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## The formation and the study of a collection of the *Miscanthus* resource species gene pool in the conditions of the West Siberian forest steppe

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Several species of the genus *Miscanthus* Anderss. (elephant grass) characterized by a high rate of growth of the above-ground vegetative mass are currently in the focus of attention due to their high practical application as a source of bioethanol and cellulose. The main goals of this study were: (1) molecular genetic identification and (2) histochemical analysis of the genus *Miscanthus* Anderss. species in the collection of Central Siberian Botanical Garden SB RAS in order to identify the most perspective and technically valuable individuals. To study the collection of *Miscanthus* samples, a multi-disciplinary approach was applied. To collect the samples of different species from native habitats, traditional systematic and geobotanical methods (comparative morphological and phytocenological) were used. According to the results of the ISSR-analysis, 16 samples of three *Miscanthus* species were divided into two clades: Sinensis and Sacchariflorus, the former including two subclades. For the samples of *M. purpurascens* I and II, a hybrid origin of this species was confirmed by ISSR data. The molecular data obtained from the study allowed us to hypothesize that the samples involved in the subclade I of the Sinensis clade could be used as donors of resistance to adverse environments, and the samples of the subclade II, as donors of high biomass productivity. Based on histochemical analysis, sclerenchyma cells were characterized by the most lignin-rich thickened membranes, so the most appropriate direction in *Miscanthus* selection should be based on identification and using less lignin-containing samples.

Key words: *Miscanthus*; ISSR analysis; histochemical analysis; biomorphology; microecology; cluster dendrogram; bioethanol.

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## Формирование и изучение коллекционного генофонда ресурсных видов рода *Miscanthus* Anderss. в условиях лесостепи Западной Сибири

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В настоящее время особый интерес представляют растения, характеризующиеся высокой скоростью нарастания надземной вегетативной массы и имеющие практическое применение в качестве источника биоэтанола и целлюлозы, например, виды рода *Miscanthus* Anderss. (веерник). Цель данного исследования – молекулярно-генетическая идентификация и гистохимический анализ видов рода *Miscanthus*: *M. sacchariflorus* (Maxim.) Benth., *M. sinensis* Anderss. и *M. purpurascens* Anderss. коллекционного генофонда Центрального сибирского ботанического сада СО РАН для выявления перспективных технических сырьевых растений. Для формирования, изучения и идентификации коллекционного генофонда рода *Miscanthus* применялся мультидисциплинарный подход, заключающийся в следующем: при сборе образцов в природе использовались традиционные методы классической систематики и геоботаники (сравнительно-морфологический и фитоценологический); при выделении форм под конкретные ресурсные (биоэнергетические), технологические задачи применялись биоморфологические подходы и проводилось описание микроэкологических условий с акцентом на наименее благоприятные для произрастания факторы. Молекулярно-генетические исследования были направлены на уточнение таксономической принадлежности и анализ генетических дистанций между представителями трех видов *Miscanthus*. По результатам ISSR-анализа изученные образцы трех видов мискантуса разделились на две клады: Sinensis и Sacchariflorus, что хорошо согласуется с видовой при-

надлежностью большинства образцов. Исключение составили растения *M. purpurascens*\_I и II, которые по данным молекулярно-генетического анализа отнесены к кладе *Sacchariflorus*, что может служить подтверждением гибридного происхождения данного вида. Клада *Sinensis* разделена на две субклады. В субкладу I вошли образцы, произрастающие в наименее благоприятных микроэкологических условиях – на более засоленных почвах, а в субкладу II сосредоточены наиболее габитуально мощные образцы. Эти данные позволяют предположить, что среди образцов субклады I могут оказаться доноры устойчивости, а среди образцов субклады II – доноры продуктивности. Известно, что из-за повышенного содержания лигнина снижается технологическая ценность сырья. Согласно проведенному нами гистохимическому анализу, мощность механической лигнин-содержащей ткани различается у разных растений мискантуса, поэтому наиболее целесообразен селективный отбор особей, накапливающих меньше лигнина (с наименее развитой склеренхимой). Выявленные молекулярные признаки разных видов мискантуса могут быть использованы для идентификации и паспортизации перспективных форм и линий в качестве альтернативного источника биотоплива и целлюлозы.

Ключевые слова: род *Miscanthus*; ISSR-анализ; гистохимический анализ; биоморфология; микроэкология; кластерная дендрограмма; биоэтанол.

## Introduction

*Miscanthus* is considered to be one of the most efficient solar energy accumulators among representatives of the Earth vegetative kingdom (Dohleman, Long, 2009). High cellulose content and significant plant biomass make it possible to treat *Miscanthus* as a promising alternative energy source (Lewandowski et al., 2000; McCalmont et al., 2017; Van Der Weijde et al., 2017). Physiologists and biochemists deem *Miscanthus* species to be unique highly productive sources of renewable raw materials for producing ethylene and cellulose (Slynko et al., 2013).

*Miscanthus* is a valuable ameliorative culture as well. High plant growth rates, unpretentiousness to soil conditions, pronounced drought resistance have contributed to *Miscanthus* wide use to stabilize and reduce the intensity of soil erosion processes (Kahle et al., 1999, 2001).

In the late 20 century, many domestic and foreign botanical gardens started introducing *M. sacchariflorus* and *M. sinensis* into the culture as decorative cereals. The criteria for *ex situ* selection – rhythmological and ontogenetic ones – have been developed in conformity with the prospects for their use in landscape architecture. In the 21st century, the above-mentioned species, as well as *M. × giganteus*, are recognized by the world scientific community as the main resource species for elaborating the alternative energetics sphere.

The Central Siberian Botanical Garden of the Siberian Branch of the Russian Academy of Sciences (CSBG SB RAS, Novosibirsk) have been investigating *Miscanthus* species as a part of the lawn and decorative cereals collection since the late 1990s. Forming and studying of the collection gene pool of the genus *Miscanthus* complex have recently carried out in accordance with the world trends – decorative and bioenergetic directions. Searching perspective forms in nature and further studying their bioenergetic potential outside the natural range have required the additional selection criteria development, and economic and biological traits and properties evaluation.

The Russian Far East is the main region of field works to study the intraspecific polymorphism of *Miscanthus* species and search promising samples, where *M. sacchariflorus* occupies northern habitats, but *M. sinensis* spreads southward. In 2013, the collection gene pool was replenished with *M. sinensis* samples from the Gamov Peninsula (Khasan District, Primorsky Krai). In 2017, *M. sacchariflorus* was collected

in the Chuguev District, and *M. sinensis* and, presumably, *M. purpurascens*, in the Khasan District of Primorsky Krai. In addition, living material was collected on the Kuril islands – Shikotan, Kunashir, Iturup.

The biomorphological and microecological criteria have been allotted as main ones to gather samples in nature. During the expeditions, the vegetative parts (rhizome) were selected from plants with the most powerful aboveground vegetative mass. At the same time, it was marked microecological conditions of growing, among which the following considered as unfavorable: saline splash zone, sites with arid compacted soil, open windy hilltops. Plants with normal shoot formation in such habitats should obtain high adaptive potential under more severe climatic conditions of cultivation.

It became necessary to study genetic polymorphism and DNA identification as a result of cumulating the intraspecific and form diversity in the collection gene pool. The study objective is molecular-genetic identification and histochemical analysis of the CSBG SB RAS' collection gene pool of the genus *Miscanthus* species to reveal promising forms as technical raw plants.

## Materials and methods

An integrated multi-disciplinary approach to the collection gene pool formation and certification was used in the study. The traditional techniques of classical systematics and geobotany were applied to choose field sites, and collect samples of various species in nature. While isolating forms in natural habitats, biomorphological approaches were employed to solve specific resource problems, microecological conditions are described. The geographical coordinates of collecting sites were recorded when selecting material for molecular genetic and histochemical research.

16 samples of three species (*M. sinensis*, *M. sacchariflorus* and *M. purpurascens*) were studied (Table 1). *M. sacchariflorus* samples were gathered in the Chuguev District of Sikhotealin, as well as the northern part of the National Park “Zov Tigra (Call of the Tiger)” vicinities situated in the Chuguev, Olgin, and Lazov Districts. This species grows here in open flat areas and non-arable lands, which are secondary successions.

*M. sinensis* and *M. purpurascens* were collected in the Khasan District (Primorsky Krai). These species grow as a part of shrub-grass groupings, some specimens are taken in

**Table 1.** The origin of the samples of three *Miscanthus* species analyzed in the paper

Species	Place of collection, geographical coordinates
<i>M. sinensis</i>	
<i>M. sinensis_I</i>	Gamow Peninsula, Khasansky region. Observation deck on the road, overlooks Vityaz Bay. 42°36'24" N, 131°09'56" E
<i>M. sinensis_II</i>	Gamow Peninsula, Khasansky region. On the way to the lighthouse
<i>M. sinensis_III</i>	Gamow Peninsula, Khasansky region. On the way to the lighthouse
<i>M. sinensis_IV</i>	Gamow Peninsula, Khasansky region. The Bay Vityaz. 42°60'65" N, 131°16'59" E
<i>M. sinensis_V</i>	Gamow Peninsula, Khasansky region. Plateau above Vityaz Bay. 42°60'88" N, 131°19'48" E
<i>M. sinensis_VI</i>	Gamow Peninsula, Khasansky region. Plateau above Vityaz Bay. 42°61'51" N, 131°17'89" E
<i>M. sinensis_VII</i>	Gamow Peninsula, Khasansky region. Supralittoral in Telyakovsky Bay. 42°35'25" N, 131°17'74" E
<i>M. sinensis_VIII</i>	Gamow Peninsula, Khasansky region
<i>M. sinensis_IX</i>	Gamow Peninsula, Khasansky region
<i>M. sinensis_X</i>	Gamow Peninsula, Khasansky region. Gas Stop at the turn into the village Andreevka. 42°40'33" N, 131°06'17" E
<i>M. sacchariflorus</i>	
<i>M. sacchariflorus_I</i>	Sykhote-Alin. One of the most Northern Checkpoints of the area. Chuguevsky region, not reaching the river Pokrovka. Route A181. 44°51'21" N, 134°52'95" E
<i>M. sacchariflorus_II</i>	Sykhote-Alin. In the same population
<i>M. sacchariflorus_III</i>	Sykhote-Alin. In the area surrounding the National Park "Call of the Tiger". 43°33'3" N, 134°66'7" E
<i>M. sacchariflorus_IV</i>	Sykhote-Alin. 43°53'24" N, 134°12'57" E
<i>M. purpurascens</i>	
<i>M. purpurascens_I</i>	Far East of the Russian Federation. Collection of living plants CSBG (No. USU 440534)
<i>M. purpurascens_II</i>	Gamow Peninsula, Khasansky region. Mountain Tumannaya (foggy). 42°33'54" N, 131°12'34" E

a light shrub-forb oakery (*Quercus dentata* Thunb.) on the Telyakovsky Bay slope. The main works were carried out at the Gamov Peninsula, 14–112 m altitudes, with a wide range of microecological conditions in *M. sinensis* and *M. purpurascens* habitats.

The molecular genetic analysis to clarify samples taxonomic position and identification was carried out using plant dried leaves of three *Miscanthus* species: *M. sinensis*, *M. sacchariflorus*, and *M. purpurascens* collected of 15 plants in natural populations (see Table 1). Besides, *M. purpurascens* sample from the Living Plant Collection of the CSBG SB RAS (see Table 1, *M. purpurascens\_I*, UNU No. USU 440534) was analyzed.

DNA extraction was performed by the NucleoSpin Plant II kit (Macherey and Nagel, USA). The purity and concentration of DNA extracts were determined with spectrophotometry (Spectrophotometer kinetic and  $\mu$ -cuvette, Eppendorf, Germany). DNA purity was calculated as the ratio of the solution optical power at 260 and 280 nm wavelength.

The 25  $\mu$ l reaction PCR mixture consisted of: 2.7 mM  $MgCl_2$ , 1.25 mM primer, 0.4 mM mononucleotides, 1x PCR buffer, 1.5 units Taq DNA polymerase (Medigen, Russia) and 20–30 ng matrix. The amplification program included the following steps: DNA denaturation: 90 s at 94 °C; 35 amplification cycles: 40 s at 94 °C, 45 s at 41–58 °C (primer annealing),

and 90 s at 72 °C; 5 min at 72 °C. The amplification was made in C 1000 Thermal Cycler (BioRad Laboratories, USA), its products were separated by electrophoresis in a 0.8 % agarose gel in 1x TBE buffer. The obtained ISSR fragments were stained with SYBR-Green (Medigen, Russia), visualized using the Gel Doc XR + gel documentation system, analyzed by Image Lab Software (Bio-Rad Laboratories, USA).

The size of the identified ISSR fragments was determined with a molecular mass marker (Medigen, Russia). Each amplified fragment was considered as a dominant marker, and its presence (1) or absence (0) was noted for every compared samples.

Statistical data processing, cluster dendrogram construction and principal component analysis (Principal Components, PCO) were carried on applied the PAST software (Hammer et al., 2001).

As for the histochemical analysis, dry vegetative shoots were selected at the final vegetation stage from 3 samples (IV, V and VI) of *M. sinensis* and 1 sample (II) of *M. purpurascens*, taken as live rhizomes from 4 natural populations in the Khasan District, Primorsky Krai, Russia. Their subsequent vegetation took place in similar conditions as a part of CSBG SB RAS' Bioresource Scientific Collection USU No. 440534. A stem part was taken from a minimal industrial height – 10 cm from the ground level (10 cm length).

Further studies were carried out at CSBG SB RAS' Center for Collective Use. The bottom side shoots were longitudinally cut with a scalpel into bars of about 3 mm, placed on a freezing microtome, and 60–90 µm longitudinal sections were made. Staining was practiced in two variants: phloroglucinol in hydrochloric acid, alcian blue in acetic acid according to standard techniques (Barykina et al., 2004). Besides, the possibility of differentiated staining with these dyes was checked.

Microcopying with photography was performed by Carl Zeiss Axio Scope A1 light microscope.

## Results

### Molecular genetic analysis

The study had previously tested 16 ISSR primers, nine of which were used for the analysis (Table 2). They are characterized by the greatest amount and polymorphism of the amplified fragments, and are suitable to investigate the genetic variability of the genus *Miscanthus* plants at the intra- and interspecific levels.

The extracts of *Miscanthus* DNA obtained from dried leaves had 3–24 ng/µl concentration, 1.25–1.83 purity expressed with  $A_{260}/A_{280}$  ratio. The analysis of intermicro-satellite DNA sections of the studied samples using nine ISSR primers allowed identifying 177 amplified fragments of 270–1800 bp length. Figure 1 shows the ISSR profile of samples obtained by amplification with primer 17899B.

According to the ISSR analysis results, 16 *Miscanthus* samples were divided into two clades: Sinensis and Sacchariflorus (Fig. 2), that is consistent with the species of each sample (the bootstrap support value is 55). At the same time, *M. purpurascens* I and II were distributed inside the clade Sacchariflorus (see Fig. 2). Taking into account the admittedly hybrid origin of *M. purpurascens* species, the authors tend to evaluate the result as a confirmation of this hypothesis (Jiang et al., 2013).

The extremely close genetic relationship found for samples of *M. sacchariflorus* I and II is remarkable (see Fig. 1, 2). These samples, collected in one population, have almost

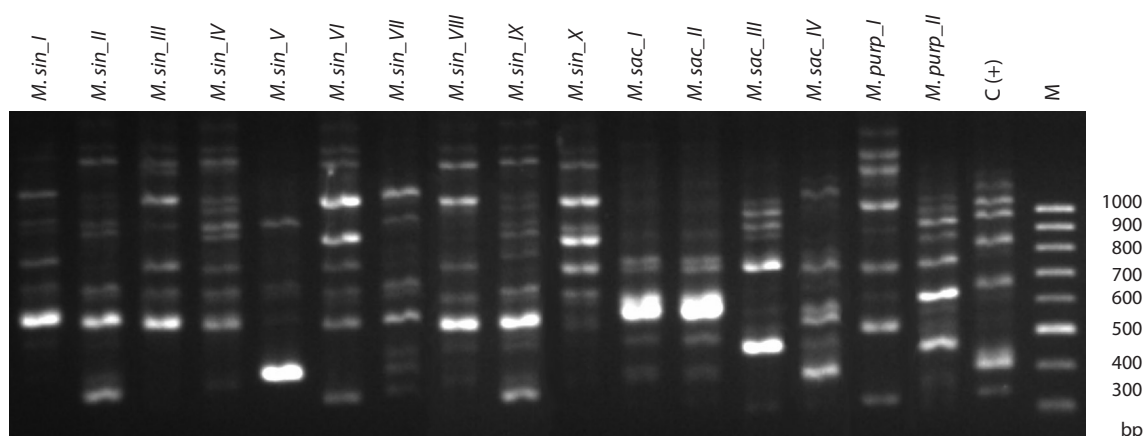
**Table 2.** Characteristics of ISSR primers tested and selected (in bold)

to study the genetic polymorphism of *Miscanthus* species

Primer	Nucleotide sequence, 5'-3'	Temperature of annealing, °C
<b>814</b>	(CT) <sub>8</sub> TG	51
17898A	(CA) <sub>6</sub> AC	45
<b>17898B</b>	(CA) <sub>6</sub> GT	48
<b>17899A</b>	(CA) <sub>6</sub> AG	48
<b>17899B</b>	(CA) <sub>6</sub> GG	42
<b>844A</b>	(CT) <sub>8</sub> AC	44
<b>844B</b>	(CT) <sub>8</sub> GC	42
<b>M1</b>	(AC) <sub>8</sub> CG	56
<b>M2</b>	(AC) <sub>8</sub> YG	58
M7	(GAC) <sub>5</sub>	46
M11	(CA) <sub>6</sub> AR	39
M14	(GACA) <sub>4</sub>	47
<b>HB10</b>	(GA) <sub>6</sub> CC	48
HB12	(CAC) <sub>3</sub> GC	41
HB14	(CTC) <sub>3</sub> GC	42
UBS826	(AC) <sub>8</sub> C	53

identical ISSR patterns, that may be evidence of these two individuals origin as a result of vegetative reproduction of the original plant.

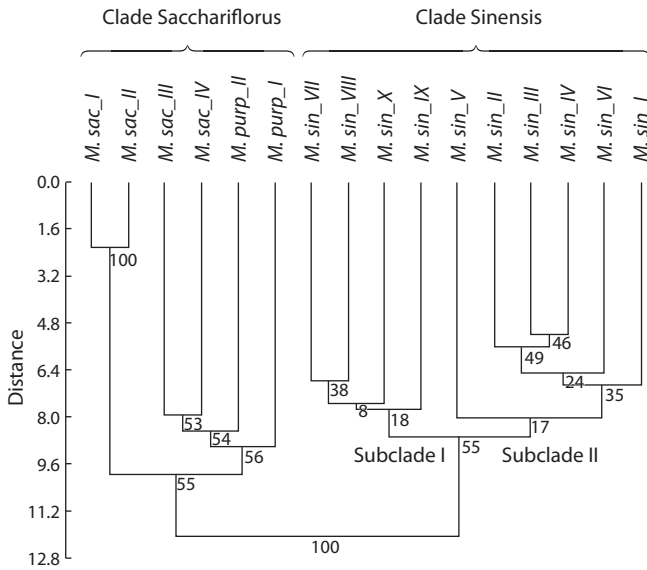
Based on the principal component analysis (PCA) of ISSR-marking data, the distance between two groups of samples inside Sacchariflorus clade has been determined (Fig. 3). The first group includes *M. sacchariflorus* I and II, the second – *M. sacchariflorus* I, II and *M. purpurascens* I, II.



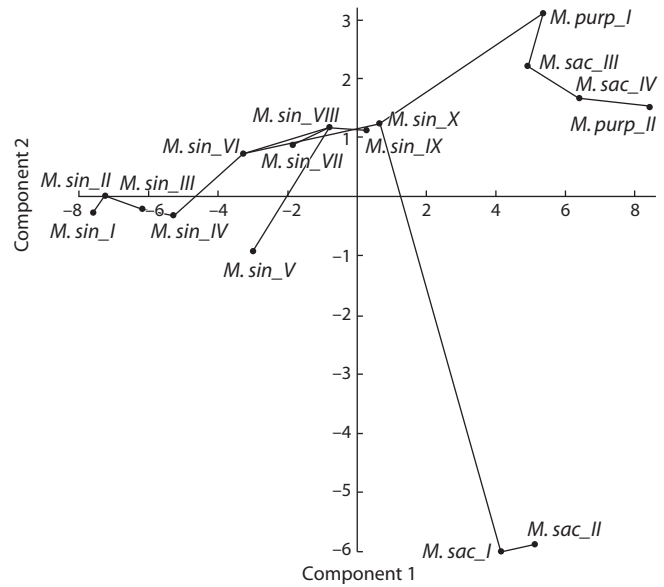
**Fig. 1.** ISSR-PCR profile of three *Miscanthus* species: ISSR primer 17899B was used for amplification.

C(+), positive control; M, weight marker. Designation of the samples of *Miscanthus* see in Table 1.





**Fig. 2.** Cluster dendrogram reflecting the value of genetic distances between 16 samples of three *Miscanthus* species.



**Fig. 3.** The scheme of genetic relationships between 16 specimens of three *Miscanthus* species resulted by the Principal Component Analysis (PCA) of ISSR data.

### Histochemical analysis

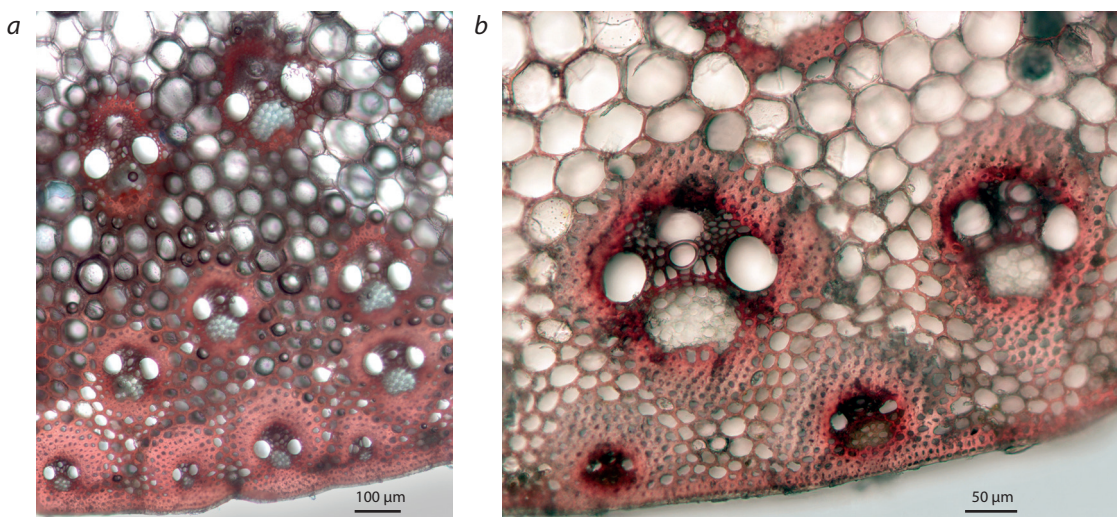
Histochemical studies aimed to investigate the seasonal dynamics and peculiarities of straw lignification were undertaken related to chemical analysis results carried out at the Institute of Problems of Chemical and Energetic Technologies SB RAS (IPChET SB RAS, Biysk city) showed some samples with elevated lignin content reaching  $28.1 \pm 0.5$  % in terms of absolutely dry weight (Dorogina et al., 2018). It has been found that high lignin content decreases the technological value of the raw material (Dorogina et al., 2018).

Based on histochemical analysis, it is revealed that the shoot structure of *M. sinensis* representatives is similar to that of the family Poaceae. The straw outside is covered with thin

single-layer epidermis; this species layers are radially spread mechanical tissue with thick lignified cell walls (Fig. 4). The thickened cell walls of the sclerenchyma are richest in lignin arranged in two layers around the conducting beams (see Fig. 4). The mechanical lignin-bearing tissue thickness differs in various plants: the less pronounced lignified sclerenchyma is developed in plant of population No. 3 compared with the sample of population No. 24 (see Fig. 4, a, b).

### Discussion

Based on the clade Sinensis analysis, it was found that the clade was divided into two subclades (see Fig. 2). Subclade I included samples growing under the most unfavorable micro-



**Fig. 4.** The cut stem of the samples *M. sinensis\_VI* (a) and *M. sinensis\_IV* (b). Staining with phloroglucinol – altianalis blue die.

ecological conditions. So, the sample of *M. sinensis\_VII* is collected in the Telyakovsky Bay splash or supralittoral zones located on the sea-land border, above the maximum tide level, which implies more saline microecological conditions (Fig. 5).

*M. sinensis\_X* sample grew in a technogenically disturbed habitat, in the gas station environs at the crossway to Andreevka village not excluding high exhaust gases effect (see Table 1).

It is noteworthy that the samples entering subclade II are plants with the most powerful habitus. *M. sinensis\_V* and *M. sinensis\_VI* growing in the shrub-grass grouping on a plateau above Vityaz Bay stood out by their morphological parameters (Fig. 6).

## Conclusion

The foresaid suggests that there are plants characterizing with high resistant in subclades I samples, and that's why they might be donors of resistance, while subclades II samples – donors of productivity. The revealed molecular signs of *Miscanthus* various species should be used to identify and certify *Miscanthus* forms and lines perspective to get economically available plant materials suitable for applying as environmentally safe promising alternative biofuels.

Based on the histological analysis of shoots of *M. sinensis* representatives, it should be assumed that some *M. sinensis* specimens store a large amount of lignin in dry straws after vegetation, which could impede industrial processing, therefore additional studies of young shoots are needed to investigate the lignin dynamics accumulation. In this regard, feasibly earlier harvesting of the vegetative mass should be more productive, and selection of individuals accumulating less lignin (with the least developed sclerenchyma) is the most appropriate.

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Fig. 5. General view of the natural habitat of *M. sinensis\_VII* in the Telyakovsky Bay splash zone.



Fig. 6. General view of the plants *M. sinensis\_V* and *M. sinensis\_VI* growing in the shrub-grass group on the plateau above the Bay Vityaz (Primorsky Region).

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