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Potential Use of SSR and ISSR Markers in Estimation of DNA Polymorphism Within Genus *Vicia*

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Summary: The development of novel markers usually requires high costs and is time consuming. Instead of developing new markers, their transfer within a genus is an alternative particularly suitable in less studied species. Hence the goal of this study was to evaluate the ability of SSR markers, previously developed from *Vicia faba*, and ISSR markers to estimate DNA polymorphism within the genus *Vicia*. Eight *Vicia* species were used for cross-species amplification. Transferability of SSR markers in the studied species ranged from 25% to 100%, with an average of 56.25%. Although three SSR primers were polymorphic, the nature of the amplification products indicated that their further usefulness is questionable. ISSR primers generated polymorphic products of amplification in all tested *Vicia* species. The average polymorphism of ISSR primers was 93.75%, which implies that these primers can be used within the genus *Vicia*.
Keywords: DNA polymorphism, ISSRs, molecular markers, SSRs, *Vicia*

Introduction

The genus vetch (*Vicia* L.) belongs to one of the largest families of flowering plants, Fabaceae (Leguminosae). It comprises a large and certainly not a definite number of species, presently estimated at more than 200 (ILDIS, 2005). Vetches are herbaceous annual and perennial species spread out in temperate zones of Euro-Asia, North and South America and Africa (Maxted 1995). They are known as food and forage legumes.

Regarding world production of 3.5 million tons (FAOSTAT, 2013), faba bean (*Vicia faba*) is categorized as the most important species in the *Vicia* genus, while in Serbia it is grown only locally (Mihailović et al. 2005). Common vetch (*Vicia sativa* L.), hairy vetch (*Vicia villosa* Roth), Hungarian vetch (*Vicia pannonica* Crantz), Narbonne vetch (*Vicia narbonensis* L.) and bitter vetch (*Vicia ervilia* L.) are grain legumes commonly used as forage (Jaiwal & Singh 2003). In Serbia, vetches are grown on the area of around 7,000 ha (Mikić et al. 2006) with average yield 1,200 kg/ha (Karagić et al. 2010). *Vicia sativa* L., *Vicia villosa* Roth and *Vicia pannonica* Crantz are economically most important vetches grown on Serbian fields (Vujaković et al. 2011). Certain *Vicia* species are also used as cover crops, green manure or livestock forage (Allen & Allen 1981).

There are about 30 species of vetches in the Serbian flora. Recently it has been demonstrated that some of these, such as narrow-leaved vetch (*V. sativa* ssp. *nigra* L.) and large-flowered vetch (*V. grandiflora* L.), have considerable potential for agricultural production (Ćupina et al. 2007). Beside high forage yields, *V. grandiflora* possesses some preferences, such as tolerance to low temperatures and earliness, allowing it to fit well into any model of crop rotation (Mikić et al. 2008).

SSRs (simple sequence repeats) are abundant, codominant, reproducible markers with great genome coverage (Kalia et al. 2011). SSR analysis has not been used extensively for molecular studies of species that lack information on DNA sequence because of the high costs of development. Thus, markers from well-studied species can be used in species with no, or low amount of available molecular data. Inter simple sequence repeat (ISSR) is fast and simple PCR-based method that amplifies sequences located between two microsatellite regions. They are not sequence-specific and amplify

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sequences of 200-2000 bp in length (Pradeep Reddy et al. 2002). Although only one primer of 16-25 bp in length is used (Zietkiewicz et al. 1994), ISSRs proved to be stable and reproducible (Bornet & Branchard 2001).

So far, DNA markers like RAPDs, ISSRs, AFLPs and SSRs were extensively used in assessing genetic diversity in faba bean (Link et al. 1995, Zeid et al. 2003, Román et al. 2004, Wang et al. 2012), while in common vetch only few studies were published (Potokina et al. 2000, 2002, Raveendar et al. 2015). There is low amount of available information considering molecular markers in other species within the *Vicia* genus. The above mentioned is the reason why possibility of transferring markers from closely related species was exploited in this study. The aim of this study was to investigate the possible use of previously developed SSR markers from *Vicia faba* and ISSR markers used in three *Vicia* species (*V. ramuliflora, V. unijuga* and *V. cracca*) in estimation of DNA polymorphism within the genus *Vicia*.

Materials and Methods

Eight *Vicia* species were used in this study. Six species, each with three accessions, were taken from the Institute of Field and Vegetable Crops vetch collection: *Vicia villosa*, *Vicia pannonica*, *Vicia ervilia, Vicia faba, Vicia narbonensis* and *Vicia sativa* ssp. *sativa*. Samples of *Vicia grandiflora* were collected at localities in Novi Sad, Kruševac and Belgrade, while *Vicia sativa* ssp. *nigra* samples were collected at Arhangel (Macedonia), and two localities in Novi Sad.

Leaves from single plant were used for *Vicia grandiflora* and *Vicia sativa* ssp. *nigra* DNA isolation from every locality. Genomic DNA from other *Vicia* species was isolated from approximately 25 g of seed bulk from every accession. Total DNA was extracted using Nucleo Spin Plant (Machery-Nagel) kit. DNA concentration was estimated by visualization on 1% agarose gels in 0.5xTBE buffer with ethidium bromide (0.5 g/ml) added to the gel. The λ phage DNA concentrations of 25 ng, 50 ng, 100 ng and 250 ng were used as standards. Images were taken under UV light.

A set of three SSR markers (VfG7, VfG14, VfG24) from *Vicia faba* was selected for the analysis, since they were known to have amplification products in at least one of five European genotypes (Zeid et al. 2009). Another three SSRs (GA4, GAI1, GATA5) were chosen from Požárková et al. (2002) because of the large number and size of their repeat motifs. SSR primer sequences are listed in Table 1. In order to amplify reaction product, polymerase chain reaction (PCR) was performed in volume of 25 µl, containing 1 x PCR buffer (Thermo Scientific, including 20 mM $MgCl_2$), 0.2 mM each dNTP (Thermo Scientific), 0.4 µM primer (Thermo Scientific), 1.5 U Dream Taq DNA polymerase (Thermo Scientific) and 50-100 ng DNA. Tpersonal thermocycler (Biometra) was used for DNA amplification. Touchdown program was applied to amplify GA4, GAI1 and GATA5 primers. After initial denaturation at 95°C for 3 min and denaturation at 95°C for 50 s, annealing was performed during 30 s at 68°C for GA4, 65°C for GAI1 and 66°C for GATA5. Primer extension lasted 1 min at 72°C. Annealing temperatures decreased for 1°C per cycle until reaching final annealing temperatures (62°C, 55°C and 58°C for GA4, GAI1 and GATA5, respectively). After reaching final annealing temperature, amplification ran for another thirty cycles for GAI1 and 35 cycles for GA4 and GATA5. Final elongation was done at 72°C for 10 min. PCR conditions for VfG7, VfG14 and VfG24 primers included: initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 60 s and elongation at 72°C for 60 s. In the end, final elongation was performed at 72°C for 7 min.

Table 1. SSR primers used for cross-species transferability testing within *Vicia* genus

Primer	Forward primer sequence	Reverse primer sequence	Repeat motif	Expected size (bp)
VfG7	AGGCATGTGGTGTTTTGATT	GCAATAAAATATGCTTTTCTGGA	$(AG)_{12}$	284
VfG14	CAAGCTTGTTGAGAGCCAAA	GAACGAGGCTCACGAAAATA	$(AG)_{10}$	229
VfG24	GTGTCATCGACCACATGGTTA	CAGCTGAGCACGAGACTAA	$(AG)_9$	273
GA4	GAACTAAGGTGTACACGCGGG	GGGGGGTAGATCTTGTTTTTTCC	$(CT)_{16}$	232
GAI1	GTTGAGGCAGGTTAGGAGGG	GTTTGCCAGTTACTTTGTTCACG	$(GA)_{14}$	242
GATA5	GAAACGTGCCTATGTAAAGGGA	GAATCTGATTCTGCTCAAAAGCA	$(GATA)_2GGT(GATA)_6$	291

Table 2. ISSR primers used for testing DNA polymorphism within *Vicia* genus

Primer	Primer sequence
$(GACA)_4$	GACAGACAGACAGACA
$(AG)_8T$	AGAGAGAGAGAGAGAGT
$(AC)_8T$	ACACACACACACACACT
$(GA)_8T$	GAGAGAGAGAGAGAGAT

Amplified fragments were separated on 2% agarose gel containing ethidium bromide (0.5 g/ml) and visualized under UV light. GeneRuler 50 bp DNA Ladder and O'RangeRuler 200 bp DNA Ladder (Termo Scientific) were used as molecular standard.

Four ISSR markers (Table 2) that proved to give clear polymorphic bands in three *Vicia* species (*Vicia ramuliflora, V. unijuga, V. cracca)* were chosen (Han & Wang 2009). For each reaction 50-100 ng DNA was used, with 10x reaction buffer (Thermo Scientific, including 20 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 µM primer (Metabion international ag) for $(AG)_8T$, $(AC)_8T$, and 2 µM for $(GACA)_4$ and $(GA)_8T$, 1.5 U Dream Taq polymerase. PCR was performed as follows: 1 cycle for 3 min at 94°C, 35 cycles of 92°C for 30 s, 50°C (55.2°C for $(AC)_8T$) for 30 s and 72°C for 1 min, followed by 1 cycle for 5 min at 72°C.

Products of ISSR marker amplification were separated on 1.5% agarose gels. Ethidium bromide (0.5 g/ml) was used to make bands visible under UV light. 200 bp O`Range Ruler (Termo Scientific) was utilized as molecular standard.

Bands were analysed visually in order to observe whether the amplification occurred. SSR bands were scored with "+" for amplicons of different size then in *Vicia faba*, "(+)" for amplicons of the same size as in *Vicia faba* and "–" for no amplification. ISSR banding patterns of *Vicia* sp. were compared with each other and marked as "+" for different banding patterns and "(+)" for the same banding patterns.

Results and Discussion

This study evaluated the ability of SSR markers from *V. faba* to amplify in other *Vicia* species and possible use of ISSRs in *Vicia* sp. was investigated. Four out of six SSR primers generated amplification products in *V. faba* of the same size as in Zeid et al. (2009) and Požarkova et al. (2002) (Table 3). Although *V. faba* in the study did not amplify with VfG24, the size of products obtained in *V. sativa* ssp. *nigra* and *V. grandiflora* was as expected in *V. faba*. The same was with *V. grandiflora`s* amplification product with primer GATA5. Amplification products GA4, and GAI1 in all *Vicia* species were monomorphic, except in *V. sativa* ssp. *nigra*. Therefore, these primers could not be considered suitable for further variability studies in *Vicia* genus. Amplification products of primers VfG7, VfG14 and GATA5 were polymorphic, but their poor quantity and quality calls their usefulness into question.

Primer VfG14 amplified in all tested species (Figure 1), while GAI1 primer amplified with success rate of 75%. Primer GATA5 amplified DNA of 62.5% tested *Vicia sp*. The lowest amplification was observed for VfG24 primer, which generated products only in *V. sativa* ssp. *nigra* and *V. grandiflora*. Average transferability of SSR markers was 56.25% which was in concordance with the previously published results on legumes (Reddy et al. 2010, Datta et al. 2012, Gupta et al. 2013).

Table 3. Transferability of SSR primers within *Vicia* genus

Vicia sp	VfG7	GA4	VfG14	GAI1	VfG24	GATA5
Vicia faba*	290 bp	230 bp	230 bp	240 bp	-	-
Vicia narbonensis	+	(+)	+	(+)	-	+
Vicia ervilia	+	(+)	+	(+)	-	+
Vicia pannonica	-	-	+/-	-	-	-
Vicia villosa	-	-	+/(+)	-	-	-
Vicia sativa* ssp. *sativa	-	-	+	(+)	-	+
Vicia sativa* ssp. *nigra	+	(+)	+	+	(+)	+
Vicia grandiflora	-	-	+	(+)	(+)	(+)
Transferability (%)	50	50	100	75	25	62,5

* observed product size, + different size than in V. faba, (+) same size as in V. faba, - no amplification

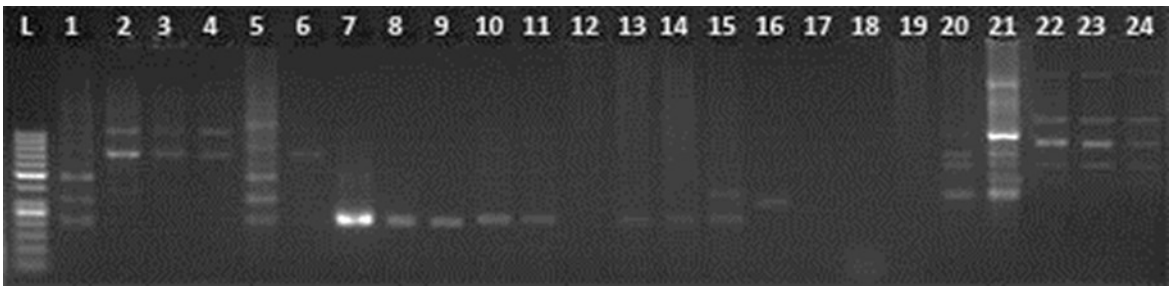

Figure 1. Amplification products with primer VfG14 in *Vicia* sp. (L-50bp ladder, 1, 2, 3-*V. narbonensis,* 4, 5, 6-*V. ervilia,* 7, 8, 9-*V. faba,* 10, 11, 12-*V. pannonica,* 13, 14, 15-*V. villosa,* 16, 17 18-*V. sativa* ssp. *sativa,* 19, 20, 21- *V. grandiflora,* 22, 23, 24 - *V. sativa* ssp. *nigra*)

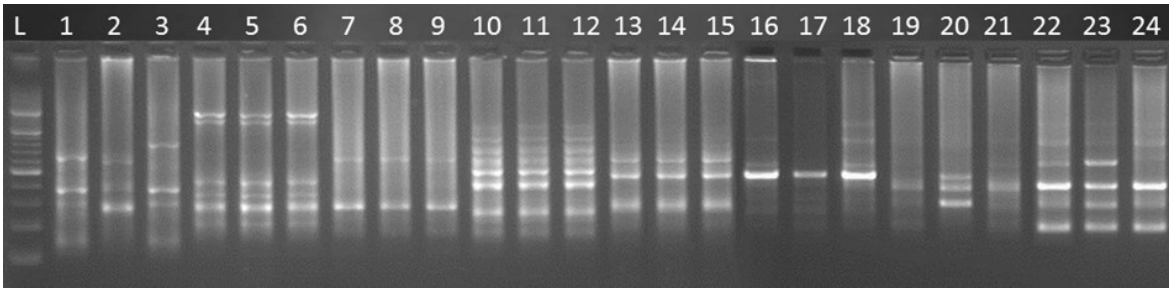

Figure 2. Amplification products with primer $(AC)_8T$ in *Vicia* sp. (L-200bp ladder, 1, 2, 3-*V. narbonensis,* 4, 5, 6-*V. ervilia,* 7, 8, 9-*V. faba,* 10, 11, 12-*V. pannonica,* 13, 14, 15-*V. villosa,* 16, 17 18-*V. sativa* ssp. *sativa,* 19, 20, 21-*V. sativa* ssp. *nigra,* 22, 23, 24-*V. grandiflora)*

Table 4. Polymorphism of ISSR primers in *Vicia* genus

Vicia sp	**$(GACA)_4$**	**$(AG)_8T$**	**$(AC)_8T$**	**$(GA)_8T$**
Vicia narbonensis	+	+	+	(+)
Vicia ervilia	+	+	+	+
Vicia faba	+	+	+	(+)
Vicia pannonica	+	+	+	+
Vicia villosa	+	+	+	+
Vicia sativa* ssp. *sativa	+	+	+	+
Vicia sativa* ssp. *nigra	+	+	+	+
Vicia grandiflora	+	+	+	+
Polymorphism (%)	100	100	100	75

+ different banding patterns, (+) same banding patterns

All tested ISSR primers generated different amplification profiles in *Vicia* sp. (example in Figure 2), with exception of *Vicia narbonensis* and *Vicia faba,* which had the same banding pattern with primer $(GA)_8T$ (Table 4). The average polymorphism of ISSR primers was very high (93.75%), confirming results that this type of markers could be useful in species differentiation and diversity studies in *Vicia* sp.

Conclusions

The development of novel markers usually requires high costs and is time consuming. Instead of developing new markers, their transfer within genus could be alternative, particularly suitable in less studied species. In this study we assessed the potential for cross-species amplification of SSR markers developed from *Vicia faba.* Although primers VfG7, VfG14 and GATA5 were polymorphic, quantity and quality of the amplification products indicates that their usefulness in further variability studies is questionable. The conditions of amplification reactions would have to be extensively modified which could take a lot of time and resources. Therefore, it is our opinion that in variability studies of *Vicia* sp., some other approach should be considered, such as development of EST-based markers, which are easier and less expensive to develop and possess high rate of transferability to related species.

Within ISSR set, reactions resulted in different amplification profiles for each investigated *Vicia* species. Our results imply that these primers can be used within the genus *Vicia.*

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Mogućnost primene SSR i ISSR markera u proceni DNK polimorfizma unutar roda *Vicia*

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Sažetak: Razvoj markera obično zahteva vreme i velika materijalna ulaganja. Umesto razvijanja novih markera za manje proučavane vrste, alternativa može biti transfer sa vrsta unutar istog roda. Cilj ovog rada je bio da se proceni mogućnost primene SSR prethodno razvijenih na *Vicia faba* i ISSR prajmera u proceni DNK polimorfizma u rodu *Vicia*. Prenosivost SSR markera se kretala u intervalu 25-100% sa prosečnom vrednošću od 56,25%. Iako su produkti tri SSR prajmera bili polimorfni, zbog dobijene količine i kvaliteta, njihovo korišćenje u daljim istraživanjima je diskutabilno. Svi ispitivani ISSR prajmeri su davali različite profile amplifikacije za svaku ispitivanu vrstu. Prosečni procenat polimorfizma ISSR prajmera je bio vrlo visok (93,75%), što sugeriše da ovi prajmeri mogu biti korišćeni u proceni DNK polimorfizma unutar roda *Vicia*.

Ključne reči: DNK polimorfizam, ISSR, molekularni markeri, SSR, *Vicia*