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G Lokesh

Central Sericultural Germplasm
Resources Centre, Central Silk
Board, Hosur, Tamil Nadu,
India

Geetha N Murthy

Central Sericultural Germplasm
Resources Centre, Central Silk
Board, Hosur, Tamil Nadu,
India

Gargi

Central Sericultural Germplasm
Resources Centre, Central Silk
Board, Hosur, Tamil Nadu,
India

M Maheswari

Central Sericultural Germplasm
Resources Centre, Central Silk
Board, Hosur, Tamil Nadu,
India

Jayaprakash Pandey

Central Tasar Research &
Training Institute, Central Silk
Board, Ranchi, Jharkhand,
India

Alok Sahay

Central Tasar Research &
Training Institute, Central Silk
Board, Ranchi, Jharkhand,
India

Correspondence**G Lokesh**

Central Sericultural Germplasm
Resources Centre, Central Silk
Board, Hosur, Tamil Nadu,
India

Semen collection and artificial insemination in wild silk moth *Antheraea mylitta* Drury for effective conservation of Tasar genetic resources

G Lokesh, Geetha N Murthy, Gargi, M Maheswari, Jayaprakash Pandey and Alok Sahay

Abstract

The techniques of cryopreservation of semen and artificial insemination of the female can be helpful in solving the reproductive problems and is also desirable for the selective breeding based on desired traits in wild silkworms. Moreover the genetic resources can be conserved *ex situ* for a longer period. In this context, a collection protocol for cryopreservation of semen from the wild silkworm *Antheraea mylitta* a tropical tasar silkworm at -196°C in liquid nitrogen was standardized. Further, artificial insemination of virgin female moth was also pursued to get viable embryos. Under standardization, the semen was collected from the seminal vesicle of freshly emerged male moth and from the bursa copulatrix (BC) and spermatheca of the female moth after 1 hour mating. The morphology and behaviour of semen was recorded through microscopic examination. The semen consisting of sperms entangled in a membrane in the form of bundles. Hence, the sperm bundles were treated with trypsin to dissociate the sperm bundles for activation of sperms before introducing into the female reproductive system for further fertilization. Microscopic observation of semen sample recovered from the bursa copulatrix showed rigorous motility of the spermatozoa. Artificial insemination using freshly collected semen sample treated with trypsin, preserved semen sample (-80°C) and semen collected from bursa copulatrix was carried out through the vaginal opening of the female virgin moth. Fecundity was recorded in each group which did not reveal significant difference. However, hatching of larvae recorded significant difference with 82% hatching under natural mating on the day of hatching (82%) compared to under artificial insemination of freshly collected semen (26%), preserved semen (25%) and semen from bursa copulatrix (39%). The study enumerates that, standardization of cryopreservation techniques followed by artificial insemination lead to the development of hybrids in tropical tasar through crossing different ecoraces.

Keywords: *Antheraea mylitta*, semen processing, conservation, seminal vesicle, artificial insemination

1. Introduction

The wild silk moth *Antheraea mylitta* has vast genetic resources distributed widely in the tropical zones of India with remarkable diversity expressed in the form of ecoraces adapted to different ecological niches [1]. The genetic improvement of commercially exploited semi-domesticated races of *A. mylitta* through hybridization and breeding to obtain desired breeds are not explored due to limitations such as incompatibility; less amenable to human handling, change of behaviour under *ex situ* conditions in addition, non-synchronization of moth emergence and difficulties in mating among different variability. Moreover, the genetic diversity of wild silkworms is facing earnest threat due to indiscriminate use of bio resources, damage to the environment and destruction of eco-systems [2, 3]. Realising this, many efforts are being made to conserve the vast seri-biodiversity in the country [4].

Cryopreservation of male sperm and artificial insemination offer better strategies for preservation of biologically active samples like semen at sub-zero temperature conditions (-196°C) for longer duration [5-7]. Many studies on artificial insemination have been carried out in insects and the techniques developed have been used for breeding and maintenance of honey bee colonies [8-10]. The silkworm *Bombyx mori* has been widely used to study artificial insemination [11-16]. It was demonstrated that virgin females artificially inseminated using seminal fluid collected from a seminal vesicle of male reproductive organ and subsequent treatment with the glandula prostatica secretion could oviposit fertilized eggs at a rate almost similar to that of the females mated with males [17]. Further, artificial insemination was carried out using sperm treated with trypsin and could obtain fertilized eggs without the secretion

of the glandula prostatica in *B. Mori* [15]. As evidenced earlier, many efforts were made on artificial insemination in *B. mori* but the study in wild silkworms such as *Antheraea mylitta* is very limited [18]. There is an urgent need for introduction of these techniques to conserve invaluable wild tasar silkworm genetic resources. In view of this in the present study, attempts were made to develop/standardize the protocol for collection of sperm from male *A. mylitta* moth, processing to get viable semen for cryopreservation and artificial insemination which can help solve the reproductive problems as well as for selective breeding to develop wild silkworm breeds with desired traits. Moreover, the technique will be helpful in the conservation of the vast tasar silkworm genetic resources for future use.

2. Materials & Methods

Cocoons of the tropical tasar silkworm *A. mylitta* were collected from CTR&TI, Ranchi, India and preserved at CSGRC, Hosur grainage following all standard protocols until the emergence of moths. The semen samples were collected during first and second grainage seasons i.e., July and September – 2016-17.

2.1 Dissection and collection of Semen: The male tasar moth (Fig.1a) was used for collection of sperm by dissecting seminal vesicle (SV) from the male reproductive organ (Fig.1b). The seminal fluid was collected in a micro centrifuge tube containing Grace's insect culture medium (GICM), centrifuged at 3000 rpm for 3 min. and was stored under refrigerated conditions until further use.

2.2 Recovery of semen from female moth: Mating of the moths was allowed and after 1 hr. female moths were decoupled and collected for dissection. The bursa copulatrix (BC) and spermatheca (Fig. 1c & 1d) were carefully dissected out and placed in a 1ml tube, the membrane was ruptured and contents collected. The BC fluid was subjected to microscopic study.

2.3 Semen processing with protease enzyme: Different concentrations of trypsin solution were prepared in cell culture medium (GISM). The seminal fluid recovered from SV was separately mixed with different concentrations of trypsin solution i.e., 0.05µg/ml, 0.1µg/ml, 0.2µg/ml, 0.3µg/ml, 0.4µg/ml and 0.5µg/ml. The mixture was then incubated at 25°C for 15 min to digest the spermatophore membrane and release sperms. The efficiency of trypsin activity was examined through microscopic observations separately for different concentrations [15].

2.4. Semen Preservation: Semen samples collected from SV were further diluted with the GISM consists of cryoprotectants (CPAs) such as 5% glycerol and 7% DMSO to prepare sperm medium to protect the sperm from effects of freezing. Initially the sperm medium was preserved at -80 °C and later it was transferred to liquid nitrogen until used for the artificial insemination.

2.5. Artificial insemination of different types of semen samples: Different types of semen samples were prepared such as 1] Freshly collected semen from the male SV and treated with 2mg/ml trypsin, 2] Semen sample recovered from the BC & spermatheca without trypsin treatment and 3] Cryopreserved and trypsin treated semen samples for

insemination of virgin female moths. Insemination was done by taking semen sample into a 0.5 ml syringe attached with micro-needles. About 400 µl Semen was transferred in to virgin female through the vaginal opening. The inseminated moths were allowed for the egg laying for 72 hrs (Fig.4). The efficiency of different types of semen samples were examined through assessment of fecundity and hatching.

3. Results and Discussions

The results of present study revealed that, the seminal fluid collected from SV of an adult male reproductive system consists of dimorphic bundle like structures. Large and longer bundles were identified as eupyrene sperm bundles which are less in number and long, thin filaments more in numbers as apyrene bundles. There was no movements were observed in the large bundles. However, apyrene showed rapid motility (Fig.2 a&b). Similar observations have been earlier reported in *A.mylitta* [18] and *B.mori* [19, 20]. Eupyrene and apyrene sperms derive from bipotential spermatogonia. In both types, 256 sperm cells are surrounded by a layer of cyst cells after maturation division, later forming a sperm bundle [21, 22]. The bursa copulatrix (BC) of the female silkworm is filled with various secretions and during mating the seminal fluid is transferred from the male reproductive system. The contents are all heterogeneous, since they are formed by the partially mixed, viscous streams of the male secretions. Apyrene and eupyrene spermatozoa move towards spermatheca after their maturation [23]. The microscopic analysis of BC content of adult female *A. mylitta* revealed high viscosity with dense spermatozoa. The spermatozoa were active and showing vigorous motility in different forms. The apyrene movement was observed for a short while, whereas, eupyrene movement was constant for a longer period. Since the eupyrene sperms samples collected from the SV were not showing any movement and are in the bundle form. They have been subjected to trypsin treatment to dissolve the sperm bundles and facilitate sperm activation. Among the various concentrations of trypsin it was observed that, higher concentration of trypsin > 0.4µg/ml affected the digestibility and no appearance of either sperm bundles or active spermatocytes, could only observe dispersed inactive spermatocytes. At lower concentration of 0.1µg/ml, there was no effective on dissolution of the bundles. However, the trypsin concentration at 0.2µg/ml to 0.3µg/ml revealed free and active spermatocytes (Fig.3). As per earlier reports [24], the sperm requires to be activated before it is used for insemination. The secretion of the glandula prostatica is essential for the acquisition of motility and fertility of the sperm in *B. mori*. It was demonstrated that, in *Antheraea pernyi* a peptide of molecular weight ranging between 1600 and 4500 was responsible for sperm activation [25]. When sperms were incubated without trypsin, eupyrene sperm remained in bundle form and apyrene sperm moved slowly [15]. On the other hand, when the sperms were treated with 0.3 µg/ml concentration of trypsin, eupyrene sperm bundles completely dissociated, like those collected from the BC of normally mated females. Artificial insemination using freshly collected semen sample treated with trypsin, preserved semen sample (-80°C) and semen collected from BC through the vaginal opening of the female virgin moth was carried out. After 72 hours of oviposition of mother moths, fecundity of eggs was recorded and found that there was difference in the egg laying capacity of different moths inseminated with different types of semen samples. However, no significant

difference in the fecundity among different groups was observed (Fig.5). The fecundity and egg fertility is considered as one of the most desired quantitative characters of commercial importance in silkworms [26]. The healthy and robustness of the mating male-female moths are very important for subsequent quality and quantity of egg laying [27]. Moreover, the number of eggs produced in the ovary of the female silkworm moth invariably lays most of the eggs. Hence the quality of the sperm not always influence the fecundity however in certain cases the male factors triggers more laying of eggs [28]. Hatching of larvae recorded significant difference with 82% hatching under natural mating compared to under artificial insemination of freshly collected semen (26%), preserved semen (25%) and semen from bursa

copulatrix (39%). The viability of the semen with active sperms is very important for the successful fertilization. In addition the mode of transfer of sperms from male to female and quality of the semen with respect to sperm activity and density are the critical. Sperm fertility depended on both the concentrations of a mixture of sperm and secretion from the glandula prostatica in silkworms [15 29]. In the present case, normally mated female laid eggs were recorded higher fertility compared to other type of semen samples inseminated. However, in the semen samples collected from BC of mated female, sperms were activated naturally while transfer from male to female moth and hence recorded better rate of fertility (39%) compared to other artificially inseminated semen samples.

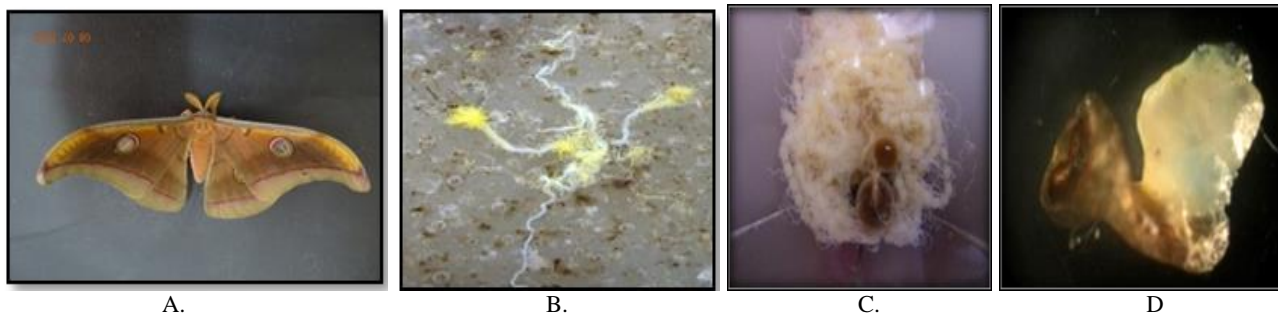


Fig 1: a. Male *A.myltta* moth, b, Male reproductive system, c&d. Bursa copulatrix of female adult

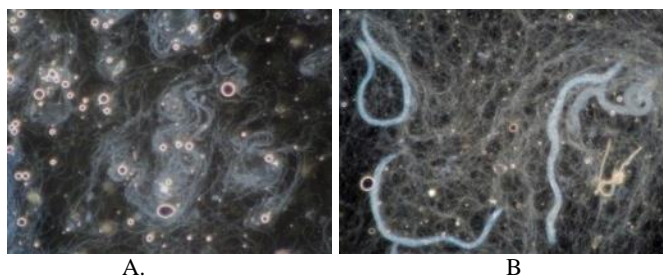


Fig 2: a. Microscopic observations (200X) of Seminal fluid., b. SV fluid consisting Eupyrene sperm bundles and apyrene sperms.

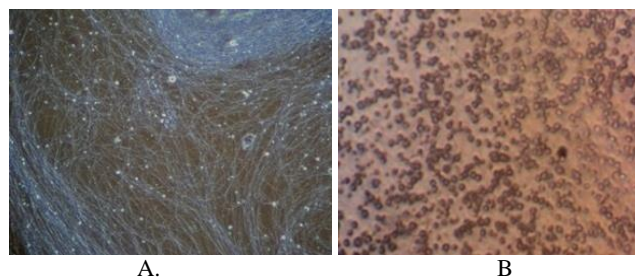


Fig 3: a. Trypsin treated semen sample collected from SV. b. Semen sample collected from BC after 1 hr. of mating.



Fig 4: Artificially inseminated moths

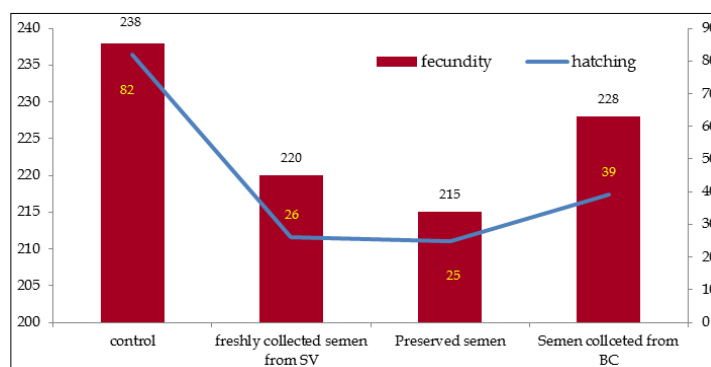


Fig 5: Fecundity & hatching% from insemination with different types of semen

4. Conclusion

Based on the outcome of the present study it is inferred that standardization of sperm collection and processing to get viable and active sperms is pertinent for efficient and successful insemination that can yield fertile offspring. The standardized techniques for successful artificial insemination will not only go a long way in solving the reproductive compatibility problems in tasar silkworm ecoraces but will also be beneficial in long term conservation of these invaluable genetic resources for future use.

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