Silkworm genetics

Genetics of the qualitative traits.

It was estimated that silkworm genome consist of about 4.8×10^8 bp, its genetic information volume is about one sixth of human being. There are over 450 morphological, physiological and biochemical characters recorded at present, among them 300 (including multi-allele) had been located on 27 groups of the total 28 chromosomes.

Voltinism: According to Lea(1993) two genetic mechanisms seem to be involved in voltinism. One is sex-linked multiple alleles (Hs,Hs^2 , hs) as modified by a number of autosomal genes , each with minor effects. The second mechanism is three alleles(V^1, $+^v$, V^3) of a major gene on the sixth chromosome. Although maternal inheritance has been demonstrated in many genetic experiments for voltinism , the mode of inheritance is much more complicated , due to the influence of the temperature and light in each development stage from egg to moth. When bivoltine eggs were incubated at 15 ^{o} C after blastokinesis of the embryo , the moths which developed laid non-hibernating eggs. When eggs were incubated at a temperature above 24 ^{o} C , the resulting moths laid hibernating eggs.

In other physiological experiments , light as well as temperature strongly affected voltinism , especially during the egg incubation period. Light longer than 16 hours produced hibernating eggs , and light shorter than 12 hours produced non- hibernating eggs. When the incubation temperature is 25-26 ^{o} C the light doesn't have influence on the voltinism except for the case of feeding the larvae on artificial diet. In general dominance is recognized in the order of univoltine> bivoltine > multivoltine. The multivoltine races always lay non-hibernating eggs. The breeds between multi and bivoltine could lay some non-hibernating eggs under high incubation temperatures. The bivoltine strains lay non-hibernating eggs under low and hibernating eggs under high temperature of egg incubation. The breeds between bi and univoltine could lay both non and hibernating eggs , even under low incubation temperatures. The typical univoltine races always lay hibernating eggs.

The embryonic diapause in the silkworm , Bombyx mori L. is controlled by several genes. One of them is the so called ***pnd*** , or pigmented but non-diapausing egg, which is linked on the 11^{th} chromosome(Yoshitake and Takei, 1975;Takei and Yoshitake, 1975;Sonobe and Okada,1984; Sonobe,1984;Sonobe and Odake,1986). According to Murakami and Ohtsuki(1989),Tzenov and Petkov (1998) if the silkworm egg is homozygous for the ***pnd*** gene they develop in moths laying only non-diapausing eggs irrespective of incubation and larval rearing environmental condition , therefore the diapause character is of an obligatory type. It is considered that most of uni and bivoltine breeds are heterozygous for the ***pnd*** gene , while some of multivoltine races are

homozygous. Tzenov and Petkov (1998) detected that the percentage of layings with ***pnd*** gene manifestation and the percentage of non-diapausing eggs in the laying were higher in the Bulgarian pure line of Japanese type Super 1 , compared with the Bulgarian pure line Hesa 2 , of Chinese type. A positive dominance for the higher parent (HP) in the crosses between Super 1 and Hesa 2 lines and a negative dominance for the lower parent (LP) in the reverse cross were detected.

There is also a ***pnd-2*** gene , which is linked on the 12^{th} chromosome, and having an action similar to those of ***pnd*** gene(Murakami , 1989) .

Other gene controlling the voltinism is ***V***, which is linked on the 6^{th} chromosome(Morohoshi , 1969).

The fourth gene of voltinism is ***npnd*** or non- pigmented and non- diapausing egg. This gene is linked with the female sex chromosome and have a maternal effect on the phenotypic expression of voltinism. The same gene is also connected with the hormonal mechanism of diapause. In fact the non-diapausing eggs laying , provoked by low temperature incubation is due to ***npnd*** gene action. According to Tzenov et al. (2000) , Lazarov et al.(2000) the percentage of layings with ***npnd*** gene manifestation in F_1 crosses between uni-bivoltine and multivoltine races is inherited intermediately , while the percentage of non-pigmented and non-diapausing eggs in the laying is inherited by a negative dominance of the uni-bivoltine race. Our investigations (Tzenov et al.,1995;Tzenov, 1996;) manifested that the ***npnd*** gene could have a phenotypic expression in uni-bivoltine breeds only after low temperature egg incubation. However the low temperature incubation can not provoke the expression of the other gene , namely ***pnd*** , because of the epistasis of gene ***npnd*** over ***pnd***. We proved also that since the ***pnd*** gene expression is not influenced by the environmental conditions during incubation and larval rearing its expression could be restricted by a strict rejecting of all batches , having even low percentage of pigmented , but non- diapausing eggs.

Moultinism: Three different types of moulters are known in the silkworm(Lea , 1993): trimoulters(M^3) , tetramoulters (M^4) and pentamoulters (M^5) .Each type is linked on the sixth chromosome in the multiple allelic relationship. Trimoulters are considered the original form. However almost all varieties used for cocoon production are tetramoulters , that is the larvae moult four times before reaching the fifth instar. The dominant/recessive relationship of the major genes is in the order of tri-> tetra- > pentamoulters, but is also affected by minor modifier genes under the influence of temperature and nutrition. Tri – or pentamoulting individuals may appear from tetramoulting varieties , as influenced by temperature conditions during the time of egg incubation and larval rearing. The major moulting genes also interact with the sex-linked maturity genes of $\boldsymbol{Lm}^{e}$ (early as found in multivoltine races) , $+^{\mathbf{Lm}}$ (intermediate as found in bivoltines) and ***Lm*** (late as found in univoltines).

Epistatic gene interaction along with monohybrid segregation is also demonstrated in the crosses between 3-moulters x 4-moulters and 3-moulters x 5 – moulters .

Inter-relationship among moultinism, maturity and voltinism: Three separate alleles of ***V*** , ***Lm*** and ***M*** genes appear to be physiologically inter-related through coordinating the organ of brain , expressed as diapause hormone (suboesophageal ganglion) , juvenile hormone (Corpoara allata) and ecdyson (prothoracic gland) . As a result , it is possible that even when modifier gene effects are small the characteristics of moultinism and voltinism may almost be segregated quantitatively in a certain complicated heterozygotic gene combination of an experimental strain.

Other qualitative characters of breeding interest: According to Lea(1993) there are at least five quantitative characters that are of interest to silkworm breeders. First is egg color , which is determined in a combination of shell color(maternal origin , genes normally colorless to semi-transparent to opaque chorion and mutant yellow(***ye***) and green ***Gre*** , etc.) and serosa color (embryonic origin , genes normally dark brown and mutants white ***we*** – alleles , pink ***pe*** , red ***re*** , brown ***b*** – alleles etc.) . Pigments appear in the serosa cells of the hibernating egg on the second day after egg – laying , and the final coloring is reached in three or four days. In the non-hibernating egg , the serosa remains colorless(the ***npnd*** gene).

Second is larval markings which are normally ***p***+ and ***p***, ***S-***, ***Ze*** – alleles with various markings to plain ***p*** without any markings. The gene ***p*** is in multiple allelic series condition.

Third , blood colors are determined by two major independent genes , yellow ***Y*** and white $+^{y}$, and yellow inhibitors ***I/I***^{s}, plus red haemolymph ***rb*** .

Fourth is cocoon colors which are normally white. Green and yellow are also popular. A variety of colored cocoons can be obtained by combining cocoon color genes : ***C***-alleles - $+^{c}$, golden yellow ***C*** , light yellow ***C***^{d}, inner layer yellow ***C***^{i}. Dominant white ***I*** inhibits the action of ***Y***, and thus is colorless when ***YC*** ;flesh colored ***F*** when combined as genotype ***YCF***; pink ***Pk*** when ***YCFPk*** and greens can be ***Ga, Gb, Gc***. (Tzenov et al. , 1997,1998,1999,2000).

Fifth is cocoon shapes. This is heritable , but it is difficult to designate a gene to a certain shape due to an almost continuous quantitative – like variation of the various topological factors involved. The shape is commonly elliptic. More spherical cocoons are found in Chinese varieties , more peanut – shaped in Japanese and more peaked cocoons in tropical varieties. The cocoon shape inheritance is controlled by polygenic additive gene actions.(Tzenov et al., 1999). There is also a wide range of variation in size and surface texture , with double cocoon semi-dominant over single.

Genetics of quantitative characters

Heritability: Most of economic characters are of quantitative type , and each is expressed by cumulative action and interaction of many genes in either an additive or non-additive mode (Lea , 1993). Resemblance between relatives and inbreeding depression are two basic genetic phenomena associated with quantitative characters. Resemblance through genetic causes only is defined as heritability . The reverse effect of inbreeding depression is called heterosis , or hybrid vigor. In general , the closer the relationship, the closer the resemblance. Selective breeding is based on the resemblance between offspring and parents. The average level of a certain character in the next generation will be improved when the more desirable individuals are used as parents. The extent of resemblance varies with the character. Some characters which show more resemblance are more responsive to selection than others. One characteristic of a population is the degree of resemblance between relatives. A quantitative genetic approach such as heritability estimation will show how the degree of resemblance between different sorts of relatives can be used to predict the outcome of selective breeding. It can also lead a breeder to the best method of selection. Heritability expressed in quantitative terms is the proportion of total variance due to the average effects of genes and also determines the degree of resemblance between relatives. Its predictive role is of important use to breeders , because heritability expresses the reliability of the revealed phenotypic values as a guide to the concealed heritable breeding value of additive gene action, or the degree of correspondence between phenotypic value and breeding value.

If a breeder choose parental silkworms according to their phenotypic values , improvement of characters in the subsequent generation can be predicted only by knowing the extent of correspondence between phenotypic values and breeding values. Therefore , the magnitude of heritability is very important as breeders select breeding methods and determine breeding procedures. A distinction is usually made between heritability in “broad” and “narrow” sense, reflecting the components of variation (variance in statistical terms) included in their estimation. Broad sense heritability can be described as the ratio between the genetic variance and phenotypic variance. Narrow sense heritability is a more meaningful term, and thus used almost exclusively by silkworm breeders. It is defined as the ratio of additive genetic variance to total phenotypic variance.

Heritability is a property of a certain character as well as of the population and of the environment to which the individual silkworms are subjected. The value of heritability varies depending on the magnitude of all the components of variance. A change in any one of these components will therefore influence heritability estimates. All the genetic components are influenced by gene frequencies , which may be different from one population to another , according

to their history. Large populations will show higher heritability than small populations, in which a significant amount of fixation has occurred for some time. More variable environmental rearing conditions are expected to reduce heritability , while more uniform conditions will increase it. Whenever a value is stated for the heritability of a given character , it refers to a particular population under particular conditions. A considerable range of variation is thus commonly shown among different estimates of heritability for the same character, although it may be partly due to statistical sampling. The grater the heritability value , in general , the smaller the variation of the character because of environmental differences.

The relatively high values of h^2 that were found out (Nasirillaev, 1975, 1981) for some leading selection traits such as cocoon shell weight , filament length etc. are informative for the big genetic variation in populations of *Bombyx mori L.* and for the perspectiveness of phenotype selection. In the same time however, the relatively low values of h^2 for the trait number ot eggs in one batch determine the considerably trait magnitude by the environmental conditions (Singh et al., 1998).

For traits with low h^2 is recommend inclusion in the breeding programs of other closely correlated traits with relatively high values of h^2. Relatively higher values of heritability were found for cocoon weight (73.60%), pupal weight (78.50%), shell weight (80.20%), growth rate (79.30%), raw silk yield (79.00%), and shell ratio (72.40%) in the studies of Gamo and Hirobayshi (1983),Gamo (1993), Singh at al. (1998), Yan (1989) , Rajana, Reddy (1990). Middle values for larval period duration were found , while low heritability was detected for the traits pupation rate (28.00%) and reelability (19.00%) (Gamo, Hirobayshi, 1983).

Inbreeding: Both inbreeding and hybridization are forms of nonrandom mating or selective mating , but operate in opposite ways(Lea,1996). Inbreeding is a kind of genetic assortative mating as compared with phenotypic assortative mating of hybridization. While gene frequencies do not change on the whole, genotypic frequencies do change toward the production of more homozygotes and fewer heterozygotes. Thus, any change in the population mean as a result of inbreeding must be related to a difference in genotypic value between homozygote and heterozygote.

If M_o and M_f indicate the original and subsequent population means, respectively , the result from inbreeding is expressed as follows:

$M_f = M_o - 2hpqF$, where p and q represent gene frequencies of a population for a given character, F is the inbreeding coefficient and h is the value of the heterozygote.

If the value of heterozygote , h , is zero, then there is no change in the population mean. That is , there is no dominance and inbreeding shows no effect on the means. When “h” does have a value , there is a change in the

population mean with inbreeding . This change is a consequence of dominance with the direction of change toward the most recessive allele. In general , the characters that are important for fitness of the individual will show a reduction due to inbreeding. Those characters unrelated to fitness will show little or no inbreeding depression. When inbreeding is accompanied by directional selection , the resulting families will be similar phenotypically , and total genetic variance among them will be decreased.
Since the self – fertilization in the silkworm is not possible the most intense form of inbreeding is full – sib mating. Less intense forms of inbreeding can be obtained from cousin, second cousin and other types of family mating. The relative effects can be seen in the inbreeding coefficient , F , in consecutive generations. For the mating type of full – sib , inbreeding coefficients increase from 0.250 , 0.375 , 0.500 , 0.594 and up to 0.672 as inbred generations proceed from generation 1,2,3,4 and go up to 5 , respectively.
In practical terms , the breeding expectation is that inbreeding leads to isolation of homozygous lines of generally reduced vigor. The reduction in vigor is not in a direct relationship with generation , but generally parallels the reduction in heterozygosity. Inbreeding can be expedited by selecting for the ultimate inbred characters , if they are defined. This system of mating will exhaust its effect in a few generations and has little impact thereafter . Many authors, on analyzing *Bombyx mori L.* breeding methods, assume that the manifestation of inbred depression depends mainly on biological features, geographic and genetic origin of the utilized initial population. For example, monovoltine populations are less resistant to depression compared to bi- and polyvoltine ones due to their weaker adaptability to unfavourable environmental conditions. At the same time inbreeding in combination with intensive systematic selection led to creation of highly productive lines of *Bombyx mori L*.

Correlation between characters: Two major causes of correlation between characters are genetic and environmental factors which result in correlation between the genotypic values of the two characters and the correlation between the environmental deviations (Lea , 1996). The primary cause of genetic correlation is pleiotropic gene action. Pleiotropy refers to the gene's attribute which affects more than one character, so that if one gene is segregating , it causes simultaneous variation in the character it affects. However , linkage is also a cause of genetic correlation , especially in populations derived from crosses between varieties with divergent genetic backgrounds.

Such actions of a gene or interactions gene to gene as well as genotype to environment result in phenotypic , genetic , or environmental correlations between the characters of economic interest. The extent of correlation usually varies among silkworm breeds and between male and female due to differences in cross – over rates which are higher in the male than in the female.

For all polygenic traits , the guiding principle for any silkworm breeding scheme is information on correlation and linkage in the female , along with prior knowledge about heritability . Before beginning actual breeding experiments , it is important to know how improvement of one character will cause simultaneous change , either in a positive or negative direction, in other characters. In that aspect the ignorring of mutual genetic link between leading productive traits led to decrease in the genetic progress.

According to Singh et al., (1992) correlation between cocoon weight and pupal weight was high and positive (+0.994), between cocoon weight and shell weight was also positive but lower (+0.614), as well between pupal weight and cocoon shell weight (+0.527). Considerable negative correlation was found between pupal weight and shell ratio (-0.827).

Fecundity correlates positively with pupal weight, but negative with productivity, shell ratio and strength of fiber .The shell ratio is a better trait for assessment the quality of cocoons for reeling because of higher shell ratio led to higher silk yield, due to the proved positive correlation between shell ratio and quality of cocoons.

The phenotypic correlation not always corresponded to the genetic nature and accurately reflected the degree and character of inherited link between productive traits in *Bombyx mori L.* For example this is the indicated negative phenotypic correlation between traits shell ratio and total egg weight in a batch. In that case negative phenotypic correlation informed that carrying the selection on shell ratio in positive direction will affect negatively on weight of eggs in batches. In the same time however positive genetic correlations show possibilities for differentiation of lines that combine as high shell ratio, as relatively high reproductive ability of silkworm.(Greiss, 2003).

Heterosis: According to Lea (1993) the phenomena of heterosis is the reverse of inbreeding and is defined as a restoration of vigor, often to a point exceeding the performance of the parents. The less related the strains are , the more heterotic the reaction is. This can be demonstrated in crosses of inbreeds with one , or no parents in common. The increase in heterosis corresponded to the reduction in common parentage. Several explanations or theories have been proposed to explain heterosis . The first is the dominant favorable gene hypothesis which begins with the idea that there are deleterious genes that build up in random mating populations. These are not expressed , however due to the masking effect of the dominant alleles. When inbreeding is imposed on this population , the deleterious genes are unmasked , and weakened individual lines appear. However , when two inbred pure lines are crossed , the dominant favorable alleles suppress the appearance of the effect of the recessives , and a superior line results. This explanation does not exclude the possibility of complementary gene action.

The second explanation is the theory of the superiority of the heterozygote . It is proposed that heterosis could reflect an interaction of multiple alleles at a locus such that the heterozygote would have a level of performance exceeding either homozygote. This would thus relate more to a physiological stimuli that would increase with greater diversity of gametes. The term heterobeltiosis is used to define the heterotic effect over the better parent, and is used to distinguish heterosis over the mid-parent value. Current thinking has arrived at a blend of both theories.

Based on detailed studies Strunnikov (1986) proposed a new scientifically based hypotesis. The statement that depression and heterosis are mutually dependent and related is laying in its fundament, as arising of highly heterozygous forms is determined by the depression in the preceding generations. Low combining ability of highly depressed individuals the author explained with the lack of sufficient reserve for formation of gene complexes that would compensate the effect of polynucleotides. This hypothesis, called "of compensatory complex" give explanation not only to diverse manifestation of heterosis, but also showed the ways for creation of heterosis forms.

It is also important to consider the physiological bases for heterosis . Much of the evidence accords with the assumption that the primary heterotic effect is concerned with growth substances such as regulatory proteins and hormones, the predominant activity of which is registered in the early part of the developmental cycle of the silkworm. Greater metabolic efficiency in mitochondria of the heterotic hybrids was also observed , while no such changes occurred in those not exhibiting heterosis.

Heterosis in *Bombyx mori L.* was used the first time by Italian biologist Cacimile in 1846 for increasing the yield of raw silk, and later on for increase in resistance to the flachery disease.

It has to be stated with sufficient reason, that now-a-day successes in the world silkworm science and practice are build just on the most rationalized utilization of heterosis and hybridization. The selection is applied , in principle , to the crosses, with the aim of finding pairs of lines that cross well , so that the lines may be perpetuated and provide cross – bred individuals for commercial use.

In genetics , crosses made at random between lines inbred without selection are expected to have a mean value equal to that of the base population. This is the reason why inbreeding or crossing alone cannot be expected to lead to an improvement , but must be supplemented by selection. In practice , some improvement can be expected from the effects of natural selection which eliminates lethal and severely deleterious genes during inbreeding . To the extent that these genes affect the desired character , an improvement of the cross-bred mean can be expected over that of the base population. However this improvement will not be very great (< 5 %) , because

the deleterious genes that have been eliminated would have been at low frequencies in the base population. Therefore , any significant amount of improvement must come from applying artificial selection to the characters that are economically desirable (Lea , 1996).

Combining ability : One of the most important research activities for development of better hybrid varieties is the identification of inbred lines for specific cross combinations (Lea , 1993). Good combiners are distinguished by means of the combining ability test. General combining ability is usually measured by inbred variety or top crosses. The top cross test for general combining ability followed by specific single crosses of inbreeds is used. The tester strain is an important factor for evaluating results. Generally , the preferable tester is one that is recessive at all loci at which improvement is desired , since the evaluation could more easily discern those loci where dominant favorable alleles had been added. A diallele crossing block may also be used since it allows a silkworm breeder to identify combiners that are superior in both general and specific combining ability. A topcross is essentially a method for comparing the general combining abilities of different lines , leading to the choice of the lines most likely to yield the best cross , among all crosses that might be made between the available lines. However , if specific combining ability causes much variation between crosses , then the general combining ability of two lines will not be a reliable guide to the performance of their cross. The relative general and specific combining ability can be measured by analyzing data from carefully designed crossing blocks. Quantitative genetic studies have also related general combining ability to predominantly additive gene action. Specific combining ability has been related to dominance , epistatic and other gene interaction. Inbreeding does not seem to affect the combining ability of derived lines. However the breeder would have an advantage if combining ability was determined as early as possible in order to develop good combining inbreeds .

Expression of combining ability is influenced by the environment.

Silkworm breeds may form simple (A x B), triple [(A x B)x C] and double crosses [(A x B) x (C x B)]. Usually the simple cross hybrids have (display) a stronger hybrid vigor. On studying different types of crosses, it was found that double and triple hybrids do not show bigger variation on account of the commercial traits of cocoons compared with simple ones. For triple hybrids it is difficult to chose between hybrids of the [(Japanese x Japanese) x Chinese] type and these of the [(Chinese x Chinese) x Japanese] type, though the quality of silk from the latter is considered better. Double crosses of the [(Japanese x Chinese) x (Japanese x Chinese)] type produce cocoons not enough uniform in shape and in thread length compared with those from the [(Japanese x Japanese) x (Chinese x Chinese)] type, which is preferable.

Present status of silkworm germplasm at global level

Though accurate data are not available on the silkworm germplasm in different countries of the world, an approximate information indicate that there are more than 4000 silkworm germplasm accessions available in different countries **(Table 6)**. There is every likelihood that some of these silkworm accessions are duplicated ; for instance the silkworm germplasm from China, Japan,Italy, France, Ex- Soviet union countries might be represented in the germplasm collection of other countries since these are the principal source of sericultural germplasm and also several countries might have exchanged some silkworm germplasm for silkworm breeding and hence a proper documentation on the availability of silkworm germplasm in different countries is very much required.

A very recent compilation of silkworm genetic stocks indicate that there are around 3000 genotypes of *Bombyx mori* at the global level, which includes mutants, parthenoclones, polyploids and geographical races (Nagaraju *et.al* 2001). In fact much of the genetic diversity of *Bombyx mori* is derived from the inbred lines of land races and elite stocks evolved by the silkworm breeders and also from hybridisation of different geographical races; mainly the Japanese, Chinese, European and tropical races, which are distinct for several economic characters (Thangavelu , 2002).The geographical races also possess several heritable characters for a variety of morphological, biochemical and quantitative characters. Among the four geographical races, the bivoltine and univoltine races of temperate origin and multivoltine races of tropical origin differ widely and exhibit contrasting characters. The bivoltine and univoltine races produce high quantity of good quality silk, whereas the multivoltine races are hardy, tolerant to pathogen load and thereby resistant to diseases compared to the bivoltines but produce low amount of poor quality silk. Thus, these geographical races are very valuable genetic stocks for further improvement of silkworm races and evolution of superior breeds of *B. mori* . Apart from a rich biodiversity of geographical races, there are also a large number of mutants. The silkworm genetic stocks include more than 500 mutants for a variety of characters viz., serosal colours; larval and adult integument colours; skin markings and body shapes; cocoon colours and shapes; physiological traits such as diapause, number of larval moults and timing of larval maturity; food habits and biochemical features such as digestive amylase, blood and egg esterases, larval integument esterase, alkaline and acid phosphatases; haemolymph proteins; silk production and fibroin secretion; homeoproteins and body plan determination etc. and the various mutants, gene locus and phenotype were documented recently (Nagaraju et.al, 2001; Thangavelu ,2002). Apart from the geographical races and mutants there is a large genetic stock of *B.mori* evolved by the breeders mostly utilising the geographical races and mutants of larval, pupal and cocoon color variants of sex limited races, particularly in China,

Japan, Korea ,India , Bulgaria and erstwhile Ex- Soviet Union and some of these breeds are commercially exploited in these countries for silkworm rearing to produce raw silk and the remaining breeds are maintained in the silkworm germplasm of these countries as breeders genetic stocks and they are utilised as the genetic material in the silkworm breeding programmes for evolution of more superior and elite races. Thus, the geographical races, mutants and the elite breeders stock constitute the major portion of the present day silkworm germplasm at the global level apart from the parthenoclones, triploid, polyploids and wild relatives of *Bombyx* and *Bombycidae* **(Fig. 52 , 53,54 and 55)**.

Characterization and evaluation of silkworm genetic resources

The primary characters needed to authorize a silkworm race as genetic stock and the characteristics of geographic races are shown in **Tables 7 and 8**
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Methodology for obtaining the data and calculation of the main breeding characters values:

Qualitative characters

-**egg serosa color**.It is determined visually on all silkworm layings, produced from each accession. The color is green ,gray, brown, mixed.

-**egg chorion color**.It is determined visually on 3 replicates having 200 eggs or 2 layings each. The color is white ,yellow and mixed.

-larval markings.It is determined visually on the 5^{th}-7^{th} day of the 5^{th} instar on the all larvae reared per each accession. The larval markings are plain, normal marking and zebra.

-cocoon shape. It is determined visually after harvesting and floss removal , on the whole amount of good quality cocoons produced per each accession. Cocoon shape is oval, elongated oval, oval with constriction, elongated, elongated with constriction, spindle.

-cocoon color. It is determined visually after harvesting and floss removal on the whole amount of good quality cocoons produced per each accession. Cocoon color is white and colored.

-cocoon size. It is determined on random sample of 100 good quality cocoons .Cocoon size is big, medium, small.

-cocoon nature of grains. It is determined on random sample of 100 good quality cocoons. It is fine, medium, coarse and flossy.

As main qualitative characters for silkworm genetic resources at SES-Vratza, Bulgaria characterization were accepted only egg serosa color and chorion color, larval markings, cocoon shape and color.

Quantitative characters

-hatchability in %. It is determined on 6 layings per accession and on each laying ,selected for incubation in P3 category pure lines. It is calculated on the 3rd day after hatching by the following formula:
number of normal eggs in the layings – number of unhatched eggs in the layings / number of normal eggs in the layings x 100
-larval duration in h. The beginning is day and hour of larval brushing ,the end is day and hour when the feeding is stopped and larvae mounted.
-5th instar duration in h. The beginning is day and hour of the first feeding after the 4th molt, the end is day and hour when the feeding is stopped and larvae mounted.
-pupation rate in %. It is calculated by the formula: Number of cocoons with alive pupa/number of larvae counted x 100.
-number of normal eggs in the laying. It is determined on random sample of 15 layings, after 20th October of the current year.
-weight of the normal eggs in the laying in g. It is determined on random sample of 15 layings, after 20th October of the current year.
-abnormal eggs in the laying in %. It is calculated by the formula :number of abnormal eggs in the laying/number of normal eggs in the laying x 100.
-number of normal eggs per 1 g. It is determined on 3 replicates ,having 0.5 g loosing eggs each.
-fresh cocoon grades in %. It is determined immediately after cocoon harvesting and floss removal. The cocoons are assorted in good quality(having alive pupae),double cocoons and unreelable cocoons. After the assorting all the three categories are weighed .
-fresh cocoon weight, and shell weight in g. There are two methods:1.All good quality cocoons/shells in the laying/replicate are weighed and after that divided by their number.2.A random sample consisted of 30 female and 30 male good quality cocoons/shells is taken and after weighting their weight is divided by the number.
In the silkworm breeding the measuring is individual for each cocoon/shell.
-shell percentage .It is calculated by the formula: shell weight/fresh cocoon weight x 100.
-fresh cocoon yield by one box of eggs(20000 eggs) in kg. It is calculated by the following formula: fresh cocoon yield by laying/replicate in kg x 20000 x (hatchability x 0.01)/ number of larvae counted.
-filament length in m. It is determined on a random sample of 30 good quality cocoons after single cocoon reeling test.
-filament weight in g. After the cocoon reeling the filament is dried to constant weight and weighed.
-filament size in denier. It is calculated by the formula: filament weight in mg/filament length in m x 9.
-reelability in %. It is calculated by the formula: filament weight/the weight of filament+frizen+pelet x 100

-raw silk percentage. The formula used is: filament weight/dry cocoon weight x 100.
-raw silk yield by one box of silkworm eggs. The following formula is used: fresh cocoon yield by 1 box of eggs x(good quality cocoons percentage x 0.01) x (percentage of dried cocoons obtained from the fresh cocoons after drying to constant weight x 0.01) x (raw silk percentage x 0.01).
-consumption index(CI) = dry weight of the mulberry leaf ingested in mg during the feeding period/feeding period duration in days x average dry weight of one larva during the feeding period in mg
-growth rate(GR) =body gain during the feeding period/ feeding period duration in days x average dry weight of one larva during the feeding period in mg.
-leaf ingestibility(AI) = dry weight of the mulberry leaf ingested in mg/mulberry leaf supplied in mg dry weight x 100.
-food digestibility(AD)= dry weight of the mulberry leaf ingested in mg – dry weight of the excrements in mg/ dry weight of the mulberry leaf ingested in mg x 100.
-efficiency for conversion of the food supplied in products(ECS) = dry weight of the products obtained ,namely body gain during the feeding period or cocoon shell or pupa or eggs / mulberry leaf supplied in mg dry weight x 100.
-efficiency of conversion of the food ingested in products(ECI) = dry weight of the products obtained ,namely body gain during the feeding period or cocoon shell or pupa or eggs / dry weight of the mulberry leaf ingested in mg during the feeding period x 100.
-efficiency of conversion of the food digested in products(ECD) = dry weight of the products obtained ,namely body gain during the feeding period or cocoon shell or pupa or eggs / dry weight of the mulberry leaf ingested in mg – dry weight of the excrements in mg x 100.

Characterization and evaluation of silkworm genetic resources:

Since it is too difficult to make a characterization of the global silkworm germplasm , here we shall characterize the silkworm genetic resources , maintained in Bulgaria as sufficiently representative.

Most of the silkworm accessions have gray or/and green egg serosa color, white and/or yellow egg chorion color, marked or/and plain larvae ,elongated with or without constriction or oval cocoon shape .The breeds with white cocoons are prevailing. The data regarding the 5^{th} instar duration in the pure lines show that it vary from 180 h in the breed Gergana 2 to 218 h in the breed AS.

Most of the silkworm accessions manifest comparatively high hatchability. 80 % of the silkworm accessions have pupation rate higher than 85%,namely 26 % of the strains have pupation rate from 85 to 90%, 39 % have pupation rate from 90 to 95 % ,and 15 % of the breeds manifest pupation rate higher than 95

%. Since the data for pupation rate were obtained by rearing of silkworms under optimal conditions they do not give correct information about the survivability of the races under adverse environment.

Tzenov et.al.(2000) studied the pupation rate and fresh cocoon yield in different Bulgarian silkworm races and hybrids under optimal and adverse rearing conditions during the last two larval instars. It was estimated that there existed clearly expressed genetically determined differences between the races and hybrids for pupation rate under adverse silkworm rearing conditions. However no any correlation was detected between the pupation rate in the optimal and adverse rearing conditions in one the same strain.

Under the standard rearing regime most of the accessions manifested cocoon weight from 1.8 to 2 g –43 %, and 35 % –cocoon weight higher than 2 g. 75 % of the races have shell weight higher than 0.350 g, namely from 0.350 g to 0.400 g – 35 %,from 0.400g to 0.450 g – 26%,from 0.450 to 0.500 g – 8 % and higher than 0.500 g – 6 %. 70 % of the accessions displayed shell ratio higher than 19 % and 29 %– shell ratio higher than 21 %.

93 % of silkworm accessions have cocoon yield by one box of eggs higher than 25 kg. Most of the races(37%) manifested cocoon yield from 30 to 35 kg,29 % -cocoon yield from 35 to 40 kg and 5 % -from 40 to 45 kg.

57 % of the accessions manifested cocoon filament length higher than 900 m , 17 % -from 900 to 1000 m,21 % - from 1000 to 1100 m,12 % - from 1100 to 1200 m, 6 % - from 1200 to 1300 m and 1 % from 1300 to 1400 m.

Vassileva and Tzenov(2001) tested 31 accessions from silkworm genetic resources for their ability for parthenogenetic development.The results showed that the highest parthenogenetic eggs hatchability(over 20 %) was detected in the races ShV,TV,Hesa 2,Siria 2,Mysore 1,70-42,MNB and China.

Tzenov(1996) studied the fecundity in 16 highly productive silkworm races and detected that in the Japanese type races the moth emergence percentage was the highest in Kom 1(93.32%) and the lowest in AS(61.88%).In the Chinese type races the moth emergence was the highest in ShV(88.04%),Hebar 2(83.18 %) and TV(92.51%) and the lowest in Merefa 2(55.24%).In average the Chinese type races have higher values of the traits number and weight of normal eggs in the laying than the Japanese type.

Other important traits are those characterizing the food ingestion, digestion and utilization. From his study with the same 16 silkworm races ,the tropical polyvoltine Bonde 517 and their hybrids Tzenov(1996) detected that there were significant differences between the races regarding the amount of dry mulberry leaves supplied during the whole fifth larval instar. The highest amount of leaf was supplied to the race AS(5.227 g dry matter per 1 larva during the 5^{th} instar) and the lowest amount – in multivoltine Bonde 517 – only 3.170 g.

In the Japanese type races the amount of food ingested was the highest in Vratza 51(3.688 g) and the lowest in Hebar 1(3.137 g).In the Chinese type races

the food ingested was the highest in Vratza 54(3.784 g) and the lowest in Hebar 2(2.858 g).

The food ingested and digested was the lowest in polivoltine race- 0.943 and 0.347 g respectively. The data manifested that most of the hybrids studied expressed different in degree ,positive heterosis for the amount of food ingested and digested. The leaf ingestibility was the highest in the Chinese type race Vratza 54(73.53 %) and the lowest in the polivoltine race Bonde 517 – only 29.75 %.The highest food digestibility was detected in the tropical race (36.80 %),Super 1 (31.74 %), and Vratza 53(30.04%).The food digestibility was the lowest in Hebar 2(25.72%),KS(26.07%) and Merefa 2(26.65%).

Most of the hybrids studied showed different in degree positive heterosis for the leaf ingestibility, but the heterosis manifested for the food digestibility was low or negative.

The consumption index was the highest in Vratza 51(0.829 mg),Vratza 53(0.825 mg),Vratza 52(0.827 mg),Vratza 54(0.809 mg), Bonde 517(0.823 mg) and the lowest in Super 1(0.718 mg),Vratza 35(0.732 mg),AS(0.744 mg) and Merefa 2 (0.741 mg).Unlike the other races who have high leaf ingestion and consumption index, the polivoltine race combined high consumption index with low food intake. As regards the growth rate character values the inter-racial differences were negligible .The polivoltine race manifested very high growth rate (0.267 mg) .The efficiency of conversion of the food supplied(ECS) for body gain was the highest in Super 1(17.83%), Merefa 2(17.37%) , Vratza 35(17.16%) and the lowest in Hebar 2(13.53%) and Bonde 517(9.65 %).

The utilization of the food supplied for silk shell was the highest in Super 1(7.65%),Vratza 54(8.18%) ,Vratza 35(7.87%) and the lowest in Hebar 2(6.54%) and Bonde 517(2.08%).

The utilization of the food supplied for eggs was the highest in Super 1(2.62%),Hesa 2(2.66%) and the lowest in AS(2.18%) and Hebar 2(1.84 %).

The data for efficiencies of utilization of the food ingested and digested, manifested that in most of the cases the races having comparatively high leaf ingestion and digestion showed lower food utilization and vice versa. The heterosis expression in F1 was low or negative.

From their study on the food utilization inheritance in hybrids between uni-bivoltine and polyvoltine breeds Tzenov et al. (1999) detected an incomplete dominance in F1 for the higher parent values as regards the amount of food ingested, digested , ingestibility and digestibility.

Bozchkova et al.(1996) investigated the haemolymph lysozime activity in 16 silkworm races and its inheritance in 10 F1 hybrids .Lysozime is an important agent of the natural resistance of the insects. The results obtained clearly bring out that there are significant differences between the races and hybrids as regards the haemolymph lysozime activity. Generally the races, having plain larvae and oval-shaped cocoons(Chinese type) displayed a higher lysozime activity than that observed in the races with larvae having markings and

elongated cocoons(Japanese type).However there are some races of the Japanese type ,having a higher lysozime activity. The F 1 hybrids showed a partial dominance or overdominance for the higher parent or lower parent value.

Molecular characterisation of silkworm germplasm: There is no comprehensive method available for genetic characterisation of the silkworm gene pool at the molecular level. However, the well known molecular markers viz., Restriction Fragment Length Polymorphic (RFLP) markers, Minisatellite DNA markers, Microsatellite DNA markers, Random Amplification of Polymorphic DNA (RAPD) markers, Inter Simple Sequence Repeat (ISSR) markers and Amplified Fragment Length Polymorphic (AFLP) markers provide valuable information to identify duplicate accessions apart from trait based mapping and molecular tags (Thangavelu,2002). Molecular characterisation helps to identify the genetic distinctness of a race / breed / stock and thereby helps to eliminate duplicates and reduce the cost of germplasm maintenance and volume of work. Molecular markers that are linked to metric and resistance traits and their subsequent mapping in silkworm is essential. Also these genetic markers can be applied periodically to monitor changes in heterogeneity and heterozygosity in the accessions, as they are routinely reproduced for maintenance.

Management and utilization of silkworm germplasm resources

Methodology for maintenance of silkworm accessions: The main purpose in the silkworm accessions maintenance is to conserve their characters closed to the characteristics in the passport, therefore the selection is made only for the qualitative characters, but the main quantitative traits values are also checked. Other important purpose of the maintenance methodology is to avoid inbreeding.

Now all silkworm accessions from the germpalsm, maintained at SES-Vratza, Bulgaria are reared only once per year, namely during the most favorable spring season-May/June.

There were some investigations regarding the methods for maintenance of the polyvoltine races(Tzenov,1998;Tzenov et al.,2001).It was proved that in Bulgaria is possible to maintain the polyvoltine race Bonde 517 by only one rearing per year within 4 years .

During the papionage 21 layings are produced per each accession and preserved under the standard regime up to the next spring. Before putting into incubation 8 layings ,having biggest number of normal eggs are selected and treated by 2 % formaline solution for disinfection.

The layings are hatched mixed and the larvae are grown also mixed. After incubation the hatchability is only checked but no any selection is made for

higher hatchability .Only the larvae, hatched on the day of "mass" hatching are brushed for rearing.

After the 2nd molt 200 larvae are counted per each accession and grown together up to the cocooning.

After cocoon harvesting the following quantitative characters are checked: average fresh cocoon weight, shell weight, shell percentage, pupation rate, fresh cocoon yield by 1 box of silkworm eggs. Out of 150-190 cocoons, produced per race 80-100 cocoons are selected for the qualitative characters, namely cocoon color, shape and nature of grains.

The cocoons selected are put mixed for the papionage and the eggs are produced on cartoons.

Once per 3 years a random cocoon sample is taken from each accession in order to investigate the cocoon filament characters by reeling test.

Methodology for silkworm pure lines maintenance: The main purposes in the maintenance of the pure lines ,which are parents of the commercial hybrids is to keep the values of the main breeding characters at least at the level of their selection by the breeders and to improve the grainage characters like, moth emergence percentage and fecundity. Also the inbreeding must be avoided obligatory.

When the amount of egg production is comparatively small (less than 2000 boxes of F_1/year) the pure lines of the commercial F_1 hybrids could be maintained in two categories, namely P_3 and P_1.In the case of bigger egg production there must be produced P_2 category as well.

The pure lines which are parents of single hybrids are maintained in 2 sublines each by the following methodology:

	Super 1		**Hesa 2**	
P_3	Line 1	Line 2	Line 1	Line 2
P_2	Line 1	Line 2	Line 1	Line 2
P_1	Line 1xLine 2		Line1xLine2	
F_1		Super 1 x	Hesa 2	

The pure lines which are parents of four-way hybrids are maintained as population, without any sub - lines ,following the methodology:

P_3	**KK**	**Hesa 1**	**Vesletz 2**	**Gergana 2**

P_2 KK Hesa 1 Vesletz 2 Gergana 2

P_1 KKxHesa 1 Vesletz2 xGergana 2

F_1 (KK x Hesa 1) x (Vesletz 2 x Gergana 2)

The P3 category of pure lines is grown once per year ,during the most favorable season in April/May/June. During the papionage 200 layings are produced per each pure line/sub-line. Before incubation 120 layings ,having the biggest number of normal eggs are selected in each line/sub-line.

The layings are disinfected by 2 % formaline solution. After the hatching,55 layings, having hatchability more than 98 % are selected for silkworm rearing. Only the larvae, hatched on the day of "mass" hatching are brushed for rearing.

Each laying is reared separately. After the 2nd moult 200 larvae are counted from each laying for growing up to cocooning. During the larval period the layings(if any) having not typical for the race larval marking are rejected(separated for production of P_1 or F_1 or for reeling).

Also the layings having not uniform larval growth or some diseases(NPV) are rejected.

After cocoon harvesting, pupation rate, the cocoon yield, average cocoon weight, shell weight and shell percentage are checked for each laying.

Usually from the 55 layings reared ,20-25 layings ,having the highest pupation rate are selected for reproduction of P_3.After that only 8-12 layings out of them are selected based on the highest cocoon weight, shell weight and shell percentage. The cocoons of the selected 8 layings are assorted for alive pupa, cocoon color, shape , texture and shell hardness manually by an experienced researcher in silkworm breeding and after that cut and the pupae are separated by sex. Then all the selected cocoons are measured individually for checking their cocoon weight , shell weight and shell percentage. Only those having the highest cocoon/shell weight and sufficiently high shell ratio are selected for papionage. Practically out of about 180 cocoons produced per a laying only about 50-60 are selected for P_3 multiplication. If having 8 selected layings per line , 400-500 cocoons are selected for papionage and about 200 layings of P_3 are produced for multiplication. The female pupae of the first four layings are mixed for papionage with the male pupae from the rest 4 layings and vice versa in order to avoid the inbreeding.

Once per 3 years random samples for making silk reeling test are taken from each pure line.

For control of pebrine disease after hatching , the egg shells of each laying are microscoped, samples of silkworm skins after each molt, early spun cocoons etc. are taken for microscope examination.
The cocoons of the rest layings, produced are assorted and used for production of P_2 , P_1 or directly for F_1 if it is a single hybrid.
The maximum amount of silkworm eggs which could be produced following this scheme is as follows:
P_3: 200 layings per pure sub-line(or pure line in the parents of four - way hybrids)
P_2: 35 boxes per pure sub-line
P_1: 1200 boxes per pure line(or 2400 boxes P_1)
F_1: 96 000 boxes

The flow chart on collection, introduction and maintenance of silkworm germplasm in India is presented in **Fig. 56 .**

Methods of silkworm breeding

Main criteria for the desired level of breeding characters and selection
The breeding targets are generally the following:
Japanese type races: marked or plain larvae, but most of them have marked larvae , elongated with or without constriction shaped cocoons, white in color.
Chinese type races :plain larvae, oval and white in color cocoons.
Hatchability: as high as possible. Checked for each laying.
Larval duration:27-29 days. Checked for the laying.
Fifth instar duration:7-9 days. Checked for the laying.
Pupation rate: as high as possible. Checked for the laying.
Number of normal eggs in the laying: more than 550.Checked for each laying.
Weight of the normal eggs in the laying: more than 0.330 g. Checked for each laying**.**
Moth emergence percentage: to be more than 80 % . Checked for the race.
Good quality cocoons: more than 95 %.Checked for the laying.
Fresh cocoon weight: the individuals having the highest or medium cocoon weight for the race are selected. Checked individually.
Cocoon shell weight: the individuals ,having the highest for the race shell weight ,the highest or average cocoon weight and the highest or average shell percentage are selected. Checked individually.
Shell percentage: the individuals, having the highest or average for the race shell percentage are selected. Checked individually.
Fresh cocoon yield by 1 box of eggs.: The layings, having the highest values are selected. Checked for the laying.

Filament length: This character is checked during the primary evaluation of the initial population for breeding and every year/generation during the breeding process. During the primary evaluation are selected only populations/races/lines having total cocoon filament length more than 950 m and unbreakable filament length more than 800 m. During the breeding process if any line showed values of the trait less than the limit for more than 2 generations is rejected.
Raw silk percentage.: This character is checked during the primary evaluation of the initial population for breeding and every year/generation during the breeding process. During the primary evaluation are selected only populations/races/lines having raw silk percentage more than 39 %. During the breeding process if any line showed values of the trait less than the limit for more than 2 generations is rejected.
Neatness defects: It is better, as it is little. It has to be over 93 points on the defects.
Lousiness defects: should score above 75 % in the lousiness test.
Degumming loss of cocoon shell: It is better, as it is little. It has to be lower than 26 percent.

Breeding methods

The first step in the silkworm breeding process is the primary evaluation of the initial breeding material(breeds, lines, hybrids etc.).
Usually the following evaluation is made:
-Phenotypic evaluation of the main qualitative and quantitative breeding characters.
-Phenotypic variation of the values of the quantitative characters.
-Heritability of the main quantitative characters.
-Correlation between the main quantitative breeding characters.
-Regressions between the main quantitative breeding characters.

For breeding new silkworm races the following different methods are used:

Segregation from foreign F1 hybrids: By this method in Bulgaria were selected most of the older silkworm breeds , like Vratza 1,Vratza 2,Vratza 3,Vratza 4, Vratza 5, Vratza 6 etc.
Generally the method is as follows:
1.Initial population:**J-124 x C-122**(Japanese F_1hybrid)
2.Selection of larvae, having markings and elongate shaped cocoons in F_2
3.Mating the selected moths
4.Batch rearing, selection for larval markings, cocoon shape, texture, main quantitative characters for 11-16 generations.
5.New breed: **Vratza 1**

Separation from foreign F1 hybrids: By this method the pure line KK was selected in Bulgaria. It is common that in the F1 hybrids can be found some individuals from the mother pure line due to some mistakes in the sex discrimination , leading to mating within the pure line.
The method is the following:
1.Initial population: **South Korean hybrid**
2.Selection of cocoons ,having elongated with constriction shape and mating between them.
3.Inbreeding for 4 generations, batch rearing, selection for cocoon shape and main quantitative characters.
4.Autbreeding for 9 generations, selection for cocoon shape and main quantitative characters.
5.New breed: **KK**

Using populations of Japanese races: By this method in Bulgaria were selected many breeds like 157 K, Vratza 7,Super 1,Super 2,Hesa 1,Hesa 2,Gergana 1,Gergana 2,Vesletz 1,Vesletz 2,Kom 1,Kom 2 etc.
As an example we will give the breeding of the line Super 1/11by Petkov(1976):
1.Initial population; **J 124**
2.Selection of 6 layings as initial parents for future sub-lines.
4.Inbreeding for 7-8 generations, batch rearing, selection for larval marking, cocoon shape, texture, main quantitative characters.
5.Checking the combining ability of each line by crossing with the race of Chinese type 157 K(general combining ability) and rejection 2 of the lines due to poor combining ability.
6.Inbreeding for another 7-8 generations, batch rearing, selection for larval marking, cocoon shape, texture, main quantitative characters, with simultaneous checking the combining ability of each line with 3 other promising races of Chinese type(specific combining ability).Some of the sub-lines are rejected due to inbreeding depression.
7.It was detected that the best specific combining ability had the line Super 1/11.
8.New race :**Super 1/11,**ready for direct hybridization.

Other example is the breeding of pure lines Hebar 1/18 and Hebar 2/1(Petkov,1976) by the following method:
1.Initial population :**Kinshu**
2.Selection of 3 sub-lines
3.Inbreeding for 3 generations ,batch rearing, selection for larval marking, cocoon shape, texture, main quantitative characters.
4.Rejection 2 of the sub-lines
5.Inbreeding in another 3 generations, batch rearing, selection for larval marking, cocoon shape, texture, main quantitative characters.

6.New breed :**Hebar 1/18**

By making initial hybrid populations for further breeding :By this method were selected the pure lines Vratza 35,Merefa 2 ,the breeds Vratza 51,Vratza 53,Vratza 52,Vratza 54 etc.
Here we are giving an example with the breeding of line Vratza 35 (Nacheva et al.,1998):
1.Initial population: **Hebar 1 x Tashkent 11**
2.Inbreeding in 2 generations, batch rearing, selection for larval marking, cocoon shape ,texture, main quantitative characters.
3.Autbreeding for 16 generations, batch rearing, selection for larval marking, cocoon shape, texture, main quantitative characters.
4.New breed : **Vratza 35**

Methods of breeding sex limited for egg color , larval marking and cocoon color races: It is well known that the main reason to use sex - limited silkworm lines is in order to make easier ,and more effective silkworm egg production, especially the sex discrimination. The first attempts to create sex-limited silkworm breeds were made by Tichomirov(1891) ,followed successfully by other researchers like Astaurov(1933),Tazima(1944),Hasimoto(1948) , Lee at al. (1989) etc.

The first silkworm strain ,having marked female larvae and plain male was created by Tazima(1944) by treatment of newly laid silkworm eggs with X – rays and translocation of the P^+ allele and its connection with the sex W chromosome .Later , using the same method Hasimoto(1948) managed to create a new sex-limited breed having zebra female and plain male larvae.By this method as initial material were used sex limited races , crossed with other races, having plain larvae or yellow eggs. After that the hybrid population was maintained by batch rearing for 4 generations and with inside batch mating(inbreeding).On the 5^{th} generation an inter batch crossing was made ,followed again by inbreeding in the 6^{th} and 7^{th} generations. At the 8^{th} generation the two inbred lines were crossed and the new breed was created.
Using this method Petkov(1995) selected 2 sex limited for egg color lines (T 15/4 and HT-215/38) and 5 sex limited for larval marking lines (B2/6,BTV-2/64,TBV-2/24,HB-2/22 and TV-3/2.)
Sex-limited cocoon color. Female cocoon is yellow and male white. It is easily for sex-discrimination and the male cocoon can separately be reeled to produce high grade silk. According to Chen (2002) , CY_{2c}(Chinese race) and JY_{628} are one of this type of commercial varieties. Their pureline performance are 1.543 g and 1.694g in cocoon weight, 0.388 and 0.419g in shell weight, 25.15 and 24.71% in shell ratio, 83.09 and 93.05% in survival rate of larva-pupa respectively. The performance of corresponding hybrid was close to the control variety, and its male cocoon produced much better quality silk.

Method of breeding sex limited for larval marking races as analogues of pure lines of approved commercial hybrids: In the recent time the efforts of silkworm breeding at Sericulture Experiment Station in Vratza, Bulgaria were directed to create lines/hybrids having higher tolerance to adverse rearing conditions. The reasons were the necessity to intensify the cocoon production in Bulgaria by adoption of summer and autumn rearing as well as the possibilities to export silkworm eggs to some tropical countries.

As a result a new hardy silkworm four-way commercial hybrid (Hesa 1x KK) x (Gergana2xVesletz2) was selected , authorized by the government in 1999 and widely adopted in the field during the period 2000-2003.

In order to improve the silkworm egg production technology we came to the idea to use the parental pure lines of the already created hybrid for breeding new sex-limited for larval markings pure lines. It was expected that if we use the backcrossing method , combined with a proper selection the newly bred sex-limited pure lines would demonstrate similar qualitative and quantitative characters values to those of the parental pure lines of the commercial hybrid.

The breeding process had been started since the spring season of 1998 and 3 generations were reared in each year for four years.

The breeding strategy aimed to use the existing pure lines , parents of the hardy commercial hybrid (Hesa 1xKK) x (Gergana2xVesletz2) as well as four introduced recently from Japan sex-limited for larval markings breeds in order to create new sex-limited lines, having similar qualitative and quantitative characters values to those of the parental pure lines of the commercial hybrid.

The breeding method was based completely on the back crosses and by using it had been selected 4 new lines, namely SN1,Iva 1,Nova 2 and Magi 2,analogues of the pure lines Hesa 1,KK,Vesletz 2 and Gergana 2 ,but sex -limited for larval marking.

Since the breeding methodology was one the same in all the 4 lines ,here we give an example only with the breeding of the line Iva 1:

Step 1.Initial population: cross between females of **Nig 1**(sex limited for larval marking-female marked , male plain, cocoons of Japanese type- elongated with constriction)and males of **KK**(having plain larvae, cocoons of Japanese type-elongated with high constriction).

Step 2.Backcrosses for 9 generations, using females of the above initial population , mated with selected males of the line KK and mass rearing.

Step 3.Backcrosses for other 3 generations, but with batch rearing.

Step 4.New silkworm line : Iva 1 ,maintained every year by batch rearing and using selected female individuals only, mated with selected males of the line KK.

Practically the selection was made only with the female individuals of Iva 1,using selected males of KK. In this case the basic pure line (KK) was

maintained in big volume ,allowing to make the necessary selection and the sex-limited analogue Iva 1was maintained in lower volume(8-10 batches during rearing).
By using 11 backcrosses , combined with proper selection methods were created 4 new silkworm sex-limited for larval markings pure lines , having similar qualitative and quantitative characters values to those in the original pure lines of the recognized by the government and widely adopted in the field commercial hybrid (Hesa1xKK) x (Gergana2xVesletz2).

Method of breeding tolerant to adverse rearing conditions silkworm breeds: In Bulgaria ,only 3 breeds (SB1,VB1,HB 2) were selected by this method in the recent years by using crosses between the uni-bivoltine pure lines Super 1 and Hesa 2 and the polyvoltine race Bonde 517.

The method is following:
1.Super 1 x Bonde 517 F1
2.Super 1 x Bonde 517 F2.There was segregation in colored and white cocoons with different shape and texture. Seven male cocoons having white color, elongated shape, similar to those of the uni-bivoltine line texture out of 32 kg cocoons , were selected and mated with selected females of the line Vratza 35.
3.Inbreeding for 6 generations, batch rearing, selection for cocoon color, shape ,texture and the main quantitative characters.
4.Outbreeding for 5 generations, batch rearing, selection for cocoon color, shape ,texture and the main quantitative characters.
5.New breed **VB 1**,the breeding work should be continued.
The newly selected breeds manifested a higher tolerance to adverse rearing conditions, but their productivity was comparatively low. The idea is to use them as components of four-way hybrids with highly productive but ,sensitive to bad environment pure lines.

Method for virus resistant silkworm race breeding:

The healthiness of silkworm is an important theme in the breeding. In Japan(Kosegawa,2002) by the screening of the silkworm-genetic stock, the densonucleosis virus (DNV) resistant line was discovered. As a result of the genetic analysis, it is demonstrated that the resistant property is controlled by the single-dominant gene. Therefore, it is possible that the resistance expressed at the F_1 hybrid. Consequently, the resistant race "Taisei" was successfully established.
In China moreover, 200 silkworm varieties were investigated on their resistance to densonucleosis through oral administration of the pathogen. It was discovered that 28 of them had the resistance. Two resistant lines were selected and the densonucleosis resistant gene *nsd-Z* and eye spot gene *bl* liked lines were created, so that it is possible to breed densonucleosis resistant varieties with eye-

spot as genetic marker instead of administration of the pathogen to silkworms. Chen et al.(1996) found significant difference among preserved silkworm varieties to NPV-sensitivity, the resistance is of incomplete dominance to susceptibility, it is controlled by over two pairs of genes.

Breeding of tolerant to fluoride pollution silkworm strains: Fluoride pollution has been seriously disturbed commercial cocoon production due to the rapid development of rural industry since early 80's of 20th century in China. In order to stabilize cocoon production in the major sericultural regions, some highly fluoride-tolerant varieties were bred. Huafen$_{Gw}$ × Hue.A (Shen et al., 2002) can normally developed when fluoride content reached 60mg/kg in the mulberry leaves during 1st-3rd instar and 120mg/kg during 4th-5th instar.(Generally, the 30mg/kg fluoride content in mulberry leaves was considered to be the threshold concentration for normal development of silkworm larvae). Lu.Pin × Qing.Guang (Lin et al., 2001) can grow very well when fluoride content was 100mg/kg during the whole larval stage. Other popularized varieties such as Qiufeng × Baiyu, Fengyi ×54A, Xia7 × Xia6, Xinhang × Keming and etc. are all relatively less sensitive to fluoride pollution.

Method for breeding races that have characteristic silk thread:Generally, thin and fineness silk thread is fitting for the making of the high-grade textile. In Japan(Kosegawa,2002) it was established that the race, Akebono, which formed 2.2 denier of silk thread. The thread was twisted together with chemical fiber, and the product which is called as Hybrid Silk has been sold. In 1998, Hakugin race was established as the race that had the silk fiber thinner than Akebono. Surprisingly, the thread size is 1.6 denier. It is the first record by the thinner size less than 2 denier in spite of tetramolter. It is expected that the super-thin textile made form the raw silk of this race attracts the attention of the worldwide as a particular product of Japan.

In contrast to the upper paragraph, there is a demand of the thickly and short raw silk for Western clothes, and a race which produces the silk thread of 4 deniers has been improved.

Utilization of colored cocoon and double cocoon: Colored-cocoon race has well known in worldwide. In Japan(Kosegawa , 2002) , the yellow pigment of the cocoon is presently attracted because of the antibacterial activity of flavonoid that is a component of the pigment. And, the pigment of the colored cocoon is chemically fixed, and the utilization of colored raw silk has been attempted.

Hand spun silk fabric, which is called Tsumugi, formed from floss silk is very popular and traded expensively in Japan. The double cocoon, as an ingredient of the floss silk, is too scarce to use large amount, because the

mounting technique of matured silkworms has been improved. So, double cocoon race has been established.

Breeding transgenic silkworm: In Japan Tamura (2000) succeeded in generation of the transgenic silkworm in NIAS. Since exogenous protein gene could be integrated into genome by using of the technique, it will result in an increase in number of the genetic stock of silkworm.

Mutation breeding: The utilization of silkworm gene mutation in commercial production is widely noticed by silkworm breeders. First of all, some of the mutations in economical characters can be directly utilized in silkworm breeding. The polyphagous mutation had been utilized to breed silkworm varieties adaptable to artificial diet; the trimolter mutation was utilized to breed trimoult silkworm varieties to produce especially fine silk; the non-glutinous mutation was used to produce natural loose eggs. Secondly, some of the morphological characters have multiple functions which also positively effect on the economical characters, therefore, it is possible to be directly utilized in new variety breeding. Experiment had showed that *K* gene had positive effect on feed efficiency and heterosis; *PS* and black pupa(*bp*) also showed the potential to increase feed efficiency; the introduction of non-scale mutation on the wing (*nlw*) can greatly improve the silkworm seed production condition. Thirdly, sex-control can raise single sex larvae, such as to rear only male silkworm in commercial cocoon production.

Breeding some basal varieties with special characteristics: In China **a**fter a long period of continuous breeding research, several basal varieties with special characteristic were made. 651 and 652 (Rong , 1978) are the basal varieties with especially long filament length; EH_{1new} (Chen,2002) with especially high cocoon shell weight, average 0.7g and shell ratio around 27%. Breed TB with extremely high in cocoon shell ratio(Its average cocoon shell ratio by batch reached 30.58%, the highest of female individual reached 31.79%, and male 38.09%.); “125” (Luo et al., 1997) with high raw silk rate(around 28 % calculated on the basis of fresh cocoon weight); “SG” (HeY et al., 1997) with dominant trimolter (especially fine filament size, 2.0 d). Moreover, the basal variety breeding of rich egg laying and superthick filament size was one of the Chinese national silkworm breeding program in the recent years. The task of this program is to breed a variety which could produce more than 800 eggs per moth or filament size thicker than 4.0 d and cocoon shell ratio exceeds 22%. Chen et al. (1995) had reported that “He” with hyperlaying was discovered from the 400 preserved silkworm variety resources in 1986, and it was used as one the parent material to breed the basal variety HEK which lays 821 eggs per moth average, the most 983, cocoon shell ratio reached 22.08%, cocoon weight 2.208, shell weight 0.486g, larval duration 23-25days. Wu et al.(1996) also had reported that four rich egg laying varieties –GE, GCL, GLZ, GLBZ had been bred from

1991-1995 by classical breeding techniques. The highest one, GE reached 1017 eggs per moth on average, and the highest moth 1083 eggs. The new varieties with filament size 4.5d and shell ratio 25.45 were also bred. The newly bred hybrid Xinmiao × Mingri (Xu et al., 2000) can produce filament size 5.245d , and other characters are reach to the popularized varieties, that is , cocoon weight 2.67g, shell weight 0.680g, shell ratio 25.45%, filament length 1094m, reelability 79.53% and non-broken filament length 870m. Its raw silk and the made fabric tests had shown a significant increase in bending rigidity and elasticity, tensile strength, elongation and elasticity, which indicated that its raw silk should be a suitable material for the exploitation of silk fabrics with better anti-wrinkle and hand-feeling.

Silkworm breeding by using the help of computer programs: The following breeding scheme was performed by Greiss(2003):

1 Tested optimal rearing inputs for the silkworm *Bombyx mori L.* during 1995-1996 in mixed batches.

2 Reared 4-6 sib-matted inbred lines in these optimal inputs in 4 generations to increase genetic homozygosity during 1997-1998.

3 Checked the best combiner inbred lines versus a ***top cross*** of the same breed reared in a mixed batch rearing and randomly matted.

4 Crossed the best two general combiner lines (**GRAND PARENTS**) to produce the **PARENTS** egg batches.

5 Selection by "relative culling method" , having the following selection parameters:

5.1 Choosing the individuals that are within the top 30 individuals from 100 population per sex in two or more characters from each sex.

5.2 The minimum number of selected individuals should be four or more from each sex.

5.3 Priority was given to shell ratio and the cocoon weight character, if not possible the shell weight character was selected for, instead of cocoon weight.

Computer programs on Microsoft Excel 97 base were specially designed to undergo selection according to the criteria given according to the Skew and Kurt parameters of each of the male and female populations, which dictated the selection criteria in the case of the experiments.

All the genetic estimates were computer calculated using the standard formulae. The breeding scheme is shown in **Fig. 57 .**

Breeding partheno/androgenetic lines, and control the sex-balance: There are many practical methods developed to control the sex balance of silkworm under

the joint effort of scientists from different countries, these are: androgenesis, parthenogenesis balanced lethal mutation and control of incubation condition.
Ameiotic parthenogenesis: The method is based on treatment by pure water with temperature 46 °C for 18 min of silkworm eggs extracted from the ovariole . By this treatment is prevented the reductional ovarian separation and the diploid chromosome set is preserved in the nucleus. In the same time the ovaries are stimulated for development. So as a result only female copies of the mother moth are produced. This method was successfully used in Russia (Strunnikov et al. , 1986) , Georgia , Ukraine and Uzbekistan for breeding special partheno- lines. The Sericulture institutes in Georgia and Uzbekistan have a good cooperation with the Laboratory of Cytogenetics and Sex Regulation at the Institute of Developing Biology under the Russian Academy of Sciences in the field of selection new parthenogenesis lines , and F_1 hybrids between them and ordinary lines. The advantage of the parthenogenesis line x ordinary breed F_1 hybrids over the other hybrids are as follows:

*the percentage of seed cocoons used is much higher(85 % while 40 % in the ordinary).

*all the seed cocoons from the maternal breed give female moths who lay eggs .

*the uniformity in F_1 is better.

*in the maternal breed there is no any sex separation, because all the individuals are female.

*the F_1 hybrid purity is 100 %.

This new breeding method was further developed in Uzbekistan by creation of breeds , having Z lethal genes and giving male progeny only. In this case the female parent of the hybrid is parthenogenetic line , having only females and the male line is “Z lethal gene male progeny” , having only males. By this achievement was solved completely the problem with sex separation in the silkworm egg production.The values of main quantitative breeding characters of some recent Georgian parthenogenetic lines and their hybrids with ordinary lines are presented in **Table 9 .**

Meiotic parthenogenesis: This method was developed for the first time by Strunnikov (1987). The silkworm ovaries are allowed to reductive separation and the nucleus has haploid number of chromosomes. If such kind of ovaries are preliminarily stimulated by high(46 ° C) or low(-11 ° C) temperature they separate into two sister’s cells which fuse without meeting any spermatozoa . In this fusion , the two cells having haploid chromosome set each , make a cell with diploid chromosome set in the nucleus. As it is well known in the silkworm the female sex is heterogametic (WZ) and the male sex is homogametic (ZZ). In the case of meiotic parthenogenesis both the cells are heterogametic and in their

fusion the possible combinations are WW and ZZ . From the eggs having ZZ – chromosomes male individuals develop , but the eggs having WW – chromosomes die.

Androgenesis: Newly laid eggs are first treated by X-rays (12000 – 15000 R) and then they are treated by high temperature (40 ^{o} C) water for 1 h. The X – rays destroy the ovarian nucleus , so the female nucleus become incapable to fuse with spermatozoa and the development starts after fusion of two spermatozoids nucleuses with the participance of maternal cytoplasm. Only the father's genotype is inherited (ZZ) and only male individuals are obtained. Since both spermatozoids are from one the same father the progeny is with low viability.

Methods for obtaining only male progeny by sex-limited lethal genes: It was discovered by Russian(Strunnikov,1987) and Chinese scientists(Chen,2002) that the sex-linked chocolate gene mutation(*sch*) was recessive gene which is very sensitive to high temperature during incubation stage. When genotype ZW crossed with Z^{sch} Z^{sch}, the female embryos of their hybrid could be killed during the incubation stage, therefore, only male silkworm will be hatched.

Methods for creation commercial silkworm hybrids:

Some of the selected silkworm races are used as pure lines for commercial hybridization.
The first precondition one race to be selected as pure line is to cover all the breeding characters criteria mentioned above , to have low variability of the quantitative characters values and the inheritance of the characters to be stable in the generations.
The chosen(or especially selected) races are tested for the combining ability and those one showed the highest combining ability between them are used as pure lines of the commercial hybrid.
After that the new hybrid is tested at least for 3 years at the station/institute where it has been selected, and then the hybrid is tested for at least 2 years in the system of the State Executive Agency for Varieties Testing.
During the testing as control is used the most widely adopted in the field silkworm hybrid for the last 5 years. To be recognized as new and original the hybrid must display difference from other hybrids/races at least in 1-2 qualitative characters and higher than the standard at least fresh cocoon and raw silk yield by one box of silkworm eggs.
The most popular are single and four-way F_1 commercial hybrids.

Methods of creation tolerant to adverse rearing conditions F 1 hybrids

By using selected for tolerance to adverse rearing condition races:

By this method in Bulgaria were created the hybrids SB 1 x HB 2,VB 1 x HB 2 and the opposite. Really ,under highly adverse rearing conditions these hybrids manifested a high tolerance ,but under optimal rearing their productivity was lower than the control.

However the target was to create a hybrid ,tolerant to adverse environment, but under good rearing condition to give a productivity near to the control. The reason is that if the hybrid is reared by a good farmer to ensure him/her a yield like the ordinary highly productive hybrid. So the tolerant to adverse rearing condition breeds were used as components of four-way hybrids , together with highly productive breeds.

By testing accessions of the silkworm germpalsm for higher tolerance to adverse rearing conditions and further hybridization:

15 silkworm breeds of the Japanese type , 13 breeds of the Chinese type and 16 F 1 hybrids between some of the breeds were used in the study, conducted at SES- Vratza , Bulgaria. All the breeds/hybrids had white cocoon color and were uni and bivoltine.

The breeds were chosen from the silkworm germplasm, maintained at SES-Vratza for having comparatively high values of the main quantitative characters.

Then the breeds were tested for three times regarding their tolerance to adverse rearing conditions during the 4^{th} and 5^{th} instars(t 28-31 ^{0} C,RH 75-80%,feeding amount and rearing space – reduced by 50 %,very reduced ventilation).Each breed was tested in volume of 3 replicates, consisted of 300 larvae each, counted after the 2^{nd} moult , both under optimal(Kipriotis et al., 1999) and adverse rearing conditions .

The breeds who manifested the highest pupation rate under adverse larval rearing conditions were crossed between some of them and the F_1 hybrids were tested also under both standard and adverse rearing regime. As an alternative cross for comparison(control) , the hybrid Vratza 35 x Merefa 2 and the reciprocal , made between two of the most sensitive to adverse rearing breeds , one of Japanese and one of Chinese type respectively was used. Each hybrid was tested for 3 times in 3 replicates , consisted of 300 larvae each.

Then the hybrids performed the best under the adverse rearing conditions and manifested comparable to the control productivity under standard rearing conditions were chosen and further tested.

Simultaneously, each silkworm breed selected by the adverse rearing test was mass reared for 6 generations under adverse conditions and only the individuals survived and having the highest cocoon weight, shell weight and shell percentage were selected for further reproduction. For the mass rearing 50

batches of each breed were hatched separately, and only those , having a hatchability more than 98 %(20-30) were chosen for mass larval rearing. After the second moult 4 replicates , consisted of 250 larvae each were counted and reared until the cocoon spinning.

After the 6th generation the breeds have been maintained by batch rearing ,following the standard methodology for pure lines described above.

As a result two new four-way hybrids ,namely (KKxHesa 1) x (Vesletz2xGergana 2) , (KK x AS) x (Vesletz2xGergana 2) and the reciprocal crosses, manifested the highest tolerance to adverse rearing conditions together with a satisfactory high productivity under optimal rearing were created.