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NOTE
SIMPLE SEQUENCE REPEAT MARKERS (SSR) IN *ANNONA DECEPTRIX* WESTRA H. RAINER, AN ENDANGERED SPECIES OF THE ECUADORIAN COAST

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Polymorphic microsatellite markers were developed in *Annona deceptrix* (Annonaceae) to evaluate its genetic diversity and population structure. This species is a relevant agricultural and food resource for society because its edible fruit that can be considered as a consumption option. However, natural populations have a high degree of vulnerability caused for human activities and disturbances. The objective of this study was to isolate and characterize SSR loci as a tool to determine the genetic diversity in *A. deceptrix* and others in Annonaceae family. Twenty-two polymorphic Simple Sequence Repeat (SSR) loci were isolated from *A. deceptrix* using a new-generation kit Nextera of miseq illumine technology. The mean number of alleles per locus ranged from 2 to 8. Observed and expected heterozygosity ranged from 0.00 to 1.0 and 0.00 to 0.93, respectively. The average the inbreeding coefficient in the Humedad site was 0.007, while in Agua Blanca and Tachina, they obtained negative values of -0.090 and -3.222, respectively. These are the first microsatellite markers developed for *A. deceptrix*. All SSR markers developed are promising candidates for analysing genetic variation within or between natural populations and can be transferable to other species of the genus Annona.

Keywords: Cross-amplification, endemic species, Annonaceae, Nextera, heterozygosity

INTRODUCTION

Annona deceptrix Westra H. Rainer is an endemic tree of the province of Manabí and is commonly known as chirimoya de monte o anonilla (Pico-Mendoza et al. 2020). This species is threatened due to deforestation and land-use change (León-Yánez et al. 2011). *A. deceptrix* is found in moist coastal remnants, sharing its niches with woody and herbaceous species. This species presents white to yellowish flowers and conical elongated sweet fruits with different diameters and a smooth to rough appearance. This Annonaceae has excellent nutritional attributes as it can be consumed as fresh fruit or in processed products. However, there is no information on the attributes and potential of *A. deceptrix*, where the probability of disappearance and extinction is increasing. A study on the germination of *A. deceptrix* seeds reported that the seed needs a scarification process for its germination, which starts 25 days after sowing (Pico-Mendoza et al. 2020).There are studies in other commercial Annonaceae, focused on development of marker, analysis of genetic diversity, and micropropagation, even in the ploidy of different Annonaceae species (Escribano et al. 2004 & 2008, Pereira et al. 2008, van Zonneveld et al. 2012, Anuragi et al. 2016, Fabio Ernesto Martínez et al. 2016, Hasan et al. 2017, Ferreira et al. 2019, Saitwal et al. 2022)as many of these species are still

undomesticated, or in incipient stages of domestication and local populations can offer yet-unknown traits of high value to further domestication. For many outcrossing species, such as most trees, inbreeding depression can be an issue, and genetic diversity is important to sustain local production. Diversity is also crucial for species to adapt to environmental changes. This paper explores the possibilities of incorporating molecular marker data into Geographic Information Systems (GIS but none on endangered species. Therefore, it is crucial to assess the genetic diversity of *A. deceptrix* populations using molecular markers to obtain information on its genetic composition and to develop conservation strategies to preserve the species. Despite its potential for economic and medicinal uses, very little is known about the genetics of this species, making it difficult to conserve and utilise effectively. For this reason, it is necessary to evaluate the genetic pool of *A. deceptrix* contained within the populations through molecular markers, which allows researchers to obtain information on its genetic composition, and developing conservation strategies to preserve the specie. Evaluating these unexplored genetic resources will mainly help their protection and permanence in the surrounding ecosystems. For this reason, this study aimed to develop a set of polymorphic microsatellite markers to assess the diversity and genetic structure of *A. deceptrix*.

MATERIALS AND METHODS

Four samples of *A. deceptrix* were collected in two places, Cerro Pata Pajaro Protective Forest at Pedernales canton (0° 01' 40.4" N 80° 00' 50" W) and Machalilla National Park (1° 34' 45.4" S 80° 42' 25.2" W) for sequencing. Forty samples of *A. deceptrix* were selected to evaluate the diversity in the isolated loci, Agua Blanca (n = 10), Tachina (n = 18) and Humedad (n = 12). Three samples of *A. manabiensis* and *A. conica* were used for cross-amplification. Two leaves were collected from each individual, preserved and dried in silica gel. The collection of samples of *A. deceptrix* was authorized by the Ministry of the Environment of Ecuador, license number: MAAE-CMARG-2021-0442. The total genomic DNA of *A. deceptrix* was extracted from young leaves with a modified CTAB (cetyltrimethylammonium bromide) method (Murray & Thompson 1980, Doyle & Doyle 1990). The quality and quantity of isolated DNA were determined using a Nanodrop and agarose gel electrophoresis. The Nextera Library Preparation Kit was used to construct the library from 5 ng of DNA (0.5 ng ul^{-1}) following the manufacturer's protocols applied in the AUSTRAL-omics laboratory, Universidad Austral de Chile. A total of 200.00 reads were obtained, with an average length of 386 bp, which were generated by three independent runs. The sequences were assembled using the GS De Novo Assembler (V2.9) software. We used the MSATCOMANDER (Faircloth 2008) program to locate Simple Sequence Repeat (SSR). The primers were designed using the software PRIMER 3 (Untergasser et al. 2012) according to the parameters; primer size 18–24 bp, Tm (melting temperature) of 55–60 °C, and GC content of 100 40–65%. For the amplification of selected SSRs, the PCR was performed following the method developed by (Schuelke 2000), which uses three primers; a forward primer with an M13 (-21) tail at its 5' end, a standard reverse primer, the universal M13 (-21) fluorescent-labeled primer with either 6-FAM, VIC, PET or NED fluorochromes dyes. The SSR information and GenBank accession numbers are listed in Table 1. PCR reactions were performed in a 14 µL reaction mixture with 10 ng templates DNA, 0.15 mM of each dNTP; 1 × Taq polymerase reaction buffer; 1.5 mM MgCl2; 0.1 µM forward primer; 0.4 µM of reverse primer; 0.4 µM M13 fluorescent-labeled primer (FAM/VIC/NED/PET) and 1U of Taq DNA Polymerase. PCR amplifications were performed in an Applied Biosystems Veriti thermal cycler under the following conditions; initial denaturation at 95 °C for 5 min, 10 cycles of 30 s at 95 °C, annealing temperature specific to each primer pair for 45 s, extension at 72 °C for 45 s, followed by eight cycles of 30 s at 95 °C, annealing at 53 °C for 45 s, extension at 72 °C for 45 s and a final extension at 72 °C for 1 min. The annealing temperature of the primers is specified in Table 1. SSR markers and polymorphisms were resolved as follows; two µL of PCR products were mixed with two µL of primer M13 VIC, five µL of primer M13 NED, one µL of primer M13 FAM and five µL of primer M13 PET and separated by capillary electrophoresis on an

Table 1 Characteristics of 22 SSR loci and primer pair's development for *Annona deceptrix*

LOCUS	Primer sequences (5′–3′)	Repeat motif	Product size	Size (bp)	Dye	Ta (°C)	GenBank
AD01	F: TCATGTTGTGGATGTGGAGC R: TCTTTGGGAACCATCAGACC	(AG) 7	318	200-213	NED	60.8	ON469949
AD02	F: TTCACTGCATGCCATAGAAGA R: GTAATGGCCCGGAAGAGTATC	(AAAC)5	139	324-343	NED	60.75	ON469950
AD03	F: TGAAGATTTCTGTATTCCATGGTT R: TCTTGCCCGTGGTACTCAAT	(AT)6	145	135-146	FAM	60.6	ON469951
AD04	F: AAACGTAAGCAGACGCCAAC R: AAGATTCCGACCCAACACAG	(AC)6	201	152-163	FAM	61	ON469952
AD05	F: AAGAGAGGCAGAAATCACGG R: CGATGAGACTGAGGTGGTCA	(AG)14	172	326-353	PET	61.35	ON469953
AD06	F: GCGCAAATTGTGAGAAATGA R: GCCTCCACGGAAATTGTTT	(AAC)7	113	196-216	FAM	59.95	ON469954
AD07	F: TAACGATGAGCAAATCGACG R: ATCTTTCGTGTCGTGCTTCC	(AT)7	222	257-270	NED	60.6	ON469955
AD08	F: CTCTGACCAATGTCAACGGAT R: ATTCTGCAGGACAATCCCAA	(AG)7	165	357-370	PET	60.75	ON469956
AD09	F: TCGTTTCTTCTATTTATCCCGAA R: GCCGGGAGACTTGATTCTTT	(AGC)7	206	145-165	PET	59.85	ON469957
AD10	F: TCCATCTGTTCTGCTTGACG R: ACCAGACGAAGAAGGCTTGA	(AG)6	273	248-259	VIC	62	ON469958
AD11	F: GGAGTTCCCTCAAAGAAGGG R: GCTGTCTTCCTGAGGCACTT	(AAAAC)5	290	228-252	FAM	63	ON469959
AD12	F: GGTAAGGTGTTACCATGCAGC R: TACCCACATCCTCCTATGCC	(AG)5	190	110-119	NED	62	ON469960
AD13	F: CAGCAGTCTGATTGTCGGAA R: GCGAGTCATGAAAGCTTGG	(AAGC)5	234	306-325	VIC	61	ON469961
AD14	F: GAAACGAGGTATCGCAAAGG R: AGCAGAAACCGGAAATGATG	(AGC)	213	227-241	VIC	60	ON469962
AD15	F: TTTGCTGGCATTTGCTCTAC R: GAATTGTCTGCAGAACCACAAA	(AAAAC)5	293	70-94	FAM	60	ON469963
AD16	F: GGAACAGCAGTCTTCTTGGC R: CTTGTTGGATGCGGACAAT	(AGAT)6	290	271-294	NED	61	ON469964
AD17	F: GGTGGTGGAAATTGGTTGTC R: TCGAACCAACACCATGAGTC	(AGC)5	188	120-140	VIC	61.15	ON469965
AD18	F: CGAGAGATATGCAGCATCCA R. TGACCTCCCATGCACCTAGT	(AAG)5	233	153-167	VIC	62.65	ON469966
AD19	F: ACCACGAGGCAGGTCAGTT R: CACCAACAATGGTGTTCTCG	(AGC)9	205	154-180	FAM	61.55	ON469967
AD20	F: AGGAGACGGGTTACCTGGAC R: AAACGACCATTGCAGGGC	(AG)8	303	190-239	FAM	62.3	ON469968
AD21	F: ACCTCCAGTTGAGAAGAGCG R: CTGCGTGGGTCTGTGACTAA	(AAGC)5	225	129-143	PET	62.15	ON469969
AD22	F: AAGTAGTACCGGTCAGAGAGCG R: GTTGGATTATTGAAACGCGG	(AGCG)6	292	213-236	NED	59.85	ON469970

Ta = optimal annealing temperature

ABI 3500 Genetic Analyzer in AUSTRAL-omics, Universidad Austral de Chile. Allele sizes were automatically calculated with Geneious V.8.0.4..

Allelic richness (A), observed heterozygosity (Ho) and expected heterozygosity (He), and inbreeding coefficient (Fis) were estimated using PopGene (Yeh et al. 1999). Deviation from the Hardy–Weinberg equilibrium was determined with GENEPOP (Raymond & Rousset 1995). The inbreeding coefficient (Fis) (Weir & Cockerham 1984) was determined using Genetix (Belkhir et al. 2003). Null alleles were checked using MICRO-CHECKER V 2.2.3 (Van Oosterhout et al. 2004). The cross-amplification was tested on two threatened species, *A. conica*, and *A. manabiensis*. Primers were considered successful when one clear, distinct band was detected on 2% agarose gel.

RESULTS AND DISCUSSION

In the study, the most common SSRs motif were dinucleotides, accounting for 72.7% of all motifs observed. Trinucleotides were the second most abundant, making up 24.4%, followed by tetranucleotides at 2.1% and pentanucleotides at 2%. Based on their high level of polymorphism, we selected 22 SSR loci, consisting of nine dinucleotides, seven trinucleotides, four tetranucleotides and two pentanucleotides. All of these loci were found to be polymorphic, and variations between populations were observed. The average level of polymorphic loci across all populations (PPL) was 96.97%, indicating a high degree of genetic diversity. The mean number of alleles per locus varied between populations, ranging from 2 to 8, with an average of 3.77 in the Tachina population. In the Agua Blanca and Humedad populations, the mean number of alleles per locus were 2.82 and 3.68, respectively (Table 2). These results showed that dinucleotides were the most common SSRs motif in the studied population, with trinucleotides being the second most abundant. The selected SSR loci exhibited a high degree of polymorphism, with variations between populations. The results indicated a high level of genetic diversity in the populations studied, which could have important implications for conservation efforts and future research. These results may differ from other studies concerning the development of SSR markers due to the number of samples, the number of loci, and the type of sequencing (Escribano et al. 2008, Piñeiro et al. 2016). In this study, the average polymorphic information content (PIC) was 0.56, with the highest value observed at the AD08 locus (0.884) and the lowest value at the AD18 locus (0.131). On the other hand, both observed and expected heterozygosity ranged from 0.00 to 1.0 and 0.00 to 0.93, respectively, with an overall average of 0.63 and 0.54, respectively. In terms of specific loci, the AD08 and AD13 loci exhibited the highest expected hetero-zygosity value of 0.93 for the Agua Blanca population, whereas the Tachina population's AD05 locus and the Humedad population's AD12 locus displayed the highest expected heterozygosity values of 0.95 and 0.86, respectively. Additionally, the AD05 locus demonstrated the greatest inbreeding coefficient value among the Agua Blanca, Tachina, and Humedad populations, with respective values of 0.367, 0.524 and 0.554. Moreover, the AD07 locus showed a value of 1.00 across all populations, while the AD14 locus's lowest value for all populations was -1.000. It is worth mentioning that four microsatellites (AD02, AD09, AD10 and AD19) were monomorphic in the Agua Blanca population, while the AD18 locus was monomorphic in the Humedad population. These results have significant implications for the understanding of the genetic diversity and population structure of the species under investigation, as well as to aid the development of conservation strategies. Overall, the results obtained from this study provide valuable insights into the development of SSR markers and their utility in characterising the genetic diversity of populations. However, further research is required to gain a more comprehensive understanding of the genetic makeup of the species in question, particularly in the context of their natural habitats and potential ecological interactions within and between populations. The inbreeding coefficient value for the AD05 locus was found to be the highest among the three populations studied, with values of 0.367, 0.524 and 0.554 for Agua Blanca, Tachina, and Humedad populations, respectively. Additionally, Locus AD07 demonstrated a value of 1.00 across all populations, indicating complete homozygosity. In contrast, the AD14

Table 2 Estimated genetic parameters and Hardy-Weinberg test in 22 microsatellites loci in three wild populations of *Annona deceptrix*

	Agua Blanca (n = 10)				Humedad (n = 18)				Tachina (n = 12)				Cross-amplification	
Locus	N_a	H_o	H_e	F_{is}	N_a	H_o	H_e	F_{is}	N_a	H_o	H_e	F_{is}	*Annona manabiensis*	*Annona conica*
AD01	5	0.75	0.89	-0.124	3	0.83	0.59	0.190	4	0.6	0.7	-0.148	+	+
AD02	1	0	0	-0.286	2	0.83	0.53	0.091	3	0.6	0.64	-0.655	+	+
AD03	2	0.5	0.57	0.248	3	0.166	0.43	-0.039	3	0.6	0.5	0.417	+	+
AD04	2	0.25	0.25*	-0.159	4	0.666	0.56	-0.002	2	0.2	0.2*	-0.157	+	+
AD05	5	0.25	0.89*	0.367	5	0.5	0.72	0.554	8	0.6	0.95*	0.524	+	+
AD06	3	0.75	0.75	-0.308	5	1	0.74	-0.124	5	1	0.84	-0.240	+	+
AD07	2	0	0.57	1.000	2	0	0.48*	1.000	2	0	0.35	1.000	+	+
AD08	6	0.75	0.93	0.333	6	0.33	0.81*	0.073	5	0.8	0.75	0.448	+	+
AD09	1	0	0	-0.106	3	0.83	0.62	0.286	2	0.4	0.53	-0.371	+	+
AD10	1	0	0	-0.417	3	1	0.64	-0.038	5	1	0.93	-0.634	+	+
AD11	4	0.75	0.82	0.014	4	0.83	0.8	0.100	4	0.75	0.82	0.014	+	+
AD12	6	1	0.92	-0.057	6	1	0.86	0.006	6	0.8	0.86	-0.143	+	+
AD13	5	0.66	0.93	-0.159	6	1	0.84	0.055	7	1	0.91	0.002	+	+
AD14	2	1	1	-1.000	2	1	0.54	-1.000	2	1	0.55	-1.000	-	+
AD15	2	1	1	-0.233	3	0.66	0.53	-0.143	2	0.5	0.5	-0.322	-	-
AD16	2	1	1	0.176	5	0.83	0.74	0.429	4	0.4	0.78	-0.150	-	+
AD17	2	1	0.6	-0.217	5	1	0.79	-0.338	4	0.8	0.73	-0.448	+	+
AD18	2	0.33	0.33*	-0.011	1	0	0*	-0.011	2	0.2	0.2*	-0.077	-	+
AD19	1	0	0	-0.312	3	0.66	0.53	-0.304	3	0.8	0.62	-0.258	-	+
AD20	3	0.66	0.6	-0.412	4	0.83	0.63	-0.364	3	1	0.71	-0.289	-	+
AD21	3	1	0.73	-0.472	3	1	0.66	-0.382	3	0.75	0.6	-0.556	+	+
AD22	2	0.66	0.53	0.147	3	0.33	0.32	0.109	4	0.6	0.78	-0.179	-	+
Mean	2.82	0.56	0.62	-0.090	3.68	0.70	0.64	0.007	3.77	0.65	0.69	-3.222		

+ = successful PCR amplification, - = unsuccessful PCR amplification, N_a = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, Fis= inbreeding coefficient

* = significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$)

locus had the lowest value for all populations, being -1.000. The mean F_{is} value for the Tachina population was significantly lower than the values observed for the Agua Blanca and Humedad populations. This suggests that the Tachina population may have experienced genetic drift or other evolutionary forces that led to a decrease in genetic diversity compared to the other two populations. Furthermore, loci AD04, AD05, and AD18 exhibited significant deviations from Hardy-Weinberg equilibrium in the Agua Blanca and Tachina populations, while the Humedad population's AD18 locus was monomorphic. Additionally, the AD07 and AD08 loci showed significant deviations from Hardy-Weinberg equilibrium in the Humedad population. Although few loci displayed deviations from Hardy-Weinberg equilibrium, this may be typical of Annonas due to their cross-reproduction and self-pollination systems. For example, a study on *Annona crassiflora* found that seven out of ten loci showed Hardy-Weinberg significance (Pereira et al. 2008). These results denote the capacity of the SSR developed to investigate diversity studies and genetic structure of the Annona species studied. The observed deviations from Hardy-Weinberg equilibrium may have implications for the development of conservation strategies for these populations. Additionally, the study showed that of the 22 markers developed, 15 SSRs amplified for *A. manabiensis*, and 21 for *A. conica*, respectively. This result suggests that the markers are transferable and with the selection of the loci that amplify for each species, may be used to carry out genetic diversity studies in other Annonas species. These cross-amplifications of the *A. deceptrix* loci in other Annonaceae are related to other similar studies, as is the case of the annonas of the *Greenwayodendron* genus, where nuclear markers were isolated to determine cross-amplification in different species within of this genus (Piñeiro et al. 2016) as well as developed markers for *Annona cherimola*, which were transferable to related taxa (Escribano et al. 2008).

CONCLUSION

Using Nextera platform, 22 microsatellite markers specific were development for *A. deceptrix*, they were mostly polymorphic, and two other species of the genus Annona. They proved to be reproducible and suitable as molecular markers for population genetics, genetic structure and genetic variability studies. However, further research above diversity studies is needed considering all populations to elucidate the genetic makeup of the Annona species in question and to understand the evolutionary processes that have shaped their genetic diversity.

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