



Intra- and interspecific variability of *Mentha arvensis* L. and *M. canadensis* L.

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The identification of plants of the genus *Mentha* is often difficult due to significant intraspecific polymorphism, intense interspecific hybridization, and ploidy changes. An attempt was made to apply an integrated approach to the study of different parameters of two species: *Mentha arvensis* L. and *M. canadensis* L. Eight geographically dispersed populations of *Mentha* in different regions (European Russia, Khakassia and Far East, Western Ukraine, and Indochina) were studied. Diagnostic morphological characters and compositions of essential oil components were examined, and DNA was analyzed with ISSR markers. The data obtained were statistically processed by cluster, principal component, and principal coordinate analyses. The European and Asian groups of samples were clearly distinguished by the analysis of quantitative parameters of the calyx and leaves, but different methods of data processing produced different results in determining the belonging of the Far Eastern plants to a particular group. Therefore, their taxonomic positions can hardly be determined on morphological grounds. According to the composition of essential oil and ISSR fragments, a group of the genetically, morphologically, and phytochemically closest plants was identified, which included representatives of the populations of the Moscow oblast, Vladimir oblast, Kaluga oblast, the Komi Republic, and Khakassia. All these plants belonged to *M. arvensis*. Plants collected in the natural flora of the Russian Far East showed a greater resemblance in essential oil composition and ISSR markers to the European group of *M. arvensis* than to plants from Indochina, which, according to the data obtained, belonged to *M. canadensis*. It was shown that a comprehensive study of plant morphological characters, the compositions of essential oil, and ISSR fragments allows one to clarify the species identity and to assess their polymorphism and the degree of kinship between populations. A certain correlation between the data of molecular analysis and the composition of essential oil and, to a lesser extent, their correlation with morphological characters of plants was revealed.


Key words: *Mentha arvensis*; *Mentha canadensis*; polymorphism; DNA; ISSR; molecular markers; composition of essential oil.

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Внутри- и межвидовая изменчивость *Mentha arvensis* L. и *M. canadensis* L.

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При идентификации представителей рода *Mentha* L. часто возникают сложности из-за значительного внутривидового полиморфизма, активной межвидовой гибридизации, а также изменений в плоидности растений. В настоящей работе предпринята попытка комплексного подхода к изучению различных параметров растений двух трудно разделяемых видов с перекрывающимися признаками: *Mentha arvensis* L. и *M. canadensis* L. Исследованы представители восьми географически разобщенных популяций *Mentha*, собранные в разных регионах: европейская часть, Хакасия и Дальний Восток России, Западная Украина и Индокитай. Изучены диагностические для этих видов морфологические признаки, состав компонентов эфирного масла и проведен фрагментный анализ ДНК с использованием ISSR-маркеров. Полученные данные обработаны статистически с помощью кластерного анализа, метода главных компонент и метода главных координат. По результатам анализа количественных характеристик чашечки и листьев четко разделяются европейская и азиатская группы образцов, а в определении принадлежности дальневосточных растений к той или иной группе есть расхождение при использовании разных методов обработки данных и, соответственно, затруднено определение их таксономического положения с использованием только морфологических признаков. По составу эфирного масла и ISSR-фрагментов выделена группа наиболее генетически, морфологически и фитохимически близких растений, в которую вошли представители популяций Московской, Владимирской, Калужской областей, Республики Коми и Хакасии. Все они относятся к виду *M. arvensis*. Растения, собранные в природной флоре Даль-

него Востока, по компонентному составу эфирного масла и составу ISSR-фрагментов имели большее сходство с *M. arvensis* из европейской части, чем с коллекционными образцами из флоры Индокитая, которые, согласно полученным данным, относятся к *M. canadensis*. Комплексное изучение морфологических признаков растений, состава эфирного масла и фрагментного ISSR-анализа ДНК позволяет уточнить видовую принадлежность, а также оценить полиморфизм и степень родства между популяциями. Выявлена корреляция между данными молекулярно-генетического анализа и составом эфирного масла для растений *M. arvensis* и *M. canadensis* и, в некоторой степени, с морфологическими признаками растений.

Ключевые слова: *Mentha arvensis*; *Mentha canadensis*; полиморфизм; ДНК; ISSR; молекулярные маркеры; компонентный состав эфирного масла.

Introduction

Mentha L. species have a significant potential due to the content of essential oil in the aerial parts of plants. Mint is used in medicine as an antispasmodic, sedative, gastric, and choleric herb. In addition, it is employed in the food and perfume industries. In southern regions of Asia, it is cultivated to obtain technical essential oil. Special treatment of this oil yields menthol, which is a component of many important medicines.

Various sources report from 18 *Mentha* L. species and 11 hybrids (Tucker, Naczi, 2007) to 27 species and 15 hybrids (WCSP, 2019). There are different opinions as to the volumes and boundaries of morphologically similar but polymorphic species. A distinctive feature of this genus is a significant polymorphism within its species and, for all that, the similarity of a number of morphological features among the species. In this regard, it is advisable to use the composition of the essential oil as an additional feature for the characterization of species. The composition of essential oil in all *Mentha* species is genetically determined. Under favorable growing conditions, component synthesis proceeds to the full set of terpenoid compounds characteristic of the species, but unfavorable conditions result in arrest of the synthesis of these substances at early stages, which leads to the appearance of simpler essential oil components (Gouyon et al., 1986). It raises difficulties in the taxonomical discrimination of related species, such as *Mentha arvensis* L. and *M. canadensis* L.

Mentha arvensis grows in Europe and Asia. This species is common in European Russia, Ciscaucasia, and Siberia (Gubanov et al., 2004). The geographic range of *M. canadensis* in Russia includes Siberia and the Far East (Doron'kin, 1997); outside Russia, North America, and East Asia (Tucker, Naczi, 2007).

Mentha arvensis L. (field mint) is a widespread and very variable morphological species, and the habit of plants can vary significantly depending on the growing conditions. In shady damp places, tall plants with ascending or recumbent stems, large green or light green leaves, and pale lilac, sometimes almost white flowers are formed. Plants growing in drier sites are undersized, with erect stem, close internodes, small leaves with red-purple anthocyanin shade, and bright lilac color flowers.

Mentha canadensis L. (Canadian mint) was described by C. Linnaeus (Linne, 1753). It is related to *M. arvensis* and difficult to identify. *M. canadensis* differs from *M. arvensis* in having a higher, unbranched, densely pubescent stem; one-half as wide, sharp, and deeply serrated leaves; and a specific calyx shape. Different morphological criteria are used for the

discrimination of *M. arvensis* and *M. canadensis*. These species are proposed to be divided according to the shape of the calyx and the structure of the calyx teeth (Doro'nkin, 1997). *M. arvensis* calyces are bell-shaped; the teeth are short and wide-triangular, whereas *M. canadensis* calyces are tubular with long pointed teeth. Other researchers identify the two species by leaf form and flavor; however, these traits do not provide a reliable criterion (Tucker, Naczi, 2007).

Such morphological features as the type and degree of branching, the shape and size of the leaf, and shoot pubescence cause difficulties in the identification of these two species. These traits are so variable within a species that they can overlap between species. All these features vary greatly depending on growing conditions (especially in *M. arvensis*); nevertheless, there are important in identifying species.

Researchers often disagree in recognizing these two species. In particular, *M. canadensis* was considered a form or subspecies of *M. arvensis*. Holmes (1882) described mint from Japan as *M. arvensis* f. *piperascens* Maliniv. ex Holmes. Briquet (1894) identified mint from North America as *M. arvensis* var. *canadensis*, and mint from Asia as *M. arvensis* var. *haplocalyx*, whereas Hara (1956) described the latter as *M. arvensis* ssp. *piperascens*. Currently, *M. canadensis* is recognized as an independent species, and its numerous synonyms are most fully represented in the World Checklist of Selected Plant Families, Royal Botanic Gardens, Kew (WCSP, 2019).

M. canadensis is probably an amphidiploid resulting from interspecific hybridization of *M. arvensis* and *M. longifolia* (Tucker, Chambers, 2002). There is evidence from chloroplast DNA sequences that in this interspecific hybridization process *M. arvensis* may have been the maternal parent (the source of female gametes) for the resulting species, *M. canadensis* (Bunsawat et al., 2004). A comprehensive study involving flow cytometry and ISSR-analysis suggested that *M. canadensis* was an allopolyploid resulting from cytomixis between representatives of parental species with different ploidies (Jedrzejczyk, Rewers, 2018).

Our work concerns the intra- and interspecific variability and differentiation of *M. arvensis* and *M. canadensis* of several populations distant from each other. Previously, we studied polymorphism in *M. arvensis* populations on the base of morphological and molecular data (Shelepova et al., 2016b) and attempted to differentiate these species using morphological and phytochemical analysis (Shelepova et al., 2016a). However, the resulting notion was still incomplete. A number of investigators successfully undertook a combined assessment of plants by morphological characteristics, essential oil com-

position, and ISSR-analysis data to study interspecies and interpopulation variation in the genus *Mentha* (Hua et al., 2011; Rodrigues et al., 2013; Shelepova et al., 2017). Therefore, to accomplish our task; we comprehensively examined morphological features, essential oil compositions, and ISSR-markers of DNA in two mint species.

Materials and methods

Sampling in populations. Plants of *M. arvensis* and *M. canadensis* to be examined belonged to eight populations. They were collected from four regions distant from each other (Table 1).

Five to eight shoots from typical plants collected at 10–200-m intervals depending on the population size in the nature and 1–3 shoots of plants cultivated in experimental plots were taken for the study of morphological features and ISSR-analysis of DNA. In our preliminary study, samples from natural populations demonstrated a wide variety of amplified fragments (significant diversity of genotypes within a population), and the plants from the collection did not differ in the spectrum of ISSR fragments (belonged to a single clone). *Mentha spicata* L. was used as an external standard. One to five plant samples were taken to study the composition of essential oil in populations concurrently with the collection of herbarium images (in the phase of mass blossoming) from a site of 0.2 m². Herbarium specimens are stored at the Laboratory of Plant Physiology and Immunity and in the Herbarium of GBS RAS (MHA).

Study of morphological features. The collected plants were identified to the species level. Most of the samples were attributed to *M. arvensis*, but plants from the natural flora of the Russian Far East and collection samples from the Indochina flora were previously identified as *M. canadensis*. The following quantitative indices were studied: calyx length, calyx tooth length, calyx tooth width at the base, the tooth length : width ratio, the calyx length : tooth length ratio, the calyx tube length : tooth length ratio; the length and maximum width of the leaf, the distance from the basis to the maximum width of leaf; the density of secretory granules on the lower and upper surfaces of the leaf per 1 cm², the ratio between the numbers of secretory granules on the lower and upper surfaces of the leaf. The structure of the calyx was examined with a light stereomicroscope at 400× magnification. Images for comparative analysis were taken with a Lumenera Infinity 2 video camera and processed with the Infinity Analyses 5.0.2 program. Secretory granules were counted as in (Shelepova et al., 2012).

Methods of studying the essential oil composition. Essential oil was isolated from an average sample of the above-ground mass (a mixture of inflorescences and leaves) of plants. The oil was obtained by hydrodistillation of crushed air-dry material (Ginsberg, 1932). Plant essential oil consists of a mixture of natural compounds, mainly isoprenoids (monoterpenes, diterpenes, hemiterpenes, sesquiterpenes, and their oxides). The most detailed report on their classification, biosynthesis, and detailed study of each class of compounds was provided by V.V. Plemenkov (2007). The qualitative composition of the oil was determined by gas chromatography in the “Biotechnology” Shared Access Center, RAS (RFMEFI62114X0002) in a Shimadzu GS 2010 gas chromatograph with a GCMS-QP

2010 mass detector as previously described in (Shelepova et al., 2017).

DNA extraction and PCR. DNA was isolated from dry herbarium specimen leaves by the CTAB method (Doyle J.J., Doyle J.L., 1987). DNA polymorphism was analyzed by the ISSR approach. Primers for PCR analysis were synthesized and purified by PAAG by Syntol Company (Moscow, Russia). Eight primers were chosen after pilot tests (Table 2). Two series of PCR were carried out for 46 and 42 samples, and some of the samples were retested by PCR. ISSR-PCR and the separation of PCR products were carried out by previously reported methods (Shelepova et al., 2016b, 2017).

Analysis of molecular data and statistical data processing. The band profiles of the ISSR fragments were compared visually and with the CrossChecker program. Only bright and distinct fragments were taken into consideration, and unclear bands were discarded. Each band in an electrophoretic gel was considered a countable character and used as a binary code in the matrix of the presence/absence of fragments. Then the results were analyzed in the PAST program using cluster analysis, principal coordinate method, and principal component method (Hammer et al., 2001). Bootstrap analysis was carried out with 1000 replicas to assess the stability of the dendrograms obtained.

Results

The analysis of quantitative morphological features by the principal component method shows that the samples can be divided into two main groups (Fig. 1, a). The first group includes all samples from European Russia, the Republic of Komi, Khakassia, the Russian Far East, and Ukraine and one plant from Indochina (Ind2). Plants from the Far East are grouped together to form part of a large group, and other samples form a mixed cloud. An individual cloud is formed by plants from Indochina, which were identified as *M. canadensis*. Two main clusters with somewhat different compositions of samples therein were obtained when processing the same data by cluster analysis using the Gower distance (Fig. 1, b). The first cluster includes all plants from the Russian Far East and Indochina, and the second, plants from Europe and Khakassia.

Essential oil composition can be an independent criterion for the identification of *Mentha* plants. About 47 components were found in *Mentha* essential oil. All components constituting more than 0.1 % of the total amount were easily identified by retention time and mass spectra. The proportions of individual components in the essential oil vary significantly among plants from different regions.

The distribution of samples according to essential oil composition proved to be consistent with morphological data. However, the plant group from the Russian Far East shows a more distinct and stable position among others regardless of the statistical methods applied (Fig. 2). When using the principal components method (see Fig. 2, a), the samples are divided into three main groups: 1, *M. spicata* (the most abundant component is menthone); 2, Indo-Asian plants (menthol); and 3, a large group of samples, which included plants from Moscow, Kaluga, and Vladimir regions, the Russian Far East, Komi, Khakassia and Ukraine (the predominant components are *trans*- and *cis*- β -ocimenes, γ -terpinene, 1,8-cineole, α - and β -pinenes and pulegone).

Table 1. Sites of *Mentha* collection

Sample identifier	Number of plants	Sampling locality	Geographical coordinates
M-1,2	2	Moscow, territory of the Main Botanical Garden, RAS. Dry bed of a stream on the left bank of the Likhoborka River	55°50'35.7" N, 37°36'52.3" E
M-3	1	Moscow region, Znamenskoye Sadki manor, shore of a pond over the Bitsa River	55°34'44.8" N, 37°33'19.3" E
M-4	1	Moscow region, the first river terrace of the Bitsa River, 3 km downstream from the Znamenskoye Sadki manor	55°34'38.1" N, 37°33'47.1" E
M-5	1	Moscow region, 1.5 km south-west of Mar'ino village, Krasnogorsk district, at the water edge of a dam on the Sinichka River	55°51'07.6" N, 37°18'35.6" E
M-6	1	Moscow region, Valuevsky forest park, near Meshkovo village	55°35'03.5" N, 37°18'31.6" E
M-7	1	Moscow, Vorobyevy Gory Nature Reserve	55°42'33.4" N, 37°33'16.6" E
Kh	5	Khakassia, Altai Kray, high right bank of the Abakan River, about 1.5 km west of Izykhskie Kopi village, on a pebble shallow	53°33'4.54" N, 91°14'38.15" E
F	5	Vladimir region, Kovrovskiy district, floodplain of the Klyazma River	56°24'59.1" N, 41°23'06.0" E
K	2	Kaluga region, Zhukovskiy district, outskirts of Okorokovo village	55°03'57.3" N, 36°42'55.5" E
Komi	3	Collection plot of the Laboratory of Plant Physiology and Immunity, Main Botanical Garden of the RAS, obtained from Syktyvkar, Komi natural flora	55°50'06.5" N, 37°35'17.4" E
Ind1-5	5	Collection plot of the Laboratory of Plant Physiology and Immunity, Main Botanical Garden of the RAS, obtained from Vietnam, Indochina natural flora	55°50'06.5" N, 37°35'17.4" E
<i>Mentha spicata</i>	1	Collection plot of the Laboratory of Plant Physiology and Immunity, Main Botanical Garden of the RAS, obtained from the Nikitskiy Botanical Garden, Yalta	55°50'06.5" N, 37°35'17.4" E
U-1	1	Ukraine, Lviv, within the city, Pogulyanka Park, marshy lowland near a spring below a slope of beech forest	49°49'11.0" N, 24°02'14.3" E
U-2	1	Ukraine, Lviv, Pogulyanka Park, swamp edge	49°49'22.9" N, 24°03'52.4" E
U-3,4	2	Ukraine, northwestern outskirts of Lviv, Belogorsha village, peatland	49°50'28.0" N, 23°55'02.5" E
U-5	1	Ukraine, Ivano-Frankivsk region, Galich district, Tustan' village, floodplain of the Gnilaya Lipa River	49°08'00.0" N, 24°45'20.4" E
U-6	1	Ukraine, Ivano-Frankivsk region, Galich district, between the villages of Vodniki and Dubovtsy, dead arm of the Dniester River	49°04'33.1" N, 24°47'53.1" E
RFE-1-5,8	6	Primorsky Krai, Khasanskiy district, Vityaz Bay	42°35'36" N, 131°11'13" E
RFE-6,7	2	Primorsky Krai, Khasanskiy district, Astafyev's Bay	42°37'7" N, 131°11'54" E
RFE-9,10	2	Primorsky Krai, Lazovskiy district, Zapovednoye Station	42°50'27" N, 133°41'56" E

Table 2. List of ISSR-primers used for PCR

Primer	Sequence	Primer annealing temperature, °C	Number of polymorphic ISSR fragments
M2	(AC) ₈ (C/T)G	50.0	12
M3	(GA) ₈ (C/T)C	52.7	17
M4	(AG) ₈ YC	50.0	17
M7	(CAG) ₅	52.7	16
M8	(GTG) ₅	52.7	13
M12	(CA) ₆ (A/G)(C/T)	50.0	13
UBC 840	(GA) ₈ AYT	50.0	12
UBC855	(AC) ₈ CYT	50.0	17

Euclidean distance-based cluster analysis of the essential oil composition divided samples into two clusters with a high degree of support (bootstrap support level 84–87 %) (see Fig. 2, b). *M. spicata*, taken as an external group, assumes a basal position in relation to two clusters, the predominant compound of its essential oil being menthone (67.9 %) (Table 3). The first cluster includes plants from Indochina, which are characterized by the accumulation of menthol (46.9–67.7 %) and its derivatives: menthone (3.11–30.3 %) and isomenthone (2.33–22.7 %) (see Table 3).

The second cluster includes all other plants (from Central Russia, Komi, Khakassia, and the Far East). This cluster has a bootstrap support below 84 %, and it is divided into three well-supported subclusters. Within the second cluster, a subcluster is distinguished, consisting of plants of *M. arvensis* from Moscow (M-6), Kaluga (K-1), and Vladimir (F) regions and Ukraine (U-2). The mints from the Russian Far East (RFE),

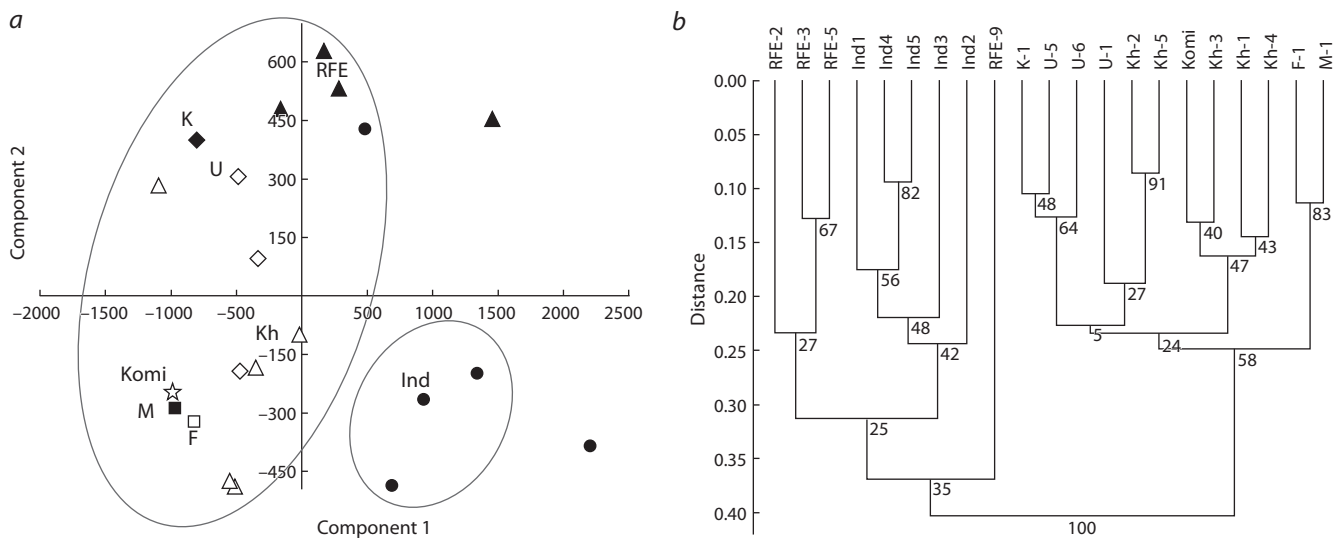


Fig. 1. Distribution of *M. arvensis* and *M. canadensis* samples based on morphological characters: *a*, by the principal component method; *b*, by cluster analysis.

Hereinafter: ■, Moscow region (M-6); ◆, Kaluga region (K-1); □, Vladimir region (F); △, Republic of Khakassia (Kh); ▲, Russian Far East (RFE); ☆, Republic of Komi (Komi); ◇, Ukraine (U-2); ●, Indochina (Ind1-5).

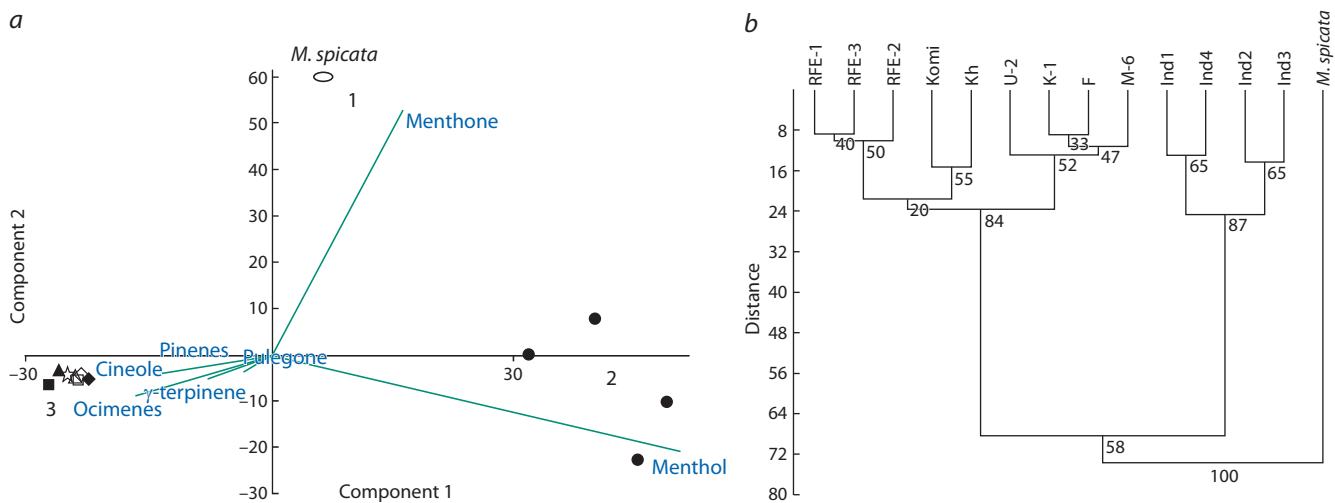


Fig. 2. Distribution of *M. arvensis*, *M. canadensis*, and *M. spicata* samples based on essential oil composition analysis in the aerial parts: *a*, by the principal component method; *b*, by the cluster analysis of the data.

as well as the Republic of Komi (Komi) and Khakassia (Kh) formed two independent sister subclusters within it.

It appears from data in Table 3 that the populations M-6, K-1, F, and U-2 are distinguished mainly by the contents of *trans*- and *cis*- β -ocimenes (4.2–23.8 %) and γ -terpinene (11.8–21.8 %); RFE, Komi, and Kh populations, by 1,8-cineole (20.1–28.8 %) and *trans*- and *cis*- β -ocimenes (15.6–24.0 %); and in the Komi and Kh populations, in addition to 1,8-cineole (24.7 and 12.8 %) and *trans*- and *cis*- β -ocimenes (12.1 and 17.3 %), pulegone and its predecessor, isopulegone are abundant (15.5 and 21.1 %, respectively). Hotelling paired tests indicate a significant variation ($p < 0.02$ with the Bonferroni

correction) among all populations. Thus, the revealed differences between *Mentha* plants allow recognition of the following chemotypes: menthol (plants from Indochina), *trans*- and *cis*- β -ocimenes and γ -terpinene (*M. arvensis* plants from Moscow, Kaluga, and Vladimir regions and from Ukraine); 1,8-cineole and *trans*- and *cis*- β -ocimene (plants from the Russian Far East) and, provisionally, pulegone (plants from the Republic of Komi and Khakassia).

DNA fragments were examined by the ISSR method for better elucidation of the genetic similarity of populations. PCR was performed according to two schemes. The first scheme of experience included plants from Indochina, Khakassia,

Table 3. The essential oil composition of *Mentha*

Component name	Percentage in the whole essential oil													
	<i>M. spicata</i>	M-6	K-1	F	Kh	Komi	U-2	RFE-1	RFE-2	RFE-3	Ind1	Ind2	Ind3	Ind4
α-Pinene	0.53	2.20	1.15	1.91	2.04	2.86	0.98	5.24	3.85	4.19	0.25	0.41	0.95	0.45
β-Pinene	0.02	4.51	1.59	2.19	1.44	6.36	3.75	7.08	6.82	7.84	0.26	0.47	1.00	0.48
Sabinene	1.01	2.46	1.00	0.90	4.36	3.73	1.84	6.03	7.84	6.79	0.23	0.20	0.32	0.21
β-Myrcene	0.61	3.18	3.68	2.33	6.68	1.13	4.68	t*	6.16	2.85	0.39	0.36	0.73	0.39
Limonene	0.69	0.65	2.09	1.02	1.88	0.82	6.05	1.24	1.30	1.19	1.99	0.93	2.04	2.13
1,8-Cineole	3.62	13.89**	3.28	8.87	12.75	24.73	15.28	20.06	28.82	20.15	0.20	0.31	0.41	0.20
<i>trans</i> -β-Ocimene	0.08	11.17	8.98	2.01	7.59	5.91	7.26	10.85	8.85	6.62	0.02	t	0.02	0.15
<i>cis</i> -β-Ocimene	0.04	12.61	11.73	2.19	9.73	6.18	8.54	13.12	9.51	8.95	t	0.28	0.01	0.06
γ-Terpinene	1.11	21.81	19.32	13.59	4.23	3.21	11.79	0.74	0.52	3.54	0.01	t	t	t
Terpinolene	0.19	1.97	13.46	5.72	3.46	0.12	3.15	0.30	0.20	0.78	0.01	t	0.01	0.01
Linalool	0.08	0.45	1.02	2.79	2.60	1.20	1.02	0.45	0.78	0.51	0.92	0.28	0.02	0.011
Menthol	3.16	0.58	1.60	1.81	0.21	0.58	0.98	0.42	0.63	0.71	46.92	67.67	67.32	52.96
α-Terpineol	0.32	1.58	1.65	1.79	3.19	1.72	2.74	2.81	1.84	1.21	0.17	t	0.12	t
Menthone	67.88	0.22	0.56	0.85	0.37	0.56	0.64	t	t	t	19.25	3.11	16.36	30.33
Isomenthone	4.68	0.48	0.11	0.27	0.43	0.44	0.36	0.19	0.28	0.12	22.69	6.29	5.90	2.33
Isopulegone and pulegone	1.17	0.98	3.13	0.79	21.14	15.51	1.63	0.87	0.56	0.61	2.54	5.79	0.49	0.28
Methyl acetate	0.36	0.11	0.63	0.81	0.47	0.10	0.72	0.39	0.21	0.78	8.94	7.05	0.42	1.95
Isomenthol	1.03	0.21	0.22	0.25	0.18	0.18	0.22	0.38	0.42	0.73	0.38	1.03	0.04	1.51
β-Caryophyllene	1.69	2.99	1.55	1.96	2.29	7.96	6.97	5.24	2.60	2.41	0.61	0.55	0.65	0.40
Germacrene D	1.08	4.38	5.07	3.22	3.84	5.28	3.45	0.98	0.80	1.47	0.60	0.93	0.94	0.32
Piperitone	0.70	0.12	0.13	0.36	0.10	0.06	5.25	1.03	0.55	0.54	0.84	0.68	0.46	2.77

* Absent from the essential oil.

** Major essential components are shown in boldface.

European Russia, and Western Ukraine with *M. spicata* as an external standard. Samples from the Far East flora were added to this group in the second experiment.

A matrix including 117 polymorphic fragments was constructed in the first scheme of PCR. Plants were divided into two main groups when analyzing the data by the principal coordinate method involving the Dyce index (Fig. 3, a). One of these groups includes plants from Indochina, and the other, all plants from European Russia and Khakassia. *M. spicata* and one sample of Asian flora (Ind1) occupied a separate position. Plants from Ukraine formed a group hardly separated from other European and Khakassian samples. According to the results of cluster analysis (Fig. 3, b) there are two large groups of plants with a medium level of bootstrap support (41–56 %) and a fairly high degree of similarity (0.6). The first group included Indo-Asian plants, and the second, European and Khakassian.

A matrix based on 80 polymorphic fragments was compiled according to the results of the second scheme of PCR. As a result of processing the data by the principal coordinate method and cluster analysis with the Dice index (Fig. 4), the plants were also divided into two groups. The first group included plants from Indochina, and the second, all the other plants. Some of them formed a common cloud/cluster, and others formed separate subgroups adjoining the general. Plants from Indochina contained specific amplicons, absent from other plants, including samples from the Russian Far East.

Plants of the second group fall into several subgroups. Plants from the Moscow, Vladimir, and Kaluga regions, as well as from the Republic of Komi and Khakassia, form a common cloud in which samples are practically inseparable. This indicates a significant genetic similarity between these populations. Two relatively separate groups of plants adjoin

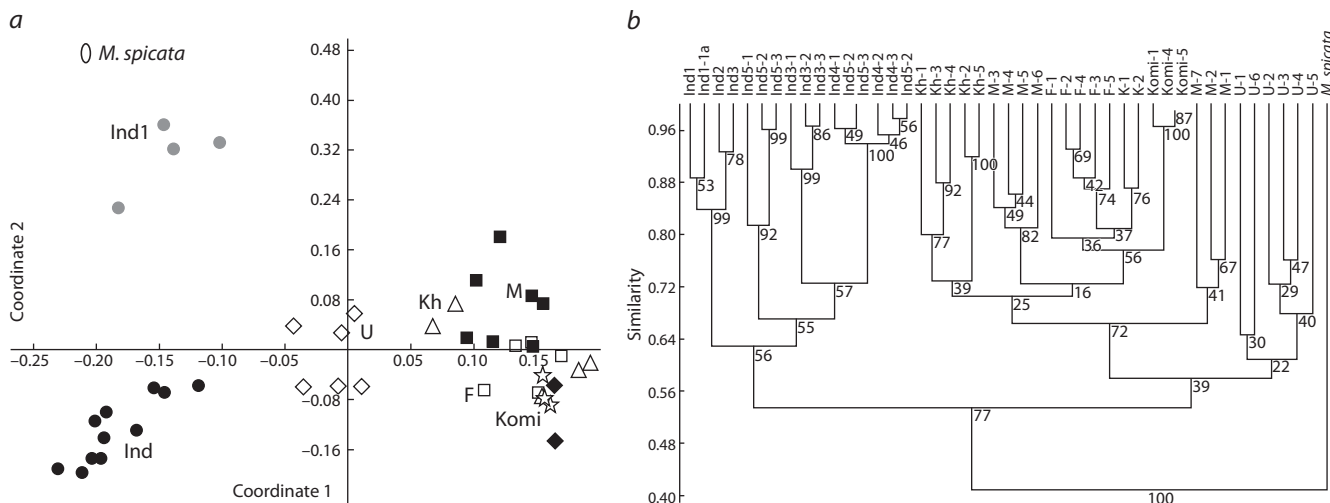


Fig. 3. Distribution of samples based on ISSR marker composition analysis in *M. arvensis*, *M. canadensis*, *M. spicata* plants: *a*, by the method of principal coordinates; *b*, by cluster analysis.

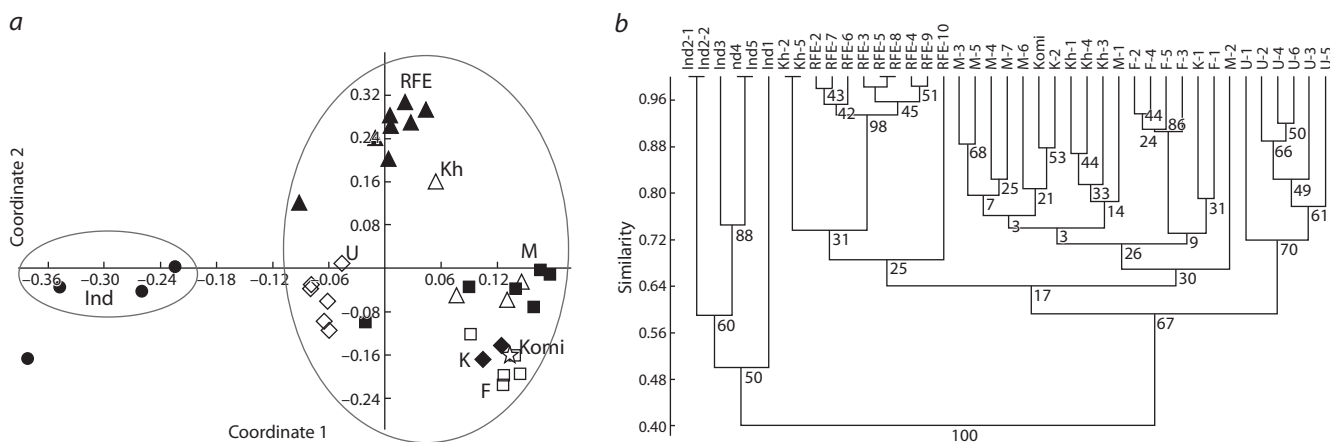


Fig. 4. Distribution of samples based on ISSR markers composition analysis in *M. arvensis* and *M. canadensis* plants: *a*, by the method of principal coordinates; *b*, by cluster analysis.

the mixed group. One of them is formed by the population of the Russian Far East and two plants from Khakassia. The other consist of samples from Western Ukraine, which form a cloud adjoining to plants from European Russia. All plants collected in the Russian Far East have a very high measure of similarity (0.9) and cluster bootstrap support (98 %), thus indicating a close relationship among all the studied Far Eastern local populations.

Discussion

On the base of quantitative calyx and leaf characters, the European and Indo-Asian groups of plants are clearly recognized, but the assignment of Far Eastern plants to one or the other group is ambiguous. Accordingly, it is difficult to determine their taxonomic position solely by morphological features. An additional criterion for the identification of *M. arvensis*

and *M. canadensis* might include such qualitative indicators as the ultrastructure of the seed surface (Sheleпова et al., 2016a), but seeds were not available from plants of Indochinese origin, as seeds rarely ripen when these accessions are grown in Central Russia.

According to essential oil composition in the aerial parts of the plants, all the samples were distributed into different chemotypes related to the genetic characteristics of the plants. The literature mentions many chemotypes for both *M. arvensis* and *M. canadensis* isolated on the basis of components with shares above 10 % (Tucker, Chambers, 2002). Nine chemotypes are mentioned for *M. arvensis*: limonene, pulegone, 1,8-cineole and/or β -pinene, and others. *Trans*- and *cis*- β -ocimenes and γ -terpinene dominated in the essential oils of *M. arvensis* plants examined. These are acyclic limonene precursors and cyclic terpenoid intermediates. Limonene is

known to be transformed through a series of biochemical reactions into isopulegone, which, in turn, is a precursor of pulegone (Bugaenko, 2011). This is recorded in *M. arvensis* plants from Komi and Khakassia (provisionally pulegone chemotype).

There have been identified, among others, 1,8-cineole and β -ocimene chemotypes in *M. canadensis* from North America, and numerous chemotypes with menthol and menthone as major components in *M. canadensis* from Asia (Tucker, Chambers, 2002). Similar data were obtained in our studies: the menthol chemotype was identified in plants *M. canadensis* from Indochina and the 1,8-cineole and *trans*- and *cis*- β -ocimene in plants from the Russian Far East.

Analyzing the composition of ISSR fragments, we infer that *M. arvensis* plants from different populations of European Russia have significant genetic similarity and are almost indistinguishable from each other. On the contrary, Ukrainian and Far Eastern populations clearly differ from others. Far Eastern plants form a distinct group different from European populations, although they belong to the same cluster/cloud according to the ISSR analysis. Plants from Indochina differ significantly from other samples and belong to another species according to the results of ISSR-PCR.

A similar pattern is observed for *M. arvensis* and *M. canadensis* plant distribution according to molecular analysis, essential oil composition, and, somewhat, morphological characters. Thus, there is a correlation between the molecular, phytochemical, and, to some extent, morphological data. On the basis of our results, a group of the genetically, morphologically, and phytochemically closest plants can be recognized. It includes representatives of the populations of the Moscow, Vladimir, and Kaluga regions; the Komi Republic; and Khakassia. All of them belong to *M. arvensis*.

Conclusion

According to our data on the compositions of essential oils and ISSR fragments, the plants collected in the natural flora of the Far East were more similar to *M. arvensis* from European Russia than to the collection samples from Indochina. With all that, a certain similarity to *M. canadensis* was noted in plants of the Far Eastern population according to some morphological features (lengths of the calyx teeth were intermediate between *M. arvensis* from European Russia, and *M. canadensis* from Indochina) and essential oil components, which were characteristic of *M. canadensis* from North America. Also, genetic isolation from the rest of the *M. arvensis* plants was detected. Perhaps the Far Eastern population includes plants of hybrid origin and has a closer relationship to *M. arvensis* than to plants from Indochina, which, according to the data obtained, belong to *M. canadensis*. It follows from our results that a comprehensive analysis of morphological, phytochemical, and molecular data allows the most complete view of species polymorphism and clarifies the species identification of plants.

References

Briquet J. Fragmenta monographiae Labiatarum. Fascicule troisieme. Decades Mentharum novarum. Bull. Herb. Boiss. 1894;2:691-709.

- Bugaenko L.A. Genetic Patterns of Terpenoid Biosynthesis in Mint. Simferopol: Business Inform Publ., 2011. (in Russian)
- Bunsawat J., Elliott N., Hertweck K., Lawrence A. Phylogenetics of *Mentha* (Lamiaceae): evidence from chloroplast DNA sequences. Syst. Bot. 2004;29(4):959-964. DOI 10.1600/0363644042450973.
- Doron'kin V.M. *Mentha* L. In: Malyshev L.I. (Ed.) Flora of Siberia. Novosibirsk: Nauka Publ., 1997;11:222-225. (in Russian)
- Doyle J.J., Doyle J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 1987;19:11-15.
- Ginsberg A.S. A simple method for essential oil assay in essential-oil-bearing plants. Khimiko-Farmatsevticheskaya Promyshlennost = Chemical-Farm. Industry. 1932;8(9):326-329. (in Russian)
- Gouyon P.H., Vernet Ph., Guillermin J.L., Valdeyron G. Polymorphisms and environment: the adaptive value of the oil polymorphisms in *Thymus vulgaris* L. Heredity. 1986;57:59-66.
- Gubanov I.A., Kiseleva K.V., Novikov V.S., Tikhomirov V.N. *Mentha arvensis* L. s. l. – Field Mint. Illustrated Guide to Plants of Central Russia. Moscow: KMK Publ.; Institute of Technological Research, 2004;3:133. (in Russian)
- Hammer O., Harper D.A.T., Ryan P.D. PAST: PAleontological STatistics software package for education and data analysis. Palaeontologia Electronica. 2001;4(1):4. Available at: https://palaeo-electronica.org/2001_1/past/issue1_01.htm
- Hara H. Contributions to the study of variations in the Japanese plants closely related to those of Europe or North America. Part 2. J. Fac. Sci. Univ. Tokyo Sect. 3, Bot. 1956;6:343-391.
- Holmes E.M. The Japanese peppermint plant. Pharm. J. Trans. 1882; 13:381-382.
- Hua C.X., Wang G.R., Lei Y. Evaluation of essential oil composition and DNA diversity of mint resources from China. Afr. J. Biotechnol. 2011;10(74):16740-16745. DOI 10.5897/AJB11.1428.
- Jedrzejczyk I., Rewers M. Genome size and ISSR markers for *Mentha* L. (Lamiaceae) genetic diversity assessment and species identification. Ind. Crops Prod. 2018;120:171-179. Available at: <https://doi.org/10.1016/j.indcrop.2018.04.062>
- Linne C. *Mentha*: Species Plantarum. Holmaiae, 1753.
- Plemenkov V.V. Chemistry of Terpenoids. Barnaul: Altay University Publ. 2007. (in Russian)
- Rodrigues L., Póvoa O., Van den Berg C., Figueiredo A.C., Moldão M., Monteiro A. Genetic diversity in *Mentha cervina* based on morphological traits, essential oils profile and ISSRs markers. Biochem. Syst. Ecol. 2013;51:50-59. DOI 10.1016/j.bse.2013.08.014.
- Shelepova O.V., Semenova M.V., Enina O.L., Schantser I.A. Genetic, phenotypic and phytochemical polymorphism of Eastern European populations *Mentha arvensis* L. Russ. J. Genet. 2017; 53(1):59-66. DOI 10.1134/S1022795416120139.
- Shelepova O.V., Semenova M.V., Schantser I.A., Stepanova N. Yu. Genetic and morphological features of South Siberian *Mentha arvensis* L. populations. In: Proc. of the XV Int. practical and sci. conf. "Problems of Botany of South Siberia and Mongolia". Barnaul, 2016b;15:446-450. (in Russian)
- Shelepova O.V., Semenova M.V., Voronkova T.V., Olekhovich L.S., Bidyukova G.F. Anatomical, morphological and phytochemical differences between *Mentha canadensis* L. and *Mentha arvensis* L. Bulletin Botanicheskogo Sada-Instituta DVO RAN = Bulletin of the Botanical Garden-Institute, Far-Eastern Branch of the RAS. 2016a;15:89-92. (in Russian)
- Shelepova O.V., Voronkova T.V., Smirnova I.M., Enina O.L. Features of the glandular apparatus of *Mentha arvensis* L. plants from different geographical regions. Izvestiya Samarskogo

- Nauchnogo Tsentra Rossiyskoy Akademii Nauk = Proceedings of the Samara Research Center of the Russian Academy of Sciences. 2012;14(1):1884-1887. (in Russian)
- Tucker A.O., Chambers H.L. *Mentha canadensis* L. (Lamiaceae): a relict amphidiploid from the Lower Tertiary. Taxon. 2002;51(4):703-718.
- Tucker A.O., Naczi R.F.C. *Mentha*: an overview of its classification and relationships. In: Lawrence B.M. (Ed.). *Mint. The Genus Mentha*. CRC Press, 2007;3-39.
- WCSP. 2019. World Checklist of Selected Plant Families. Facilitated by the Royal Botanic Gardens, Kew. Available at: <http://apps.kew.org/wcsp/Retrieved>

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