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Full Length Research Paper

Optimization of ISSR marker for African edible-seeded *Cucurbitaceae* species' genetic diversity analysis

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We applied a molecular approach using inter-simple sequence repeat (ISSR) markers on three African edible-seeded cucurbits (*Citrullus lanatus* L. Matsumura and Nakai, *Cucumeropsis mannii* L. Naudin and *Cucumis melo* var. *agrestis* L. Naudin). To obtain clear and reproducible bands on 1.5% agarose gels, we screened 21 ISSR primers and three parameters (annealing temperature, gel tray, and voltage and running time). The resolution of 11 ISSR markers was performed, with optimal annealing temperature (Ta) varying from 50 to 52°C. The best combination to obtain clear and well-distinguished band patterns was 1.5% agarose gel with a 20-lanes tray (6 mm width) at 80 V for 5 h. Applying the 11 ISSR primers on DNA extracted from an accession of *C. lanatus*, 66 bands with 4 to 11 bands per primer was observed.

Key words: ISSR, Cucurbits, genetic diversity, molecular markers, optimization.

INTRODUCTION

As the high loss of global biodiversity continues, it is worth to devote efforts to the conservation of genetic resources, particularly for indigenous crops. The first step in achieving this objective is a better knowledge of the genetic diversity of the target crop. Such analyses allow the identification of most valuable populations on the basis of the allelic richness. One of the two conservation approaches (*in situ* or *ex situ* methods) could then be applied to representative sample of populations in order to conserve a high level of genetic diversity. Biochemical methods such as isoenzyme (Vallejos, 1983) or molecular approaches based on DNA (Evett and Weir, 1998) have been developed several years ago and are extensively used to evaluate genetic diversity in crop species. These methods allowed a better understanding of the genetic variability among plants. Allozymes are good tools for genetic diversity studies, due to the availability of an extensive literature for several crops species (Hamrick and Godt, 1997). More interestingly, allozymes give sometimes concordant results with these from DNA markers (Djè et al., 1999). In spite of this, DNA markers show a high degree of variability, making them a valuable set to understand genetic diversity in species. Consequently, these genetic markers are used in increasing number of recent studies. Several methods are available in molecular markers Studies and scientists can choose a method of particular interest, depending on available materials and objectives. Among the existing molecular approaches, single sequence repeats (SSR) or microsatellites markers tend to be the most variable for discriminating among genotypes (Russel et al., 1997; Djè et al., 2000). Microsatellites are widely represented in the genome. Nevertheless, microsatellites require complex methods to obtain reliable results, making the use of such markers time consuming. Microsatellites have not been widely used to study lesser-known species. Random Amplified Polymorphic DNA (RAPD) is one of the most largely used molecular methods in genetic study and has been applied for lesser-known species. But this method is less reproducible and shows low degree of variability

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Table 1. List of 21 ISSR primers used in optimization process.

S/N	Primers	Tm	Sequences
1	Sola1	50	BDB-$(ACA)_5$
2	Sola2	56	DD-$(CGA)_5$
3	Sola3	58	DBH-$(CGA)_5$
4	Sola4	56	VHV-$(GT)_7$G
5	Sola5	50	DBD$(AC)_7$
6	Sola6	60	BDB-$(CAC)_5$
7	Sola7	52	$(AG)_8$YT
8	Sola8	56	$(GA)_8$YC
9	Sola9	52	$(AC)_8$G
10	Sola10	56	$(AC)_8$YG
11	Sola11	50	GAG-$(CAA)_5$
12	Sola12	58	CTG-$(AG)_8$
13	B2	50	$(AG)_8$T
14	B3	50	$(GA)_8$A
15	B4	52	$(AC)_8$YA
16	B5	50	$(GA)_8$T
17	B10	52	$(AG)_8$G
18	P2	60	DDC-$(CAC)_5$
19	P3	52	$(GT)_8$C
20	P4	56	$(GT)_8$YC
21	CBCT5	52	$(AC)_8$YT

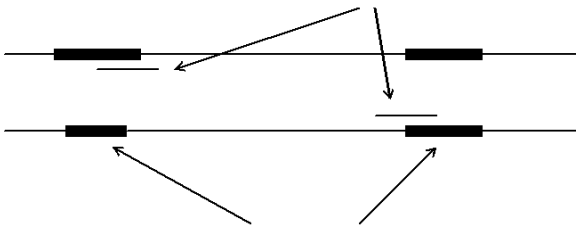

Figure 1. Amplification region of genome targeted by ISSR primers.

than microsatellites. Few years ago, a method combining a wide RAPD markers and high polymorphism and reliability of microsatellite markers was published by Zietwiecki et al. (1994). The method called Inter Simple Sequence Repeat (ISSR) is based on the amplification of DNA region located between two microsatellites locus. In practice, when the primer successfully locates two microsatellite regions within an amplifiable distance away on the DNA strands, the PCR reaction generates a band of a particular size for that locus (Figure 1). ISSR is a dominant marker like RAPD (scored using presence or absence of band at a locus) but with greater robustness in repeatability and extremely high variability. These features make ISSR better than other readily available marker systems in investigating the genetic variation among very closely related individuals and in crop cultivar classification (Fang and Roose, 1997; Nagaoka and Ogihara, 1997).

African edible-seeded cucurbits locally called “pistachio” or “egusi” are popular in several West and Central Africa (Agbagwa and Ndukwu, 2004; Zoro Bi et al., 2003). These species are important source of income for rural people. The cultivation is mainly carried out by women, annually, as minor crops intercropped with staples. According to Zoro Bi et al. (2003), at least five species from these cucurbits are cultivated for seed consumption in Côte d’Ivoire. Some morphological studies are available, but to our knowledge no study concerning the genetic diversity of that species has been undertaken. In the present study, we explored experimental conditions for the use of ISSR in genetic diversity analysis of African edible-seeded cucurbits.

MATERIAL AND METHODS

Plant material

For this study, 11 accessions representing three African cucurbit species (*Citrullus lanatus* L. Matsumura and Nakai, *Cucumeropsis mannii* L. Naudin and *Cucumis melo* var. *agrestis* L. Naudin) were

used. This consists of 5 accessions of *C. lanatus*, (NI 056, NI 098, NI 101, NI 103, and NI 110), 3 accessions of *C. mannii* (NI 089, NI 097, and NI 100), and 3 accessions of *C. melo* (NI 095, NI 102, and NI 104). Seeds of each species were grown in a greenhouse at 25°C and a 16 h day 8 h night photoperiod. We selected one individual plant per accession for DNA extraction, except for accession NI 056 for which 7 plants was sampled.

DNA extraction

DNA was extracted directly in Eppendorf tubes using about 75 to 80 mg of leaves from 21 day-old plants following Murray and Thompson (1980) with few modifications. Leaf tissues were directly grounded in Eppendorf tubes in 500 µL of cethyltrimethylammonium bromide (CTAB) extraction buffer containing 2% CTAB, 2 M NaCl, 2% PVP, 20 mM EDTA pH 8.2, 100 mM Tris-HCl pH 8.0, and 1% β-mercaptoethanol. The crude extract was then heated at 55°C for 1 h. DNA was extracted twice with 500 µL of isoamylalcohol 24:1 (v:v) and centrifuged at 13,000 rpm for 10 min. DNA was then precipitated with 350 µL of isopropanol for 1 h at room temperature and washed twice with 70% ethanol. Finally DNA was dissolved in 20 µL Tris-EDTA buffer (10 mM Tris-Hcl pH 8.0 and 1 mM EDTA pH 8.0) at –20°C. The extracted DNA was quantified on 0.8% agarose gel, with a run time of 2 h at 90V in 1x Tris Borate EDTA (TBE: 40 mM Tris-HCl, 20 mM boric acid, 1 mM EDTA pH 8.0). We used Smart Ladder^{TM} (Eurogentec) as internal size standard for which DNA concentration were correlate with bands intensity.

PCR and electrophoresis

PCR were performed using 10 µL of a solution containing 0.2 µL (0.2 unit per reaction) of Taq DNA polymerase (Eurogentec), 1 µL of PCR buffer (Eurogentec: system II), 0.4 µL of 2 mM dNTPs, 0.16 µL of each ISSR primer, 1 µL of extracted DNA, and 7.24 µL of water. A preliminary screening was carried out, using 21 ISSR primers in order to select those that could amplify the extracted DNA. PCR cycling conditions were 5 min initial denaturation at 95°C, 30 cycles of (30 s at 95°C, 45 s at the annealing temperature (Ta), 2 min elongation at 72°C) and final elongation of 5 min at 95°C). All PCR reactions were performed with thermocycler PTC-100 from Nalgene. The result of each amplification reaction was analyzed on agarose gel (Molecular grade II from Eurogentec) in 1x TBE buffer. The gels were stained by adding 1.4 µL of ethidium bromide (10 mg/ml) in 100 mL of agarose. Amplified fragments were submitted to ultraviolet illuminator (Fisher Scientific Bioblock) and visualised on Video Copy Processor (Model P91E, Fisher Scientific Bioblock). This array of PCR amplification was aimed at the selection of ISSR primers giving few discrete, large bands or smears. ISSR primers giving positive results from PCR trials (few discrete, large band or smears) were then selected to optimize the amplification, using several annealing temperature (Ta) determined on the basis of the values of Tm indicated by the manufacturer (Table 1). Using the optimal Ta value for each ISSR primer selected, we investigated two additional parameters in order to refine the resolution and reproducibility of bands. First, we tested two gel trays: 20 and 26 lanes per gel. Second, we searched the best voltage and running time by analyzing four sets of values: 110 V for 2 h, 100 V for 3 h, 90 V for 4 h, and 80 V for 5 h. All tests were performed twice, and three times if results were conclusive.

RESULTS AND DISCUSSION

Of the 21 ISSR primers screened, 11 gave bands varying from discrete to large. In many cases, stained gels showed smears (Figure 2).

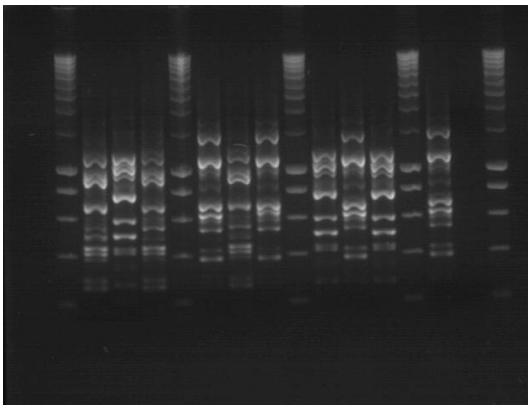

Figure 2. ISSR primer SOLA 2 (DD-(CGA)$_5$) showing irregular and intensive bands on agarose gel.

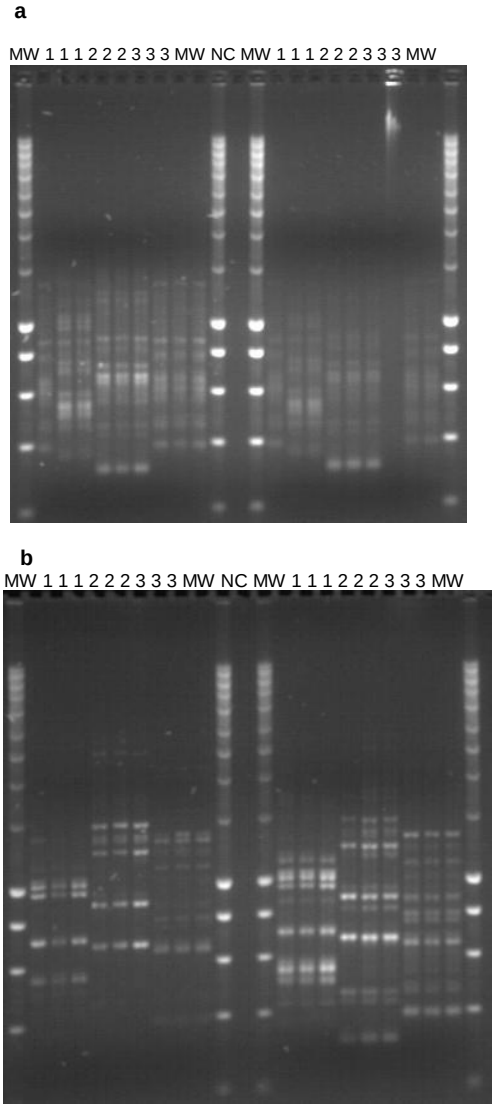

Figure 3. Results of amplification with primer Sola5 at 48 and 49°C (**a**) and at 51 and 52°C (**b**). MW: molecular weight; 1: *Cucumis melo* var. *agrestis*; 2: *Citrullus lanatus*; 3: *Cucumeropsis mannii*; NC: negative control.

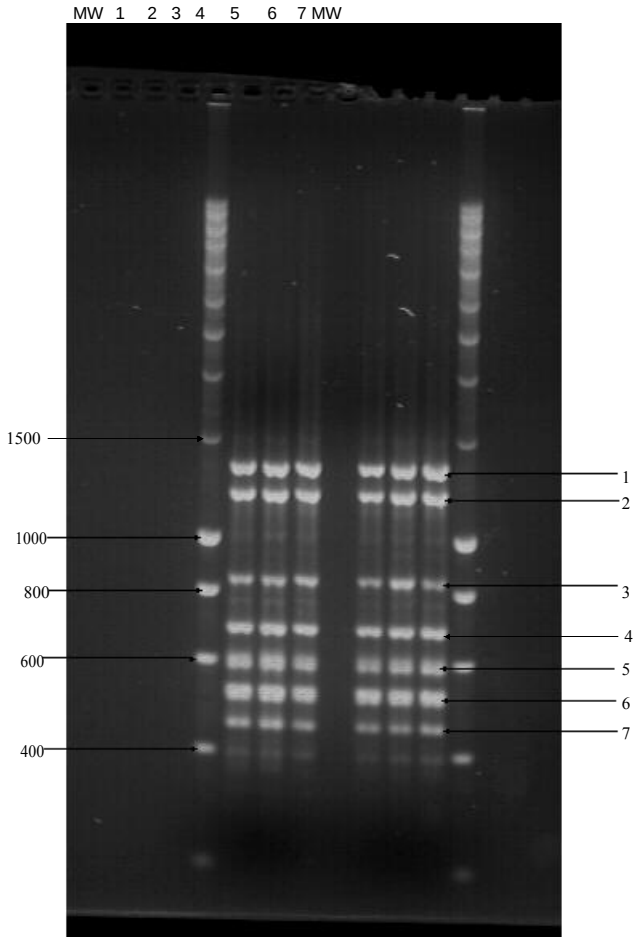

Figure 4. ISSR primer B5 (GA) $_{8T}$ at Ta=52°C run on 1.5% agarose with 26 lanes gel tray. MW=molecular weight; 1-7: individuals of *C. lanatus* (accession NI056).

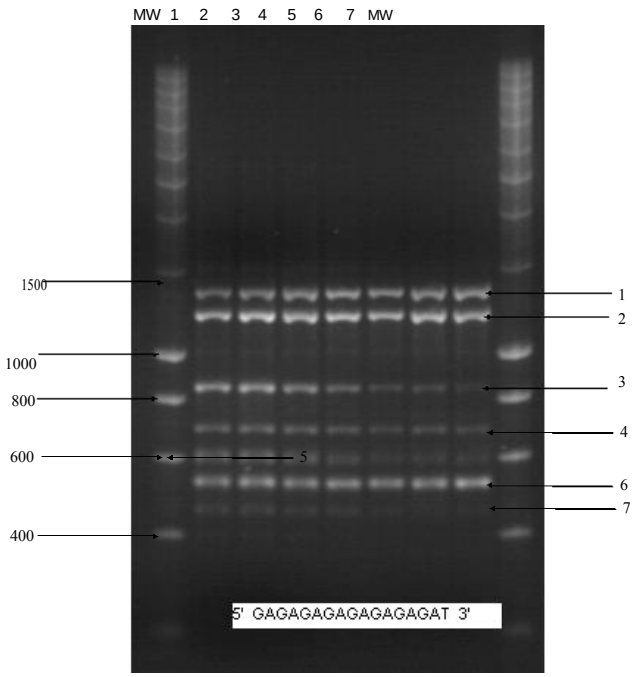

Figure 5. ISSR primer B5 (GA) $_{8T}$ at Ta=52°C run on 1.5% agarose with 20 lanes gel tray. MW=molecular weight; 1-7: individuals of *C. lanatus* (accession NI056).

Annealing temperature

For the 11 primers selected after the preliminary screening of 21, we observed clear, intensive and thin bands with annealing temperatures (Ta) ranged from 50°C to 52°C. Using samples from *C*. *lanatus*, we noted 66 bands varying from 4 to 11 per primer, with the mean number of 6 (Table 2). For each primer, we noted a curve of progression in observed band intensity. In fact, the intensity of bands became better as the conditions tends to the best annealing temperatures (50°C to 52°C) and then decreased and became large to finally disappeared (Figures 3a,b). According to Bornet and Branchard (2001), annealing temperature (Ta) is primer specific and is always superior to Tm because of the need of high stringency to facilitate annealing of primers. These authors reported from their study that Ta values for optimal hybridization was 2 to 14°C higher than Tm. Similar results were also reported by Charters and Wilkinson (2000). Indeed, studying the tropical cocoa tree, the authors founded that Ta was 2 to 5°C above the mean Tm of each primer. It is worth noting that controversial results were reported by Gilbert et al. (1999) that used low values of Ta (3°C lower than Tm). The discrepancy in trends toward optimal values of Ta in comparison with Tm indicated the need of optimization. In our study, we founded no particular trends in optimal hybridization temperature (Table 2).

Gel tray

One of the principal objectives in molecular markers optimization is to obtain well-separated bands, allowing a reliable interpretation. To achieve this goal, it is important to apply the best combination for running conditions. Each aspect of running process is important and can enhance or reduce the quality of bands. One of them is the gel tray used to load PCR products. Figures 4 and 5 show two gels obtained from the same PCR products but loaded with two different gels trays. In Figure 4, we used a gel tray with 26 lanes, each measuring 3 mm width. In Figure 5, we used a 20 lanes gel tray with 6 mm width. The two gels presented intensively colorized bands but with the 26 lanes gel, we observed large bands. The interpretation of such large bands was sometimes difficult, due to the fact that some of them, resulting from fragments with closed length, were incorrectly separated. With gels loaded using 20 large lanes (6 mm width), we

Table 2. List of 11 selected ISSR primers with annealing temperature (Ta) and number of bands observed in indigenous *C*. *lanatus*.

S/N	Primers	Sequences	Tm	Ta	Number of bands
1	Sola1	BDB-$(ACA)_5$	50	52	5
2	Sola5	DBD$(AC)_7$	50	52	6
3	Sola11	GAG-$(CAA)_5$	50	50	7
4	B5	$(GA)_8$T	50	51	6
5	B2	$(AG)_8$T	50	50	5
6	B10	$(AG)_8$G	52	50	6
7	Sola7	$(AG)_8$YT	52	51	4
8	Sola9	$(AC)_8$G	52	50	4
9	Sola2	DD-$(CGA)_5$	56	51	11
10	Sola4	VHV-$(GT)_7$G	56	51	8
11	Sola8	$(GA)_8$YC	56	51	4

obtained intensive, thin and well-separated bands that could be interpreted without ambiguity.

Voltage and running time

We observed that high voltage (110 V for 2 h) allowed rapid migration of amplified fragments, but under such condition bands separation was poor (Figure 2). The bands tend to be thin and well-separated when we reduce voltage and increase running time. Running gels at 80 V for 5 h (with the migration distance around 11 cm) revealed well-separated and thin bands. Using the combination of the best parameters, we resolved the electrophoresis products for 11 ISSR primers presenting 4 to 11 bands per primer with DNA from *C*. *lanatus* (Table 2).

CONCLUSIONS AND PERSPECTIVES

Molecular markers represent new tools for a better understanding of genetic diversity. Many studies devoted to several aspects of genetic diversity and structure are available in literature. Unfortunately, optimization stage cannot be avoided due to the diversity of the target species. In addition, the reproducibility of experimental methods in different laboratories is not guarantied. Our study indicates that ISSR markers could be powerful tools to study genetic diversity among African edible cucurbits. Eleven ISSR markers describing the genetic diversity of the indigenous cucurbit species is now available.

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REFERENCES

Agbagwa IO, Ndukwu BC (2004). The value of morpho-anatomical futures in the systematic of *Cucurbita* L. (*Cucurbitaceae*) species in Nigeria. Afr. J. Biotechnol. 3:541-546.

Bornet B, Branchard M (2001). Nonanchored intersimple sequence repeat (ISSR) markers: reproducible and specifics tools for genome fingerprinting. Plant Mol. Biol. Rep. 19:209-215.

Charters YM, Wilkinson MJ (2000). The use of self pollinated progenies as “in group” for the genetic characterization of cocoa germplasm. Theor. Appl. Genet. 100:160-166.

Djè Y, Forcioli D, Ater M, Lefèbvre C, Vekemans X (1999). Assessing population genetic structure of sorghum landraces from north-western Morocco using allozyme and microsatellites markers. Theor. Appl. Genet. 99:157-163.

Djè Y, Heuertz M, Lefèbvre C, Vekemans X (2000). Assessment of genetic diversity within and among germplasm accessions in cultivated sorghum landraces using microsatellites markers. Theor. Appl. Genet. 100:918-925.

Evett IW, Weir BS eds. (1998) Interpreting DNA evidence. Sinauer Associates Inc.: Sunderland, Massachusetts, USA.

Fang DQ, Roose ML (1997). Identification of closely relative Citrus cultivars with inter simple sequence repeat markers. Theor. Appl. Genet. 95:408-417.

Gilbert JE, Lewis RV, Wilkinson MJ, Caligari PDS (1999). Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor. Appl. Genet. 98:1125-1131.

Hamrick JL, Godt MJW (1997). Allozyme diversity in cultivated crops. Crop Sci. 37:26-30.

Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 10:4321-4325.

Nagaoka T, Ogihara Y (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RAPD and RFLP markers. Theor. Appl. Genet. 94:597-602.

Russel J, Fuller J, Young G, Thomas B, Taramino G, Macauley M, Waugh R, Powell W (1997). Discriminating between barley genotypes using microsatellites markers. Genome 40:442-450.

Vallejos CE (1983). Enzyme activity staining. In Tanksley SD, Orton TJ (eds.) Isozymes in plant genetics and breeding, part A. Elsevier: Amsterdam, The Netherlands, pp. 469-516.

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20:176-183.

Zoro Bi IA, Koffi KK, Djè Y (2003). Caractérisation botanique et agronomique de trois espèces de cucurbites consommées en sauce en Afrique de l’ouest, *Citrullus* sp, *Cucumeropsis mannii* Naudin, *Lagenaria siceraria* (Molina) Standl. Biotechnol. Agron. Soc. Environ. 8:189-199.