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GENOTYPING OF FLAX GENETIC RESOURCES BY MIRNA-BASED MOLECULAR MARKERS AND MORPHOLOGY

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MicroRNAs (miRNAs) are a class of non-coding RNAs about 20–24 nucleotides long. They play an important role in the gene regulation at the post-transcriptional level. They affect the plant genome response to environmental stress. The miRNA-based molecular markers is type of functional markers reported in very few plants. However, the information connected to the evaluation of genotypes by this type of markers within a single species is missing. Considering the stability, polymorphism, functionality and transferability potential of miRNA-based markers, the research was conducted to apply selected types of them (miR156b, miR408a and the combined type of miR156b/miR408a) for the genotyping analysis of eight flax genotypes of different origin together with the morphology analyses. A total of 145 miRNA loci were identified, of which 19 were unique. The highest numbers of miRNA loci (57) and unique fragments (9) as well as the highest percentage of polymorphism and the extent of polymerase chain reaction (PCR) amplification of miRNA fragments have been observed with the combination of miR156b-F and miR408-F markers. By means of the miRNA markers has been recorded the unique profile of the miRNA loci for individual accessions. The morphology study has shown that the genotypes are the same in the expression of selected morphological traits despite the different use and different places of origin. However, we have identified an interface between some of morphological traits and miRNA-based markers for genotyping the genetic resources of flax. By mutually linking these two types of markers, we were able to determine unique genotypes of flax.

Key words: miRNA156b, miRNA408a, polymorphism, qualitative traits

The expansion of the genetic base of cultivated crop varieties is a prerequisite for the reduction of their vulnerability to adverse environments and diseases, since the genetic improvement largely depends on the extent of genetic variability present in the population (Ganie & Mondal 2015). The genetic

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resources of plants should be evaluated by an integrated approach implementing several methods, including not only the most advanced molecular methods, but also the classic methods (morphological analysis) (FAO 1996; Hammer 2003). Further research of the morphological and molecular markers and their application will be beneficial to quantify the genetic diversity and genetic erosion (Rao & Hodking 2002; Hammer *et al*. 2003).

The microRNAs (miRNAs) are endogenous 20–24 nucleotide long non-coding RNAs derived from single-stranded RNA precursors that can form stem-loop structures (Barvkar *et al*. 2013). The miRNA molecules are produced from the non-coding mRNAs, which undergo various processing steps to form mature miRNA. Their ability to bind to the target mRNAs, resulting either in a translation delay or mRNA degradation (Erson-Bensan 2014), is making them a very intersting research subject. The molecules of the miRNA control gene expression of plants under various biotic and abiotic stress as well as in different developmental stages (Mondal & Ganie 2014).

The plant miRNAs are embedded in regulatory networks that coordinate different gene expression programmes in support of developmental plasticity (Rubio-Somoza & Weigel 2011). The miRNA-target node is an interface that comprises the regulatory relationship between a specific locus from the miRNA family and the direct targets under its control. In specific cases, a node reflects the interaction between one miRNA locus and one target. The modification of miRNA-target nodes might underlie morphological and physiological diversity. Most annotated miRNAs are located in the intergenic regions (Fu *et al*. 2013; Zhang *et al*. 2009). However, the intergenic regions are more liable to mutation than the genic ones, including the untranslated regions (UTRs), which result in the presence of more repeat regions within the miRNA sequences (Ganie & Mondal 2015).

One of the extensively rewiewed miRNA networks includes the miR156, which regulates the developmental timing. The family of miR156 targets the squamosa promoter binding protein (SBP), transcription factor in monocot and dicots, which is involved in controlling the flowering time and controls the transition from the juvenile to adult vegetative phase (Barvkar *et al*. 2013). The study of Kulcheski *et al*. (2010) provided evidence that the expression stability of miR156b was the highest across the soybean tissue and apllied stress conditions.

The miRNA, miR408a of *Linum usitatissimum* L., is included in the regulation of metabolism and transport procceses and it also targets some of the transcription factors. Its expression is tissue specific (Barvkar *et al*. 2013; Neutelings *et al*. 2012). The high level of expression of miR408a indicates that this miRNA might play an important role in flax development (Barvkar *et al*. 2013). However, miR408a is considered a potential biomarker in plant stress response (Bej & Basak 2014; Sunkar 2010), specifically under the conditions of drought, UV-B radiation, mechanical and nutrition stress.

The high conservation of miRNA sequences provides an opportunity to develop a novel type of molecular markers, which have so far been applied in two species, *Brassica sp.* (Fu *et al*. 2013) and *Se-*

T a b l e 1

List of *Linum usitatissimum* L. genotypes used in molecular and morphological study

Code	Genotype designator	Species	Subsp.	Type	Origin
1	kk-1127	*L. usitatissimum*	*mediterraneum* Vav. et Ell.	Intermediate	Tunisia
2	kk-1195	*L. usitatissimum*	*indo-abyssinicum* Vav. et Ell.	Linseed	Ethiopia
3	k-1288	*L. usitatissimum*	*eurasiaticum* Vav. et Ell.	Linseed	Afghanistan
4	k-3002	*L. usitatissimum*	*hindustanicum* Ell.	Intermediate	India
5	k-3730	*L. usitatissimum*	*eurasiaticum* Vav. et Ell.	Intermediate	China
6	k-7015	*L. usitatissimum*	*mediterraneum* Vav. et Ell.	Linseed	Tunisia
7	k-7130	*L. usitatissimum*	*mediterraneum* Vav. et Ell.	Intermediate	Morocco
8	u-562348	*Linum crepitans*	–	Linseed	Portugal

taria italica, including some related grass species (Yadav *et al*. 2014). The miRNA-based primers might amplify the regions between the neighbouring miRNAs, resulting in additional variation. The miRNA-based microsatellite marker system was used in rice genotyping applications (Ganie & Mondal 2015) and for the identification of salt responsive miRNA-SSR (Simple-Sequence Repeats) markers in rice (Mondal & Ganie 2014).

Flax has recently gained importance because of its high contents of health-promoting compounds such as alpha-linolenic acid and lignan. These unique health attributes stimulated research interests to understand the biology of this crop (Barvkar *et al*. 2013). However, in terms of the genetic resources of flax, it is still a problem to find adequate and appropriate genetic material for breeding and improving the varieties of flax for food use. There are institutions and scientific teams dealing with the characterisation and evaluation of genetic resources of flax (Diederichsen 2001; Diederichsen & Raney 2006; Brutch & Porokhovinova 2011a; Brutch & Porokhovinova 2011b).

The research was conducted to apply selected types of miRNA-based markers (miR156b, miR408a and the combined type of miR156b/miR408a) for the genotyping analysis of eight flax genotypes of different origin together with the morphology analyses.

MATERIAL AND METHODS

A total of seven genotypes of origin and commercial types of *L. usitatissimum* L. and one genotype of *Linum crepitans* were used in the present study. The genotypes were grown on experimental fields of N. I. Vavilov Research Institute of Plant Industry at Department of Oil and Fibre Crops in Russia-Pushkin. The selected genotypes were classified into subspecies (Table 1) based on the classification suggested by E. V. Elladi (1940).

From the molecular point of view, we were interested in the specificity of miRNA loci profile in the wild type of flax, *L. crepitans*. According to the place of origin, the genotypes have typical phenological performance of traits on the plant, flower, capsule or seed. For our purpose, we selected the following traits: petals and reproduction organ colour (Table 2).

The total genomic DNA was extracted from the roots of the germinated seeds using the modified method according to Saghai-Maroof *et al*. (1984). The extracted DNA was quantified by the Implen NanoPhotometer®, and diluted to 70 ng/µl with nuclease-free water for PCR amplification. The primers for the miRNA-based markers were designed according to the mature miRNAs sequences, originating from the miRNA database (http://www.mirbase.org/). The single forward primers and the universal miRNA reverse primer (Chen *et al*. 2005; Kulcheski *et al*. 2010) were combined to perform a marker assay (Table 3). For the assay, we selected two types of miRNA-based primers: gm-miR156b and lus-miR408a. The effectiveness and transferability of the gm-miR156b primer have been confirmed in previous studies (Hlavačková *et al*. 2015; Ražná *et al*. 2015).

T a b l e 2

List of morphological traits

Trait name	colour of petals in bud just before flowering
	colour of petals on fully opened flower
	colour of veins on flower petals
	colour of anthers
	colour of anther filament
	colour of stigma
	colour of style

T a b l e 3

Primer combinations used for miRNA-based marker assay

Primer name	Sequences
miR156b-F miR-R	5′-TGACAGAAGAGAGAGAGCACA-3′ 5′-CCAGTGCAGGGTCCGAGGTA-3′
miR408a-F miR-R	5′-GGCTGGGAACAGACAGAGCATGGA-3′ 5′-CCAGTGCAGGGTCCGAGGTA-3′
miR156b-F miR408a-F	5′-TGACAGAAGAGAGAGAGCACA-3′ 5′-GGCTGGGAACAGACAGAGCATGGA-3′

miR – microRNA; F – forward primer; R – reverse primer

The miRNA-based markers were PCR amplified in a 20-µl reaction mixture containing 70 ng of genomic DNA, 10 pmol/dm^3 of each primer, 2 units of DreamTaq DNA polymerase, 0.8 mmol/dm^3 dNTPs (Bioline) and 1 × DreamTaq Buffer (KCl, $(NH_4)_2SO_4$, 20 mmol/dm^3 $MgCl_2$). The PCR amplification programme used the 'touchdown' method as follows: initial denaturation at 94°C for 5 min; 5 cycles of 30 s at 94°C, 45 s at 64°C (with a 1°C decrease in annealing temperature per cycle) and 60 s at 72°C; 30 cycles of 30 s at 94°C, 45 s at 60°C and 60 s at 72°C; and the final extension at 72°C for 10 min. The samples were subsequently stored at 8°C. The PCR products were separated using 15% TBE-PAGE gels, running in 1 × TBE Running Buffer at a constant power 90 V, 25 mA for 120 min. The polyacrylamide gels were stained with the GelRed™ Nucleic Acid Gel stain and were visualised in the G-Box Syngene electrophoresis documentation system. For the recording of loci number and unique identification of fragments, the gels were analysed by the GeneTools software (Syngene) (figures not shown). It should be noted that the resolution of electrophoreograms placed in the text is not such as to allow the discernment of individual fragments that have been identified through the previously mentioned software.

RESULTS AND DISCUSSION

A total of 145 miRNA loci were identified with three miRNA-based primers. Of this number, 19 loci were unique (Table 4). The highest number of loci (57) was shown by the combination of miR156b-F/miR408-F markers, whereas the combination of miR156b-F/miR-R primers produced least 39 loci. The most monomorphic loci was produced by the combination of miR408-F/miR-R primers. The lowest, but also the highest amplicon size was produced by the combination of miR156b-F/miR408-F markers.

The extent of PCR amplification of miRNA fragments amplified by miR156b-F/miR-R primer pair, ranged from 35 bp to 150 bp and it amplified 39 miRNA loci, out of which 6 were unique (Figure 1). Each of eight accessions had their own unique profile of the miRNA loci, we could therefore differentiate these genotypes using this type of combination of primers. The unique miRNA loci were identified in two oily genotypes, no. 2 from Ethiopia and no. 8 from Portugal, wherein one of the genotypes (no. 8) represents primitive flax form with dehiscent capsules of *L. usitatissimum* convar. *crepitans* (Boenn.) Dumort. The other four specific miRNA fragments were detected in three intermediate genotypes from India, China and Marocco, where two unique fragmenst were present. Our results support the data of Melnikova *et al*. (2014) where the accessions of dehiscent flax differed significantly from the accessions of flax landrace and the accessions of winter flax based on the cluster analysis derived from the retrotransposon-based molecular markers. Both accessions of dehiscent flax (one of our research) had species-specific SSAP (Sequence Specific Amplified Polymorphism) markers.

The highest number of miRNA loci (7) was detected in the intermediate genotype (no. 5) from China and the oily genotype of *L. crepitans* (no. 8) from Portugal. On the other hand, the lowest number of

T a b l e 4

Description of PCR amplification parameters

Primer combinations	Extent of PCR amplification of miRNA fragments	Number of amplified miRNA fragments	Number of unique fragments	Percentage of polymorphism
miR156b-F/miR-R primer pair	30–150 bp	39	6	15.38
miR408-F/miR-R primer	60 bp above 330 bp	49	4	8.16
miR156b-F/miR408-F	20 bp above 330 bp	57	9	15.79
Total	–	145	19	13.10

F – forward primer; R – reverse primer; bp – base pair

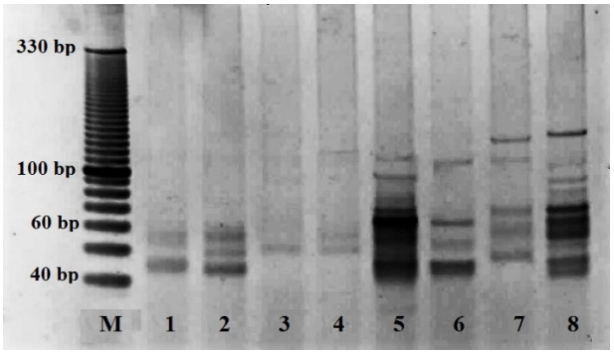


Figure 1. PCR amplification profiles generated with miR156b-F/miR-R primer pair in flax genotypes

Legend: M – DNA Ladder; 1 – kk-1127; 2 – kk-1195; 3 – k-1288, 4 – k-3002; 5 – k-3730; 6 – k-7015; 7 – k-7130; 8 – u-562348

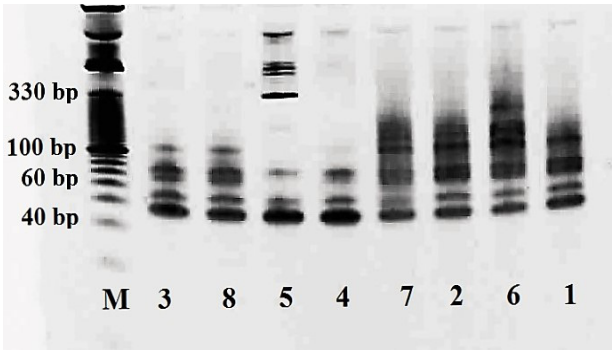


Figure 2. PCR amplification profiles generated with miR408a-F/miR-R primer pair in flax genotypes

Legend: M – DNA Ladder; 1 – kk-1127; 2 – kk-1195; 3 – k-1288, 4 – k-3002; 5 – k-3730; 6 – k-7015; 7 – k-7130; 8 – u-562348

miRNA loci was observed in the intermediate genotype (no. 1) from Tunisia. Two genotypes from Tunisia, one (no. 1) of intermediate type and one (no. 6) of oily type were included into the analysis. Their miRNA profiles were genotype specific. The group of oily flax genotypes (no. 2, 3, 6 and 8) as well as the group of intermediate flax genotypes (no. 1, 4, 5 and 7) were characterised by a specific miRNA loci profile depending on their origin.

The extent of PCR amplification of the miRNA fragments amplified by miR408a-F/miR-R primer pair, ranged from 60 bp to 330 bp and it amplified 49 miRNA loci in total, out of which 4 were unique (Figure 2). The highest number of loci (8) was observed in the intermediate genotype of Ethiopian origin and the lowest number of miRNA loci (5) was amplifiend in the two oily genotypes (no. 3 and 8), one of which represents the wild type of flax *(L. crepitans)* and the intermediate genotype from India. Four unique miRNA loci were recorded in the oily genotype (no. 2) from Ethiopia and two intermediate genotypes from India (no. 4) and China (no. 5), while these two genotypes (no. 4 and 5) were characterised by a significantly different miRNA profile.

A total of 57 miRNA loci were amplified by the combination of two applied primers, miR156b and miR408a, out of which 9 were unique (Figure 3). Each of the genotypes was characterised by a specific miRNA profile. Three out of four oily genotypes generated six unique miRNA loci. Three unique fragments were observed in the Ethiopian genotype no. 3, two unique fragments were observed in the genotype originating in Afganistan and one unique fragment was observed in Portuguese wild type of *L. crepitans*. The above-mentioned genotype (*L. crepitans*) was also characterised by the highest number of miRNA loci and genotype no. 5 from China had the lowest number of loci. Although the two genotypes from Tunisia (no. 1 of intermediate type and no. 6 of oily type) had the same number of miRNA loci (7), their profile was markedly different.

In general, we can conclude that in comparison with the profiles generated by the miR156b primer

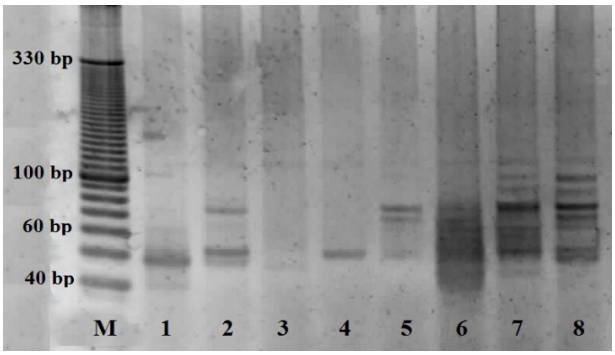


Figure 3. PCR amplification profiles generated with miR156b-F/miR408a-F primer pair in flax genotypes

Legend: M – DNA Ladder; 1 – kk-1127; 2 – kk-1195; 3 – k-1288, 4 – k-3002; 5 – k-3730; 6 – k-7015; 7 – k-7130; 8 – u-562348

and by combining the two mentioned primers, the miRNA loci profile generated by the miR408a primer was much less polymorphic (Table 5, Figure 4). This research finding is supported the study of Neutelings *et al*. (2012) concerning the role of miRNA408a in the transport procceses mediated by the roots. The unique miRNA loci in oily genotype originated from Ethiopia were amplified by all three applied primer pairs. The unique fragments in the intermediated genotype from Tunisia (no. 1) and the oily genotype from Afganistan (no. 3) were amplified only by the combination of miR156b/miR408a primers. Using the primer pairs miR156b-F/miR-R and miR156b-F/miR408a-F, it was possible to distinguish the individual genotypes based on their origin.

Apart from the analysis of miR156b, miR408a and miR156b/miR408a polymorphism, the morphological traits on flowers and reproduction organs were also traced on selected genotypes. The phenopype performance was analysed by a multilevel data sorting of flax genotypes according to the selected morphological traits. The statistical units (morphological traits) were put into a pivot table and the absolute count of follow-up objects (genotypes) was determined by frequency classes.

The tables 6 and 7 show the results of multilevel sorting of colour on buds, petals and petal nervation. We found that genotypes 5, 7 and 3 have same expression in the observed traits. The genotypes 1, 6 and 8 are the same in the expression of bud colour. Genotypes 4 and 2 had a unique expression of all three characteristics.

Table 8 shows the results of a multilevel sorting of characteristics of colour on the reproduction organs (anther, anther filament, stigma and style).

T a b l e 5

Number of amplified loci in miRNA-based marker assay

Combination of primers	Genotype number							
	1	2	3	4	5	6	7	8
Primer miR156b-F/miR-R	3	4	4	4	7	4	6	7
Primer miR408a-F/miR-R	5	5	7	5	7	8	6	6
Primer miR156b-F/miR-408a-F	7	9	7	3	6	7	9	10

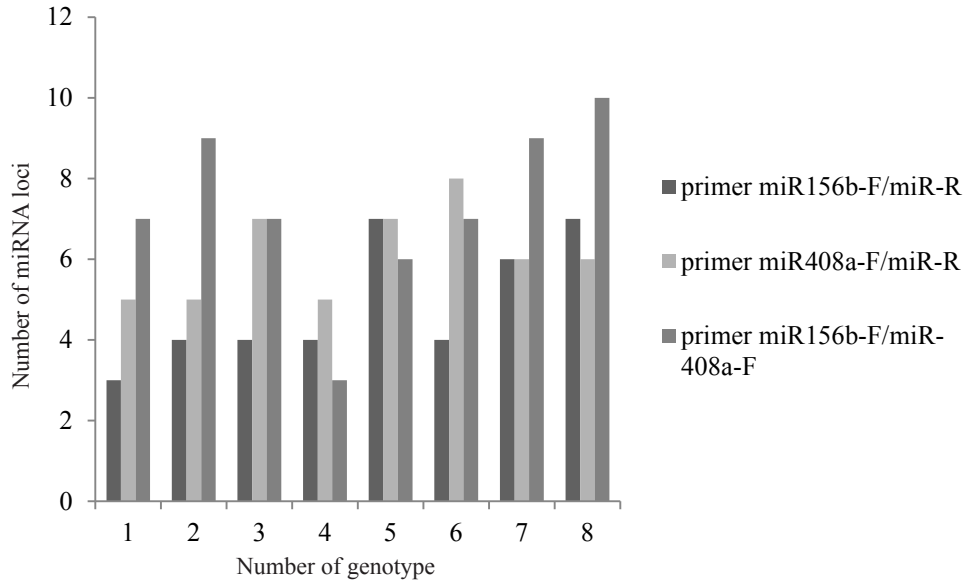


Figure 4. Number of amplified miRNA loci of eight genotypes depending on applied primer combination

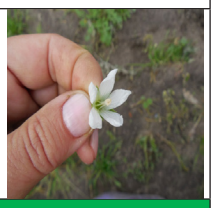
We found that genotypes 5 and 7 have yet again the same expression in the observed traits. Genotypes 1 and 3 also have the same phenotype expression and genotype 4 only differs in the stigma colour trait. Genotypes 6 and 8 differ from each other only in the style colour trait. Genotype 2 is the same as genotypes 3, 6 and 8 only in the anther colour trait. This genotype has again unique expression in other two traits as in the previous Table 7.

The results show that the genotypes are the same in the expression of selected morphological traits despite the different use (intermediate, oily) and different places of origin. The phenological expression of the studied traits, compared with E. V. Elladi classification, in many cases coincides despite the divergent classification of genotypes to subspecies. For example, genotypes 1, 3 and 4 (Table 8) are from different places of origin but consistent in all three studied traits. The same applies to genotypes 5 and 7 (Table 8). Genotype 8, which belongs to another species (*L. crepitans*), is identical with genotype 6 in three traits (Table 8).

Following the variability of the traits for colour of bud, flower petals and reproduction organs with the polymorphism of miRNA loci, we were able to identify unique genotypes. Genotypes 2 and 4 showed a unique expression of some flower characteristics (buds, petals and petal nervation colour) and in some characteristics of colour on the reproduction organs (anther, anther filament, stigma and style). These two genotypes were also characterised by a unique miRNA loci profile amplified by the miR156b and miR408a molecular markers and in the case of genotype 2 also by the combination of

T a b l e 6

Results of multilevel data sorting of genotypes (intermediate type) in qualitative traits: colour of buds, petals and nerves

Colour of flower parts		Genotypes – intermediate type			
		1	4	5	7
					
Bud	white				
	pale violet-blue				
	pale violet				
	dark blue				
Petals	white				
	very pale violet				
	very pale red-violet-blue				
	blue				
	dark blue-violet				
	pale dark blue-violet				
Nerves	white				
	blue				
	violet with red blue				
	dark blue				
	dark blue violet				
Subsp.		ME	HI	EA	ME

Legend: ME – subsp. *mediterraneum* Vav. et Ell.; HI – subsp. *hindustanicum* Ell.; EA – subsp. *eurasiaticum* Vav. et Ell.; photos by Hlavačková and Porokhovinova

both types of markers. The polymorphism profile of *L. crepitans* as a representative of wild type of flax included the unique miRNA loci determined by the combination of miR156b-F/miR-R and miR156b-F/miR408a-F primer, although this specificity was not determined at the morphological level because this genotype had identical identity with genotype 6 in three traits (Table 8).

Recently, considerable attention has been given to linking morphological and molecular markers of selected characters and properties of plants (Soto-Cerda *et al*. 2013; Shedai *et al*. 2014). For example, between the higher concentration of oil and higher seed weight was observed moderate positive correlation. The yellow-coloured flax seeds had higher seed weight and concentration of oil compared with brown flax seeds. The results indicate the possibility of indirect selection of genotypes of flax with higher concentration of seed oil with regard to the higher weight and the yellow seed colour (Diederichsen & Fu 2008).

The central role of the miRNA networks (i.e., the connection of miRNA sequences and target sequences) in the control of key agronomic traits makes them appealing biotechnological targets for the production of varieties with improved performance (Rubio-Somoza & Weigel 2011). The allelic variation is a determining factor (Ganie & Mondal 2015) for the persistance of species and organisms adapting to the changing environments. Our results confirm the suitability of miRNA-based molecular markers for genotyping analyses, which corresponds

T a b l e 7

Results of multilevel data sorting of genotypes (oily type) in qualitative traits: colour of buds, petals and nerves

Colour of flower parts		Genotypes – oily type			
		2	3	6	8
					
Bud	white				
	pale violet-blue				
	pale violet				
	dark blue				
Petals	white				
	very pale violet				
	very pale red-violet-blue				
	blue				
	dark blue-violet				
	pale dark blue-violet				
Nerves	white				
	blue				
	violet with red blue				
	dark blue				
	dark blue violet				
Subsp.		IA	EA	ME	CREP

Legend: IA – subsp. *indo-abyssinicum* Vav. et Ell.; EA – subsp. *eurasiaticum* Vav. et Ell.; ME – subsp. *mediterraneum* Vav. et Ell.; CREP – *Linum crepitans*; photos by Hlavačková and Porokhovinova

T a b l e 8

Results of multilevel data sorting of genotypes in the qualitative trait: reproduction organ colour

Reproduction organ colour		Genotypes							
		Intermediate type				Oily type			
		1	4	5	7	2	3	6	8
Anther	yellow								
	blue								
Anther filament	white								
	pale violet								
	pale blue								
Stigma	white								
	blue								
	pale violet								
Style	white								
	pale blue								
	blue near ovary								
	dark blue								
Subsp.		ME	HI	EA	ME	IA	EA	ME	CREP

Legend: ME – subsp. *mediterraneum* Vav. et Ell.; HI – subsp. *hindustanicum* Ell.; EA – subsp. *eurasiaticum* Vav. et Ell.; IA – subsp. *indo-abyssinicum* Vav. et Ell.; CREP – *Linum crepitans*

to the results of several studies of Fu *et al*. (2013), Yadav *et al*. (2014), Mondal and Ganie (2014) and Ganie and Mondal (2015), who used this type of molecular markers.

CONCLUSIONS

From the applied miRNA-based molecular markers, the combination of miR156b-F/miR-R and miR156b-F/miR408a-F primer was proven to be more suitable for this type of analysis. It was possible to identify the unique miRNA loci in all three types of miRNA-based molecular markers. By mutually linking the morphological and molecular markers, we were able to determine the unique genotypes that originated from Ethiopia (linseed) and India (intermediate).

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