CMA is unable to locate them as a result of the presence of additional GC-rich chromosome regions besides the ribosomal clusters.

As in other organisms (Pendás et al. 1993a,b; Phillips and Reed 1996), as well as G. sturmi, the most appropriate method for detection of rDNA cistrons remains rDNA FISH. In fact, this method of investigation revealed rRNA genes corresponding to the terminal areas of the heterochromatic regions of a maximum of three subtelocentric chromosome pairs, indicat-
ing that just a small portion of the GC-rich C-positive material consists of rDNA. We also used silver nitrate to assess the rRNA gene activity, as silver binds acidic protein(s) associated with the DNA of the decondensed chromatin of the NORs (Jordan 1987; Medina et al. 1983). In G. sturmi, silver is unable to individuate active rDNA clusters, since it stains both constitutive heterochromatin and heterochromatin associated with the NORs. Within Scarabaeoidea, this unusual heterochromatin reaction is also shown by the geotridid Thorectes intermedius (Vitturi et al. 1999), the melolonthid Phyllophaga natus excavatus (Colomba 1998), and the lucanid Dorcus parallelopipedus (personal, unpublished data), which represent three phyletic branches of the superfamily, thus suggesting that such a feature is quite common within this taxon. On the contrary, among other animal groups, a similar reaction is reported only for the hedgehog Eira (Aethuscus algirus) (Sánchez et al. 1995). Moreover, the extensive silver stainability of the heterochromatin within Scarabaeoidea is quite independent from its base pair composition. In fact, the AT-rich heterochromatin of Bubas bison (Scarabaeidae) is as silver positive (Colomba et al. 1996) as the GC-rich heterochromatin of the other scarabaeoid species studied so far (Vitturi et al. 1999, this article).

Finally, results arising from in situ hybridization using the all-human telomeric sequence (TTAGGG), reveal the lack of homology between the telomeric probe and the telomeres of G. sturmi. Taking into account that in the same hybridization experiments, the chromosomes of the earthworm Eisenia fetida gave a positive reaction, the possibility that the results obtained for G. sturmi may be due to a technical shortcoming can be discarded. This assumption seems to be further supported by the fact that no evident hybridization signals have been detected in Drosophila (Mason and Brissmann 1995). On the other hand, Okazaki et al. (1993) isolated, from the Bombyx mori genomic library, a telomeric sequence (TTAGGG), which does not hybridize to vertebrate DNAs, but, on the contrary, seems to be conserved among insects. In fact, this pentanucleotide hybridized to DNAs from 8 of 11 orders of tested insect species. All this might suggest that the hesanucleotide telomeric sequence (TTAGGG), is not as widely conserved within euakaryotes as it has been stressed so far (Blackburn 1991). From the Dipartimento di Biologia Animale, Università di Palermo, Via Archirafi 18, 90123 Palermo, Italy. This work was supported by a research grant from MURST. Address correspondence to R. Vitturi at the address above or e-mail: zuvitcol@unipa.it.

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264 The Journal of Heredity 2000:91(3)