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MOLECULAR TOOLS FOR UTILIZATION OF MITOCHONDRIAL DIVERSITY IN FABA BEAN (*Vicia faba*)

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We performed *in silico* PCR analyses utilizing complete mitochondrial (mtDNA) genome sequences of faba bean (*Vicia faba*) and two related species, *Vigna angularis* and *Vigna radiata*, currently available in GenBank, to infer whether 15 published universal primer pairs for amplification of all 14 *cis*-spliced introns in genes of NADH subunits (*nad* genes) are suitable for *V. faba* and related species. Then, we tested via PCR reactions whether seven out of 15 primer pairs would generate PCR products suitable for further manipulation in 16 genotypes of *V. faba* representing all botanical varieties of this species (*major*, *minor*, *equina* and subsp. *paucijuga*) of various levels of improvement (traditional and improved cultivars) originating from Europe, Africa, Asia and south America. We provide new PCR primers for amplification of *nad*1 intron 2/3 in *V. faba*, and demonstrate intraspecific variability in primary nucleotide sequences at this locus. Based on outcomes of both *in silico* predictions and PCR amplification, we report a set of PCR primers for amplification of five introns in *nad* genes that are promising molecular tools for future phylogeographic and other studies in this species for which unambiguous data on wild ancestors, centre of origin and domestication are lacking.

Key words: mitochondrial genome, NADH dehydrogenase, introns, *in silico* PCR, universal primers, faba bean

INTRODUCTION

Faba bean (*Vicia faba* L.) is a grain crop legume used mainly for food and feed predominantly in eastern and northern Africa and western Asia (AKIBODE and MAREDIA, 2011).

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Archaeological evidence reveals that *V. faba* is one of the first domesticated crops (TANNO and WILLCOX, 2006; see also DUC *et al*., 2010 and references therein). However, its origin and wild ancestors are still unknown (MURATOVA, 1931; CUBERO, 1974; MAXTED *et al*., 1991; MAXTED, 1995) and furthermore, its taxonomic placement and evolutionary relations are still unresolved despite numerous traditional morphology-based taxonomies and systematics (more than 20 since Linnaeus, reviewed by MAXTED, 1993) and available molecular phylogenies (e.g. POTOKINA *et al*., 1999; VAN DE VEN *et al*., 1993; FENNELL *et al*., 1998; SCHAEFER *et al*., 2012). New insights into these essential aspects of *V. faba* history, which are also required for breeding programs and crop improvement, may be gained via new approaches such as phylogeography (AVISE *et al*., 1987; AVISE, 2009). Phylogeography is a relatively new discipline which makes inferences based on spatial arrangements of genealogically linked lineages detected mainly at haploid, non-recombining and predominantly uniparentally inherited cytoplasmic genomes (chloroplast - cpDNA and mitochondrial - mtDNA genomes in plants).

Given the lack of cpDNA diversity in *V. faba* (SHIRAN and MASHAYEKH, 2004; HAIDER *et al*., 2012) and high mtDNA diversity obtained via PCR-RFLP method and random mtDNA clones used as probes for hybridisation (SCALLAN and HARMEY, 1996) as well as nucleotide and length variability at intron 2/3 of the first gene of the NADH dehydrogenase (*nad*1 intron 2/3) in the genus *Vicia* (RYZHOVA *et al*., 2012), it appears that mtDNA may be a promising tool not only for phylogeographic studies in *V. faba* that may shed more light on species origin and domestication but also for phylogenetic surveys that may resolve species evolutionary unfolding. However, utilization of maternally inherited (MOGENSEN, 1996) and slowly evolving mtDNA genome (WOLFE *et al*., 1987; LAROCHE *et al*., 1997) in diverse studies in *V. faba* may not be straight-forward because plant mtDNA genomes are exceptionally variable in size (200 to 2.900 kb) and organization frequently altered by insertions, deletions and structural re-arrangements (LAROCHE *et al*., 1997; see also NEGRUK, 2013 and references therein). This implies that it may be challenging not only to delineate informative regions but also to generate PCR products which can be used for further manipulation. This is because so-called universal primers, which are expected to generate PCR products in a vast majority of plant taxa (TABERLET *et al*., 1991), may simply fail to amplify desired mtDNA regions in taxa of interest due to alternative organization of their genomes. Nonetheless, a recent report on complete mtDNA sequence of *V. faba* (NEGRUK, 2013) represents an essential milestone which would facilitate broader utilization of mtDNA variability in diverse studies in this species.

The aim of this study is to provide molecular tools for broader utilization of mtDNA diversity in diverse studies in *V. faba*. We firstly test via *in silico* PCR predictions whether universal mtDNA primers reported by DEMESURE *et al*. (1995), DUMOLIN-LAPEGUE *et al*. (1997) and DUMINIL *et al*. (2002) are suitable also for *V. faba* and related species *Vigna anglularis* and *Vigna radita* whose complete mtDNA genome sequences are currently available in GenBank. We focus primarily on 15 primer pairs which amplify 14 *cis*-spliced introns in genes of NADH subunits (*nad* genes) because mtDNA non-coding regions are evolving faster than their adjoining coding regions, and introns in *nad* genes are amongst the most commonly used non-coding mtDNA regions in diverse studies in higher plants (e.g. FREUDENSTEIN and CHASE, 2001; WON and RENNER, 2003; ZHANG *et al*., 2006; TOLLEFSRUD *et al*., 2009; ALEKSIĆ and GEBUREK, 2010; HAVANANDA *et al*., 2010, FROELICHER *et al*., 2011; RYZHOVA *et al*., 2012; LOCKWOOD *et al*., 2013). Secondly, we test via PCR reaction whether seven out of 15 tested primer pairs would generate PCR products suitable for further manipulation in 16 genotypes of *V. faba* originating

from Europe, Africa, Asia and south America which represent all botanical varieties of this species (*major*, *minor*, *equina* and subsp. *paucijuga*) of various levels of improvement (traditional cultivars and improved cultivars). We provide new PCR primers for amplification of *nad*1 intron 2/3 in *V. faba*, demonstrate intraspecific variability in primary nucleotide sequences at this locus, and based on outcomes of both *in silico* predictions and PCR amplifications, report a set of PCR primers for amplification of five introns in *nad* genes that are promising molecular tools for future phylogeographic and other studies in *V. faba*.

MATERIALS AND METHODS

Complete mtDNA genome sequences and in silico PCR

Mitochondrial genome sequences of *Vicia faba* cultivar Broad Windsor (GenBank accession number KC189947, NEGRUK, 2013), *Vigna radiata* (HM367685, ALVERSON *et al*., 2011) and *Vigna angularis* (AP012599, NAITO *et al*., 2013) have been downloaded from NCBI database on 14 January 2014. They were used for *in silico* PCR amplification of 14 cis-spliced introns in genes of NADH subunits (*nad*1 introns 2/3 and 4/5; *nad*2 introns 1/2, 3/4, and 4/5; *nad*4 introns 1/2, 2/3 and 3/4; *nad*5 introns a/b and d/e; and *nad*7 introns 1/2, 2/3, 3/4 and 4/5) utilizing 15 universal primer pairs published by DEMESURE *et al*. (1995), DUMOLIN-LAPEGUE *et al.* (1997) and DUMINIL *et al.* (2002). *In silico* PCR was performed utilizing FastPCR 6.3 trial version (KALENDAR *et al*., 2009). The maximal length of expected PCR products was set to 8 kb and allowed mismatch at 3'-end was set to two nucleotides. The positions of all primer binding sites of forward (F) and reverse (R) primers, the complementarity of primers and template DNA expressed through the percentage of binding nucleotides, melting temperatures (*Tm*) of individual primers (in °C), annealing temperatures (*Ta*) of primer pairs (in °C), and lengths of obtained PCR products were recorded in all legumes.

Plant material for PCR reactions

Plant material used for PCR amplification of seven introns in *nad* genes comprised 16 genotypes of *V. faba* representing different botanical varieties (*major* – abbreviation Ma, *minor* – Mi, and *equina* - Eq) and subsp. *paucijuga* (Pau) which are traditional cultivars (TC) or improved cultivars (IC) originating from China (cultivar name: Kingpi – characteristics: Eq, TC), India (CH182 –Pau, TC), Ethiopia (E14b – Eq, TC), Morocco (BPL4225 – not available), Sudan (Silian – Mi, TC), Greece (Apsalos –Eq, IL), Italy (Violetta di policoro – Ma, TC), Spain (Feve Seville – Ma, TC), Denmark (Troy – Eq, IC), Finland (Mikko – Mi, TC), Germany (Diana – Mi, IC), United Kingdom (CH170 – Eq, IL), Peru (Rojo – Ma, TC) and Serbia (three traditional cultivars of *major* type - Bački Petrovac 2 and Deligrad, and of *minor* type – 159B). Seeds of all accessions, obtained from the French National Institute for Agricultural Research (INRA) *ex situ* collection, were sawn and grown to seedlings in a greenhouse at Institute of Molecular Genetics and Genetic Engineering (IMGGE). Leaves from young seedlings were collected and desiccated with silica gel for c. two weeks prior to DNA extraction. Total genomic DNA was extracted using CTAB method, modification according to ALEKSIĆ *et al*. (2012).

PCR primers and PCR amplification of seven introns in nad genes in V. faba

Introns in *nad* genes (*nad*1 intron 2/3; *nad*4 introns 1/2 and 2/3; *nad*5 intron d/e; and *nad*7 introns 1/2, 2/3 and 4/5) were PCR amplified in our source material. The reasoning for selecting

these seven out of 14 introns in *nad* genes for further PCR analyses, which was based on outcomes of *in silico* PCR analyses and aimed at resolving obstacles evident from those analyses that may hamper PCR amplification and further utilization of these loci, is given in Results section upon providing outcomes of *in silico* PCR analysis for each out these seven loci.

We used mtDNA genome sequence of *V. faba* (NEGRUK, 2013) and FastPCR 6.3 trial version (KALENDAR *et al*., 2009) to design new primers for PCR amplification of *nad*1 intron 2/3 in this species. The difference in melting temperatures of F and R primers was set to 4 °C, CG clump was not opted, and primers of c. 20 bp length and CG content around 50% were generated. Out of several F and R primers which can be used for amplification of this particular intron, only those located within exon 2 (F primers) and exon 3 (R primers) and displaying quality above 75% were further used. Thus, we designed two F primers [*nad*1e2f15 (5'- TCTAGGAGCATTACGATCTGCAG – 3') and *nad*1e2f108 5'- ATCCGCGAAGGCAATCGCTCG - 3)'] and three R primers [*nad*1e3r1484 (5'- AGTTTCCCTGCATGTGGCTCG – 3'), *nad*1e3r1602 (5'- GGAACAAGGGAATACCGGACC – 3') and *nad*1e3r1667 (5'- TGGGAGATCAAACGGAGCTCG – 3')]. Each out of two F primers was employed with each out of three R primers for PCR amplification of *nad*1 intron 2/3 in *V. faba*.

PCR reactions were carried out in 25 µl volumes containing: 100 ng template DNA; 1 × *Taq* Buffer with $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania); 2.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.4 µM of each Forward (F) and Reverse (R) primer; 0.80 % BSA (Bovine Serum Albumin, Promega, St Louis, U.S.A.); and 0.025 U of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). Reactions were performed in the Mastercycler Gradient machine (Eppendorf AG, Hamburg, Germany) under following conditions: denaturation at 95 °C for 5 min., 39 cycles of denaturation at 95 °C for 45 sec., annealing at temperatures 55 °C (*nad*5 intron d/e, *nad*7 introns 1/2 and 2/3), 58 °C (*nad*1 intron 1/2, *nad*4 introns 1/2 and 2/3, *nad*7 intron 4/5) or 67 °C (used for all six combinations of new F and R primers for amplification of *nad*1 intron 2/3), extension at 72 °C for 2 min., final extension at 72 °C for 10 min.. PCR products were subjected to electrophoresis through 2% agarose gels followed by ethidium-bromide staining and were visualised under the UV light utilizing BioDoc Analyze (Biometra, Göttingen, Germany).

PCR products obtained with new primers *nad*1e2f15 and *nad*1e3r1602 for amplification of *nad*1 intron 2/3 were sequenced commercially with the F primer by Macrogen Europe, Amsterdam, Netherlands (http://dna.macrogen.com/eng/) via Sanger sequencing using 96-capillary 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Inc., U.S.A.). Obtained sequences of the 5'-end of this intron in 16 *V. faba* genotypes were edited and aligned manually along with *V. faba* cultivar Broad Windsor sequence of this intron (NEGRUK, 2913) using Muscle (EDGAR, 2004) in MEGA 5.04 (TAMURA *et al*., 2011).

RESULTS

In silico PCR analyses

The positions of all primer binding sites of 15 published primer pairs used for *in silico* PCR amplification of 14 cis-spliced introns in *V. faba*, *V. angularis* and *V. radiata*, the complementarity of primers and template DNA, *Tm* of individuals primers, *Ta* of primer pairs and lengths of obtained PCR products are presented in Table 1. With regard to the complementarity of primers and template DNAs of three legumes, *in silico* PCR analysis revealed that F and/or R primers from six primer pairs (for amplification of *nad*2 intron 4/5, *nad*4 introns 1/2, 3/4 and

primer pair of DEMESURE *et al*. (1995) for amplification of the entire *nad*4 gene, *nad*7 introns 2/3 and 3/4) harboured single nucleotide mismatches in their binding sites. Lower complementarity of primers and template DNAs was observed also for degenerate primers comprising up to three ambiguous nucleotides (F primers from primer pairs for amplification of *nad*2 introns 1/2 and 3/4, and *nad*7 intron 4/5).

Table 1 Results of in silico PCR amplification of 14 cis-spliced mitochondrial introns in nad genes in Vicia faba, Vigna angularis and Vigna radiata.

Species	F primer	5'- 3'	Position	Com (%)	*Tm* (°C)	R primer	5'- 3'	Position	Com (%)	*Tm* (°C)	Fr. len.	*Ta* (°C)
Locus 1: *nad*1 intron 2/3 (reference: 1, 3)												
V. faba	*nad*1 exon2	GCATTACGATCTGCAGCTCA	380141 <- 380160	100	55	*nad*1 exon3	GGAGCTCGATTAGTTTCTGC	378525 -> 378544	100	52,9	1636	60
			449926 <- 449945	100	55			448310 -> 448329	100	52,9	1636	60
								178244 <- 178263	65	9,2		
								493293 <- 493312	65	9,2		
V. angularis	*nad*1 exon2	GCATTACGATCTGCAGCTCA	222894 -> 222913	100	55	*nad*1 exon3	GGAGCTCGATTAGTTTCTGC	224527 <- 224546	100	52,9	1653	60
			35027 <- 35046	65	40,6							
V. radiata	*nad*1 exon2	GCATTACGATCTGCAGCTCA	3065 -> 3084	100	55	*nad*1 exon3	GGAGCTCGATTAGTTTCTGC	4698 <- 4717	100	52,9	1653	60
			179257 <- 179276	65	40,6							
								202612 -> 202631	75	34,1		
Locus 2: *nad*1 intron 4/5 (reference: 2, 3) (note: contains *mat*K gene)												
V. faba	*nad*1/4	GCCAATATGATCTTAATGAG	89487 -> 89506	100	46,8	*nad*1/5	TCACCTTGATACTAAACCAG	92731 <- 92750	100	48,4	3264	54
								4953 -> 4972	70	22,1		
								457758 -> 457777	70	22,1		
V. angularis	*nad*1/4	GCCAATATGATCTTAATGAG	67987 <- 68006	100	46,8	*nad*1/5	TCACCTTGATACTAAACCAG	64738 -> 64757	100	48,4	3269	54
V. radiata	*nad*1/4	GCCAATATGATCTTAATGAG	65531 <- 65550	100	46,8	*nad*1/5	TCACCTTGATACTAAACCAG	62282 -> 62301	100	48,8	3269	54
Locus 3: *nad*2 intron 1/2 (reference: 3)												
V. faba	*nad*2/1	AATGTGGGTTGGCTTGGWTT	27785 -> 27804	98	54,6	*nad*2/2	AATATGTAAAATTGTCCCTC	29101 <- 29120	100	47,1	1336	53
			2331 <- 2350	78	34,7							
			99499 -> 99518	62	41,6							
			455136 <- 455155	78	34,7							
V. angularis	*nad*2/1	AATGTGGGTTGGCTTGGWTT	147624 -> 147643	98	54,6	*nad*2/2	AATATGTAAAATTGTCCCTC	148978 <- 148997	100	47,1	1374	53
			142527 -> 142546	78	37,9							
			402176 -> 402195	68	17,5							
V. radiata	*nad*2/1	AATGTGGGTTGGCTTGGWTT	193922 <- 193941	98	54,6	*nad*2/2	AATATGTAAAATTGTCCCTC	192568 -> 192587	100	47,1	1374	53
			163456 <- 163475	78	37,9							
Locus 4: *nad*2 intron 3/4 (reference: 3)												
V. faba	*nad*2/3	AGAAARGAATGCTGTAACCG	415414 -> 415433	98	50	*nad*2/4	ATGGGGATTKTYARTATCGC				No product	
			332368 <- 332387	70	40,7							
								152757 <- 152776	58	4		
V. angularis	*nad*2/3	AGAAARGAATGCTGTAACCG	99140 <- 99159	98	50	*nad*2/4	ATGGGGATTKTYARTATCGC				No product	
								324667 <- 324686	62	23,8		
V. radiata	*nad*2/3	AGAAARGAATGCTGTAACCG	96655 <- 96674	98	50	*nad*2/4	ATGGGGATTKTYARTATCGC				No product	
								267633 <- 267652	62	23,8		

Locus 5: *nad2* intron 4/5 (reference: 3)												
V. faba	*nad2/4*	TTCATATAGAATCCATGTCC	413337 –> 413356	95	41,2	*nad2/5*	CTATTTGTTCTTCGCCGCTT	414960 <– 414979	100	56,3	1643	54
			139973 <– 139992	70	22,2							
								356348 –> 356367	70	28,6		
V. angu-laris	*nad2/4*	TTCATATAGAATCCATGTCC	101195 <– 101214	95	41,2	*nad2/5*	CTATTTGTTCTTCGCCGCTT	99594 –> 99613	100	56,3	1612	54
								89580 –> 89599	75	29,3		
V. radi-ata	*nad2/4*	TTCATATAGAATCCATGTCC	98711 <– 98730	95	41,2	*nad2/5*	CTATTTGTTCTTCGCCGCTT	97109 –> 97128	100	56,3	1622	54
								87090 –> 87109	75	29,3		
Locus 6: *nad4* intron 1/2 (reference: 1, 3)												
V. faba	*nad4* exon1	CAGTGGGTTGGTCTGGTATG	353611 –> 353630	95	53,1	*nad4* exon2	TCATATGGGCTACTGAGGAG	355634 <– 355653	100	53,9	2043	61
								418785 –> 418804	70	33,1		
V. angu-laris	*nad4* exon1	CAGTGGGTTGGTCTGGTATG	86853 –> 86872	95	53,1	*nad4* exon2	TCATATGGGCTACTGAGGAG	88877 <– 88896	100	53,9	2044	61
			13644 –> 13663	80	41,8							
								141750 –> 141769	70	33,1		
V. radi-ata	*nad4* exon1	CAGTGGGTTGGTCTGGTATG	84363 –> 84382	95	53,1	*nad4* exon2	TCATATGGGCTACTGAGGAG	86387 <– 86406	100	53,9	2044	61
			211303 –> 211322	80	41,8							
								164233 <– 164252	70	33,1		
Locus 7: *nad4* intron 2/3 (reference: 2, 3)												
V. faba	*nad4/2*	CTCCTCAGTAGCCCATATGA	355634 –>355653	100	52,5	*nad4/3*	AACCAGTCCATGACTTAACA	358879 <– 358898	100	53,4	3265	60
			82203 <– 82222	70	21,7							
V. angu-laris	*nad4/2*	CTCCTCAGTAGCCCATATGA	88877 –> 88896	100	52,5	*nad4/3*	AACCAGTCCATGACTTAACA	92197 <– 92216	100	53,4	3340	60
			206274 –> 206293	75	22,7							
			134816 <– 134835	70	21,7							
V. radi-ata	*nad4/2*	CTCCTCAGTAGCCCATATGA	86387 –> 86406	100	52,5	*nad4/3*	AACCAGTCCATGACTTAACA	89708 <– 89727	100	53,4	3341	60
			132278 <– 132297	70	21,7							
Locus 8: *nad4* intron 3/4 (reference: 2, 3)												
V. faba	*nad4* exon3	GGAGCTTTCCAAAGAAATAG	359082 –> 359101	100	49,7	*nad4* exon4	GCCATGTTGCACTAAGTTAC	362092 <– 362111	100	52,8	3030	57
			319535 –> 319554	65	16,3							
								38312 –> 38331	70	34,5		
V. angul-aris	*nad4* exon3	GGAGCTTTCCAAAGAAATAG	92400 –> 92419	100	49,7	*nad4* exon4	GCCATGTTGCACTAAGTTAC	95180 <– 95199	95	50,6	2800	57
			125508 –> 125527	75	34,9							
V. radi-ata	*nad4* exon3	GGAGCTTTCCAAAGAAATAG	89911 –> 89930	100	49,7	*nad4* exon4	GCCATGTTGCACTAAGTTAC	92694 <– 92713	95	50,6	2803	57
								122970 –> 122989	75	35,6		
Locus 9: *nad4* subunit (reference: 1) (note: F primer is located within exon1 of *nad4* gene)												
V. faba	nad4 exon2	TGTTTCCCGAAGCGACACTT	355516 –> 355535	95	52	nad4 exon4	GGAACACTTTGGGGTGAACA	362048 <– 362067	100	53,2	6552	64
V. angu-laris	nad4 exon2	TGTTTCCCGAAGCGACACTT	88759 –> 88778	95	52	nad4 exon4	GGAACACTTTGGGGTGAACA	95136 <– 95155	100	53,2	6397	64
V. radi-ata	nad4 exon2	TGTTTCCCGAAGCGACACTT	86269 –> 86288	95	52	nad4 exon4	GGAACACTTTGGGGTGAACA	92650 <– 92669	100	53,2	6401	64

Locus 10: *nad5* intron a/b (reference: 2, 3)												
V. faba	*nad5/1*	TTTTTTCGGACGTTTTCTAG	71584 –> 71603	100	49,1	*nad5/2*	TTTGGCCAAGTATCCTACAA	73834 <– 73853	100	50,3	2270	57
V. angu-laris	*nad5/1*	TTTTTTCGGACGTTTTCTAG	120992 –> 121011	100	49,1	*nad5/2*	TTTGGCCAAGTATCCTACAA	118744 <– 118763	100	50,3	2268	57
V. radi-ata	*nad5/1*	TTTTTTCGGACGTTTTCTAG	118473 –> 118492	100	49,1	*nad5/2*	TTTGGCCAAGTATCCTACAA	116224 <– 116243	100	50,3	2269	57
Locus 11: *nad5* intron d/e (reference: 2, 3)												
V. faba	*nad5/4*	CCAATTTTTGGGCCAATTCC	279736 –> 279755	100	55,4	*nad5/5*	CATTGCAAAGGCATAATGAT	281047 <– 281066	100	49,2	1331	57
			563870 –> 563889	100	55,4			562559 –> 562578	100	49,2	1331	57
			309974 <– 309993	70	29,4							
			367879 <– 367898	70	29,4							
			437664 <– 437683	70	29,4							
V. angu-laris	*nad5/4*	CCAATTTTTGGGCCAATTCC	183939 –> 183958	100	55,4	*nad5/5*	CATTGCAAAGGCATAATGAT	185255 <– 185274	100	49,2	1336	57
								367370 –> 367389	70	26,4		
								401251 –> 401270	70	20,2		
V. radi-ata	*nad5/4*	CCAATTTTTGGGCCAATTCC	324793 <– 324812	100	55,4	*nad5/5*	CATTGCAAAGGCATAATGAT	323477 –> 323496	100	49,2	1336	57
								35055 –> 35074	70	26,1		
								292633 –> 292652	70	20,2		

Locus 12: *nad7* intron 1/2 (reference: 2, 3) (note: R primers generate non-specific PCR product of 3244 bp in *V. angularis*, *Ta* = 56°C)												
V. faba	*nad7/1*	ACCTCAACATCCTGCTGCTC	226324 <– 226343	100	59,2	*nad7/2*	CGATCAGAATAAGGTAAAGC	225275 –> 225294	100	48,8	1069	55
			302233 –> 302252	100	59,2			303282 <– 303301	100	48,8	1069	55
			541373 <– 541392	100	59,2			540324 –> 540343	100	48,8	1069	55
								34233 –> 34252	75	11,3		
								94184 –> 94203	70	27,9		
V. angu-laris	*nad7/1*	ACCTCAACATCCTGCTGCTC	356308 –> 356327	100	59,2	*nad7/2*	CGATCAGAATAAGGTAAAGC	357341 <– 357360	100	48,8	1053	55
								354117 –> 354136	75	27,9		
								199933 <– 199952	70	13,5		
								115191 –> 115210	65	22,8		
								266002 <– 266021	75	11,3		
V. radi-ata	*nad7/1*	ACCTCAACATCCTGCTGCTC	343682 –> 343701	100	59,2	*nad7/2*	CGATCAGAATAAGGTAAAGC	344715 <– 344734	100	48,8	1035	49
								308812 –> 308831	70	13,5		
								284540 –> 284559	75	24,9		
								112666 –> 112685	65	22,8		
								141365 –> 141384	75	11,3		
								126095 <– 126114	65	16,5		

Locus 14: *nad7* intron 3/4 (reference: 2, 3)

V. faba	*nad7*/3	TCTATGATGGCCCAAGAACA	223919 <– 223938	95	48,7	*nad7*/4	ACACCAAATTCTCCTTTAGG	220057 –> 220076	100	52,5	3882	58
			304638 –> 304657	95	48,7			308500 <– 308519	100	52,5	3882	58
			538968 <– 538987	95	48,7			535106 –> 535125	100	52,5	3882	58
								436190 <– 436209	100	52,5		
								366405 <– 366424	100	52,5		
V. angu-laris	*nad7*/3	TCTATGATGGCCCAAGAACA	358711 –> 358730	95	48,7	*nad7*/4	ACACCAAATTCTCCTTTAGG	362576 <– 362595	100	52,5	3885	58
			266477 <– 266496	65	35,9							
								214018 <– 214037	80	16,4		
V. radi-ata	*nad7*/3	TCTATGATGGCCCAAGAACA	346086 –> 346105	95	48,7	*nad7*/4	ACACCAAATTCTCCTTTAGG	349940 <– 349959	100	52,5	3874	58
			140890 –> 140909	65	35,9							
								395462 <– 395481	75	7,4		

Locus 13: *nad7* intron 2/3 (reference: 2, 3) (note: F primers generate non-specific PCR product of 3990 bp in *V. faba*, *Ta* = 56°C)

V. faba	*nad7*/2	GCTTTACCTTATTCTGATCG	225275 <– 225294	100	49,3	*nad7*/3	TGTTCTTGGGCCATCATAGA	223919 –> 223938	95	49,8	1376	55
			303282 –> 303301	100	49,3			304638 <– 304657	95	49,8	1376	55
			540324 <– 540343	100	49,3			538968 –> 538987	95	49,8	1376	55
			221305 –> 221324	70	17,9							
			307252 <– 307271	70	17,9							
			536354 –> 536373	70	17,9							
V. angu-laris	*nad7*/2	GCTTTACCTTATTCTGATCG	357341 – >357360	100	49,3	*nad7*/3	TGTTCTTGGGCCATCATAGA	358711 <– 358730	95	49,8	1390	55
			344425 –> 344444	75	12,3							
			324826 –> 324845	70	23,3							
V. radi-ata	*nad7*/2	GCTTTACCTTATTCTGATCG	344715 –> 344734	100	49,3	*nad7*/3	TGTTCTTGGGCCATCATAGA	346086 <– 346105	95	49,8	1391	55
			231076 –> 231095	75	12,3							
			267792 –> 267811	70	23,3							

Locus 15: *nad7* intron 4/5 (reference: 3)

V. faba	*nad7*/4	TGTCCTCCATCACGATVTCG	222223 <– 222242	97	52,8	*nad7*/5	CCAAATTCTCCTTTAGGTGC	220060 –> 220079	100	51,8	2183	60
			306334 –> 306353	97	52,8			308497 <– 308516	100	51,8	2183	60
			537272 <– 537291	97	52,8			535109 –> 535128	100	51,8	2183	60
			17125 –> 17144	72	13,3							
			67213 –> 6723272	72	13,3							
								366402 <– 366421	100	51,8		
								436187 <– 436206	100	51,8		
V. angu-laris	*nad7*/4	TGTCCTCCATCACGATVTCG	360433 –> 360452	97	52,8	*nad7*/5	CCAAATTCTCCTTTAGGTGC	362573 <– 362592	100	51,8	2160	60
								59241 <– 59260	70	25,3		
V. radi-ata	*nad7*/4	TGTCCTCCATCACGATVTCG	347810 –> 347829	97	52,8	*nad7*/5	CCAAATTCTCCTTTAGGTGC	349937 <– 349956	100	51,8	2147	60
								56773 <– 56792	70	25,3		

References: 1 – DEMESURE *et al*. (1995); 2 – DUMOLIN-LAPEGUE *et al*. (1997); 3 – DUMINIL *et al*. (2002); Com – complementarity of a primer and template DNA expressed through the percentage of binding nucleotides; *Tm* – melting temperature of a primer in °C; Fr. len. – the length of the PCR product obtained via *in silico* PCR method; *Ta* – annealing temperature of a primer pair in °C.

Primer pair for amplification of the *nad*2 intron 3/4 failed to generate PCR product in all three legumes because its R primer, which comprised three ambiguous nucleotides, lacked primer binding sites within exon4 of the *nad*2 gene, and primed loosely (complementarity between 58% and 62%) to alternative locations in all taxa. The remaining 14 primer pairs generated desired PCR products in all three legumes and their sizes ranged from c. 1 kb (*nad*7 intron 1/2) to 3.9 kb (*nad*7 intron 3/4), while the length of the PCR product obtained with primers F: *nad*4 exon2 and R: *nad*4 exon4 of DEMESURE *et al.* (1995) was c. 6.5 kb in all three legumes. We found that F primer from this primer pair was located not within exon2 but within exon1.

Out of 13 primer pairs that generated desired PCR products long up to 4 kb, only F and R primers from a primer pair for amplification of the *nad*5 intron a/b displayed single binding sites with maximal complementarity with template DNAs of all three species. Many primers from the remaining 12 primer pairs, however, had not only highly complementary binding sites at expected locations within mtDNA genomes of studied legumes, but also additional (multiple) binding sites of lower complementarity (between 65% and 98%). We tested via PCR amplification in 16 genotypes of *V. faba* whether multiple binding sites of primers would lead to the occurrence of multiple amplification products.

The highest number of multiple binding sites was observed for R primer from a primer pair for amplification of the *nad*7 intron 1/2 in all legumes (up to five additional binding sites of lower complementarity). On the other hand, the entire *nad*7 gene was triplicated within *V. faba* mtDNA genome and exon5 of this gene was copied on two alternative locations (NEGRUK, 2013). Also, loci *nad*1 intron 2/3 and *nad*5 intron d/e were duplicated in this species (NEGRUK, 2013) and R and F primer from a primer pair for amplification of the former and the latter locus, respectively, also displayed additional binding sites of lower complementarity. We PCR amplified above listed loci (*nad*1 intron 2/3, *nad*5 intron d/e, *nad*7 introns 1/2 and 4/5) to test not only the potential confounding effect of the presence of two or three copies of introns on their utilization for further manipulation but also the effect of additional binding sites with maximal or lower complementarity with template DNA on occurrence of multiple amplification products.

Along with desired PCR products, non-specific amplification products were obtained via *in silico* PCR at two loci, *nad*7 intron 2/3 in *V. faba* (c. 4 kb) and *nad*7 intron 1/2 in *Vigna angularis* (c. 3.3 kb). They were generated with F primers (at the former locus) and with R primers (at latter locus). The occurrence of these multiple amplification products predicted via *in silico* PCR was tested via PCR amplification in 16 genotypes of *V. faba*.
> Table 1

PCR amplification of seven introns in nad genes in V. faba

Based on nucleotide content of mtDNA sequences of studied legumes comprising binding sites of primers, we replaced mispairing nucleotides in three primers (F primer from a primer pair for amplification of the *nad*7 intron 4/5 – we introduced nucleotide G at position 17 instead of an ambiguous nucleotide V; F primer from a primer pair for amplification of the *nad*4 intron 1/2 - T

at first position instead of C; and R primer from a primer pair for amplification of *nad*7 intron 2/3 - G at position 16 instead of A), and used such improved primers for PCR amplification in 16 genotypes of *V. faba*.

The outcomes of PCR amplification of seven introns in *nad* genes in 16 genotypes of *V. faba* are presented in Figure 1 (a to g). Surprisingly, PCR amplification of *nad*1 intron 2/3 was rather poor because PCR products of expected length (c. 1.6 kb) were obtained in five out of 16 individuals, while in other individuals, either faint bands of expected length or lack of PCR products were detected (Figure 1a). Furthermore, smears, which may occur due to various reasons including high DNA concentrations, low annealing temperatures, mispriming caused by secondary structure template, etc., were persistent even with altered DNA concentrations and PCR conditions (data not shown). Faint bands of expected length were obtained in one cultivar at locus *nad*5 intron d/e (Rojo, Peru) and in two cultivars at locus *nad*7 intron 4/5 (BPL4225, Morocco, and Diana, Germany), while lack of PCR products was observed in one cultivar at each out of two loci (Deligrad, Serbia at locus *nad*4 intron 2/3, and Rojo, Peru at locus *nad*7 intron 1/2). Overall, single bands of expected length suitable for further manipulation were obtained at three loci, *nad*4 intron 2/3, *nad*7 introns 2/3 and 4/5 (Figure 1c, f and g, respectively), while multiple amplification products were obtained at three loci, *nad*4 intron 1/2, *nad*5 intron d/e and *nad*7 intron 1/2 (Figure 1b, d and e, respectively). At locus *nad*7 intron 4/5, variation in length in 16 genotypes of *V. faba* was evident even on agarose gels (Figure 1g).

PCR amplification of *nad*1 intron 2/3 with PCR primers provided in this study (two F and three R primers) was successful, and only outcomes of PCR amplification with *nad*1e2f15 and *nad*1e3r1602 are presented in Figure 1h.

Readable electropherograms of PCR products in 16 *V. faba* genotypes obtained with primers *nad*1e2f15 and *nad*1e3r1602 and sequenced with F primer were of c. 700 bp, and were alignable among themselves and with the 5'-end of this intron from *V. faba* cultivar Broad Windsor whose full length is 1439 bp (NEGRUK, 2013). Within the 5'-end of the intron, two transversions were observed while length mutations were lacking. With regard to the primary nucleotide sequence of this intron reported by NEGRUK (2013), T/G transversion was detected at position 128, and A/C transversion at position 236.

DISCUSSION

A burst of complete mtDNA sequences of flowering plants especially over the past few years is essential for facilitating broader utilization of this haploid and predominantly uniparentally-inherited genome in diverse studies in plants. This is because available mtDNA sequences of taxa of interest or those of their close relatives may be used for detection of regions displaying variability in primary nucleotide sequences or in length, as well as for delineation of more conserved regions suitable for designing primers for PCR amplification of those informative regions. However, although mtDNA genomes of higher plants have rather slow evolution of primary nucleotide sequences (WOLFE *et al*., 1987; LAROCHE *et al*., 1997), they are exceptionally variable in size and organization and are prone to frequent insertions, deletion and structural re-arrangements (LAROCHE *et al*., 1997; NEGRUK, 2013 and references therein). Due to such fluid nature of plant mtDNA genomes, it is possible that mtDNA sequences available in public databases may differ to the certain extent from those of studied individuals and thus, the predictions based on known mtDNA sequences may not be fully applicable to unknown sequences.

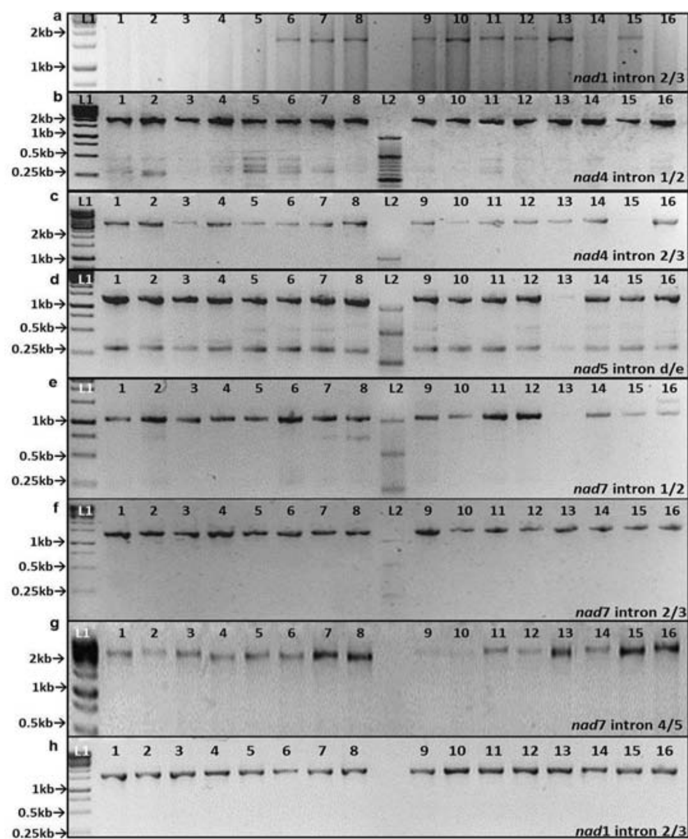

Figure 1 Results of PCR amplification of seven introns in *nad* genes in 16 genotypes of *Vicia faba* representative of the worldwide gene pool of this species; a to g – PCR products obtained with published original or improved PCR primers; h - PCR products obtained with primers developed in this study (*nad*1e2f15 and *nad*1e3r1602) for amplification of *nad*1 intron 2/3.

A negative image of an ethidium bromide-stained agarose gel is shown. The size markers L1 and L2 in the lanes 1 and 10, respectively, are fragments of a 1 kb DNA ladder and 50 DNA bp ladder; reference bands of the size marker L2 correspond to fragments of 0.25 kb, 0.5 kb and 1 kb, respectively; PCR products in *Vicia faba* traditional cultivars (TC) or improved cultivars (IC) of *major* (Ma), *minor* (Mi) and *equina* (Eq) type and subsp. *paucijuga* (Pau) are presented in lanes 2 - 8 and 11 - 18, and comprise cultivars: 1 – Troy (country of origin: Denmark, characteristics: Eq, IC); 2 - CH170 (United Kingdom – Eq, IL); 3 - E14b (Ethiopia – Eq, TC); 4 - Mikko (Finland – Mi, TC); 5 - Apsalos (Greece –Eq, IL); 6 - CH182 (India - Pau, TC); 7 - Violetta di policoro (Italy – Ma, TC); 8 - Kingpi (China – Eq, TC); 9 - BPL4225 (Morocco – not available); 10 - Diana (Germany – Mi, IC); 11 - Silian (Sudan – Mi, TC); 12 - Feve Seville (Spain – Ma, TC); 13 – Rojo Peru Ma, TC); 14 - Bački Petrovac 2 (Serbia – Ma, TC); 15 – Deligrad (Serbia – Ma, TC); and 16 - 159B (former Yugoslavia – Mi, TC).

In this study, we utilized both predictions based on *in silico* PCR analyses and PCR amplification in our source material in order to provide a set of PCR primers suitable for amplification of potentially informative introns in *nad* genes in *V. faba* which would facilitate broader utilization of mtDNA genome in phylogeographic and other studies in this species for which unambiguous data on wild ancestors, origin and domestication are lacking.

In silico PCR analyses

In silico PCR analyses revealed that six out 15 tested universal primer pairs of DEMESURE *et al*. (1995), DUMOLIN-LAPEGUE *et al.* (1997) and DUMINIL *et al.* (2002) used for predictions of amplification of all 14 *cis*-spliced introns in *nad* genes in mitochondrial genomes of *V. faba* and two *Vigna* species were not suitable for these species because they harboured mispaired nucleotides within their binding sites. Although this obstacle can be overcome, this questions universality of those primers which, however, may be increased via introduction of ambiguous nucleotides. On the other hand, we found that utilization of degenerate primers harbouring ambiguous nucleotides in primers from three out of 15 primer pairs was not necessary in studied legumes. Furthermore, we argue that in legumes, characterized by large and repetitive mtDNA genomes (e.g. the size of mtDNA genome in *V. faba* was estimated to 588 kb, NEGRUK, 2013; 404466 bp in *Vigna angularis*, NAITO et al., 2013; and 401262 bp in *Vigna radiata*, ALVERSON *et al*., 2011), utilization of degenerate primers should be avoided because they may either fail to generate PCR product (e.g. at locus *nad*2 intron 3/4), or alternatively, may display additional (multiple) binding sites with lower complementarity with template DNAs that may lead to the occurrence of multiple amplification products in PCR reactions (e.g. at loci *nad*4 intron 1/2, *nad*5 intron d/e and *nad*7 intron 1/2).

With the exception of a primer pair for amplification of the *nad*2 intron 3/4, the remaining primer pairs generated desired PCR products of up to c. 4 kb in three legume species, while the length of the PCR product obtained with primers F: *nad*4 exon2 and R: *nad*4 exon4 of DEMESURE *et al*. (1995) was c. 6.5 kb in all three legumes. DEMESURE *et al*. (1995) reported that these primers generated a PCR product of c. 4 kb in eight plant species. However, we found that such a long products were obtained because F primer was located not within exon2 but within exon1, and when employed with the R primer located within exon4, amplified the entire *nad*4 gene comprised of four exons and three introns. Such a long PCR products, although suitable for studies with restriction enzymes (DEMESURE *et al*., 1995), are rather challenging for the assessment of their primary nucleotide sequences via sequencing reactions. For instance, even mtDNA stretches of c. 2 kb have been PCR amplified via multiple PCR reactions whose products have been used to assess the nucleotide content of the entire 2 kb long region (e.g. HAVANANDA *et al*., 2010). In *V. faba*, multiple PCR reactions or sequencing with internal primers may be required for seven loci whose lengths estimated via *in silico* PCR analyses were 2 kb to 3.9 kb (*nad*1 intron 4/5, *nad*4 introns 1/2, 2/3 and 3/4, *nad*5 intron a/b and *nad*7 introns 3/4 and 4/5).

Overall, out of 15 published universal primer pairs of DEMESURE *et al*. (1995), DUMOLIN-LAPEGUE *et al*. (1997) and DUMINIL *et al*. (2002), *in silico* PCR analyses revealed that only one (for amplification of the *nad*5 intron a/b) is suitable for utilization in *V. faba* and representatives of the genus *Vigna*. Primers from this primer pair displayed single maximally complementary binding sites in all studied species and generated PCR products of c. 2.3 kb suitable for further manipulation. The main obstacles hampering utilization of other primer pairs comprised generation of exceptionally long PCR prodtucts and multiple binding sites which may lead to the

occurrence of multiple amplification products. However, as already mentioned, predictions obtained via *in silico* PCR method may not necessarily be concordant with patterns obtained via PCR reactions, as discussed below.

PCR amplification of seven introns in nad genes in V. faba

The most unexpected outcome of PCR amplification of seven introns in *nad* genes in 16 genotypes of *V. faba* was poor amplification of locus *nad*1 intron 2/3 with universal primers of DEMESURE *et al*. (1995). This is because this locus was successfully PCR amplified with same primers in 13 representatives of the genus *Vicia* including *V. faba* (RYZHOVA *et al*., 2012). Nonetheless, *nad*1 intron 2/3 was successfully PCR amplified in our material as well but with primers reported in this study.

PCR amplification of other six loci in our material revealed that intron 2/3 of the *nad*4 gene and introns 2/3 and 4/5 of the *nad*7 gene (the latter two being triplicated within mtDNA genome of *V. faba*, NEGRUK, 2013) are also suitable for future studies in this species because at these loci, multiple amplification products were lacking and PCR products of expected length were generated in all individuals with one exception only (Deligrad, Serbia at locus *nad*4 intron 2/3). Thus, it appears that duplications and triplications of mtDNA introns within mtDNA genome of *V. faba* may not represent an obstacle for their utilization. This is also supported by comparison of primary nucleotide sequences of all duplicated and triplicated mtDNA introns in *V. faba* cultivar Broad Windsor because all corresponding copies of introns harboured identical nucleotide content (data not shown). Although further work is required to reveal whether these three loci are informative for future studies in *V. faba*, variability in length has been observed at *nad*7 intron 4/5 even on agarose gels (Figure 1g). We assume that length variation at this locus was not related to the presence of five maximally complementary binding sites of the R primer from a primer pair for amplification of this locus.

Loci *nad*4 intron 1/2, *nad*5 intron d/e and *nad*7 intron 1/2 are not recommended for future utilization in *V. faba* because primers that amplify these loci are likely to generate not only desired PCR products of expected length but also additional non-specific amplification products of various lengths. Such an outcome, however, was not fully concordant with predictions on occurrence of multiple amplification products obtained in *in silico* analyses which revealed multiple amplification products at locus *nad*7 intron 2/3 only. Nonetheless, multiple binding sites of primers within mtDNA genome of *V. faba* cultivar Broad Windsor, which signify the presence of several oligonucleotide stretches within mtDNA genome of this individual that are more or less complementary with the primary nucleotide sequence of utilized primers and may contribute towards the occurrence of multiple amplification products, were observed for majority of primers used in this study. For instance, at locus *nad*4 intron 1/2, *in silico* PCR revealed only one additional binding site of R primer used for amplification of this locus, while in PCR reaction, the highest number of non-specific bands was observed at this locus (Figure 1b).

The observed discordance with regard to the occurrence of multiple amplification products which were predicted via *in silico* analyses and obtained in PCR reactions may be due to the more technical issues, such as limitations of the utilized *in silico* method which may or may not be able to delineate all more or less complementary binding sites of primers that may generate multiple amplification products, or alternatively, to the differences in organization and/or primary nucleotide sequences of mtDNA genomes used for *in silico* analyses and those used for PCR amplification. The latter is possible owing to the exceptionally fluid organization of mtDNA

genomes in plants (LAROCHE *et al*., 1997; NEGRUK, 2013 and references therein) and potential hampering effects of heteroplasmy (WOLOSZYNSKA, 2010). Thus, in case that our plant material used for PCR amplification was also used for *in silico* PCR analyses, it is possible that additional binding sites of primers, which generated multiple amplification products at loci *nad*4 intron 1/2, *nad*5 intron d/e and *nad*7 intron 1/2, may have been detected. Therefore, we suggest that *in silico* PCR method, which utilizes mtDNA sequences that may differ to the certain extent from those of studied taxa, is not only suitable but also recommended for overcoming obstacles with regard to the mispairing of nucleotides within primer binding sites especially when based on mtDNA genome sequences of several related taxa. *In silico* PCR method is also suitable for estimating the length of PCR products, while its utility in predicting the occurrence of multiple amplification products is limited.

Finally, we support previous reports on intraspecific variability at the mtDNA level in *V. faba* (SCALLAN and HARMEY, 1996) because variability in length was detected at *nad*7 intron 4/5, while variability in primary nucleotide sequence was found within the 5'-end of the second intron of the *nad*1 gene based on analyses in 16 genotypes of *V. faba* representative of the worldwide gene pool of this species. The latter locus is one of the most commonly employed non-coding mtDNA regions in various studies in higher plants (e.g. FREUDENSTEIN and CHASE, 2001; WON and RENNER, 2003; ZHANG *et al*., 2006; TOLLEFSRUD *et al*., 2009; ALEKSIĆ and GEBUREK, 2010; HAVANANDA *et al*., 2010; RYZHOVA *et al*., 2012; LOCKWOOD *et al*., 2013) and its future utilization in *V. faba* requires PCR amplification with primers provided in this study.

Mitochondrial primers for amplification of introns in nad genes in V. faba

The aim of our study was to provide molecular tools for broader utilization of variability of the mtDNA genome in phylogeographic, phylogenetic and other studies in *V. faba* that may shed more light on its origin, domestication and evolutionary relations. We focused primarily on introns in *nad* genes because non-coding mtDNA regions are evolving faster that their adjacent coding regions, and, as mentioned above, introns in *nad* genes are amongst the most commonly used mtDNA regions in various studies in higher plants.

Out of 15 universal primer pairs published by DEMESURE *et al*. (1995), DUMOLIN-LAPEGUE *et al*. (1997) and DUMINIL *et al*. (2002) which amplify all 14 *cis*-spliced introns in *nad* genes, only one (for amplification of *nad*5 intron a/b) is suitable for *V. faba*, as inferred from *in silico* analyses. Based on PCR amplification of seven introns in 16 genotypes of *V. faba* originating from Europe, Africa, Asia and south America which represent all botanical varieties of this species (*major*, *minor*, *equina* and subsp. *paucijuga*) of various levels of improvement (traditional and improved cultivars), however, either original or improved primers from three primer pairs (for amplification of *nad*4 intron 2/3 and *nad*7 introns 2/3 and 4/5) generated desired PCR products suitable for further manipulation. Along with PCR primers for amplification of *nad*1 intron 2/3 provided in this study, we report a set of primers suitable for PCR amplification of five introns in *nad* genes in *V. faba*. Although further studies are required to reveal whether these introns are informative, variability in primary nucleotide sequence was observed within 5'-end of the *nad*1 intron 2/3, while length variability was detected at locus *nad*7 intron 4/5. We also suggest further efforts to infer whether the remaining introns in *nad* genes that were not PCR amplified in this study may be utilized in *V. faba* as well, and also testing of other non-coding mtDNA regions for potential utilization in this species.

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MOLEKULARNI MARKERI ZA ISTRAŽIVANJA DIVERZITETA NA NIVOU MITOHONDRIJALNOG GENOMA KOD BOBA (*Vicia faba*)

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Izvod

Kompletni mitohondrijalni genomi (mtDNK) boba (*Vicia faba*) i dve srodne vrste, *Vigna angularis* i *Vigna radiata*, trenutno dostupni u javnoj banci gena GenBank, su korišćeni za *in silico* PCR analizu kojom je utvrđena primenljivost 15 publikovanih univerzalnih pari prajmera za amplifikuju svih 14 *cis*-isecajućih introna u genima NADH subjedinica (*nad* gena) mitohondrijalnog genoma kod *V. faba* i srodnih vrsta. Zatim je putem PCR amplifikacije analizirano da li su PCR produkti generisani primenom sedam od 15 testiranih pari prajmera kod 16 genotipova *V. faba*, koji su predstavljali sve botaničke varijetete ove vrste (*major*, *minor*, *equina* i subsp. *paucijuga*) različitog stepena oplemenjivanja (tradicionalni i poboljšani kultivari) poreklom iz Evrope, Afrike, Azije i južne Amerike, pogodni za dalju manipulaciju. Dizajnirani su i novi prajmeri za PCR amplifikaciju *nad*1 introna 2/3 kod *V. faba* za koji je takođe utvrđena intraspecijska variajbilnost na nivou primarne sekvence nukleotida. Na osnovu rezultata *in silico* predikcija i PCR amplifikacija, definisan je set prajmera za PCR amplifikaciju pet introna u *nad* genima kod *V. faba* koji predstavljaju molekularne markere pogodne za buduće filogeografske i druge studije kod ove vrste za koju ne postoje pouzdani podaci o divljim srodnicima, centru poreklu i domestikaciji.

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