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Determination of *Sambucus* Interspecific Hybrid Structure using Molecular Markers

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ABSTRACT

Phenotypic and genotypic variations within the genus *Sambucus* are limited. They could be efficiently increased by genetic recombination involving different species. The aim of the presented investigation was to assess the possibility of using molecular approach (i.e., microsattelites) in determination of unknown hybrid structures. The study involved 47 *Sambucus* genotypes (parental species and intespecific hybrids), and six microsatellite loci were analysed. The clustering method grouped the analysed genotypes into four main groups. The first main group involved two sub-groups: one with taxons and hybrids involving *S. racemosa* (in broad sense) and the other involving two botanical varieties of *S. nigra.* In the second main group, there was a sub-group involving hybrids between *S. javanica* and *S. nigra*, and a sub-group involving F_1 hybrids between *S. javanica* and *S. ebulus.* The third main group contained a sub-group with hybrids between *S. javanica* and *S. nigra* 'Black Beauty', a sub-group with hybrids involving *S. javanica*, *S. nigra* and *S. racemosa* (*miquelii*), a sub-group with backcrosses *S. javanica* × (*S. javanica* × *S. ebulus*), and un unknown hybrid. The fourth main group included a sub-group with F_1 hybrids *S. javanica* × *S. ebulus,* a sub-group involving various taxons of *S. racemosa* (in broad sense), and a sub-group involving hybrids between *S. cerulea* and *S. javanica,* with and without *S. nigra.* Our study shows that molecular analysis can be helpful in determining some of the unknown but simple interspecific hybrids of *Sambucus*. In the cases of complex hybrid combinations, the use of SSRs is most probably not the best solution.

Key words: *Sambucus*, SSR markers, interspecific hybrids, clustering method, molecular markers

INTRODUCTION

Elderberries belong to the genus *Sambucus* and the family Adoxaceae (Bolli,1994). Since ancient times, they have been considered as very useful, especially as medicinal and food plants. The most valuable are their fruits and inflorescences, although useful substances can be found also in roots, bark, leaves and shoots (Shokrzadeh et al., 2009, Atkinson and Atkinson, 2002, Charlebois et al., 2010, Vlachojannis et al., 2010, Mikulič-Petkovšek et al., 2015a, Mikulič-Petkovšek et al., 2015b, Todorović et al., 2017). Elderberries can be found in almost all regions of the world, except deserts and extremely cold areas. They grow as small deciduous trees or shrubs of various shapes, or herbs characterised by pinnately compound leaves borne oppositely along stems, and flat to roundish clusters of small white-yellowish or sometimes pinkish-purple flowers and, during maturity, by small brown-black, blue, red, orange or yellow berries (Fernald, 1950, Bolli, 1994). The taxonomy of the genus *Sambucus* is highly sophisticated due to enormous phenotypical diversity and geographical distribution. The genus includes from less than 10 to more than 30 species, depending on the taxonomical approach. Von Schwerin (1920) recognised 28 species and several varieties. R. Bolli (1994), in his PhD dissertation, reduced the number of species to only nine: *Sambucus ebulus* L., *S. wightiana* Wall. ex Wight et Arnott, *S. adnata* DC., S.

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gaudichaudiana DC., *S. australasica* (Lindley) Fritsch, *S. javanica* Blume, *S. nigra* L., *S. australis* Cham. et Schlecht. and *S. racemosa* L. Several, previously independent species became subspecies or botanical varieties. Five taxa, formerly considered as distinct species (i.e., *S. canadensis*, *S. cerulea*, *S. peruviana*, *S. maderensis* and *S. palmensis*, are now being considered as subspecies within *S. nigra*. His reduction was most probably too drastic and probably needs several corrections (Applequist, 2015). Proper taxonomy is especially important for breeders conducting interspecific crosses.

Following the traditional taxonomy, the most important species are *S. cerulea* Raf. (blue elder), *S. javanica* (Javanese or Chinese elder), and *S. nigra* (common or black elder/ elderberry). All elderberries are characterised by extremely small flowers, forming relatively dense inflorescences. Due to small sizes of flowers (when they are ready for emasculation, they measure 1.8-2.2 mm in diameter), artificial hybridisation of elderberries is very difficult. The emasculation usually takes place late in the afternoon, one day before anthesis, or in early hours in the following morning, when the flowers are still closed. The breeder has to remove all (5) anthers, together with corolla, without damaging the pistil. The pollination usually takes place immediately after all selected flowers of an inflorescence have been emasculated and other flowers carefully removed. For pollination, one can use fresh or properly stored pollen. To promote faster germination of pollen, low concentrated sugary–water solutions can be used. The pollinated inflorescences have to be protected from uncontrolled pollination by semi-transparent bags, which are removed after approximately a week (Ivančič, personal experience).

Elderberry breeding is relatively new and most of the present cultivars are probably direct selections from locally grown populations, or hybrids between locally grown genotypes. To our knowledge, most of the breeders have been using intraspecific hybridisation in order to create genetic variation. Interspecific hybridization, so far, has been rarely used. The earliest systematic and documented attempts to produce hybrid plants were reported by Böcher (1941), Winge (1944), Chia (1975), Koncalová et al. (1983) and Nilsson (1987). The main problems were difficulties in hybridisation technique, low number of successful fertilisations and sterility among progenies. Higher level of fertility was achieved when using closely related species, e.g., *S. nigra* × *S. canadensis*, compared to the cross between *S. nigra* and *S. ebulus.*

The large scale elderberry breeding programme based on interspecific hybridisation, at the Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia, began in 2003 by the lead author of this article. As elderberries, in Slovenia, are not considered to be important fruit bearing plants, the programme has never been officially funded. The first experimental crosses based on normal hand emasculation technique took place several years earlier. Those crosses were based on classical manual emasculation and resulted in a limited number of successful interspecific hybrids. In 2001, a self-incompatible genotype of *S. javanica* was introduced from the Island of Espiritu Santo, Vanuatu, and two years later, included in the hybridisation programme. Due to its almost complete self-incompatibility, emasculation of the female parent was not necessary.

Our hybridisation programme involved 5 crucial species (*S. cerulea, S. javanica, S. ebulus, S. nigra, S. racemosa*) and two botanical varieties (S. *nigra* L. var. *laciniata* L., *S. nigra* L. var. *viridis* Weston). *S. racemosa* was represented by various taxons which had been previously recognised as species, but in the revised taxonomy of Bolli (1994) they are all included to *S. racemosa* (i.e., *S. koreana, S. miquelii, S. sibirica, S. tigranii*). The two above mentioned botanical varieties are probably the oldest botanical varieties of elderberries documented in the literature. The scientific name of *S. nigra* subsp. *nigra* var. *laciniata* (parsley-leaved or cut-leaved variety) appeared for the first time in Species Plantarum (Linaeus, in 1753, vol.1, p. 270). Few years later, it was also mentioned by Richard Weston (1775), p. 39, who was also the author of the scientific name of the *S. nigra* subsp. *nigra* var. *viridis*. For this variety, he also used the common name 'green-berried elder tree'. Parsley-leaved genotypes were also described in other species (or subspecies), e.g., *Sambucus canadensis* L. var. *laciniata* A. Gray. They were also observed among our interspecific hybrids (Ivančič, personal observations).

The main objective of this programme was to recombine the most important positive morphological and physiological characteristics of *S. cerulea, S. javanica, S. ebulus, S. nigra* and *S. racemosa.* The final aim was to create dwarf or semi-dwarf, herbaceous or semi-herbaceous plants which would enable plantations with a higher density of plants, simple maintenance and mechanical harvest (Simonovik, 2007, Simonovik et al., 2007). *S. ebulus* was considered as a source of genes for dwarf herbaceous growth, while *S. nigra* and *S. cerulea* were representing the main genetic resources for productivity and desired chemical composition of fruits and inflorescences. Because of dwarf herbaceous growth harvesting would be easy and pruning would not be needed. At the beginning of each growth season, plants would be cut to the ground (mulched) and new stems would regrow from underground shoots.

S. javanica from Espiritu Santo, due to its tropic (or sub-tropic) origin, was mainly used as a source of self-incompatibility. Later, additional genotypes of this species were introduced from Africa, but they were not self-incompatible. New reports (e.g., Wenga et al., 2019), however, indicate that stem ethanol extracts of *S. formosana* Nakai (which is one of the synonyms of *S. javanica*) could be a valuable source of substances fighting human coronavirus NL63 (HCoV-NL63), one of the main circulating HCoVs worldwide, which causes difficulties in respiratory tract such as runny nose, cough, bronchiolitis and pneumonia.

The breeding programme at the Faculty of Agriculture and Life Sciences followed the scheme of modified phenotypic recurrent selection adapted to specific characteristics of different elderberry species. The programme was divided in cycles and each cycle consisted of three phases: (1) genetic recombination of selected genotypes, (2) morphological and chemical evaluation of the offspring individuals and (3) selection of superior individuals for new genetic recombinations (crosses) in order to obtain a new generation of hybrids, belonging to a new cycle. The number of hybrid combinations per cycle varied between 100 and 120, while the number of seeds per cycle generally exceeded 4,000. The average germination rate was approximately 45% and

individual cycles were completed within 4 to 6 years (Ivančič, unpublished).

Chemical analyses of the obtained interspecific hybrids and their parental species revealed tremendous variations of chemical composition and many of the hybrids were superior when compared to their parental species (Mikulič-Petkovšek et al., 2014, Mikulič-Petkovšek et al., 2015a, Mikulič-Petkovšek et al., 2015b, Mikulič-Petkovšek et al., 2016, Todorović et al., 2017, Imenšek et al., 2020). The first molecular evaluation of hybrids (i.e., hybrid origin) took place in 2006 and were published by Simonovik et al. (2007) and was based on the sequenced cpDNA region, genome size differences and PCR–RFLP analysis of the nrDNA ITS region.

At the beginning (in the first cycle), molecular evaluation of the interspecific structure was not necessary. The hybrids had several intermediate characteristics and used to be more winter hardy when compared to the tropical female parent (*S. javanica*). It was not difficult to see the differences in growth types, leaf shapes and inflorescences. The examples were the hybrids *S. javanica* × *S. ebulus, S. javanica* × *S. cerulea, S. javanica* × *S. racemosa* and *S. javanica* × *S. nigra.* The difficulties, however, arose later when complex interspecific hybrids were formed. The hypothetical example can be: ((*S. javanica* × *S. cerulea*) × (*S. javanica* × *S. racemosa*)) × ((*S. cerulea* × *S. nigra*) × (*S. javanica* × *S. nigra*)). This complex hybrid involves 4 species and it is practically impossible to determine its hybrid structure using morphological traits. Following the comments published by Applequist (2015), we consider *S. cerulea* as an independent species.

The main objective of this paper was to check the possibility of using SSRs for determining the complex hybrid structure in various complex elderberry interspecific hybrids. We assumed that molecular markers could probably be very helpful for finding out which species were incorporated in an unknown, complex interspecific hybrid. The exact hybrid structure (i.e., in which way individual species were incorporated in the hybrid structure) most probably could not be defined.

MATERIALS AND METHODS

Plant material (elderberry genotypes)

The list of the species, varieties and interspecific hybrids is presented in Table 1. Samples of all analysed hybrids were collected in May 2016 from plants growing in the Plant Gene Bank of the Faculty of Agriculture and Life Sciences of the University of Maribor. Samples of the majority of parental species (i.e., *S. cerulea, S. javanica,* two genotypes of *S. ebulus*, two genotypes of *S. nigra* and *S. racemosa* - including its taxons named as *koreana*, *miquelii, sibirica*, *tigranii*), however, were collected in SE Slovenia where the initial hybridisation had taken place. Almost all hybrid plants and some of the parental genotypes, originated directly from seed. The exceptions were two botanical varieties (*S. nigra* var. *laciniata*, *S. nigra* var. *viridis*) and ornamental cultivar 'Black Beauty' (BB)), all belonging to *S. nigra*. There was no clonal multiplication of hybrids; the only exception was JA×CER No3 which had been clonally propagated for experimental purposes. This hybrid was found to have several very useful and attractive traits such as medium height, high yield, large infructescences, stable wine-red colour of juice, better taste when compared to black elderberry, pleasant odour of inflorescences (different from other genotypes) and plants were less affected by aphids. The analysed hybrids originated from the first three cycles of crossings (first three cycles of the modified phenotypic recurrent selection). In each cycle, the selection of the parental material for the following series of crosses was based on plant vigour, growth characteristics, and fruit and floral characteristics (fruit size and colour, taste and chemical composition). Since the parental material used in interspecific crosses was assumed to be highly heterozygous, each offspring individual originating from the same cross most probably represented a different genotype. This means that two or more hybrids with the same hybrid structure (e.g., JA×(JA×EB), Table 1) should be considered as genetically different.

Molecular analysis

DNA isolation

DNA was extracted from fresh, young leaves using the CTAB protocol (Doyle and Doyle, 1987). To approximately 2-3 square centimetres of fresh leaf tissue, one ml of preheated (68 °C) CTAB extraction buffer [2 % (w/v) CTAB, cetyltriammonium bromide (Sigma), 1.4 NaCl, 20 mM EDTA, 100mM Tris-HCl (pH 8.0), 0.2 % 2-mercaptoethanol] was added and well homogenized with a mortar and pestle and transferred to a 2 ml tube. Samples were incubated for 1.5 h at 68°C in a water bath. After incubation, 600 µl of chloroform: isoamyl alcohol in a 24:1 proportion were added, and the samples were thoroughly mixed. The mixtures were centrifuged at 11.000 rev./min for 10 min. After centrifugation, the supernatant was transferred to a fresh 1.5 ml tube and the DNA was precipitated by the addition of 0.1 vol. of 3 M sodium acetate and 1 vol. of ice cold isopropanol and kept at –20 °C for 30 min. Samples were again centrifuged at 11.000 rev./min for 10 min. The pellet was washed in 70 % ethanol for 20 min, air dried and rehydrated in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA concentration was estimated by DNA fluorometer (Hoefer, TKO 100). Two replicate extractions per sample were performed.

Microsatellites

Six microsatellite loci (Table 2) developed earlier by Clarke and Tobutt (2006) were used: EMSn002, EMSn003, EMSn010, EMSn019, EMSn023 and EMSn025. Ten µl of PCR mixture contained 2 ng DNA (0.5 µl), 5 µl Qiagen Master Mix Kit, 0.5 µl of each primer (forward and reverse), 2 µl H_2O and 1.5 µl Q solution. PCR condition consisted of an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, Ta (different annealing temperatures for different loci) for 60 s, and 72 °C for 60 s, and a final step 10 min at 72 °C. The annealing temperatures were different for the loci EMSn019, EMSn002, EMSn003 (Ta = 60°C), EMSn010 (Ta = 59°C) and EMSn023, EMSn025 (Ta = 58 °C). The polymerase chain

reaction (PCR) was performed using a Whatman Biometra T-Gradient thermocycler (Goettingen, Germany). Capillary electrophoresis of PCR products was performed on Beckman Coulter CEQ8000 according to manufacturer's instructions. Fragment size analysis was done with the in-build software. A fluorescent-labeled size marker (Beckman Coulter DNA Size Standard Kit 400 bp) was used as a molecular weight reference.

Data analysis

All unambiguous fragments were scored for the presence (1) or absence (0) of each band. Only clear and reproducible fragments were taken for data analysis. The binary data matrix was used to calculate Dice's similarity coefficients (Dice, 1945). Values of Dice's coefficients are between 0 (there is no common band) and 1 (two genotypes have identical markers, so they are identical). Dice similarity coefficients were calculated using the DARWIN computer package (Perrier and Jacquemond-Collet, 2006). For each microsatellite locus, the number of alleles per locus (n), allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E) and polymorphic information content (PIC) were calculated using the Cervus 3.0.7 computer program (Marshall et al., 1998; 2014 version). The average distance between pairs of accessions was obtained by taking into account microsatellite data and a neighbor-joining tree was constructed using the DARWIN computer package (Perrier and Jacquemond-Collet, 2006). A matrix of Dice similarity coefficients was used for assessing relationships among 47 genotypes using the neighbour-joining algorithm developed by Saitou and Nei (1987).

Table 1: Plant material included in molecular analysis

Sample No.	Plant Material	Abbreviation
131	*S. racemosa*	RAC
132	*S. nigra* 1	NI 1
133	*S. miquelii*	MIQ
134	*S. javanica* 1	JA 1
136	*S. nigra* 2	NI 2
137	*S. cerulea*	CER
138	*S. tigranii*	TIG
139	*S. koreana*	KOR
140	*S. nigra* 3	NI 3
141	c.v. Black Beauty	BB
142	JA×EB 9	JAEB 9
143	JA×EB 10	JAEB 10
144	(JA×NI)×VIR	JANI/VIR
145	JA×VIR	JAVIR
146	(JA×CER)×(MIQ+TIG)[a]	JACER/(MIQ+TIG)
147	JA×MIQ 1	JAMIQ 1
148	JA×TIG	JATIG
150	(JA×NI)×(RAC+TIG) 1	JANI/(RAC+TIG) 1
152	(JA×NI)×BB 3	JANI/BB 3
153	JA×CER	JACER
155	(JA×NI)×(CER+MIQ)	JANI/(CER+MIQ)
156	(JA×NI)×(RAC+TIG) 2	JANI/(RAC+TIG) 2
157	JA×MIQ 2	JAMIQ 2
158	(JA×NI)×MIQ	JANI/MIQ
162	JA×MIQ 3	JAMIQ 3
165	*S. javanica* 2	JA 2
166	Hybrid 5	Hyb 5
167	Hybrid 3	Hyb 3
168	*S. nigra* var. *laciniata*	LAC
170	Hybrid 8	Hyb 8
171	Hybrid 4	Hyb 4
172	NI(Bg)	NI(Bg)
173	Hybrid 2	Hyb 2
174	Hybrid 6	Hyb 6
175	Hybrid 7	Hyb 7
176	Hybrid 1	Hyb 1
177	Hybrid 9	Hyb 9
179	*S. ebulus* 2	EB 2
180	*S. javanica* 3	JA 3
182	JA×EB 7	JAEB 7
183	JA×(JA×EB) 1	JA/JAEB 1
184	JA×(JA×EB) 2	JA/JAEB 2
186	JA×(JA×EB) 4	JA/JAEB 4
187	JA×(JA×EB) 5	JA/JAEB 5
194	JA×CER No. 3	JACER No. 3
195	(JA×NI)×CER	JANI/CER
197	*S. sibirica*	SIB

Note: a - (JA×CER)×(MIQ+TIG) means (*S. javanica* × *S. cerulea*) × (*S. racemosa – miquelii* + *S. racemosa – tigranii*); + is indicating pollination with a mixture of pollen of 'MIQ' and 'TIG'. The names *S. nigra* (NI), *S. cerulea* (CER) and *S. racemosa* (RAC) correspond to the names *S. nigra* subsp. *nigra*, *S. nigra* subsp. *cerulea* and *S. racemosa* subsp. *racemosa,* respectively, according to the revised classification of Bolli (1994). *S. racemosa* subsp. *racemosa* also includes the taxa named as "*miquelii*" (MIQ), "*sibirica*" (SIB) and "*tigranii*" (TIG). The taxon "*koreana*" (KOR) is included in *S. racemosa* subsp. *kamtschatica. S. javanica* (JA) was represented by 3 genotypes. BB = *S. nigra* ´Black Beauty´; LAC = *S. nigra* subsp. *nigra* var. *laciniata;* VIR = *S. nigra* subsp. *nigra* var. *viridis*; Bg = plants growing in the Maribor University Botanical Garden; Hyb = interspecific hybrids having partly known or unknown parental species.

RESULTS AND DISCUSSION

The number of alleles (Table 2) detected per locus ranged from 11 (EMSn025) to 18 (EMSn010, EMSn019), with an average of 15.17 alleles per locus. The observed heterozygosity ranged between 0.511 (locus EMSn023) and 0.787 (locus EMSn010), with an average of 0.656. The expected heterozygosity ranged between 0.791 (locus EMSn023) and 0.901 (locus EMSn003), with an average of 0.850. The differences between the observed and expected heterozygosity were observed on all loci. The largest difference was observed on locus EMSn003 (0.284) and the lowest on locus EMSn010 (0.084). The number of microsatellite markers sufficient for reliable variety identification depends on the nature and discriminating power of each marker (Tessier et al., 1999), normally six markers are sufficient for differentiating between genotypes (Zulini et al., 2002).

Table 2. SSR loci analyzed and parameters of genetic variability calculated for different microsatellite loci of the 47 *Sambucus* genotypes: number of alleles (n), effective number of alleles (n_e), observed (H_o) and expected (H_e) heterozygosity, and polymorphic information content (PIC)

Locus	n	n_e	H_o	H_e	PIC
EMSn002	15	8.87	0.702	0.838	0.809
EMSn003	16	9.22	0.617	0.901	0.882
EMSn010	18	7.21	0.787	0.871	0.850
EMSn019	18	9.00	0.745	0.898	0.880
EMSn023	13	4.60	0.511	0.791	0.758
EMSn025	11	4.86	0.574	0.803	0.774
Average	**15.167**	**6.79**	**0.656**	**0.850**	**0.825**

The reliability of microsatellite markers in genotyping of varieties was determined on the basis of the following criteria: the complexity of the banding pattern, the amplification of quality PCR products, the stability of the microsatellite repeated structure, and the polymorphic information content of markers. On the basis of PIC values, all the microsatellite loci were classified as very informative loci (PIC > 0.5), and all loci proved suitable for mapping (PIC > 0.7).

The allele sizes, frequencies and variability parameters calculated for each locus are shown in Table 3.

Table 3. Allele size (bp) and allele frequencies (in parenthesis) of the 47 *Sambucus* genotypes. at six microsatellite loci

	Locus						
Alelles		EMS n002	EMS n003	EMS n010	EMS n019	EMS n023	EMS n025
	1	70 (0.170)	135 (0.064)	189 (0.021)	142 (0.043)	182 (0.011)	127 (0.011)
	2	100 (0.255)	138 (0.011)	193 (0.032)	187 (0.021)	186 (0.053)	142 (0.096)
	3	110 (0.011)	191 (0.138)	245 (0.021)	189 (0.160)	218 (0.011)	161 (0.117)
	4	121 (0.011)	193 (0.149)	248 (0.085)	191 (0.213)	249 (0.192)	165 (0.032)
	5	253 (0.032)	195 (0.106)	250 (0.287)	193 (0.043)	253 (0.383)	179 (0.032)
	6	255 (0.011)	197 (0.053)	253 (0.096)	197 (0.021)	255 (0.032)	183 (0.032)
	7	265 (0.021)	200 (0.181)	260 (0.170)	201 (0.043)	258 (0.011)	185 (0.043)
	8	273 (0.011)	205 (0.011)	268 (0.021)	203 (0.085)	262 (0.138)	193 (0.383)
	9	275 (0.128)	209 (0.011)	270 (0.032)	207 (0.096)	264 (0.032)	195 (0.096)
	10	277 (0.043)	213 (0.096)	272 (0.043)	211 (0.021)	266 (0.096)	199 (0.149)
	11	279 (0.032)	216 (0.043)	276 (0.032)	215 (0.011)	268 (0.011)	210 (0.011)
	12	281 (0.021)	219 (0.032)	280 (0.032)	219 (0.011)	270 (0.021)	/
	13	283 (0.234)	221 (0.053)	287 (0.032)	221 (0.011)	293 (0.011)	/
	14	298 (0.021)	264 (0.021)	298 (0.021)	227 (0.032)	/	/
	15	/	282 (0.021)	300 (0.011)	229 (0.117)	/	/
	16	/	283 (0.011)	305 (0.011)	231 (0.032)	/	/
	17	/	/	309 (0.011)	233 (0.032)	/	/
	18	/	/	320 (0.043)	242 (0.0119	/	/
	Σ	14	16	18	18	13	11

The molecular analysis indicates that the dendrogram (Fig. 1) encompasses four main groups of genotypes. The first main group involves two sub-groups. The first sub-group includes five taxons which, according to Bolli (1994), belong to *S. racemosa* (i.e. *S. koreana* (KOR), *S. miquelii* (MIQ), *S. racemosa* (in narrow sense - RAC), *S. sibirica* (SIB) and *S. tigranii* (TIG)), and three interspecific hybrids. The interspecific hybrid JANI/(CER+MIQ) (the accession 155), which is the result of pollination with a mixture of CER and MIQ pollen, is obviously JANI/MIQ. For the hybrid JACER/(MIQ+TIG)) (the accession 146), it is not possible to find out which of the parents (MIQ or TIG) participated in fertilisation. Following Bolli (1994), we can write JACER/RAC (meaning RAC in a broad sense). The second sub-group involves LAC (*S. nigra* subsp. *nigra* var. *laciniata*), two undetermined hybrids (most probably JANI/LAC or JALAC, and two hybrids involving VIR (*S. nigra* subsp. *nigra* var. *viridis*) – JAVIR and JANI/VIR. In the second main group (Fig. 1), there are two sub-groups. The first one involves NI 1 and its three hybrids, most probably with one of the *S. javanica* genotypes. Considering the background of these two hybrids (they originate from the first two cycles) of crosses, their parental structure is

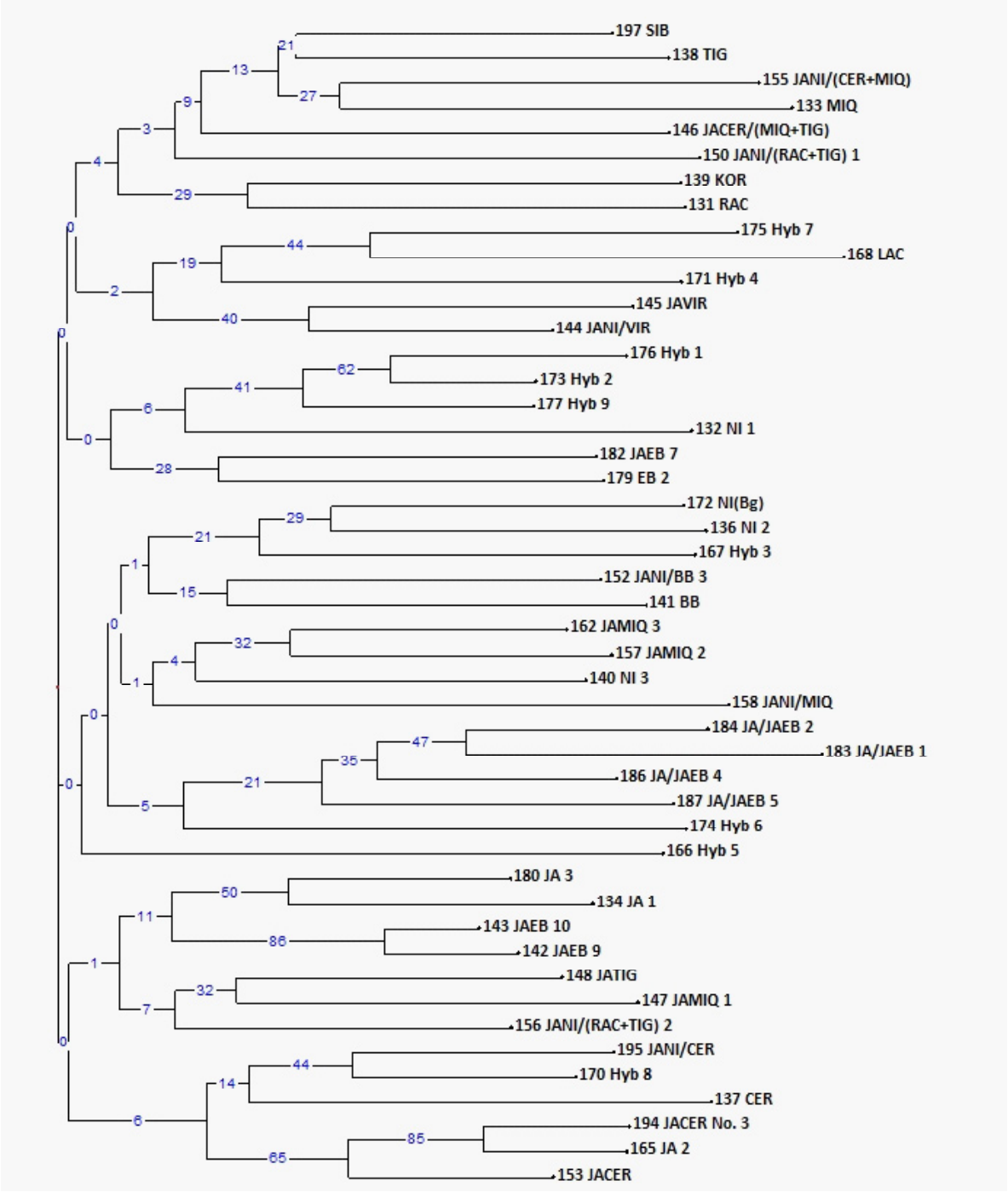

Figure 1: NJ dendrogram describing genetic relationships among 47 *Sambucus* genotypes based on SSR markers

probably JANI or JANI/NI. The second sub-group includes only two genotypes; one accession of *S. ebulus* and its hybrid with one of the genotypes of *S. javanica* (most probably the same JA genotype as in the first sub-group) – JAEB. The third main group contains three sub-groups and one hybrid (the accession 166 – Hyb 5) of which parents remain unknown. The first sub-group encompasses three *S. nigra* genotypes (NI Bg, NI 2, BB), the accession JANI/BB 3 and undetermined Hyb 3 (the accession 167). This vigorous hybrid was probably JANI/NI, involving two different *S. nigra* genotypes. If it was JANI, the plants would be shorter because the genetic influence of (smaller) *S. javanica* parent would be stronger. The second sub-group includes two JAMIQ, one JANI/MIQ and *S. nigra* genotype NI 3. This special accession of NI is probably the reason that the two JAMIQ and JANI/MIQ hybrids are not included in the first main group. The third sub-group includes backcrosses JA/JAEB. The F_1 hybrids originating from crosses *S. javanica* × *S. ebulus* were female sterile, however, they produced some fertile pollen and therefore it was possible to use them as male parental components. The resulting hybrids (JA/JAEB) were partly fertile and morphologically very attractive. The accession 174 (Hyb 6) is most probably JA/JAEB because hybrids resulting from further crosses involving this genotype were, at the time of sampling, not available.

The fourth main group includes three sub-groups. The first contains two accessions of *S. javanica*, both from Vanuatu, and two F_1 hybrids with *S. ebulus* – JAEB. The second sub-group

includes three hybrids: JATIG, JAMIQ 1 and the accession 156 – JANI/(RAC + TIG). As we applied the mixture of *S. racemosa* (in narrow sense) and *S. tigranii* pollen, it is not possible to conclude which of these two taxons participated in the fertilisation. Following the sytematics of Bolli (1994), *S. racemosa* (in broad sense) encompasses also the taxon *S. tigranii* and therefore the hybrid structure should be JANI/RAC. The third sub-group includes two main parental species (*S. cerulea* and one of the *S. javanica* genotypes), F_1 hybrids between *S. javanica* and *S. cerulea,* and two 'three-species' hybrids between F_1 progenies *S. javanica* × *S. nigra* and *S. cerulea* (the undetermined hybrid 170 (Hyb 8) is most probably JANI/CER).

The positions of main groups, sub-groups and individual hybrids in the presented dendrogram (Fig. 1) depend on several factors: (a) accuracy of crosses, including labelling, (b) accuracy of molecular analysis, (c) genetic structure of individual parents, (d) their contribution of nuclear genes to the final hybrid structure (e.g., *S. nigra* in JANI/MIQ contributes 25 % of nuclear genes while in JANI/NI 75 %), (e) complexity of a particular interspecific hybrid (e.g., JAEB vs. JA/JAEB) and (f) genetic uniformity of parental species which are used two or more times in forming a hybrid (e.g., JANI/NI was formed in two steps – F_1 (JANI) in the first year, while JANI/NI two, three or even four years later – usually involving another (completely different) *S. nigra* genotype).

The accuracy is probably the biggest problem in elderberry crosses due to sensitivity and small size of flowers distributed in relatively very dense inflorescences. During the first two years of interspecific hybridisation, when using several plants/genotypes of a particular species (e.g., of *S. nigra* or *S. ebulus*), we documented all essential details about parental components. It was much easier when there was only one genotype of a particular parental species, as it was in the case of S. *cerulea, S. koreana, S. miquelii, S. sibirica* and *S. racemosa* in narrow sense (considering the classical systematics of the genus *Sambucus*). When conducting the first crosses, we also documented the use of substances promoting pollen germination (i.e., different although very low concentrations of honey or sugar) which were used in more than 90 % of successful crosses. In later generations, the number of crosses increased and they became more complex. The labels became too small and we could document only the date of hybridisation, the chemical treatment of stigma (if any) and abbreviations of the parents (NI, EB, JA. etc.). Another problem arising in later cycles of the recurrent selection, when the programme intensified, was a need to extend crossings over a longer period (i.e., from mid-May to the beginning of October). Consequently, we had to look for late and very late flowering parental species. Late flowering *S. ebulus* and *S. nigra* could be found only in shade, on higher altitudes (in our case between 800 and 900 m asl.). Interspecific hybrids were generally characterised by late flowering, and more than half of crosses were conducted in the third decade of August and in September.

Our study shows that molecular analysis can be very helpful in determining some of the unknown interspecific hybrid structures. However, when using different genotypes of the involved parental species, it is very important to record their details. Interspecific hybrids involving only two species should not cause serious problems, as it was demonstrated by Simonovik et al. (2007). The putative interspecific hybrid structure of F_1 hybrids can also be checked phenotypically. In most cases, the comparison of offspring plants with their parental species (especially the female parent) appears to be sufficient to confirm their hybrid nature. The situation, however, can become completely unclear in the case of complex hybrids. One of the examples was already mentioned in Introduction: ((*S. javanica* × *S. cerulea*) × (*S. javanica* × *S. racemosa*)) × ((*S. cerulea* × *S. nigra*) × (*S. javanica* × *S. nigra*)). Such a complex hybrid structure involving four different species probably cannot be resoved neither by molecular nor by morphological approaches.

CONCLUSION

The phenotypic and genotypic variations within the genus *Sambucus* are very limited, especially when considering the traits associated with fruits and inflorescences. The most efficient way to increase the variation is most probably the genetic recombination involving different species. Interspecific hybridisation of elderberries, at the Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia, has a relatively long tradition. It began in 2003, although the first experimental crosses based on normal hand emasculation technique took place several years before. The work became more productive when a self-incompatible genotype of *S. javanica* was introduced from the Island of Espiritu Santo, Vanuatu. Due to almost complete self-incompatibility, emasculation of the female parent was not necessary. The result were numerous hybrids, involving two, three or four elderberry species. The aim of the presented investigation was to assess the possibility of using molecular approach (i.e., microsatellites) in determination of unknown hybrid structures. Samples of analysed hybrids were collected in May 2016 and DNA was extracted from fresh, young leaves. The molecular analysis indicated that the dendrogram consisted of four main groups of genotypes. The first main group involved two sub-groups: one including five taxons belonging to *S. racemosa* (in broad sense), and the other involving *S. nigra* subsp. *nigra* var. *laciniata* and *S. nigra* subsp. *nigra* var. *viridis*. In the second main group, there was a sub-group involning hybrids between *S. javanica* and *S. nigra*, and a sub-group involving hybrids between *S. javanica* and *S. ebulus.* The third main group contained a sub-group with hybrids between *S. javanica* and *S. nigra* 'Black Beauty', a sub-group involving hybrids with *S. javanica*, *S. nigra* and *S. racemosa* (*miquelii*), a sub-group with backcrosses *S. javanica* × (*S. javanica* × *S. ebulus*), and un unknown hybrid. The fourth main group included a sub-group with F_1 hybrids *S. javanica* × *S. ebulus,* a sub-group involving various taxons of *S. racemosa* (in broad sense), and a sub-group involving hybrids between *S. cerulea* and *S. javanica,* with or without *S. nigra.* Our study shows that molecuar analysis can be very helpful in determining some of the unknown, but not complex interspecific hybrid structures. In the cases of complex interspecific hybrid combinations (e.g., complex backcrosses, hybrids involving three or more species, convergent or successive crosses), SSRs were most probably insufficient for the determination the exact hybrid structure.

REFERENCES

1. Applequist, W. L. (2015). A brief review of recent controversies in the taxonomy and nomenclature of *Sambucus nigra* sensu lato. *Acta Horticulturae*, *1061*, 25-33. Retrieved from: https://doi: 10.17660/ActaHortic.2015.1061.1
2. Atkinson, M. D., & Atkinson, E. (2002). *Sambucus nigra* L. – Biological flora of the British Isles. No. 225. *Journal of Ecology*, *90*, 895-923.
3. Böcher, T. W. (1941). Højsommerekskursionen til Brædstrup. Bryrup og Vrads. *Botanisk Tidsskrift*, *45*, 433-439.
4. Bolli, R. (1994). Dissertationes Botanicae Band 223. Revision of the Genus *Sambucus*. J. Cramer, Berlin, Stuttgart.
5. Charlebois, D., Byers, P. L., Finn, C. E., & Thomas, A. L. (2010). Elderberry: botany, horticulture, potential. *Horticultural Reviews*, *37*, 213-280.
6. Chia, C. L. (1975). A chromosome and thin-layer chromatographic study of the genus *Sambucus* L. (Doctoral dissertation). Cornell University. College of Agriculture and Life Sciences. Department of Plant Biology. New York. 50 pp.
7. Clarke, J. B., & Tobutt, K. R. (2006). Development of microsatellite primers and two multiplex polymerase chain reactions for the common elder (*Sambucus nigra*). *Molecular Ecology Notes*, *6*, 453-455.
8. Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, *26*, 297-302.
9. Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*,19, 11-15.
10. Fernald, M. L. (1950). Gray's manual of botany, 8th ed. New York, NY: American Book Company.
11. Imenšek, N., Kristl, J., Sem, V., & Ivančič, A. (2020). Elderberry (*Sambucus* spp.) interspecific hybridization and its impact on fruit oxalates. *Plant Breeding*, *139*(2). Retrieved from: https://onlinelibrary.wiley.com/doi/full/10.1111/pbr.12808
12. Koncalová, M. N., Hrib, J., & Jicínská, D. (1983). The embryology of the *Sambucus* species and hybrids. In Fertilization and Embryogenesis in Ovulated Plants. Proceedings of the VII International Cytoembryological Symposium; Erdelská. O. Ed.; August 1982, Bratislava, Czechoslovakia, 43-47.
13. Marshall, T. C., Slate, J., Kruuk, L. E. B., & Pemberton, J. M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology Notes*, *7*, 639-655.
14. Mikulič-Petkovšek, M., Ivančič, A., Schmitzer, V., Veberič, R., & Štampar, F. (2016). Comparison of major taste compounds and antioxidative properties of fruits and flowers of different *Sambucus* species and interspecific hybrids. *Food Chemistry*, *200*, 134-140.
15. Mikulič-Petkovšek, M., Ivančič, A., Todorović, B., Veberič, R., & Štampar, F. (2015a). Fruit phenolic composition of different elderberry species and hybrids. *Journal of Food Science*, *80*, C2180-C2190.
16. Mikulič-Petkovšek, M., Samoticha, J., Eler, K., Štampar, F., & Veberič, R. (2015b). Traditional elderflower beverages: a rich source of phenolic compounds with high antioxidant activity. *Journal of Agricultural and Food Chemistry*, *63*, 1477-1487.
17. Mikulič-Petkovšek, M., Schmitzer, V., Slatnar, A., Todorović, B., Veberič, R., Štampar, F., & Ivančič, A. (2014). Investigation of anthocyanin profile of four elderberry species and interspecific hybrids. *Journal of Agricultural and Food Chemistry*, *62*(24), 5573-5580.
18. Nilsson, A. (1987). Hybriden mellan fläder och druvfläder funnen i Skåne. *Svensk Botanisk Tidskrift*, *81*, 174-175.
19. Perrier, X., & Jacquemoud-Collet, J. P. (2006). DARwin software. Retrieved from: http://darwin.cirad.fr/darwin.
20. Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*, *4*, 406-425.
21. Shokrzadeh, M., & Saravi, S. S. S. (2010). The chemistry, pharmacology and clinical properties of *Sambucus ebulus*: a review. *Journal of Medicinal Plants Research*, *4*, 95-103
22. Simonovik, B. (2007). Genetic evaluation of interspecific hybrids in the genus *Sambucus* and establishment of selected plant tissue culture techniques in common elder (*S. nigra* L.) (Doctoral dissertation). Biotechnical Faculty, University of Ljubljana, Ljubljana.
23. Simonovik, B., Ivančič, A., Jakše, J., & Bohanec, B. (2007). Production and genetic evaluation of interspecific hybrids within the genus *Sambucus*. *Plant Breeding, 126*(6), 628-633.
24. Tessier, C., David, J., This, P., Boursiquot, J. M., & Charrier, A. (1999). Optimization of the choice of molecular markers for varietal identification in *Vitis vinifera* L. *Theoretical and Applied Genetics*, *98*, 171-177.
25. Todorović, B., Mikulič-Petkovšek, M., Štampar, F., & Ivančič, A. (2017). Phenolic compounds in floral infusions of various *Sambucus* species and their interspecific hybrids. *Turkish Journal of Agriculture and Forestry*, *41*(2), 154-164.
26. Vlachojannis, J. E., Cameron, M., & Chrubasik, S. (2010). A systematic review on the sambuci fructus effect and efficacy profiles. *Phytotherapy Research*, *24*(1), 1-8.
27. von Linné, C. (Caroli Linnaei). 1753. Species plantarum: exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas... Holmiae; Impensis Laurentii Salvii. Retrieved from: https://doi.org/10.5962/bhl.title.669
28. von Schwerin, F. G. (1920). Revisio generis *Sambucus*. *Mitteilungen der Deutschen dendrologischen gesellschaft*, *29*, 194-231.
29. Wenga, J. -R., Linb, C. -S., Laic, H. -C., Line, Y. -P., Wange, C. -Y., Tsaie, Y.-C., Wuf, K. -C., Huangg, S. -H., & Line, C. -W. (2019). Antiviral activity of *Sambucus Formosana Nakai* ethanol extract and related phenolic acid constituents against human coronavirus NL63. *Virus Research*, *273*, 1-8.
30. Weston, R. (1775). The English flora: or, a catalogue of

trees, shrubs, plants and fruits, natives as well as exotics, cultivated, for use or ornament, in the English nurseries, greenhouses and stoves: arranged according to the Linnaean system: with the Latin trivial, and common English names, and an English index referring to the Latin names. Also a general catalogue of seeds for the kitchen-garden, flower-garden, grass-lands etc., usually raised for sale, and those annually imported from America. Printed for the Author and sold by: Millan, J., Robson and Co. New Bond-Street, Carnan, T., St. Paul's Church-Yard, Dilly, E., and C., London.

31. Winge, Ö. (1944). The *Sambucus* hybrid *S. nigra* × *S. racemosa*. Comptesrendus des travaux du laboratoire Carlsberg. *Sér Physiolog*, *24*, 73-78.
32. Zulini, L., Russo, M., & Peterlunger, E. (2002). Genotyping wine and table grape cultivars from Apulia (Southern Italy) using microsatellite markers. *Vitis*, *41*, 183-187.

Določitev strukture medvrstnih križancev iz rodu *Sambucus* z uporabo molekulskih markerjev

IZVLEČEK

Fenotipska in genotipska raznolikost znotraj rodu *Sambucus* je sorazmerno zelo omejena. To raznolikost lahko učinkovito povečamo z medvrstnimi križanji. Namen raziskave je bil uporabiti molekulske markerje (mikrosatelite) za določanje strukture vzorčenih medvrstnih križancev bezga oz. določiti, katere vrste bezga so bile uporabljene pri oblikovanju posameznih medvrstnih križancev. V raziskavo je bilo vključenih 47 genotipov rodu *Sambucus* (starševske vrste in/ali medvrstni križanci) in uporabljeno je bilo šest mikrosatelitskih lokusov. Metoda združevanja je analizirane genotipe razvrstila v štiri glavne skupine. Prva glavna skupina je vključevala dve podskupini: eno s taksoni in križanci, ki vključujejo *S. racemosa* (v širšem smislu), druga pa je vključevala dve sorti *S. nigra*. V drugi glavni skupini je bila podskupina, v kateri so bili križanci med *S. javanica* in *S. nigra*, in podskupina, ki je vključevala F_1 križance med *S. javanica* in *S. ebulus*. Tretja glavna skupina je obsegala podskupino s križanci med *S. javanica* in *S. nigra* 'Black Beauty', podskupino s križanci, ki vključujejo *S. javanica*, *S. nigra* in *S. racemosa* (miquelii), podskupino s povratnim križanjem *S. javanica* × (*S. javanica* × *S. ebulus*) in en nedoločljivi medvrstni križanec. Četrta glavna skupina je vključevala podskupino z F_1 križanci *S. javanica* × *S. ebulus*, podskupino, ki vključuje različne taksone *S. racemosa* (v širšem smislu), in podskupino, ki vključuje križance med *S. cerulea* in *S. javanica*, z in brez *S. nigra*. Naša raziskava kaže, da je molekulska analiza lahko zelo koristna za določanje nekaterih neznanih, a strukturno enostavnih medvrstnih križancev iz rodu *Sambucus*. V primeru bolj zapletenih kombinacij medvrstnih križanj pa uporaba mikrosatelitov verjetno ni najboljša rešitev.

Ključne besede: *Sambucus*, mikrosatelitski markerji, medvrstni križanci, metoda razvrščanja v skupine, molekulski markerji