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Original Article

Screening for eri silkworm (*Samia ricini* Donovan) ecoraces using morphological characters, growth, yields, and ISSR marker

Duanpen Wongsorn[1], Weerasak Saksirirat[2,3]*, Sivilai Sirimungkararat[1,2,3], and Hathairat Urairong[4]

[1] *Entomology Section, Department of Plant Science and Agricultural Resources,*

[2] *Cultivation and Product Development Research Group of Wild Silkmoths and Economic Insects for Value Added Creation,*

[3] *Agricultural Biotechnology Research Center for Sustainable Economy and Center of Agricultural Biotechnology (AG- BIO/PERDO-CHE), Faculty of Agriculture, Khon Kaen University, Mueang, Khon Kaen, 40002 Thailand.*

[4] *Biotechnology Research and Development Office, Sirinthorn Genetic Resources Building, Department of Agriculture, Ministry of Agriculture and Cooperatives, Pathum Thani, 12110 Thailand.*

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Abstract

The selection of eri silkworm ecoraces with high yield and distinct morphological characters is necessary for variety improvement. The five ecoraces SaKKU1, SaKKU2, SaKKU3, SaKKU4 and SaKKU5 were derived mostly by international academic cooperation. They were cultured using castor leaves of TCO 101 cultivar as food plant at 25±2°C, 80±5% R.H. Based on morphological characters, they are similar, except the body of the 5th instar larva of SaKKU1 is clearly covered with more creamy white powder and the mature larva has a shiny dominant yellow color. The duration of the life cycle among ecoraces was also similar; 46-53 days (SaKKU1), 42-53 days (SaKKU2), 42-52 days (SaKKU3), 40-56 days (SaKKU4) and 41-52 days (SaKKU5). SaKKU1 had the highest survival rate at larval stage (1st – 5th instar) (100.00%) and larva (1st – 5th instar) - adult (88.89%), including the predominant heaviest average larva weight of all instars, 0.0317 g (2nd instar), 0.2206 g (3rd instar), 1.0788 g (4th instar), 4.0102 g (5th instar), and 8.9940 g (5 days of 5th instar), which was significantly different ($P<0.05$) to other ecoraces. Moreover, this ecorace gave the highest average yields: fresh cocoon weight (3.8016 g), pupa weight (3.2532 g), shell weight (0.5287 g), shell ratio (14.01%), fresh cocoon weight/10,000 larvae (38.01 kg), eggs/moth (531.13 eggs), total eggs (6,375.27 eggs) and total hatching eggs (6,006.13 eggs), which was also significantly different ($P<0.05$) than other ecoraces. Of those properties, especially survival rates and yields, this ecorace (SaKKU1) is favored for further varietal improvement program. In parallel, genetic relationship analysis of eri silkworm ecoraces using inter-simple sequence repeat (ISSR) technique was also carried out. The result revealed from dendrogram analysis that SaKKU1 was the farthest distance than other ecoraces, especially against SaKKU3. Based on all above results, the SaKKU1 ecorace was considered to be the most suitable for heat tolerant variety improvement.

Keywords: screening, ecorace, eri silkworm, yield, ISSR marker

* Corresponding author.
Email address: weerasak@kku.ac.th

1. Introduction

Eri silkworm (*Samia ricini* Donovan), Lepidoptera: Saturniidae), a commercial silk producing insect, is believed to have originated in the Brahmaputra Valley of India (Jolly *et al*., 1979) and had restricted distribution in India, China, and Japan for two centuries (Peigler, 1993; Singh and Benchamin, 2002). The primary food plant of this polyphagous insect is castor (*Ricinus communis* L.), but it also feeds on a wide range of food plants such as Kesseru (*Heteropanax fragrans*), Tapioca (*Manihot esculenta*), Ceara rubber tree (*Manihot glaziovii*), Variegated tapioca (*Manihot esculenta variegata*), and Gulancha Phool/Champa (*Plumeria acutifolia*) (Sirimungkararat *et al*., 2005). Besides, the new food plants and the alternative food plant are also very suitable to feed eri silkworm in some cases (Sirimungkararat *et al*., 2005; Buamuangpia *et al*., 2014). The wild *S. canningi* silkworm (the progenitor of the domesticated *S. ricini*) completes one to three generations per year depending on geographical position and climatic condition of region; however, up to six generations occur in the domesticated cultures (Neupane *et al*., 1990). Populations of eri silkworm that have been commercially exploited are present in different regions of north-east India showing wide variations in morphological and quantitative characters such as absolute silk content, larval weight, cocoon weight, cocoon shell weight, and silk ratio (Siddiqui *et al*., 2000). Eri silkworm was introduced to Thailand around 1974 by technical cooperation of the Department of Agricultural Ministry of Agriculture and Cooperatives, for cultivation test of highland project in northern Thailand (Wongtong *et al*., 1980). However, due to the density of host plants in mountainous area, the eri silkworm rearing was proposed for other parts of the country where the host plants were sufficient and the farmers have skills also with indigenous knowledge on sericulture. The Northeast of Thailand is a largest planting area of cassava (*Manihot esculenta*), a host plant of eri silkworm, and the farmer has knowledge on sericulture as the way of life. Therefore, several research projects have focused on rearing technique, machine and appliance development, textile, eri food and cosmetic products including exploitation of eri rearing waste. Of those, our studies were published in series (Sirimungkararat *et al*., 2002; 2005; 2007a. 2007b; 2010; 2012; 2013a; 2013b; 2014). In order to preserve the natural biodiversity present among these populations, attempts are being made to know the basic morphological characters, growth, yield and the genetic variation of each population. As molecular markers are stable and environmentally independent, they are increasingly being preferred to phenotypic traits to detect genetic variation not only among populations but also between individuals within a population. There are a number of DNA marker systems to be applied for this purpose such as RFLP (restriction fragment length polymorphism) (Mahendran *et al*., 2006), RAPD (random amplified polymorphic DNA) (Lui *et al*., 2010), SCAR marker (sequence characterized amplified region) (Saha and Kundu, 2006), and ISSR (Chatterjee *et al*., 2004; Kar *et al*., 2005; Vijayan *et al*., 2006; Pradeep *et al*., 2011). The objective of this study aimed to evaluate the eri ecoraces from basic morphological characters, growth, some quantitative traits and genetic basis variability, leading to the most suitable ecorace for heat tolerance improvement program.

2. Materials and Methods

2.1 Eri silkworm ecoraces

Five ecoraces of eri silkworm were derived by local collecting and international academic cooperation between Thailand, India, and Japan, which were coded as SaKKU1, SaKKU2, SaKKU3, SaKKU4 and SaKKU5. The larvae of all ecoraces were fed with castor leaves cultivar TCO 101, at room temperature 25±2°C and 80±5% relative humidity (R.H.). The rearing method was modified from Sirimungkararat *et al.* (2002).

2.2 Morphological characters, life cycle, survival and yields of eri silkworm

Eri silkworm ecoraces were investigated for morphological characters of larva, life cycles, survival rates, and also the yield components of cocoons and eggs, based on rearing conditions described previously. The following data were recorded: weight and size of each instar (24 hrs after molting), survival rates from $1^{st} - 5^{th}$ larva instar and larva ($1^{st} - 5^{th}$ instar)– adult, cocoon yields (fresh cocoon weight, pupa weight, shell weight, shell ratio, total cocoon shell weight and fresh cocoon weight/10,000 larvae), and egg yields (eggs/moth, hatching eggs, total eggs, and total hatching eggs). Weighing of cocoon or pupa with four decimal, a balance Sartorius BP 210S, Germany was used. The experimental design known as completely randomized design (CRD) was used. The ecoraces served as treatments with three replications. Each replication contained 50 larvae of eri silkworm. Analysis of variance (ANOVA) was done and means of the treatments were compared statistically using Duncan's multiple range test (DMRT).

2.3 Genetic relationship study of eri silkworm using inter-simple sequence repeat technique

Ten mature 5^{th} instar larvae of each ecorace were taken for DNA extraction using silk glands. The glands were washed with 70% ethanol to remove traces of sericin and then crushed in pre-chilled mortar and pestle with liquid nitrogen. Genomic DNA from 10 individual larvae of each ecorace was extracted separately by using phenol:chloroform extraction method (Chatterjee *et al*., 2004). Five inter-simple sequence repeat (ISSR) primers were used for the study UBC811 ($(GA)_8C$), UBC818($(CA)_8G$), UBC848($(CA)_8AGG$), UBC857 ($(CA)_8CTG$) and UBC888(CGTAGTCGT $(CA)_7$). The polymerase chain reaction (PCR) was performed in an MJ Research

thermal cycler, PTC200, using 20 µl reaction mixture containing 30 ng DNA, 2.0 µl of 10xPCR buffer (MBI Fermentas), 0.2 mM dNTP, 2.5 mM $MgCl_2$, 0.15 µl M primer and 0.10 µl *Tag*DNA polymerase (recombinant) (5 U/µl). The PCR products were resolved on 1.5% agarose gel, in Tris-Boric Acid-EDTA buffer, stained with ethidium bromide (0.5 µg/ml). The genetic variability in the population was analyzed using phylogenetic tree based on Neighbor-Joining method.

3. Results

3.1 Morphological characters, life cycle, survival and yields of eri silkworm

The eri silkworm is a multivotine (polyvoltine) wild silkmoth, and has complete metamorphosis. The five ecoraces had similar morphological characters in all development stages: egg, larva, pupa and adult, both in shape and color having similar characteristics. There was only one that was somewhat different. The ripe larva of SaKKU1 showed shiny distinct yellow color than other ecorace (Table 1).

In the cultivation of eri silkworm using castor leaves cultivar TCO 101, it was shown that all ecoraces had similar life cycle durations of 46-53, 42-53, 42-52, 40-56 and 41-52 days for ecorace SaKKU1, SaKKU2, SaKKU3, SaKKU4, and SaKKU5, respectively. The shortest life cycle was 40 days (ecorace SaKKU4). The age of both female and male moths of SaKKU1 (10-14 days) was longer than other ecoraces, with values between 4-12 days for males (4-12 days) and females (5-12 days).

The survival of the larval stage (1^{st}–5^{th}) (Table 2), the rate of SaKKU1 was the highest (100.00%), which differed statistically from other ecoraces ($P<0.05$). Whereas, the survival of larval stage (1^{st}–5^{th}) – adult, the ecorace SaKKU1 still had a maximum rate (88.89%) with no statistical difference compared to ecorace SaKKU4 (85.56%)

Table 1. Phenotypic characters of five different ecoraces of eri silkworm (*Samia ricini*) fed with leaf of castor cultivar TCO 101.

Characters	Ecorace				
	SaKKU1	SaKKU2	SaKKU3	SaKKU4	SaKKU5
Voltinism	Multivotine	Multivotine	Multivotine	Multivotine	Multivotine
Egg color	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white
Larval color (5-days-old 5^{th} instar)	Greenish light blue with more white powder cover body	Greenish light blue with slight white powder cover body	Greenish light blue with slight white powder cover body	Greenish light blue with slight white powder cover body	Greenish light blue with slight white powder cover body
Larval color (ripe larva)	Distinct yellow	Yellow	Yellow	Yellow	Yellow
Larval marking	Plain	Plain	Plain	Plain	Plain
Cocoon color	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white
Cocoon shape	Oval	Oval	Oval	Oval	Oval
Moth color (both sexes)	Blackish gray	Blackish gray	Blackish gray	Blackish gray	Blackish gray

Table 2. Survival (mean±SD) of eri silkworm (*Samia ricini*) fed with leaf of castor cultivar TCO 101.

Ecorace	Survival (%)	
	Larva (1^{st} – 5^{th} instar)	Larva(1^{st} – 5^{th} instar) – Adult
SaKKU1	100.00±0.00 a	88.89±5.09 a
SaKKU2	64.44±1.92 d	64.44±1.92 d
SaKKU3	74.44±5.09 c	71.11±1.92bc
SaKKU4	85.56±5.09 b	85.56±5.09 a
SaKKU5	76.67±8.82 bc	73.33±5.77 b
F-test	**	**
C.V. (%)	**6.44**	**5.61**

Means followed by the same letter within a column are not significantly different (DMRT, $P>0.05$). ** = Significantly different at 99% level.

The average weights of silkworms of various ages were shown in Table 3. The 1st instar in all ecorace was similar and did not differ statistically. But the 2nd, 3rd, 4th, 5th, and 5day of 5th instar of ecorace SaKKU1 had the highest values (0.0317, 0.2206, 1.0788, 4.0102, and 8.9940 g, respectively), which differed statistically significant ($P<0.05$) from other ecoraces. The weight of mature silkworms (5 days of 5th instar) of ecorace SaKKU2 - SaKKU5 ranged from 5.4544 to 6.0649 g, which was not significantly different.

As well as the average body size of the silkworm in each instar and growth, it was found that the ecorace SaKKU1 had still larger body size than other ecoraces, with means of 0.10 × 0.48, 0.23 × 1.14, 0.42 × 2.31, 0.86 × 3.97, 1.25 × 6.61 and 1.63 × 9.02 cm for 1st, 2nd, 3rd, 4th, 5th, and 5 day of 5th instar, respectively. The body size of eri larvae of ecorace SaKKU2, SaKKU3, SaKKU4 and SaKKU5 in each instar were similar: 1st instar (0.10 × 0.45 - 0.47 cm), 2nd instar (0.15 × 0.89 - 0.98 cm), 3rd instar (0.38 - 0.40 × 1.96 - 2.02 cm), 4th instar (0.63 - 0.71 × 3.11 - 3.28 cm), 5th instar (1.00 - 1.09 × 5.20 - 5.33 cm), and 5 day of 5th instar (1.39 - 1.44 × 7.17 - 7.49 cm).

The average output of cocoon yields (Table 4) showed that ecorace SaKKU1 had the highest in various yields of fresh cocoon weight (3.8016 g), pupa weight (3.2532 g), shell weight (0.5287 g), shell ratio (14.01%), total cocoon shell weight (15.86 g) and fresh cocoon weight/10,000 larvae (38.01 kg), which is still higher and the difference is highly significant compared to the other ecoraces.

For egg yields (Table 5), the number of eggs/moth, total eggs, and total hatching eggs, SaKKU1 remained the most effective ecorace (531.13, 6,375.27 and 6,006.13 eggs, respectively) and were significantly different ($P<0.05$) compared to the others. The hatching egg percentage of all ecoraces were similar and did not differ statistically, whereas ecorace SaKKU1 had the highest value (94.33%), followed by ecorace SaKKU2 (93.74%) and SaKKU5 (92.57%), respectively.

Table 3. Mean (mean±SD) weights of eri larva (*Samia ricini*) of different instars fed with leaf of castor cultivar TCO 101.

Ecorace	Mean weights of larva (g)					
	1st instar	2nd instar	3rd instar	4th instar	5th instar	5 days of 5th instar
SaKKU1	0.0020	**0.0317±0.001 a**	**0.2206±0.002 a**	**1.0788±0.09 a**	**4.0102±0.13 a**	**8.9940±0.42 a**
SaKKU2	0.0020	0.0120±0.001 c	0.1371±0.008 c	0.5487±0.04 b	2.5116±0.23 b	6.0649±0.24 b
SaKKU3	0.0020	0.0166±0.001 b	0.1338±0.004 c	0.6055±0.06 b	2.4689±0.05 b	5.5984±0.61 b
SaKKU4	**0.0021**	0.0123±0.001 c	0.1573±0.008 b	0.5303±0.05 b	2.4963±0.38 b	5.4544±0.11 b
SaKKU5	0.0020	0.0136±0.002 c	0.1323±0.016 c	0.6422±0.07 b	2.3978±0.20 b	5.6790±0.37 b
F-test	**ns**	**	**	**	**	**
C.V.(%)	**4.30**	**6.93**	**5.76**	**9.74**	**8.09**	**6.13**

Means followed by the same letter within a column are not significantly different (DMRT, $P>0.05$).
ns = Non significantly different at 95% level
** = Significantly different at 99% level

Table 4. Yields (mean±SD) of eri silkworm (*Samia ricini*) fed with leaf of castor cultivar TCO 101.

Ecorace	Average yield					
	Fresh cocoon weight (g)	Pupa weight (g)	Shell weight (g)	Shell ratio (%)	Total cocoon shell weight (g)	Fresh cocoon weight/ 10,000 larvae (kg)
SaKKU1	**3.8016±0.41 a**	**3.2532±0.37 a**	**0.5287±0.04 a**	**14.01±0.59 a**	**15.86±1.07 a**	**38.01±1.42 a**
SaKKU2	2.4591±0.21 b	2.1584±0.18 b	0.2879±0.03 b	11.64±0.23 b	5.57±0.65 c	24.59±2.37 b
SaKKU3	2.0100±0.14 b	1.8695±0.12 b	0.2570±0.03 b	11.93±0.59 b	5.71±0.23 c	20.10±1.67 b
SaKKU4	2.4169±0.16 b	2.1094±0.14 b	0.2943±0.02 b	12.16±0.12 b	7.34±0.23 b	24.17±3.11 b
SaKKU5	2.3943±0.14 b	2.0971±0.13 b	0.2856±0.01 b	11.94±0.32 b	6.57±083 bc	23.95±2.04 b
F-test	**	**	**	**	**	**
C.V. (%)	**8.93**	**9.07**	**7.67**	**3.39**	**8.42**	**8.93**

Means followed by the same letter within a column are not significantly different (DMRT, $P>0.05$).
** = Significantly different at 99% level.

Table 5. Egg yields (mean±SD) of eri silkworm (*Samia ricini*) fed with leaf of castor cultivar TCO 101.

Ecorace	Average yield				
	Eggs/moth (eggs)	Hatching eggs (%)	Total eggs (eggs)	Total hatching eggs (eggs)	Male : Female
SaKKU1	**531.13**±30.28 **a**	94.33±2.60	**6,375.27±320.11 a**	**6,006.13±277.33 a**	1: 2.32
SaKKU2	353.87±45.67 b	93.74±3.27	4,027.87±297.22 b	3,762.07±321.00 b	1:1.31
SaKKU3	272.40±37.32 b	88.87±5.73	2,413.07±263.37 b	2,174.13±321.00 b	1:1.72
SaKKU4	322.87±35.62 b	91.75±5.26	3,990.20±311.01 b	3,663.20±272.33 b	1:1
SaKKU5	310.20±27.65 b	92.57±2.96	3,194.60±255.41 b	2,602.27±266.13 b	1:1.30
F-test	**	**ns**	**	**	-
C.V.(%)	**12.62**	**4.52**	**25.79**	**24.57**	-

Means followed by the same letter within a column are not significantly different (DMRT, P >0.05).
ns = Non significantly different at 95% level. ** = Significantly different at 99% level.

3.2 Genetic relationship study of eri silkworm using inter-simple sequence repeat technique

The five primers of inter-simple sequence repeat (ISSR) produced 34 different DNA fragments of all ecoraces varying from 200 to 2,700 bp (data not shown). The data of DNA fragments were analyzed to obtain a dendrogram using Neighbor-Joining method as illustrated in Figure 1. Based on the dendrogram, the ecorace SaKKU3 was the most closely related to ecorace SaKKU5. Obviously, the ecorace SaKKU1 resulted in an outgroup against SaKKU3 and other ecoraces.

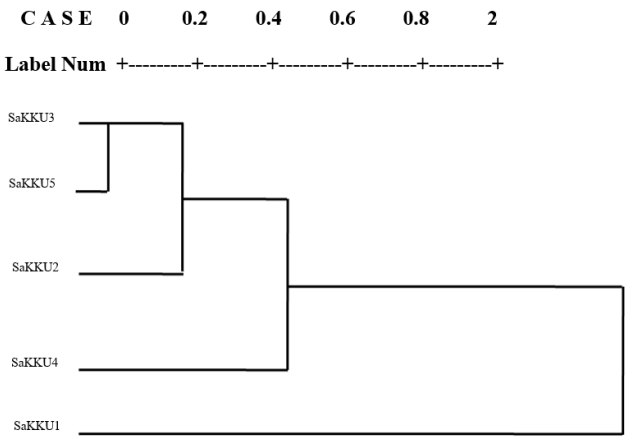

Figure 1. Dendrogram showing grouping of the five ecoraces of eri silkworm (*Samia ricini*)

4. Conclusions and Discussion

Our results on morphological study clearly indicated that the 5th instar larva of ecorace SaKKU1 showed more white powder covering larva body, with distinct yellow color than other ecoraces. These characters were supported by the ISSR data, which exhibited the outgroup of SaKKU1 with the farthest distance from SaKKU3 and other ecoraces. Five ecoraces have been also studied for their genetic relationships using simple sequence repeat (SSR) by our previous study (Sirimungkararat *et al*., 2013a) using primer SCO 1293 and could be characterized by the genetic difference of SaKKU1 among other ecoraces. For non-mulberry silkworms, molecular techniques have been used to identify differences in populations, such as RAPD for eri silkworm (Zuo *et al*., 2001; Shivashankar *et al*., 2013) and RFLP (restriction fragment length polymorphism) for the wild tasar silkmoth (Mahendran *et al*., 2006). Moreover, in the present study the ISSR technique was reliable due to specific primer of repeat bases.

In general, the objective of variety improvement in sericulture is to develop the pure variety or hybrid variety to withstand environmental conditions and to provide highest yields in both quality and quantity. The present study focuses on the screening of the best ecorace of eri silkworm for improvement against high temperature and other unfavorable environment. Therefore, the selection of ecorace is necessary to conduct to achieve the most suitable ecorace. Our result indicated that the ecorace SaKKU1 is the best ecorace, showing the highest survival rates (larval stage 100%, larva – adult 88.89%), the heaviest larva weight of 2nd – 5th instar and of 5th instar day 5. Both cocoon yields and eggs yield were statistically significant among other ecoraces (P<0.05). It is possible that the ecorace SaKKU1 responded to the food plant (castor cultivar TCO 101) better than other ecoraces. Among ecoraces the response may depend on different food plants. However, feeding one ecorace eri silkworm with the same plant but not the same variety resulted in different growth and yield expression (Sirimungkararat *et al*., 2013b; Sirimungkararat *et al.,* 2012). Whereas, Chandrashekhar *et al*. (2013) revealed that different varieties of castor had varying nutritional properties. Besides, environmental factors such as temperature, light, humidity and larval density in the rearing containers can affect the life cycle of eri

silkworm (Hans and Sundaramoorthy, 2003). In addition, Kalpana and Reddy (1988) reported that the yield of mulberry silkworm was less dependent on genetic potential of each line than on rearing environments.

Analysis of genetic difference of eri ecoraces by using ISSR exhibited that the SaKKU3 and SaKKU5 were closely related. Obviously, the SaKKU1 was the most different from other ecoraces, especially from the SaKKU3. Although the yields obtained from all ecoraces are similar, the SaKKU1 showed the best performance and potential to be subjected in the variety improvement program. The ISSR technique can be characterized on different genetic information, which correlated to the morphological characterization e.g. the SaKKU1 5th instar larva had color and cuticle powder different to other ecoraces, and also the genetic divergence was the most distinct. Besides the ISSR, the SSR (Simple sequence repeat or microsatellite) could be also applied in eri silkworm ecorace for population characterization by using SCO 1293 primer, the SaKKU1 and SaKKU4 had no inter-population polymorphism, whereas SaKKU5 had the most polymorphism (Sirimungkararat *et al*., 2013a). However, other technique, e.g. RAPD (Random amplified polymorphic DNA) for genetic characterization of silkworm (Zuo *et al*., 2001; Shivashankar *et al*., 2013) including eri silkworm, mulberry silkworm and oak silkworm (*Antheraea pernyi*) (Liu *et al*., 2010) and the RFLP (restriction fragment length polymorphism) were also applied in the genetic characterization study. Based on our result, the SaKKU1 ecorace is the most appropriate to be used in the variety improvement program.

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