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Suspension-Type Cloned Cell Lines from Embryo Tissues of *Bombyx mori*

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Abstract

The present paper outlines the establishment of four additional cloned cell lines derived from embryo tissues of *Bombyx mori*, which were designated as SES-BoMo-J125K1, SES-BoMo-J125K2, SES-BoMo-J125K5, and SES-BoMo-J125K6. These cell lines were characterized by their suspension-type, karyology, isozyme pattern, and amino acid consumption pattern. Among these cell lines, the SES-BoMo-J125K2 cell line was susceptible to *Bombyx mori* nuclear polyhedrosis virus, and could be cultured in the serum-free medium (MM-SF). This cell line could be used for the production of useful proteins or to control pests using baculoviruses.

Discipline: Biotechnology/Sericulture
Additional key word: cell cloning, characterization, nuclear polyhedrosis virus, serum-free medium, virus multiplication

Introduction

Culture of insect cell lines is one of the most effective techniques for the production of useful proteins or to control pests using baculoviruses[9,10]. For the mass production of proteins *in vitro* and multiplication of natural enemy viruses, it is necessary to establish cell lines highly susceptible to insect viruses and culture them on a large-scale with a low-cost medium. Since Grace[3] first established an insect cell line, more than 51 cell lines of Lepidoptera have been reported. Recently, Inoue et al.[7] have established a cell line which was susceptible to *Bombyx mori* nuclear polyhedrosis virus (Bm NPV). However, since the cells produced adhered to the bottom of the culture flask, they were not suitable for large-scale culture. The present paper attempts to outline the characteristics of the suspension-type cloned cell lines of SES-BoMo-J125K1, SES-BoMo-J125K2, SES-BoMo-J125K5, SES-BoMo-J125K6, which were isolated from the continuous cell line SES-BoMo-J125 of *Bombyx mori*.

Establishment of insect cell line

Eggs of *B. mori*, 20-hr-old, were treated with hydrochloric acid (specific gravity 1.075) for 5 min at 48°C to avoid the diapause and incubated at 25°C for 4 days. Then the eggs were sterilized by submersion in 70% ethyl alcohol for 5 min, washed 3 times in sterile distilled water, and transferred to a plastic petri dish (35 mm dia.) containing Carlson fluid[1]. Approximately 50 embryos in blastokinesis were separated from the chorion and adherent yolk using a scapel, and then minced into several pieces. They were placed in a culture flask (Falcon plastic, Los Angeles) containing MGM-448[11] medium supplemented with 10% fetal bovine serum (FBS) and incubated at 28°C. Half of the medium was renewed at 1 to 2 week intervals. Cell migration from the embryonic tissues started within 24 hr after initiation of the primary culture. The migrated cells were epithelial-like. They migrated actively over the base of the culture vessel, and then proliferated actively within 170 and following days after the primary culture was initiated. When the number of cells increased sufficiently, subculture was made by

transferring the cells to another flask containing 4 m*l* of a fresh medium. By the subculture, the number of cells increased 70-fold in approximately 660 days after the first subculture. The cell line was designated as SES-BoMo-J125[4].

Cell cloning

Thirty-seven cloned cells were originally isolated from the SES-BoMo-J125 cell line by dilution plating in two dishes of a 96-well micro test plate, out of which 12 cloned cells proliferated and could be subcultured. This procedure was repeated to establish additional four cloned cell lines, which were designated as SES-BoMo-J125K1, SES-BoMo-

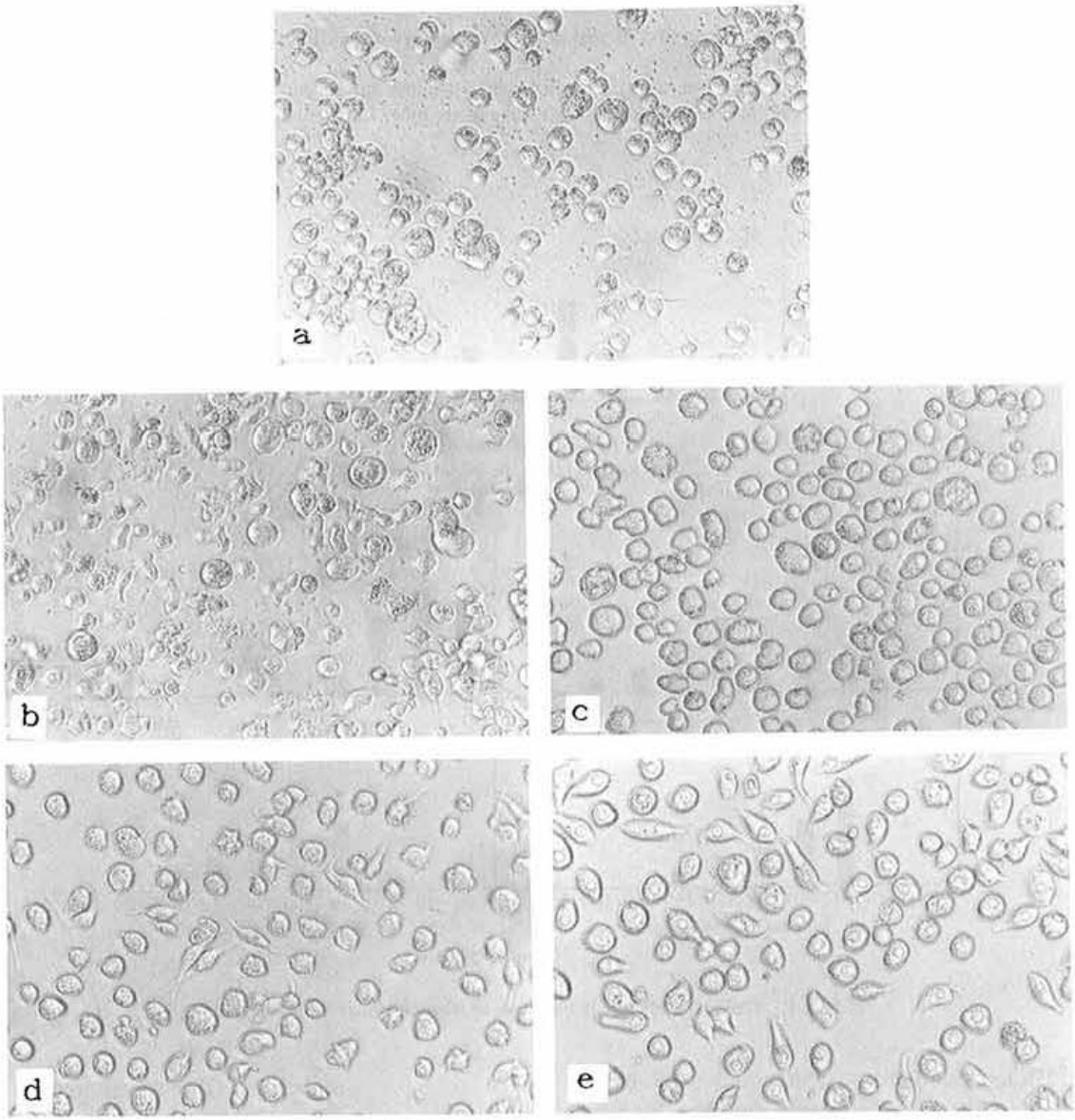

Plate 1. Magnification of cell lines of *B. mori*
a: SES-BoMo-J125, b: SES-BoMo-J125K1, c: SES-BoMo-J125K2, d: SES-BoMo-J125K5, e: SES-BoMo-J125K6.

Table 1. Cell cloning procedures from an established cell line

Step	Procedure
1.	The cell cultures to be cloned are selected among the materials in an active state of growth.
2.	Count the number of viable cells by the trypan blue exclusion assay.
3.	Dilute the cell suspension to a concentration of 10 cells per m/ of medium.
4.	Add 0.1 m/ of medium containing cells per micro test well (Falcon micro plates containing 96 wells). Use medium in which cells are cultured for 1 day.
5.	Incubate the cultures at 28°C.
6.	During the growth of colonies, supply a small amount of fresh medium into wells to prevent its decrease.
7.	When the colonies reach 50–100 cells in number, remove the medium. Add 0.1 m/ of pancreatin solution per well.
8.	Allow the cells to round, and then add cloning medium to suspend the cells.
9.	Transfer the cells to a culture tube (Nunc tissue culture tube; 15 m/ volume) and then to a flask.

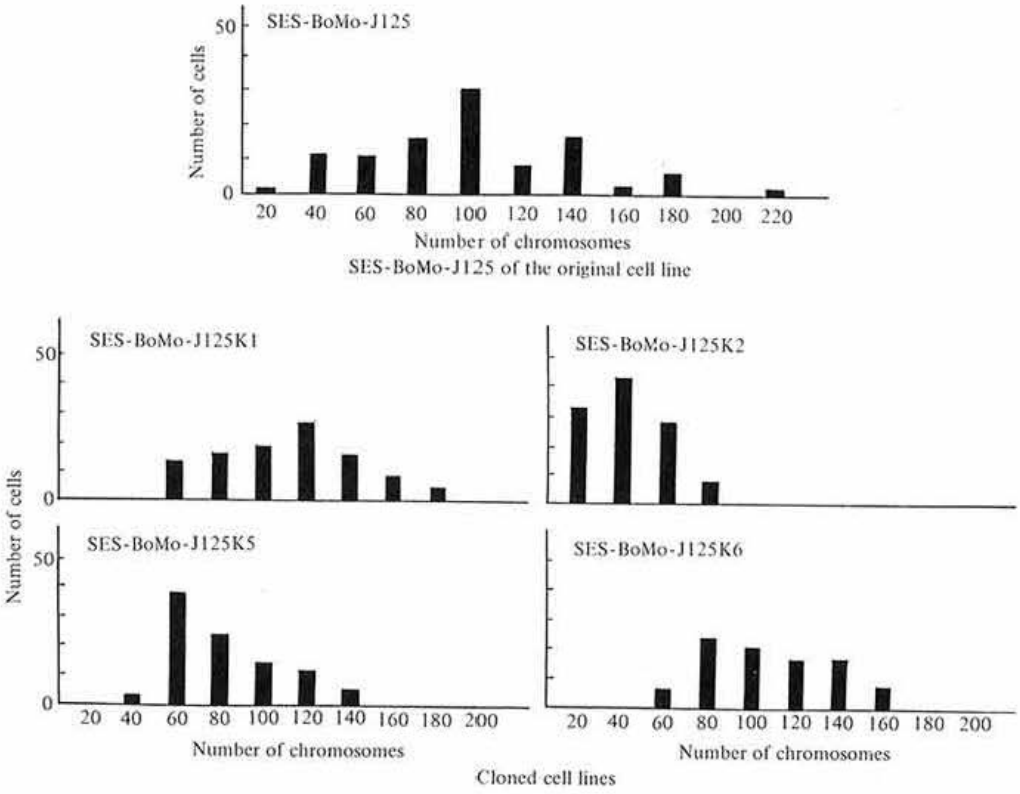

Fig. 1. Cells with different numbers of chromosomes in cell lines

J125K2, SES-BoMo-J125K5 and SES-BoMo-J125K6 (Plate 1). This paper describes the results of the study on the multiplication of Bm NPV in the SES-BoMo-J125K2 cells. The method of cell cloning is indicated in Table 1.

Results

1) Morphology and growth

All the cell lines exhibited a highly uniform mor-

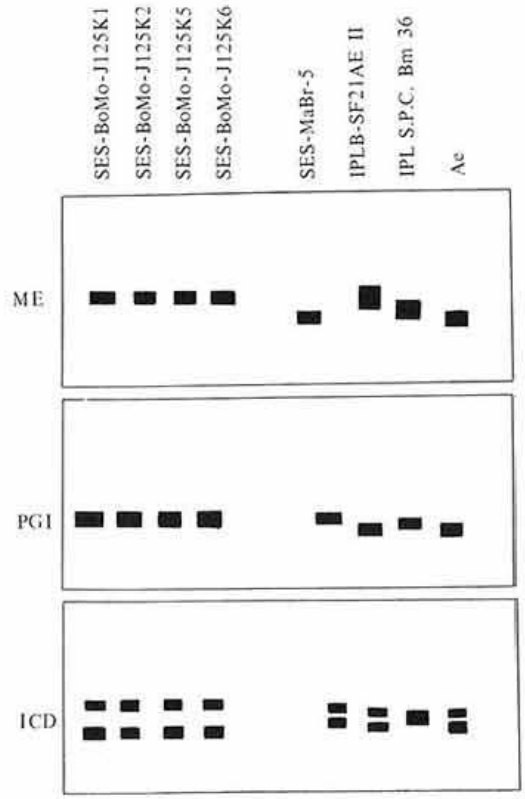

Fig. 2. Comparison of isozyme banding patterns for 8 insect cell lines
Cell lines were electrophoresed using the Corning Authentikit System (Corning Medical and Scientific, East Walpole, MA).

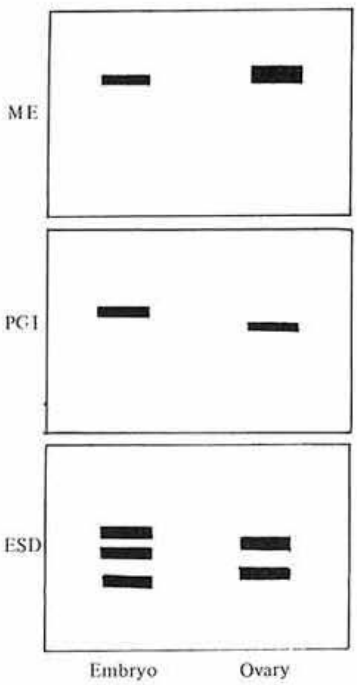

Fig. 3. Comparison of isozyme banding patterns for two tissues of Cambodge race

phology with predominantly ellipsoid-shaped cells of the suspension type. Cell generation periods for each line were as follows: BoMo-J125K1 and BoMo-J125K6, 5.41 days; BoMo-J125K2, 1.96 days; and BoMo-J125K5, 4.12 days.

2) Karyology

The method of Earley[2] was used for determining the chromosome number of the cloned cell lines. Chromosomes were stained with Giemsa and 50 spreads were counted. The chromosome number varied from one cell line to another. As shown in Fig. 1, the chromosome number of the original BoMo-J125 cell line ranged from 20 to 220. In the case of the cloned cell lines, however, the number ranged as follows: 60 to 180 in BoMo-J125K1, 20 to 80 in BoMo-J125K2, 40 to 140 in BoMo-J125K5, and 60 to 160 in BoMo-J125K6 (for reference, 2N of *B. mori* = 56).

3) Biochemical characteristics

To distinguish these cloned cell lines from one another, the staining patterns of isozymes ICD (isocitric dehydrogenase), PGI (phosphoric glucoisomerase), ME (malic enzyme), and ESD (esterase) were compared. Results of isozyme analysis of the cloned cell lines using the Corning Authentikit are shown in Fig. 2. For ICD, PGI, and ME, migration distances of each band for the cloned *B. mori* cell lines were identical, but the cell lines of other species as well as an independently established cell line of *B. mori* differed from the cloned cell lines reported here. They included the following lines: SES-MaBr-5[6] (derived from larval fat body of *Mamestra brassicae*), IPLB-SF21AEII[14] (from pupal ovary of *Spodoptera frugiperda*), Ae[3] (from pupal ovary of *Antheraea eucalypti*), NISES-BoMo-Cam 1[8] (from ovary of *B. mori*), and IPL S.P.C. Bm 36[13] (from ovary of *B. mori*). The Bm 36 cell line was

Table 2. Changes in percentage of free amino acids contained in the medium for culture of each cell line

(%)

Amino acid	Cont.	Cell line				
		J125	K1	K2	K5	K6
α-Alanine	59	125	136	143	129	112
β-Alanine	53	54	70	60	55	44
Arginin	63	64	79	71	67	52
Aspartic acid	66	33	29	14	27	40
Asparagine	77	34	56	26	45	25
Cystine	73	31	69	86	42	66
Glutamine	0	0	0	0	0	0
Glycine	55	58	72	65	61	47
Histidine	50	55	67	60	57	43
Isoleucine	64	52	76	61	58	50
Leucine	63	58	76	61	58	50
Lysine	58	60	76	66	63	50
Methionine	68	59	77	59	59	47
Ornithine	72	69	92	72	75	58
Phenylalanine	66	66	83	72	69	55
Phosphoserine	101	125	105	86	117	98
Proline	63	66	79	72	68	53
Serine	52	57	70	62	58	45
Taurine	103	85	107	—	101	84
Threonine	64	65	82	72	68	53
Valine	63	58	75	64	60	49
Ammonia	49	35	61	13	33	38

The initial content was taken as 100%.
The cells were cultured at 25°C for 6 days.

established from pupal ovarian tissues of *B. mori*, while the cloned cell lines were established from embryonic tissues. The isozyme patterns of the ovarian and embryonic tissues of the Cambodge race of *B. mori* were identical (Fig. 3), indicating that the observed differences might have been caused by genetic divergence in the original silkworm strains used as a source of cultured cells.

4) Amino acid consumption in cultured cells

The BoMo-J125, BoMo-J125K1, BoMo-J125K2, BoMo-J125K5 and BoMo-J125K6 cell lines were cultured at 2×10^5/m*l* cell density at 25°C for 6 days. Thereafter the contents of free amino acids in the MGM-448 medium were determined. The quantity of most amino acids decreased, and the changes were characterized by a significant decrease in the contents of asparagine, aspartic acid, and ammonia, and by a significant increase in the content of α-alanine, while the contents of phosphoserine and taurine did show any changes (Table 2). In a comparison of the quantitative changes in amino acids at culture temperatures of 25 and 28°C, most amino acids showed a greater decrease at 28°C than at 25°C (data not shown).

5) Adaptation of cultured cells to a low-cost medium and virus multiplication

(1) Adaptation of culture cells from MGM-448 medium (10% FBS) to MM-SF medium (0% FBS)

As the BoMo-J125K2 cell line consists of cells in suspension, large-scale culture is more convenient. Although a serum-free medium[11] is required for large-scale culture, it is not easy to adapt cultured cell lines to the MM-SF medium, which is less costly. In the present study, the suspension-type cell lines were successfully adapted to MM-SF medium after they were transferred from MM medium containing 3% FBS. These cells were subcultured continuously

Table 3. Effect of the susceptibility of Bm NPV on the various media

Cell line	Media	$TCID_{50}$ / ml [a]	
		7 days post inoculation	10 days post inoculation
BoMo-15A	MGM-448 (10%FBS)	$10^{-4.75}$	$10^{-4.75}$
BoMo-J125K2	MGM-448 (10%FBS)	$10^{-5.00}$	$\leqq 10^{-6.38}$
BoMo-15A	MM (3%FBS)	$10^{-2.50}$	$10^{-3.00}$
BoMo-J125K2	MM (3%FBS)	$10^{-3.38}$	$10^{-3.75}$
BoMo-J125K2	MM-SF (0%FBS)	0	$10^{-0.88}$

a): $TCID_{50}$ was calculated by the method of Behrens-Karber.
$TCID_{50}$ of the supernatant of culture medium in which Bm NPV-infected cells were taken as 10^0.

with MM-SF medium, in which the content of FBS decreased gradually in volume. After six months, the cells could be subcultured with MM-SF medium only. However, the generation period was approximately two weeks.

(2) Virus multiplication in the low-cost medium

The study on the susceptibility of the BoMo-J125K1, BoMo-J125K2, BoMo-J125K5 and BoMo-J125K6 cell lines to Bm NPV (P6E clone strain)[7] revealed that BoMo-J125K2 showed the highest susceptibility to virus multiplication. Accordingly, an experiment on virus multiplication was carried out using the BoMo-J125K2 cell line and BoMo-15A[5] cell line derived from embryonic tissues as the control. Both cell lines were subcultured in MGM-448 (10% FBS), MM (3% FBS) and MM-SF (serum-free) media, and 2 days later, 150 μl of each culture was inoculated with 25μl the Bm NPV solution. Approximately 1,000 cells were observed with time and the number of cells that formed polyhedra was counted (Table 3).

The susceptibility of the BoMo-J125K2 cells cultured in the MM medium to Bm NPV was lower than that of the cells cultured in the MGM-448 medium. Compared to the BoMo-15A cell line, the BoMo-J125K2 cell line showed a higher susceptibility to Bm NPV, not only in the MGM-448 medium (10% FBS), but also in the MM medium (3% FBS). However, in the MM-SF medium (serum-free), none of the cells formed polyhedra. These results suggested that FBS markedly promoted the formation of Bm NPV polyhedra in a culture medium, and that the BoMo-J125K2 cell line was highly susceptible to Bm NPV.

Conclusion

In order to produce useful proteins *in vitro* using a baculovirus-insect culture cell system, it is necessary to establish a cell line that is highly susceptible to Bm NPV. For this reason, the BoMo-J125 cell line originating from embryonic tissues was first established, and used to isolate several cloned cell lines. These cloned cell lines were characterized by their morphology, growth, karyology, isozyme pattern and amino acid consumption. When Bm NPV was inoculated into these cloned lines, the BoMo-J125K2 line was found to be highly susceptible to Bm NPV. This cloned cell line grew in suspension, and could be cultured in a serum-free medium (MM-SF). Although the BoMo-J125K2 cells were less susceptible to Bm NPV in the serum-free medium, the addition of FBS[15] and insect hemolymph[6] to the low-cost medium improved polyhedra formation. The establishment of the BoMo-J125K2 cell line provides a promising new system for achieving a high yield of virus production.

References

1) Carlson, J. G. (1946): Protoplasmic viscosity changes in different regions of the grasshopper neuroblast during mitosis. *Biol. Bull.*, **90**, 109-121.
2) Earley, E. M. (1975): Chromosomal preparations from monolayer cell cultures. *TCA Manual*, **1**, 31-35.
3) Grace, T. D. C. (1962): Establishment of four strains of cells from insect tissues grown *in vitro*. *Nature*, **195**, 788-789.
4) Imanishi, S. & Ohtsuki, Y. (1988): Characteristics of cell lines established from embryonic tissues of several

races of the silkworm, *Bombyx mori*, cultured *in vitro*. *J. Seric. Sci. Jpn.*, **57**, 184–188.
5) Inoue, H. & Mitsuhashi, J. (1984): A *Bombyx mori* cell line susceptible to a nuclear polyhedrosis virus. *J. Seric. Sci. Jpn.*, **53**, 108–113.
6) Inoue, H. (1989): Establishment of insect continuous cell lines and its utilization for virus multiplication *in vitro*. *JARQ*, **23**, 31–36.
7) Inoue, H., Taniai, K. & Kobayashi, J. (1990): Establishment and characterization of substrate depending cell lines of *Bombyx mori*. *Bull. Nat. Inst. Seric. Entomol. Sci.*, **1**, 13–25 [In Japanese with English summary.]
8) Kobayashi, J. & Inoue, H. (1989): Establishment of cell line derived from ovary of *B. mori* and its susceptibility to Bm NPV. *In* 59th Ann. Meet. Seric. Sci. Jpn. [In Japanese].
9) Luckow, V. A. & Summers, M.D. (1988): Trends in the development of baculovirus expression vectors. *Bio/Technology*, **6**, 47–55.
10) Maeda, S. et al. (1985): Production of human α-interferon in silkworm using a baculovirus vector. *Nature*, **315**, 592–594.
11) Mitsuhashi, J. (1982): Advances in cell cultures (vol. 2). ed. Maramorosch, K., Academic Press, N.Y., 133–196.
12) Mitsuhashi, J. (1984): Isolation of a continuous cell line from larval fat bodies of an Arctiid moth, *Spilarctia seriatopunctata* (Insecta, Lepidoptera, Arctiidae). *Zool. Sci.*, **1**, 415–419.
13) Quiot, J. M. (1982): Establishment d'une ligne cellulaire (S.P.C. Bm 36) a partir d'ovaires de *Bombyx mori* L. (Lepidoptera). *Sericologia*, **22**, 25–31.
14) Vaughn, J. L. et al. (1977): Establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera, Noctuiidae). *In Vitro*, **13**, 213–217.
15) Watanabe, H. (1987): Effect of the concentration of fetal bovine serum in a culture medium on the susceptibility of *Bombyx mori* cells to a nuclear polyhedrosis virus. *Appl. Entomol. Zool.*, **22**, 397–398.

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