Russian Journal of Genetics, Vol. 41, No. 5, 2005, pp. 518–528. Translated from Genetika, Vol. 41, No. 5, 2005, pp. 646–656.

PLANT GENETICS

Genomic Configuration of the Autotetraploid Oat Species *Avena macrostachya* Inferred from Comparative Analysis of ITS1 and ITS2 Sequences: on the Oat Karyotype Evolution during the Early Events of the *Avena* Species Divergence

A. V. Rodionov[1], N. B. Tyupa[1], E. S. Kim[1], E. M. Machs[1], and I. G. Loskutov[2]

[1] *Komarov Botanical Institute, Russian Academy of Sciences, St. Petersburg, 197376 Russia; e-mail: biocyt@rednet.ru*
[2] *Vavilov Institute for Plant Industry, Russian Academy of Agricultural Science, St. Petersburg, 190000 Russia*

Received April 30, 2004

Abstract—To examine the genomic configuration of *Avena macrostachya*, internal transcribed spacers, ITS1 and ITS2, as well as nuclear 5.8S rRNA genes from three oat species with AsAs karyotype (*A. wiestii, A. hirtula*, and *A. atlantica*), and those from *A. longiglumis* (AlAl), *A. canariensis* (AcAc), *A. ventricosa* (CvCv), *A. pilosa*, and *A. clauda* (CpCp) were sequenced. All species of the genus *Avena* examined represented a monophyletic group (bootstrap index = 98), within which two branches, i.e., species with A- and C-genomes, were distinguished (bootstrap indices = 100). The subject of our study, *A. macrostachya*, albeit belonging to the phylogenetic branch of C-genome oat species (karyotype with submetacentic and subacrocentric chromosomes), has preserved an isobrachyal karyotype, (i.e., that containing metacentric chromosomes), probably typical of the common *Avena* ancestor. It was suggested to classify the *A. macrostachya* genome as a specific form of C-genome, Cm-genome. Among the species from other genera studied, *Arrhenatherum elatius* was found to be the closest to *Avena* in ITS1 and ITS sequence. Phylogenetic relationships between *Avena* and *Helictotrichon* remain intriguingly uncertain. The HPR389153 sequence from *H. pratense* genome was closest to the ITS1 sequences specific to the *Avena* A-genomes (*p*-distance = 0.0237), while the *p*-distance between this sequence and the ITS1 of *A. macrostachya* reached 0.1221. On the other hand, HAD389117 from *H. adsurgens* was close to the ITS1 specific to *Avena* C-genomes (*p*-distance = 0.0189), while its differences from the A-genome specific ITS1 sequences reached 0.1221. It seems likely that the appearance of highly polyploid ($2n = 12x–21x$) species of *H. pratense* and *H. adsurgens* could be associated with interspecific hybridization involving Mediterranean oat species carrying A- and C-genomes. A hypothesis on the pathways of *Avena* chromosomes evolution during the early events the oat species divergence is proposed.

INTRODUCTION

Avena macrostachya Bal. ex Coss. et Dur., an endemic grass of the Atlas Mountains (northeast of Algeria) [1, 2], attracts the attention of breeders and geneticists due to its resistance to barley yellow dwarf virus (BYDV), to crown and stem rust, halo blight, to the attacks of aphids and powdery mildew, and also due to its increased winter hardiness [2–6]. *Avena macrostachya* is the only perennial, cross-pollinating plant among all oat and wild oat species (genus *Avena*) [2–12]. These features, which for *Avena* are usually considered archaic [3, 8–13], are typical of the genus *Helictotrichon* related to *Avena* [12, 13]. Karyotype of *A. macrostachya* also resembles that of some *Helictotrichon* species [12]. Based on these and other data, *A. macrostachya* is sometimes attributed to the genus *Helictotrichon* under the name of *Helictotrichon macrostachyum* (Bal. ex Coss. et Dur.) Henrard [2]. However, cladistic analysis carried out by Baum [8, 9], which involved more than 100 variations of 29 morphological characters of *Avena* and related genera, showed that *A. macrostachya* was an archaic member of the genus *Avena*. It was suggested to assign *A. macrostachya* to a special section of the genus *Avena*, named *Avenotrichon* (Holub) Baum, or *Avenastrum* Koch, placing this section near the root of the *Avena* phylogenetic tree [6, 9].

Genomic, or more correctly, since it refers to a polyploid species, subgenomic configuration of *A. macrostachya* is unclear (see [14]). According to the chromosome morphology, in *Avena* four genome types are usually distinguished, A, B, C, and D [6, 11, 15–17]. The A- and C-genomes in diploid state are found in the wild oat species, and, in addition, they form the karyotypes of hexaploids (AACCDD). To date, the B-genome has been found only in the karyotypes of three tetraploid species, *A. barbata*, *A. abyssinica*, and *A. vaviloviana* (AABB). It is very close to the A-genome [18, 19]. The D-genome is found only in hexaploids. According

1022-7954/05/4105-0518

Table 1. List of species examined, their genomic configuration, and origin

No.	Species	Genomic configuration	Number of chromosomes (2*n*) in the karyotype	Accession number in the catalog of VIBP and the initial sampling locality
1	*Avena canariensis* Baum	AcAc	14	K-1916, Spain
2	*A. longiglumis* Dur.	AlAl	14	K-1811, Morocco
3	*A. atlantica* Baum et Fedak	AsAs	14	K-1894, Morocco
4	*A. hirtula* Lag.	AsAs	14	K-2, Israel; K-2034, Tunisia
5	*A. wiestii* Steud.	AsAs	14	K-95, Israel
6	*A. clauda* Dur.	CpCp	14	K-267, Azerbaijan
7	*A. pilosa* M.B. (syn. *eriantha*)	CpCp	14	K-210, Azerbaijan
8	*A. ventricosa* Bul.	CvCv	14	K-1909, Cyprus
9	*A. macrostachya* Bal. ex Coss. et Dur.	?	28	K-1856, Algeria
10	*A. sativa* L.	AACCDD	42	K-11840, Germany, Borrus variety
11	*A. sterilis* L.	AACCDD	42	K-171, United States
12	*Agrostis capillaris* L.		28	Lamin-Suo, Russia, Leningrad oblast
13	*Colpodium versicolor* (Stev.) Schmalh.		4	Teberda, Russia
14	*Triticum aestivum* L.		42	Banatka cultivar*

* Kindly provided by P.P. Strel'chenko and O.P. Mitrofanova.

to GISH data, it is similar to the A-genomes, but differs from C-genomes [16, 20]. On the one hand, based on the fact that all chromosomes of *A. macrostchya* are meta- and submetacentrics, this species is considered to be an autotetraploid with the A-genome [6, 17]. On the other hand, C-banding pattern of its chromosomes is remarkably different from the C-banding of A-genome chromosome sets [21, 22].

Comparative analysis of *Avena* chromosomes using GISH showed that subgenomes of *A. macrostachya* were closer to the genomes of diploid *A. pilosa* (= *A. eriantha* Dur.) with the genomic formula of CpCp than to *A. strigosa* with the genomic formula of AsAs [16].

To examine the genomic configuration of *A. macrostachya*, and to determine the position of this species on the phylogenetic tree relative to the diploid *Avena species* with the A- and C-genomes, internal transcribed spacers, ITS1 and ITS2, as well as nuclear 5.8S rRNA genes from *A. macrostachya*, eight diploid, and two tetraploid *Avena* species were sequenced. The sequence data obtained were compared with ITS1 and ITS2 sequences from the representatives of Aveneae and Poeae tribes from the NCBI database.

Genomes of the *Avena* species examined were of the A-type, specifically, AsAs (*A. wiestii*, *A. hirtula*, and *A. atlantica*), AlAl (*A. longiglumis*), AcAc (*A. canariensis*), and of the C-type (CvCv in *A ventricosa* and CpCp in *A. pilosa* and *A. clauda*). The choice of the DNA region for sequencing was based on the on the fact that ITS sequences, due to their evolutionary variability, proved to be informative in gene systematic studies at the interspecific level [23–27], and also in analyses of the phylogenetic relationships between genera, families, and higher taxa [28, 29]. At the same time, these genomic regions have been sequenced only in *A. longiglumis* (karyotype AlAl) [30], *A. sativa* (AACCDD) [26], and partly (ITS1, 5.8 rDNA, and a fragment of ITS2) in *A. barbata* (AABB) [31].

MATERIALS AND METHODS

The nuclear genome ITS sequences from eleven *Avena* species, one *Agrostis* species, one *Colpodium* species, and one *Triticum* species (outer group) were amplified and sequenced. A list of the species studied is presented in Table 1.

Genomic DNA was isolated from the leaves using the method described in [32] with modifications. Dried (37°C) leaves were grinded in a mortar with Al_2O_3, and 0.5 ml of the homogenate obtained were placed into microcentrifuge tube, carefully mixed with 500 μl of TES buffer (100 mM Tris–HCl, pH 8.0; 10 mM EDTA; 2% SDS) and 50 μl of β-mercaptoethanol, and incubated at 60°C for 60 min with periodic agitation. Then, 140 μl 15 M NaCl and 70 μl of 10% CTAB buffer (10% CTAB; 50 mM Tris–HCl, pH 8.0; 0.7 mM EDTA) were added and the mixture was incubated at 65°C for 10 min. The lysate was then mixed with an equal volume of isoamyl alcohol : chlorophorm (1 : 24) solution and incubated at 22°C for 60 min with constant agitation. After 10-min centrifugation (10 000 rpm) using the Eppendorf centrifuge, the DNA-containing phase was collected and placed in a separate tube. For DNA precipitation, 0.1 volume of 3 M sodium acetate

(pH 5.5) and 1 volume of cold (–20°C) isopropyl alcohol were added, and the mixtures were incubated at –20°C for 2 to 24 h. DNA pellets were obtained by centrifugation (10 000 rpm) for 10 min. The supernatants were discharged, and DNA pellets were dried. DNA specimens were dissolved in 50 μl of deionized water, or TE buffer (pH 8.0) (10 mM Tris–HCl; 1 mM EDTA).

Polymerase chain reaction (PCR) was performed with the primers ITS1F (5'-cttggtcatttagaggaagtaa-3') [33] and ITS4(5'-tcctccgcttattgatatgc-3') [34]. The amplification conditions consisted of one cycle of 10 min at 97°C; 35 cycles of 1 min at 94°C; 1 min at 48°C, 1 min at 72°C, and 10 min at 72°C. Sequencing was performed according to Sanger *et al.* [35] using fluorescent dye-labeled terminator nucleotide analogues technology. Both strands of each DNA fragment were sequenced. Fluorescently labeled 2',3'-ddNTP used were from the Big Dye Terminator Kit v.2.0 (Perkin–Elmer Life Sciences, United States). Sequencing PCR was carried out using an automated sequencer ABI Prizm 377 (Applied Biosystems, United States) at the Kheliks NPO (St. Petersburg, Russia). The sequences determined were deposited with NCBI database (www.ncbi.nlm.nih.gov).

Sequences were compared using DAMBE software package [36]. To establish phylogenetic relationships, in addition to the sequences determined in the present study, comparisons were made for the sequences of *Avena barbata* (AF494348 [31]), *Alopecurus vaginatus* (AVA96923, AVA96922, AVA96921, and AVA96920 [26]), *Arrhenatherum elatius* (AEL96883 [26]), *Beckmannia eruciformis* (BER389163 [37]), *Brachypodium distachyon* (BRH58SITS [38]), *Bromus catharticus* (BCAITS1 and BCAITS2 [25]), *Calamagrostis epigejos* (CEP306448 and CEP306449 [39]), *Deschampsia alpina* (AY237845 [40]), *D. antarctica* (AF521900 [41]), *D. caespitosa* (AF532929 [42]), *D. christophersenii* (AF486267 [43]), *D. flexuosa* (AY237846 [40]), *D. mejlandii* (AF486268 [43]), *D. sukatschewii* subsp. *borealis* (AY237844 [40]), *Helictotrichon adsurgens* (HAD389119, HAD389117, HAD389115, HAD389113, and HAD389111 [37]), *H. pratense* (HPR389153, HPR389155, HPR389151, HPR389149, HPR96860, and HPR96858 [37]), *Holcus lanatus* (HLA96918, HLA96917, HLA96916, HLA96915, and HLA96914 [26]), *Koeleria pyramidata* (KPY96913, KPY96912, KPY96911, and KPY96910 [26]), *K. digorica* (KDI96909, KDI96908, KDI96907, KDI96906, KDI96905, and KDI96904 [26]), *Lagurus ovatus* (LOV389165 [37]), *Poa trivialis* (AJ240161 [44]), *Poa pratensis* (AF171183 [45]), *Pseudarrhenatherum longifolium* (PLO389161, PLO389157, and PLO389159 [37]), *Trisetum turcicum* (Z96900, TTU96901, TTU96903, and TTU96902 [26]), *T. flavescens* (TFL96899, TFL96898, TFL96897, and TFL96896 [26]), *T. spicatum* subsp. *ovatipaniculatum* (TSP389167 [37]), *Zingeria biebersteiniana* (ZBI428836 [46]), *Z. trichopoda* (AJ428835 [46]).

Statistical testing of the phylogenetic trees was performed using bootstrap analysis [47, 48]. Predictions on the ITS1, ITS2 and 5.8S rRNA secondary structures were made using the GArna software program designed by Titov *et al.* [49].

RESULTS AND DISCUSSION

A set of DNA fragments sequenced in the present study was comprised of the 18S rDNA fragment, ITS1, 5.8S rDNA, ITS2, and 26S rDNA fragment. The 5.8S rRNA gene sequences in the oat species examined were identical and had the size of 163 bp. They contained the flowered plants-typical conservative motifs considered to be essential for the ribosome functioning [50, 51], and represented by the 5-bp unpaired motif from the A-loop of 5.8S rRNA, 5'-AAGAA-3', [50], 14-bp motif 5'GAATTGCAGAATC-3' [51], and the 5.8S rRNA terminal region, forming a hairpin with 5'-terminal region of 26S rRNA gene [50–52], (Fig. 1). The sizes of ITS1 and ITS2 sequences appeared to be similar to those in the members of other Poaceae species [23–27, 29–31, 37–44] and constituted 219 to 220 bp for ITS1 and 213 to 215 bp for ITS2, respectively. The secondary structure of these genomic regions (ITS2 in particular) was found to be substantially more conservative, compared to their nucleotide sequences (compare, for example, Fig. 1 and [52]).

Comparison of the *Avena* ITS1 and ITS2 sequences with those of the other genera of the tribe Aveneae (Table 2) provided deduction of the ITS sequence of an *Avena* common ancestor (Fig. 1). Comparison of ITS sequences from *Avena* with those in other sequenced Aveneae showed that nucleotide substitutions and indels (deletions and insertions) were present in 118 positions. They are shown in Table 2.

Comparison of the ITS1 and ITS2 sequences from the modern *Avena* species showed that two groups of oat species, those with A-genomes (including polyploids) and those with C-genomes (including *A. macrostachya*) remarkably differ from one another (Table 2). The dG + dC content in the ITS of *A. macrostachya* was 62.5%, while in the C-genome oats, the value of this index varied from 60.8 to 61.7%, and in other A-genome species, it ranged from 59.6 to 60.2%. The ITS sequences of the *Avena* oats with A-genomes examined differed from the ancestral sequence by ten transversions, sixteen transitions (nine of which were C ⟶ T transitions), and three indels. The number of changes in the species with C-genome and *A. macrostachya* was lower; only five to six transversions, ten transitions, and one to three deletions typical of this group were described. Between *A. macrostachya* and C-genome *Avena* species, far more synapomorphies than differences were observed. The latter, however, were also detected. These were three transversions and four transitions typical exclusively of *A. macrostachya*, along with two transversions, four transitions, and two indels, typical of diploid *Avena* species with Cp- and Cv-genomes.

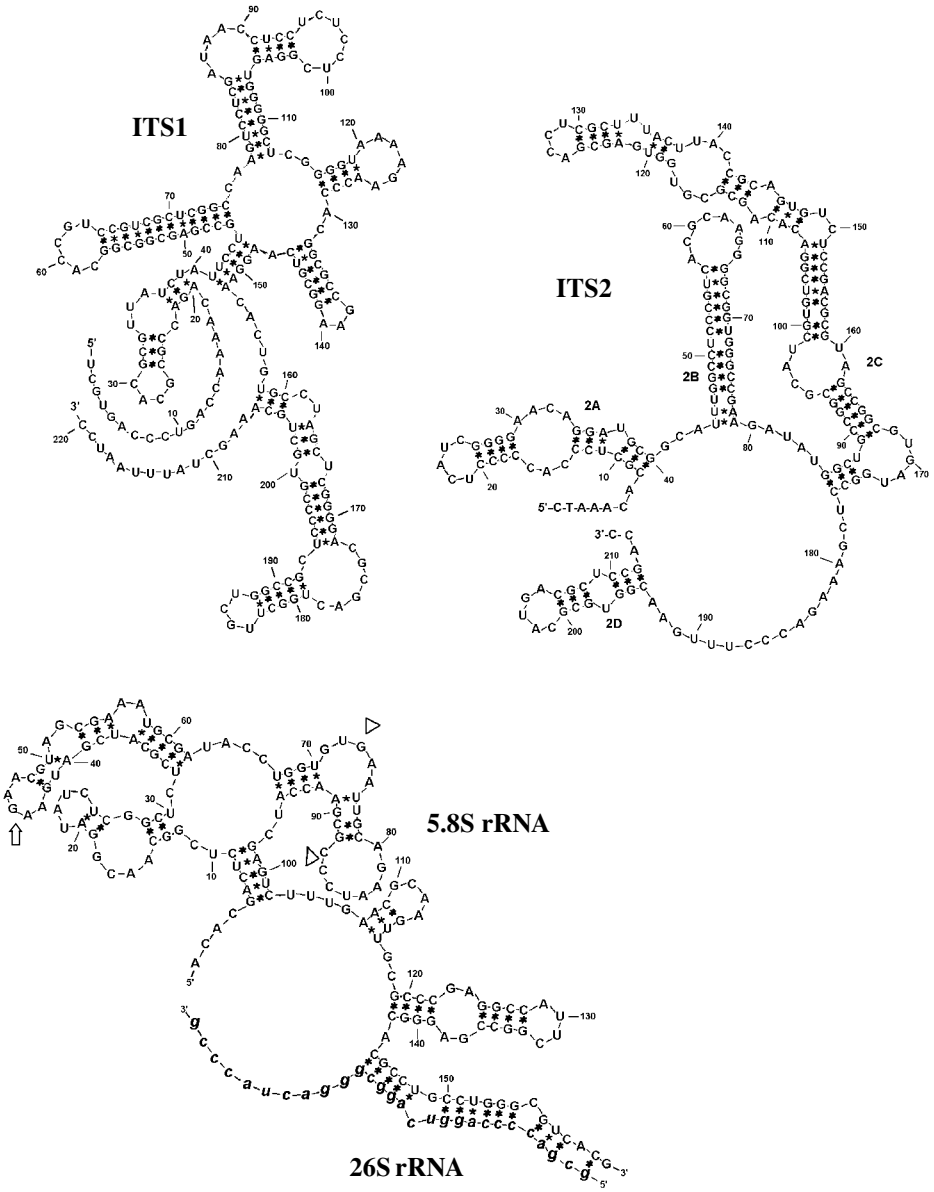

Fig. 1. The ancestral sequences of ITS1, 5.8S rDNA, and ITS2 of *Avena* based on comparative analysis of these sequences in Aveneae. The secondary RNA structure was constructed using the GArna program [49]. In the ITS2 diagram, evolutionarily conserved "hairpins" are designated as 2A–2D. In 5.8S rRNA, arrows mark 5-nucleotide unpaired motif 5'-AAGAA-3' [50] and 14-nucleotide motif 5'-GAATTGCAGAATC-3' [51], which is conserved in flowered plants. The 3'-terminal region of 5.8S rRNA is complementary to the 5' end of 26S rRNA (shown in lowercase letters).

All *Avena* representatives examined were characterized by the presence of mutations, which distinguished them from the other Aveneae and were represented by three transitions, seven transversions, and one insertion (Table 2).

Using DAMBE software package, the proportion of substitutions in ITS1 and ITS2, distinguishing the species examined at the pairwise comparisons (the so-called *p*-distance), were calculated [48]. Comparison of the ITS from *A. macrostachya* with the ITS of any of the oat species with C-genome showed that the *p*-distance between them ranged from 3.2 to 4.2%, while the *p*-distance between *A. macrostachya* and the species with A-genomes was twice higher, ranging from 8.75 (*A. hirtula*) to 9.4% (*A. atlantica*) (Table 3). The most probable tree topology uniting species from the tribes Poeae and Aveneae was calculated using the Fitch–Margoliash [36, 53], and the maximum parsimony methods [36, 48]. The tree is presented in Fig. 2.

Our findings showed that all *Avena* species examined, both with A- and C-genomes, represented a monophyletic group (bootstrap index = 98). In turn, A-genome species (including polyploids) and C-genome species formed separate clades within the genus *Avena*

Table 2. Varyable positions of nucleotides ITS1 and ITS2 in members of tribes Aveneae, Phleeae, and Poeae

Tribe	Genome	Species, genus	Nucleotide positions
			ITS1
Triticeae		*T. aestivum*	t c c c t c t –c–c a c c c c c g–t c c c c c a g c c c t a g g a c c g g c a g t a c c c c g t a
Brachypodiae		*Brachypodium*	t c c c t c–a c–t g c t c c c g–g c c a c c a g c c c a g g a c c g g c g g t t c c c c g t t
Phleeae		*Beckmannia*	. t . t –t . . . –c . . c t . t . a . t g . . . . a . . . . . . a t . . . . . a . . . . . t
		Alopecurus	. . t t –a . . . –c . . c . . t . a . t . . . . . c . . . . . t . t . . . . . . a . . . . . t
Poeae		*Colpodium*	. t t t . . . . . –t . . t . . t . a . . . . . t . . . . . a . . . . . a . . . . . t
		Poa	. t t t . . . . . c . . a . . t . a . a . . g t . . c . . . . . t . . a –. . . a . . . . . t
Aveneae	(?)	*Z. biebersteiniana*	. t . . . . . –t . . t t . t . a . . . . . t –. . . . . a . . . . . t
	(?)	*Z. trichopoda*	. t . t . . . . . –t . . t t . t . a . . . . . t . . . . . a . . . . . t t
		Pseudarrhenatherum	. . . . –. c . . . c . . c . . . . . t g . . . c . . . . . g . . . . g . . . . . c a
		Deschampsia	. t . . –a . . . –c . . c . . . . . t . . . . . t . t . . . . . g . . . . . t
		Holcus	. . . . –t g . . . c a . t . . . . . t . . . . . t . t . . . . . g . . . . . t
		Koeleria	. . . . –. c . . . c . –c . –. . . . . c . . . . . g t . . . . . g . a . . . c t
		Trisetum	. . . . –. c . . . c . –c . –. . . . . c . . . . . g . . . . g . a . . . c t
		Lagurus	. t . . –t c . t . t . –c . –. . . . –. . . a . . c . . . . . a g . . . . g . . . . . c t
		Calamagrostis	. . . . –. . . . –c . . Y t . . . . . t . . . . . t . . . . . t g . . . . g . . . . . c t
		Agrostis	. . a . –. . . . –c a . t . . . . . t . . . . a t g . . . . g . . . . . c t
		Arrhenatherum	. . a . –. . . t . c . . c . . . . . t . t g a . . c . . . . . g t g . . . . . a . . . . a c a
		Avena	**t c t t –t t g c a c g c c c c c g–g t a c t c a g t c c t a g g g t c g g c g g t g c t c c g c a**
			1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2
			2 3 3 4 4 4 4 4 5 5 5 5 6 6 6 7 7 –7 8 8 9 9 0 0 0 0 1 1 1 2 3 3 6 6 6 7 7 7 7 8 8 9 9 9 9 9 0 0
			4 6 5 9 1 2 5 6 8 0 2 4 8 0 4 8 2 3 –9 7 8 3 5 1 4 5 6 2 4 9 0 3 8 4 6 7 0 1 3 4 7 7 8 0 4 5 6 9 2 8
	?	***A. macrostachya***	**. . . . –. c . . . . a . . . . . g . . . . . t . t . . c . t . t a . . .**
	Cv	***A. ventricosa***	**. . . . –. –. . . . . t . . . . . g a . . . a t a a . . . t . t . . . . . t . . . .**
	Cp	***A. clauda***	**. . . . –. –. Y . . . . . t . . . . . g a . M . . a a . . . t . t . . c . t . t . K . .**
	Cp	***A. pilosa***	**. . . . –. –. Y . . . . . t . . . . . g a . M . . a a . . . t . t . . c . t . t . K . .**
	Ad	*A. damascena*	. a . y t . . . t . t . . . –. . a . a . . . . t . t . . t . . . . . t . . . a a . . . . . t . .
	As	*A. atlantica*	. a . Y t . . . t . t . . . –. . a . a . . . . t . t . . t . . . . . t . . . a a . . . . . t . .
	As	*A. hirtula*	. a . . t . . . t . t . . M–. . a . a . . . . t . K . . t . . . . . t . . . a a . . . . . t . .
	As	*A. wiestii*	. a . . t . . . t . t . . M–t . a . a . . . . t . . . . t . . . . . t . . . a a . . . . . t . .
	Al	*A. longiglumis*	a a . . t . . . t . t . . . –. . a . a . . . . t . . . . t . . . . . t . . . a a . . . . . t . .
	Ac	*A. canariensis*	W a . . t . . . t . t . S . –. . a . a . . . . t . . . . t . . . . . t . –. a a . . t . . . t . .
	AB	*A. barbata*	. . t . . . t . t . . . –. . a . a . . . . t . . . . t . . . . . t . . . a a . . . . . t . .
	ACD	*A. sterilis*	a a . . t . . Rt . t . S . –. . a . a . . . . t . . . . t . . . . . t . . . a a . . . . . t . .
	ACD	*A. sativa*	. a . . t . . Rt . t . . . –. . a . a . . . . Y . . . t . . . . . t . . . a a . . . . . t . .

Table 2. (Contd.)

Tribe	Genome	Species, genus	Nucleotide positions
			ITS2
Triticeae		*T. aestivum*	c a c a t c c a g t c g c c c t c t t g a g t g c t c g t c t a g c a t c c g c g g t – g t t t t g a a g a c c –
Brachypodiae		*Brachypodium*	c c c t t c t c g g c a a t c t c t c g g g c g c t t g a t t c g c – t g c g g t c c – g c a c a a – a g a t c –
Phleeae		*Beckmannia*	. . . . . t . . . t . g . . c . . . c . . g t t t t . a c . . . . . t c t . . – – . . a . t
		Alopecurus	. . a . c – t . . . t . g . . c . . . a . . g c . t c . a c . . . . . a t g – – t – – . . a . t
Poeae		*Colpodium*	. . . . . a c . . . c – t . . . . . t . . g c . t t . a t . . . t . . . . a . t . . . . t c t . . – . . . . . t
		Poa	. . . . . t . . . – – g . . c . c . a t . g t . . c . a a . . . . g . . . . a c . . . . t g t – . – . . . g . t
Aveneae	(?)*	*Z. biebersteiniana*	. . . . . a c . . . t – t . t . . . . t . . g c . t t . a t . a . . . . . a . t . . . . t c t . . – . . . . . t
	(?)	*Z. trichopoda*	. . . . . a c . . . c – t . . . . . t . . g c . t t . a t . a . . . . . a . t . . . . t c t . . – . . . . . t
		Deschampsia	. . . . . t . . – t t g . . c . . . c a . g t t t c . a t . . . . . t . . . . t g c a . – . . . . a c c
		Pseudarrhenatherum	t . . . . . t . . t c . g . . c . . . t . . a t . . c . a c . . . . . t g c . . a . . t . . t
		Holcus	. . . . . t t . . . t . g . . c . . . t . . t t t t c . a c . . . t . . . . t c g . t a . . t a t .
		Koeleria	t . . . c . t . . . c . g . t c . . . . . a t . t c . t c a . . . . . t . . . . . c g c – – a . . t . . .
		Trisetum	t Y . . c - t M . . c . g . t c . . . K . . a t . t c . t c a . . . . . K . . . . . c g c K . – . . t . t .
		Lagurus	– . . . . . t . . t c . g . t c . . . . . a t t t c t c – . c . . . . . a a . . . . c g t . . t . – t . . .
		Calamagrostis	t . . . . . t . . . t t g . t c . . . . . a t . t c . a c . . . . g . . . a . . a . . . t g c . . – . . . . .
		Agrostis	– . . . . . t . . . c . g . . c . . . c . . a t . t c a a c . . . . . g . . . . . t g c t . – . . t . . c
		Arrhenatherum	t . . . . . t – . . t a g . t c . . . . . a t . t t . a c . . . . c . . . a g . . . . . t a c t . a . . t . . c
		Avena	**t c c c t c t c g a c a t c t c c t g g g g a t c c t a a c t t g c c t c c a a g g c – a t g c g a a a – t t c t**
			1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 – 1 1 1 1 1 1 1 1 – 1 1 1 2
			1 1 1 2 2 2 2 2 3 3 3 4 5 5 5 5 5 6 6 6 7 8 8 9 9 0 2 2 2 3 3 4 4 5 5 5 5 5 5 5 6 – 6 6 7 7 7 8 8 8 – 9 9 9 0
			2 6 7 9 1 2 4 5 7 1 2 3 5 0 1 3 4 6 0 5 7 2 3 4 4 6 3 2 6 7 4 9 6 9 0 1 2 3 5 6 7 9 4 – 6 9 0 8 9 0 1 2 – 0 7 9 3
	?	***A. macrostachya***	. . a . . . . . a . . . . c . . . . c . . . . a . . a – . . . . . t . . t . . . . .
	Cv	***A. ventricosa***	. . a t . . . . a . . . . c . . . . g – . . . . . – t . . . . . a
	Cp	***A. clauda***	. . a . c . . . a . . . . a . . . . t . t . . . R . . – . . . c . . t t . . . . . – t . . . . . a
	Cp	***A. pilosa***	. . a . c . . . a . . . . a . . . . t . t . . . . . g – . . . c . . t . . . . . . – t . . . . . a
	Ad	*A. damascena*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a . . . g . . a . . a . . g . . .
	As	*A. atlantica*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a . . . g . . a . . a . . g . . .
	As	*A. hirtula*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . Y . c a . . . g . . a . . a t . g . . .
	As	*A. wiestii*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a . . . g . . a . . a t . g . . .
	Al	*A. longiglumis*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a a . . g . . a . . a t . g . . .
	Ac	*A. canariensis*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a . . . g . . a . . a . . g . . .
	AB	*A. barbata*	. a . . . t c g . . . . . t c . – . c
	ACD	*A. sterilis*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . Y . . c a a . . g . . a . . a t . g . . .
	ACD	*A. sativa*	. a . . . t c g . . . . . t c . . . c . – . . . . . t . t . a . . t . . . . . c a a . . g . . a . . a t . g . . .

Note: Designations: M = A, R = A and G, W = A and T, S = C and G, Y = C and T, and K = G and T.
* The genus *Zingeria* is traditionally assigned to the tribe Aveneae, but, according to the ITS sequence characteristics, this genus is a typical member of Poeae.

Table 3. The level of differences between sequences ITS1 and ITS2 in species from the genus *Avena* (*p*-distance, %)

Species	*A. macrostachya*	***A. clauda***	***A. ventricosa***	***A. pilosa***	*A. longiglumis*	*A. wiestii*	*A. hirtula*	*A. canariensis*	*A. atlantica*	*A. sterilis*
A. clauda	4.21									
A. ventricosa	3.28	2.88								
A. pilosa	3.72	0.89	2.19							
A. longiglumis	**9.19**	**10.02**	**9.67**	**9.45**						
A. wiestii	**9.19**	**9.35**	**9.01**	**8.79**	1.31					
A. hirtula	**8.75**	**9.58**	**9.23**	**9.01**	0.87	0.44				
A. canariensis	**8.77**	**9.38**	**9.25**	**8.81**	0.87	1.09	0.87			
A. atlantica	**9.43**	**9.82**	**9.47**	**9.25**	1.97	1.31	1.31	1.31		
A. sterilis	**9.19**	**10.02**	**9.67**	**9.45**	0.65	1.31	0.87	0.87	1.97	
A. sativa	**8.75**	**9.35**	**9.23**	**8.79**	0.87	1.09	0.87	1.09	1.75	0.65

Note: In bold are set out p-distances between the nucleotide sequences of ITS1 and ITS2 species carrying C genomes and species carrying A genomes.

(bootstrap index = 100). All 35 most probable phylogenetic trees constructed using the maximum parsimony method pointed to monophyly of the *Avena* genus and to its subdivision into two phylogenetic branches, representing the species with A- and C-genomes, respectively. These data are consistent with the results of comparative analysis of RAPD and AFLP patterns of diploid oat species with A- and C-genomes, where it was demonstrated that these species formed two separate clades with C-genome species forming an outer group relative to the *Avena* oats with AA, AABB, AACC, and AACCDD genomes [54, 55].

Diploid *Avena* species with A-genome are characterized by a low level of interspecific divergence of ITS1 + ITS2 (mean *p*-distance = 0.010, standard deviation = 0.0027). Within the clade of A-genome species, topology of the tree presented in Fig. 2 was not statistically valid, which is demonstrated by low bootstrap indices, but interesting in some respects. For example, it contained a clade formed by two morphologically similar species of *A. hirtula* and *A. wiestii*, usually considered by the taxonomists as two subspecies, *A. barbata* subsp. *wiestii* (Steud.) Mansf. and *A. barbata* subsp. *hirtula* (Lag.) Tab.-Mor. [10].

Relative to the ITS sequences, all C-genome species of *Avena* can be divided into two groups: *A. pilosa* and *A. clauda* are close to one another with the outer group of *A. ventricosa*. Moreover, judged by the ITS analysis, reality of the *pilosa–clauda* clade is highly statistically significant (bootstrap = 99), which is consistent with both the data of morphological analysis [7] and karyological observations [11, 15, 56], as well as with the results of interspecific crosses [11, 17], RAPD analysis, and AFLP patterns [54, 55].

Among the species belonging to the other genera examined, *Arrhenatherum elatius* was found to be closest to *Avena* in the ITS1 and ITS2 structure (Fig. 2). Phylogenetic relationships between *Avena* and *Helictotrichon* remain intriguingly uncertain. A large set of cloned and sequenced ITS1 from *Helictotrichon* species [26, 37] contains HPR389153 sequence, which is very similar to *Avena* A-genome spesies (*p*-distance = 0.0237), while the differences between this sequence and ITS1 from *A. macrostachya* reach 0.1221. By contrast, HAD389117 from this set is close to ITS1 from C-genome *Avena* (*p*-distance between it and C-genome-specific ITS1 sequences is equal to 0.0189, while its differences from the A-genome-specific ITS1 sequences reach 0.1221). HPR389153 (A-genome-relative) is one of the sequences isolated from the genomic DNA of *H. pratense* (L.) Besser ($2n$ = 84–140 [13]), and HAD389117 was isolated from *H. adsurgens* (Schnur ex Simonk.) Conert ($2n$ = 120–126) [13]. Both of these species belong to highly polyploid ($2n = 16–18x$) group of northern Mediterranean representatives of *Helictotrichon* [12, 13]. This genus is thought to be polyphyletic, since other cloned ITS sequences from these and other species are similar to the ITS sequences from other Aveneae members, but are substantially different from the *Avena*-specific ITS [26]. It is suggested that the appearance of highly polyploid *H. pratense* and *H. adsurgens* was associated with interspecific hybridization involving Mediterranean oats with A- and C-genomes.

The genome of *A. macrostachya* contains rRNA genes typical of the C-genome oats, which is congruent with the data of GISH analysis by Leggett and Markhand [16]. At the same time, the chromosome structure

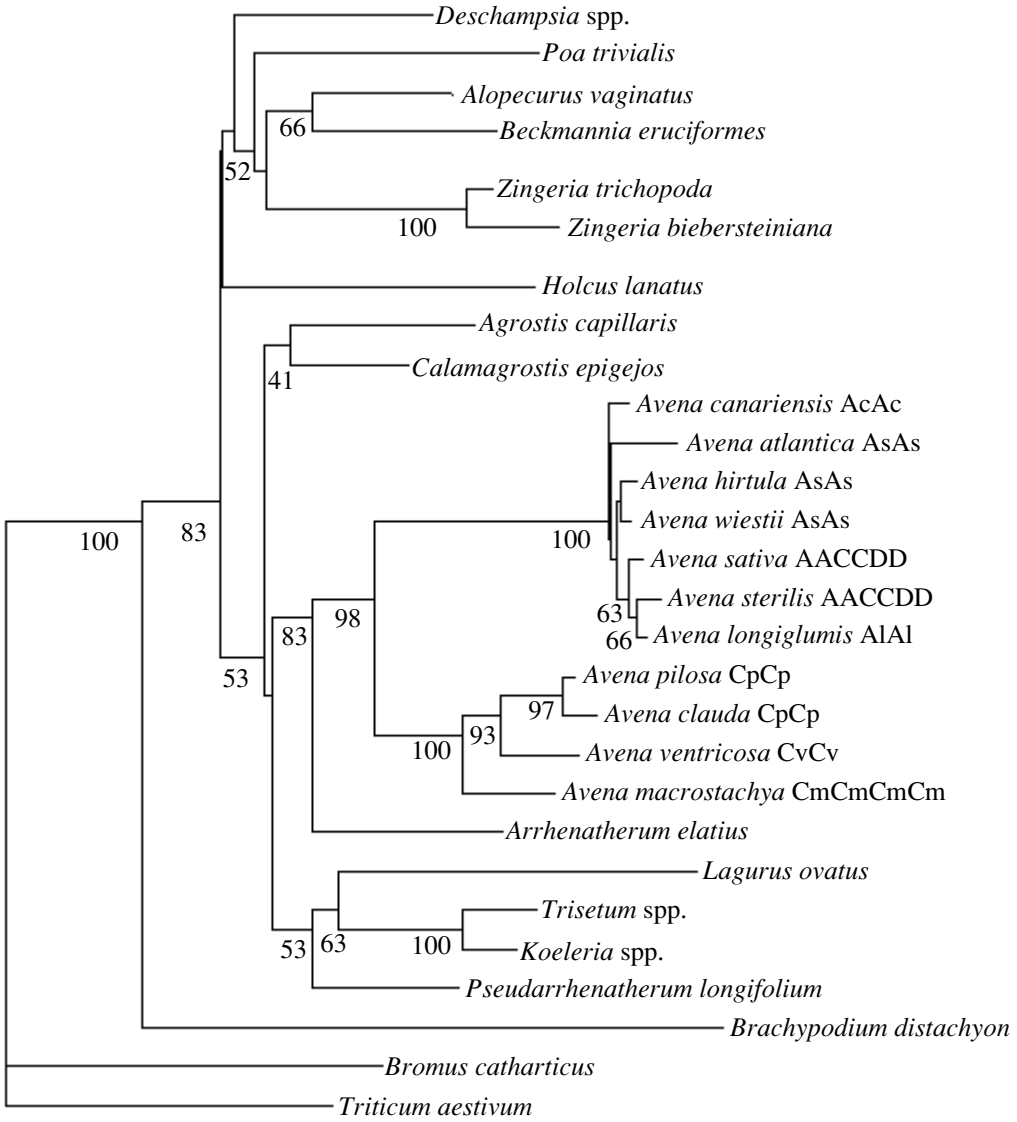

Fig. 2. Phylogenetic tree reflecting the divergence of the ITS1 and ITS2 sequences in the tribe Aveneae and some members of the tribe Poeae. Numerals give bootstrap values.

of *A. macrostachya*, especially that revealed upon C-banding [21, 22], is unique and is found in no other *Avena* species examined so far, provides identification of this genome type as a special C-genome variant, Cm-genome. An isobrachyal character of Cm-genome specific chromosomes brings *A. macrostachya* karyotype close to the karyotypes of A-genome *Avena* species.

Based on our and literature data it can be hypothesized that the earlier stages of oat karyotypes evolution were as follows (Fig. 3): the ancestor of *Avena* had a diploid isobrachial chromosome set with "symmetrical" chromosomes, similar to the chromosome set of *A. macrostachya* $(Cm)_4$ and to the karyotypes of diploid oat species with the A-genome [11, 15]. Next, the divergence of phylogenetic oat lineages with A- and C-genomes occurred. This event was accompanied by accumulation of the differences in dispersed repeat sequences [57, 58] (which may explain the results of GISH hybridization [16]), as well as by accumulation of transitions and transverions specific to each lineage. After that, within C-genome, *A. macrostachya* ancestral phylogenetic brunch diverged from the C-genome species ancestor. At the next stage, the chromosome set of *A. macrostachya* was duplicated, and large C-heterochromatic block appeared in the pericentric chromosome regions [21, 22]. At the same time, in the ancestors of other species (*A. clauda*, *A. pilosa*, and *A. ventricosa*) with C-genomes chromosomal rearrangements, a change of the centromere position took place. In addition, chromosomal "desymmetrization" in the ancestor of *A. clauda*, *A. pilosa*, and *A. ventricosa* was accompanied by the appearance of a great number of small interstitial C-heterochromatin blocks along with relatively

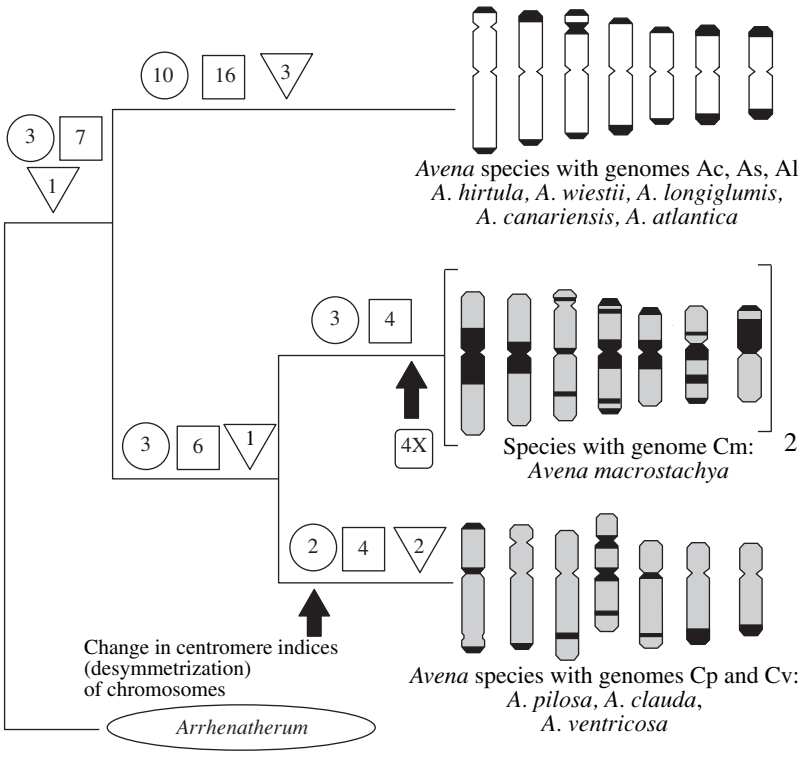

Fig. 3. The karyotype evolution at the early divergence stages of the *Avena* with the A and C genomes. The circled numerals indicate the number of transversions; the boxed numerals, the number of transitions; the numberal in triangles, the indel number. 4X, tetraploidization event.

intense Giemsa staining of chromosome arms ([21, 22, 59]; E. Badaeva, personal communication).

ACKNOWLEDGMENTS

The authors thank E.D. Badaeva for the discussion of the results, A.V. Konarev and P.P. Strel'chenko for their help and support at the initial stages of the study, and A.M. Efimov and E.O. Punina for their help and valuable comments on the manuscript.

This work was supported by the Russian Foundation for Basic Research (grant nos. 03-04-49477 and 02-04-49667).

REFERENCES

1. Cosson, E. and Durieu De Malsonneuve, M.C., Notes sur guelqies Graminees d'Algerie, *Bull. Soc. Bot. Fr.*, 1855, vol. 1, pp. 1–318.
2. Guarino, L., Chadja, H., and Mokkadem, A., Collection of *Avena macrostachya* Bal. ex Coss. et Dur. (Poaceae) Germplasm in Algeria, *Econ. Bot.*, 1991, vol. 45, pp. 460–466.
3. Baum, B.R. and Rajhathy, T., A Study of *Avena macrostachya, Can. J. Bot.*, 1976, vol. 54, pp. 2434–2439.
4. Hoppe, H.-D. and Pohler, W., Successful Hybridization between *Avena prostrata* and *Avena macrostachya, Cereal Res. Commun.*, 1988, vol. 16, pp. 231–235.
5. Leggett, J.M., A New Triploid Hybrid between *Avena eriantha* and *A. macrostachya, Cereal Res. Commun.*, 1990, vol. 18, pp. 97–101.
6. Loskutov, I.G., Species Diversity and Breeding Potential of the Genus *Avena* L., *Extended Abstract of Doctoral (Biol.) Dissertation*, St. Petersburg, 2003.
7. Malzew, A.I., *Ovsyugi i ovsy* (Wild and Cultivated Oats), Leningrad, 1930.
8. Baum, B.R., Delimitation of the Genus *Avena* (Gramineae), *Can. J. Bot.*, 1968, vol. 46, pp. 121–132.
9. Baum, B.R., Classification of the Oat Species *Avena* (Poaceae) Using Various Taximetric Methods and an Information-Theoretic Model, *Can. J. Bot.*, 1974, vol. 52, pp. 2241–2262.
10. Tsvelev, N.N., *Zlaki SSSR* (Cereals of the Soviet Union), Leningrad: Nauka, 1976.
11. Rodionova, N.A., Soldatov, V.N., Merezhko, V.E., *et al.*, in *Kul'turnaya flora. Oves* (Cultivated Flora: Oat), Moscow: Kolos, 1994, vol. 2, part 3.
12. Röser, M., Patterns of Diversification in Mediterranean Oat Grasses (Poaceae: Aveneae), *Lagascalia*, 1997, vol. 19, pp. 101–120.
13. Röser, M., Character of Evolution of the Genus *Helictotrichon* (Poaceae: Aveneae) Reconsidered in View of Recent Results in Ibero-Mauritanian and Eurasian Species, *Flora*, 1998, vol. 193, pp. 425–447.
14. Zoshchuk, N.V., Badaeva, E.D., and Zelenin, A.V., History of Modern Chromosome Analysis: Chromosome

Banding in Plants, *Ontogenez*, 2003, vol. 34, no. 1, pp. 5–18.

15. Thomas, H., Cytogenetics of *Avena, Oat Science and Technology*, Marshall, H.G. and Sorrels, M.E., Eds., Madison, Wis.: ASA and CSSA, 1992, no. 33, pp. 473–508.
16. Leggett, J.M. and Markhand, G.S., The Genomic Configuration of *Avena* Revealed by GISH, *Kew Chromosome Conf. IV.*, Brandham, P.E. and Bennett, M.D, Eds., Kew: Royal Botanic Gardens, 1995, pp. 133–139.
17. Loskutov, I.G., Interspecific Crosses in the Genus *Avena* L., *Rus. J. Genet.*, 2001, vol. 37, no. 5, pp. 467–475.
18. Ladizinsky, G., Genetic Control of Bivalent Pairing in the *Avena strigosa* Polyploid Complex, *Chromosoma*, 1973, vol. 42, pp. 105–110.
19. Katsiotis, A., Hagidimitriou, M., and Heslop-Harrison, J.S., The Close Relationship between the A and B Genomes in *Avena* L. (Poaceae) Determined by Molecular Cytogenetic Analysis of Total Genomic, Tandem and Dispersed Repetitive DNA Sequences, *Ann. Bot.*, 1997, vol. 79, pp. 103–109.
20. Linares, C., Ferrer, E., and Fominaya, A., Discrimination of the Closely Related A and D Genomes of the Hexaploid *Avena sativa* L., *Proc. Natl. Acad. Sci. USA*, 1998, vol. 95, pp. 12 450–12 455.
21. Jellen, E.N., C-Banding of *Avena macrostachya*, http://pas.byu.edu/Faculty/enj/oatsite/aventaxa.htm.
22. Hutchinson, J. and Postoyko, J., C-Banding of *Avena* Species, *Genetic Manipulation in Plant Breeding: Proc. Int. Symp., EUCARPIA*, Berlin, 1986, pp. 157–160.
23. Hsiao, C., Chatterton, N.J., Asay, K., and Jensen, K.B., Phylogenetic Relationships of 10 Grass Species: An Assessment of Phylogenetic Utility of the Internal Transcribed Spacer Region in Nuclear Ribosomal DNA in Monocots, *Genome,* 1994, vol. 37, pp. 112–120.
24. Hsiao, C., Chatterton, N.J., Asay, K., and Jensen, K.B., Molecular Phylogeny of the Pooideae (Poaceae) Based on Nuclear rDNA (ITS) Sequences, *Theor. Appl. Genet.,* 1995, vol. 90, pp. 389–398.
25. Ainouche, M.L. and Bayer, R.J., On the Origins of the Tetraploid *Bromus* Species (Section *Bromus*, Poaceae): Insights from Internal Transcribed Spacer Sequences of Nuclear Ribosomal DNA, *Genome*, 1997, vol. 40, pp. 730–743.
26. Grebenstein, B., Röser, M., Sauer, W., and Hemleben, V., Molecular Phylogenetic Relationships in Aveneae (Poaceae) Species and Other Grasses As Inferred from ITS1 and ITS2 rDNA Sequences, *Plant Syst. Evol.*, 1998, vol. 213, pp. 233–250.
27. Wang, J.-B., Wang, C., Shi, S.-H., and Zhong, Z., Evolution of Paternal ITS Regions of Nuclear rDNA in Allopolyploid *Aegilops* (Poaceae) Species, *Hereditas* (Lund, Swed.), 2000, vol. 133, pp. 1–7.
28. Troitsky, A.V., Melekhovets, Yu.F., Rakhimova, G.M., *et al.*, Angiosperm Origin and Early Events of Seed Plant Evolution Deduced from rRNA Sequence Comparisons, *J. Mol. Evol.*, 1991, vol. 32, pp. 253–261.
29. Hershkovitz, M.A. and Lewis, L.A., Deep-Level Diagnostic Value of the rDNA ITS Region, *Mol. Biol. Evol.*, 1996, vol. 13, pp. 1276–1295.
30. Chatterton, N.J., Hsiao, C., Asay, K.H., *et al.*, Nucleotide Sequence of the Internal Transcribed Spacer Region of rDNA in the Primitive Oat Species, *Avena longiglumis* Durieu, *Plant. Mol. Biol.*, 1992, vol. 20, pp. 163–164.
31. Moore, L.A. and Field, C.B., see http://www.ncbi.nlm.nih.gov.
32. Doyle, J.J. and Doyle, J.L., A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue, *Phytochem. Bull.*, 1987, vol. 19, pp. 11–15.
33. Gardes, M. and Brunes, T.D., ITS Primers with Enhanced Specificity for Basidiomycetes—Application to the Identification of Mycorrhizae and Rusts, *Mol. Ecol.*, 1993, vol. 2, pp. 130–138.
34. White, T.J., Bruns, T., Lee, S., and Taylor, J., Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics, *PCR Protocols: A Guide to Methods and Applications*, Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., Eds., San Diego: Academic, 1990, pp. 315–322.
35. Sanger, F., Niclein, S., and Coulson, A.R., DNA Sequencing with Chain-Terminating Inhibitors, *Proc. Natl. Acad. Sci. USA*, 1977, vol. 74, pp. 5463–5467.
36. Xia, X. and Xie, Z., DAMBE: Data Analysis in Molecular Biology and Evolution, *J. Hered.*, 2001, vol. 92, pp. 371–373.
37. Hemleben, V., Grebenstein, B., and Herges, H., see http://www.ncbi.nlm.nih.gov.
38. Hsaio, C. and Chatterton, N.J., see http://www.ncbi.nlm.nih.gov.
39. Jakob, S.S. and Blattner, F.R., see http://www.ncbi.nlm.nih.gov.
40. Brysting, A.K., Fay, M.F., Leitch, I.J., and Aiken, S.G., see http://www.ncbi.nlm.nih.gov.
41. Corach, D., Fernandez Souto, D., and Bernasconi, P., see http://www.ncbi.nlm.nih.gov.
42. Catalan, P., Torrecilla, P., Rodriguez, J.-A., and Olmstead, R., see http://www.ncbi.nlm.nih.gov.
43. Chiapella, J.O., see http://www.ncbi.nlm.nih.gov.
44. Charmet, G., Ravel, C., and Balfourier, F., Phylogenetic Analysis in the *Festuca–Lolium* Complex Using Molecular Markers and ITS rDNA Sequences, *Theor. Appl. Genet.,* 1997, vol. 94, pp. 1038–1046.
45. Gaut, B.S., Tredway, L.P., Kubik, C., *et al.*, see http://www.ncbi.nlm.nih.gov.
46. Kotseruba, V., Gernand, D., Meister, A., and Houben, A., Uniparental Loss of Ribosomal DNA in the Allotetraploid Grass *Zingeria trichopoda* (2*n* = 8), *Genome*, 2003, vol. 46, pp. 156–163.
47. Felsenstein, J., Confidence Limits on Phylogenesis: An Approach Using the Bootstrap, *Evolution*, 1985, vol. 39, pp. 783–791.
48. Nei, M. and Kumar, S., *Molecular Evolution and Phylogenetics*, New York: Oxford Univ. Press, 2000.
49. Titov, I.I., Vorobiev, D.G., Ivanisenko, V.A., and Kolchanov, N.A., GArna: Predicting 2D Structure of RNA by Genetic Algorithm, http://www.icg.sbras.ru/rus/Resources Rus.html.
50. Suh, Y., Thien, L.B., and Zimmer, E.A., Nucleotide Sequences of the Internal Transcribed Spacers and 5.8S rRNA Gene in *Canella winterana* (Magnoliales; Canellaceae), *Nucleic Acids Res.,* 1992, vol. 20, pp. 6101–6102.

51. Jobes, D.V. and Thien, L.B., Conserved Motif in the 5.8S Ribosomal RNA (rRNA) Gene Is a Useful Diagnostic Marker for Plant Internal Transcribed Spacer (ITS) Sequences, *Plant Mol. Biol. Rep.*, 1997, vol. 15, pp. 326–334.

52. Goertzen, L.R., Cannone, J.J., Gutell, R.R., and Jansen, R.K., ITS Secondary Structure Derived from Comparative Analysis: Implications for Sequence Alignment and Phylogeny of the Asteraceae, *Mol. Phylogenetics and Evolution,* 2003, vol. 29, pp. 216–234.

53. Fitch, W.M. and Margoliash, E., Construction of Phylogenetic Trees, *Science*, 1967, vol. 155, pp. 279–284.

54. Perchuk, I.N., Loskutov, I.G., and Okuno, K., Study of the Species Diversity in Oat by Means of RAPD Analysis, *Agrarn. Ross.*, 2002, no. 3, pp. 41–44.

55. Drossou, A., Katsiotis, A., Leggett, J.M., *et al.*, Genome and Species Relationships in Genus *Avena* Based on RAPD and AFLP Molecular Markers, *Theor. Appl. Genet.*, 2004, vol. 109, pp. 48–54.

56. Rajhathy, T. and Thomas, H., Chromosomal Differentiation and Speciation in Diploid *Avena*: III. Mediterranean Wild Populations, *Can. J. Genet. Cytol.*, 1967, vol. 9, pp. 52–68.

57. Linares, C., Serna, A., and Fominaya, A., Chromosomal Organization of a Requence Related to LTR-Like Elements of *Ty1–copia* Retrotransposons in *Avena* Species, *Genome,* 1999, vol. 42, pp. 706–713.

58. Linares, C., Irigoyen, M.I., and Fominaya, A., Identification of C-Genome Chromosomes Involved in Intergenomic Translocation in *Avena sativa* L., Using Cloned Repetitive DNA Sequences, *Theor. Appl. Genet.*, 2000, vol. 100, pp. 353–360.

59. Jellen, E.N., Phillips, R.L., and Rines, H.W., C-Banded Karyotypes and Polymorphisms in Hexaploid Oat Accessions (*Avena* spp.) Using Wright's Stain, *Genome*, 1993, vol. 36, pp. 1129–1137.