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ZI Buhroo
Temperate Sericulture Research Institute, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, India

MA Bhat
Department of Studies in Sericulture science, University of Mysore, Mysuru, Karnataka, India

NA Ganai
Directorate of Planning and Monitoring, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar India

AS Kamili
Temperate Sericulture Research Institute, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, India

GK Bali
Department of Zoology, Savitribai phule Pune University, Pune, Maharashtra, India

A Aziz
Department of Sericulture, Poonch campus, University of Jammu, Poonch, India

Correspondence
MA Bhat
Department of Studies in Sericulture science, University of Mysore, Mysuru, Karnataka, India

An efficient protocol for the inter-simple sequence repeat (ISSR) marker approach in population genetic studies

ZI Buhroo, MA Bhat, NA Ganai, AS Kamili, GK Bali and A Aziz

Abstract
Molecular tools opened a new vista to understand nature's bio-diversity and its relevance and the same approach was availed to build-up the foundation work on the bio-diversity. Screening the available biodiversity germplasms and evaluating their genetic diversity could optimize and facilitate the breeding process. ISSRs within a species can be highly variable regions of DNA.. Consequently, this technique has been used for DNA fingerprinting and assessing genetic diversity in germplasm. ISSRs are presumably noncoding loci and are dispersed throughout the genome, and their association with agronomic traits is influenced by the breeder only in regions under selection. The production of large numbers of fragments, the reproducibility, and the low costs are advantages of using ISSR markers. Thus, ISSR could be an unbiased tool for evaluating changes in diversity in agronomically important crops and genetically diverse pant and animal species. Here we devise the protocol for ISSR marker identification in relation to genetic divergence Studies.

Keywords: Approach, efficient, marker, protocol, inter simple sequence

Introduction
Understanding and preserving biodiversity is one of the most important global challenges that biologists are facing. Widespread realization that biodiversity is strongly modified by changes in global environment has generated strategies to conserve and protect biodiversity in many parts of the world. Assessment of the genetic diversity present within a species is a prerequisite for developing a sustainable conservation program. The advent of molecular biological techniques clearly showed the advantages of molecular markers over morphobiochemical markers to analyze population diversity. As the 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. The conservation of genetic diversity both within and among natural populations is a fundamental goal for conservation of biological science. Henceforth, the knowledge utilizing different biotechnological methods are prioritized and also an essential component of plant and animals (as well as insects) resource management and they are becoming increasingly important for the conservation strategies utilizing different markers. However, a DNA molecular marker in essence detects nucleotides sequence variation at a particular location in the genome. The genetic variation/diversity must be found between the parents of the chosen cross for the marker to be informative among their offspring and to allow its pattern of inheritance to be analyzed. DNA markers can generate fingerprints, which are distinctive patterns of DNA fragments resolved by PCR- agarose-electrophoresis and detected by staining or labeling. The advent of the PCR was a breakthrough for molecular marker techniques and made possible many fingerprinting methods. A number of DNA marker systems such as simple sequence repeats (SSR; Kimpton *et al*. 1993; Estoup *et al*. 1993; Reddy *et al*. 1999a; Prasad *et al*. 2005) [12, 9, 19, 17], random amplified polymorphic DNA (RAPD; Williams *et al*. 1990; Nagaraja and Nagaraju 1995; Chatterjee and Pradeep 2003) [24, 15, 2], inter-simple sequence repeats (ISSR; Zietkiewicz *et al*. 1994; Ehtesham *et al*. 1995; Reddy *et al*. 1999b; Chatterjee *et al*. 2004; Kar *et al*. 2005; Pradeep *et al*. 2005) [26, 8, 18, 3, 4, 10, 16], expressed sequence tag (EST; Vlachou *et al*. 1997; Ciolfi *et al*. 2005) [22, 6] and amplified fragment length polymorphism (AFLP; Vos *et al*. 1995; Reineke *et al*. 1998; Katiyar *et al*. 2000) [23, 20, 11] have been used to study the population genetics of different organisms

including insects. However, Inter simple sequence repeats (ISSR) marker system has been extensively used for genetic analysis of plants and animals (Zeitkiewicz *et al*., 1994; Reddy *et al*., 1999b; Vijayan, 2004) [26, 18, 3, 4] as the ISSR-PCR technique permits to screen quickly a wider part of genome without prior knowledge of DNA sequence. Further, the technique of ISSR amplification is sensitive enough to differentiate closely related individuals (Zietkiewicz *et al*., 1994) [26] and assess the genetic diversity in germplasm (Wolfe *et al*., 1998) [25]. ISSRs are presumably noncoding loci and are dispersed throughout the genome. The production of considerable numbers of fragments, their reproducibility, and the low costs are advantages of using ISSR markers. This paper focuses on the development of effective and efficient techniques in dealing with ISSR marker studies in population genetic studies.

2. DNA Extractions

Use a method which will give a good quality of relatively clean DNA. The standard CTAB extraction will likely be sufficient for most groups. Specific groups with high levels of tannins or phenolics may require additional cleaning. Generally fresh tissue is best but frozen and silica-gel dried material can work equally well.

3. Generating ISSR Data

3.1 PCR Master Mix

Make a standard PCR reaction Master Mix with one of the primers, aliquot 25 µl of the Master Mix into individual tubes, put 1-1.5 µl of undiluted DNA sample into each tube (you should do a series of DNA amounts and $MgCl_2$ amounts for a couple of your samples, to determine the optimum concentrations to give you good bands when you start the project), and amplify them.

The constituents are a modification of those originally proposed by Wolfe *et al*. (1998) [25] which allow for "dirtier" DNA extracts in each 25 µl reaction. The following are two slightly different recipes which work for different taxa. We will test both or variations of these with our samples.

Table 1: PCR Constituents for 25 µl ISSR reactions

#	H_2O	buffer	$MgCl_2$*	dNTPs**	BSA^+	$primer^{++}$	Taq
1	19	2.5	2	2	0.5	0.25	0.25
2	38	5	4	4	1	0.5	0.5
3	57	7.5	6	6	1.5	0.75	0.75
4	76	10	8	8	2	1	1

Table 2: PCR Constituents for 25 µl ISSR reactions

#	H_2O	buffer	$MgCl_2$*	dNTPs**	BSA^+	$primer^{++}$	Taq
1	17	3	2.5	4	2.5	0.5	0.1
2	34	6	5	8	5	1	0.2
3	51	9	7.5	12	7.5	1.5	0.3
4	68	12	10	16	10	2	0.4

*Assums $MgCl_2$ concentration at 50m M; with 25mM you'd use 2.5ul and reduce water by 1.25 µl each rxn
**dNTPs--10mM concentration in a mix; includes 40µl of each dNTP plus 240µl water
$^+$BSA (bovine serum albumin)--4mg/ml concentration
$^{++}$primers--20mM concentration.

Test the quantity of DNA in at least a few sets of extracts (corresponding to many individuals in a population) to determine whether the concentration is roughly comparable across extracts. This can be done with a spectrophotometer or by comparison in an agarose gel. If different extracts within and among populations show high variability in DNA concentration, all the extracts should be routinely measured for concentration, and then DNA must be standardized approximately (by dilution, or drying and rehydration in less water) across samples and populations. This isn't always necessary.

It is important to amplify each set of samples and a particular primer twice--giving two amplification replicates. A few bands appear and disappear at random, depending on conditions and the probabilistic nature of PCR. Bands are scored as "present" for a sample and a given primer only where they occur in both replicates, and "absent" where they occur in only one replicate or neither of them. Each fragment scored as "present" is treated as a "dominant" (amplified) band for that locus, while one scored as "absent" is treated as a "recessive" (null) band; note that homozygous dominant and heterozygous genotypes can't be distinguished in diploid individuals. This must be accommodated in statistical formulas in arriving at F-equivalent "phi" statistics.

3.2 Primer sequences

The following is a set of commonly used dinucleotide and trinucleotide primers which work for various plant groups; you could also invent your own primers (e.g., $(CAC)_4GT$). Not all may work for a particular plant group, but over 85% should. Data from 3-4 of the "best" for your group should be sufficient for all purposes. Most of these will give fragments ranging between 750bp and 1750bp in size. The primers below in bold are those which give some amplification for the majority (or all) of the vascular plant groups I have used this technique with; these would be the first primers you should try with your group.

ISSR Primers

Name*	Sequence
814	$(CT)_8TG$
844A	$(CT)_8AC$
844B	$(CT)_8GC$
17898A	$(CA)_6AC$
17898B	$(CA)_6GT$
17899A	$(CA)_6AG$
17899B	$(CA)_6GG$
HB 15	$(GTG)_3GC$

The first seven primers were designed by Wolfe *et al*, 1998 [25] where she incorporated ambiguous nucleotides. These are shown here in a "decomposed" state providing specific primers without ambiguities. The different A and B primers from the decompositions actually amplify considerably different sets of primers, which means they're both useful independently. You should initially test most or all primers with very different repeat unit combinations, with a handful of individuals from different populations or species, to find out which primers give the sharpest bands and the variation best suited to your question. (Even sticking to the repeat units given above, many more primers could be designed and tested by simply changing the two end nucleotides that "anchor" the primer [avoiding lots of As or Ts, however, since these don't provide stable annealing and don't amplify that well]). You would ideally settle on 3-4 primers to use with all the samples you've collected. It is also a good idea to conduct a pilot study of one or more species, using one to three populations of many individuals, to determine empirically the minimum number of individuals you should sample to capture 95% or more of the genetic variation in a given population.

3.3 Thermal cycling

The following amplification program is the general program used for running ISSRs.:

94 ºC for 2 minutes; 35 cycles of: 94 ºC for 30 secs, 44 ºC for 45 secs, 72 ºC for 1 min 30 secs; 72 ºC for 20 min; and 4 ºC hold forever. In certain cases, one may want to change the primary "core" program from 35 to 40 cycles if amplification is weak, perhaps also with a concomitant slight increase in the amount of DNA template used.

3.4 Gel visualization

Run out 5 µl of the 25 µl products on a 1.3% agarose "mini-gel" stained with ethidium bromide included in the agarose, just until the tracking dye has traveled 1/4-1/3 of the gel length, to check for good amplification. Batches of good amplification products can be combined and run (using 10 µl of each product) in 1.5% to 2% agarose on "maxi-gel" rigs to provide the greatest separation. Use a 250 base-pair ladder on both sides and in the middle of your running samples to compare bands across samples on the gel. Ethidium bromide (EtBr) can be included in the gel when run although during long runs the EtBr can run off the end of the gel. For even staining it is best to run the gel and later stain in a solution of EtBr. A large gel should run 2-4 hours depending on the voltage (needs testing for your setup). The gel is then visualized in UV light and digital images made of the gel. Automatic band detection and sizing is best and any standard gel imaging system will be able to do that.

3.5 DNA sequencer visualization

An alternative to gel visualization is using an automatic DNA sequencer to view the ISSR fragments. This is much more costly as it requires the purchase of florescent primers and sequencer supplies, but it does give more exact size determinations. (This would be difficult and overly costly on the equipment we have here at FLC).

3.6 Scoring the data

Data points are the presence/absence of each distinct (not "ghost") band across all samples for the same primer, in both replicate sets of amplifications.

3.7 Statistical analysis

ISSRs are dominant markers thus information on recessive and dominant alleles is lost. The nature of the data prevents its use in common parsimony analyses. You can see this performed occasionally in published works but it technically violates the assumptions of the analysis. Tree building can be performed using UPGMA and Neighbor Joining methods. Other phenetic methods and ordination, most frequently PCoA are also applicable. Standard genetic analyses can be performed if the dominant marker statistic is used. Further analysis of gene sharing and genetic structure can also be performed using Bayesian estimation in the program HICKORY for determining F statistics and the program STRUCTURE for inferring gene pool differentiation. This last method which is particularly good for hybrid studies is used very well in the paper for class discussion.

4. Conclusion

It may be concluded that the present communication can be used as an ideal approach in undertaking ISSR studies based on which a great deal of study can be planned for detailed investigation on the bio-diversity of agriculture crop plants and animal species in India and elsewhere. The ability of ISSR markers to screen many polymorphisms in a single assay makes it suitable technique for large scale screening of diverse germplasm species. Hence, It is suggested that more and detailed researches on ISSR technique needs to be undertaken for further improvement this technique for undertaking population genetic studies.

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