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Research note

Study of genetic diversity in *Indigofera suffruticosa* crops in El Salvador

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Abstract

Añil is a woody shrub that belongs to the Fabaceae family. It is recognized as native and contributes to the sustainable agriculture of many rural regions of El Salvador due to its ability to fix nitrogen to the soil and is preferred because it produces quality indigotine, which is exported to different parts of the world. Currently, its genetic status is unknown; this research aimed to analyze the genetic variability of añil in crops in El Salvador. The samples were collected in five locations with cultivars of the species in 2021. DNA extraction was performed using the CETAB protocol with modifications, followed by PCR amplification of the ITS2 ribonuclear region. The PCR products were sent to Macrogen (Korea) for sequencing. The sequences were edited and analyzed using bioinformatics programs. The analyses revealed two haplotypes distributed in the Salvadorean territory and low genetic diversity indices (Hd= 0.46, #= 0.004). In addition, very high genetic differentiation has been found, mainly between the CPB locality and the rest of the localities (Fst# 0.5). The results are conclusive regarding a low genetic diversity, the presence of very few haplotypes, and a gene structure based on differences mostly between populations.

Keywords:

Indigofera suffruticosa, haplotypic diversity, ITS2 ribonuclear region.

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The cultivation of añil and the production of its dye were the backbone of Central America's export economy towards the end of the Colonial period. Although dye and its export had been carried out since the sixteenth century in the Kingdom of Guatemala, it was not until the second half of the eighteenth century that it became the driving product of the Central American Economy (Erquicia, 2019). The same author pointed out that the production, commercialization, and export of añil dye was fundamental for the economic, social, and political development of the region, especially for the Republic of El Salvador.

The word añil is assigned to the extract of the indigo blue dye that can be obtained from different varieties of plants, but this name has commonly been assigned to the group of Fabaceae used in agriculture, with *Indigofera suffruticosa* being one of those that predominate in Salvadoran crops (Stevens *et al*., 2009).

In El Salvador, there is a large amount of germplasm or seed of *I. suffruticosa*, which is saved from each harvest or exchanged between farmers with the aim of maintaining or improving the quality and concentration of indigotine; nevertheless, some farmers are currently incorporating wild seeds into their agricultural practices, which could have implications at the genetic level in this species.

On the other hand, Ahuja and Jain (2015) specify that, at the level of the agricultural landscape, monoculture systems and the simplification of agroecosystems represent one of the threats that lead to genetic erosion. For this reason and given that there is no information on the genetic status of this species in the country that allows a better use of this resource, the main objective of this research project was to analyze the genetic diversity in crops of *I. suffruticosa* in El Salvador.

DNA sampling and extraction

The samples of *I. suffruticosa* were collected in the cultivars of Chalchuapa (Casa Blanca), Department of Santa Ana; Jardín Botánico La Laguna, Department of La Libertad; Hacienda Los Nacimientos, Department of Cuscatlán; Cantón San Francisco and Cooperativa Pinares de Berlín, Department of Usulután, and a control sample of the species *I. guatemalensis* was collected in the Department of San Miguel.

All collections were made in 2021. A total of 49 leaf tissue samples were selected. For DNA extraction, the CETAB extraction protocol was applied with slight modifications. The quality of the samples was verified by electrophoresis in 1% agarose gel (w/v). DNA samples were stored at -20 °C (Borges *et al*., 2012).

PCR, sequencing, and genetic analysis

Polymerase chain reaction (PCR) was carried out in a MultiGene™ Mini thermal cycler, using the standard primer ITS2 ribonuclear region, sequence (5'-3'): FACGAATTCATGGTCCGGTGAAGTGTTCG/ RTAGAATTCCCCGGTTCGCTCGCCGTTAC (Fazekas *et al.,* 2012).

The conditions for PCR were: initiation 95 °C for 3 min, denaturation 94 °C for 0.3 min, annealing 59 °C for 0.3 min, elongation 72 °C for 0.45 min, extension 72 °C for 10 min, for a total of 35 cycles and a final extension stage at 4 °C. PCR products were evaluated in 2% agarose gel electrophoresis (w/v) at 150 volts for 15 min. The size of the amplified fragments was determined with the help of a commercial DNA marker of 100bp DNA Ladder. The product obtained was sent to the company called Macrogen Inc. (Korea) for sequencing.

The electropherograms resulting from the sequencing were analyzed and edited with the SnapGene® 5.3.1 software, from which a fasta file per sequence was obtained. The taxonomic classification of each individual was performed by comparing sequences reported in GenBank. The annealing of sequences was done in Mega. DnaSP v6.12.03 was used to calculate the genetic diversity indices. Haplotypic analysis was performed in PopART 1.7, and the analysis of molecular variance (Amova) was performed using the Harlequin 3.5 program.

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Regarding the indices of genetic diversity, the number of haplotypes (Hn) found in the populations of *I. suffruticosa* was two. The global haplotypic diversity (Hd) was 0.45883. The mean number of nucleotide (K) differences was 1.3765 at the level of individual populations. The total nucleotide diversity (π) is represented by a value of 0.004, where Cantón San Francisco stands out with a nucleotide diversity of 0.005; therefore, it is the population that showed the highest polymorphism among its individuals (Table 1).

Table 1. Indices of genetic diversity of *I. suffruticosa* by locality.

Populations	N	Hn	Hd	K	Π
Casa Blanca	9	1	0	0	0
Cooperativa Pinares de Berlín	11	1	0	0	0
Cantón San Francisco	11	2	0.56	1.64	0.005
Hacienda Los Nacimientos Aguilares	10	1	0	0	0
Jardín Botánico	6	1	0	0	0
Total	47	2	0.46	1.38	0.004

Sample size (n); number of haplotypes (Hn); haplotypic diversity (Hd); average number of nucleotide differences per haplotype (K); and nucleotide diversity (π).

These results reflected a gene flow associated with very low values of haplotypic diversity (Hd= 0.46) and nucleotide diversity (π= 0.004) and consequently, a low genetic variability, presumably linked to inbred and self-pollination processes typical of monoculture systems (Ruíz *et al*., 2018), which should undoubtedly be considered as erosive of the gene pool of this species in cultivated conditions, which leads to the loss of resilience against pests and diseases (Vera, 2017).

In the estimation of the population pairwise fixation index (F_{ST}) and its significance *p*-values (Table 2), the pairs of populations that have an F_{ST} value of 1 are CPB/CB, HN-A/CPB, and JB/CPB; therefore, their differentiation is very high, the F_{ST} values of 0.5 for CSF/CPB, 0.4 for CSF/CB, and HN-A/CSF, respectively, 0.35 for JB/CSF correspond to moderate genetic differentiation. The pairs of populations HN-A/CB, JB/CB, and JB/HN-A reflect an F_{ST} estimate of 0, whose genetic differentiation is null.

Table 2. F_{ST} fixation index. *p*-values on the diagonal and F_{ST} values below the diagonal.

Populations	CB	CPB	CSF	HN-A	JB
Casa Blanca	-	0*	0.0105*	0.0994	0.0994
Cooperativa Pinares de Berlín	1	-	0.0023*	0*	0*
Cantón San Francisco	0.4	0.52	-	0.0062*	0.0457
Hacienda Los Nacimientos Aguilares	0	1	0.42	-	0.099
Jardín Botánico	0	1	0.35	0	-

CB= Casa Blanca; CPB= Cooperativa Pinares de Berlín; CSF= Cantón San Francisco; HN-A= Hacienda Los Nacimientos Aguilares; JB= Jardín Botánico; *= When the *p*-value< 0.05.

These results will allow us to anticipate a directly proportional relationship between geographical distance and genetic distance. Consistent with the above, in the case of Hacienda Los Nacimientos and Casa Blanca, an F_{ST}= 0 has been found due to the fact that, geographically, they are a few kilometers apart. Contrary to the above, Cantón San Francisco and Cooperativa Pinares de Berlín showed moderate genetic differentiation F_{ST}= 0.5 despite the fact that these crops are in the same

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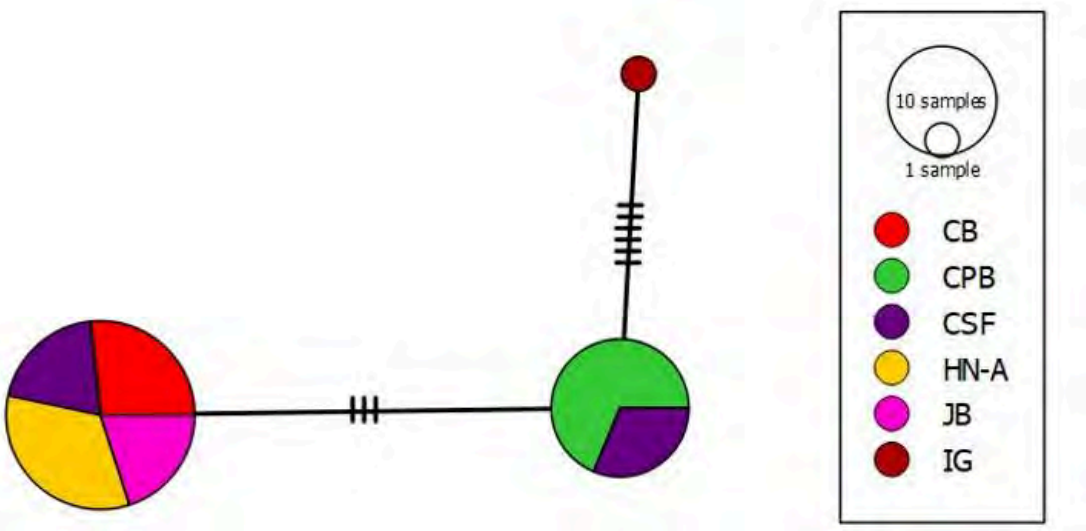
geographical area, which can be explained by a reduced gene flow between both populations in addition to being from different haplotypic groups.

When comparing the genetic diversity by locality, in the case of Cantón San Francisco, which presents higher diversity values (Hd= 0.56, π= 0.005) compared to the other localities and the presence of two haplotypes in its samples, it reveals evidence of the introduction of wild germplasm to the añil crops in these localities (as confirmed by the farmers of that site).

Regarding the population pairwise fixation index (F_{ST}), very high genetic differentiation was clearly found, mainly between the CPB locality and the rest of the localities (F_{ST}≥ 0.5), which again could be explained by the introduction of wild germplasm in that region of the country. Figure 1 shows the network of haplotypes, where each circle represents a haplotype and its size is proportional to the number of sequences that share it.

Figure 1. Network of haplotypes with sequences of *I. suffruticosa*. Each circle represents a haplotype, each color a locality, and the transverse lines the mutational steps between haplotypes.



The analysis showed that haplotype one is shared by four populations (colors: purple CSF, red CB, pink JB, and yellow HN-A) that bring together 31 sequences. For its part, haplotype two has 16 sequences from the populations of CPB (green) and CSF (purple). Haplotype three represents the control sample of the species *I. guatemalensis* (ochre).

The topology of the network shows that haplotype 1 is the most widely distributed, with a presence in almost all the sampled regions, so it is deduced that it has been present in the Salvadorean territory for longer than haplotype 2 (Leigh and Bryant, 2015). On the other hand, the Amova test (Table 3) revealed that most of the genetic variation observed resides in differences between populations (77.15%) and fewer differences within populations (22.85%). These results could be due to self-pollination since, according to (Hariri *et al*., 2017), self-pollinating species commonly exhibit less genetic variation within their population than between populations.

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Table 3. Analysis of molecular variance (Amova) of 47 sequences of *I. suffruticosa* from five culture sites.

Source of variation	Sum of squares	Variance components	Percentage variation (%)
Between populations	46.956	0.62067	77.15
Within populations	16.364	0.18386	22.85
Total	63.319	0.80453	100

Conclusions

The analyses carried out on the ITS2 marker of the different populations of *I. suffruticosa* crops showed a low genetic diversity, the presence of very few haplotypes, and a gene structure based on differences mostly between populations. This indicates a notable decrease in genetic diversity in the germplasm used for añil cultivation in El Salvador, which can be explained by the pressure of artificial selection typical of monocultures and a self-pollinating system.

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