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Phylogenetic study of Lac Insects of *Kerria* spp. using intron length polymorphism (EPIC-PCR)

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Abstract
Lac insects belonging to *Kerria* species are the most commonly used species for commercial lac production. They are also harnessed for the production of lac dye and wax. Using five Exon Primed Intron Crossing (EPIC) PCR primers for *Kerria* spp., we studied the intra- and interspecific variation among a population of forty eight lac insect lines. The study separated *K. chinensis* from rest of the lines and also made differentiation between the infrasubspecific forms of K. lacca *i.e.*, kusmi and rangeeni.

Keywords: lac insect, Kerria, EPIC-PCR

1. Introduction
The development of molecular techniques for genetic analysis has led to a great increase in our understanding of insect genetics. Variations or polymorphisms in DNA sequences can be exploited as genetic markers (usually called molecular markers), which are very useful tools for genetic research (e.g. construction of genetic maps, mapping of genes or quantitative trait loci) and breeding (e.g. marker-assisted selection). Intron polymorphisms have been successfully utilized in population genetics surveys [1-3] and gene mapping [4]. Introns are widespread and abundant in eukaryotic genomes [5-6]. For example, introns constitute approximately 11 and 24% of the fruit fly [7] and human [8] genomes, respectively. The non-coding introns are more variable than coding sequences and the intron length polymorphism (ILP) is easy to recognize. Lessa (1992) [1] introduced intron-targeted PCR, in which a non-coding intron was amplified using primers designed from highly conserved exon sequences. This approach, called Exon-Primed Intron-Crossing (EPIC)-PCR by Palumbi and Baker (1994) [9], has been shown to yield substantial variability, mainly from intron length polymorphism and was successfully used in several population genetic surveys [3]. Introns have also been successfully used in species-level studies [10-13]. The advantage of EPIC-PCR is that exon sequences are relatively more conservative and therefore the primers designed in exons may have more extensive applications than those designed in non-coding sequences. EPIC-PCR has several advantages in populations genetic studies: (i) by using primers from heterologous genes, cloning and sequencing of target can be avoided; (ii) cross-species amplification should be easier than when primers are designed in non-coding sequences because exon sequences are more conserved across species; (iii) for the same reason, within species, PCR artifacts such as null alleles are expected to be less frequent. Further advantage of EPIC markers is that having both the exon and intron fragments, it can be useful for examining genetic variation at the intraspecific and interspecific levels simultaneously, a feature that is particularly useful when studying species complexes. It also helps in assessing the orthology of collected sequences [14].
The present investigation involves the study of lac insect phylogeny using EPIC primers. Lac insect belongs to the family Tachardiidae (=Kerriidae), a specialized group in Superfamily Coccoidea (Hemiptera: Sternorrhyncha) which comprises of about 7,500 species under variable number of families (20 and above) [15]. The lac insect thrives on specific plant species and secretes a protective resinous covering from the epidermal glands. Lac insects are widely used and produced in India for resin as well as for dye and wax; intensive research has resulted into the application of lac in areas such as food, cosmetics, pharmaceutics, ethnic jewellery,

varnishes and paints, adhesives, perfumes, food coloring, etc [16-18]. The lac insects are mainly distributed in the tropical and subtropical regions between the latitudes 40° N and 40° S [19] and it confines the production of lac to a few countries in the tropical forest region [18]. Twenty-one species of *Kerria* have been reported so far [20]. Out of the 14 species of *Kerria* reported in India, only a few are exploited for commercial lac production [21]. The lac production in India is derived practically from the Indian lac insect *Kerria lacca* (Kerr), which is represented by two infra-subspecific forms, *viz*., *rangeeni* and *kusmi*, which differ for their host preference, life cycle patterns and quality of resin secreted [19]; and maintain their genetic identity due to their preference of host and asynchronous life cycle pattern restricting crossbreeding under natural conditions. Other minor species are *K. sharda* [22] and *Kerria chinensis*. *Palas* (*Butea monosperma*), *ber* (*Ziziphus mauritiana*) and *kusum* (*Schleichera oleosa*) are the common hosts used for lac production in India [23]. There is a long felt need for the study of phylogenetic study of lac insects. Among the various markers available for this purpose, EPIC markers have been proved useful. Hence the present study was taken up with following objectives (1) to develop EPIC primers for lac insects using the published green pea aphid, *Acyrthosiphon pisum* genome; (2) to survey a set of lac insect lines using the primers; (3) to investigate the relationship between intron size, amplifiability and inter- and intra-specific genetic distance of target insects in order to predict how distantly related two insects of same species can be.

2. Material and Methods

2.1 Primer designing

For an year long phylogenetic study of lac insects using EPIC-PCR, intron flanking primers were designed to amplify introns of five genes *viz*. ATP synthase-alpha subunit (eUN1), Cathapsin (CA4), Actin, Myosin (MY2), lw-Rh gene for long wavelength opsin (eAP3) and S7 Ribosomal protein (RP1) coding gene sequences. Four universal EPIC markers for nuclear genes of multicellular organisms were also used for the amplification of introns from ATPSα, Adenine Nucleotide Translocator (ANT), Signal Recognition Particle 54 (SRP54) and Tata Binding Protein (TBP) coding gene sequences [24]. The primers were designed using PRIMER-BLAST, an online primer designing tool from NCBI, where the organism for specificity checking was limited to *A. pisum*, Green pea aphid.

2.2 Insect Materials and DNA Isolation

Mature female insects were collected from lac insect cultures being maintained at the Gene Bank of Indian Institute of Natural Resin and Gum (IINRG), Ranchi, India. The insects of 48 different lines were kept in 100% ethanol for 48 hours at room temperature to dissolve the resinous covering followed by cleaning the insects with sable hair brush under a stereo zoom microscope. The cleaned insects were kept at –80 ºC overnight prior to the genomic DNA isolation. Genomic DNA was isolated using HipurA Insect DNA Purification Spin Kit (Himedia), as per manufacturer's instructions.

2.3 PCR Amplification and Sequencing

The primers were screened against 48 lac insect lines and the amplification was observed only in five primer sets. All the amplified loci obtained from Cathapsin, Myosin, lw-Rh gene for long wavelength opsin, S7 Ribosomal protein and ATPSα were easily resolved by agarose or acrylamide gel electrophoresis and sizes ranged from 100 bp up to 2000 bp.

The PCR reactions were performed in 50 µl reaction mixtures containing 40 ng of template DNA, 1X Taq buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% (v/v) Nonidet P40; Thermo Scientific, USA], 2.0 mM $MgCl_2$ (Thermo Scientific, USA), 0.2 mM of each dNTP mix (Affymetrix USB, Cleveland), 10 picomoles of each primers, 3 units of Taq DNA polymerase (Thermo Scientific, USA). The PCR reactions were carried out with the following cycling conditions. Initial denaturation of template DNA was carried out at 95 ºC for 5 min followed by 35 cycles programmed for denaturation step at 95 ºC for 30 sec, primer annealing step at specific temperature (Table 1) for 30 sec, and DNA extension step at 72 ºC for 1 min. The final extension of the PCR products was carried out at 72 ºC for 10 min.

Table 1: Primers used for the EPIC-PCR against lac insect DNA

Primer ID	Sequences (5'- 3')	Tm (ºC)	Gene amplified	Amplicon length (bp)
CA4_F	TTT TTG TTG TTC TCC CAT GC	42	Cathapsin	250
CA4_R	CGA CTT CAC TTG TAG AAT TA			700 1000 **2000***
MY2_F	GTC CTC TTT GCC CAA AAT GC	50	Myosin	**300***
MY2_R	GCA GCC GTA AAA AGT TCA TCC			350
eAP3_F	GGG TCA CAT CAA TAT GGA CAA	40	lw-Rh gene for long wavelength opsin	100 175 400 **450*** 600 1000
eAP3_R	GTC ATA GGT TTT GCA GAT A			
eUN1_F	GAG CCM ATG CAG ACT GGT ATT AAG GCY GT	54	ATPSα	625 **1200***
eUN1_R	TTG AAN CKC TTC TGG TTG ATG ATG GTG TC			
RP1_F	AAT GGA CTT ATC TTT TTC GC	40	S7 Ribosomal protein	**1100***
RP1_R	GTA GTT ATA CTG AAA AAA GC			2000

*Amplicon size having the most common presence in the majority of insect lines.

Table 2: List of lac insect lines used in the study along with their description

Sl. No.	Institute ID	Place of collection
1.	IINRG-LIK 0001	Korba, Chhattisgarh
2.	IINRG-LIK 0002	Ranchi, Jharkhand
3.	IINRG-LIK 0003	Sundergarh, Orissa
4.	IINRG-LIK 0004	Palamau, Jharkhand
5.	IINRG-LIK 0005	Bokaro, Jharkhand
6.	IINRG-LIK 0006	Medinipur, West Bengal
7.	IINRG-LIK 0007	Sarat, Mayurbanj, Orissa
8.	IINRG-LIK 0008	Bangalore, Karnataka
9.	IINRG-LIK 0010	Thrissur, Kerala
10.	IINRG-LIK 0011	Udaipur, Rajasthan
11.	IINRG-LIK 0012	Jhalod, Rajasthan
12.	IINRG-LIK 0013	Ludhiana, Punjab
13.	IINRG-LIK 0014	Jammu, J&K
14.	IINRG-LIK 0015	Banaskantha, Gujarat
15.	IINRG-LIK 0016	Chhotaudepur Gujarat
16.	IINRG-LIK 0017	Ahmednagar, Maharashtra
17.	IINRG-LIK 0018	Aurangabad, Maharashtra
18.	IINRG-LIK 0019	Maharajganj,Uttar Pradesh
19.	IINRG-LIK 0020	Echoda, Andhra Pradesh
20.	IINRG-LIK 0021	Experimental line
21.	IINRG-LIK 0023	Thailand
22.	IINRG-LIK 0024	Experimental line
23.	IINRG-LIK 0025	Ranchi, Jharkhand
24.	IINRG-LIK 0027	Silli, Jharkhand
25.	IINRG-LIK 0028	Bokaro, Jharkhand
26.	IINRG-LIK 0029	Korba, Chhattisgarh
27.	IINRG-LIK 0031	Nangpoh, Meghalaya
28.	IINRG-LIK 0032	Ahmedabad, Gujarat
29.	IINRG-LIK 0037	Ranchi, Jharkhand
30.	IINRG-LIK 0038	Bhopal, Madhya Pradesh
31.	IINRG-LIK 0039	Nawadih
32.	IINRG-LIK 0041	Bandagaon
33.	IINRG-LIK 0042	Selection
34.	IINRG-LIK 0043	Experimental line
35.	IINRG-LIK 0044	Experimental line
36.	IINRG-LIK 0045	Experimental line
37.	IINRG-LIK 0046	Experimental line
38.	IINRG-LIK 0047	Experimental line
39.	IINRG-LIK 0048	Experimental line
40.	IINRG-LIK 0049	Experimental line
41.	IINRG-LIK 0059	Dhanbad, Jharkhand
42.	IINRG-LIK 0060	Purulia, West Bengal
43.	IINRG-LIK 0061	Bankura, West Bengal
44.	IINRG-LIK 0062	Medinipur, West Bengal
45.	IINRG-LIK 0063	Patiala, Punjab
46.	IINRG-LIK 0064	Varanasi, UP
47.	IINRG-LIK 0065	Bankhedi, MP
48.	IINRG-LIK 0066	Imphal, Manipur

2.4 Binary data analysis

The consistent bands produced from repetition of the EPIC amplification for each primer was considered for scoring through binary method. The clear and unambiguous bands present across the DNA samples from forty-eight *Kerria* lines at particular locus (based on size) were scored as 1 while for absence or very faint were scored as 0 to generate a binary matrix, which was used for all the analysis. In order to study the genetic relationships among the *Kerria* lines, the scored binary matrix was analyzed using the software program DARwin 5. The scores were used for computing dissimilarity based on "presence/absence" dissimilarity index using Jaccard's coefficient, and the dendrogram was generated using neighbor-joining method.

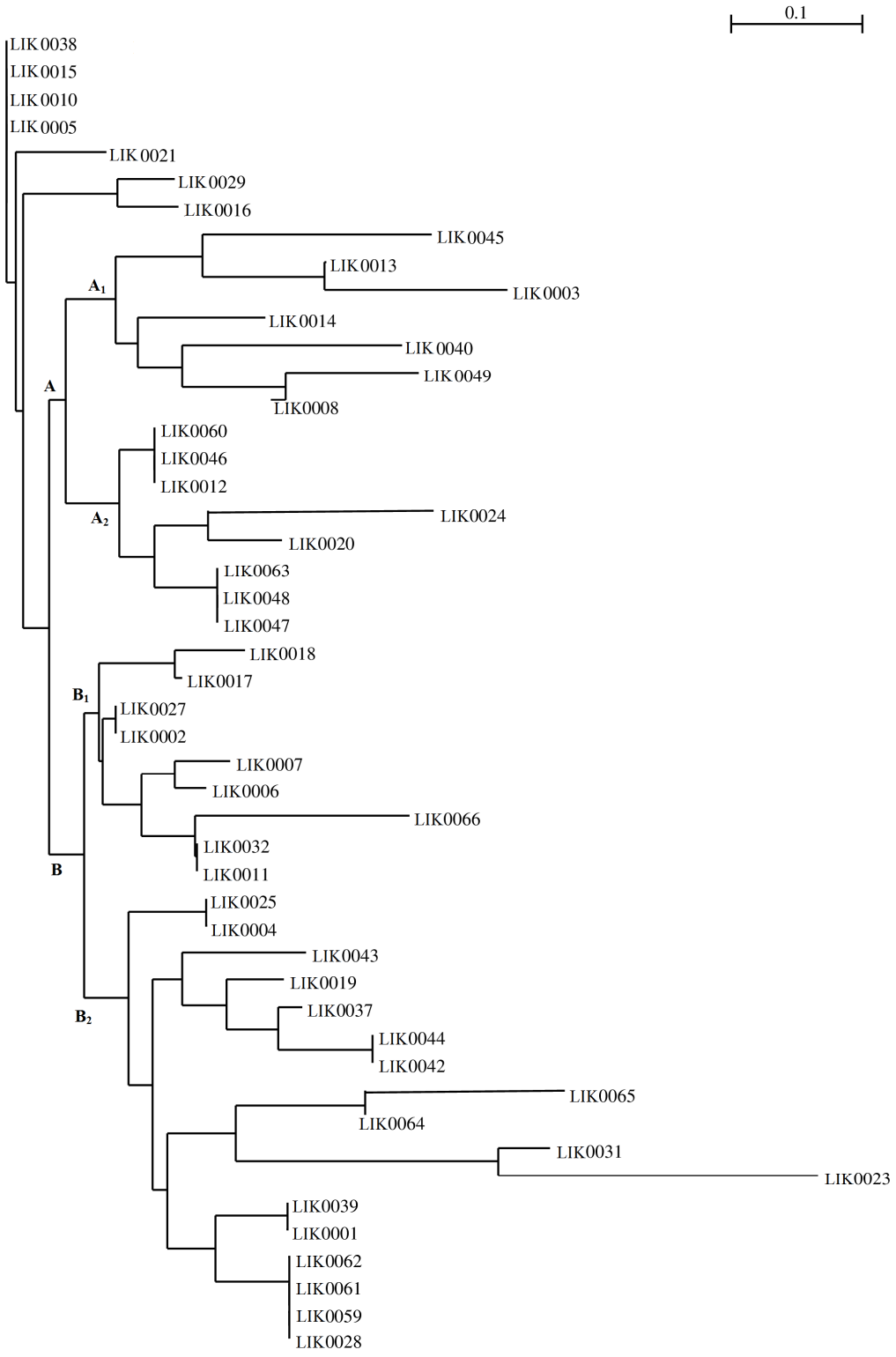

Fig 1: Dendrogram of 48 lac insect lines showing phylogenetic relationship generated from Jaccard's similarity coefficients based on EPIC PCR data, using neighbor-joining method.

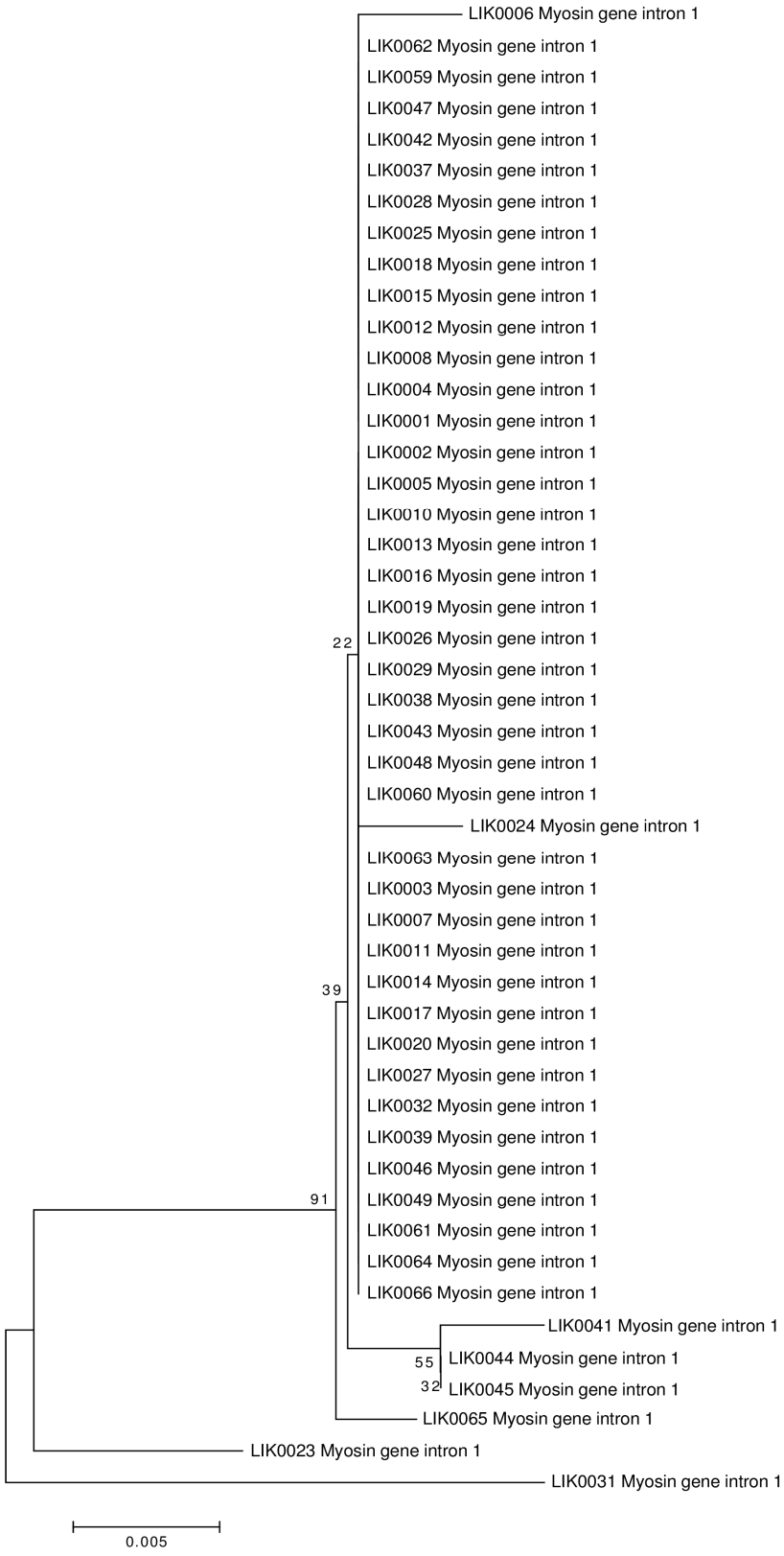

Fig 2: The evolutionary tree of 48 lac insect lines showing phylogenetic relationship generated using the Neighbor-Joining method (Saitou and Nei 1987). The analysis involved 48 nucleotide sequences from intron region of myosin gene.

3. Results and Discussion

The Jaccard's similarity matrix using neighbour-joining method generated a dendrogram (Fig. 1) which resolves the insect lines into two major clusters (nodes A and B) and outgroups comprising seven lines under three branches. The cluster originating from node A comprises of 15 lines, which

includes mainly the *rangeeni* forms of *K. lacca*. This cluster is further differentiated into two subclusters (A_1 and A_2). The subcluster A1 is heterogeneous group of seven lines, comprising of two cultivated *rangeeni* lines (LIK0045 and LIK0049), two *kusmi* lines (LIK0003 and LIK0041) and three lines of cultivated populations from Punjab, Jammu and Karnataka. The subcluster A2 comprises of eight lines, which includes four lines of cultivated populations from West Bengal, Rajasthan, Punjab and Andhra Pradesh (LIK0060, LIK0012, LIK0063 and LIK0020), rest of the lines are cross bred lines.

The node B comprises of twenty six lines, which include two *K. chinensis* lines along with five *kusmi* and other identified *rangeeni* forms of *K. lacca*. The subcluster B_1 comprises of nine lines which are the cultivated lines of Maharashtra, Jharkhand, Orissa, West Bengal, Gujarat and Rajasthan. The subcluster B_2 is a major branch with seventeen lines, which includes the *rangeeni* forms, five lines from Jharkhand (LIK0025, LIK0004, LIK0037, LIK0059 and LIK0028), two lines from West Bengal (LIK0061 and LIK0062), two from Uttar Pradesh (LIK0019 and LIK0064), it also includes two inbred lines (LIK0043 and LIK0044) and a line from Bankhedi, Madhya Pradesh. The three *kusmi* forms (LIK0042, LIK0039 and LIK 0001) and two *Kerria chinensis* lines from Meghalaya and Thailand (LIK0031 and LIK0023) can also be observed in this subcluster. The remaining seven lines form outgroup, which is dominated by the *rangeeni* infra-subspecific form of *K. lacca*.

The evolutionary history involving 48 intronic sequences of myosin gene was inferred using the Neighbor-Joining method [25]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches in Fig 2 [26]. The evolutionary distances were computed using the Maximum Composite Likelihood method [27] and are in the units of the number of base substitutions per site. Evolutionary analyses conducted in MEGA5 [28] separated *K. chinensis* (LIK0031 and LIK0023) from rest of the lines; no significant sequence variation was observed among other strains of *K. lacca*.

Study of inter and intraspecific variation of indigenous lac insect races to determine the genetic variability of lac insect is essential in order to understand, document and harness the biodiversity of such an economically important insect for lac production in India. Inter- and intra-specific genetic variation using RAPD markers have been studied previously [29] among the lac insect populations. The present study using EPIC markers supports the status of infra-subspecific forms of *K. lacca* and separates *K. chinensis* from other insect lines, in accordance with the previous findings [29].

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5. References

1. Lessa EP. Rapid surveying of DNA sequence variation in natural populations. Molecular Biology and Evolution 1992; 9:323–330.
2. Côrte-Real HBSM, Dixon DR, Holland PWH. Intron-targeted PCR: a new approach to survey neutral DNA polymorphism in bivalve populations. Marine Biology 1994; 120:407-413.
3. Daguin C, Borsa P. Genetic characterisation of *Mytilus galloprovincialis* Lmk. in North West Africa using nuclear DNA markers. Journal of Experimental Marine Biology and Ecology 1999; 235:55-65.
4. Wydner KS, Sechler JL, Boyd CD *et al.* Use of an intron length polymorphism to localize the tropoelastin gene to chromosome 5 in a region of linkage conservation with human chromosome 7. Genomics 1994; 23:125–131.
5. Hawkins JD. A survey on intron and exon lengths. Nucleic Acids Research 1988; 16:9893–9908.
6. Deutsch M, Long M. Intron–exon structures of eukaryotic model organisms. Nucleic Acids Research 1999; 27:3219–3228.
7. Adams MD, Celniker SE, Holt RA *et al.* The genome sequence of *Drosophila melanogaster*. Science 2000; 287:2185–2195.
8. Venter JC, Adams MC, Myers EW *et al.* The sequence of the human genome, Science 2001; 291:1304–1351.
9. Palumbi SR, Baker CS. Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. Molecular Biology and Evolution 1994; 11:426–435.
10. Chow S, Nakadate M. PCR primers for fish G6PD gene intron and characterization of intron length variation in the albacore *Thunnus alalunga*. Molecular Ecology Notes 2004; 4:391–393.
11. Fujita MK, Engstrom TN, Starkey DE, Shaffer HB. Turtle phylogeny: insights from a novel nuclear intron. Molecular Phylogenetics and Evolution 2004; 31:1031–1040.
12. Berrebi P, Boissin E, Fang F, Cattaneo-Berrebi G. Intron polymorphism (EPIC-PCR) reveals phylogeographic structure of *Zacco platypus* in China: a possible target for aquaculture development. Heredity 2005; 94:589–598.
13. Willows-Munro S, Robinson TJ, Matthee CA. Utility of nuclear DNA intron markers at lower taxonomic levels: phylogenetic resolution among nine *Tragelaphus* spp. Molecular Phylogenetics and Evolution 2005; 35:624–636.
14. Ishikawa G, Yonemaru J, Saito M, Nakamura T. PCR-based landmark unique gene (PLUG) markers effectively assign homoeologous wheat genes to A, B and D genomes. BMC Genomics 2007; 8:135.
15. Dave KN. Lac and the lac insect in the Atharva-veda. International Academy of Indian Culture, Nagpur, 1950.
16. Resh VH, Carde RT. Encyclopedia of Insects, Edn 2, Elsevier, San Diego, 2009.
17. Sarkar PC. Applications of lac- past, present and emerging trends. In: Recent Advances in Lac Culture. (ed. Kumar KK, Ramani R and Sharma KK),. ILRI, Ranchi, India, 2002, 224–230
18. Ramani R, Baboo B, Goswami DN. Lac- an Introduction, Indian Lac Research Institute, Ranchi, 2007.
19. Kapur AP. The lac insect. In: A Monograph on Lac. (ed. Mukhopadhyay B and Muthana MS), Indian Lac Reseach Institute, Ranchi, India, 1962, 59–89.
20. Varshney RK. Revised synoptic catalogue of the lac insects of the world (Hemiptera: Coccoidea: Tachardiidae). Bionotes 2009; 11:6–10.
21. Sharma KK, Ramani R. Genetic variability in lac insects. In *Recent Advances in Lac Culture*. (ed. K.K. Kumar, R. Ramani and K.K. Sharma), ILRI, Ranchi, India, 2002, 22–33.
22. Mishra YD, Sushil SN. A new trivoltine species of *Kerria*

Targioni-Tozzetti (Homoptera: Tachardiidae) on *Scheichera oleosa* (Lour) Oken from Eastern India. Oriental Insects 2000; 34:215–220.
23. Roonwal ML. Lac Hosts. In: A monograph on Lac. (ed. Mukhopadhyay B and Muthana MS), Indian Lac Research Institute, Ranchi, India, 1962, 14–58.
24. Jarman SN, Ward RD, Elliot NG. Oligonucleotide primers for PCR amplification of Coelomate introns. Marine Biotechnology 2002; 4:347–355.
25. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 1987; 4:406–425.
26. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 1985; 39:783–791.
27. Tamura K, Nei M, Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences of the USA, 2004; 101:11030–11035.
28. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 2011; 28:2731–2739.
29. Ranjan SK, Mallick CB, Saha D, Vidyarthi AS, Ramani R. Genetic variation among species, races, forms and inbred lines of lac insects belonging to the genus *Kerria* (Homoptera, Tachardiidae). Genetics and Molecular Biology 2011; 34:511-519.