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Identification of molecular markers (SSR) associated with thermo tolerance in silkworm *Bombyx mori*

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Introduction

- In India about 90% of the silk comes from polyvoltine x bivoltine hybrids due to prevailing of tropical climate conditions.
- To increase the bivoltine silk production in India, there is an urgent need for the development of thermo-tolerant bivoltine silkworm breeds/ hybrids, which can be reared throughout the year.
- Hence the situation warrants the need to reorient the breeding approaches using molecular biological tools on the available genetic stocks to supplement the conventional practices adopted in the breeding programmes.
- DNA-based molecular markers hold great promise in breeding for improvement of complex traits such as thermotolerance.

- **Simple Sequence Repeats (SSRs) show high reproducibility, genomic covering, co-dominance, neutrality and are highly polymorphic (Spooner *et al*., 2005) .**
- **SSR have been extensively used to study genetic variability in different organisms, mapping and tagging of genes or QTL (Quantitative Trait Loci) controlling economical /agronomical and disease resistance traits etc., (Witcombe and Hash, 2000; Asins, 2002; Liu et al., 2004; Zhang et al., 2005).**
- **A molecular marker very closely linked to the target gene can act as a tag, which can be used for indirect selection of the gene(s) in a breeding programme.**

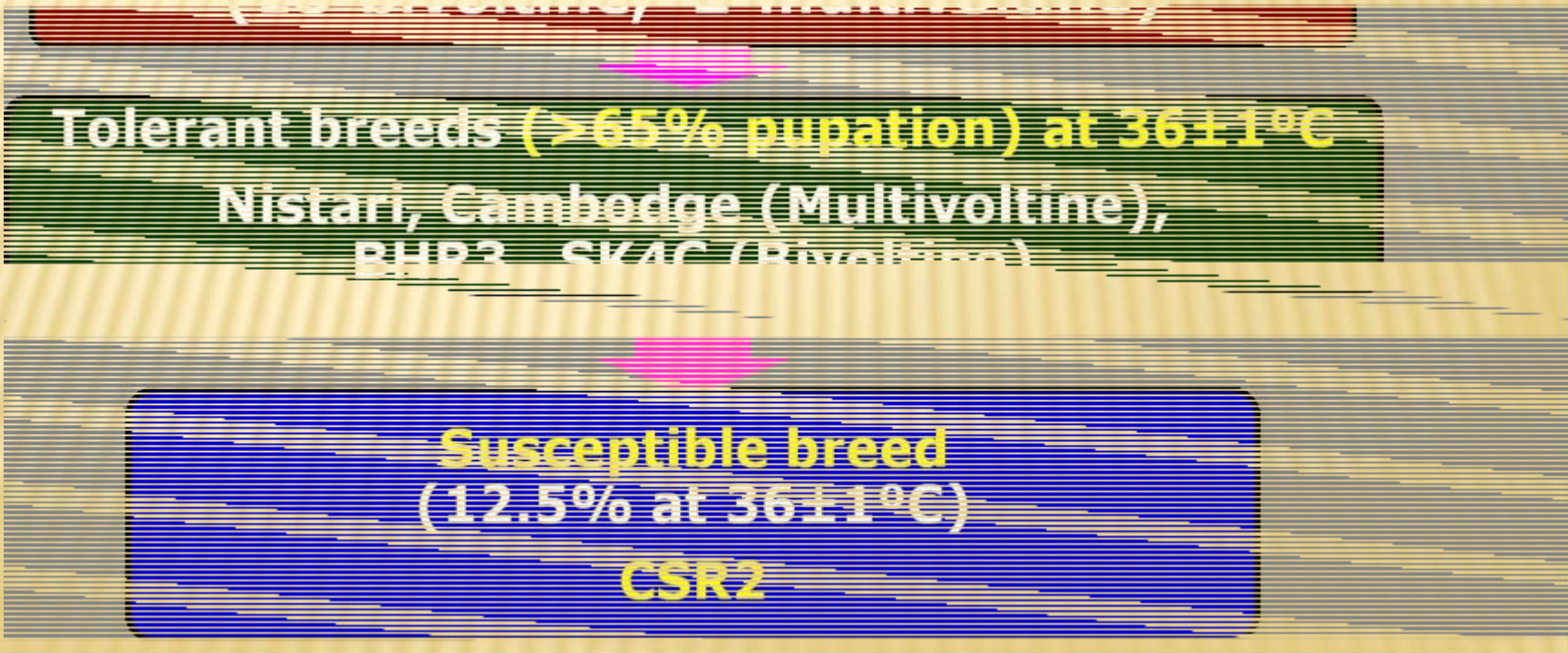
- Recently Zhao *et al* (2010) mapped thermo tolerant gene KN and reported that it is present in 8th linkage group using Dong34, a thermo tolerant strain, and Ou17, a highly susceptible strain and identified five SSR markers linked to thermo tolerance silkworm.

- The present study was undertaken to validate these SSR markers in different genetic backgrounds and also to identify other SSR markers if any, associated with thermo-tolerance in silkworm, which will be further used to develop thermo-tolerant silkworm breeds by employing MAS.

Materials and Methods

a)Evaluation of thermo tolerance

Screening of silkworm breeds at high temperature (32,34,36,38±1ºC & RH:85 ± 5%) (5th instar , 3rd day to till spinning for 6 hrs)



b)DNA extraction & PCR amplification

- **DNA extraction and PCR amplification was carried out by following the standard protocol suggested by Nagaraja and Nagaraju (1995) and Nagarju *et al* (2001).**

c)Sample collection (tolerant and susceptible progeny)

- **The larvae of four F2 combinations (BHR3 x CSR2, SK4C x CSR2, Nistari x CSR2 and Cambodge x CSR2) prepared from the tolerant and susceptible parental breeds were exposed to high temperature (36±1ºC) for six hours daily from third day, 5th instar till spinning.**

- **The larvae which were about to die were considered as susceptible and the larvae, which spun, and subsequently metamorphsed into**

d)Bulked segregant analysis & Confirmation of polymorphic markers

- **Bulked segregant analyses (BSA) was used in conjunction with SSR analysis (Michelmore *et al*., 1991).**
- **Tolerant and sensitive bulks were prepared from F_2 individuals by pooling aliquots, containing equivalent amounts of total DNA, approximately, 50 ng/µl from each of twenty sensitive and twenty tolerant F_2 progeny.**
- **To confirm the result obtained through BSA, 10 individuals each of tolerant and susceptible from each combination were run with each marker.**
- **The amplified products were resolved on 3% gels of Metaphor agarose as well as in PAGE by silver staining as per the requirement for the resolving of the bands.**

e)Statiscal analysis:

One way ANOVA and Regression and stepwise regression analysis carried out

d)Linkage map construction

- Hundred individuals of F2 progenies of Nistari x CSR2 combination were used for linkage map construction.
- MAPMAKER/EXP 3.0 was used to make the genetic map.
- Map distances were calculated with Kosambi function by considering two point linkage analyses (Lander et al., 1987; Lincoln et al., 1992).

Results

83 primers (Different linkage groups)

11 are polymorphic (CSR2, SK4C, BHR3, Nistari, Cambodge)

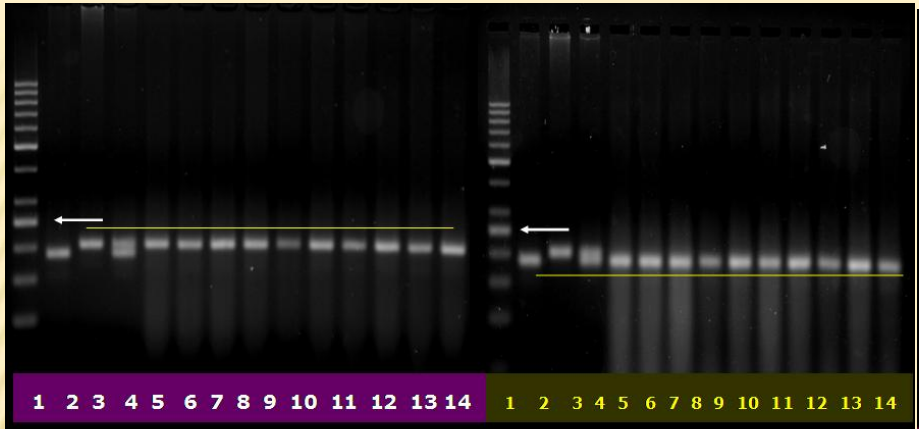
5 are polymophic (in tolerant & susceptible progeny)

3 shown 4 genetic background (LFL0329,LFL1123, SO813) (BHR3 x CSR2, SK4C x CSR2, Nistari x CSR2, Cambodge x CSR2)

2 in 3 genetic background (LFL0407-not in BHR3 x CSR2 ; SO801- not in SK4C x CSR2)

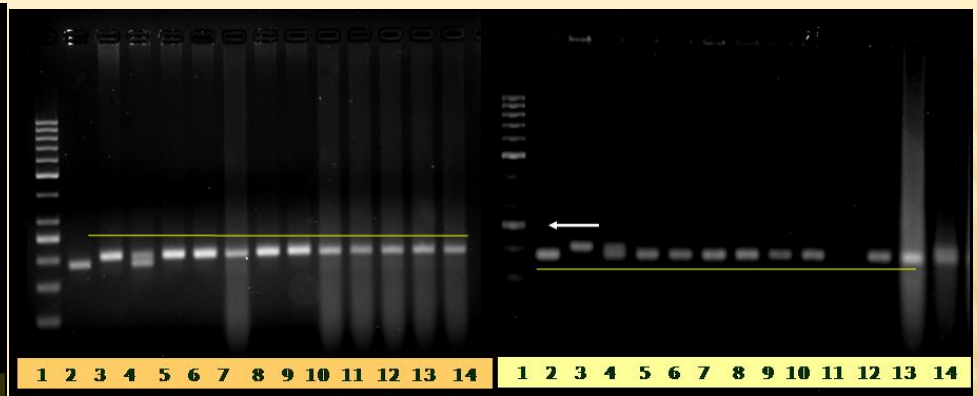
LFL0329: Tole ~190bp: Sus: ~210 bp
LFL1123: Tole ~225bp: Sus:~250 bp
S0813:Tole; ~520bp: Sus:~500bp

LFL0407: Tole ~230bp: Sus: ~225bp
S0801:Tole; ~240bp: Sus:~250bp)



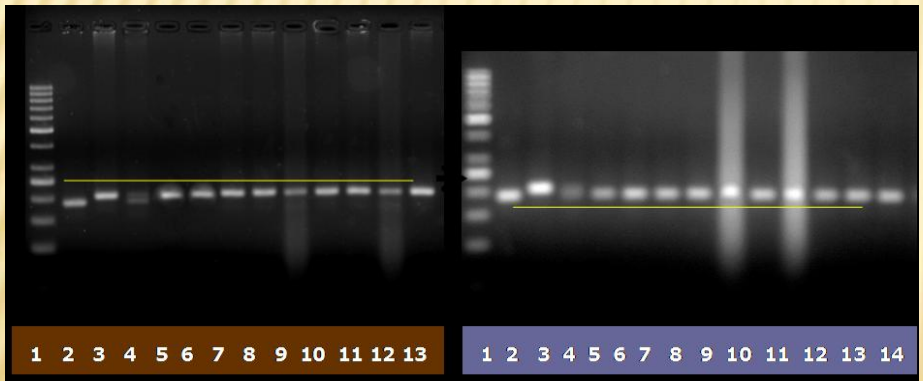
BHR3 x CSR2- F2
1.Marker; 2. BHR3; 3.CSR2;
4: BHR3 x CSR2- F1;
5-14 are susceptible progeny

BHR3 x CSR2- F2
1.Marker; 2. BHR3; 3.CSR2;
4: BHR3 x CSR2- F1;
5-14 are tolerant progeny



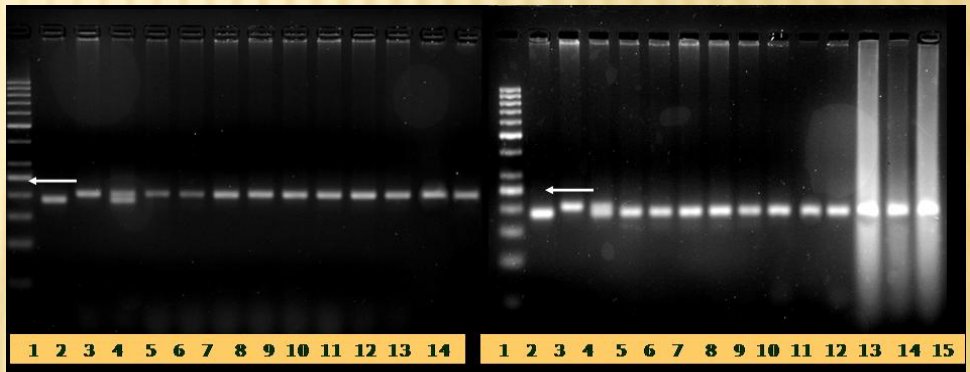
SK4C x CSR2 -F2
1.Marker; 2: SK4C; 3.CSR2;
4: F1-SK4C x CSR2;
5-14 are susceptible progeny

SK4C x CSR2- F2
1.Marker; 2: SK4C; 3.CSR2;
4: F1-SK4C x CSR2;
5-14 are tolerant progeny



Nistari x CSR2- F2
1. Marker; 2.Nistari; 3. CSR2;
4: N x CSR2-F1;
5-13 are susceptible progeny

Nistari x CSR2- F2
1. Marker; 2.Nistari; 3. CSR2;
4: N x CSR2-F1;
5-14 are tolerant progeny

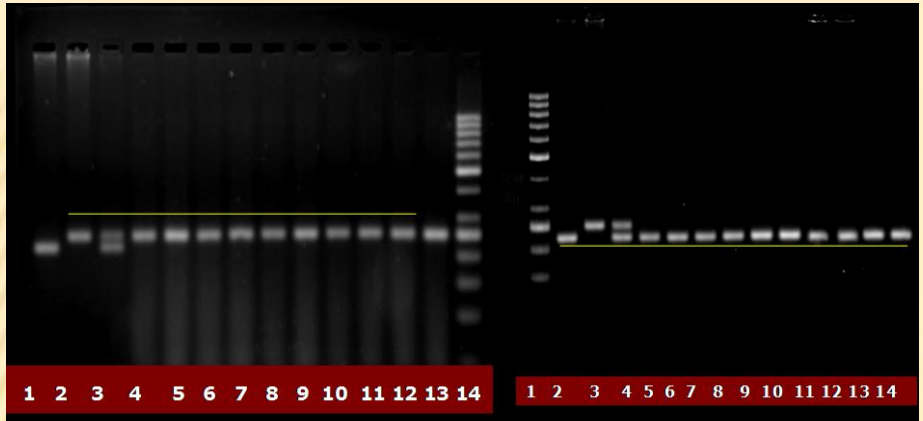


Cambodge x CSR2 F2
1.Marker; 2. Cambodge;3. CSR2;
4: F1- Cambodge x CSR2;
5-14 are susceptible progeny

Cambodge x CSR2-F2
1.Marker; 2. Cambodge;3. CSR2;
4: F1-Cambodge x CSR2;
5-15 are tolerant progeny

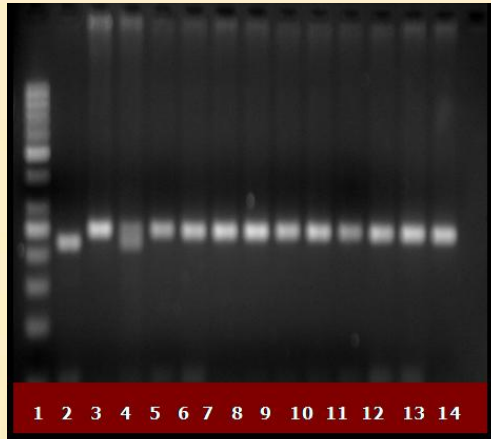
Amplification of SSR primer LFL0329

(Tolerant: ~190bp: Susceptible: ~210 bp)

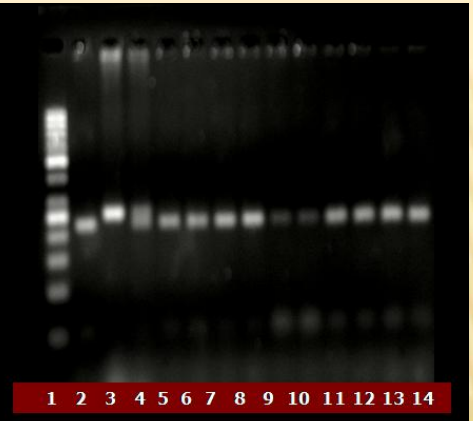


SK4C x CSR2 -F2
1. SK4C; 2. CSR2;
3: F1-SK4C x CSR2;
4-13 are susceptible progeny
14. Marker

SK4C x CSR2- F2
1.Marker; 2: SK4C;
3. CSR2;
4: F1-SK4C x CSR2;
5-14 are tolerant progeny



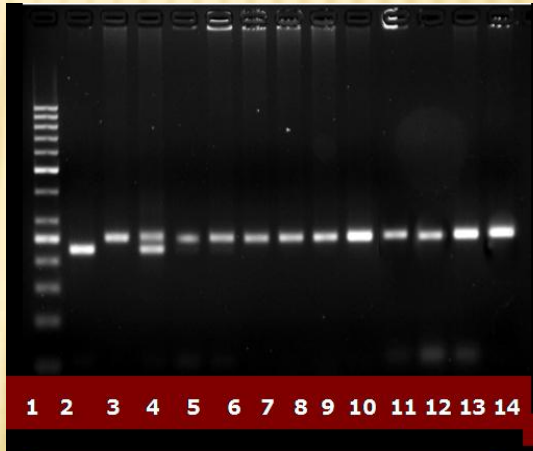
BHR3 x CSR2-F2
1.Marker; 2: BHR3; 3. CSR2;
4: F1 -BHR3 x CSR2;
5-14 are susceptible progeny



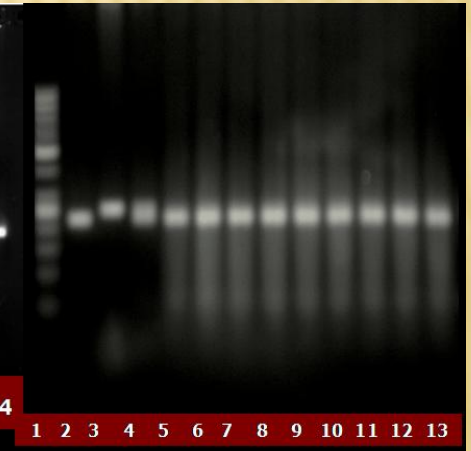
BHR3 x CSR2-F2
1.Marker; 2: BHR3; 3.CSR2;
4: F1-BHR3 x CSR2;
5-14 are tolerant progeny

Nistari x CSR2- F2
1. Nistari; 2. CSR2;
3: F1-N x CSR2;
4-13 are susceptible progeny

Nistari x CSR2 F2
1:Marker; 2. Nistari;
3. CSR2; 4: F1-N x CSR2;
5-14 are tolerant progeny



Cambodge x CSR2 F2
1.Marker; 2. Cambodge; 3. CSR2;
4: F1- Cambodge x CSR2;
5-14 are susceptible progeny

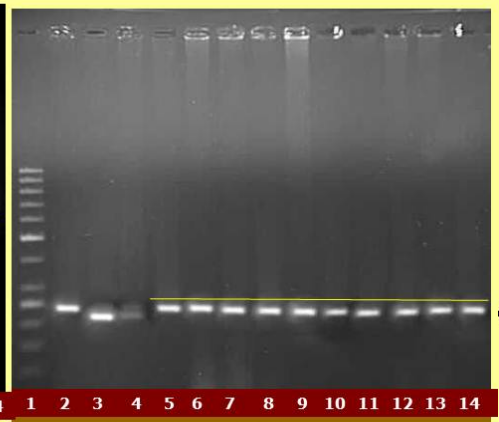


Cambodge x CSR2 F2
1.Marker; 2. Cambodge; 3. CSR2;
4: F1- Cambodge x CSR2;
5-13 are tolerant progeny

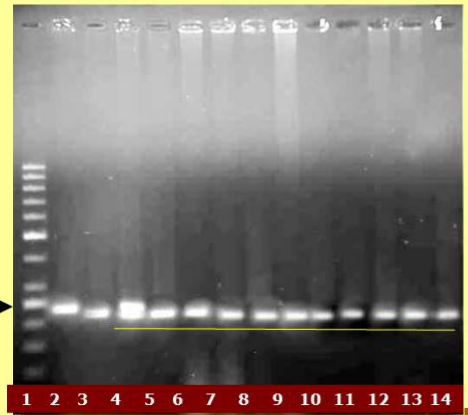
Amplification of SSR primer LFL1123

(Tolerant: ~225bp: Susceptible:~250 bp)

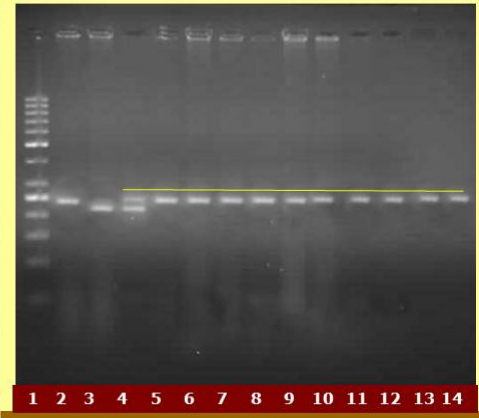
Amplification of SSR primer no. LFL407 in Nistari x CSR2 F2
1.Marker; 2. Nistari;
3. CSR2; 4: F1- Nistari x CSR2; 5-14 are susceptible progenies



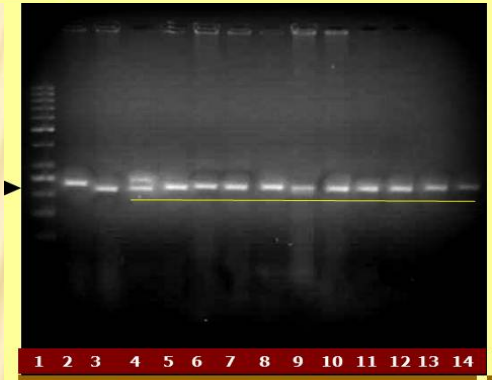
Amplification of SSR primer no. LFL407 in Nistari x CSR2 F2
1.Marker; 2. Nistari;
3. CSR2; 4: F1- Nistari x CSR2; 5-14 are tolerant progenies



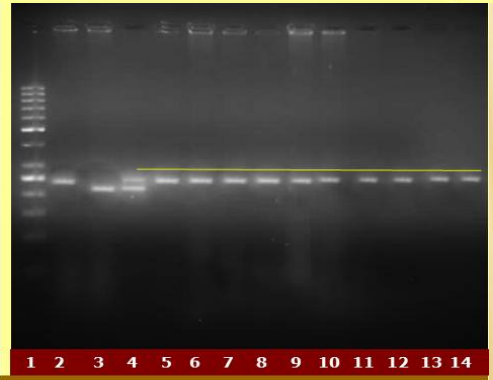
Amplification of SSR primer no. LFL407 in Cambodge x CSR2 F2
1.Marker; 2. Cambodge;
3. CSR2; 4: F1- Cambodge x CSR2; 5-14 are susceptible progenies



Amplification of SSR primer no. LFL407 in Cambodge x CSR2 F2
1.Marker; 2. Cambodge;
3. CSR2; 4: F1- Cambodge x CSR2; 5-14 are tolerant progenies



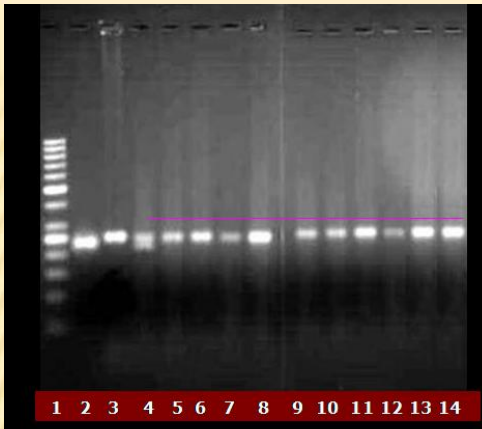
Amplification of SSR primer no. LFL407 in SK4C x CSR2 F2
1.Marker; 2. SK4C;
3. CSR2; 4: F1- SK4C x CSR2; 5-14 are susceptible progenies



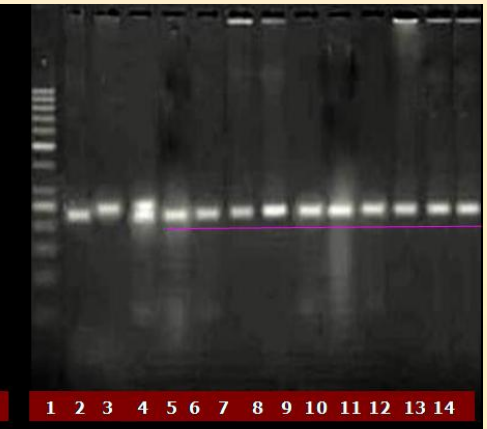
Amplification of SSR primer no. LFL407 in SK4C x CSR2 F2
1.Marker; 2. SK4C;
3. CSR2; 4: F1- SK4C x CSR2; 5-14 are tolerant progenies

Amplification of SSR primer LFL0407

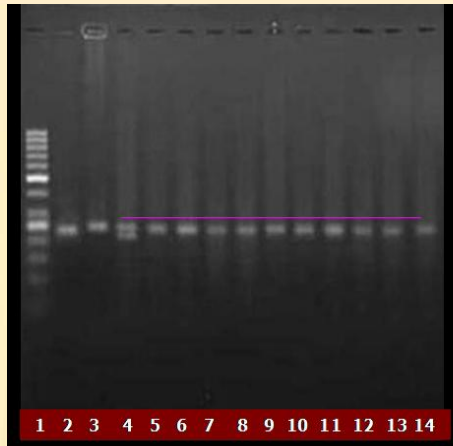
(Tolerant: ~230bp: Susceptible: ~225bp)



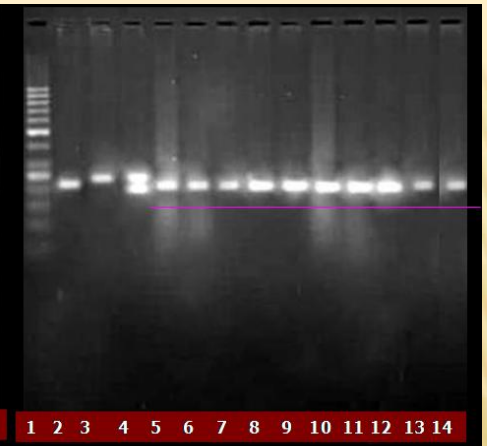
Nistari x CSR2 - F2
1.Marker; 2. Nistari;3. CSR2;
4: F1- Nistari x CSR2;
5-14 are susceptible progeny



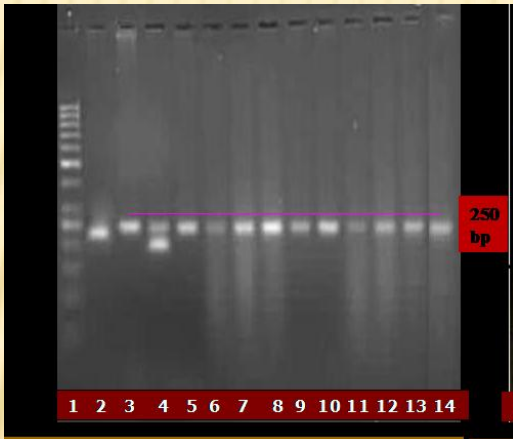
Nistari x CSR2 - F2
1.Marker; 2. Nistari; 3. CSR2;
4: F1- Nistari x CSR2;
5-14 are tolerant progeny



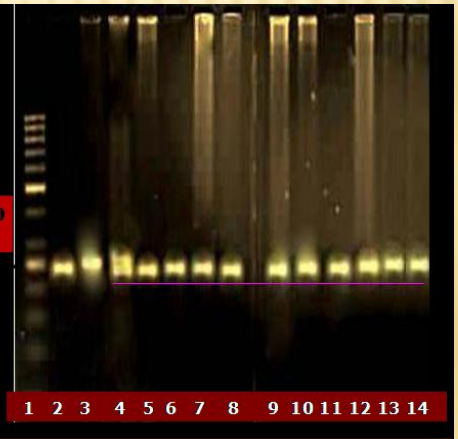
Cambodge x CSR2 - F2
1.Marker; 2.Cambodge; 3. CSR2;
4: F1- Cambodge x CSR2;
5-14 are susceptible progeny



Cambodge x CSR2 - F2
1.Marker; 2. Cambodge; 3. CSR2;
4: F1- Cambodge x CSR2;
5-14 are tolerant progeny



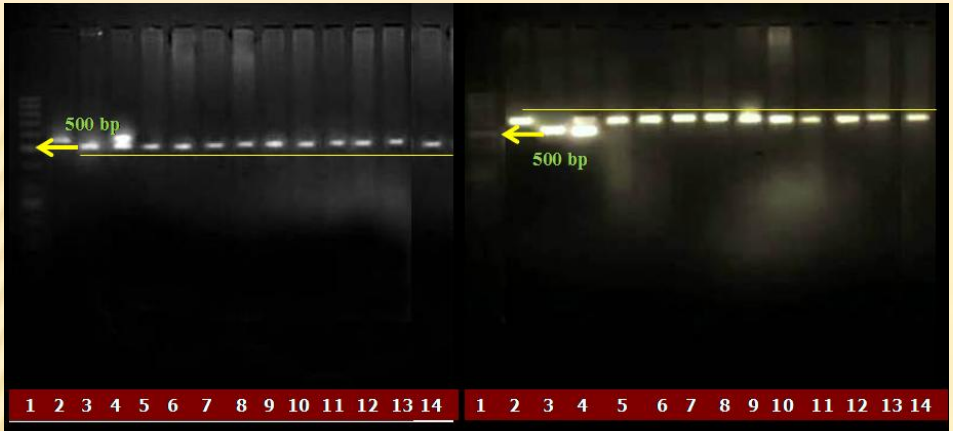
BHR3 x CSR2 - F2
1.Marker; 2. BHR3;
3. CSR2; 4: F1- BHR3 x CSR2;
5-14 are susceptible progeny



BHR3 x CSR2 - F2
1.Marker; 2. BHR3;
3. CSR2; 4: F1- BHR3 x CSR2;
5-14 are tolerant progeny

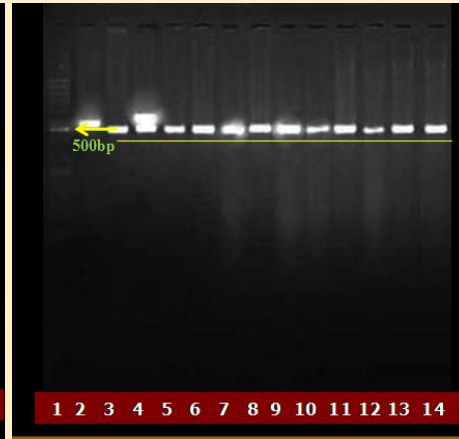
Amplification of SSR primer S0801

(Tolerant: ~230bp: Susceptible: ~225bp)



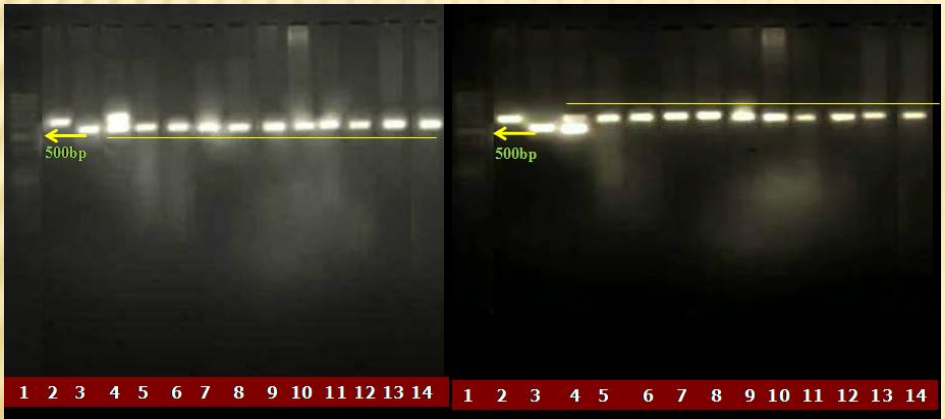
BHR3 x CSR2- F2
1.Marker; 2. BHR3;
3. CSR2; 4: F1- BHR3 x CSR2;
5-14 are susceptible progeny

BHR3 x CSR2-F2
1.Marker; 2. BHR3;
3. CSR2; 4: F1- BHR3 x CSR2;
5-14 are tolerant progeny



SK4C x CSR2 - F2
1.Marker; 2. SK4C; 3. CSR2;
4: F1- SK4C x CSR2;
5-14 are susceptible progeny

SK4C x CSR2- F2
1.Marker; 2. SK4C; 3. CSR2;
4: F1- SK4C x CSR2;
5-14 are tolerant progeny



Nistari x CSR2 - F2
1.Marker; 2. Nistari;3. CSR2;
4: F1- Nistari x CSR2;
5-14 are susceptible progeny

Nistari x CSR2 - F2
1.Marker; 2. Nistari; 3. CSR2;
4: F1- Nistari x CSR2;
5-14 are tolerant progeny

Cambodge x CSR2 - F2
1.Marker; 2. Cambodge; 3.
CSR2; 4: F1- Cambodge x CSR2;
5-14 are susceptible progeny

Cambodge x CSR2 -F2
1.Marker; 2. Cambodge; 3. CSR2;
4: F1- Cambodge x CSR2;
5-14 are tolerant progeny

Amplification of SSR primer S0813

(Tolerant: ~520bp; : Susceptible:~500bp)

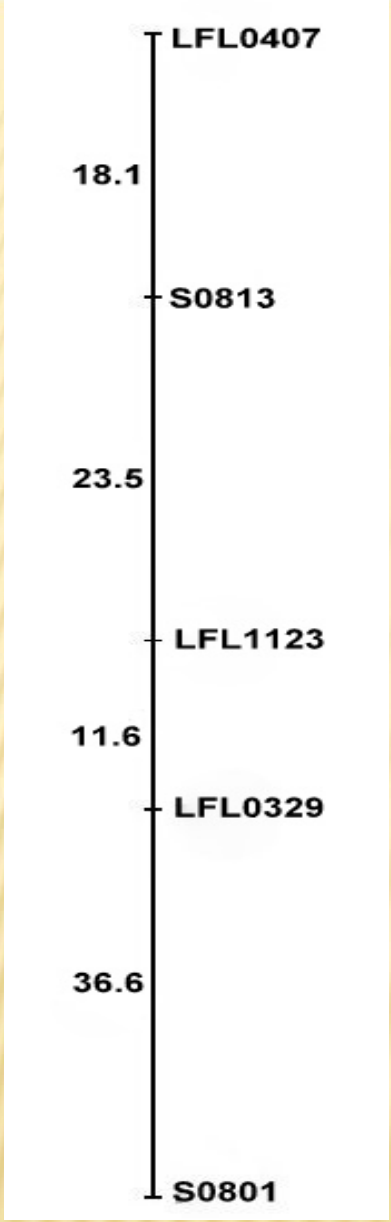
Analysis of variance and regression analysis between markers

Primer	Pearson correlation coefficient	R^2 (%)	F-value	Significance (P-value)
LFL1123	0.467	21.8	16.148	0.0001
LFL0329	0.575	33.0	28.604	0.0001
LFL0407	0.259	6.7	4.167	0.046
S0801	0.259	6.7	4.158	0.046
S0813	0.468	21.9	16.240	0.0001

Stepwise Regression analysis

Primer	R^2 (%)	Significance (P-value)
LFL0329	33	0.000
Excluded variables		
LFL1123	-	0.442
S0801	-	0.972
LFL0407	-	0.454
SO813	-	0.399

- Mapmaker analysis indicated that LFL0329 and LFL1123 were closely linked with a distance of 11.6cM.
- LFL1123 were SO813 were linked with a distance of 23.5cM



Map distance between markers (in cM)

From	To	Distance in Centi morgans
LFL407	S0813	18.1
S0813	LFL1123	23.5
LFL1123	LFL0329	11.6
LFL0329	S0801	26.6
LFL407	S0801	79.7

Linkage map of 5 SSR markers in the 8th linkage group

Conclusion :

- On surveying the linkage map, it was found that all polymorphic markers identified in this study were found in 8th linkage group.
- Using single genetic background Zhao *et al*.(2010), reported that five markers (LFL1123, LFL0407,LFL0329, LFL0658 and LFL0944) in the 8th linkage group are linked to thermo tolerance gene.
- But in our study we used four different genetic back grounds and found that S0803 and S0813 are polymorphic between the tolerant and susceptible bulk which was not reported by Zhao *et al* .(2010).
- Out of the above two markers, S0813 has shown similar pattern in all four genetic back ground tested.

- Moreover we also found that LFL0329 was probable marker which is concurrent with results of Zhao *et al* (2010).
- Accordingly using these markers, 20 genotypes were screened and identified
- Two donor parents-(Tolerant: APS110-Oval; SK4C- Dumbbell)
- Four recurrent parents (Sensitive: SK3, CSR27-Oval; CSR26, CSR6-Dumbbell)
- For developing thermo tolerant productive silkworm breeds using marker assisted back crossing.

Thank U

Results:

83 SSR markers were used for assessing variation between the parents.

Of these 83 markers, 11(13.5%) generated polymorphism between the parents.

Out of 11, five SSR markers LFL1123, LFL0329, LFL0407, SO801, and SO8013 were only, generated polymorphic fragments in DNA bulks.

- **Among the five markers which have shown polymorphism in the DNA bulks-**

  - three viz., LFL1123, LFL0329 and SO813-

  have generated polymorphic fragments of ~225 bp, 190 bp and 300 bp respectively in all the (four) genetic backgrounds-

  - which were present only in the tolerant bulk and tolerant parents (Nistari, Cambodge, SK4C, BHR3) and were absent in the susceptible bulk and susceptible parent(CSR2).

- **Similarly ~250bp, 210bp and 225bp was present only in susceptible bulk and sensitive parent (CSR2).**

- **LFL407, generated polymorphic fragment only in three genetic backgrounds i.e., SK4C x CSR2, Nistari x CSR2 and Cambodge x CSR2 not in BHR3 x CSR2 (~230 bp in tolerant; ~225 bp in susceptible).**
- **In the same way SO801 generated polymorphic fragment only in three genetic backgrounds i.e., BHR3 x CSR2, Nistari x CSR2 and Cambodge x CSR2 not in SK4C x CSR2 (~240 bp in tolerant; ~250 bp in susceptible).**
- **To confirm the result obtained through BSA, 10 individuals each of tolerant and susceptible from each combination were run with each marker which showed identical profile as observed by BSA.**