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—Review—
Review Series: Animal Bioresource in Japan

The Silkworm—An Attractive BioResource Supplied by Japan

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Abstract: Silkworms have played an important agricultural role in supporting Japan's modernization, and traditionally, Japan has led the world as a repository of silkworm bioresources. The silkworm is a small and highly domesticated insect, which is ideal as a laboratory tool, although it is a bioresource that is relatively infrequently used in experiments at present. In this review, we describe the potential for silkworm resources to contribute to life sciences.
Key words: bioresource, *Bombyx mori*, domesticated animal, NBRP-silkworm

Silkworm, a Unique BioResource from Japan

Japan has a long history of sericulture, which encompasses various races and strains of silkworm. Race can be defined as a regional line uniquely reared in different locations for the purpose of sericulture. Conversely, strain can be defined as a sub-classification of race reared to conserve natural mutant variants discovered during the breed improvement process or within a race, and mutant variants artificially induced by exposure to radiation or chemical agents. Around 500 races are bred, mainly for sericulture, at the National Institute of Agrobiological Sciences (the former National Institute of Sericultural Science of The Ministry of Agriculture, Forestry and Fisheries of Japan) and about 820 strains are stored at Kyushu University for use in research.

It is estimated, based on the production volume of cocoons, that around 235 billion silkworms were reared domestically in 1930 when the sericulture activity of Japan peaked. Postwar, however, despite a decline in the cocoon production volume, 50 to 60 billion silkworms per year were still reared up to around 1975, while 200 million silkworms a year are currently being reared, despite the production volume having declined still further. Mutant varieties, which represent useful starting material for research work, emerge at a certain rate based on the population of a group. Therefore the larger the population, the more often the mutant variant emerges. Silkworms, meanwhile, are organisms in which many mutant have been discovered; largely because of the sheer numbers reared. Because sericulture thrived in Japan, silkworms have become an original bioresource of Japan.

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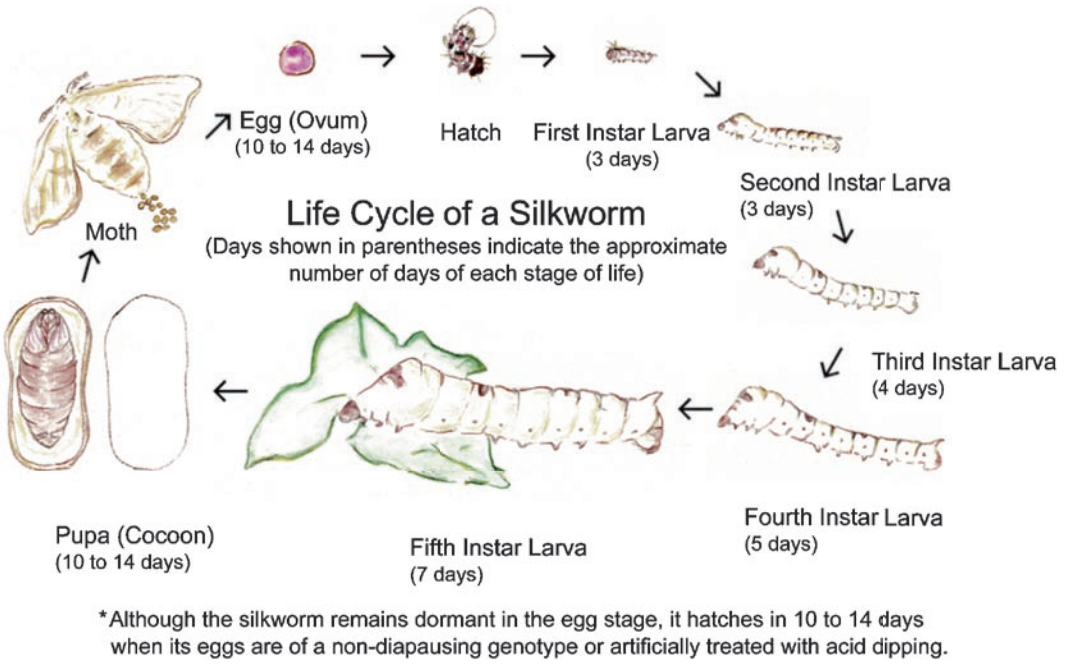

Fig. 1. Life cycle of the silkworm. Each stage of the silkworm life cycle as shown herein progresses when the silkworm is reared under temperatures ranging from 25 to 27°C.

From an international perspective, the country now possessing silkworm bioresources on a large scale is China, which may also hold more races for sericulture than Japan. The latter, however, retains a more favorable position than China with respect to gene mutant strains, which are scientifically more useful. Korea and India also have a certain scale of resources, while national resource centers in Europe are found in Bulgaria, Italy, and France, housing 220, 120, and 60 strains respectively. In the US there is no stock center; hence the silkworm as a resource available from Asia, including Japan, plays a central role.

Characteristics of the Silkworm as an Experimental Animal for Research Work

The life cycle of a silkworm can be primarily divided into 4 stages—namely the egg (ovum), larva, pupa, and adult (moth). The number of days required for each stage is 10 to 14 days for the egg, 20 to 25 days for the larva, 10 to 14 days for the pupa, 7 days for the adult and 50 to 60 days for 1 cycle (Fig. 1). At the maximum rate, 7 generations of silkworms a year can be reared. Silkworms, the ultimate in domesticated organisms, cannot survive in the open and perish unless fed mulberries, their sole diet, by humans. Adult (moth), although winged, are flightless. This characteristic makes them easy to manage and presents a convenient advantage as an experimental animal. As shown in Table 1, there are many advantages unique to silkworms, which have contributed to their use in academic research. One example deserving particular attention was the 1906 discovery by Kametaro Toyama, a Japanese researcher, that Mendel's principles of genetics could be applied to animals for the first time [11]. At the time, many mutant traits had already been recorded, such as the color of pupa, skin color and variegated skin patterns of larva, and colors of eggs, and Kametaro Toyama engaged in pioneering research work utilizing these variants. Currently about 260 phenotype mutations are known and researchers in Europe and the United States are focusing on silkworms as insects suitable for genetic study [1]. Although mutant variants of drosophila often emerge at the adult stage, those of silkworms often emerge at the egg and larva stages (Table 2 and Fig. 2). Another noteworthy contribution to scientific research made by silkworms has been

work concerning the physiological functions of insect hormones and sex pheromones. Crustaceans and insects grow by molting during the process of growth, while holometabolous insects grow through dramatic morphological changes into pupa and moths. Substances that play leading roles in such changes are known as insect hormones, namely molting or juvenile hormones, while the substance controlling the secretion of such hormones is called a brain hormone. Discovery, isolation and studies of the action mechanisms of such substances have been conducted using silkworms as and key research material. Why and for what reason? The major reason lies in the fact that the silkworm is an animal that can be reared in bulk. For silkworms, it is possible to prepare millions of individual organisms that are genetically purified, at the same developmental stage, enabling the development of bioassays of good reproducibility and the purification of minute quantities of hormone. Dr. Butenandt, a German biochemist, imported a few million silkworms from Japan and was awarded the Nobel Prize for his research work on sex pheromones. Thus, the silkworm can be considered a suitable animal for research work in the field of genetics and physiology. In the following sections, we introduce recent examples of the utility of silkworms as experimental animals and their future potential.

Recent Applications of Silkworms as an Experimental Animal

Application as an animal for primary bioassays

To develop new drugs it is common to use mice and other mammals to measure the biological effectiveness of chemical compounds. The associated expense, however, is large and attempts to reduce such expenses or switch to the use of non-mammalian animals are under-

Table 1. Biological features of the silkworm

- They can survive only under control of human beings.
- One pair lays 400 to 600 eggs in one day.
- Body size is within a suitable observable range.
- There is no stock center in Europe or the United States.
- There are abundant mutants.
- There is accumulated research information in Japan.
- One generation can easily be reared within a cycle time of approx. 50 days.
- Rearing is possible in large quantities in a small space.

Table 2. Number of mutant variants expressed in a given developmental stage

Developmental stage	Number of mutants
Eggs (including embryo)	45
Larva	142
Pupa	9
Cocoon	23
Adult	14
Expressed in multiple stages	31
Total	264

Fig. 2. Mutants of silkworm (left: egg stage, right: larval stage). The egg color is determined the colors of the yolk, serosa, and chorion. The standard egg color is dark purple. A white skin is the standard color of the larval body, but many kinds of variation exist.

way. When screening many chemical compounds, the use of silkworms in the initial step of experiments to measure biological effectiveness is convenient [2, 5]. Silkworms can be used for experimental inoculation with bacteria (e.g., *Staphylococcus aureus*, *Pseudomonas aeruginosa*). After confirmation of infection, test compounds can be administered to examine if they have curative effect. Although examination to verify whether the efficacy of drugs in silkworms is the same as that in humans is vital, silkworms can be used as a convenient model to narrow down the number of candidates from many chemical compounds. In such experiments, strains derived from different origins or with different historical backgrounds are useful.

Utilization as a protein production system

Silkworms are utilized to obtain targeted proteins by expressing cloned genes. One utilization method obtains targeted proteins by integrating cloned DNA into the genome of viruses living within host silkworms. The transgenic virus proliferates in the silkworm body and the silkworms usually die in a few days, because of the proliferation of the virus. We need to produce proteins derived from exogenous genes more efficiently, but the productive efficiency of the exogenous gene depends significantly on the host silkworm strain. By screening silkworm strains collected by NBRP, we discovered some which are virus-resistant [6]. This production system of targeted proteins has already been commercialized by some business corporation [3, 4, 10].

Preparation of resources for the functional analysis of genes

Germline transformation technology of silkworms became available in 2000 [8]. It is a complex operation, and was problematic because only skilled operators could prepare the resources. However, its application has gradually become widespread, together with its improvement. NBRP evaluates transgenic (TG) silkworm lines mainly developed domestically and collects TG strains that are useful for researchers. Now, enhancer trap, gene trap and RNAi lines are being produced as TG silkworm lines. Although the development of gene traps and RNAi has just got underway, improvements including system developments are required. In this project we are mainly focusing on enhancer trap strains [12] (Table 3).

Silkworms as a model for the domestication of wild animals

Humans have domesticated wild animals and have utilized them as food and for clothes. While we tend to refer to such animals as domesticated animals, cows and horses can survive even after being released into the open. Silkworms cannot do the same. This is because silkworms have lost their ability to fly and find their food, the mulberry, by themselves during their long process of domestication. *Bombyx mandarina*, an insect that inhabits East Asia is thought to be an important ancestral species of the silkworm, *Bombyx mori* [9]. In Fig. 3 the relationships between the two species and the historical domestication process are shown. Research work is underway to determine the genomic differences between the wild silkworm, *B. mandarina*, and the silkworm. In this research, a consomic line is useful. We constructed a consomic line from a hybrid of *B. mori* and *B. mandarina*. By repeated back-crossing with the male *B. mori* of the same strain, a series of consomic lines in which the *B. mori* chromosome except that of interest has been replaced with the chromosome of the *B. mandarina* is almost complete and its use for research work is underway. So far, 26 consomic lines corresponding to 26 chromosomes of the silkworm have been established. The chromosome responsible for traits lost or gained during the domestication process and the genes responsible may be identified. Once this is done, we anticipate their exploitation for insect pest management and the rearing of silkworms.

Development of Custom-Designed Strains Responding to Requests from Researchers

Many researchers apparently think that mulberry leaves are essential for rearing silkworms, and also that it is not possible to rear silkworms in with the winter season due to a lack of leaves. To solve this problem, an artificial diet soy bean protein as the main component has successfully been developed. Rearing various mutant variant strains on an artificial diet, however, has proved difficult. To solve this problem, NBRP has bred

Table 3. Stock list of transgenic silkworms

No.	Strain names	Vectors*	Names of vectors in references	Purposes: transgenes	Selection markers
1	BmA3-GFP	pBac[BmA3-EGFP]	pPIGA3GFP	Reporter: *Bombyx mori* Cytoplasmic Actin A3 (BmA3) promoter and EGFP	BmA3-EGFP
2	FibL-GFP	pBac[FibL-EGFP/3xP3-DsRed2]	pBac(3xP3-DsRed2+L-chain-GFP)	Reporter: Fibroin light chain gene fused with EGFP	3xP3-DsRed2
3	UAS-GFP	pBac[UAS-EGFP]	pBacUAS-GFP	Reporter: UAS-EGFP homozygous strain, with no marker	no marker
4	UAS-CBP	pBac[UAS-CBP/3xP3-EGFP]	pBacMCS[UAS-CBP-3xP3-EGFP]	Effector: UAS and carotenoid binding protein (CBP)	3xP3-EGFP
5	UAS-JHE	pBac[UAS-JHE/3xP3-ECFP]	pBac{UAS-JHE-3xP3-ECFP}	Effector: UAS and juvenile hormone esterase (JHE)	3xP3-ECFP
6	Js14	pMi[BmA3pigTP/3xP3-ECFP]	pMiBmA3pigTP/3xP3ECFP	Jumpstarter: BmA3 promoter with *piggyBac* transposase in *Minos* vector	3xP3-ECFP
7	Js15	pMi[BmA3pigTP/3xP3-ECFP]	pMiBmA3pigTP/3xP3ECFP	Jumpstarter: BmA3 promoter with *piggyBac* transposase in *Minos* vector	3xP3-ECFP
8	Js18	pMi[BmA3pigTP/3xP3-ECFP]	pMiBmA3pigTP/3xP3ECFP	Jumpstarter: BmA3 promoter with *piggyBac* transposase in *Minos* vector	3xP3-ECFP

*pBac: *piggyBac* transposon vector, pMi: *Minos* transposon vector.

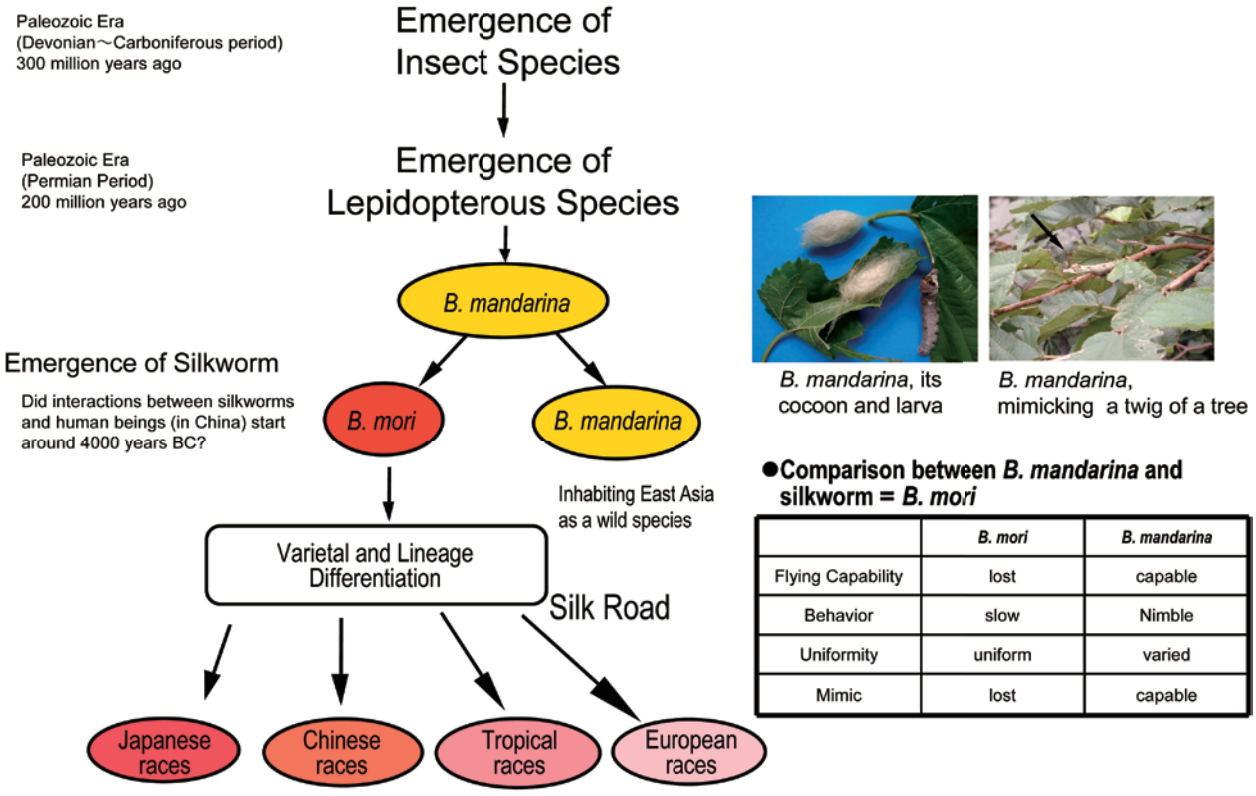

Fig. 3. A model of the domestication process of the silkworm (from *B. mandarina* to silkworm = *B. mori*).

Fig. 4. A fifth instar larva feeding on mulberry leaves (left), and third instar larva feeding on an artificial diet (right).

a group of silkworm strains that will feed on artificial diet without problems and has created many silkworm strains that are available allyear round. Currently, researchers who are unable to obtain mulberries can utilize silkworms for research work anytime with a commercial artificial diet (Fig. 4). Only one company provides the artificial diet, Nosan corporation (http://www.nosan.co.jp).

NBRP Silkworm: Implementation System and Organization

This project involves the collaboration of Kyushu University as a representative institution, the University of Tokyo, the Shinshu University and the National Institute of Agrobiological Sciences as group partners. The tasks shared by each institution are outlined as follows:

Kyushu University handles standard (reference) strains and mutant strains of silkworms, as well as *B. mandarina*, which is considered to be an ancestor of silkworm. There are approx. 820 silkworm strains comprising 450 mutant strains, 300 improved strains that can feed on an artificial diet without problems and 70 transgenic strains. The p50 strain in our stocks is the most popular among researchers, and is used for genome sequencing. The p50 has the standard silkworm phenotype and is highly resistant to disease. It is suitable for developmental biology research. Currently a few strains of *B. mandarina* can be supplied in live form. *B. mandarina* is widespread in Japan and Kyushu University receives delivery requests for *B. mandarina* which have been collected in different areas and are of vital academic value as described hereinafter. Because of the difficulty, however, in maintaining such strains, Kyushu University stores and supplies the DNA of the majority of *B. mandarina* collected in the field. A database containing detailed information of strains, "Silkwormbase", has been established and made public in cooperation with Dr. Yamazaki *et al*. of the Genetics Research Institute, NBRP Information Center.

The University of Tokyo shares the task of collection, storage and supply of genome resources, i.e., approx. 50 genomic-DNA/cDNA libraries of *B. mori*, *B. mandarina*, and *Samia cynthis ricini*. In particular, it stores and supplies more than 200,000 cDNA clones, derived from various developmental stages and tissues of *B. mori* and *S. c. ricini*. Detailed information is published at http://morus.ab.a.u-tokyo.ac.jp.

Shinshu University collects, stores and supplies strains (approx. 70) and DNA of individual organisms of *Antheraea yamamai* (the Japanese oak silkworm), *Antheraea pernyi* (Chinese oak silkworm), *Rhodinia fugax* and *Samia cynthia pryeri* that are closely-related wild silkworms. Detailed information about them is published in the "Okaikosama" newsletter (in Japanese) and it is scheduled for publication on the "Silkwormbase" database. The newsletter and database are available at http://www.nbrp.jp/index.jsp.

The National Institute of Agrobiological Sciences (NIAS) collects and evaluates transgenic silkworms, the storage and supply of which is handled by Kyushu University. Currently there are 70 strains available for dis-

Table 4. NBRP project has enabled an all-year supply of silkworms to researchers

	1	2	3	4	5	6	7	8	9	10	11	12
Prior to distribution period	×	×	×	○	○	○	△	×	×	×	×	×
Distribution period of eggs treated with refrigeration and acid dipping	×	×	×	×	×	×	×	○	○	○	○	△
Distribution period of eggs having entered artificial hibernation	○	○	○	△	×	×	×	×	×	×	△	○
Distribution period after NBRP started the distribution of silkworms	○	○	○	○	○	○	○	○	○	○	○	○

tribution and NIAS plans to add 20 new strains every year. Detailed information of available strains is published at http://sgp.dna.affrc.go.jp/ETDB/ [7].

Access to Silkworms, an NBRP BioResource

As described above, silkworms can be fed on an artificial diet. Distribution of requested silkworm races or strains, however, is usually carried out during spring time only. This is because the silkworm strains are maintained in the spring and only eggs prepared in spring are available for distribution. Limited utilization during springtime alone, however, has been a major obstacle for users. To overcome this obstacle, NBRP promotes a project to prepare eggs treated with refrigeration and acid dipping and eggs having entered into artificial hibernation. The favorable hatching rate from August to December of eggs treated with refrigeration and acid dipping and from January to April of those having entered artificial hibernation has been verified. Therefore, NBRP could start the year-round supply of silkworms from in 2005. Silkworms are currently widely available to users and have become available allyear round through this system (Table 4). Researchers in need of silkworms can obtain them from NBRP by ordering through the homepage http://www.nbrp.jp/index.jsp. NBRP also responds to direct inquiries at the following address:

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