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RAPD, ISSR AND SSR MOLECULAR MARKERS APPLICATIONS IN *Vaccinium* spp.

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Abstract

The consumption of berries on a global level, either from wild or cultivated species, is on an ascending trend, being linked to the fruits' high nutraceutical qualities. Therefore, the demand to create robust cultivars adapted to the various environmental conditions worldwide is becoming higher. In order to reduce the time needed to obtain new cultivars, breeders have started to use more and more molecular methods and techniques. Molecular markers, such as RAPD, ISSR and SSR, specific DNA regions linked to genes responsible for various traits such as colour, shape, taste, firmness, tolerance to biotic and abiotic stresses, are some of the molecular tools used in genotype-assisted breeding programs. The current review presents data related to the use of RAPD, ISSR and SSR molecular markers in Vaccinium species.

Key words*: Vaccinium corymbosum, Vaccinium myrtillus, Vaccinium macrocarpon, Vaccinium ashei, Vaccinium angustifolium, genetic diversity, breeding.*

INTRODUCTION

Nowadays, the consumption of cultivated and wild berries is on the rise due to their high nutraceutical qualities and organoleptic properties (Asănică, 2018; Mudd et al., 2013). Numerous studies demonstrated the effect of berries in treating or preventing various diseases, such as high blood pressure, diabetes and cancer (Afrin et al., 2016; Bouyahya et al., 2022; Golovinskaia & Wang, 2021; Hameed et al., 2020; Wang et al., 2021).

Among the numerous berry fruits, genus *Vaccinium* from Ericaceae Family is very well represented, with 450 species and a worldwide distribution, covering the Globe from the arctic/subarctic area to the tropics (Edger et al., 2022; Kloet & Avery, 2010).

With the evident climate changes, the pressure to faster create new *Vaccinium* cultivars adapted to the present environment conditions is higher, so marker assisted breeding nowadays is a must, as it greatly increases the selection efficiency and reduces the time needed for cultivar release (Iwata et al., 2016; Lobos & Hancock, 2015). Breeding strategies to create new cultivars are greatly supported by the molecular techniques to reduce the time, space and biological materials used in the breeding programs. Currently, molecular markers such as RAPD, ISSR and SSR are some of the molecular tools used for plant breeding (MAS - marker assisted selection), and multiple other purposes in various fields: genetic variability studies, accession identification in collections, germplasm management, checking genetic stability after micropropagation, etc.

Preserving and increasing the genetic variability of the possible genitors' pool is extremely important, as it gives a better chance to find genitor combinations that would ensure the production of new cultivars with traits adapted to the environmental changes and customers' demands. One way to increase this pool is to look into the wild relatives of the cultivated species (Migicovsky & Myles, 2017). Considering the fact that the abundance of some wild relatives of the cultivated blueberry species has declined or become more variable (Hupp et al., 2015; Vega-Polo et al., 2020), is important to preserve these genetic resources *in situ* and *ex situ*, in collections.

Plant collections management also makes use of molecular markers, as they can be utilised to identify duplicates and mislabelled accessions,

especially when there are little differences at morphological level.

Micropropagation technique is used for clonal mass propagation of genotypes. Berry crops are well suited for this technique, as they are heterozygous, thus their genetic characteristics are preserved using vegetative reproduction. However, plants propagated in vitro could still be the object of somatic mutations, so the genetic stability of micropropagated plants can be checked using molecular markers (Debnath et al., 2012).

Current review presents recent data on the use of three types of molecular markers, RAPD, ISSR and SSR for species belonging to *Vaccinium* genus.

RAPD, ISSR and SSR marker development and uses in *Vaccinium*

RAPD markers were first developed to assess DNA polymorphism based on the amplification of random DNA fragments with a single, short (~10 bp) primer with an arbitrary nucleotide sequence (Williams et al., 1990). The technique is simple, cost effective, it does not need prior knowledge of the genome studied, and it can be used for a variety of purposes: estimation of genetic diversity, genetic mapping, germplasm management, monitoring of genetic erosion, cultivar identification, hybrid verification, genetic fidelity testing for *in vitro* grown plants, etc. (Babu et al., 2021). A summary of the RAPD marker uses in *Vaccinium* spp. is presented in Table 1.

Table 1. RAPD marker uses in *Vaccinium* spp.

Species	Use of RAPD marker	Reference
V. macrocarpon	Identification of varietal misclassification; genetic diversity	(Novy et al., 1994)
V. darrowi; V. elliottii	Linkage map	(Rowland & Levi, 1994)
V. ashei	Cultivar identification	(Aruna et al., 1995)
V.darrowi; V. corymbosum	Inheritance mode in interspecific hybrids	(Qu & Hancock, 1995)
V. macrocarpon	Genetic variability	(Stewart & Nilsen, 1995)
V. macrocarpon	Genetic variability	(Stewart Jr. & Excoffier, 1996)
V. corymbosum, V. ashei, V. darrowi	Cultivar identification	(Levi & Rowland, 1997)
V.darrowi; V. corymbosum	Linkage map	(Qu & Hancock, 1997)
V. stamineum	Genetic variability	(Kreher et al., 2000)
V. hiepii	New taxon discovery	(Vander Kloet & Paterson, 2000)
V. cilindraceum	Genetic variability	(Martin-Clemente et al., 2001)
V. vitis-idaea	Genetic variability	(Persson & Gustavsson, 2001)
V. myrtillus	Genetic variability	(Albert et al., 2003)
V. myrtillus	Genetic variability	(Albert et al., 2004)
V. macrocarpon; V. angustifolium; V. vitis-idaea	Cultivar identification	(Debnath, 2005)
V. vitis-idaea	Genetic diversity; Selection for ex-situ conservation	(Garkava-Gustavsson et al., 2005)
V. oxycoccos	Genetic variability	(Areškevičiūtė et al., 2006)
V. macrocarpon	Genetic variability	(Debnath, 2007)
Vaccinium oxycoccos	Genetic variability	(Cesoniene et al., 2013)
V. bracteatum; V. corymbosum	Hybrid confirmation	(Tsuda et al., 2013)
V. corymbosum	Cultivar identification	(Carvalho et al., 2014)
V. padifolium; V. corymbosum	Hybrid confirmation	(Ehlenfeldt & Polashock, 2014)
V. myrtillus; V. uliginosum; V. vitis-idaea	Genetic variability; population dynamics	(Bjedov et al., 2015)
V. corymbosum	Genetic variability	(Wach et al., 2016)
V. corymbosum	Genetic variability	(Gawroński et al., 2017)
V. corymbosum	Genetic stability	(Nowakowska & Pacholczak, 2017)
V. myrtillus	Genetic variability	(Giordani et al., 2018)
V. corymbosum	Genetic stability	(Clapa et al., 2019)
V. myrtillus	Genetic variability	(Nin et al., 2019)

One of the most common uses of the **RAPD technique** is the study of ***genetic diversity*** among cultivars, or within populations in the case of wild species.

Genetic variation in the case of cultivated cranberry (*Vaccinium macrocarpon*) was studied in United States among samples picked from sites in Massachusetts, New Jersey, and Wisconsin, North Carolina, Tennessee, West Virginia, New York, Michigan, USA (Novy et al., 1994; Stewart & Nilsen, 1995; Stewart Jr. & Excoffier, 1996). In Canada, a genetic diversity assessment of 43 wild cranberry clones and 5 cultivars from 4 Canadian provinces was done using the RAPD technique (Debnath, 2007).

Genetic variability of highbush blueberry, *Vaccinium corymbosum*, was assessed in cultivars grown in Poland (Gawroński et al., 2017; Wach et al., 2016).

For wild *Vaccinium* species, genetic variation was studied on local populations of lingonberry, *Vaccinium vitis-idaea,* in Sweden (Garkava-Gustavsson et al., 2005; Persson & Gustavsson, 2001), and Central Balkans (Bjedov et al., 2015), on wild cranberry, *Vaccinium oxycoccos*, in Lithuania (Areškevičiūtė et al., 2006; Cesoniene et al., 2013), on bilberry, *Vaccinium*

myrtillus, in Belgium (Albert et al., 2003, 2004), in Tuscan Apennines, Italy (Giordani et al., 2018), and in Central Balkans (Bjedov et al., 2015), on *Vaccinium stamineum* L. in USA (Kreher et al., 2000), on Azores archipelago endemic *Vaccinium cylindraceum* Smith (Martin-Clemente et al., 2001) and on *Vaccinium uliginosum* L. on Central Balkans (Bjedov et al., 2015).

A genetic diversity assessment study was done also on Tuscan Apennines wild bilberry seedlings to check the preservation of variability following micropropagation, to aid in the species conservation (Nin et al., 2019).

Cultivar identification is another benefit of using RAPD technique. In USA, using 15 rabbiteye blueberry (*Vaccinium ashei* Reade) cultivars and 4 wild selections, Aruna et al. (1995) developed a cultivar key based on 11 RAPD markers, and Levi and Rowland (1997) used RAPD and SSR-anchored primers to identify highbush and rabbiteye blueberry cultivars. In Canada, Debnath (2005) used 22 decamer primers to differentiate genotypes of three *Vaccinium* species: cranberry, lowbush blueberry (*V. angustifolium* Ait), and lingonberry. Carvalho et al. (2014) differentiated northern cultivars types from the southern types of highbush blueberry using RAPD and ISSR markers from fruits and leaves. Going beyond the scope of simply cultivar identification for its own purpose, Tsuda et al. (2013) used RAPD and CAPS markers to confirm the hybrid nature of plants resulted from the crosses of *Vaccinium bracteatum* (♀) and *Vaccinium corymbosum* (♂), and Ehlenfeldt & Polashock (2014), used RAPD markers to confirm the hybrid nature of plants resulted from the crosses of *Vaccinium padifolium* (♀) and *V. corymbosum* (♂). Also, in an earlier study, Qu and Hancock (1995) used the RAPD markers to determine the mode of inheritance and the level of heterozygosity transmitted by 2n gametes in the hybrid plants US75 resulted from crosses of *Vaccinium darrowi* (Florida 4B) and *V. corymbosum* (cultivar 'Bluecrop').

Linkage map construction based on molecular markers is useful to indicate gene loci linked to useful traits such as fruit quality indicators, disease tolerance or abiotic stress resistance. Based on RAPD markers, linkage maps were constructed from a cross between an F1 interspecific hybrid, *Vaccinium darrowi* Camp x *V. elliottii* Chapm, and a *Vaccinium darrowi* plant (Rowland & Levi, 1994), and from a cross between hybrid US75 and *V. corymbosum* cultivar 'Bluetta' (Qu and Hancock, 1997).

Genetic stability of plants propagated ex vitro was assessed on microcuttings of *V. corymbosum*, cultivars 'Bluecrop' and 'Duke'. Genetic stability of the cuttings was not affected regardless of the type of rooting enhancer used (0.2% Goteo, 50 mg/l auxin indole-3-butyric acid IIBA), or Rhizopon AA containing 1% IBA) (Nowakowska & Pacholczak, 2017). In another study, genetic stability of cuttings of *V. corymbosum* cultivars 'Aurora', 'Draper' and 'Liberty,' micropropagated in vitro for 10 subcultures, was tested using RAPD and SRAP markers, revealing no genetic variations (Clapa et al., 2019).

Last but not least, Vander Kloet & Paterson (2000) reported the **discovery of a new taxon**, *Vaccinium hiepii* vander Kloet, sp. nov., following RAPD and morphological assessment.

Microsatellites, or simple sequence repeats markers consisting of repetitions of 1-6 bp DNA sequences, have been used for several decades for assessment of genetic diversity, QTL discovery, marker assisted selection for desired traits (MAS), cultivar DNA fingerprinting, germplasm characterization, genome organization, etc. (Nybom & Lācis, 2021; Taheri et al., 2018).

SSR technique is based on amplifying DNA sequences containing simple sequence repeats by using primer pairs designed from the conserved flanking sequences (Gupta et al., 1996).

If initially the discovery and development of microsatellite loci has been cumbersome, next generation sequencing (NGS) techniques that allowed faster whole genome sequencing and resequencing, greatly increased the easyness of detecting SSRs in plants (Zalapa et al., 2012).

Presently there are four reference genomes publicly available in the National Center for Biotechnology Information (NCBI) database, that can be mined for molecular markers (Table 2).

Table 2. *Vaccinium* reference genomes published to date publicly available in the NCBI database

Species	Genome coverage	Sequencing technology	Reference
V. macrocarpon cv. 'Ben Lear'	100.0x	Oxford Nanopore GridION; Illumina NovaSeq	(Kawash et al., 2022)
V. darrowii F1 hybrid NJ 8807/NJ 8810	64.0x	Illumina; PacBio	(Yu et al., 2021)
V. corymbosum cv. "W8520"	40.0x	454	Direct submission to NCBI
V. myrtillus ecotype "North-Norwegian"	100.0x	Illumina; Oxford Nanopore	Direct submission to NCBI

After sequencing *de novo* the cranberry genome, *Vaccinium macrocarpon*, cultivar 'HyRed', over 100000 SSR loci were detected, with the dinucleotide AG being the most frequent repeat detected (34% of the total SSRs). From the 96 loci tested in 25 cranberry genotypes, 48 proved to be polymorphic (Zhu et al., 2012). Another study on *V. macrocarpon* identified ~700 polymorphic loci located in transcribed and genomic regions, and suggested ~500 loci for genetic diversity and segregation analyses (Schlautman et al., 2015).

For non-model plants in which reference genomes are not yet available, SSR mining in transcriptomes is a viable option (Taheri et al., 2018).

In *Vaccinium corymbosum*, cultivar 'Bluecrop', almost 16000 EST-SSR loci were identified from the leaf, developing fruit, and flower buds at different stages of cold acclimation transcriptomes. Based on these loci, 100 primer pairs were tested for amplification and polymorphism, the results being a 68% amplification rate and a 43% polymorphism rate. Among the SSRs discovered, AG repeats accounted for 38% of the total SSRs (Rowland et al., 2012).

A genetic variability study of 24 populations of *V. macrocarpon* and 21 populations *V. oxycoccos* from United States National Forests using 32 SSRs, revealed over 600 for the first and almost 900 highly heterozygous alleles for the second, identifying a unique population of *V. macrocarpon* outside its native range, and helping decide conservation actions priorities (Rodriguez-Bonilla et al., 2020). A study with a similar purpose was done for the Andean blueberry, *Vaccinium floribundum* Kunth., using 16 SSR to characterize 100 individuals from 27 collection sites from ten provinces in the Ecuadorian Highlands, the analysis yielding 4 genetic cluster, distributed according to their geographic location (Vega-Polo et al., 2020).

Another use for SSR markers is genetic "fingerprinting". Two sets, one with 5 and the other with 10 SSRs containing three nucleotide repeats, were enough to genotype 367 Vaccinium samples from National Clonal Germplasm Repository (NCGR) (Corvalis, Oregon, USA), and confirm the accession identities by detecting true-to-type cultivars, homonyms and synonyms (Bassil et al., 2020).

ISSRs, inter simple sequence repeats, are DNA sequences located between two identical SSRs. **ISSR technique** also uses microsatellites, however, as opposed to SSR technique it is not specific, as it uses for amplification a single primer – the microsatellite itself, usually with a length of 16-25 bp (Pradeep Reddy et al., 2002). The technique is used similarly to RAPD technique, and it has the additional advantage of higher reproducibility, due to the longer primer's size (Grover & Sharma, 2016).

Intra and inter-population genetic diversity of 32 bilberry individuals belonging to populations from Iceland, Norway, Sweden, Finland and Germany, were studied using four ISSR primers (UBC-825, UBC-857, UBC-873 and UBC-881), that amplified 127 polymorphic loci, permitting the identification of 85% of the **genetic variation** within these populations (Zoratti et al., 2015).

(Debnath & An, 2019) used ISSR markers together with EST-SSR and EST-PCR markers to **study biodiversity** within a group of 75 wild cranberry clones, in an attempt to correlate biochemical (antioxidant properties) and genetic clustering. However, clustering differed, probably due to markers' degree of genomic coverage. A similar study, this time of blueberry cultivars and hybrids using three types of SSR markers (EST-SSR, G-SSR and EST-PCR), confirmed the poor correlation between genetic and biochemical data, however some of the markers proved to be associated with antioxidant properties (Bhatt & Debnath, 2021).

EST-PCR, EST-SSR and ISSR markers have also been used to monitor and **confirm clonal fidelity** of micropropagated lingonberry plants (Arigundam et al., 2020). ISSR markers have

been used as well **for confirming the hybrid nature** of interspecific hybrids of *V. uliginosum* × *(V. corymbosum* × *V. angustifolium*) propagated *in vitro* (Erst et al., 2021).

CONCLUSIONS

Molecular markers such as RAPD and microsatellites have been employed for decades to facilitate plant breeding, wild species conservation efforts, plant collections management, micropropagation industry, and much more. The advent of next generation sequencing made easier the discovery and development of novel markers based on microsatellites, especially in the light of affordable resequencing of genotypes for which there are reference genomes available, and this is the case in the present for four *Vaccinium* species. However, for those species that are not yet sequenced, RAPD markers are still an option, as they are easy to use and relatively not expensive.

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