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5S rDNA OF *DACTYLIS GLOMERATA* (POACEAE): MOLECULAR ORGANIZATION AND TAXONOMIC APPLICATION

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Aim. *During the last decades, extensive use of molecular methods resulted in significant elucidation of evolution and taxonomy of Poaceae, but determination of boundaries between related tribes still represent an actual problem. Especially, application of variable genes like 5S rDNA is necessary for further improvement of existing knowledge about the phylogeny of Poaceae. In order to clarify the phylogenetic position of the subtribe Dactylidinae within the tribe Poeae, we describe molecular organization of 5S rDNA of Dactylis glomerata.* ***Methods****. The 5S rDNA repeated units were amplified by PCR, cloned and sequenced.* ***Results****. It was shown that only one 258 bp-long variant of 5S rDNA repeats is present in the genome of D. glomerata and the level of intragenomic similarity of the repeats is high, ranging from 96,8 to 98,8%. The repeats appear to be functionally active since they contain all known elements of external promoter for RNA polymerase III. According to sequence comparison the 5S rDNA of D. glomerata appears to be similar to that one of distantly related Aveninae species.* ***Conclusions****. The data indicate a possible hybridization between representatives of subtribes Dactylidinae and Aveninae.*

Key words*: Dactylis, 5S rDNA, intergenic spacer, molecular evolution and taxonomy, hybridization.*

Introduction. Poaceae, which comprise more than 11 000 species and dominate in many terrestrial ecosystems, is one of the biggest and economically most important monocot families [1]. During the last decades much effort has been put into elucidating the evolution and taxonomy of monocots, which resulted in significant reclassification of the group at different taxonomic levels [2, 3, 4]. Nevertheless, numerous questions still remain to be unresolved.

Determination of boundaries between related tribes and genera and reconstruction of phylogeny within Poaceae represent a complicated and interesting task. Earlier, a comparison of plastid *trnT-F* and nuclear *ITS* sequences was performed in order to determine borders between closely related tribes Aveneae and Poeae and to clarify evolutionary relationships in the groups [4]. It was confirmed the insights of Soreng and Davis [5], which indicated that Aveneae and Poeae were intermingled and should be combined in a single tribe Poeae [4, 6]. However, the results obtained applying plastid and nuclear sequences were partially controversial due to presumptive intertribal hybridization events. Hence,

further analyses using other genes are necessary in order to detect the origin of each group and the nature of potential horizontal gene transfer. Especially, it was demonstrated that nuclear loci encoding 5S rRNA (or 5S rDNA) represent an appropriate tool for such purposes [3, 7].

5S rDNA belongs to the class of head-to-tail tandem-arranged repeated sequences. Each 5S rDNA repeated unit consists of an evolutionary conserved coding region and a variable intergenic spacer (IGS). 5S rDNA is transcribed by RNA polymerase III (Pol III). The 5S rDNA repeats are organized in the genome in clusters, which are localized on one or a few chromosomes. The number of repeats per genome ranges in higher plants from hundreds to thousands [8, 9].

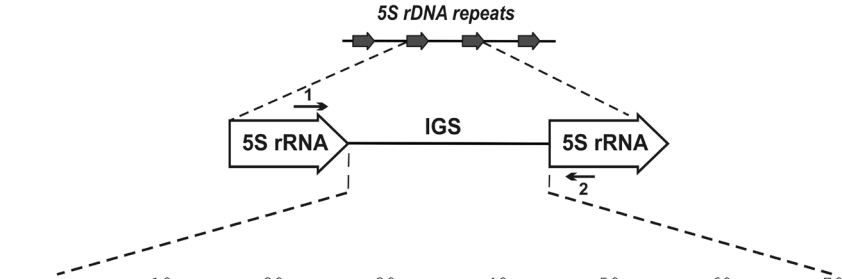
Although numerous copies of rDNA repeats co-exist in the same genome, they tend to be nearly identical in many diploid species; i.e., individual copies of the repeated elements evolve not independently, but in a concerted manner as a result of sequence homogenization or periodical elimination of some repeat variants [10]. Mutations that occur in the IGS have a predominantly neutral character, since they escape from the effects of selection. As a result, the rate of IGS evolution is relatively high and comparable to the rate of speciation [12, 13]. Due to variation of the IGS several classes of repeats with different lengths (200–900 bp) may be found within the same species. Respectively, differences in the IGS sequence can be used for discrimination of closely related species and even populations [14, 15].

Up to now the molecular organization of 5S rDNA has been studied in several genera of Poaceae representing the tribes Triticeae (*Kengyilia* [16], *Hordeum* [17] and *Triticum/Aegilops* [18, 19]) and Poeae (subtribe Aveninae: *Avena, Helictotrichon, Helictochloa, Lagurus, Tricholemma*, *Trisetum* and subtribe Phalaridinae: *Phalaris* [3, 20]). Here we describe the molecular organization of 5S rDNA of the widely distributed species *Dactylis glomerata*, a representative of the small subtribe Dactylidinae (Poeae) [6], which comprise only two genera, *Dactylis* and *Lamarckia.*

Materials and methods

The plant samples of *Dactylis glomerata* were collected on the territory of Cecyno Natural Monument near Chernivtsi city, Ukraine. Genomic DNA was isolated from leaves according to a standard protocol [21].

The complete 5S rDNA units of *Dactylis glomerata* were amplified by PCR using hot-start Maxima Taq polymerase (Thermo Fisher Scientific, Inc.) and primers 5S-14a-Not (5'-CAATGCGGCCGCGAGAGTAGTACT AGGATGCGTGAC-3') + 5S-15-Not (5'-CAT TGCGGCCGCTTAACTTCGGAGTTCTGATG GGA-3') complementary to the 5S rRNA coding region [7]. The reaction was performed in 50 µl of reaction mixture containing the following components: 0.1 µg of the genomic DNA, 1.0 U of DNA polymerase, 1 × PCR buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP and 1 µM of each primer. The amplification was carried out applying the following program: (1) initial DNA polymerase activation at 95°C, 2 min; (2) DNA denaturation at 94°C, 45 s; (3) primer annealing at 57°C, 40 s; (4) DNA synthesis at 72°C, 1 min; (5) amplification completion at 72°C for 10 min. The total number of amplification cycles was 35. For subsequent cloning, *Not*I recognition sites (GCGGCCGC, underlined above) were added at the 5' ends of both primers. The applied primers provide the amplification of the IGS and the flanking regions of the coding sequence (Fig. 1).

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          10        20        30        40        50        60        70
     ---------+---------+---------+---------+---------+---------+---------+
CON  --------TCTTTTTT-GCGTCACGCGACGAC----GTGGCACGGCT-----GG-CGTTAT---ATTTTT
DG   --------.........T.....-------------------T.-----------------..---......   76
AC   --------.........-.........-.CGC.T-----C.TT.---.A-----------...---T.A...   38
TJ   ----TCTT.T.A....-...........ACG.-----C..G.GC..A----------...---..A....   46
HA   --------..A.....-..C...T........GACCTGC......T.AATCC..C..G...ATAT.....   61
AM   --------..C.....-..............----.GA.T....TG-----.ACTT.CT.TTCT....C   52
HC   --------A.C.....-..............G-GC.......A...---GG..G......---.....A   54
LO   --------.........-........T...A.---AA.A..GCAC...---CT.T-...CGG---...A..   51
TS   --------..C.....-..............G-GC......-C...---CGA.-.....G---......   52
PC   TCTTTTTA.T......-...G..AC.......GCAA.A....G..TC---GG.A-..A..CT--...G..  153

          80        90        100       110       120       130       140
     ---------+---------+---------+---------+---------+---------+---------+
CON  TTGCGGCCCCTCGTGCTCGCCTTTTGGCCATGGTAAAA--TG-GGTTTTGC--GGTGG--TAAACTCGCG
DG   .........GC...AGA..TA......GGGC.......--..G..G.AA..GT...-------------.  131
AC   .-A...TA.....A.G.G.TT....T..TGG.A.....AA..G..A..C..GA...AAGCA.G..CAT..  107
TJ   ......T.GTGG....A.......G.....A......T--..A..A..G..AA...AACT.C.--.G.A.  112
HA   GC......G............G....CG.G....G..C--CC-..G...T--GAAC..--....T.....  124
AM   GC.G...A---....G...TG......G.........G--..T.........--A-...--GG.G....G.  112
HC   .......................C.....C......T--..-..G.....--A-...--....TCG.GT  116
LO   .A......G..............C.....A.......--..-.T......--.-...--.G..G...T.  113
TS   G..................G..C.............--..-.T.....T--.-...--....G...T.  114
PC   GC.T...A--C.....AT.T.AA.C..TG.C........--AC-.T.AGC.T--..C..--.........A  214

          150       160       170       180       190       200
     ---------+---------+---------+---------+---------+---------+------
CON  TGTAGGCACGA---GAATGAGCTGGA-GGGGCAAGCATAAAGGGA-AAACGGCGTGCAGAACATGTC
DG   C..G.......---...G........-............T..T.A.CT-C..TC.........A..      192
AC   ...G....T..---.............-..............C.TCTC.........C........      169
TJ   .......G.T.---............-.A..............G...-..................      173
HA   C..........---.........C..-...C.............-......G.C.A.......T..      185
AM   ..........---T...........-..T.GTG.........G--...................A.      172
HC   GCAT.......---.......G....-...............GG......A..............      178
LO   C.....G.A..---.......G.AA.-T...-C..G......AG-.G.............T.....      173
TS   ........A..---.....G......-........G......TG-.G...................      175
PC   .T.T..T.A..TGA...C...GC...GC...-..........T.-...AAT......T.C......      278
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Figure 1. Sequence comparison of 5S IGS of *Dactylis glomerata* and related representatives of Poaceae. Putative transcription termination site and Pol III external promoter elements are printed, respectively, in bold italic and bold underline. Abbreviations: CON – consensus sequence, DG – *Dactylis glomerata* (clone pDagl-2), AC – *Avena clauda*, TJ – *Tricholemma jahandiezii*, HA – *Helictochloa aetolica*, AM – *Avena macrostachya*, HC – *Helictotrichon convolutum*, LO – *Lagurus ovatus*, TS – *Trisetum spicatum*, PC – *Phalaris coerulescens*

The PCR products were digested with *NotI* (Fermentas, Lithuania), ligated into pBluescript KS and transformed into *E. coli* strain XL-blue. The clones that contained recombinant plasmids were identified by the blue-white colony selection method and validated by restriction mapping. Plasmid DNA isolation, restriction mapping and other standard procedures were carried as described [22]. Inserts of selected clones were sequenced using the Big Dye Terminator Cycle Sequencing Kit

on ABI Prism 310 sequencer (PE Applied Biosystems, USA). The sequences obtained appeared in the GenBank nucleotide database under the accession nos. KF743553-KF743556, KF496941.

Database search was performed in GenBank at the National Center of Biotechnology Information server (http://www.ncbi.nlm.nih.gov) with Entrez, BLAST [23]. The following 5S rDNA sequences were used for dendrogram construction: *Avena clauda,* GenBank Acc. No DQ823492; *A. macrostachya,* AJ390215; *Helictochloa pratensis*, AJ390197; *Helictochloa aetolica,* AJ390137; *Helictotrichon convolutum*, AJ390078; *Lagurus ovatus,* AJ390222; *Phalaris coerulescens,* Y09573; *Tricholemma jahandiezii,* AJ390218; *Trisetum spicatum,* AJ390233 [3, 20]. Sequence alignment was obtained by Clustal W method [24] applying MEGALIGN software [25].

Maximum likelihood (ML) and neighbor-joining (NJ) minimal distance dendrograms were generated with PAUP, version 4.0b10 [26]. The ML dendrograms were produced using heuristic search by "random" stepwise addition and tree bisection-reconnection (TBR) branch swapping. Zero-length branches were collapsed. Gaps were treated as "missing". To produce the NJ dendrograms, the distance measure option was specified as (i) uncorrected (or p-distance), (ii) Felsenstein [27] or (iii) Tamura and Nei [28]. Statistical node support was established by non-parametric bootstrapping (BS) [29] under NJ and ML with PAUP. Bootstrap values were obtained from 1000 replicates.

Results and discussion

Separation of PCR products using gel electrophoresis showed that only one type of 5S rDNA, which is 260 bp long, is present in the genome of *D. glomerata*. The PCR products were cloned into the bacterial vector pBluescript KS.

Twenty colonies bearing the recombinant plasmid were identified by blue-white colony selection and used for further plasmid DNA extraction. Digestion with the restriction endonuclease *Not*I and further electrophoretic analysis showed two DNA fragments. The bigger fragment always had the length of 2900 bp, while the smaller one was 260 bp long, which corresponded to the size of the linearized vector pBluescript KS and the 5S rDNA insert of *D. glomerata*, respectively. In total, twenty plasmids carrying the insert were identified, of which five were sequenced.

Sequence analysis showed that the five clones contain inserts that are flanked by sequences of primers used for PCR. Using our novel sequences for BLAST search in Genbank [23], we found that they demonstrate the highest level of similarity to 5S rDNA of several species of subtribe Aveninae [3]. Therefore, in order to clarify the phylogenetic position of *D. glomerata* in more detail, we have selected 5S rDNA sequences of the most divergent representatives of the group and used them for further analysis (Fig. 1, Table).

Comparing the 5S rDNA sequences of *D. glomerata* with the sequences of other species we have determined the border between the 5S coding region and IGS. It was found that the total length of the coding region, which flanks the IGS of our clones (including the primers 5S-14a-Not, 5S-15-Not), was 113 bp. Taking into account the fact that 6 bp of the coding region remain unamplified using our primers, we can suggest that the total size of the 5S rDNA of *D. glomerata* is 119 bp, which is equal to the coding region size in other species of subtribe Aveninae [3]. The length of IGS amounts to 139 bp in all five sequenced clones. Consequently, only one 258 bp-long variant of 5S rDNA repeats is present in

Table. Similarity (in %) of 5S IGS of *Dactylis glomerata* and related representatives of Poaceae.

Species name	*D. glomerata*	*A. clauda*	*T. jahandiezii*	*H. aetolica*	*A. macrostachya*	*H. convolutum*	*L. ovatus*	*H. convolutum*	*Ph. coerulescens*
Dactylis glomerata	100	67.0	63.6	52.9	57.3	58.7	56.3	59.7	46.1
Avena clauda		100	68.4	53.4	60.2	61.7	58.7	63.6	46.6
Tricholemma jahandiezii			100	57.3	61.7	68.0	64.6	68.0	52.9
Helictochloa aetolica				100	67.5	71.8	64.1	72.8	54.9
Avena macrostachya					100	70.9	67.5	71.4	55.8
Helictotrichon convolutum						100	74.8	84.0	60.2
Lagurus ovatus							100	83.0	59.7
Trisetum spicatum								100	64.6
Phalaris coerulescens									100

the genome of *D. glomerata*. Earlier, it was demonstrated that the length of 5S rDNA repeats in representatives of tribe Poeae ranges from 285 to 329 bp [3]. Hence, *D. glomerata* possesses the shortest 5S rDNA repeat among Poeae.

Sequence alignment using the ClustalW method showed a high similarity among the 5S rDNA repeats of *D. glomerata*: from 96,8 to 98,8%. Comparing to the consensus sequence the individual clones contain two (pDagl-2 and -4), three (pDagl-3 and -5) or five (pDagl-1) transitions, which probably emerged due to the deamination of 5-methyl-C.

In contrast to the high level of intragenomic sequence homology, the similarity between *D. glomerata* and several representatives of the tribe Poeae ranges from moderate (67,0%) to low (46,1%). Among the analysed species the lowest level of similarity was found between *Ph. coerulescens* (subtribe Phalaridinae [6]) and other species studied. Respectively, *Ph. coerulescens* was taken as out-group species during the construction of phylogenetic dendrograms (Fig. 2). Application of boot-strap analysis demonstrated that at the ML-cladogram (i) *A. clauda* is a sister taxon to *D. glomerata* and that (ii) *D. glomerata* and the majority of representatives of subtribe Aveninae – exept *Lagurus ovatus* and *Trisetum spicatum* – are combined in a clade with a weak boot-strap support of 64. The similarity between *D. glomerata* and *A. clauda* is further supported at the NJ-cladogram, where these two species are placed in a clade with a moderate boot-strap support of 76. In contrast to our data, for plastid *trnT*-F and nuclear *ITS* sequences Quintanar et al. [4] found that *Dactylis* represent an out-group to all representatives of subtribe Aveninae. The unusual similarity between 5S rDNA of *D. glomerata* and Aveninae species could be explained by intertribial hybridization, which resulted in transfer of 5S locus from subtribe Aveninae to *Dactylis* species. However, this hypothesis should be additionally tested.

It was demonstrated earlier that the 5S IGS in plants can be divided into three different parts: the 3' and 5' flanking sequences (FS), located respectively downstream and upstream of 5S rRNA coding region, and a variable region (VR) in

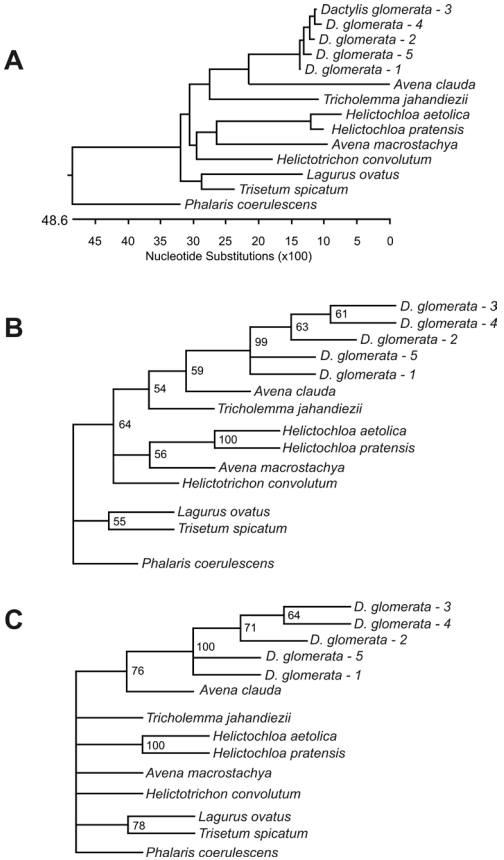

Figure 2. Dendrograms derived from 5S IGS sequence comparison of *Dactylis glomerata* (five clones), and related Poaceae species. A – the best distance tree generated by Clustal W; B and C – majority-rule (50%) consensus trees obtained by maximum parsimony heuristic search with gaps treated as "missing", "random" stepwise addition, and tree bisection-reconnection branch swapping (MP-cladogram) or by neighbor-joining search with uncorrected distance measure (NJ-cladogram). Values near the nodes indicate bootstrap support higher than 50% for the corresponding branching point

the middle of IGS [3, 7, 8]. Our comparative sequence analysis (Fig. 1) showed that the 57 bp-long fragment of the IGS immediately upstream of the coding region demonstrates an increased level of similarity among the species of tribe Poeae. Similar observation was made previously for 5S IGS of genus *Solanum*, where the increased level of similarity was taken as criterion by definition of 3' and 5' FS [7]. Remarkably, the length of 5' FS in *Solanum* amounts to 56-59 bp, i.e., the 5' FS appears to have the same size in distantly related families like Solanaceae (dicots) and Poaceae (monocots).

The reason for the relative decrease of base substitution rate and conservation of the 5' FS length appears to be the functional importance of this region since elements of external promoter involved in Pol III transcription initiation are located here. In Arabidopsis, such signals include the TATATA hexanucleotide motif localized at position –28 bp from the 5' end of the coding region, as well as the GC and C nucleotides at positions – 12 and –1 bp, respectively [11, 30]. Similar AT-rich motifs, TATATA and TTAATA, were detected in 5S IGS of other dicots, *Rosa* and *Solanum* at positions –28 and –29, respectively. Also, the GC nucleotides at position –12 were found to be highly conserved. However, C at position –1 bp is substituted by T in *Solanum* [31, 32]. Comparative analysis of the 5' FS (Fig. 1) revealed that in all species of tribe Poeae the GC and C nucleotides are present at the same positions, –12 and –1 bp. In contrast, only tetranucleotide AT-rich motif, ATAT surrounded by GC bases was found at position –28 bp (see Fig. 1).

Formerly it was also shown that in Aveneae the 5' FS ends with an AACATGTC motif, and the CATGTC sequence is conserved for all pooid grass species [3]. However, in *D. glomerata* the AACATGTC sequence was found only in one (pDagl-1) of five sequenced clones, whereas in the other four clones the AACATGAC sequence is present, indicating that the T nucleotide at position –2 bp appears to be not critical for transcription.

A specific feature of the 3' FS in different plants is a T-rich oligonucleotide sequence that functions as a terminator for Pol III [11,

30, 33]. In Aveneae the termination sequence appears to be CTTTTT(ATTTT), which is followed by a GC-rich region of different lengths, from 12 to 28 bp [3]. Our novel data show that in the 3' FS of *D. glomerata* the conserved T-rich termination motif is TCTTTTTTT followed by GCGTC sequence, whereas the main portion of the GC-rich region is deleted (Fig. 1). A similar motif, TATTTTTTTGCGGC, is present further downstream in the IGS of *D. glomerata*. Interestingly, this sequence is not totally conserved in other representatives of Aveneae, but different combinations of AT-rich and GC-rich motifs of similar length are present in the IGS of this species (Fig. 1).

Thus, all known "standard" signals involved in 5S rDNA transcription initiation and termination are present in the obtained clones. Also, the sequenced fragments of coding region contain only few mutations. Taking together, the data show that our novel sequences probably represent "normal" functionally active copies of the 5S rDNA of *D. glomerata*.

Conclusions

D. glomerata possesses 258 bp-long 5S rDNA repeats, which is the shortest variant among representatives of tribe Poeae. According to IGS sequence comparison the 5S rDNA of *D. glomerata* appears to be similar to that one of distantly related Aveninae species indicating a possible intertribial hybridization.

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5S рДНК *DACTYLIS GLOMERATA* (POACEAE): МОЛЕКУЛЯРНА ОРГАНІЗАЦІЯ ТА ЗАСТОСУВАННЯ У СИСТЕМАТИЦІ

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Мета. Використання молекулярних методів протягом останніх десятиліть привело до суттєвого прояснення еволюції та таксономії Poaceae, проте визначення границь між спорідненими трибами все ще залишається актуальною проблемою. Зокрема, використання варіабельних генів, таких як 5S рДНК, є необхідним для подальшого поглиблення існуючих знань стосовно філогенії Poaceae. Для з'ясування філогенетичного положення субтриби Dactylidinae в межах триби Poeae ми вивчали молекулярну організацію 5S рДНК *Dactylis glomerata*. **Методи.** Повторювані одиниці 5S рДНК було ампліфіковано за допомогою ПЛР, клоновано і сиквеновано. **Результати.** Було показано, що в геномі D. *glomerata* присутній тільки один варіант повтору 5S рДНК довжиною 258 нп, причому рівень внутрішньо-

геномної подібності повторів виявився високим: від 96,8 до 98,8%. Ці повтори мають бути функціонально активними, оскільки вони містять всі відомі елементи зовнішнього промотора для РНК-полімерази III. Порівняння послідовностей 5S рДНК *D. glomerata* показало, що вони є подібними до послідовностей віддалених видів підтриби Aveninae. **Висновки.** Отримані дані вказують на можливу гібридизацію між представниками підтриб Dactylidinae та Aveninae.

Ключові слова: *Dactylis,* 5S рДНК, міжгенний спейсер, молекулярна еволюція і систематика, гібридизація.

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Цель. Использование молекулярных методов в течение последних десятилетий привело к существенному прояснению эволюции и таксономии Poaceae, однако определение границ между родственными трибами все еще остается актуальной проблемой. В частности, использование вариабельных генов, например, 5S рДНК, является необходимым для дальнейшего углубления существующих знаний относительно филогении Poaceae. Для выяснения филогенетического положения субтрибы Dactylidinae в пределах трибы Poeae мы изучали молекулярную организацию 5S рДНК *Dactylis glomerata*. **Методы.** Повторяющиеся участки 5S рДНК были амплифицированы при помощи ПЦР, клонированы и секвенированы. **Результаты.** Было показано, что в геноме *D. glomerata* присутствует только один вариант повторов 5S рДНК длиной 258 нп, причем уровень внутригеномного сходства повторов высокий и составляет от 96,8 до 98,8 %. Повторы должны быть функционально активными, так как они содержат все известные элементы внешнего промотора для РНК-полимеразы III. Сравнение последовательностей 5S рДНК *D. glomerata* показало, что они сходны с последовательностями отдаленных видов подтрибы Aveninae. **Выводы.** Полученные данные указывают на возможную гибридизацию между представителями подтриб Dactylidinae и Aveninae.

Ключевые слова: *Dactylis*, 5S рДНК, межгенный спейсер, молекулярная эволюция и систематика, гибридизация.