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The genetic diversity of reed canarygrass (*Phalaris arundinaceae* L.) assessed by isozyme markers

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The reed canarygrass (*Phalaris arundinacea* L.) is a wild-growing rhizomatous perennial cereal plant. This is a valuable forage and decorative crop, widely spread over all the continents except for Antarctic. So far, the reed canarygrass has become rather demanded in many European countries as a source of bioenergy. Among the major advantages of the reed canarygrass are high biomass yield, ecological stability, tolerance, and high seed production. Similar to most of wild-growing plants, the reed canarygrass is poorly studied. In the current study, the genetic diversity of a reed canarygrass collection (42 populations collected in meadow biocenoses of several regions in Russia and some other countries) was investigated using isozyme markers IDH (isocitrate dehydrogenase), GDH (glutamate dehydrogenase), MDH (malate dehydrogenase), ME (malic enzyme), and SKDH (shikimate dehydrogenase). Genetic control of these enzymes was determined in reed canarygrass for the first time. IDH and ME are controlled each by one locus (*Idh* and *Me*, respectively), SKDH and GDH have digenic control (loci *Skdh1* and *-2*; *Gdh1* and *-2*, respectively), MDH is controlled by 3 loci (*Mdh1*, *-2* and *-3*). A number of alleles per locus varied from 1 to 3. High activities in different organs and tissues, as well as codominant inheritance make isozymes convenient genetic markers in various studies into ecological and population genetics, especially in plant species, like reed canarygrass, with unsequenced genome. Cluster analysis based on isozyme data distinguished 22 diverse groups. The degree of genetic similarity was not related with geographical origin of the material.

Key words: *Phalaris*, canarygrass, bioenergy source, genetic diversity, genetic markers.

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Генетическое разнообразие канареечника (*Phalaris arundinaceae* L.), выявленное с помощью изоферментных маркеров

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Канареечник тростниковидный (*Phalaris arundinaceae* L.) – многолетний корневищный дикорастущий злак. Эта ценная кормовая и декоративная культура, широко распространенная по всем континентам (кроме Антарктиды), рассматривается в последнее время во многих европейских странах еще и как перспективный источник биотоплива. Основными достоинствами канареечника являются высокая продуктивность биомассы, экологическая стабильность и устойчивость к абиотическому стрессу, высокая семенная продуктивность. По сравнению с большинством других дикорастущих растений тростниковидный канареечник изучен слабо. В настоящей работе с помощью изоферментных маркеров изоцитратдегидрогеназы (ИДГ), глутаматдегидрогеназы (ГДГ), малатдегидрогеназы (НАД-МДГ), малик-энзима (МЭ) и шикиматдегидрогеназы (ШДГ) изучена коллекция канареечника тростниковидного, представленная 42 популяциями луговых биоценозов нескольких регионов России и ряда других стран. Данные ферменты тростниковидного канареечника впервые описаны в настоящем исследовании с генетической точки зрения. Установлено, что ИДГ и МЭ кодируются каждый одним локусом (*Idh* и *Me* соответственно), ШДГ и ГДГ имеют дигенный контроль (локусы *Skdh1* и *Skdh2*, *Gdh1* и *Gdh2* соответственно). МДГ соответствуют 3 локуса (*Mdh1*, *Mdh2* и *Mdh3*). Число аллелей на локус варьировало от 1 до 3. Высокая активность в различных органах и тканях, а также кодоминантный тип наследования делают изоферменты удобными маркерами в эколого- и популяционно-генетических исследованиях, особенно у видов растений с неизученным геномом, к которым относится и канареечник тростниковидный. В статье обсуждаются результаты кластерного анализа, выполненного на основе данных исследования изоферментов в коллекции канареечника. Кластерный анализ выявил 22 различные группы. Сделан вывод о том, что степень генетического сходства образцов из изученной коллекции не связана с географическим происхождением материала.

Ключевые слова: канареечник тростниковидный (*Phalaris arundinaceae* L.), источник биотоплива, генетическое разнообразие, изоферментные маркеры.

In the 21st century, the humankind encounters the problem in increasing energy consumption on the background of reducing resources of fossil fuel. In addition, it is commonly accepted that one of the factors involved in changing climate is greenhouse gas discharges into the atmosphere resulting from fuel combustion. This has induced the research into renewable energy sources and design of novel technologies for energy production. Biogas production via anaerobic cleavage of various raw plant materials is ever increasing worldwide as an alternative energy source. More promising is utilization of green mass of various perennial plants as a raw material for biogas stations, including, *Miscanthus* Anderss., *Galega* Tourn. ex L. and *Polygonum sachalinense* F. Schmidt ex Maxim. However, the reed canarygrass (*Phalaris arundinacea* L.) is most demanded for manufacturing biogas in the United States (Tahir et al., 2011), Canada (Wrobel et al., 2008) and several European countries: Latvia (Dubrovskis et al., 2009), Poland (Kacprzak et al., 2012), and Denmark (Kandel et al., 2013). Among the major advantages of the reed canarygrass are high biomass yield, ecological stability, tolerance, and high seed production (Wrobel et al., 2008; Dubrovskis et al., 2009; Tahir et al., 2011; Kacprzak et al., 2012; Kandel et al., 2013).

Similar to most of wild-growing plants, the reed canarygrass is poorly studied. It is known that the breeding success for any agricultural species is determined by the level of knowledge about its specific genetics. With all the evident success of DNA technologies applied to studies into plant genetic diversity, which have become most widespread during the last two decades (Khlestkina et al., 2004a, 2004b; Van De Wouw et al., 2010; Börner et al., 2012), isozyme analysis still holds its grounds as a simple, reliable, and reasonable method for distinguishing the loci and alleles of the genes detectable by this method (Sikdar, 2010; Siva et al., 2013). Isozymes also remain most useful genetic markers, since they provide reliable and comprehensive genetic information over a short time period with relatively small labor and material expenditures. The goal of this work was to detect and examine the variation in isozyme markers in the reed canarygrass genetic collection.

Materials and Methods

Totally, forty-two *Phalaris arundinacea* populations from the ICG stock collection were assayed. The material had been collected in meadow biocenoses of several regions in Russia (Altay, Arkhangelsk, Chelyabinsk, Komi, Krasnoyarsk, Leningrad, Novgorod, Novosibirsk, Omsk, Sverdlovsk, Tomsk, Volgograd and Vologda regions), as well as in other countries (Canada, Germany, Kazakhstan, Norway and USA). Patterns of the following five enzymes have been analyzed: isocitrate dehydrogenase (EC 1.1.1.42, IDH), glutamate dehydrogenase (EC 1.4.1.3, GDH), malate dehydrogenase (EC 1.1.1.37, MDH), malic enzyme (EC 1.1.1.40, ME), and shikimate dehydrogenase (EC 1.1.1.25, SKDH). Isozymes were separated using a standard system for horizontal electrophoresis in 14 % starch gel Tris–citrate system with subsequent histochemical detection of enzyme activities (Levites, 1986). Homogenate was prepared with 0.15 M Tris–HCl buffer (pH 8.3). The gel buffer (pH 7.0) contained 0.0125 M Tris and 0.041 M citric acid and the electrode buffer, the same components in the following proportions: 0.0375 M Tris and 0.0125 M citric acid. Electrophoresis was conducted for 6 h at a voltage of 160 V. At least 50 individuals from each population have been assayed. The isozymes were assayed in seeds, seedlings, and leaves (over the entire vegetation period). The presence or absence of each allele in population was coded by 1 or 0, respectively, and was scored for a binary data matrix. The binary data were used to compute a pairwise similarity matrix using the DICE similarity index (Dice, 1945). The similarity matrix was subjected to cluster analysis using the UPGMA (unweighted pair-group method with arithmetic average) algorithm (Sokal, Michener, 1958) on NTSYS-pc, version 2.0 (Rohlf, 1998).

Results

Isozyme analysis

Starch gel electrophoresis has been used to detect the isozyme patterns of isocitrate dehydrogenase (IDH), glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), malic enzyme (ME), and shikimate dehydrogenase (SKDH). As an example, IDH spectra are presented at Fig. 1. The data on enzyme activity, designation and number of detected loci and alleles are summarized for all five isozymes in Table.

IDH. The IDH pattern of the reed canarygrass displays one anode enzyme activity zone with fast and slow migrating enzyme variants (Fig. 1). It was mainly detectable in leaves and was also expressed in seedlings. The fast migrating enzyme variant (FF) was widespread in various reed canarygrass populations and the slow migrating variant (SS) was rare. Some plants display a three-band pattern, comprising FF, SS, and FS (the variant with an intermediate mobility). These results and known dimeric quaternary structure of plant IDH suggest monogenic control of the IDH synthesis in the reed canarygrass. Two alleles of the *Idh* locus were designated *Idh-F* and *Idh-S.*

MDH. Electrophoretic pattern of the reed canarygrass SKDH displayed three activity zones (see Supplementary materials[1]). Three-band phenotypes (NNLLFF and NNLLSS according to our designations) as well as five-band ones (NNLLFS) were observed. The enzyme variants in the first two anode slow migrating zones were assumed to be monomorphic and controlled by two monomorphic loci *Mdh1* and *Mdh2*. The third zone (the corresponding locus was designated *Mdh3*) displayed polymorphism: two one-band enzyme variants with fast (FF; allele *Mdh3-F*) and slow (SS; allele *Mdh3-S*) mobilities and a hybrid three-band variant (FS; heterozygous). The reed canarygrass displayed a high activity in the leaves over the entire vegetation period as well as in seedlings. It was also detectable in seeds (Table).

SKDH. Electrophoretic pattern of the reed canarygrass SKDH displayed two activity zones, anode slow migration zone 1 and fast migration zone 2 (see Supplementary materials). A low enzyme activity in zone 2 interfered with interpretation of the patterns. Several types of patterns are detectable in the slow migration zone 1, namely, three types of one-band patterns differing in the electrophoretic mobility (FF, fast migrating phenotype; NN, intermediate; and SS, slow) and three types of two-band patterns, also differing from each other (NS, FS, and FN phenotypes). Since SKDH is a monomer, the

[1] Supplementary materials see in Appendix 2: http://www.bionet.nsc.ru/vogis/download/pict-2016-20/appx3.pdf

detected two-band patterns represent three different types of heterozygotes formed by different alleles. Correspondingly, the observed SKDH variants in zone 1 are the products of the *Skdh1* locus with the alleles *Skdh1-F*, *Skdh1-N*, and *Skdh1-S*. SKDH was detectable in leaves only (Table).

GDH. In the GDH isozyme pattern, two isozyme activity zones were identifiable (see Supplementary materials). Both zones house two types of one-band patterns with fast and slow mobilities (FF and SS). Any hybrid patterns have been undetectable. Presumably, the observed isozymes are the products of two loci, *Gdh1* with the alleles *Gdh1-F* and *Gdh1-S* and *Gdh2* with the alleles *Gdg2-F* and *Gdh2-S*, which control GDH in the reed canarygrass. GDH was expressed in leaves only (Table).

ME. The reed canarygrass ME has one anode activity zone (see Supplementary materials). It was detectable in leaves only. Two types of one-band patterns differing in their mobilities are detectable in this zone, namely, fast (FF) and slow (SS) variants. No geterozygotes were observed. Presumably, these ME isozymes are products of the *Me-F* and *Me-S* alleles of the *Me* locus (Table).

Cluster analysis

Comparison of the forty-two populations by the isozymes allelic composition (presence/absence of certain alleles in the populations) is presented as dendrogram (Fig. 2). Analysis distinguished 22 groups combined into six major clusters. Cluster I included six populations from Russia (Altay, Komi, Novosibirsk (3 populations) and Sverdlovsk regions) and one from Germany. Cluster II combined three populations from West Siberia (Novosibirsk, Omsk and Tomsk regions) and one from Eastern Kazakhstan. Four populations from distinct parts of Russia were included into each cluster III and IV. Cluster V contained two similar populations from Novosibirsk and Krasnoyarsk regions (Russia), whereas the biggest cluster VI included twenty-one populations from Canada, Kazakhstan, Norway, Russia and USA (Fig. 2). Thus, the genetic similarity established between populations was not related with their geographical origin.

Discussion

IDH is a dimeric enzyme, with genetic control considerably differing among plant species. The rye IDH is monomorphic (Mitra, Bhatia, 1971). Two loci (*Idh1,* comprising five alleles, and *Idh2,* comprising eight alleles) have been detected in the maize (Goodman, Stuber, 1980), while this enzyme of the sugar beet is controlled by three loci, including two polymorphic diallelic loci (Levites, 1986). Genetic control of this enzyme in reed canarygrass is different from the described above. The presence of plants with one- and three-band patterns in populations of reed canarygrass as well as the dimeric quaternary structure of plant IDH suggests that the IDH synthesis in this species is controlled by one locus, *Idh,* with two alleles *Idh-F* and *Idh-S*.

The plant MDH is a rather well-studied enzyme. Differences in the number of loci, degree of polymorphism, and interactions between various alleles and loci have been detected in different plant species. Note that polymorphism and specificity of multiple MDH molecular forms are characteristic of both different tissues within one plant and different cell compartments (Goodman et al, 1980; McMillin, Scandalios, 1981; Newton, 1983; Tarasova, 1988; Zoro et al, 1999; Yudina, Levites, 2007). Several plant genes involved in the MDH control have been localized on chromosomes (Goodman et al, 1980; Newton, Schwartz, 1980;Wijsman, 1983). As has been shown, the MDH molecule is a dimer in its quaternary structure (Levites et al, 1980; Goodman et al, 1980; McMillin, Scandalios, 1982; Benito, Salinas, 1983; Arus, Orton, 1984). Several researchers have used MDH isozymes as a genetic marker in population genetic studies of incense-cedar (Harry, 1983), maize (Levites, 1986), cultivated peach forms (Arulsekar et al, 1986), sugar beet (Tarasova et al, 1988), and amaranth (Yudina et al, 2005). Based on the MDH dimer structure in different plant species and having conditionally separated the mobility of isoforms into three zones, we have assumed the presence of the two monomorphic loci *Mdh1* and *Mdh2* (Table). The presence of hybrid isozyme in the MDH pattern of the polymorphic zone 3 suggests a dimeric nature of the reed canarygrass MDH, and the detected MDH pattern in zone 3 is a typical pattern for a dimeric enzyme, controlled by the locus *Mdh3* with the alleles *Mdh3-F* and *Mdh3-S*.

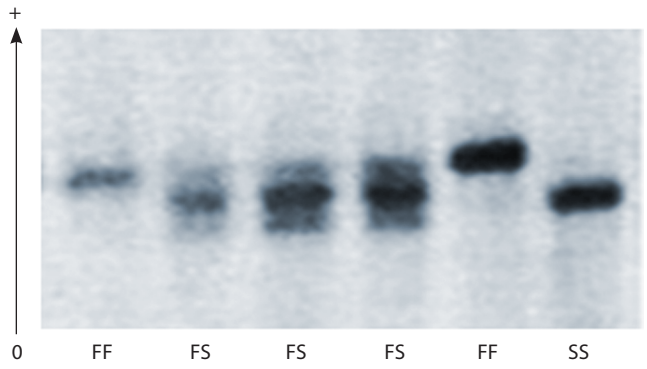

Fig. 1. Spectra of IDH in reed canarygrass homozygous (FF and SS) and heterozygous (FS) plants.

SKDH is a monomer in its quaternary structure. The wheat SKDH is controlled by three homoeologous genes (Koebner, Shepherd, 1982) and the maize SKDH, by only one gene (Wendel et al, 1985). This enzyme is widely used as a marker for detecting genetic variation in various plant species, such as the larch (Larionova, 2004), English oak (Mullagulov et al, 2008), tulip (Kutlunina, Belyaev, 2008), and water lotus (Koren et al, 2012). From the two activity zones displayed by the reed canarygrass SKDH, the zone 2 had too low enzyme activity for proper interpretation of the patterns, while the observed SKDH variants in zone 1 were the products of the *Skdh1* locus with the alleles *Skdh1-F*, *Skdh1-N*, and *Skdh1-S* (Table).

GDH in various plant species is controlled by different number of loci distinct in their expression. In its quaternary structure, GDH is a hexamer. The maize GDH is controlled by two loci, *Gdh1* and *Gdh2*. Interaction of the products of these loci gives a distinct seven-band pattern, confirming a hexamer nature of the enzyme (Suchorzhevskaya, 1980; Goodman, Stuber, 1983). The rice has an analogous GDH system (Endo, Morishima, 1983). A single polymorphic locus has been

Isozymes loci identified in the study of reed canarygrass

Enzyme	Abbreviation	EC number	Tissue	Loci	Alleles
Isocytrate dehydrogenase	IDH	1.1.1.42	Seedlings and leaves	*Idh*	*Idh-F* *Idh-S*
Malate dehydrogenase	MDH	1.1.1.37	Seedlings, leaves and seeds	*Mdh1*	*Mdh1*
				Mdh2	*Mdh2*
				Mdh3	*Mdh3-F* *Mdh3-S*
Shikimate dehydrogenase	SKDH	1.1.1.25	Leaves	*Skdh1*	*Skdh1-F* *Skdh1-N* *Skdh1-S*
				Skdh2	–*
Glutamate dehydrogenase	GDH	1.4.1.3	Leaves	*Gdh1*	*Gdh1-F* *Gdh1-S*
				Gdh2	*Gdh2-F* *Gdh2-S*
Malic enzyme	ME	1.1.1.40	Leaves	*Me*	*Me-F* *Me-S*

* The products of *Skdh2* display a very weak activity, interfering with data interpretation.

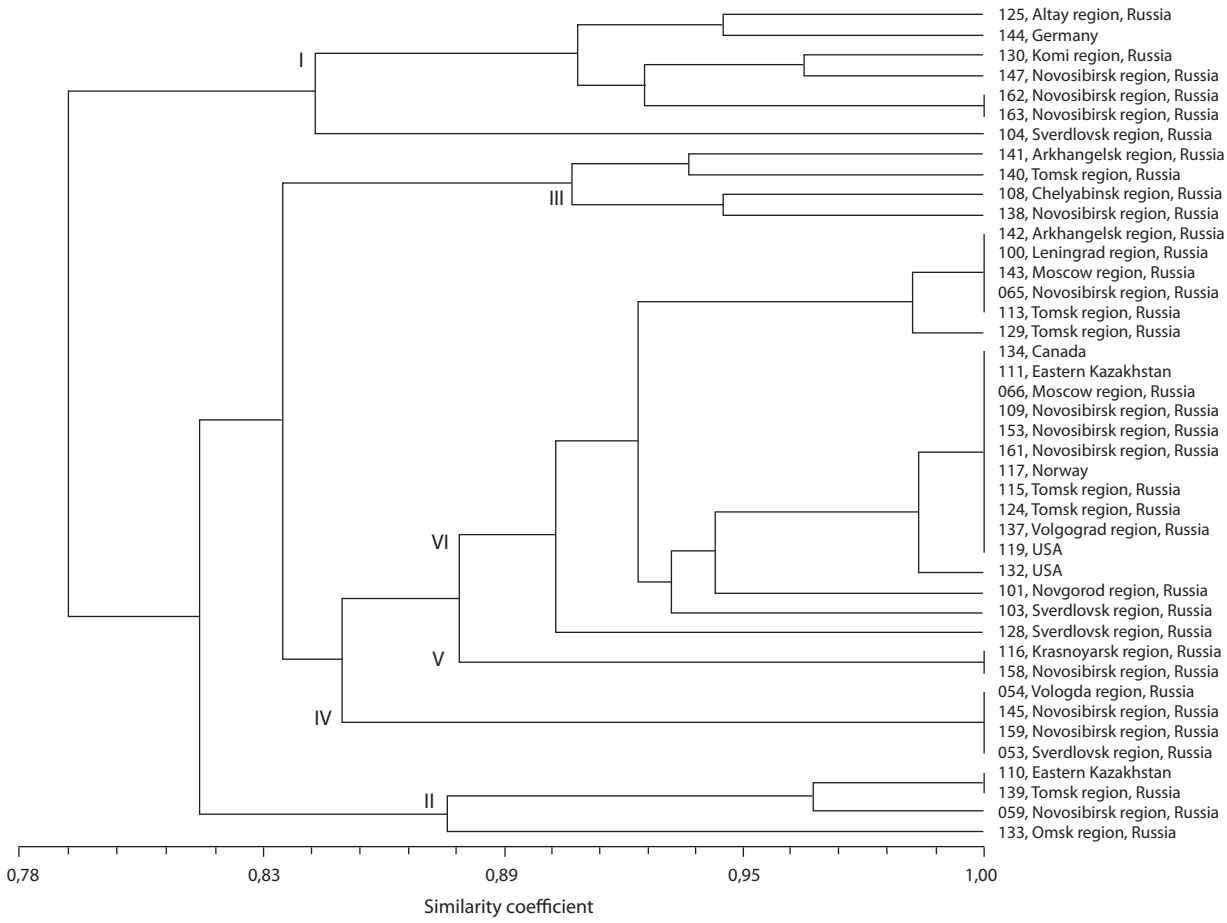

Fig. 2. Genetic diversity of 42 reed canarygrass populations collected in meadow biocenoses.

identified in the pine *Pinus taeda* L. (Adams, Joly, 1980a, b) and barley (Brown, Munday, 1982). In the reed canarygrass GDH isozyme pattern two isozyme activity zones (1 and 2) were identifiable, corresponding two loci, *Gdh1* with the alleles *Gdh1-F* and *Gdh1-S* and *Gdh2* with the alleles *Gdg2-F* and *Gdh2-S* (Table).

Malic enzyme has been studied in the maize, and two correspondingly loci, *Me1* and *Me2*, have been identified. The products of *Me1* locus are present in the seedling tissues, while the *Me2* products appear in adult plant. Four alleles have been identified, namely, three very rare alleles and one null allele (Larionova et al, 2004). The maize ME is a tetramer. Two loci, *Mod1* and *Mod2*, are involved in the ME genetic control in the sugar beet (Levites, 1986). Six alleles – C, D, F, E, S, and L, differing in electrophoretic mobility of the encoded products – have been identified in the locus *Mod1*. The heterozygotes for this locus display five isozymes, suggesting a tetrameric nature of ME. The reed canarygrass ME has one anode activity zone, corresponding to *Me* locus with *Me-F* and *Me-S* alleles (Table).

As is mentioned above, we have assayed different reed canarygrass tissues for isozymes, namely, seeds, seedlings, and leaves. Tissue specificity of the studied enzymes in the reed canarygrass development has been observed. Only NAD-dependent MDH is detectable in seeds; MDH and IDH appear in seedlings; and the remaining enzymes, ME, SKDH, and GDH, as well as MDH and IDH are present in leaves (Table). Further on, high activities of all the examined enzymes are retained during the overall vegetation period until harvesting. The data on genetic control of the studied isozymes allows the tissue specificity to be interpreted as a result of differential gene activity during the reed canarygrass development.

The results obtained suggest that the polymorphism in the studied enzymes detected in the reed canarygrass (*Phalaris arundinacea* L.) is genetically determined by the presence of several loci with multiple alleles. Cluster analysis performed in the current study using isozyme markers distinguished 22 diverse groups among 42 reed canarygrass populations collected in meadow biocenoses of several regions in Russia and some other countries. The degree of genetic similarity was not related with geographical origin of the material (Fig. 2).

Overall, a distinct phenotypic manifestation, high activities in different organs and tissues, and codominant inheritance make isozymes convenient genetic markers in various studies into specific, ecological, and population genetics.

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Conflict of interests

The authors declare no conflict of interests.

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