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Evaluation of genetic variability in *Crataegus ×macrocarpa* using ISSR markers for the selection of high-value forms for breeding purposes

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INTRODUCTION & AIM

Hawthorns (*Crataegus* L., *Rosaceae*) possess numerous health-promoting and medicinal properties affecting the circulatory system. It has been known in phytotherapy since the late 19th century. Currently, hawthorn inflorescences and fruits are considered important herbal raw materials widely used in the prevention of coronary heart disease, cardiac disorders, and in the stabilization of blood pressure. Hawthorns are difficult to identify due to their high variability, intense hybridization, and the phenomena of introgression and polyploidization. It's also important that hawthorn raw material is still largely obtained from the wild, from various, often unrecognized taxa. High morphological variability and the presence of many spontaneous hybrids make it necessary to evaluate such diverse plant material from a genetic perspective.

Fig. 1. *C. ×macrocarpa* genotype D-1

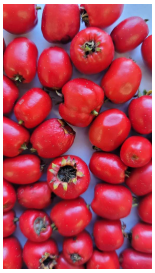

Fig. 2. Fruits of *C. ×macrocarpa* genotype E-12

The study aimed to preliminarily assess the differentiation of native hawthorns in Poland using ISSR markers on samples obtained from the collection of the Garden of Medicinal Plants in Plewiska near Poznań (Western Poland, INF&MP-NRI). In this work, we present our findings on the natural hybrid *Crataegus ×macrocarpa* Hegetschw. (Fig. 1), which is considered suitable for cultivation due to its large fruits. The variation in fruit shape and sepal arrangement in the *C. ×macrocarpa* hybrid is shown in Fig. 2.

METHODS

DNA extraction

Total genomic DNA (gDNA) was isolated from 7 hawthorn populations, 6 samples each, collected in the Garden of Medicinal Plants in Plewiska (INF&MP-NRI). Fresh plant material (0.1 g) was used for isolation and subjected to automatic homogenization using metal beads and a grinding frequency of 30 for 1 min. Mechanical homogenization was performed using the Millmix20 DOMEL device (Slovenia). The ground plant material was subjected to cell lysis with the use of 20 µl of proteinase K enzyme (A&A Biotechnology, Poland), followed by incubation at 65°C (ThermoMixer, Eppendorf) and centrifugation for 2 min at 10,000 rpm (Eppendorf). The obtained supernatant was applied to MagnifiQ™ 16 Genomic DNA instant kit plates and automated DNA isolation was performed. DNA extraction was performed using the Auto Pure Mini automated RNA/DNA isolation system from A&A Biotechnology. Modern automatic systems for nucleic acid isolation (robotic station) allow for obtaining homogeneous gDNA preparations. After isolation, the genetic material was suspended in 100 µL of TRIS dilution buffer and subjected to quantitative and qualitative spectrophotometric analysis using the NanoDrop2000 spectrophotometer (Eppendorf), then stored at -80°C.

Inter-simple sequence repeat (ISSR) analysis

Eleven primers according to Table 1 were used to perform the ISSR-PCR reaction. The 25µL PCR reaction mixture used in the experiments contained: PCR Mix (A&A Biotechnology, Poland) with 20 ng DNA, 200 µM of each dNTP, 1.0 U of polymerase, 1x PCR buffer and 1 µM of each ISSR primer (Genomed, Poland). Amplifications were performed in a PCR Thermocycler Mastercycler® (Ependorff, Germany) and the cycling conditions were as follows: 94°C for 5 min for initial denaturation, then 94°C for 60 s, 44/49°C for 60 s and 72°C for 2 min for 45 cycles, followed by a final extension step at 72°C for 10 min. After amplification, each PCR reaction was analyzed by electrophoresis on a 1.5% agarose gel with Midori Green Advance (Nippon Genetics, Germany), visualized under UV light, scanned, and analyzed on a Bio-Doc-ItTM Imaging System transilluminator (UVP, UK). The sizes of the PCR products were compared with the Gene Ruler Plus DNA Ladder (Thermo Fisher Scientific, USA). Two independent replicates were performed to confirm the re-producibility of the results. Well-defined bands were scored as present or absent for the ISSR analyses. Data were scored as “1” for the presence and “0” for the absence of DNA bands in each of the tested samples. Based on the results of molecular markers data we evaluated the genetic diversity parameters: number of alleles detected from each marker, number of examined genotypes, number of different bands in each primer, number of polymorphic bands in each primer, total number of bands in the gene pool and frequencies of alleles. The following information uploaded on the software DEMoMa (Determination of Effectiveness of Molecular Markers) application to generated indices of polymorphism: PIC (Polymorphic Information Content), EMR (Effective Multiplex Ratio), M1 (Marker Index) and RP (Resolution Power) [1].

RESULTS & DISCUSSION

One of the most commonly used molecular techniques for determining genetic variation between plants are ISSR (Inter-simple sequence repeat) markers. ISSR analysis, due to high annealing temperature and longer primer sequence, can give reliable and repeatable results at relatively lower costs compared to other genetic markers. In the literature, there are reports on the use of ISSR markers for assessing genetic diversity and population structure of *Crataegus* [2-3]. In our study, each primer used produced a unique set of amplification products.

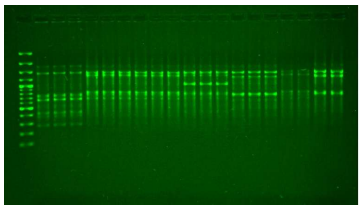

Fig. 3. ISSR profile of *C. ×macrocarpa* for UBC840 starter. Path: 1 - Gene ruler plus size marker; 2 - 20 selected hawthorn genotypes

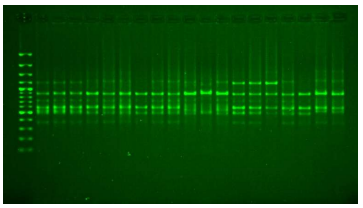

Fig. 4. ISSR profile of *C. ×macrocarpa* for UBC818 starter. Path: 1 - Gene ruler plus size marker; 2 - 20 selected hawthorn genotypes

The number of bands generated and the approximate size of the amplified products were dependent on the primer used but also on the *C. ×macrocarpa* genotype analysed (Table 1).

Table 1. Polymorphism analysis using the ISSR method for *C. ×macrocarpa* genotype

No.	Tm [°C]	Primer	Genotype Sequence [5'-3']	*C. ×macrocarpa* TNB	ROA	PB	MB	PP	PIC	EMR	MI	RP
1	44	ISSR14	ATGATGATGATG ATGATG	7	1500-300	6	1	85.71	0.49	5.14	2.53	6.11
2		UBC825	ACACACACACAC ACACT	9	1000-300	8	1	88.89	0.46	7.11	3.29	6.53
3		857	ACACACACACAC ACACyA	7	1500-400	4	3	57.14	0.47	2.29	1.06	5.16
4		W1-UBC	CACACACACACA CAGT	6	1200-400	5	1	83.33	0.37	4.17	1.54	2.95
5		W2-UBC	CACACACACACA CAAT	7	1500-400	6	1	85.71	0.21	5.14	1.09	1.68
6		1-PASS	GAGAGAGAGAG ACC	7	900-300	3	4	42.86	0.42	1.29	0.54	4.21
7	49	ISSR13	CTCTCTCTCTCT CTCTAC	9	2000-400	8	1	88.89	0.48	7.11	3.42	7.26
8		856	ACACACACACAC ACACCTA	7	1500-300	6	1	85.71	0.43	5.14	2.22	4.42
9		UBC840	GAGAGAGAGAG AGAGACTT	10	2000-200	9	1	90.00	0.49	8.10	4.00	8.84
10		UBC818	CACACACACACA CACAG	8	1500-300	6	2	75.00	0.33	4.50	1.50	3.37
11		UBC824	TCTCTCTCTCTC TCTCG	11	1500-300	10	1	90.91	0.49	9.09	4.47	9.58
Total				88		71	88	874.16	4.65	59.08	25.66	60.11
Average				8.00		6.45	4	**79.47**	**0.42**	5.37	2.33	5.46

TNB - total number of bands; ROA - range of amplicons (bp); PB - Polymorphic bands; MB - Monomorpic bands; PP - Percent of polymorphism (%), PIC - polymorphic information content; EMR - Effective multiplex ratio; MI - Marker index; RP - Resolution power, Tm - Annealing temperature

Molecular analysis enabled the generation of 88 bands, both monomorphic and polymorphic, using 11 primers. The total number of bands (TNB) per primer ranged from 6 using primer W1-UBC to 11 using primer UBC824; and the amplification range was on average from 2000 bp to 200 bp. High genetic variability of the genotypes was identified, with an average percentage of polymorphism of 79.47%. The polymorphic information content (PIC) in the genetic variation analysis was on average 0.42, with the highest value being 0.49 for three of the 11 primers used (ISSR14, UBC840, UBC824). These primers are therefore considered the most effective in identifying polymorphisms.

Analysis of the genetic variation of the natural hybrid *Crataegus ×macrocarpa* revealed high levels of polymorphism among the genotypes studied from seven populations. Hybrid populations with increased genetic variation may have a greater potential for faster adaptation to new, changing environmental conditions (e.g., in the face of climate change) or possess other improved traits. These results provide a basis for the identification of species-specific genetic markers and will facilitate the selection of forms with favourable yield-related traits for further breeding and cultivation of hawthorns.

CONCLUSION

The research contributed to understanding the range of variability of native hawthorns and provided valuable data on the genetic profiles of forms with high utility value. This opens up the possibility of further in-depth research, including the identification and search for species-specific markers within the *Crataegus* genus. The obtained results will enable a comprehensive assessment of the genetic variability of species within the *Crataegus* genus, enabling the future propagation of the most valuable genotypes for further comparative studies under growing conditions and potential breeding efforts.

ACKNOWLEDGMENTS / REFERENCES

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