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FAMILY-SPECIFIC VS. UNIVERSAL PCR PRIMERS FOR THE STUDY OF MITOCHONDRIAL DNA IN PLANTS

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Mitochondrial genomes (mtDNAs or mitogenomes) of seed plants are characterized by a notoriously unstable organization on account of which available so-called universal or consensus primers may fail to fulfil their foreseen function - amplification of various mtDNA regions in a broad range of plant taxa. Thus, the primers developed for groups assumed to have similar organization of their mitogenomes, such as families, may facilitate a broader usage of more variable non-coding portions of these genomes in group members. Using *in silico* PCR method and six available complete mitogenomes of *Fabaceae*, it has been demonstrated that only three out of 36 published universal primer and three *Medicago sativa*-specific primer pairs that amplify various mtDNA regions are suitable for six representatives of the *Fabaceae* family upon minor modifications, and develop 21 *Fabaceae*-specific primer pairs for amplification of all 14 *cis*-splicing introns in genes of NADH subunits (*nad* genes) which represent the most commonly used non-coding mtDNA regions in various studies in plants. Using the same method and six available complete mitogenomes of representatives of related families *Cucurbitaceae*, *Euphorbiaceae* and *Rosaceae* and a model plant, *Arabidopsis thaliana*, it has further been demonstrated that applicability of newly developed primer pairs for amplification of *nad* introns in more or less related taxa was dependent not only on species evolutionary distances but also on their genome sizes. A reported set of 24 primer pairs is a valuable resource which may facilitate a broader usage of mtDNA variability in future studies at

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both intra- and inter-specific levels in *Fabaceae*, which is the third largest family of flowering plants rarely studied at the mtDNA level, and in other more or less related taxa.

Keywords: Fabaceae, mitochondrion, NADH dehydrogenase, introns, PCR primers, *in silico* PCR method

INTRODUCTION

Cytoplasmic genomes of an endosymbiotic origin - mitochondrial genomes (mtDNAs or mitogenomes) in mammals and mtDNA and chloroplast genomes (cpDNAs) in plants, differ from nuclear genomes (nrDNAs) in several aspects among which are non-Mendelian inheritance pathways (e.g. HUTCHINSON *et al.*, 1974; MOGENSEN, 1996), overall size and structure (e.g. BENDICH, 1993; 2004), replication mode (e.g. FALKENBERG *et al.*, 2007; WOLOSZYNSKA, 2010) and evolutionary rates and rules (e.g. WOLOSZYNSKA, 2010). While genes of the small (c. 16 Kbp) and simple-designed mammalian mtDNA genome are evolving 10 times faster than mammalian nuclear genes (BROWN *et al.*, 1979; GRAUR and LI, 2000; BALLARD and WHITLOCK, 2004), the opposite trend and different rates of evolution of cytoplasmic genes are typical in seed plants (WOLFE *et al.*, 1987; LAROCHE *et al.*, 1997) in which the overall relative rate of synonymous substitutions of nrDNA, cpDNA and mtDNA genes are 10:3:1 (DROUIN *et al.*, 2008). Therefore, current predominant usage of cpDNA over mtDNA variability in various studies in seed plants is mainly due to the faster evolution of their genes which may provide better resolution even at lower taxonomic levels. In addition, due to the relatively stable architecture, gene order and gene intron content of c. 200 Kbp large cpDNA genomes (reviewed in JANSEN and RUHLMAN, 2012), it is possible to develop so-called universal or consensus primers (e.g. TABERLET *et al.*, 1991; SHAW *et al.*, 2007; HAIDER, 2011 and references therein) which amplify various cpDNA regions in a broad range of plant taxa. Such primers are usually applicable in species of interest, and thus, it is not necessary to invest resources into the development of *de novo* primers.

Universal mtDNA primers have also been reported in plants, but it seems that only a small number of them are truly universal (e.g. TABERLET *et al.*, 1991). For instance, FROELICHER *et al.* (2011) demonstrated recently that 35 out of 44 published and commonly used universal mtDNA PCR primer pairs failed to generate PCR amplicons in citrus, while the performance of some of these primers was slightly better in, for instance, spruces (JARAMILLO-CORREA *et al.*, 2003). This may be due to the numerous molecular peculiarities of mtDNA genomes of seed plants, such as frequent insertions, deletions and structural re-arrangements via homologous recombination between direct and inverted repeat sequences (LAROCHE *et al.*, 1997; KUBO and MIKAMI, 2007; KUBO and NEWTON, 2008; KITAZAKI and KUBO, 2010; MARÉCHAL and BRISSON, 2010) which have led to the exceptional variability in size (200 to 2.900 Kbp), rather fluid organization, non-conserved and variable gene order and organization of their mtDNA genomes (reviewed in KNOOP, 2012). Such unstable structure of these genomes across plant kingdom, shown to preclude the existence of universal intergenic spacers (ADAMS and PALMER, 2003; LYNCH *et al.*, 2006), is likely to preclude the development of primers with broad taxonomic spread as well.

Thus, instead of a rather comfortable usage of universal primers, some additional efforts for time- and resource-consuming development of species-specific primers are usually required in studies that employ mtDNA variability in plant species (e.g. HAVANANDA *et al.*, 2010; FROELICHER *et al.*, 2011; LOCKWOOD *et al.*, 2013). Another obstacle discouraging workers to use mtDNAs in their surveys especially at lower taxonomic levels is the above mentioned low rate of synonymous substitutions in mtDNA genes (with few exceptions, see KNOOP, 2012 for references).

However, the non-coding portion of mtDNA genomes - introns and intergenic spacers, are evolving faster than their adjoining coding-regions (KNOOP, 2012 and references therein) and may display inter- and intraspecific variability in length and primary nucleotide sequence (see references given below). Since groups of more related species, such as families, are expected to have a similar evolution and organization of cytoplasmic genomes (e.g. WU *et al.*, 2011; GHIMIRAY and SHARMA, 2014; WANG *et al.*, 2015), development of family-specific primers may facilitate a broader usage of more variable non-coding mtDNA regions in group members. A growing number of publically available complete mtDNA genome sequences of seed plants especially over the past decade (more than 40, http://www.ncbi.nlm.nih.gov/genome/) provides an excellent genomic resource for comparative analysis of mtDNA genomes in members of different families, detection of variable and potentially informative non-coding parts and their adjoining, more conserved regions suitable for the development of family-specific primers.

Family *Fabaceae* (legumes) is the third largest family of flowering plants with c. 20.000 described species distributed worldwide (DOYLE, 1994). To date, introns of the nine subunits of the key respiratory chain proteins - NADH dehydrogenase (*nad* genes) have only occasionally been employed in various studies in members of the *Fabaceae* or closely related taxa (e.g. ZHANG *et al.*, 2006; HAVANANDA *et al.*, 2010; RYZHOVA *et al.*, 2012). However, introns in these genes are amongst the most commonly used non-coding mtDNA regions in plants, not only in diverse studies at the higher taxonomic level (e.g. BAKKER *et al.*, 2000; FREUDENSTEIN and CHASE, 2001; GUGERLI *et al.*, 2001a, b; WON and RENNER, 2003; GUO and GE, 2005; ZHANG *et al.*, 2006; RYZHOVA *et al.*, 2012; LOCKWOOD *et al.*, 2013; HAO *et al.*, 2015) but also at the species level (e.g. SPERISEN *et al.*, 2001; GAMACHE *et al.*, 2003; MENG *et al.*, 2007; TOLLEFSRUD *et al.*, 2009; ALEKSIĆ and GEBUREK, 2010; 2014; HAVANANDA *et al.*, 2010; AIZAWA *et al.*, 2015).

The aim of the present study is to provide mitochondrial PCR primers for amplification of potentially variable introns in genes of the NADH subunits suitable for members of the *Fabaceae*. To achieve this aim, complete mtDNA sequences of six *Fabaceae* species currently available in GenBank have been used: (i) to test via *in silico* PCR method whether 36 commonly used universal mtDNA primer pairs reported by DEMESURE *et al.* (1995), DUMOLIN-LAPEGUE *et al.* (1997) and DUMINIL *et al.* (2002) and three primer pairs of HAVANANDA *et al.* (2010) specific for *Medicago sativa*, which amplify various coding and non-coding mtDNA regions, are suitable for usage in *Fabaceae* species; and (ii) to design new *Fabaceae*-specific primers for PCR amplifications of all 14 *cis*-splicing introns in *nad* genes. It has also been test via *in silico* PCR method whether *Fabaceae*-specific primers are suitable for six members of three related families, *Cucurbitaceae*, *Euphorbiaceae* and *Rosaceae*, and a model species, *Arabidopsis thaliana*, whose complete mitogenomes are available in GenBank.

MATERIAL AND METHODS

MtDNA genome sequences

Complete mitochondrial genome sequences of all members of the *Fabaceae* family available in NCBI database on March 10th 2014 (six species), as well as those of representatives of the *Cucurbitaceae* (four species), *Euphorbiaceae* (one species), *Rosaceae* (one species), and a model plant species, *Arabidopsis thaliana*, were used, yielding a total sample of 13 complete mitogenomes. Accession numbers, genome sizes and authors were as follows: 1) *Fabaceae*:

Glycine max (JX463295, size: 402558 bp, CHANG *et al.*, 2013), *Lotus japonicus* (JN872551, size: 380861 bp, KAZAKOFF *et al.*, 2012), *Millettia pinnata* (JN872550, size: 425718 bp, KAZAKOFF *et al.*, 2012), *Vicia faba* cultivar Broad Windsor (KC189947, size: 588 Kbp, NEGRUK, 2013), *Vigna angularis* (AP012599, size: 404466 bp, NAITO *et al*., 2013), and *Vigna radiata* (HM367685, size: 401262 bp, ALVERSON *et al.*, 2011); 2) *Cucurbitaceae*: *Citrullus lanatus* (GQ856147, size: 379236 bp, ALVERSON *et al.*, 2011), *Cucumis melo* subsp. *melo* (JF412792, size: 2428112 bp, RODRIGUEZ-MORENO *et al.*, 2011), *Cucumis sativus* mitochondrion chromosomes 2, 1 and 3 (NC_016004, NC_016005 and NC_016006, respectively, size: 83817 bp, 1555935 bp and 44840 bp, respectively, ALVERSON *et al.*, 2011), and *Cucurbita pepo* (GQ856148, size: 982833 bp, ALVERSON *et al.*, 2010); 3) *Euphorbiaceae*: *Ricinus communis* (HQ874649, size: 502773 bp, RIVAROLA *et al.*, 2011); 4) *Rosaceae*: *Malus* x *domestica* cultivar Golden delicious (FR714868, size: 396947 bp, GOREMYKIN *et al.*, 2012); and 5) *A. thaliana* (NC_001284, size: 366924 bp, MARIENFELD *et al.*, 1996).

***In silico* PCR testing of published mtDNA primer pairs in six *Fabaceae* species**

Along with 36 unique universal mtDNA primer pairs of DEMESURE *et al.* (1995), DUMOLIN-LAPEGUE *et al.* (1997) and DUMINIL *et al.* (2002), which amplify various coding and non-coding mtDNA regions, three primer pairs specific for *Medicago sativa* (HAVANANDA *et al.*, 2010) were used as well, yielding a total sample of 39 published mtDNA primer pairs. All primers were used for *in silico* PCR amplification in six *Fabaceae* species using FastPCR 6.3 trial version (KALENDAR *et al.*, 2009). The maximal length of expected PCR products was set to eight Kbp and allowed mismatch at 3'-end was set to two nucleotides. The positions of all primer binding sites, 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, and the performance of individual primers and primer pairs in six *Fabaceae* species was evaluated.

Development of Fabaceae-specific PCR primers for amplification of cis-splicing introns in nad genes

MtDNA sequences of six *Fabaceae* species were used for designing primers for amplification of all 14 *cis*-splicing 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. Forward (F) and Reverse (R) primers of each primer pair were obtained using FastPCR 6.3 trial version (KALENDAR *et al.*, 2009). The differences in melting temperatures of F and R primers from a particular primer pair were set to four °C, CG clump was not opted, and primers of c. 20 bp length and CG content above 50% were generated. In cases when several different primer pairs may be used for the amplification of a particular intron, only those having the least number of multiple binding sites (*mbs*) in all six *Fabaceae* species were further used despite somewhat lower CG content and larger differences in melting temperatures of such primers. The positions of all primer binding sites, their location with respect to the coding and non-coding portion of the mtDNA genome [i.e. within exon (e), within intron (i), spanning over 3'-end of the exon and 5'-end of the intron (e/i) or spanning over 3'-end of the intergenic spacer and 5'-end of the exon (s/e)], and the other relevant parameters regarding primers mentioned above were recorded.

Transferability of Fabaceae-specific mtDNA primers into related species and A. thaliana

Fabaceae-specific mtDNA primers were tested via *in silico* PCR method in six species belonging to *Cucurbitaceae*, *Euphorbiaceae*, *Rosaceae*, and in a model species, *Arabidopsis thaliana*. All relevant parameters regarding primers mentioned above were recorded and their performance in studied species was estimated as well.

RESULTS

In silico PCR testing of published mtDNA primer pairs in six Fabaceae species

The outcomes of *in silico* PCR amplification of 39 published mtDNA primer pairs in six *Fabaceae* species (positions of all primer binding sites, the complementarity of primers and template DNA, *Tm* of individual primers, *Ta* of primer pairs and lengths of generated PCR products) are given in Supplementary material Table S1, and the statistics on performance of individual primers is given in Fig. 1A. Regarding the binding sites of individual primers (78 individual primers from 39 primer pairs, which have 468 primer binding sites in six *Fabaceae* species, treated as 468 primers with 468 primer binding sites), 216 out of 468 primers (46.15%) had unique binding sites at expected positions (complementarity with template DNA ranging from 63% to 100%), 245 primers (52.35%) had additional, usually poorly complementary binding sites along with the binding site at the expected position (*mbs*, not including primers that amplify duplicated and triplicated mtDNA regions, see later), and seven primers (1.50%) lacked primer binding sites in all species. Primers with the highest number of *mbs* were F and R primers from a primer pair for amplification of *rps*12-2/nad3-1 (10 to 11 binding sites for each of these primers in all six species) and F and R primers for amplification of *orf*25 (four to six binding sites for each of these primers in more or less all six species). Regarding the complementarity of individual primers with template DNA, misprimed nucleotides were present in 182 out of 468 primer binding sites (38.89%, including all 20 degenerate primers that have 120 primer binding sites in six species).

Based on the criteria that *in silico* PCR amplification of target mtDNA regions was successful if: 1) binding sites of individual primers were at expected positions and unique (i.e. lacking *mbs* that may contribute towards occurrence of multiple amplification products); 2) primers were highly complementary with template DNA; and 3) the lengths of generated PCR product were suitable for further manipulation (up to a few Kbp), none of 39 primer pairs would meet these criteria and thus, they cannot be characterized as suitable for usage in six members of *Fabaceae*. Nonetheless, one primer pair specific for *Medicago sativa* (for amplification of the c. 1 Kbp long 5'-end of the fourth intron of *nad*7 gene, *nad*7inner F1/R1) was successfully amplified in five species, while in *Lotus japonicus*, a single nucleotide mismatch was observed in the binding site of the R primer. Regarding 36 universal primers, four primer pairs (11.11%, for amplification of loci *ccb*256, *nad*1 intron 4/5, *nad*5 intron a/b and *rpS*14-*cob*) may be characterized as suitable not for all but for majority of studied species because these primers were highly complementary with template DNA (95% to 100%), they had the least number of *mbs*, and generated PCR products of 529 bp to 3.3 Kbp. Although the primers of DEMESURE *et al.* (1995) for amplification of *nad*4 subunit had unique binding sites and were highly complementary with template DNA, they generated PCR products of c. 6 Kbp in all species because the F primer was not located within exon2, as expected, but within exon1, and thus, the entire *nad*4 gene, comprised of four exons and three introns, was amplified with these primers. Although such long fragments may be suitable for analyses with restriction enzymes, PCR amplification of such long products may be difficult as well as their sequencing.

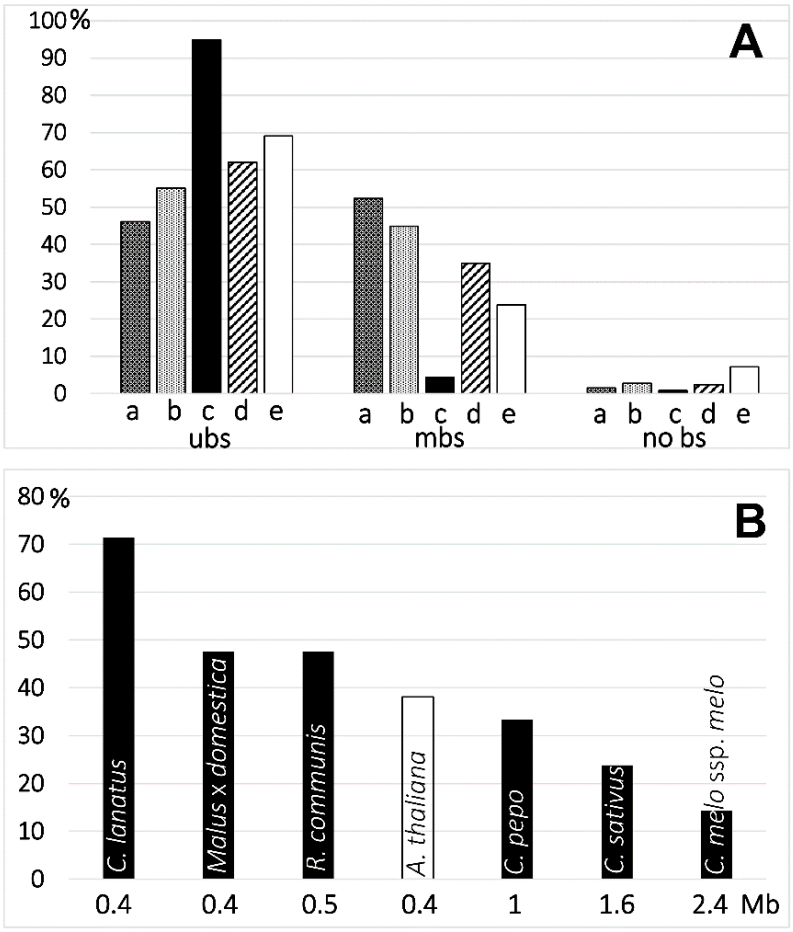

Figure 1. The performance of mitochondrial PCR primers in studied species (A), and transferability of Fabaceae-specific primers into more or less related species (B).

ubs, the percentage of individual primers having unique binding sites; mbs, the percentage of individual primers having multiple binding sites; no bp, the percentage of individual primers lacking binding sites; a -- e in fig. 1A, the transferability of: published primers which amplify various coding and non-coding mtDNA regions into *Fabaceae*, published primers which amplify introns in *nad* genes into *Fabaceae*, *Fabaceae*-specific primers into *Fabaceae*, *Fabaceae*-specific primers into members of *Cucurbitaceae*, *Euphorbiaceae Rosaceae*, and *Fabaceae*-specific primers into *Arabidopsis thaliana*, respectively; the sizes of mtDNA genomes of studied species in fig. 1B are given at x-axis; Mb, mega base-pairs.

The remaining 31 universal primer pair and two primer pairs specific for *Medicago sativa* (84.61% of a total of 39 primer pairs) did not perform well in six *Fabaceae* species due to various reasons. PCR products were not generated at four loci (*atp*6-2, *ccb*206, *nad*2 intron 3/4 and *nad*7

intron 4/5 inner F2/R2) in all species due to the lack of a primer binding site of one of the primers, or displaced binding sites of F and R primers from a particular primer pair (too distant or less than 100 bp distant, see below). At three loci (*cox*2/1–2, *cox*2/2–3 and *nad*9), PCR products were not obtained in one or two species which lacked primer binding sites for one of the primers. Multiple amplification products obtained with a single primer pair were observed at two loci (*rps*3 and r*ps*12-2/*nad*3-1). However, at five loci (*cox*1, *cox*2/1-2, *cox*3, *nad*7 intron 1/2 and *nad*7 intron 2/3), they were generated also with two F or two R primers from a particular primer pair along with the target PCR product, and their lengths ranged from 3 Kbp to 7 Kbp.

Out of 18 universal and *Medicago sativa*-specific primer pairs that amplify 14 *cis*-splicing introns in *nad* genes (36 individual primers treated as 216 primers with 216 primer binding sites in six species), 119 primers (55.09%) had unique and highly complementary binding sites in six legumes, 97 primers (44.91%) had additional *mbs* with various complementarity with template DNA, and six primers (2.78%) lacked primer binding sites (Fig. 1A). The highest number of *mbs* was observed for R primer used for amplification of *nad*7 intron 1/2 (up to eight binding sites in six species). Regarding the complementarity of these primers with template DNA, 63 out of 216 primers (29.16%, including all degenerate primers) harboured mismatch nucleotides within primer binding sites. PCR products were not obtained at two loci (*nad*2 intron 3/4 and *nad*7 innerF2/R2) in all species because binding sites of F and R primers were far away from each other (at the former locus) or distant less than 100 bp (at the latter locus). Based on the above mentioned criteria for estimating the successfulness of *in silico* PCR amplification, only three out of 18 primer pairs (16.67%) may be characterized as suitable for majority but not for all studies species (universal primers for amplification of *nad*1 intron 4/5 and *nad*5 intron a/b, and *Medicago sativa*-specific primers for amplification of c. 1 kb of the 5'-end of the fourth intron of *nad*7 gene, *nad*7inner F1/R1).

***Fabaceae*-specific PCR primer pairs for amplification of *cis*-splicing introns in *nad* genes**

Twenty one *Fabaceae*-specific PCR primer pairs were provided for amplification of 14 *cis*-splicing introns in *nad* genes, and the outcomes of *in silico* PCR amplification in six *Fabaceae* species are given in Table 1.

One primer pair per locus was provided for nine loci (introns 2/3 and 4/5 of *nad*1 gene, introns 1/2 and 4/5 of *nad*2 gene, introns 1/2, 2/3 and 3/4 of *nad*4 gene, intron a/b of *nad*5 gene, and intron 3/4 of *nad*7 gene), and the F primer from a primer pair used for amplification of *nad*4 intron 3/4 (F: *nad*4e3f202) was in addition used with the R primer *nad*4 exon4 of DEMESURE *et al.* (1995) which performed well in six *Fabaceae* species. It is worth mentioning that *nad*1 intron 2/3 was duplicated, and the complete *nad*7 subunit of NADH dehydrogenase, comprised of five exons and four introns, was triplicated in *Vicia faba*. Therefore, duplicated (*nad*1 intron 2/3) and triplicated (*nad*7 intron 3/4) maximally complementary primer binding sites and PCR products having identical lengths and primary nucleotide sequences were obtained at these loci in this species.

Two primer pairs per locus were given for four loci, *nad*5 intron d/e, *nad*2 intron 3/4 and introns 1/2 and 2/3 of *nad*7 gene, and each set of primers was suitable for a subset of species. For instance, at locus *nad*7 intron 2/3, two different F primers (*nad*7e2f218 and *nad*7e2f236) were used with the same R primer (*nad*7e3r1715), because the first F primer had an additional binding site of 62% complementarity and *Tm* of 26.1 °C in *Lotus japonicus* while the second F primer had an additional binding site of 67% complementarity and *Tm* of 43.2 °C in *Milletia pinnata*. Due to the duplications (*nad*5 intron d/e) and triplications (*nad*7 introns 1/2 and 2/3) of loci in *Vicia faba*,

additional primer binding sites and PCR products of identical lengths and primary nucleotide sequences were observed.

Table 1. The outcomes of in silico PCR amplification of 14 cis-splicing introns in nad genes in six Fabaceae species with 21 Fabaceae-specific mitochondrial PCR primer pairs, primer characteristics and PCR products lengths.

Glycine max

gene	intron *Ta* (°C)	F primer location	5' - 3' position and orientation	R primer location	5' - 3' position and orientation	Len (bp)
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	53720 <– 53742	e	55340 –> 55362	1643
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	340728 <– 340747	e	337292 –> 337312	3456
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	134701 <– 134720	i	133643 –> 133663	1078
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	320467 –> 320486	e	323171 <– 323192	2726
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(66°C)	i	320686 –> 320705	i	323002 <– 323023	2338
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	
	(60°C)	e	318400 –> 318420	e	320467 <– 320486	2087
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	102723 <– 102742	e	100652 –> 100673	2091
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	100652 <– 100673	e	96898 –> 96919	3776
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	96898 <– 96919	e	94102 –> 94123	2818
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	96898 <– 96919	e	94095 –> 94114	2825
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	252177 –> 252197	e	253947 <– 253967	1791
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	267658 –> 267676	i	268843 <– 268862	1205
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	267651 –> 267670	i	268790 <– 268810	1160
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	170004 –> 170026	e	171122 <– 171142	1139
					149893 <– 149913 (*c. 73%)	
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	170049 –> 170071	e	171104 <– 171124	1076
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	171104 –> 171124	e	172610 <– 172629	1526
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(65°C)	i	171122 –> 171142	e	172610 <– 172629	1508
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	172608 –> 172630	e	174178 <– 174198	1591

	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	174177 –> 174196	e	176375 <– 176398	2222
				e	355029 <– 355052	
				e	256308 <– 256331	
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	174177 –> 174196	i	176079 <– 176098	1922
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
	(64°C)	e	174177 –> 174196		175989 <– 176009	1833

Lotus japonicus

gene	**intron** ***Ta*** **(°C)**	**F primer location**	**5' - 3' position and orientation**	**R primer location**	**5' - 3' position and orientation**	**Len (bp)**
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	3079 <– 3101	e	4691 –> 4713	1635
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	43048 –> 43067	e	46477 <– 46497	3450
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	60379 –> 60398	i	61434 <– 61454	1076
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	101612 –> 101631	e	103460 <– 103481	1870
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(65°C)	i	101832 –> 101851	i	103291 <– 103312	1481
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	
	(60°C)	e	99509 –> 99529	e	101612 <– 101631	2123
					353430 –> 353449 (c. 70%)	
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	137801 <– 137820	e	135717 –> 135738	2104
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	135717 <– 135738	e	131995 –> 132016	3744
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	131995 <– 132016	e	129199 –> 129220	2818
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	131995 <– 132016	e	129192 –> 129211	2825
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	73439 <– 73459	e	71653 –> 71673	1807
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	222198 –> 222216	i	223395 <– 223414	1217
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	222191 –>222210	i	223342 <– 223362	1172
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	24972 –> 24994	e	26103 <– 26123	1152
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	25017 –> 25039	e	26085 <– 26105	1089
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	26085 –> 26105	e	27592 <– 27611	1527
			189146 –> 189166 (c. 62%)			
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	

	(65°C)	i	26103 –> 26123	e	27592 <– 27611	1509
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	27590 –> 27612	e	29160 <– 29180	1591
	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	29159 –> 29178	e	31386 <– 31409	2251
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	29159 –> 29178	i	31090 <– 31109	1951
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
	(64°C)	e	29159 –> 29178	i	31000 <– 31020	1862

Milletia pinnata

gene	intron *Ta* (°C)	F primer location	5' - 3' position and orientation	R primer location	5' - 3' position and orientation	Len (bp)
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	3025 <– 3047	e	4641 –> 4663	1639
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	370467 –> 370486	e	373868 <– 373888	3422
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	194832 –> 194851	i	195889 <– 195909	1078
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	134741 <– 134760	e	132016 –> 132037	2745
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(66°C)	i	134522 <– 134541	i	132185 –> 132206	2357
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	
	(60°C)	e	136805 <– 136825	e	134741 –> 134760	2085
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	356015 <– 356034	e	353949 –> 353970	2086
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	353949 <– 353970	e	350192 –> 350213	3779
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	350192 <– 350213	e	347923 –> 347944	2291
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	350192 <– 350213	e	347916 –> 347935	2298
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	117077 –> 117097	e	118847 <– 118867	1791
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	241897 –> 241915	i	243082 <– 243101	1205
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	241890 –> 241909	i	243029 <– 243049	1160
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	410193 –> 410215	e	411315 <– 411335	1143
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	410238 –> 410260	e	411297 <– 411317	1080
			20538 <– 20560 (c. 61%)			
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	411297 –> 411317	e	412806 <– 412825	1529
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	

	(65°C)	i	411315 –> 411335	e	412806 <– 412825	1511
			234667 –> 234687 (c. 67%)			
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	412804 –> 412826	e	414367 <– 414387	1584
	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	414366 –> 414385	e	416573 <– 416596	2231
					286384 <– 286407 (c. 67%)	
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	414366 –> 414385	i	416264 <– 416283	1918
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
	(64°C)	e	414366 –> 414385	i	416174 <– 416194	1829

Vicia faba

gene	intron *Ta* (°C)	F primer location	5' - 3' position and orientation	R primer location	5' - 3' position and orientation	Len (bp)
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	449930 <– 449952	e	448313 –> 448335	1640
	(62°C)	e	380145 <– 380167	e	378528 –> 378550	1640
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	89446 –> 89465	e	92875 <– 92895	3450
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	27911 –> 27930	i	28932 <– 28952	1042
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	415435 –> 415454	e	417713 <– 417734	2300
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(66°C)	i	415654 –> 415673	i	417544 <– 417565	1912
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	
	(60°C)	e	413347 –> 413367	e	415435 <– 415454	2108
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	353346 –> 353365	e	355416 <– 355437	2092
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	355416 –> 355437	e	359043 <– 359064	3649
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	359043 –> 359064	e	362039 <– 362060	3018
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	359043 –> 359064	e	362048 <– 362067	3025
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	71676 –> 71696		73461 <– 73481	1806
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	279800 –> 279819	i	280980 <– 280999	1200
	(63°C)	e	563807 <– 563825	i	562626 –> 562645	1200
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	279793 –> 279812	i	280927 <– 280947	1155
	(62°C)	e	563813 <– 563832	i	562678 –> 562698	1155
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	302183 –> 302205	e	303311 <– 303331	1149
	(65°C)	s/e	541420 <– 541442	e	540294 –> 540314	1149

	(65°C)	s/e	226371 <– 226393	e	225245 –> 225265	1149
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	302228 –> 302250	e	303293 <– 303313	1086
	(63°C)	e	541375 <– 541397	e	540312 –> 540332	1086
	(63°C)	e	226326 <– 226348	e	225263 –> 225283	1086
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	303293 –> 303313	e	304791 <– 304810	1518
	(63°C)	e/i	540312 <– 540332	e	538815 –> 538834	1518
	(63°C)	e/i	225263 <– 225283	e	223766 –> 223785	1518
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(65°C)	i	303311 –> 303331	e	304791 <– 304810	1500
	(65°C)	i	540294 <– 540314	e	538815 –> 538834	1500
	(65°C)	i	225245 <– 225265	e	223766 –> 223785	1500
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	304789 –> 304811	e	306337 <– 306357	1569
	(63°C)	e	538814 <– 538836	e	537268 –> 537288	1569
	(63°C)	e	223765 <– 223787	e	222219 –> 222239	1569
	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	306336 –> 306355	e	308550 <– 308573	2238
	(63°C)	e	537270 <– 537289	e	535052 –> 535075	2238
	(63°C)	e	222221 <– 222240	e	220003 –> 220026	2238
				e	436240 <– 436263	
				e	366455 <– 366478	
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	306336 –> 306355	i	308254 <– 308273	1938
	(64°C)	e	537270 <– 537289	i	535352 –> 535371	1938
	(64°C)	e	222221 <– 222240	i	220303 –> 220322	1938
				i	325641 –> 325660 (c. 70%)	
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
	(64°C)	e	306336 –> 306355	i	308164 <– 308184	1849
	(64°C)	e	537270 <– 537289	i	535441 –> 535461	1849
	(64°C)	e	222221 <– 222240	i	220392 –> 220412	1849

Vigna angularis

gene	intron *Ta* (°C)	F primer location	5' - 3' position and orientation	R primer location	5' - 3' position and orientation	Len (bp)
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	222887 <– 222909	e	224521 –> 224543	1657
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	68028 <– 68047	e	64593 –> 64613	3455
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	147750 –> 147769	i	148809 <– 148829	1080
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	99119 <– 99138	e	96427 –> 96448	2712
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(66°C)	i	98901 <– 98920	i	96596 –> 96617	2325
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	

	(60°C)	e	101184 <– 101204	e	99119 –> 99138	2086
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	86588 –> 86607	e	88660 <– 88681	2094
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	88660 –> 88681	e	92361 <– 92382	3723
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	92361 –> 92382	e	95127 <– 95148	2788
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	92361 –> 92382	e	95136 <– 95155	2795
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	120899 <– 120919	e	119116 –> 119136	1804
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	184003 –> 184021	i	185188 <– 185207	1205
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	183996 –> 184015	i	185135 <– 185155	1160
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	356258 –> 356280	e	357370 <– 357390	1133
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	356303 –> 356325	e	357352 <– 357372	1070
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	357352 –> 357372	e	358864 <– 358883	1532
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(65°C)	i	357370 –> 357390	e	358864 <– 358883	1514
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	358862 –> 358884	e	360436 <– 360456	1595
	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	360435 –> 360454	e	362626 <– 362649	2215
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	360435 –> 360454	i	362330 <– 362349	1915
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
		e	360435 –> 360454		–	–

Vigna radiata

gene	intron *Ta* (°C)	F primer location	5' - 3' position and orientation	R primer location	5' - 3' position and orientation	Len (bp)
***nad*1**	**i 2/3**	nad1e2f15	TCTAGGAGCATTACGATCTGCAG	nad1e3r1649	TTCTGGGAGATCAAACGGAGC	
	(62°C)	e	3058 <– 3080	e	4692 –> 4714	1657
	i 4/5	nad1e4f46	CGTCTTCAATGGGGTCTGCT	nad1e5r3475	GGAAAGGTGACTGAAACACCA	
	(63°C)	s/e	65572 <– 65591	e	62137 –> 62157	3455
***nad*2**	**i 1/2**	nad2e1f311	CTAATGCGGCACCGGGTTCG	nad2e2r1352	TTCGGATTTGCGCGAACTAGC	
	(65°C)	i	193796 <– 193815	i	192736 –> 192756	1080
	i 3/4	nad2e4f535	GGTGGGTGAACCCTCATAGA	nad2e3r2138	CAATTAGCCAAGATTTTGACCG	
	(61°C)	e	96634 <– 96653	e	93945 –> 93966	2709
			228903 <– 228922 (c. 65%)			
		nad2e4f754	ATCAGACGGCTGTTCCCGTA	nad2e3r2665	GTACCGCTCAGATTGCGGCCAA	
	(66°C)	i	96416 <– 96435	i	94114 –> 94135	2322
	i 4/5	nad2e5f172	ATCCATGTCCTAGGTGTATCA	nad2e4r2260	TCTATGAGGGTTCACCCACC	

	(60°C)	e	98700 <– 98720	e	96634 –> 96653	2087
***nad*4**	**i 1/2**	nad4e1f52	TGTCCCGTGCTAGGAAGCAT	nad4e2r2155	GGTGCCTCTACATGAGCTTCGG	
	(67°C)	e	84098 –> 84117	e	86170 <– 86191	2094
	i 2/3	nad4e2f252	CCGAAGCTCATGTAGAGGCACC	nad4e3r3879	CCCCGATAAAGCTGCTAGTACC	
	(65°C)	e	86170 –> 86191	e	89872 <– 89893	3724
	i 3/4	nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4e4r3198	TTTGGGGTGAACACCCATCCGA	
	(65°C)	e	89872 –> 89893	e	92641 <– 92662	2791
		nad4e3f202	GGTACTAGCAGCTTTATCGGGG	nad4 exon4[1]	GGAACACTTTGGGGTGAACA	
	(63°C)	e	89872 –> 89893	e	92650 <– 92669	2798
***nad*5**	**i a/b**	nad5af276	ATGAAGTCGCACCGGGAGCTA	nad5br2061	GGCATGAATCACCGAACCTGC	
	(66°C)	e	118380 <– 118400	e	116596 –> 116616	1805
	i d/e	nad5d125	TGCTGCTCCAACCATTACC	nad5er1305	CCCTCATCCGGCTCTCTGCA	
	(63°C)	e	324730 <– 324748	i	323544 –> 323563	1205
		nad5df58	CCGAGTTTGCTGCTCCAACC	nad5er1192	CCTCCCTTTACCACATCATGG	
	(62°C)	e	324736 <– 324755	i	323596 –> 323616	1160
***nad*7**	**i 1/2**	nad7e1f83	CCCATGACGACTAGGAACGGGCA	nad7e2r1211	CATTCAGTGACTCCGCGATCG	
	(65°C)	s/e	343632 –> 343654	e	344744 <– 344764	1133
		nad7e1f128	TTCGGACCTCAACATCCTGCTGC	nad7e2r1193	TCGCCCTCTAAACGATCAGAA	
	(63°C)	e	343677 –> 343699	e	344726 <– 344746	1070
					188921 –> 188941 (c. 67%)	
	i 2/3	nad7e2f218	TTCTGATCGTTTAGAGGGCGA	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(63°C)	e/i	344726 –> 344746	e	346239 <– 346258	1533
		nad7e2f236	CGATCGCGGAGTCACTGAATG	nad7e3r1715	GACGGAGTTGATGCTCCCAC	
	(65°C)	i	344744 –> 344764	e	346239 <– 346258	1515
	i 3/4	nad7e3f289	ATGTGGGAGCATCAACTCCGTCC	nad7e4r1837	CATTCGACATCGTGATGGAGG	
	(63°C)	e	346237 –> 346259	e	347813 <– 347833	1597
	i 4/5	nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2552	GGTGCTCTTATTTTACGACGGTAG	
	(63°C)	e	347812 –> 347831	e	349990 <– 350013	2202
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2254	TGAGCCATTACCAAGGAGCT	
	(64°C)	e	347812 –> 347831	i	349694 <– 349713	1902
		nad7e4f336	TCCTCCATCACGATGTCGAA	nad7e5r2164	CATAGGGCTTCGGCGCTACTA	
		e	347812 –> 347831	–	–	–

i, intron; position and orientation, position and orientation of primers in mtDNA genomes; location, location of a primer with regard to the coding and non-coding portion of the mtDNA genome: e, exon; i, intron; s, intergenic spacer; * c., complementarity of a primer and template DNA expressed through the percentage of binding nucleotides for primers having complementarity lower than 100%; Len, the length of the PCR products obtained via *in silico* PCR method; *Ta*, annealing temperature of a primer pair in °C; [1], R primer of DEMESURE *et al.* (1995).

Three primer pairs per locus were provided for *nad*7 intron 4/5, each suitable for a subset of species. The same F primer (*nad*7e4f336, located within exon4), and different R primers, positioned within exon5 (*nad*7e5r2552), within 3'-end of intron4 (*nad*7e5r2254) and more upstream of the 3'-end of this intron (*nad*7e5r2164), were used. The first R primer had five maximally complementary binding sites in *Vicia faba*, three of which were found within a triplicated *nad*7 gene and were generating PCR products, while the remaining two were positioned within two additional copies of exon5 and were not giving rise to PCR products. Similarly, in *Glycine max*, this R primer had three maximally complementary binding sites (due to the

occurrence of two additional copies of exon5), and only one PCR product. In addition, this primer had an additional binding site of 67% complementarity and *Tm* of 27.2 °C in *Milletia pinnata*. The second R primer was suitable for *Glycine max* and *Milletia pinnata* but not for *Vicia faba* in which the fifth binding site was lost but the fourth binding site remained, and was characterized by several mismatch nucleotides lowering complementarity to 70% (*Tm* of 29.9 °C). The third R primer was suitable for four species including *Vicia faba*, but lacked binding sites in two species from the genus *Vigna*.

Taking into account all *Fabaceae*-specific primers (42 primers which were treated as 252 primers with 252 primer binging sites in six species), 4.36% of primers had additional *mbs* and 0.79% of primers lacked primer binding sites (Fig. 1A). However, given the availability of several primer pairs for amplification of the same locus, each suitable for a subset of species, this statistics may be neglected because all introns in *nad* genes in all species may be amplified with alternative primer pairs having unique and maximally complementary binding sites and lacking *mbs*. The lengths of PCR products obtained with all above listed primer pairs in all species ranged from 1 Kbp to 3.8 Kbp.

Transferability of Fabaceae-specific mtDNA primers into related species and A. thaliana

The outcomes of *in silico* PCR analysis of 21 *Fabaceae*-specific mtDNA primer pairs in six species belonging to *Cucurbitaceae*, *Euphorbiaceae*, *Rosaceae* and in *Arabidopsis thaliana*, are presented in Supplementary material Table S2, and the statistics on performance of individual primers, given separately for *A. thaliana* and other species, is shown in Fig. 1A. In case of six species belonging to the above mentioned families, out of 252 individual primers (42 primers treated as 252 primers with 252 primer binding sites in six species), 158 primers (62.70%) had unique and highly complementary binding sites at expected positions, 88 primers (34.92%) had additional *mbs* with lower complementarity with template DNA, while six primers (2.38%) lacked binding sites. The highest number of *mbs* was observed for various primers used in *Cucumis melo* subsp. *melo*, *Cucumis sativus* mtDNA chromosome 1 and *Cucurbita pepo* having rather large mtDNA genomes (2428112 bp, 1555935 bp and 982833 bp, respectively). Regarding the complementarity of primers and template DNA, 104 primers (41.27%) had mismatches in primer binding sites. PCR products were not obtained in nine cases (7.14%), mainly due to the lack of primer binding sites of one of the primers predominantly in species with larger mtDNA genomes. In *A. thaliana*, 69.05% of *Fabaceae*-specific primers had unique and highly complementary binding sites at expected positions, 23.81% of primers had additional *mbs*, and 7.14% of primers were lacking primer binding sites. Mismatches in primer binding sites were present in less than a quarter of tested primers (23.81%). PCR products were not obtained in five cases (23.81%) mainly due to the lack of primer binding sites. The lengths of generated PCR products in *A. thaliana* and other species ranged from 1Kbp to more than 6 Kbp.

Eight primer pairs (38.09%, for amplification of *nad*1 intron 2/3, the second pair of primers for *nad*2 intron intron 3/4, *nad*2 intron 4/5, *nad*7 intron 1/2, *nad*7 intron 3/4, the first primer pair for *nad*7 intron 4/5) may be used in majority of species including *A. thaliana* upon improving complementarity of some of the primers via the replacement of mismatched nucleotides. Interestingly, several of these primer pairs (e.g. those for amplification of *nad*1 intron 2/3, the second pair of primers for *nad*2 intron intron 3/4, and a primer pair for amplification of *nad*2 intron 4/5) are suitable for all species having smaller mtDNA genomes (less than 1 Mbp)

including *A. thaliana*, while in species with larger mtDNA genomes, *mbs* of some of the primers were observed.

Regarding the applicability of *Fabaceae*-specific primer pairs in individual species upon improving complementarity of some of the primers via the replacement of up to two mismatched nucleotides, the same trend -- larger transferability into species with smaller mtDNA genomes was observed (Fig. 1B). Up to 15 primer pairs (71.42%) is suitable for *Citrullus lanatus*, ten (47.62%) for *Malus* x *domestica* cultivar Golden delicious and *Ricinus communis*, nine (38.10%) for *Arabidopsis thaliana*, seven (33.33%) for *Cucurbita pepo*, five (23.81%) for *Cucumis sativus* mitochondrion chromosome 1, and three primer pairs (14.28%) for *Cucumis melo* subsp. *melo*.

Multiple amplification products were not observed, and PCR products generated with two F or two R primers were not detected.

DISCUSSION

In contrast to the relatively stable architecture, gene order and gene intron content of the chloroplast genome of seed plants (reviewed in JANSEN and RUHLMAN, 2012), which facilitates development of universal primers usually suitable for usage in numerous species, plant mitogenomes have a notoriously unstable organization and other peculiar features (reviewed in KNOOP, 2012) on account of which development of primers having a wide taxonomic spread may be impossible. Although in rare cases, mtDNA primers may indeed turn out to be universal (e.g. TABERLET *et al.*, 1991), it appears that many of supposedly universal primers have a much narrower range of applicability than initially thought. A few published mtDNA primers have successfully been amplified in spruces (JARAMILLO-CORREA *et al.*, 2003), but more or less the same set of currently available universal mtDNA primers was of a rather limited success in citrus (FROELICHER *et al.*, 2011) and faba bean (ALEKSIĆ *et al.*, 2014). The same holds for members of *Fabaceae*, as demonstrated in the present study. Only four out of 36 tested universal primer pairs of DEMESURE *et al.* (1995), DUMOLIN-LAPEGUE *et al.* (1997) and DUMINIL *et al.* (2002), which amplify various mtDNA regions, and one out of three *Medicago sativa*-specific primer pairs of HAVANANDA *et al.* (2010), are suitable for majority of studied *Fabaceae* species. Obstacles that hamper the usage of the remaining primers include the lack of primer binding sites and PCR products, mispriming within primer binding sites, multiple binding sites that may lead to the occurrence of undesired multiple amplification products, occurrence of rather long PCR products unsuitable for further manipulation as well as non-specific PCR products generated not only with F and R primers from a particular primer pair but also with two F and/or two R primers of a single primer pair.

Therefore, further quests for universal mtDNA primers in seed plants may be vain, and the alternative solution may be development of primers having a narrower taxonomic spread. Groups of more closely related species, such as families, are assumed to have similar evolution and organization of their cytoplasmic genomes (e.g. WU *et al.*, 2011; GHIMIRAY and SHARMA, 2014; WANG *et al.*, 2015) suggesting that a more conserved gene order, which would enable the development of primers suitable for members of those groups, is to be expected. However, as demonstrated in the present study, even the development of family-specific primers may be a difficult task due to the peculiarities of mtDNA genomes of individual species. It is worth mentioning that species-specific features of chloroplast genomes have been observed in *Asteraceae* (WANG *et al.*, 2015) while in case of mtDNA genomes, extreme size ranges of mtDNA genomes have been reported in *Cucurbitaceae* (390 Kbp to 2.900 Kbp, ALVERSON *et al.*, 2010). In *Fabaceae*, mtDNA size alternations were not that pronounced (i.e. ranging from 380 Kbp to 588

Kbp), but in case of a few *nad* introns, it was impossible to deliver a single primer pair suitable for all six studied *Fabaceae* species. Therefore, several out of 21 *Fabaceae*-specific primer pairs for amplification of 14 *cis*-splicing introns in *nad* genes are not suitable for all but for a subset of studied species, and this would most likely be the case in other members of this family not used in the present study.

Eight out of 21 *Fabaceae*-specific primer pairs (38.09%) appear to be suitable for members of *Cucurbitaceae*, *Euphorbiaceae*, *Rosaceae* and *A. thaliana*. The performance of some of these primers may be improved by the replacement of up to two nucleotides in their primary nucleotide sequences to increase their complementarity with template DNA. This would imply that the development of more universal primers was possible via the introduction of degenerate nucleotides. However, this was avoided because degenerate primers are likely to have additional (multiple) binding sites especially in species with large mitogenomes, as observed for several degenerate primers published by DUMINIL *et al.* (2002). On the other hand, the assessment of transferability of *Fabaceae*-specific primers into more or less related species revealed an interesting trend. That is, although higher transferability rates are expected into more closely related species, the size of the mtDNA genome apparently plays an important role in this aspect as well. The highest transferability of primers was observed in *Citrullus lanatus* (*Cucurbitaceae*) having a rather small mtDNA genome, while transferability into a less related *A. thaliana*, characterized also by a small mtDNA genome, was higher than into other members of *Cucurbitaceae* (*Cucurbita pepo*, *Cucumis sativus* mitochondrion chromosome 1, and *Cucumis melo* subsp. *melo*) having mtDNA genomes of 1 Mbp or larger. This would suggest that the size of mtDNA genomes may also be used as an indicator of the potential transferability of *Fabaceae*-specific primers into more or less related taxa.

Along with three previously reported primer pairs which are expected to successfully amplify introns in *nad* genes in *Fabaceae* species upon minor modifications of their primary nucleotide sequence (two universal primer pairs of DUMOLIN-LAPEGUE *et al.*, 1997, and one primer pair of HAVANANDA *et al.*, 2010 specific for *Medicago sativa*), a set of 24 mtDNA primer pairs for amplification of all 14 *cis*-splicing introns in *nad* genes is available for this large group having c. 20.000 described species distributed worldwide (DOYLE, 1994). They represent a valuable resource which would facilitate broader usage of mtDNA sequences in various studies at the species and/or higher taxonomic levels not only in *Fabaceae* species but also in other more or less related taxa. Numerous studies over the past decade or so have demonstrated that mtDNA variability of plants may successfully be used for molecular characterization (e.g. CIARMIELLO *et al.*, 2015), depicting past interspecific hybridizations (ISODA *et al.*, 2000; SONG *et al.*, 2002; XIANG *et al.*, 2015), resolving evolutionary relations (e.g. GUGERLI *et al.*, 2001a, b; GUO and GE, 2005, LOCKWOOD *et al.*, 2013; HAO *et al.*, 2015), inferring phylogeographic patterns and/or histories of plant taxa (e.g. TOMARU *et al.*, 1998; BAKKER *et al.*, 2000; GAMACHE *et al.*, 2003; MENG *et al.*, 2007; JARAMILLO-CORREA *et al.*, 2010; ALEKSIĆ and GEBUREK, 2014) and assessing population genetic structures (e.g. OLSON and MCCAULEY, 2002; ALEKSIĆ and GEBUREK, 2010; AIZAWA *et al.*, 2015). Thus, an array of possible applications of presumed variability of *nad* introns in *Fabaceae* and other species, that may be detected using the primers reported in the present study, is potentially extensive.

CONCLUSIONS

In comparison to cpDNA variability, mtDNA variability in plants is still underutilised, mainly due to the slower evolution of their genes (LAROCHE *et al.*, 1997) hampering inferences at

the lower taxonomic levels, but also due to the notoriously unstable organization (reviewed in KNOOP, 2012) preventing the development and straight-forward usage of universal or consensus primers (e.g. FROELICHER *et al.* 2011; ALEKSIĆ *et al.*, 2014; present study). Therefore, a broader usage of potentially more variable mtDNA non-coding regions in various studies in plants may be facilitated by the development of primers which are specific for groups of more closely related species, such as families, assumed to have similar evolution and organization of their cytoplasmic genomes (e.g. WU *et al.*, 2011; GHIMIRAY and SHARMA, 2014; WANG *et al.*, 2015). However, even such an approach may turn out to be a difficult task because of the species-specific features of mtDNA genomes of plants, as demonstrated in the present study, revealing also that not only evolutionary distance but also the size of mtDNA genomes may impact transferability of family-specific primers into more or less related taxa. Nonetheless, as shown in the present study, the development of family-specific primers for amplification of all 14 *cis*-splicing introns in *nad* genes, which are amongst the most commonly used non-coding regions in various studies in plants (e.g. BAKKER *et al.*, 2000; FREUDENSTEIN and CHASE, 2001; GUGERLI *et al.*, 2001a, b; SPERISEN *et al.*, 2001; GAMACHE *et al.*, 2003; WON and RENNER, 2003; GUO and GE, 2005; ZHANG *et al.*, 2006; MENG *et al.*, 2007; TOLLEFSRUD *et al.*, 2009; ALEKSIĆ and GEBUREK, 2010, 2014; HAVANANDA *et al.*, 2010; RYZHOVA *et al.*, 2012; LOCKWOOD *et al.*, 2013; AIZAWA *et al.*, 2015; HAO *et al.*, 2015) is feasible for *Fabaceae*. Reported *Fabaceae*-specific mtDNA primers are advantageous over published universal mtDNA primers, and may enable broader usage of mtDNA variability residing within *nad* introns not only in members of this large family represented by 20.000 described species distributed worldwide (DOYLE, 1994) but also in more or less related taxa.

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FABACEAE-SPECIFIČNI VS. UNIVERZALNI PCR PRAJMERI ZA ISTRAŽIVANJA MITOHONDRIJSKE DNK KOD BILJAKA

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Izvod

Mitohondrijska DNK (mtDNK ili mitogenomi) semenica se odlikuju veoma nestabilnom strukturom koja onemogućava širu primenu tzv. univerzalnih ili koncenzus PCR prajmera za amplifikaciju različitih potencijalno informativnih mtDNA regiona. Stoga PCR prajmeri dizajnirani za grupe srodnih taksona za koje se pretpostavlja da se odlikuju sličnom organizacijom mitogenoma, kao što su familije, mogu omogućiti širu primenu potencijalno varijabilnih nekodirajućih regiona mtDNK kod predstavnika datih grupa. Koristeći *in silico* PCR metod i šest dostupnih kompletnih mitogenoma predstavnika familije *Fabaceae*, pokazano je da samo tri od ukupno 36 publikovanih univerzalnih PCR prajmera i tri para prajmera specifičnih za *Medicago sativa*, koji amplifikuju različite regione mtDNA, primenljivo kod predstavnika familije *Fabaceae* nakon manjih modifikacija, i dizajnirani su *Fabaceae*-specifični PCR prajmeri za amplifikaciju svih 14 *cis*-isecajućih introna u genima NADH subjedinica (*nad* geni) koji spadaju u najčešće korišćene nekodirajuće regione mtDNK u različitim studijama kod biljaka. Koristeći isti metod i šest kompletnih mitogenoma predstavnika srodnih familija kao i mitogenom model sistema *Arabidopsis thaliana*, pokazano je da primenljivost 21 para novo-dizajniranih *Fabaceae*-specifičnih prajmera kod manje ili više srodnih taksona zavisi ne samo od evolutivne distance već i od veličine genoma. Definisan je set od 24 para PCR prajmera za amplifikaciju svih *cis*-isecajućih introna u *nad* genima koji mogu doprineti široj primeni mtDNK varijabilnosti u različitim studijama na intra- inter-specijskom nivou kod predstavnika *Fabaceae*, koja predstavlja treću najbrojniju familiju cvetnica čiji predstavnici su retko ispitivana na nivou mtDNA, i kod drugih manje ili više srodnih taksona.

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