THE STUDY OF HYBRIDIZATION PROCESSES WITHIN GENUS *SPARGANIUM* L. SUBGENUS *XANTHOSPARGANIUM* HOLMB. BASED ON DATA OF NEXT GENERATION SEQUENCING (NGS)

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❦ The study represents the results of research of intragenic polymorphism in transcribed spacer ITS1 of the *35S* rRNA genes in representatives of subgenus *Xanthosparganium* genus *Sparganium* which were obtained by means of locus-specific next generation sequencing on the platform Illumina MiSeq. It was shown that ribotype variations in studied samples generally correspond to the division of this genus into three sections — *Erecta* (subgenus *Sparganium*), *Natantia* and *Minima* (subgenus *Xanthosparganium*). High level of intragenic polymorphism was revealed in *S. hyperboreum*, with ribotypes distributed among several groups. Genome of this species includes ribotypes which are typical for other species in subgenus *Xanthosparganium*. For two investigated *S. glomeratum* samples, there were no ribotypes similar to such ribotypes in other species of *Natantia* section. *S. glomeratum* has got ribotypes identical with *S. hyperboreum* of *Minima* section. This feature may be the evidence of ancient intersectional hybridization of these two species. Characteristics of rDNA in *S. glomeratum* are in favor of putting this species into *Minima* section. It was suggested that speciation processes within the genus could be based not only on hybridization but also went on in allopatric way. The fist statement is supported by the presence of similar and identical ribotypes in *S. emersum*, *S. × longifolium*, *S. gramineum* and *S. hyperboreum*, the second — as it was mentioned by other researchers, is due to close relationship between North American and Eurasian taxa.

❦ **Keywords:** Typhaceae; bur-reed; gene systematics; molecular phylogeny; interspecific hybridization; speciation.

ГИБРИДИЗАЦИОННЫЕ ПРОЦЕССЫ В РАМКАХ РОДА *SPARGANIUM* L. ПОДРОДА *XANTHOSPARGANIUM* HOLMB. ПО ДАННЫМ СЕКВЕНИРОВАНИЯ СЛЕДУЮЩЕГО ПОКОЛЕНИЯ (NEXT GENERATION SEQUENCING — NGS)

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❦ В работе изложены результаты сравнительного исследования внутригеномного полиморфизма транскрибируемого спейсера ITS1 гена *35S* рРНК у представителей подрода *Xanthosparganium* рода *Sparganium*, полученные методом локус-специфичного секвенирования следующего поколения на платформе Illumina MiSeq. Показано, что вариации риботипов изученных образцов, в целом, соответствуют делению рода на три секции — *Erecta* (подрод *Sparganium*), *Natantia* и *Minima* (подрод *Xanthosparganium*). Высокий уровень внутригеномного полимофизма выявлен у *S. hyperboreum*, риботипы которого были распределены по нескольким группам. В геноме этого вида присутствуют риботипы, характерные для других видов подрода *Xanthosparganium*. У двух изученных образцов *S. glomeratum* нами не обнаружено риботипов, сходных с таковыми у других видов секции *Natantia*. *S. glomeratum* имеет одинаковые риботипы с *S. hyperboreum* из секции *Minima*. Такая особенность может являться признаком древней межсекционной гибридизации этих двух видов. Особенности рДНК *S. glomeratum* говорят в пользу того, что вид может быть отнесен к секции *Minima*. Высказано мнение, что видообразовательные процессы в рамках рода могли происходить не только на основе гибридизации, но и ал-

лопатрическим путем. Первое подтверждается наличием близких и одинаковых риботипов у *S. emersum*, *S. × longifolium*, *S. gramineum* и *S. hyperboreum*; второе, как ранее указывалось другими исследователями, кроется в близкой связи североамериканских и евроазиатских таксонов.

❀ **Ключевые слова:** Typhaceae; ежеголовник; геносистематика; молекулярная филогения; межвидовая гибридизация; видообразование.

INTRODUCTION

The phenomena of interspecific and intergeneric hybridization have recently been considered as an effective way of occupying new ecological niches and formation of species of vascular plants [1−4]. Among 37,000 species of flora in Europe, North America, and parts of Australia, belonging to 3,212 genera of 282 families of vascular plants, the species, which morphology suggests as their hybrid origin, were registered in 40% of families and 16% of genera, with an average frequency of nine species of hybridogenic origin per 100 species of non-hybrid origin [5]. In particular, among aquatic and coastal plants, hybrids were recorded in the genera *Potamogeton* L. [6−9], *Nuphar* Smith [10], *Nymphaea* L. [11, 12], *Carex* L. [13−15], *Ranunculus* L. [16, 17], and *Typha* L. [18, 19]. From this point of view, the genus *Sparganium* L. (bur-reed), which belongs to the sister family of Typhaceae for Pophae, is of great interest as the object of study [20−24].

The *Sparganium* genome is one of the smallest genomes among vascular plants: 1C = 490 million bps. [25]. It should be noted that, unlike gramineous plants, in which the number of chromosomes in the genome has changed repeatedly [26, 27] as a result of recent acts of polyploidization and secondary diploidization, the number of chromosomes in different species of bur-reed is stable, $2n = 30$ [28, 29]. It follows that the average size of the chromosome in bur-reeds is 32 million bps, which is four times lower than in humans, for example.

The *Sparganium* karyotype comprises two pairs of nucleolus organizer chromosomes [30]. A relatively high number of chromosomes in the karyotype ($x = 15$) suggests that all the representatives of the genus are poliploids, probably allotatraploids [31]. The available data suggest the distribution of interspecific hybridization in the bur-reed, which contributes to the creation of new genotypes and phenotypes. Because of this, the plants occupy certain ecological niches in different types of water reservoirs in a wide range of natural climatic zones. Thus, Y. Ito et al. [23] revealed the existence of a number of combinations of hybrids, namely, *S. angustifolium* × *S. emersum* (*S. engleranum* Asch. et Graebn.), *S. hyperboreum* × *S. natans*, *S. acaule* × *S. fluctuans*, and *S. fallax* × *S. japonicum*, by comparing the topology of molecular phylogenetic trees constructed on the basis of variations in the sequences of chloroplast DNA and the phytochrome C nuclear gene (*phyC*). Apparently, that the bur-reed hybrids are homoploid hybrids that preserve the ploidy level of the parent species.

As a rule, this type of hybridization, which is accompanied by the incorporation of a certain part of the genes of one parent species into the genome of another, is called introgressive hybridization [32].

We conducted a comparative analysis of intragenomic polymorphism in the subgenus *Xanthosparganium* Holmb. of the *Sparganium* genus. We also searched for traces of hybridization processes in related species of the genus using locus-specific sequencing of the transcribed spacer (ITS1) of *35S* rRNA genes repeatedly that are multiplicated in the genome and encode the sequences *18S*, *5.8S*, and *26S* rRNA of the large and small ribosome subunits. In plants, these sequences are transcribed as a single transcription unit; the molecular weight of the transcript is approximately *35S* (*45S* in animals) [33]. In each nucleolus organizer, there may be several thousands of *35S* rRNA genes [34, 35]. The study of homoploid hybrids and allopolyploids revealed that the *35S* rRNA genes obtained from their ancestors can be preserved in their genome for many generations. Their sequencing enables us to identify recent events of interspecific hybridization in the species [36, 37], which was the aim of our work.

MATERIALS AND METHODS

Because of the likely hybrid origin of the species, we investigated the intragenomic polymorphism of the ITS1 spacer of the *35S* rRNA gene using locus-specific sequencing on the Illumina MiSeq sequencer. The ITS1 region was selected as a marker due to the fact that, in some cases, rDNA isogenization does not occur completely, resulting in hybrids retaining parental sequences in their genome (for example [38]). A successful study of hybrid origin, using pyrosequencing of the ITS1 region, has previously been conducted on orchids [39].

Genomic DNA was isolated, as described in [40], and the DNA samples were transferred to the Common Use Center of the All-Russia Research Institute for Agricultural Microbiology for library preparation and sequencing.

Next-generation sequencing (NGS) primary data were processed using standard tools, such as FastQC [41], Trimmomatic [42], and Fastq-join [43]. For further data filtering, we used consensus haplotype filtering, calculation of the corresponding haplotype frequencies, sorting by decreasing haplotype frequencies (equal haplotypes are presented in the subsequent analysis as a single copy, but with the indication of a repetition frequency), the exclu-

sion of single and rare haplotypes from the analysis, reference-free alignment, and filtering of contaminants using the GenBank database (BLAST): https://www.ncbi.nlm. nih.gov/genbank/.

The NGS data on intragenomic pools of ITS1 sequences were obtained for the following herbarium specimens of *Sparganium*:

- *S. emersum* Rehm. (sample no. 55: Russia, Tver Oblast, Andreapolsky District, Parshinskoe Lake, in the outskirts of the village of Parshino and the village of Bologovo, 14.VIII.2016, collected by E. A. Belyakov and E.V. Garin, in the collection of E.A. Belyakov; sample no. 56: the Republic of Belarus, Minsk Oblast, Myadelsky District, the Stracha River, "Golubye Ozyora" Nature Reserve, 23.VI.2016, collected by E.A. Belyakov, E.V. Garin, and A.G. Lapirov, in the collection of E.A. Belyakov; sample no. 59: Russia, Nizhny Novgorod Oblast, Arzamassky District, near the village of Staraya Pustyn, in the channel between the Dolgoye and Parovoe lakes, 14.VIII.2014, collected by E.A. Belyakov, in the collection of E.A. Belyakov);
- *S. gramineum* Georgi (sample no. 61: Tyumen Oblast, Surgut District, 10 km from the village of Ugut, Ekipomykhlor (Kinyaminskoe) Lake, shallow water offshore, 28.VII.2000, collected by A.S. Baikalova, LE);
- *S. glomeratum* (Laest. ex Beurl.) Neuman (sample no. 52: Russia, Yaroslavl Oblast, Poshekhonsky District, in the outskirts of the village of Golodyayka, in a forest ditch, 10.IX.2016, collected by E.A. Belyakov, in the collection of E.A. Belyakov; sample no. 58: Russia, Vologda Oblast, Cherepovets District, Darwin Nature Reserve, in the outskirts of the village of Muravyovo, a stream, tributary of the River Chimsory, 01.VIII.1997, collected by A.A. Bobrov, IBIW);
- *S. hyperboreum* Laest. (sample no. 51: Russia, Tyumen Oblast, Tazovsky District, in the outskirts of the village of Tazovsky, in a waterlogged roadside degradation, 01.VIII.2015, collected by S.A. Nikolaenko, in the collection of E.A. Belyakov; sample no. 53: the same, plant sprouted from seeds; sample no. 57: Russia, the Republic of Komi, Izhemsky District, 76 km from the village of Izhma, crossover of B. Izhma, 2.5 km below, 02.VIII.1979, collected by Z.G. Ulle, in the collection of E.A. Belyakov);
- *S. microcarpum* (Neum.) Domin (sample no. 60: the Republic of Belarus, Minsk Oblast, Myadelsky District, the Stracha River, "Golubye Ozyora" Nature Reserve, 23.VII.2016, collected by E.A. Belyakov, E.V. Garin, and A.G. Lapirov, in the collection of E.A. Belyakov); and
- One hybrid *S. × longifolium* Turcz. ex Ledeb. (sample no. 62: Russia, Yaroslavl Oblast, Rostov District, shallow water of Lake Chashnitskoye, in the outskirts of the village of Chashnitsy, 16.VIII.2016, collected by E.A. Belyakov, in the collection of E.A. Belyakov).

In total, we obtained data for five species and one hybrid. In addition, some ITS1 sequences were taken from the GenBank database for comparative analysis [41], namely, *S. emersum* (KF265393), *S. glomeratum* (KF265386), *S. gramineum* (KF265381), *S. fluctuans* (KF265378), *S. fallax* (KF265376), *S. subglobosum* (KF265397, KF265387), *S. hyperboreum* (KF265396), *S. natans* (KF265385), and *S. stoloniferum* (KF265395) [22].

RESULTS

Our results revealed that the ribotypes of the studied samples were distributed into 10 groups (see Fig. 1). Group 1 (SAE) included KF265393, which was declared to GenBank as *S. emersum*. However, the ribotype of this sample differs from that of our sample of *S. emersum*, obtained from the Republic of Belarus, by eight nucleotide substitutions. This difference is in accordance with the opinion of Y. Ito et al. [23], that KF265393 was sequenced from a sample that should have been identified as *S. acaule* [23]. Group 2 (SGH) mainly comprised *S. glomeratum* and, to a lesser extent, *S. hyperboreum* ribotypes. The two samples of *S. glomeratum* had common ribotypes only with the samples of *S. hyperboreum*. Group 3 (SLG) included the ribotypes of *S. × longifolium* and its parent species, *S. gramineum* (KF265381). Group 4 (SFS) was represented by *S. fluctuans* (KF265378 in the GenBank database). Group 5 (SNA), the largest group, included *S. emersum*, *S. glomeratum*, *S. gramineum*, and *S. × longifolium* ribotypes belonging to the *Natantia* section. Two groups of ribotypes were represented in the Genbank sequenses only. Group 6 (SFX) was represented by *S. fallax* ribotype (KF265376), and group 7 (SSM) included *S. subglobosum* ribotype (KF265397 and KF265387). Group 8 (SNH) consisted of both *S. natans* (KF265385) and *S. hyperboreum* (KF265396) ribotypes, together with the ribotypes obtained by us from *S. hyperboreum* samples taken from Tyumen Oblast and the Republic of Komi. Group 10 (SHM) included the ribotypes characteristic of *S. hyperboreum* only. It should be noted that groups 8 and 10 were distinguished by characteristic informative nucleotide substitutions for 20 mismatched positions. Group 9 (SMH) included ribotypes of representatives of the *Erecta* section (*S. stoloniferum* (KF265395) and *S. microcarpum* from the Republic of Belarus). The presence of rare *S. hyperboreum* ribotypes was also noted here.

According to a comparative analysis of the intragenomic polymorphism of the ITS1 region, *S. hyperboreum* retained traces of hybridization processes with *S. glomeratum* in its genome. The presence of identical ribotypes in *S. glomeratum* and *S. hyperboreum* may indicate that these two species are close and are included only in rare cases of introgressive hybridization. In contrast, *S. emersum*, *S. × longifolium*, *S. gramineum*, and *S. hyperboreum* have many similar, and some identical, ribotypes.
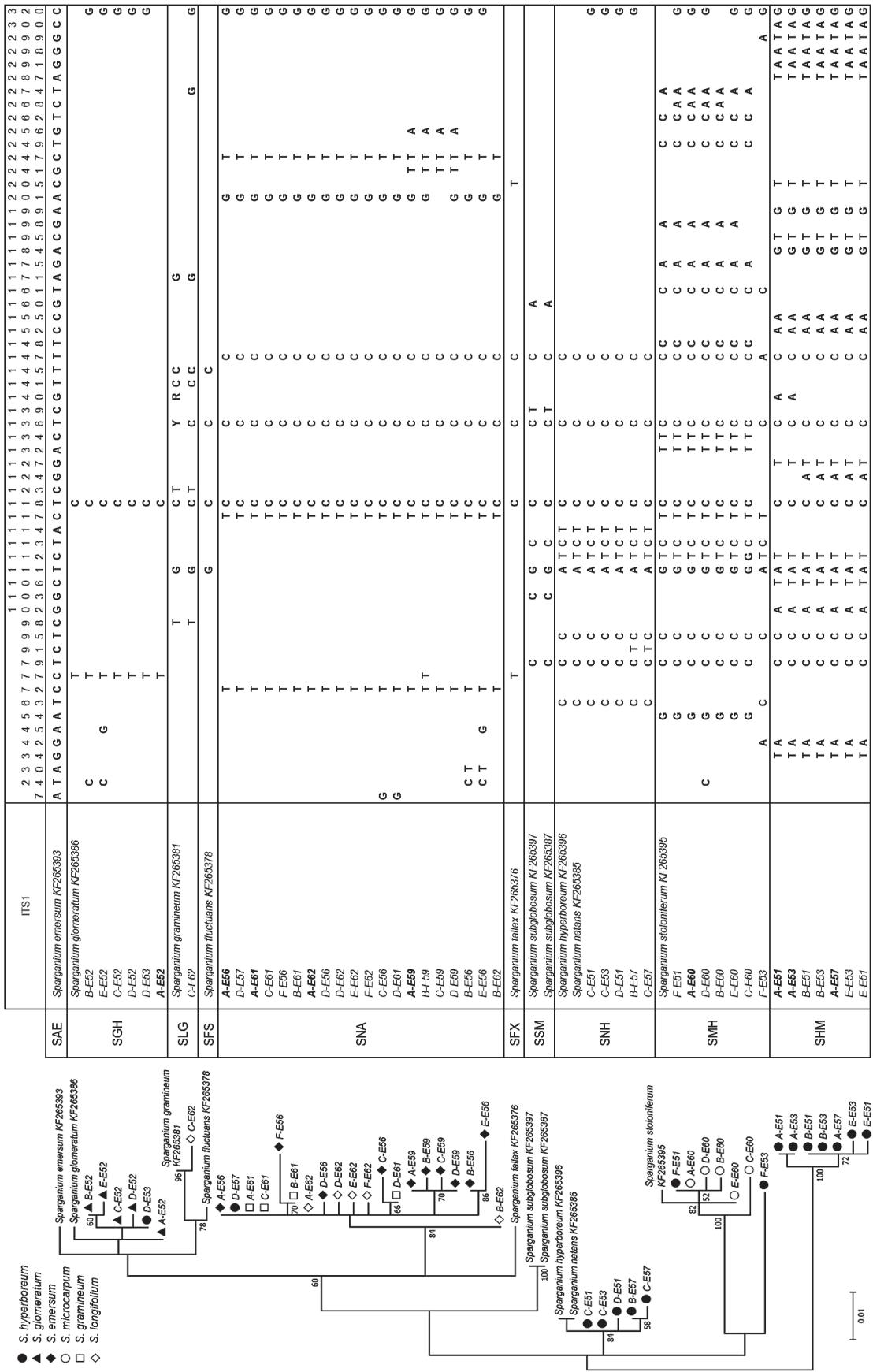
S. hyperboreum ●
S. glomeratum ◄
S. emersum ◆
S. microcarpum ○
S. gramineum □
S. longifolium ◇

Fig. Molecular phylogenetic analysis and ITS1 ribotypes within the genus *Sparganium* of the subgenus *Xanthosparganium*, according to the NGS. Molecular phylogenetic analysis was conducted applying the maximum likelihood method, based on the Tamura–Ney model [51]. The tree with the highest logarithmic probability is presented (–1211.06). The percentage of trees in which related taxa are grouped together is presented at the branch nodes. Source trees for heuristic searches were obtained based on the Neighbor–Join and BioNJ algorithms, according to the pairwise distance matrix, which is estimated by the maximum composite likelihood with the subsequent selection of the topology with the highest value of logarithmic probability. A tree was created to scale, and the length of the branches was measured by the number of substitutions per site. The analysis used 56 nucleotide sequences. In total, there were 339 positions in the final data set. An evolutionary analysis was conducted at MEGA7 [52]. The first letter (A–F) in the name of the ribotype corresponds to a decrease in the frequency of occurrence (A is the highest)

S. microcarpum is the most remote of all the studied samples. The same group includes a sample of *S. stoloniferum* from GenBank. Both species are part of the subgenus *Sparganium*. It is noted that this group also included single *S. hyperboreum* ribotypes (sample no. E51 from Tyumen Oblast).

DISCUSSION

Under unstable climatic conditions of the Oligocene and subsequent geological epochs, the degree of the bur-reeds' resistance to cooling and significant temperature fluctuations increased, which contributed to the expansion of the range of individual species. In this case, the overlapping taxon ranges and various critical situations, such as periodic climatic changes and anthropogenic transformation of biotopes, can cause the spontaneous hybridization of species [44−56]. These are hybrid plants that occupy an intermediate position by their characteristics (e. g., by the nature and structure of endocarps [47−48]). They are possibly heterotic in the first generations, highly polymorphic in genome and phenotype, therefore they had a chance to adapt to new environmental conditions.

As our study demonstrated, the presence of close and identical ribotypes in the group of *S. emersum*, *S. × longifolium*, *S. gramineum*, and *S. hyperboreum* indicates the presence of hybridization within the *Natantia* section of the *Xanthosparganium* subgenus. The conclusion regarding the hybrid origin of *S. × longifolium* is also consistent with the special aspects of its morphology, as it combines the characters of *S. emersum* and *S. gramineum* [49]. Hybridization between these species and the case of return hybrids are quite possible, since the ranges of species partially overlap. In this case, apparently, selective differentiation leads to the replacement of natural (parental) species through genetic assimilation [50]. In addition, hybridization between two highly specialized species can contribute to secondary despecialization, increasing the ecological flexibility of the hybrid. An example of this is the fact that *S. × longifolium* eventually displaces the parent species of bur-reed from the reservoir, gradually occupying their habitat [49].

Our haplotype distribution pattern is consistent with the division of *Sparganium* into two subgenera, *Sparganium* and *Xanthosparganium*. Representatives of the *Natantia* (*S. emersum*, *S. × longifolium*, and *S. gramineum*) and *Minima* (*S. hyperboreum* and *S. natans*) sections are well supported within the *Xanthosparganium* subgenus. However, the presence of common ribotypes in the studied samples of *S. hyperboreum* and *S. glomeratum* suggests the transfer of the latter from the *Natantia* section to the *Minima* section. Previously, *S. glomeratum* belonging to the *Natantia* section was primarily due to the usual similarities (size characteristics, leaves with a pronounced fin, etc.) between this plant and *S. emersum*. Note that the lectotype of the *Natantia* section is *S. angustifolium* Michx. [53]. Meanwhile, *S. glomeratum* has an intermediate position along the length of the column (at the fruitlet) between the two abovementioned sections (e. g., in *S. glomeratum*, the length of the column is 1.3 ± 0.2 mm; in *S. emersum*, it is 3.4 ± 0.6 mm; and in *S. hyperboreum*, it is 0.3 ± 0.1 mm), and upper pestillate inflorescences approximate to each other, which is often observed in representatives of the *Minima* section. Therefore, the transfer of *S. glomeratum* to the *Minima* section can be confirmed by both its molecular and morphological characteristics.

The presence of haplotypes common to all studied species of the subgenus *Xanthosparganium* may indicate the prevalence of hybridization in the subgenus. It should be noted that significant intragenomic polymorphism was found in *S. hyperboreum*, which had common haplotypes with the three other species of the *Natantia* section. The largest numbers of common haplotypes were recorded in *S. hyperboreum* and *S. glomeratum*. *S. glomeratum* itself had common haplotypes only with *S. hyperboreum*. This presentation can be explained by the introgressive hybridization between these two species. In addition, *S. hyperboreum* itself may be a species of hybrid origin; the result of the hybridization of species from different sections, *Natantia* and *Minima*.

Speciation within the genus could occur not only on the basis of hybridization but also allopathically. In this regard, confirmation of the close relationship between the North American and Eurasian pairs of species, for example, *S. fluctuans−S. gramineum*, plays an important role at the molecular level [22, 23]. There are other pairs of species that are of interest to modern researchers (e. g., *S. americanum−S. japonicum* [54]), the relatedness of which have not yet been sufficiently studied using molecular data [22]. The previously expressed point of view on the formation of taxa allopathically [22, 23] is also confirmed by the fact that, in the Eocene−Oligocene era, there was a union of the continental fragments, which led to a reduction in the number of ecological provinces; namely, Hindustan separated from Africa and joined Asia; in the Cretaceous−Paleogene era (52−60−65 megayears ago), Laurasia split and North America separated from Greenland, and Greenland from Europe, the remnants of Gondwana split, and the separation of Antarctica and Australia occurred [55]. It should be noted that confirmation of the close relationship between North American and Eurasian taxa indicates the monophyletic nature of the genus *Sparganium* [22, 56].

CONCLUSIONS

According to the NGS data, a comparative analysis of the intragenomic polymorphism of the ITS1 spacer revealed that the distribution of close haplotypes in the

studied samples corresponds to the division of the genus *Sparganium* into two subgenera (*Sparganium* and *Xanthosparganium*).

Within the *Xanthosparganium* subgenus, the *Minima* section is well supported. As for the *Natantia* section, the two studied samples of *S. glomeratum* did not exhibit the same haplotypes as the other studied species samples in this section; however, there were identical haplotypes with the two studied *S. hyperboreum* samples from the *Minima* section. Therefore, *S. glomeratum* can be relegated to the *Minima* section.

The presence of a large number of hybrid taxa, including intersectional ones, allows us to consider the genus *Sparganium* as an introgressive interspecies complex promising for molecular phylogenetic studies.

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