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Research Article

ISSR Fingerprints and ITS Restriction Cleavage Differs in Selected Native Chilli Pepper Genotypes

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Chilli peppers (*Capsicum* spp.) belong to very popular and significant horticultural crops, evaluated for their culinary, nutritional, and pharmacological properties. Knowledge about their genetic diversity is important because of their breeding strategies. In this study, restriction profiles of ITS sequences of three different chilli species were analysed for their differences together with ISSR fingerprint profile. A total of eleven genotypes of native chilli peppers from Peru were used in this study. Three different restriction patterns were obtained. Individual species, *Capsicum* chinesis Jacq, *Capsicum frutescens* and *Capsicum baccatum* were distinguished by UPGMA analysis.

Keywords: variability, DNA markers, chilli peppers

1 Introduction

Chilli peppers (*Capsicum* spp.) are among the most globally significant horticultural crops, evaluated for their culinary, nutritional, and pharmacological properties. The genus *Capsicum* encompasses over 30 species, of which *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and *C. pubescens* are the most widely cultivated and genetically studied taxa (Csilléry, 2006). Genetic variability within and among these species is the key resource for plant breeders aiming to improve traits such as fruit morphology, pungency, disease resistance, and abiotic stress tolerance (Qin et al., 2014). Genetic diversity studies across chilli peppers germplasm collections have revealed distinct clusters corresponding to species and fruit types, indicating rich inter- and intra-specific variability in *Capsicum* that can be harnessed in breeding (e.g., *C. annuum*, *C. chinense*, *C. frutescens* show clear differentiation based on genetic profiles) (Lozada et al., 2021; Swamy, 2023).

The internal transcribed spacers (ITS) evolve relatively rapidly, often showing higher sequence variation than adjacent coding regions, making them useful as phylogenetic and DNA barcoding markers in plant. They were applied to characterize chilli peppers of different species including *C. annuum*, *C. chinense*, *C. frutescens*, *C. baccatum*, and others and here, the ITS1 region generally shows higher nucleotide variability compared to ITS2, consistent with patterns seen in other plants. Variable sites, including indels and single nucleotide polymorphism (SNPs), have been reported among *Capsicum accessions* and in Naga King Chili, ITS alignments exhibited specific deletions in ITS1 and distinct deletions even in the 5.8S gene that provided phylogenetic signal (Kehie et al., 2015). ITS polymorphism within individuals were detected in most species except *C. annuum*, indicating incomplete concerted evolution of ITS arrays in the genome. Concerted evolution normally homogenizes tandem nrDNA repeats, but incomplete homogenization may occur due to hybridization or the presence of paralogous copies (Shirakagi et al., 2020).

DNA based markers provide an effective method to analyse different aspects of genetic variability of plant

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genomes and many of types were developed (Amiteye, 2021) and up to now, they were used in the study of wide range of crops. Simple sequence repeats (SSRs), single nucleotide polymorphism (SNPs), RAPDs (Random Amplified Polymorphic DNA), ISSRs (Inter Simple Sequence Repeats) and other DNA markers have been previously successfully applied in the research of chilli peppers genetic diversity (da Costa et al., 2006; Gu et al., 2019). DNA marker-based studies have described the extensive genetic variability present in chilli peppers, delineated population structure and relationships among cultivars and species, and provided essential tools for conservation and targeted crop improvement. Microsatellite or SSR markers have been frequently used due to their co-dominant inheritance and high polymorphism, enabling detailed assessments of genetic distance and population structure within germplasm collections. A total of 29 simple sequence repeat (SSR) markers, that are distributed over 12 pepper chromosomes were used to analyse chilli peppers and assisted in constructing core collections representing most allelic diversity (Gu et al., 2019). Studies using SSR markers also detect wide genetic distance ranges among chilli genotypes, reflecting extensive genetic heterogeneity fit for selection programs (Sharmin, 2018). ISSR markers, which target genomic regions between microsatellite loci, have also demonstrated considerable polymorphism among chilli accessions. These markers efficiently reveal genetic differentiation and assist in germplasm management and conservation strategies by capturing broad genetic variation patterns (Haq et al., 2022).

The aim of this study was to analyse the ITS restriction pattern and ISSR fingerprint profile and their differences in the genotypes of three native chilli peppers species originated in the Campo Verde and Pimental production zone of Peruvian Ucayali region.

2 Material and Methods

2.1 Biological Material

Eleven native chilli peppers (Table 1.) with high local and national economic and cultural importance were collected *in situ* in Peruvian Ucayali region in area of Pucallpa city in villages of the Campo Verde district. Mature fruits were collected on ecological fields of APE Pimental Association. The collected fruits were transported to Pucallpa city in boxes labelled by type, date and locality. Further, they were transported to the Czech Life University in Prague to grow them in the greenhouse followed standard growing conditions. Seeds of the collected genotypes were transported to Laboratory of genetic technologies to the Slovak University of Agriculture in Nitra for DNA analysis.

Table 1 Accessions of chilli peppers used in the study

Native name of genotype	Scientific name (Meckelmann et al., 2013)
Charapita rojo	*Capsicum chinense* Jacq
Aji Charapita	*Capsicum chinense* Jacq
Trompita rojo	*Capsicum* sp
Cerecito Amarillo	*Capsicum* sp
Malagueta	*Capsicum frutescens*
Challuaruro	*Capsicum baccatum*
Upia Ucho	*Capsicum* sp
Ayuyo	*Capsicum baccatum*
Trompito Amarillo	*Capsicum* sp
Pinchito de mono	*Capsicum frutescens*
Cerecito rojo	*Capsicum* sp

2.2 DNA Extraction and PCR and Restriction Cleavage

Seeds were homogenized using liquid nitrogen and the DNA was extracted using a commercial kit GeneJET™ Plant Genomic DNA Purification Mini Kit (Thermo Scientific) following the manufacturer instruction. Quantity and quality of extracted DNA was checked using the NanoPhotometer P300 (Implen). Extracted DNA was normalized to 30 ng microL^{-1}.

Internal transcribed spacer sequence was amplified using the universal primers ITS1 and ITS2 as described by White et al. (1990) and Dream Taq Green PCR Master mix (Thermo Scientific). Obtained amplicons were cleaved using the NlaIII, FatI, Mn1I and MseI restriction endonucleases (BioLabs) and the restriction cleavage was performed following the instructions of endonucleases provider with following temperatures: 55 °C for FatI and 37 °C for NlaIII, Mn1I and MseI.

ISSR fingerprint PCR was performed using the (GAG)3GC microsatellite sequence as primer using the 1x Dream Taq PCR MasterMix (Thermo Scientific), 350 nmol dm^{-3} primer and 60 ng of genomic DNA. Time and temperature profile was as follows: [2´ 94 °C` 45 cycles of (1´ 94°C; 1´ 55 °C; 3´ 72 °C) and final 7´ 72 °C.

2.3 Amplified/Cleaved Fragments Separating and Data Analysis

Generated restriction fragments were loaded on 2% agarose gels, separated by electrophoresis and stained by GelRed™ (Biotium). ISSR fingerprints separated by Experion™ Automated Electrophoresis System capillary electrophoresis (BioRad). Generated binary

matrices of amplicon presence were prepared, Nei-Li coefficient of genetic distance was used to calculate basic relationships among individual genotypes and a dendrogram was constructed by UPGMA method using the Statgraphics software.

3 Results and Discussion

Restriction cleavage of analysed genotypes of chilli peppers showed distinct patterns for individual species (Figure 1). NlaII and Mn1I provided monomorphic restriction pattern in all analysed chilli peppers species. In the case of FatI and MseI, differences were obtained among the analysed species that ranged from non-cleavage for *Capsicum* frutesces up to the three fragments for *Capsicum baccatum*.

ISSR primer (GAG)3GC showed a successful DNA amplification among analysed different *Capsicum* genotypes. A total of 87 DNA amplicons were obtained ranging from 5 to 10 for individual analysed chilli pepper genotypes. Amongst *Capsicum* genotypes, the maximum banding patterns were observed in *Capsicum chinense* Jacq. followed by *Capsicum* spp, then *Capsicum baccatum* and *Capsicum* frutescens (Figure 2). The used primer showed increased and reduced DNA banding profile in individual analysed genotypes, and the size of DNA amplicons ranged from 210 bp to 859 bp. Total polymorphism of 100% was obtained.

Constructed dendrogram based on binary matrix obtained from ISSR fingerprint results showed a total of four main branches, where genotypes of *Capsicum frutescens* as well as genotypes of *Capsicum baccatum* are grouped together at the level of 0.7 and 0.49, respectively (Figure 3). Both of the *Capsicum* chinensis Jacq are joined with two genotypes of *Capsicum* spp. Here, the used ISSR marker confirmed the reported

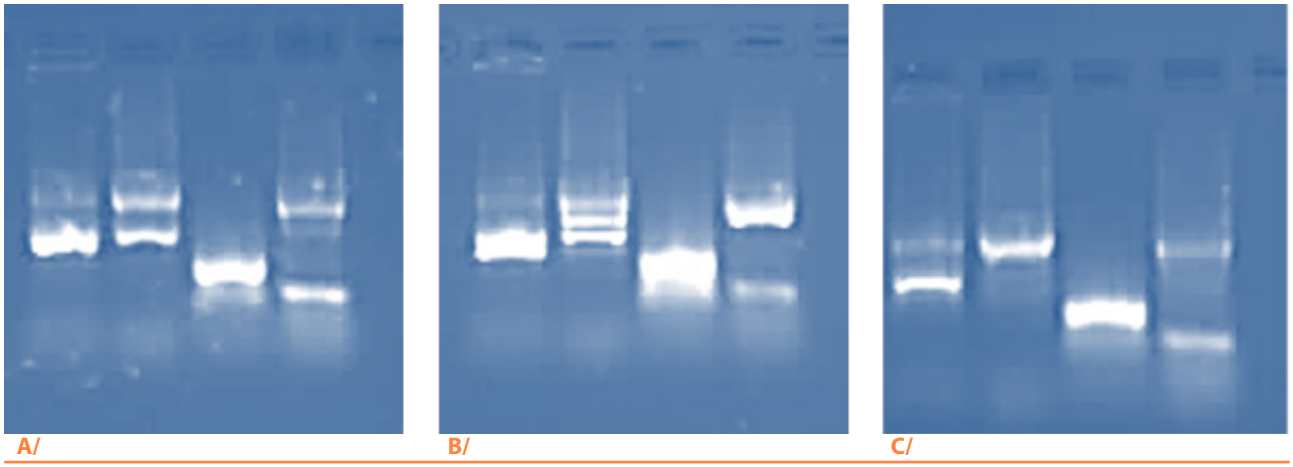

Figure 1 Restriction profiles of A/ *Capsicum* spp and *Capsicum chinensis* Jacq genotypes; B/ *Capsicum baccatum* genotypes and C/ *Capsicum frutescens* genotypes
The restriction endonucleases used from left to right: NlaIII, FatI, Mn1I and MseI.

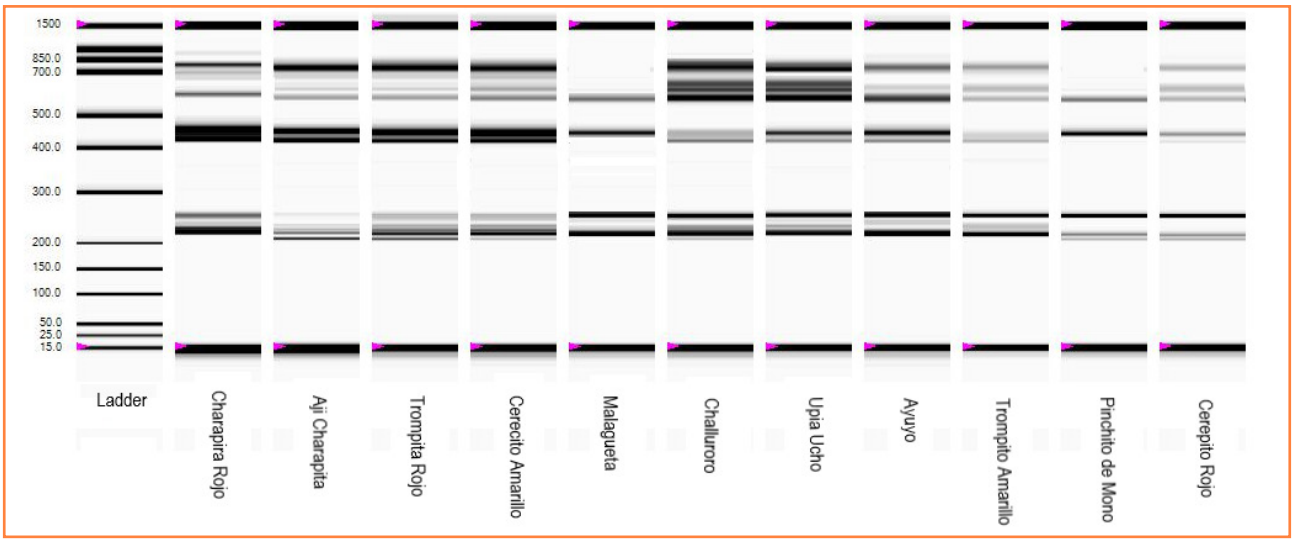

Figure 2 Visualization of capillary electrophoresis data obtained for (GAG)3GC primer for analysed chilli peppers genotypes

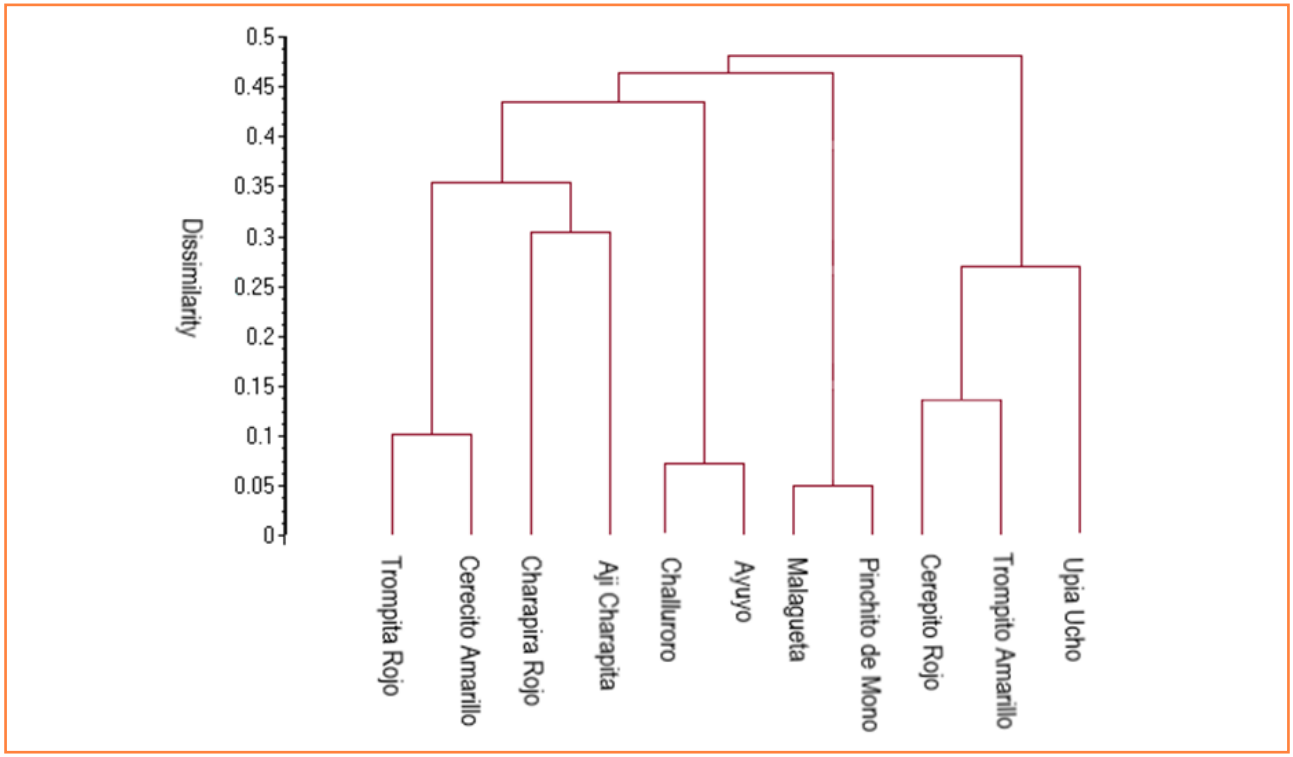

Figure 3 Dendrogram of analysed chilli peppers genotypes based on ISSR fingerprint profile

potential of this technique to distinguish different species of chilli peppers (Haq et al., 2022).

Capsicum is one of the most important horticulture crops, which comes in a variety of agromorphologically unique kinds. The size, shape, color, flavor, shelf life, and chemical makeup of capsicum fruits varied in addition to the growing regions. Furthermore, understanding variations in genome size, genetic plasticity, degree of adulteration, fruit quality, pungency, size, and color is crucial for chilli breeding efforts (Maharijaya et al., 2019). Morphological indicators have been crucial for understanding variation in *Capsicum* species, with flower and fruit traits being the most significant (Hasan et al., 2014; Naegele et al., 2016; dos Santos Pesoa et al., 2018; Jha et al., 2021). Biochemical, physiological, and molecular aspects of these indicators have also been thoroughly studied.

Up to now, different research was performed to characterize genetic diversity in *Capsicum* species using various DNA based markers, such as AFLP (Albrecht et al., 2012), SSRs (Gu et al., 2019), RAMPO (Rai et al., 2013), and RAPD (da Costa et al., 2006), but only a few studies exist using ISSR markers (Ibarra-Tores et al., 2015; Haq et al., 2022). ISSR markers are dominant markers comprising of polymorphic arbitrary primers with high reproducibility and requiring high stringency in PCR conditions (Gemmill and Grierson, 2021). The ISSR marker technique is a recognized and important method for investigating varieties for practical applications, including germplasm identification, parentage analysis, genetic diversity assessment, gene mapping, quantitative trait loci analysis, evolutionary strategies, and taxonomic studies (Milad et al., 2011; Gupta et al., 2012; Alansi et al., 2016; Nilkanta et al., 2017; Zhao et al., 2020). In the study of Haq et al. (2022), a total of 80 bands was amplified when seven ISSR primers was used with the average of 11.43 bands per primer. In the study of Ibarra-Tores et al. (2015) two ISSR primers amplified a total of 38 bands with an average of 19 bands per primer for *Capsicum*. In both of these studies, polymorphism ranged from 79.62 to 100% what corresponds with data obtained in our study. For other marker techniques used for *Capsicum*, primer polymorphism ranged from 50 to 100% with an average of 81.52% using SCoT markers (Gupta et al., 2019). Here, 100% polymorphism amongst *Capsicum accessions* was obtained, and this value is quite comparable with that of different chilli accessions using different DNA techniques.

4 Conclusions

Understanding the genetic architecture of *Capsicum* has direct implications for breeding programs. Genetic variability characterized by DNA markers supports the identification of distinct genetic pools, facilitates marker-assisted selection, and underpins the development of core germplasm collections that capture maximal diversity with minimal redundancy. Here, the specific restriction profiles of three chilli

capsicum species reported their differences. ISSR technique was proved here to have the potential to distinguish individual species of chilli peppers.

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